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Review

The acquisition of neural fate in the chick

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Abstract

Neural development in the chick embryo is now understood in great detail on a cellular and a molecular level. It begins already before gastrulation, when a separation of neural and epidermal cell fates occurs under the contol of FGF and BMP/Wnt signalling, respectively. This early specification becomes further refined around the tip of the primitive streak, until finally the anterior–posterior level of the neuroectoderm becomes established through progressive caudalization. In this review we focus on processes in the chick embryo and put classical and more recent molecular data into a coherent scenario.

Keywords: Neural fate; Chick; Neural development; Neural induction; Hensen's node

1. Introduction

The acquisition of neural fate in the chick is a complex process, which begins while the egg is still in the uterus and occupies much of the first day of extrauterine development. It involves a tightly regulated series of inductions between the different tissues and morphological structures of the early embryo, before and during gastrulation. Today, we do not only know the fine anatomy of the early chick embryo, but also obtained an understanding of the dynamics of development, thanks to the mapping efforts in many laboratories. The establishment of specification maps revealed the intrinsic states of commitment for many key tissues at different embryonic stages. In addition, a wealth of molecular information was collected in the recent years, defining the major molecular players of the neural induction process. In particular, the major signalling molecules seem to be identified and a coherent picture of their interwoven, sometimes multiple activities is emerging. It has become a common practice to evaluate findings for avian embryos in the light of models derived from other, mostly amphibian embryos. These include the organizer, or head–trunk organizer concepts of H. Spemann and O. Mangold, as well as the activation–transformation hypothesis of P. Nieuwkoop (Mangold, 1933; Nieuwkoop, 1952; Spemann and Mangold, 1924). Here, we will largely refrain from such attempts, which certainly have their benefits, but also appear difficult taking into account the different embryologies and, consequently, specific technologies. In this review, we will not summarize the large amount of information available on neural induction in amphibia, but will rather put together the extensive knowledge focussing on the chick. After reviewing briefly the early morphology, fate maps and specification status, we will summarize experimental evidence from tissue transplantation experiments and from applications of purified factors. Finally, we will try to generate a coherent scenario, and describe the current views on the sequential inductions and molecular events during the acquisition of neural fate in the chick.

2. Structural components of early chick embryos

The first cleavages of the unicellular chick embryo do not divide the complete yolk, but occur only superficially, so that a syncytium develops on top of the yolk sphere, with all early cells open to the yolk (Eyal-Giladi and Kochav, 1976). After about 10 h of intrauterine development, a disc-shaped blastoderm is recognizable for the first time. Three hours later a differentiation of the blastoderm is apparent, with

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only the peripheral cells open to the yolk, and the central cells separated by transverse membranes. Thus, a yolk-rich 'area opaca' surrounds a translucent 'area pellucida', the latter by now consisting of a single cell layer. By the time of egg laying, after about nineteen hours in the uterus, dispersed cell aggregates indicate the beginning formation of a two layered embryo consisting of an upper 'epiblast' and a lower 'hypoblast'. In such embryos, the intermediate zone between the area opaca and pellucida can be recognized as the 'marginal zone' (Fig. 1, top row).

Further incubation leads to the completion of the lower layer, the hypoblast, within the first five hours. The hypoblast spreads from the prospective posterior pole of the embryo, where a sickle shaped structure, 'Koller's sickle', is more or less obvious at the anterior border of the posterior marginal zone ('PMZ' Bachvarova et al., 1998; Callebaut and Van Nueten, 1994; Eyal-Giladi and Kochav, 1976; Izpisua-Belmonte et al., 1993). After about 6 h a triangular thickening indicates the generation of the 'primitive streak', i.e. the site of ingression for endo- and mesodermal cells. The hypoblast now becomes displaced by another tissue of the extraembryonic, primitive endoderm, the 'endoblast' (Bachvarova et al., 1998; Callebaut et al., 1998; Foley et al., 2000; Vakaet, 1970; Fig. 1, top row). Within the next twelve hours, the primitive streak elongates significantly, until it reaches a length of about 1.8 mm at the definitive streak stage. The first cells to ingress through the tip of the primitive streak become definitive endoderm, and extraembryonal mesoderm ingresses through its posterior portion. With the ongoing of gastrulation, future head and heart mesoderm is generated in the anterior streak (Garcia-Martinez and Schoenwolf, 1993; Psychoyos and Stern, 1996a; Fig. 1, third row). After the mid-streak stage, the tip of the streak thickens to form 'Hensen's node', or simply the 'node' (Hensen, 1876). The lower layer now consists of a large area of endoderm around the tip of the streak, while the endoblast is displaced towards the periphery, and the hypoblast towards the anterior embryo (Fig. 1, top row). Mesoderm has not reached the anterior part of the embryo, where the anterior head will develop. When the ingression of endoderm stops, a mesendodermal cell population, the 'prechordal mesendoderm' leaves the node and migrates to the anterior area, below the prospective forebrain. It is followed by cells, which assemble to form the midline mesoderm of the 'notochord' (Fig. 1, top row and third row). Future paraxial mesodermal cells ingress through the lateral node and the post-nodal streak. They assemble to form first the presomitic mesoderm of the segmental plate, which then is converted into the segmented, epithelially organized somites (Fig. 1, third row).

3. Fate and specification of the neuroectoderm

Fate maps of chick embryos were studied extensively in various stages of development, with numerous experimental approaches, including the application of charcoal particles, chick-quail chimeras, or fluorescent dyes (Fig. 1; Gräper, 1929; Spratt, 1942; Rosenquist, 1966; Vakaet, 1970; Schoenwolf et al., 1989; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Psychoyos and Stern 1996; Schoenwolf, 2001). Cells fated to the neuroectoderm are widely spread in the area pellucida of early chick embryos before gastrulation (Hatada and Stern, 1994). However, there is an early concentration of these cells at the posterior midline, adjacent to Koller's sickle (Fig. 1, second row). With the forward spreading of the hypoblast, the prospective forebrain field moves anteriorly, avoiding the front of the endoblast. Upon streak formation it is located anterior to the tip of the streak, where it remains also after elongation of the streak (Foley et al., 2000; Fig. 1, second row). Thus, the forebrain field is not associated with any mesoderm in the early phases of development, and the contact to the prechordal mesendoderm is its first exposure to embryonic cells from below. The neuroectodermal fate map at the definitive streak stage includes also the precursors of the midbrain and the hindbrain anteriorly and adjacent to the node, and the spinal cord flanking the primitive streak. This neuroepithelium, the 'neural plate', becomes increasingly thicker towards the node, and is surrounded by non-neural epithelium (Fig. 1, second row).

More difficult than to determine the fate of cells is to answer questions concerning their specification and commitment, i.e. to determine the developmental program a population of cells represents at a given time and location. Specified cells will maintain their developmental program in a neutral environment, but are still able to respond to signals and to change their fate. Committed cells, will exert their differentiation program even in the presence of factors or in environments that can repress or change a specific fate of yet uncommitted cells. To analyze the state of specification or commitment, tissues or cell populations are explanted out of their embryonic context and kept in culture systems, such as chorioallantoic membranes or collagen gels, or transferred to ectopic sites within cultured embryos. After a given culture period such explants/transplants are assayed for cell type specific morphology or marker gene expression (Rudnick, 1935; Rudnick, 1938; Spratt and Hass, 1960; Garcia-Martinez et al., 1997; Wilson et al., 2000; Chapman et al., 2003). Obviously, the experimental conditions are never absolutely neutral for the further development. Parameters like the size of the explanted tissue, the culture period and the choice of the markers strongly influence the outcome and conclusions of a specification study.

Wilson and colleagues studied central and lateral epiblast pieces of early chick embryos cultured in matrix for 40 h. Medial, as well as lateral explants from stage EKVIII epiblasts did not give rise to tissue expressing neural markers, indicating that a neural specification had not yet occurred. However, medial, but not lateral epiblast

explants from embryos of intrauterine stages EKIX and EKXII developed into tissues expressing pre-neural (Sox3), neural (Sox2, Pax6) and anterior neural (Otx2) marker genes (Wilson et al., 2000; Wilson et al., 2001). Such experiments indicate a separation of epidermal and neural fates already before the onset of gastrulation. In a similar assay Muhr and coworkers investigated the regional specification of the neural plate during gastrula stages (Muhr et al., 1999). Rostral and caudal explants of neural plate ectoderm at the early intermediate streak stage expressed the markers Sox2 and Otx2, but no midbrain, hindbrain or spinal chord markers. Explants taken from different anterior–posterior levels of neural plates of later stages showed a sequential specification into separate forebrain, midbrain and hindbrain territories, demonstrated by the successive activation of more posteriorly expressed molecular markers. The authors concluded that the early neural plate has a anterior character, which is then caudalized. However, Otx2 alone is not an unproblematic marker to define anterior neural specification and regionalization, since it is expressed extremely early and not restricted to the neural epiblast (Bally-Cuif et al., 1995). In chick embryos the earliest known marker exclusively expressed in the anterior neural plate is the homeobox gene GANF, which was not tested by Muhr et al. When Chapman and coworkers investigated isolates of anterior epiblast from intermediate streak stages for the activity of Sox2 and GANF, they found an early specification for Sox2 expression. However, GANF expression required signals from the lower layer, i.e. the hypoblast and the anterior definitive endoderm. Based on their results, the epiblast would first be neurally committed, and afterwards independently instructed to acquire an anterior identity (Chapman et al., 2003). Thus, there is conflicting argumentation for either a nexus between neural and anterior specification, or for a separation of these two events. Since both arguments rely on different experimental conditions, and apply either exclusively Otx2 or GANF, respectively, as a single anterior marker, a better understanding of this issue seems not impossible.

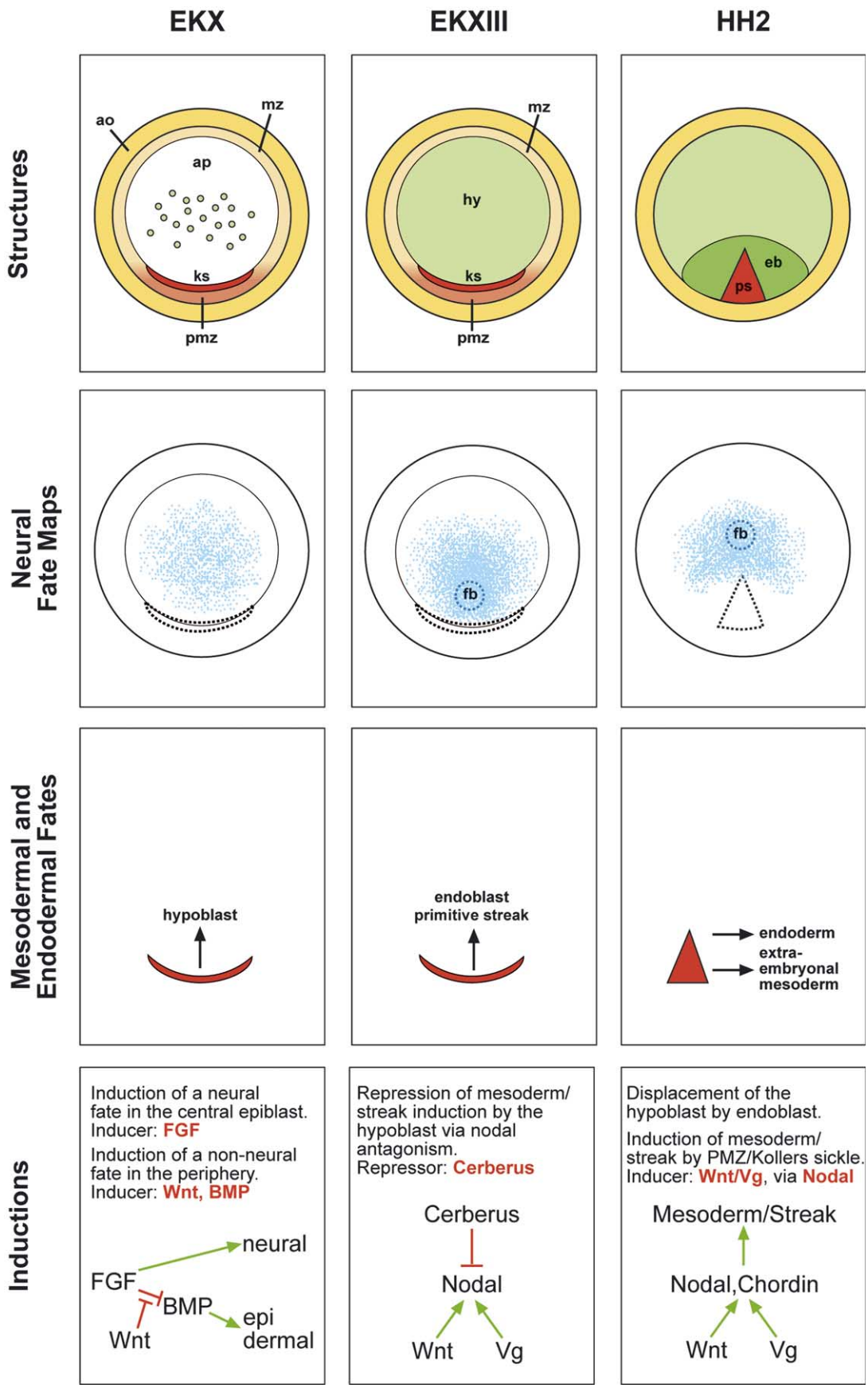
4. Inductive activities of transplanted tissues

One significant experimental advantage of the chick embryo is the possibility to create novel tissue associations by transplantation. Embryos can be made freely accessible in culture or in ovo, they are relatively large and robust. The typical induction experiments address the two major processes in the early embryo, neural and mesoderm induction. The key question is, if a graft can induce an epiblast to develop into anterior or posterior neuroepithelium, or if the induction leads to the generation of mesoderm in form of a more or less elongated primitive streak, which may or may not contain a node-like structure. Important is also the stage of the host and the area where the transplant is grafted, since the competence, i.e. the ability of the responding cells to react on inductive signals changes during development. For many experiments, the responding tissue needs to be further manipulated, e.g. by dissection or by the removal of tissues with dominating regulatory functions.

4.1. The posterior marginal zone and Koller's sickle

In the pre-streak embryo a site of major regulatory activities is the PMZ and its anterior margin, Koller's sickle. PMZ transplants can initiate the development of ectopic streaks. For a successful induction the stage of the donor has to be between EKX and EKXI, while donors of stage EKXII loose the competence to respond to a grafted PMZ (Khaner and Eyal-Giladi, 1986; Eyal-Giladi and Khaner, 1989; Bachvarova et al., 1998). PMZ grafts do not contribute cells to the induced axes, as demonstrated by the use of quail PMZ grafts to chick anterior regions (Bachvarova et al., 1998). The transplantation of Koller's sickle cells resulted similarly in the induction of secondary streaks, however, such grafts tended to contribute cells to the induced

Fig. 1. Stages of neural induction and neural patterning in the chick embryo. Staging is indicated according to Eyal-Giladi and Kochav (1976); abbreviated EK and roman numbers) and Hamburger and Hamilton (1951); abbreviated HH and arabian numbers). *Top row*: Schematic illustration of endodermal and mesodermal structures involved in neural induction and development at the respective stages. *EKX*: embryo in a freshly laid egg, cells that detach from the epiblast form islands of hypoblast cells (green circles) in the subgerminal cavity. The embryo is divided into the outer area opaca (ao) and the inner area pellucida (ap). The marginal zone (mz) constitutes an intermediate region between area opaca and area pellucida. At the posterior marginal zone (pmz), a morphological thickening is visible, Koller's sickle (ks). *EKXII*: the hypoblast (hy) has formed a sheet of cells underlying the epiblast. From the caudal part of the embryo the endoblast (dark green; eb) forms and displaces the hypoblast. *HH2*: initial streak stage: the triangular primitive streak (ps) forms. *HH3*: intermediate streak stage: the primitive streak elongates and the definitive endoderm (en) displaces hypoblast and endoblast. *HH4*: definitive streak stage: the primitive streak reaches its maximum extension, at the anterior tip a morphologically distinguishable structure is formed, Hensen's node (hn). *HH5*: head process stage: Hensen's node gives rise to the prechordal mesendoderm (pme) and the headprocess (hp). *Second row*: Schematic illustration of the ectodermal epiblast, neural fate is shown in blue. The fate maps are derived from Hatada and Stern, 1994 (EKX-HH3), Fernandez-Garre et al., 2002 (HH4) and Spratt, 1952 (HH5). At early stages (EKX-HH2) neural and epidermal fates are not completely separable from each other (compare Hatada and Stern, 1994), therefore the prospective neural area is not delineated. The dashed circle represents the prospective forebrain field, which moves away from the forming primitive streak in response to the hypoblast. From stage HH4 onwards, the anterior-posterior patterning of the neural plate becomes established: fb prospective forebrain, hb prospective hindbrain; mb prospective midbrain; sc prospective spinal chord. *Third row*: The developmental fates of the endoderm and mesoderm ingressing from Koller's sickle and along the anterior-posterior axis of the primitive streak are indicated. *Bottom row*: Description of the inductive events at the respective developmental stages.


EKX
EKXIII
HH2
Structures
ao
mz
ap
ks
pmz
hy
eb
ps
Neural Fate Maps
fb
Mesodermal and Endodermal Fates
hypoblast
endoblast
primitive streak
endoderm
extra-embryonal mesoderm
Inductions
Induction of a neural fate in the central epiblast.
Inducer: FGF
Induction of a non-neural fate in the periphery.
Inducer: Wnt, BMP
neural
FGF
BMP
epi dermal
Wnt
Repression of mesoderm/ streak induction by the hypoblast via nodal antagonism.
Repressor: Cerberus
Cerberus
Nodal
Wnt
Vg
Displacement of the hypoblast by endoblast.
Induction of mesoderm/ streak by PMZ/Kollers sickle.
Inducer: Wnt/Vg, via Nodal
Mesoderm/Streak
Nodal,Chordin
Wnt
Vg

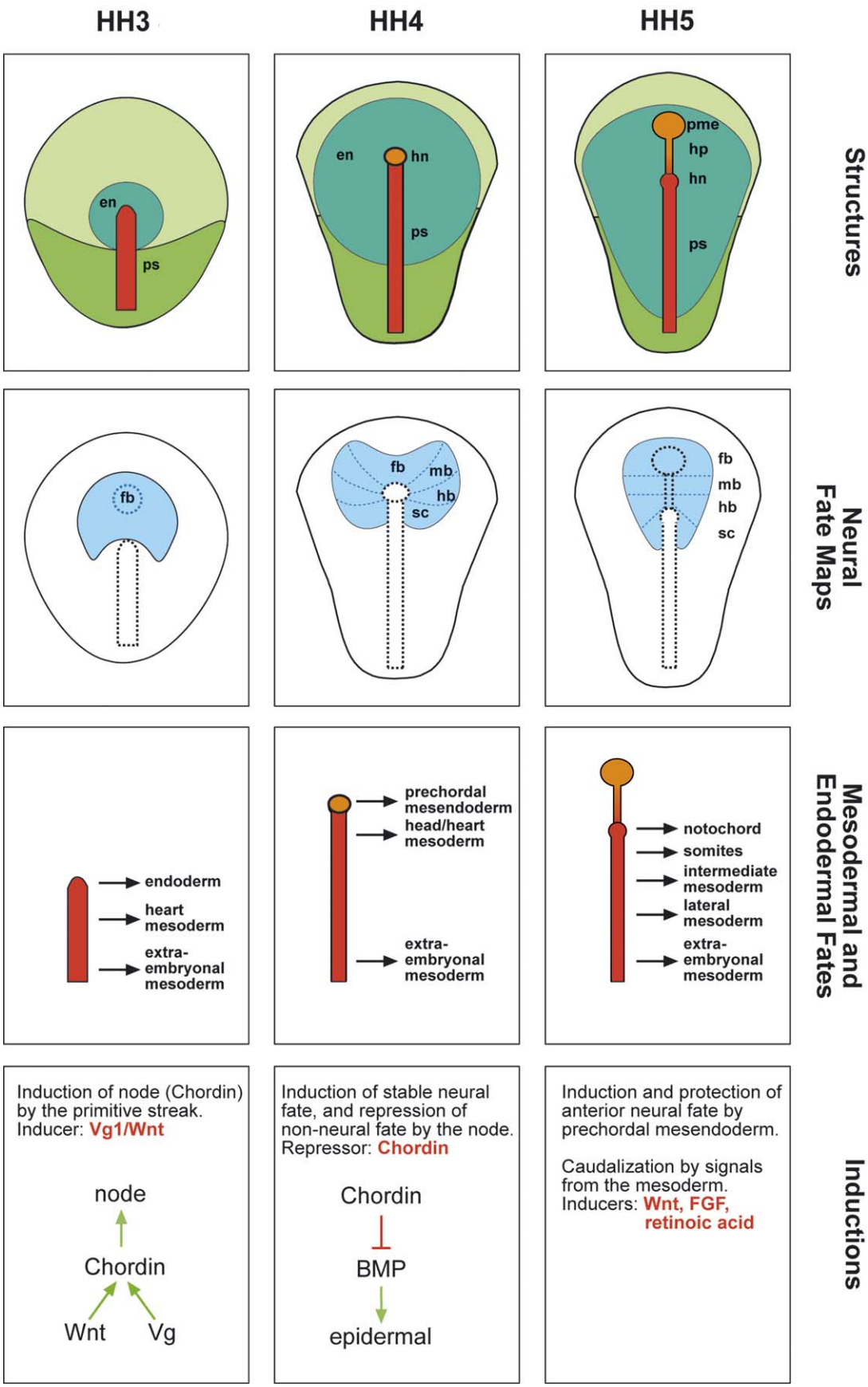


Fig. 1 (*continued*)

structures (Bachvarova et al., 1998; Callebaut and Van Nueten, 1994; Callebaut et al., 1998; Izpisúa-Belmonte et al., 1993).

4.2. Hypoblast

Studying the function of the lower layer for the induction and patterning events in the early embryo has a long, often controversial history. It was thought to determine the polarity of the embryo, but these findings were later refuted (Azar and Eyal-Giladi, 1981; Khaner, 1995). It was suspected to induce forebrain type neuroectoderm, while molecular markers (GANF, Otx2) indicated no stable neuralization in response to hypoblast grafts (Eyal-Giladi and Wolk, 1970; Knoetgen et al., 1999). Only more recently experiments differentiated cleanly between the anterior definitive endoderm, hypoblast and endoblast. Chapman and colleagues demonstrated that Sox2 expression was maintained in rostral epiblast isolates of early intermediate streak stages, from which the lower layer was removed, while GANF expression was not activated (Chapman et al., 2003). If, however, they recombined hypoblast with caudal epiblast isolates, GANF expression became induced. They argued for an instructive role of the hypoblast in anterior neural patterning, independent of a preceding neural induction. Other laboratories concluded that the hypoblast has no instructive function in stable neural induction and patterning (Foley et al., 2000; Knoetgen et al., 1999). However, a transient induction of neural markers (Otx2, Sox3) could be demonstrated, while effects on GANF were not reported (Foley et al., 2000).

As already observed by Waddington in the 1930s, rotations of the hypoblast could alter the orientation of the streak (Waddington, 1930, 1932, 1933). It remained unclear if this redirection of primitive streak development was due to inductive events or to changes in cell movements. Recent re-investigations with the use of fluorescent dyes and time-lapse movies demonstrated that the hypoblast influences cell movements in the epiblast, without changing cellular fates. By this activity the hypoblast would direct the future forebrain territory away from the emerging streak towards the anterior area of the embryo (Foley et al., 2000). Extirpation of the hypoblast can lead to the formation of supernumerary primitive streaks. These findings suggested that normally the hypoblast suppresses streak formation, and thus mesoderm induction, at the anterior margin of Koller's sickle, until it is displaced by the endoblast. (Bertocchini and Stern, 2002).

4.3. Primitive streak

The emerging primitive streak possesses different inductive capacities, leading finally to the generation of the central entity of neural induction, Hensen's node. The type of induction a grafted primitive streak fragment will evoke depends on the stage of the donor and of the host, as well as of the exact origin of the graft along the antero-posterior level of the donor streak (Dias and Schoenwolf, 1990; Storey et al., 1992). Fragments of early streaks induce the formation of an ectopic primitive streak, showing all characteristics of gastrulation (Lemaire et al., 1997). In more developed streaks, the middle region retains the capacity to induce gastrulation, as identified by Brachyury, but gains also the activity to induce node markers like chordin (Lemaire et al., 1997; Joubin and Stern, 2001). The developmental potential of the streak changes from the induction of gastrulation to the establishment of the node and therefore to neural induction.

4.4. Hensen's node

Hensen's node is morphologically characterized by a thickening at the tip of the fully extended primitive streak. Its activity was first demonstrated by Waddington, when he transplanted the node below lateral ectoderm of a cultured avian embryo and observed the induction of an ectopic, embryo-like structure including a partial neural tube, a notochord and somites (Waddington, 1932). The principal similarity of these experiments to the discoveries of Spemann and Mangold in amphibian embryos suggested a homology between the node and the amphibian dorsal blastopore lip. As the dorsal blastopore lip, also the node can recruit neighboring cells and organize them into an embryonic axis. This function of Hensen's node as an organizer in the sense of the amphibian organizer is the topic of numerous original publications and reviews (e.g. Boettger et al., 2001; Wittler et al., 2003; Smith and Schoenwolf, 1998; Stern, 2001). For a typical organizer transplantation experiment in the chick, the node of a definitive streak stage embryo would be transplanted to the anterior/lateral region at the area opaca/pellucida boundary, or within the inner area of the area opaca of a definitive streak or almost definitive streak stage embryo mounted in a culture system. In this anterior area and at this embryonic stage, the epiblast has still the competence to react on the neural inducing signals from the node (Dias and Schoenwolf, 1990; Storey et al., 1992; Lemaire et al., 1997; Knoetgen et al., 2000). Several hours of contact are necessary between node grafts and overlying epiblast. In response, the epithelium will thicken and form the typical, pseudostratified neuroepithelium. The induced structure will readily elongate and possesses an anterior–posterior patterning identifiable by molecular markers (Storey et al., 1992). The capacity to induce anterior neural markers is independent from influences from any lower layer. Self-differentiation of nodal tissue induces considerable dorsal ventral patterning, obvious by the rising of neural folds and the partial fusion to form a tube structure.

At the beginning of primitive streak formation, the cells that will contribute to the node are separated in two populations. One group of node precursors is associated with Koller's sickle and expresses the homeobox gene

Goosecoid (Izpisua-Belmonte et al., 1993). A second is located in the center of the epiblast, rostral to the tip of the early streak. With a further elongation of the primitive streak, the posterior, Goosecoid positive cells move at the tip of the streak to the center of the epiblast, where they unite with the central cell population and form the node (Izpisua-Belmonte et al., 1993; Streit et al., 2000). The ability of the node to induce anterior neural markers is restricted to stages, when it contains cells fated to the definitive endoderm or mesendoderm and correlates with the expression of Goosecoid (Dias and Schoenwolf, 1990; Izpisua-Belmonte et al., 1993). When the Goosecoid positive cells left the node to form the prechordal mesendoderm, the node can induce only posterior neuroectoderm up to the hindbrain level, or self-differentiate into elongated notochord-like structures (Dias and Schoenwolf, 1990; Storey et al., 1992).

Fate map and gene expression studies show that the cellular composition of the node changes constantly. Cells emigrate from the node and are replaced by cells from the surrounding epiblast, which then acquire node properties (Selleck and Stern, 1991; Joubin and Stern, 1999). This dynamic nature becomes also apparent, when the node is experimentally ablated: Within twelve hours after ablation, organizer markers reappear and reconstitute the node (Joubin and Stern, 1999; Psychoyos and Stern, 1996b; Yuan and Schoenwolf, 1998). This restoration depends on inductive signals from the central primitive streak. It also involves protective mechanisms, preventing the spread of organizer properties throughout the epiblast (Joubin and Stern, 1999). The autoregulatory control of organizer formation is achieved by the activity of a TGF beta factor, the anti-dorsalizing morphogenetic potein ADMP, which is expressed in the node. Beside the continuous change of the cellular composition, also constant populations of cells were described (Selleck and Stern, 1991; Selleck and Stern, 1992) that contribute to the notochord and the somites and behave as stem cells. However, it is unclear if such resident cells are reinduced during restoration of the node or if they are absolutely required for the full function of the node at all.

4.5. Endoderm

Extirpation of the endoderm from primitive streak stage embryos impedes the formation of a neural plate, indicating a function of the endoderm in neural development (Pera et al., 1999). Anterior definitive endoderm functions also in the maintenance of anterior neural specification as shown by the loss of GANF expression in endoderm deficient rostral epiblast isolates (Chapman et al., 2003). In this line is the result that removal of anterior endoderm leads to morphological defects in the development of the forebrain and the reduction of forebrain marker expression (Withington et al., 2001). In addition, an influence of the anterior endoderm on the specification of the prechordal mesoderm by TGF beta signals was reported (Vesque et al., 2000).

4.6. Prechordal mesendoderm/Head process

The prechordal mesendoderm is constituted from the Goosecoid expressing, cells that leave the node directly after the definitive endoderm has formed. Prechordal cells are a source of anteriorizing signals. Grafting of such cells to ectopic sites of the neuroepithelium activated the forebrain markers NKX2.1, Otx2 and tailless (Foley et al., 1997; Pera and Kessel, 1997). Mesendodermal tissue of the prechordal region is also able to rescue the ability to induce anterior neural tissue of head process stage nodes. If the prechordal plate itself is a source for neuralizing signals, is controversial. Transplantation of prechordal tissue to the anterior area pellucida/area opaca boundary led to the induction of neural tissue and the ectopic activation of the forebrain markers GANF, CNOT1 and NKX2.1 (Knoetgen et al., 1999; Pera et al., 1999; Pera and Kessel, 1997). In a different experimental approach, when mesendodermal cells of the prechordal region were placed into the area opaca of a more posterior level, induction of neural tissue could not be observed (Foley et al., 1997). However, in the embryo the signals of the prechordal mesendoderm reach epiblast cells, which are already neurally specified. There, they establish and stabilize the anterior neural character of the forebrain territory.

Directly after the prechordal mesendoderm, axial mesoderm leaving the node will condense to the anterior notochord, the head process. Consistent with the anterior–posterior regionalization of the neural plate, transplanted head process mesoderm induces neural tissue that is characterized by the expression of neural markers up to the midbrain level (Pera and Kessel, 1997). In headfold to one-somite stage embryos, the caudal part of the head-process refines the patterning of the neural tissue by inhibiting rostral gene expression and therefore caudalizing the developing neural tube (Rowan et al., 1999).

5. Application of purified signalling molecules

The chick embryo offers an ideal model to analyze localized effects of signalling molecules in the embryonic context. Cells secreting overexpressed proteins or beads loaded with a purified factor can be grafted into the embryo and act as an ectopic signalling source. Alternatively, tissue explants can be cultivated with one or a combination of factors and analyzed for changes in their developmental fate. The following paragraphs will present the central signalling molecules involved in neural development, and will outline experimental approaches in chick embryos to understand their function in the acquisition of neural fate (Fig. 1).

5.1. Fibroblast growth factors (FGFs)

Fibroblast growth factors are involved in numerous induction processes, both in the early embryo and later in

organogenesis. They play important roles in mesoderm induction, migration, and caudalization. Here, we will review briefly the function of FGFs in the acquisition of neural fate, and their role as one signal in the caudalization of fates along the antero-posterior body axis. FGFs have been extensively, and also controversially, disputed as instructive factors in neural induction of the frog *Xenopus laevis* (for review see Wilson and Edlund, 2001).

Several observations indicate very early functions of FGFs occurring before the initiation of gastrulation. Using an explantation approach it could be shown, that neural specification is initiated already between embryonic stages EKVIII and EKIX (Wilson et al., 2000). Central pieces from such early embryos develop in culture into neural tissue of anterior character, as defined by Otx2 expression. This neural specification depends on the presence of FGF, possibly FGF3, which is required but not sufficient (Wilson et al., 2000). Antagonists of this reaction are BMP and Wnt signals, but BMP4 is normally downregulated by FGFs in central pieces. If, however, FGF is experimentally attenuated, BMP4 is no longer repressed and epidermal tissue develops. This result is reversed in the presence of the BMP antagonists Noggin or Chordin. In lateral epiblast pieces, Wnt signalling inhibits the repressive activity of FGF on BMP transcription and thus BMP can instruct the acquisition of an epidermal fate. FGF signalling in the chick blastoderm has a dual role. It activates a pathway necessary for the progression to neural fate, and represses BMP transcription in an independ pathway. It is not clear from where the early FGF signals initiating neural specification might come, or if a cascade of different FGF molecules triggers neural specification. One possible origin of FGF signalling, e.g. FGF3, would be the epiblast of EKIX embryos, while the few hypoblast cells in this stage appear a rather unlikely source (Wilson et al., 2000). Streit and colleagues identified a second, possibly additional FGF source (FGF8) in the middle cells of Koller's sickle (Streit et al., 2000). These cells represent a population of node precursors, which migrates forward to unite with a second population in the epiblast (Izpisua-Belmonte et al., 1993). It remains unclear, if subsequently FGF8 from the spreading hypoblast can further reinforce earlier FGF signals, or if other FGFs are part of a functional cascade.

In the chick embryo several FGF genes are expressed in the primitive streak and/or the node, thus their products are present at the right time and space for neural induction (Karabagli et al., 2002). Their requirement for neural induction by the node could be shown after the positioning of specific FGF inhibitors next to a node graft (Streit et al., 2000). Localized, ectopic applications of FGF sources were performed with FGF4 or FGF8, both proteins likely to emulate the activity of other FGFs. Implantation of an FGF soaked bead below naive epiblast of primitive streak stage chick embryo elicits a neural response, recognizable in the thickening of the epithelium and the activation of posterior, but not anterior neural markers (Alvarez et al., 1998; Storey et al., 1998). The concomitant activation of Brachyury raised a concern that this neural induction occurs via a primary induction of mesoderm. It could recently be demonstrated that the zinc finger protein Churchill is involved in the decision, between neural or mesodermal induction by FGF (Sheng et al., 2003). Churchill represses Brachyury, and thus mesoderm formation, and by that favors neural development in response to FGF. The relatively late expression of Churchill indicates that this function is exerted at a time, when neural induction and specification is well under way, and the primitive streak and the node are fully formed. Since the ectopic induction of neural tissue with FGF implants activates only posterior markers, a role in the caudalization of neural tissue was considered likely. In this process one function of FGFs is exerted directly on neural cells, another activity is to induce the caudalizing activity of the paraxial mesoderm (Muhr et al., 1999).

5.2. Wnt

Wnt signalling in the early, pre-gastrulation chick embryo is confined to the periphery (Roeser et al., 1999). Most probably, Wnt8c is the responsible molecule, but also Wnt3a is expressed in the lateral epiblast (Hume and Dodd, 1993; Wilson et al., 2001). Wnt-, together with BMP signalling, is essential for the acquisition of an epidermal fate (Wilson et al., 2001). The highly expressed Wnt signals in the lateral epiblast inhibit both functions of FGF signalling, the neural inducing activity and the inhibitory effect on BMP transcription. This leads to a block of neural specification and the induction of epidermal fate in the peripheral epiblast (Wilson et al., 2001). When the hypoblast spreads anterior, it expresses several anti-Wnt molecules, in particular Cerberus, Crescent and Dkk1 (Chapman et al., 2002). Directed by the hypoblast also the cells of the prospective forebrain field move towards the anterior of the embryo, always ahead of the tip of the streak. It is likely, but not formally shown, that the anterior identity of this developmental field requires safe mechanisms prohibiting the reception of Wnt signals.

Initially Wnt8c activity defines the periphery of the early embryo. Up to the initiation of the primitive streak it becomes confined to the PMZ and from there it passes over to the developing primitive streak. When the streak approaches its final length, Wnt8c transcription retreats from the tip of the streak, allowing the formation of a new functional structure, Hensen's node. During induction and formation of the primitve streak, Wnt functions in cooperation with the signalling molecule cVg1, which will be discussed in the next paragraph (Skromme and Stern, 2001).

Once neural cells of an anterior identity are induced and specified, a caudalization of neural cells needs to take place. In this process, Wnt signalling plays a more instructive role, compared to its rather permissive role in early embryogenesis

(Nordstrom et al., 2002). It was identified as a key signal originating from the paraxial mesoderm in stages following the full extension of the primitive streak. Using explant assays, evidence for a graded effect was obtained, where higher Wnt concentrations led to more caudal neural cell fates. These Wnt effects required the permissive presence of FGF.

5.3. cVg1

The inductive capacity of the PMZ can be mimicked by the combination of two signalling molecules, cVg1 and Wnt signalling. cVg1 is expressed in the PMZ of young chick embryos at the time of egg laying (Seleiro et al., 1996; Shah et al., 1997). When cVg1 transfected tissue culture cells were grafted to peripheral positions around such early blastoderms, they initiated the formation of morphologically complete primitive streaks including a node, thus mimicking the axis-inducing ability of the PMZ. Such inductions were observed only in the marginal zone, but not after implantation to the area pellucida, indicating a requirement for additional factors. The competence of the marginal zone to initiate ectopic primitive streak formation in response to cVg1 turned out to be dependent on Wnt activity (Skromne and Stern, 2001). Indeed, co-transplantation of Wnt1 and cVg1 sources induced primitive streaks also in the area pellucida. Conversely, the Wnt antagonists Crescent and Dkk-1 blocked the primitive streak-inducing ability of cVg1 in the marginal zone. These findings suggest that a permissive role of Wnt activity in the periphery of the early embryo allows cVg1 to induce an axis. The cooperation of Wnt and cVg1 signalling is maintained in the developing primitive streak and becomes the essential component of a node-inducing center in the middle of the streak. The inductive capacity of cVg1 changes, if older, streak stage embryos are used as hosts. In such embryos, cVg1 alone is sufficient to induce a complete streak in the area pellucida (Joubin and Stern, 1999). This could be due to the presence of Wnt signals e.g. originating from the streak, but also here, the implantation of an additional Wnt source seems to reinforce the inductive capacity of cVg1.

5.4. BMP4 and BMP7

BMP expression domains in intrauterine stages have so far not been reported. In the pre-streak epiblast BMP4 is expressed at low levels in the entire embryonic and extraembryonic area, whereas BMP7 is present at high levels in the area opaca (Streit et al., 1998). With the formation of the primitive streak, both transcripts disappear from the area pellucida and demarcate just the embryonic periphery, first in the area opaca, but then increasingly in the prospective epidermis flanking the neural plate. In the posterior embryo the BMP expression domains include also the posterior streak. The boundary between prospective epidermis and neural plate is characterized by a sharp drop of BMP expression, making models involving BMP gradients highly unlikely in primitive streak stage chick embryos (Marchant et al., 1998).

FGF suppresses BMP signalling in central epiblasts of early embryos, thus allowing a neural specification (Wilson et al., 2000). The experimental exposure of central explants to BMP4 protein overcomes this regulation and strongly promotes an epidermal specification (Wilson et al., 2000). Localized application of BMP4 soaked beads below the boundary of the neural plate of cultured streak-stage embryos restricts the size and shape of the neural plate and extends prospective epidermis (Knoetgen et al., 1999; Pera et al., 1999). However, neither BMP4 nor BMP7 interfere with neural induction within the neural plate itself (Pera et al., 1999; Streit et al., 1998). It is likely, that neural development closer to the node is already irreversibly committed, whereas towards the epidermal boundary cells are only neurally specified, and can still respond to the epidermalizing signal. The most pronounced effect of ectopic BMP4 occurs on the development of the primitive streak. Applied to pre-streak embryos near the posterior site of streak initiation, or near the tip of an elongated streak, BMP4 beads inhibit further development completely.

5.5. Chordin and Noggin

In avian embryos BMP antagonism is not sufficient to elicit neural induction (Connolly et al., 1997; Streit et al., 1998). Implantation of Noggin or Chordin impregnated beads can enlarge the neural plate, when implanted near its boundary, thus giving the opposite result of BMP implantations (Knoetgen et al., 1999; Streit and Stern, 1999). Chordin can stabilize expression of neural markers in cells that already received neural inducing signals (Streit et al., 1998). These results were instrumental to demonstrate that neural induction in birds might require new models, different from the default model based on amphibian embryos. The major effect of a transplanted Chordin source is to elicit an ectopic primitive streak, complete with a molecularly identified node (Streit et al., 1998). This potency fits nicely to the earliest expression domain of Chordin in the epiblast just anterior to Koller's sickle, i.e. where the streak is induced by the PMZ, adjacent to BMP-expressing cells. Later on, Chordin, as well as Noggin expression, demarcate the node. It is unclear, what kind of function can be fulfilled by a BMP-antoginst trancribed more than 250 μm away from the sites of BMP signalling.

5.6. Nodal

Nodal is expressed at the most posterior edge of the early area pellucida epiblast, adjacent to the PMZ (Lawson et al., 2001). While this pattern suggested some similarities to Chordin, initially no streak inducing activities could be demonstrated after the implantation of ectopic sources for

Nodal. Only, when pre-streak embryos with removed hypoblasts were used as hosts, Nodal could induce streaks, complete with functional nodes (Bertocchini and Stern, 2002). These experiments point to Nodal antagonism from the hypoblast, where Cerberus, a molecule with anti-Nodal, anti-Wnt and anti-BMP activity is expressed. Implantation of nodal together with Cerberus could indeed indicate the in vivo relevance of nodal antagonism for mesoderm repression (Bertocchini and Stern, 2002).

6. Steps towards the acquisition neural fate

In the previous paragraphs we have summarized the experimental basis of early neural development in the chick based on transplantation assays and exposures to signalling molecules. In the following, we will try to integrate these findings into a coherent scenario, ordered in six steps (Fig. 1). To avoid repetitions we do not give all the references again, which can be found in the preceding text.

1. The early separation of the avian blastoderm into medial and lateral cells is tightly connected to the early steps in neural specification. It involves the establishment of a peripheral zone with Wnt and BMP signalling, and a central zone under the control of FGF signalling. FGF signalling instructs neural specification explicitly before gastrulation (Fig. 1, bottom row). Some evidence indicates that this early specification already implies an anterior regionalization, i.e. that chick cells are initially specified as rostral forebrain. Other data rather suggest initial neural specification and a later regionalization as separate events.

2. In the chick the radially symmetric separation of the intrauterine blastoderm is overlaid by a specialization of the future posterior pole of the embryo. It is manifest by the time of egg laying, when it is visible in form of Koller's sickle as a morphological landmark. Signals like Wnt8c, cVg1, and FGF8 are now shifted to the posterior embryonic margin, the PMZ or Koller's sickle. Wnt antagonism by Cerberus expression in the hypoblast blocks transiently the next step necessary for the neuralization process, namely the induction of the primitive streak (Fig. 1, bottom row).

3. The repressive function of the hypoblast at the posterior pole is abrogated, when it becomes displaced by the endoblast. Consequently, the combination of cVg1 and Wnt can induce the expression of Nodal and Chordin in the epiblast anterior of Koller's sickle. Nodal appears to be the direct trigger to induce the onset of gastrulation, i.e. the beginning of endoderm and mesoderm formation by the primitive streak (Fig. 1, bottom row). Simultaneously, the anteriorly shifting hypoblast ahead of the growing streak may exert further influences on the overlying epiblast, such as directing ectodermal cell movements away from the caudalizing influence of the primitive streak. A second influence could be a role in the stabilization and anteriorization of the neural plate.

4. While gastrulation proceeds through the elongating primitive streak, its tip, now Hensen's node, acquires the capacity to induce neural tissue. Again, this is under the control of the Wnt/cVg1 combination. Now, a thickened neural plate becomes established around the node, which is best characterized by the molecular marker Sox2. Unexpectedly in the light of data from the frog *Xenopus laevis*, this signalling could not be traced back to BMP antagonism originating from the node.

5. At the mid- to late-streak phase of development, also the molecular marker GANF indicates an anterior neural specification. Signals from the lower layer, hypoblast and anterior definitive endoderm were found necessary for its induction and maintenance, respectively. By now, however, not only specification, but also a commitment to neural development has occurred in the neural plate close to the node, as indicated by a lack of sensibility against the epidermalizing activity of BMPs.

6. After the maximal extension of the primitive streak, prospective prechordal mesendoderm cells leave the node, and with them the capacity for anterior neural induction. They migrate below the prospective forebrain, and thus under an epithelium, which is already specified for anterior neural development. They function in the patterning of the forebrain anlage, and possibly in its protection against caudalization. In subsequent development, the neuroectoderm comes under a caudalizing influence, at least partially elicited by the paraxial mesoderm. Key signals in this process are FGF- and Wnt-factors, as well as retinoic acid (Fig. 1, bottom row).

In summary, work on the chick embryo contributed much to the field of early neural development by integrating classical embryology with molecular biology. The specific properties of the chick embryo helped to transfer findings from *Xenopus* and Zebrafish to the amniote embryo and pointed out new and unexpected principles of neural induction. It was demonstrated that neural induction at least in amniota is not simply build up by BMP antagonism, but involves additional instructive signals like FGF. Additionally, the chick embryo provided insights into the requirements and dynamics of organizer formation and maintenance.

The combination of the experimental advantages of every vertebrate model organism led in the past decade to a coherent picture of the events in neural induction. Now that the major players and components involved in neuralization are known, novel challenges consist in the systemic understanding of the process. This includes the analysis of the cell intrinsic mechanisms, e.g. how the decision between neural and non-neural fate is connected to cell proliferation, cell cycle and cell differentiation. Approaches like the genomic analysis and the use of knock down techniques by morpholino antisense oligos or siRNAs will help to elucidate these issues and preserve the particular status of the chick embryo among the vertebrate model systems.

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