Wnt signaling is required at distinct stages of development for the induction of the posterior forebrain

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Summary

One of the earliest manifestations of anteroposterior patterning in the developing brain is the restricted expression of Six3 and Irx3 in the anterior and posterior forebrain, respectively. Consistent with the role of Wnts as posteriorizing agents in neural tissue, we found that Wnt signaling was sufficient to induce Irx3 and repress Six3 expression in forebrain explants. The position of the zona limitans intrathalamica (zli), a boundary-cell population that develops between the ventral (vT) and dorsal thalamus (dT), is predicted by the apposition of Six3 and Irx3 expression domains. The expression patterns of several inductive molecules are limited by the zli, including Wnt3, which is expressed posterior to the zli in the dT. Wnt3 and Wnt3a were sufficient to induce the dT marker Gbx2 exclusively in explants isolated posterior to the presumptive zli. Blocking the Wnt response allowed the induction of the vT-specific marker Dlx2 in prospective dT tissue. Misexpression of Six3 in the dT induced Dlx2 expression and inhibited the expression of both Gbx2 and Wnt3. These results demonstrate a dual role for Wnt signaling in forebrain development. First, Wnts directed the initial expression of Irx3 and repression of Six3 in the forebrain, delineating posterior and anterior forebrain domains. Later, continued Wnt signaling resulted in the induction of dT-specific markers, but only in tissues that expressed Irx3.

Key words: Chick, Forebrain, Embryonic induction, Zona limitans, intrathalamica, Wnt, Dickkopf 1

Introduction

The vertebrate forebrain develops from the anterior-most part of the neural tube, the prosencephalon. As development proceeds, a series of morphological constrictions give way to nested gene-expression patterns in subregions of the forebrain that have distinct developmental fates. Based on these observations, the forebrain is thought to develop from a series of six subregions, called prosomeres, numbered p1-p6 in a caudal to rostral fashion (Rubenstein et al., 1994). However, despite some similarities with the rhombomeres of the hindbrain, several studies show that prosomeres are not true compartments. Most importantly, individual prosomeres do not have the restricted cell lineages and border cell populations that are characteristic of rhombomere segmentation (Golden and Cepko, 1996; Larsen et al., 2002).

An exception to these findings occurs in the developing forebrain, at the zona limitans intrathalamica (zli), the interface between the future, anteriorly located ventral thalamus (vT) and the posteriorly located dorsal thalamus (dT). Before its overt formation, the location of the prospective zli is demarcated by the adjacent but nonoverlapping expression patterns of Six3 and Irx3, which encode Iroquois-type transcription factors. Six3 is expressed in neural tissue overlying the prechordal plate and Irx3 is expressed above the anterior-most portion of the notochord. In HH stage 8 chick embryos, the expression of either Six3 or Irx3 can confer anterior or posterior identity, respectively, on the developing forebrain by determining the competency of this neural tissue to differentially respond to Fgf and Shh signals (Kobayashi et al., 2002). A question that arises from these studies is how the expression domains of Six3 and Irx3 are established.

Several lines of investigation demonstrate that Wnt signaling in early forebrain tissue induces differentiation of the posterior forebrain (van de Water et al., 2001), whereas the absence of Wnt signaling allows differentiation of the anterior forebrain (Mukhopadhyay et al., 2001; Houart, 2002). These studies indicate a role for Wnts in the early anteroposterior patterning of the brain (Nordstrom et al., 2002). Our data extend these observations by demonstrating that Wnt signaling is sufficient to induce Irx3 expression and suppress Six3 expression in explanted forebrain tissue. The source of this Wnt activity remains unclear. However, somewhat later in development, Wnts are expressed in and posterior to the zli.

The zli is the first forebrain subdivision to establish, forming above the transition between the notochord and prechordal plate (Fiegler and Stern, 1993). The site of zli formation is characterized by the absence of lunatic fringe expression (Zeltser et al., 2001). The zli, a narrow strip of tissue that both expresses boundary cell markers and restricts the mixing of cell lineages (Larsen et al., 2001), defines the border between the future dT and vT. Although the role of the zli is unknown, it serves as either the site or limit of expression of several molecules with inductive capacities. Wnt3a expression deviates from its pattern along the dorsal neural tube to form a finger-
like projection that extends ventrally at the zli. Wnt3, a Wnt family member with 91% identity to Wnt3a, is expressed in the prospective dT, with its anterior limit of expression abutting the zli (Roelink and Nusse, 1991; Salinas and Nusse, 1992). In addition to Wnts, the expression patterns of several transcription factors and cell adhesion molecules have sharp borders at the zli. Gbx2 is expressed posterior to the zli in the dT (Bulfone et al., 1993) and the zli marks the posterior limit of expression for the vT markers Dlx2, R-cadherin and cadherin8 (Larsen et al., 2001; Price et al., 1991; Redies and Takeichi, 1996). Based on the timing of Wnt3 and Wnt3a expression, zli-restricted Wnts cannot account for the initial restriction of Six3 and Irx3 expression.

Here, we demonstrate that activation of the canonical Wnt signaling pathway is sufficient and required to induce dT-specific gene expression, and that the absence of Wnt signaling allows vT-specific differentiation. Blocking the Wnt response resulted in vT-specific gene expression in dT explants, and exposure of vT explants to Wnt3 resulted in the induction of both early (Irx3) and late (Gbx2) dT-specific gene expression. Furthermore, misexpression of either Six3 or the Wnt inhibitor Dkk1 in the presumptive dT initiated differentiation appropriate for the vT. These results indicate that, by determining the domains of Irx3 and Six3 expression, Wnt signaling is crucially important for the initial anteroposterior organization of the forebrain. Our observation that Wnts induced Irx3, which, in turn, allowed the dT-specific response, indicates that Wnt signaling is required at multiple stages of development of the posterior forebrain.

Materials and methods

Headfold, zli, and prospective dT and vT explant dissection

Headfold explants containing the anterior neural expression domains of Six3 and Irx3 were dissected from chicken embryos at HH stage 7-8 (Hamburger and Hamilton, 1992). Extraneous anterior endoderm was trimmed from the headfold tissue prior to culture. For co-culture and prospective dT/vT explant experiments, neural explants were taken from HH stage 8 and stage 17 chicken embryos using a modified protocol from (Yamada et al., 1993). Following dispase (Sigma) proteolysis, neural tissue was isolated and cultured as described below. The anterior limit of the notochord was used as a reference point for dissecting prospective dT and prospective vT explants. Neural explants dissected rostral to the limit of the notochord are termed prospective dT explants (Fig. 3A). Explants of the zli were obtained from HH stage-17 embryos, with the excised tissue corresponding to the Wnt3a and Shh-expressing region that lies within and just posterior to the zli (Fig. 5A).

Culture conditions and tissue fixation

Explants were embedded in collagen (Collagen Biomaterials) (Yamada et al., 1993) and cultured for 36-48 hours in neurobasal media (Gibco BRL) supplemented with 1% each of penicillin-streptomycin (Gibco BRL), nonessential amino acids (Gibco BRL), glutamine (Gibco BRL), N3 (Yamada et al., 1993) and dextrose (EM Science).

In headfold experiments, the explants were oriented in a rosette with the anterior end of the explant facing out. This orientation allowed both the anterior and posterior portion of the explants to be scored for the expression of markers following culture.

For co-culture experiments, zli explants and explants of prospective dT and vT were incubated either alone or together in collagen. To distinguish zli tissue from prospective dT or vT explant tissue, CellTracker Blue CMAC dye (Molecular Probes) was used to fluorescently label the prospective dT/vT explants according to the manufacturer's instructions. During fixation, the co-cultured explants were visualized and photographed by both fluorescent and light microscopy. These images were merged in Photoshop (Adobe) to create a map of prospective dT/vT versus zli explant tissue for each co-culture condition, thus allowing us to discriminate between zli and prospective diencephalic explant tissue (data not shown).

Wnt was supplied to headfold explants via a soluble Wnt3a-containing supernatant, generated by growing mouse fibroblast L cells stably transfected with a Wnt3a-expression construct (Shibamoto et al., 1998) in OptiMem (Gibco BRL) for 4 days. Control supernatant was obtained from mock-transfected L cells. The supernatants were added to explants at a 1:1 ratio with complete neurobasal media.

In prospective dT/vT explant experiments, Wnt was provided by one of two means, with similar results obtained using either Wnt source. First, prospective dT/vT explants were grown on a monolayer of either RatB1A cells or RatB1A cells expressing Wnt3 (Shimizu et al., 1997) in supplemented neurobasal media (see above). Following culture, the explants were placed in collagen, fixed, and processed by in situ hybridization. Alternatively, explants were exposed to the Wnt3a supernatants described above.

To inhibit Wnt signaling in vitro, casein kinase inhibitor 7 (cki7) (Seikasaku America) dissolved in DMSO was added at a final concentration of 50 μM at the start of culture. A similar dilution of DMSO was added to control wells. Alternatively, a Dkk1 supernatant was used to block Wnt signaling in headfold culture experiments. Dkk1 supernatant was generated by growing mouse 293T cells transfected with pRK5-Dkk1 in OptiMem (Gibco BRL) for 48 hours. Control supernatant was obtained from mock-transfected 293T cells transfected with pRK5 alone. The supernatants were concentrated 10-fold by filtration through 10KMWL exclusion membranes (Amicon Ultra-15), and added to explants at a 1:1 ratio with complete neurobasal media.

Explants and embryos to be processed by in situ hybridization were fixed overnight in 4% paraformaldehyde in either PBS or MEM, pH 7.4 at 4°C. After fixation, embryos to be sectioned were rinsed in DEPC PBS, followed by 30% sucrose in DEPC PBS solution, embedded in OCT (Sakura Finetechanical) and then cryosectioned.

In situ hybridization

Whole-mount and slide in situ hybridizations were performed following established procedures (Jasoni et al., 1999; Schaeren-Wiemers and Gerfin-Moser, 1993). In situ hybridizations were carried out using antisense Irx3, Six3, Gbx2, Dlx2 and Wnt3 digoxigenin-labeled (Roche) riboprobes. Antisense riboprobes were prepared from plasmids containing chicken cDNA sequences for Irx3, a gift from Dr. Jelles; Six3, a gift from Dr Shimamura; Gbx2 (Kowenz-Leutz et al., 1997), a gift from Dr Leutz; and Dlx2 (Puelles et al., 2000), a gift from Dr Rubenstein. Chicken Wnt3 cDNA was cloned (C.P.R., M.M.B. and H.R., unpublished) and used to generate an antisense Wnt3 digoxigenin-labeled riboprobe.

Headfold explants exposed to either Wnt3a, Dkk1 or cki7 were processed by in situ hybridization for Irx3 and Six3. The expression domains of these markers were scored as normal, expanded and reduced/absent compared to headfold explants cultured under control conditions.

Prospective dT and vT explants were assayed in one of two ways post in situ hybridization. In both co-culture and Wnt3a-mediated Gbx2 and Dlx2 induction experiments, explants were scored as either positive or negative, as compared to the staining of control tissue. Prospective dT and vT explants cultured in supplemented neurobasal media served as the negative control. Tissue dissected from older embryos was used for both negative and positive controls.
In ovo manipulations
Electroporations were carried out following established protocols (Watanabe and Nakamura, 2000). xDkk1 (Glinka et al., 1998), a gift from Dr Niehrs, was excised from pcS2+ and cloned into pMiwII (Watanabe and Nakamura, 2000), a gift from Dr Nakamura. eGFP (Clontech) was cloned into pcDNA3.1/Zeo (Invitrogen). A mixture of 3 µg µl−1 pMiwII-xDKK1 and 3 µg µl−1 pcDNA3.1-GFP in L-15 (Gibco BRL) supplemented with 10 mM HEPES was injected into the neuropore of HH stage 9-10 embryos. Full-length chicken xDkk1 (Kobayashi et al., 2002) was subcloned into pMES-IRES-GFP (Swartz et al., 2001). pMES-Six3-IRES-GFP resuspended in L-I5 (Gibco BRL) supplemented with 10 mM HEPES was injected into the neuropore of HH stage 9-10 embryos. The embryos were electroporated with two 25 msec pulses of 62.5 Volts cm−1. After 48 hours, embryos were fixed and processed by in situ hybridization as described above.

Results
Wnt signaling confers posterior identity and inhibits anterior identity in HH stage-7 forebrain explants
By HH stage 7, the neural plate that overlies the prechordal plate expressed Six3 and forebrain tissue overlying the anterior-most aspect of the notochord expressed Irx3 (Fig. 1A,C). The adjacent, mutually exclusive expression patterns of Six3 and Irx3 confer regional identity on the early forebrain (Kobayashi et al., 2002). The interface between anterior and posterior forebrain overlies the transition between the prechordal plate and the notochord, and predicts the recognizable structure in the diencephalon.

To address the role that β-catenin-mediated Wnt signaling plays in the establishment and maintenance of Six3 and Irx3 in the early forebrain, we isolated headfold explants from HH stage 7-8 and cultured them for 24-48 hours in either the presence or absence of Wnt3a-conditioned medium (Shibamoto et al., 1998). The tissue was then fixed and assayed for Six3 or Irx3 expression by in situ hybridization. Untreated explants expressed Six3 anteriorly and Irx3 posteriory in the explanted tissue (Fig. 2A,E). The addition of Wnt3a-conditioned supernatant resulted in a strong reduction or absence of Six3 expression in all the treated explants (Fig. 2B). By contrast, the posterior expression pattern of Irx3 following exposure to Wnt3a was not significantly different from control cultures (Fig. 2F) and an anterior expansion of Irx3 expression was detected in at least a third of the explants (Fig. 2F), indicating that the loss of Six3 can be followed by the induction of Irx3 expression.

To address whether Wnt signaling was required for Six3 or Irx3 induction and maintenance, two independent, complementary approaches were taken. First, we used the pharmacological agent cki7 to inhibit the Wnt response in culture. This blocks the transforming Wnt pathway by repressing the phosphorylation of Dsh, a crucial step in the Wnt signaling cascade (Peters et al., 1999; Sakanaka et al., 1999). The addition of 50 µM cki7 (IC50, 9.5 µM) (Chijiwa et al., 1989) was sufficient to reduce or eliminate the normal Irx3 expression domain in 100% of the treated explants (Fig. 2G). Treatment with cki7 did not significantly expand the Six3 expression domain (Fig. 2C).

To confirm the in vitro requirement for Wnt signaling on Irx3 induction in posterior forebrain tissue, medium conditioned with Xenopus Dickkopf1 (Dkk1) was added to cultured headfolds. In Xenopus, Dkk1 antagonizes Wnt action (Glinka et al., 1998) by binding to LRP and Kremen (Mao et al., 2002; Nusse, 2001), members of a receptor complex for the Wnt ligand. Treatment with Dkk1 recapitulated the cki7 results, with 90% of the headfolds tested showing a downregulation of Irx3 expression (Fig. 2H versus 2E). Again, the Six3 expression pattern was unaffected by inhibition of the Wnt-signaling pathway (Fig. 2D).

These headfold explant experiments indicated that an early, endogenous Wnt signal was required for the maintenance of the posterior forebrain determinant Irx3. Moreover, if not inhibited, Wnt signaling can preclude the proper specification of the anterior forebrain by preventing the maintenance of Six3 expression and the expansion of Irx3 expression. To determine if this Wnt activity occurs in combination with other mesoderm-derived signals, we tested if neural plate explants exhibited a similar response.

Fig. 1. Six3, Irx3, Gbx2, Dlx2 and Wnt3 expression in the developing forebrain. (A-J) In situ hybridization of Six3 (A,B), Irx3 (C,D), Dlx2 (E,F), Gbx2 (G,H) and Wnt3 (I,J) at HH stage 7-8 (upper panels) and HH stage 19 (lower panels). At HH stage 7-8, Six3 (A) and Irx3 (C) are anterior and posterior to the prospective zli, respectively. At this stage, Gbx2 (G) expression is confined to the prospective hindbrain, and Dlx2 (E) and Wnt3 (I) expression is negligible. At HH stage 19, the expression domains of Irx3 (D), Gbx2 (H) and Wnt3 (I) are posterior to the zli, whereas Six3 (B) and Dlx2 (F) are expressed anterior to this structure. Arrowheads indicate the position of the zli. Scale bar: 100 µm.
Using the axial mesoderm transition as a guide, explants containing the prospective dT and vT were dissected from HH stage 8 chick embryos (Fig. 3A), cultured for 48 hours under serum-free conditions, fixed and then assayed for the induction of Irx3, Gbx2 and Dlx2 by in situ hybridization. Prospective dT explants, and not prospective vT explants, were Irx3-positive at the time of dissection (data not shown), consistent with the expression of Irx3 in the posterior forebrain at HH stage 8.

Exposure to Wnt3a-conditioned medium increased the level of Irx3 expression (Fig. 3F) and induced the expression of Gbx2 (Fig. 3J), but only in dT explants. No significant induction of either Irx3 or Gbx2 was observed in vT explants (Fig. 3B). The anterior forebrain marker Dlx2 was not expressed in dT and vT explants in the presence or absence of Wnts (Fig. 3K-N). These data demonstrate that Wnt signaling was sufficient to maintain posterior forebrain identity in explants. The Wnt-mediated induction of Gbx2 indicates that a continuous Wnt signal is necessary for the development of the dorsal thalamus. Furthermore, the absence of Wnt does not cause expression of Dlx2, indicating that additional signals that are not provided in these in vitro cultures are required for its expression.

**Blocking the response to forebrain-derived Wnt causes specific differentiation of the anterior forebrain**

To test if zli tissue is capable of inducing vT- and dT-specific gene expression, prospective dT and vT explants from stage 8 embryos were cultured adjacent to zli explants from HH stage 17-18 embryos (Fig. 4A). Following 2 days in culture, induction of Gbx2 and Dlx2 was assayed by in situ hybridization. Gbx2 was induced in 42% of the prospective dT explants cultured in contact with zli tissue, but in none of the prospective vT explants co-cultured with zli tissue (Fig. 4B,G,C). Co-culture of zli tissue with prospective vT explants

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**Fig. 2.** Wnt signaling is sufficient to inhibit Six3 expression and is required for Irx3 expression in headfold explants. (A–H) Headfold explants were cultured in the presence or absence of Wnt3a, Dkk1 and cki7, and Six3 or Irx3 expression assayed by in situ hybridization. Representative headfold explants are shown following culture and in situ hybridization for Six3 (A–D) or Irx3 (E–H). n= the number of explants tested per condition. Scale bar: 100 μm.

**Fig. 3.** Wnt3a induces Irx3 and Gbx2 in prospective dT explants. (A) The experimental procedure. Prospective vT and dT explants from HH stage 8 embryos were embedded in collagen, cultured in the presence or absence of soluble Wnt3a, fixed and assayed for Irx3, Gbx2 and Dlx2 expression by in situ hybridization. (B) The data was quantified and analyzed statistically using the χ-square significance test. (C–N) Representative explants in the culture condition indicated following in situ hybridization for Irx3 (C–F), Gbx2 (G–J) and Dlx2 (K–N). Some panels show more than one explant. The total number of explants in each experimental condition are indicated above each bar in B. *** indicates P<0.0001. Scale bar: 250 μm in C–F, 100 μm in G–N.
induced \textit{Dlx2} in 44\% of these explants, whereas no significant \textit{Dlx2} induction was observed in prospective dT explants cultured under these conditions (Fig. 4B,D,H). Because the zli explants are taken from HH stage 17-18 embryos, \textit{Gbx2} and \textit{Dlx2} expression are sometimes observed in zli tissue (e.g. Fig. 4C). These co-culture results confirm the observation that neural tissue anterior and posterior to the prospective zli has different competencies, which is consistent with the differential Wnt response in explants from these regions.

The addition of 50 \(\mu\text{M} \text{cki7} \) altered zli-mediated inductive events in prospective dT explants. \textit{Gbx2} induction was lost completely in prospective dT explants cultured with the zli and cki7 (Fig. 4B,I). Instead, the vT marker \textit{Dlx2} was induced in 47\% of prospective dT explants cultured in the presence of zli tissue and cki7 (Fig. 4B,J). Neither \textit{Gbx2} nor \textit{Dlx2} was induced by cki7 in prospective vT or dT explants that were cultured alone (Fig. 4B) and cki7 had no significant effect on the response of vT explants to zli-derived signals (Fig. 4B,F,E).

Together, these results demonstrate that, in vitro, a Wnt signal is required for both the induction of \textit{Gbx2} and the repression of \textit{Dlx2} in prospective dT explants. Apparently, blocking Wnt-mediated signaling causes an anterior to posterior change in the response to other signals derived from the zli. To address if blocking Wnt activity in the posterior forebrain allowed an anterior forebrain-specific response in ovo, we misexpressed \textit{Xenopus Dickkopf1} (\textit{xDkk1}), \textit{pRK5-xDkk1} and \textit{pCDNA3.1-GFP} were co-injected into the neuro pore of HH stage 9-10 embryos and diencephalic misexpression of these plasmids was achieved following electroporation. Induction of \textit{Dlx2} and \textit{Gbx2} was visualized by in situ hybridization in serial sections in which GFP was present. Misexpression of \textit{xDkk1} resulted in down regulation of \textit{Gbx2} in dT tissue and a concomitant expression of \textit{Dlx2} in the same region (Fig. 5C,D). The overlapping expression of these two markers was never observed in control embryos (Fig. 5A,B). Together, these results demonstrate that Wnt can be the sole determinant that allows posterior differentiation but that the induction of \textit{Dlx2} requires other, unknown signals present in the zli or other parts of the forebrain. Because Wnt signaling efficiently represses \textit{Six3} expression, and \textit{Six3} and \textit{Irx3} are mutually inhibitory, we tested if expression of \textit{Six3} in the posterior forebrain resulted in differentiation appropriate for the anterior forebrain, as would be predicted based on a previous study (Kobayashi et al., 2002).
Six3 predicates vT-specific differentiation

Full-length chicken Six3 (Kobayashi et al., 2002), cloned into pMES-IRES-GFP, was injected into the neuropore of HH stage 9-10 embryos, and diencephalic misexpression of this plasmid was achieved via electroporation. In serial sections where GFP was present posterior to the zli, Dlx2 and Gbx2 expression was visualized by in situ hybridization. Misexpression of Six3 resulted in repression of Gbx2 in dT tissue (Fig. 6A,B). In 25% of these embryos, a concomitant induction of Dlx2 was observed in the dT (Fig. 6C). Independent electroporations demonstrated that misexpression of Six3 in the posterior forebrain alters the normal levels of Wnt3 in the dT; in regions of the dT where GFP was present, Wnt3 was repressed (Fig. 6D,E,F).

These results indicate that Six3 acts to specify late anterior forebrain differentiation, and is sufficient to allow both the induction of Dlx2 and the repression of Gbx2 and Wnt3 expression. It remains to be determined if this effect is either direct or indirectly mediated by repression of Irx3.

Discussion

Wnt signaling in forebrain patterning

Combinatorial signaling is responsible for regional patterning in the CNS. However, the precise mechanisms by which the developing vertebrate forebrain is regionalized to give rise to structures such as the cortex, basal ganglia, thalamus and hypothalamus remain largely unclear. In this study, we investigated the role of Wnt signaling in establishing and maintaining regional identities in the developing diencephalon.

Wnt family members have been implicated as posteriorizing agents during neural development. In the Nieuwkoop model, neural induction occurs via a two-step activation-transformation process (Nieuwkoop, 1952). Following the initial induction of neural tissue, all of which is anterior in nature, subsequent events underlie the induction of more caudally-fated tissue. Wnts appear capable of mediating these secondary inductive events, thereby initiating posterior neural fates (McGrew et al., 1995). In Xenopus, misexpression of xWnt8 results in loss of anterior structures, including the forebrain (Fredieu et al., 1997). Treatment with lithium, which activates the transforming Wnt pathway, has a similar effect.

In addition, the zebrafish mutant mbl−/−, which has an overactive Wnt response caused by a nonfunctional axin gene, demonstrated a role for Wnt signaling in conferring posterior identity in the developing forebrain (van de Water et al., 2001). In mbl−/− mutants, there is a loss of telencephalon and vT with a concomitant expansion of the region that gives rise to dT. Because the telencephalon and vT develop from structures that are initially localized anterior to the dT, the fate shift in mbl−/− represents a gain of posterior-forebrain fates at the expense of anterior-forebrain fates. Although the exact identity of the Wnt ligands that mediate this posteriorization are unknown,

Fig. 5. Wnt is required in ovo for proper induction of Gbx2 and repression of Dlx2 in the diencephalon. (A-D) Adjacent sections were processed by in situ hybridization to visualize the expression of Gbx2 (A,C) and Dlx2 (B,D). Electroporation was used to misexpress Dkk1 in the developing diencephalon of two HH stage-9 embryos (C,D). Ectopic expression of Dkk1 resulted in ectopic expression of Dlx2 (arrow in D) in the normally Gbx2-positive dT (A). Arrowheads indicate the position of the zli. Scale bar: 100 μm.

Fig. 6. In ovo misexpression of Six3 suppressed diencephalic Wnt3 and Gbx2 expression and upregulated Dlx2 in the dT. (A-F) Serial sections of embryos 48 hours after electroporation with Six3-IRES-GFP at HH stage 9-10. In sections in which GFP was visualized in the dT (A), Gbx2 expression was suppressed (B, n=8). In 25% of these embryos Dlx2 was induced ectopically in the dT (arrowheads in C). Misexpression of Six3-IRES-GFP in the dT (D) also results the down-regulation of Wnt3 (E,F, n=2). Scale bar: 100 μm.
several candidate molecules are present in and around the developing forebrain during early and later stages of development (Nordstrom et al., 2002; Roelink and Nusse, 1991).

How are anterior and posterior forebrain competency differences established?

Although Gbx2 and Dlx2, the markers of dT and vT forebrain fates, are not yet induced by HH stage 8, Six3 and Irx3 are expressed in distinct anterior- and posterior-forebrain domains at this time. The interface of Six3 and Irx3 expression corresponds with the boundary used to obtain prospective dT and vT explants: Six3 is expressed above the prechordal plate and Irx3 is expressed above the notochord. A study (Kobayashi et al., 2002) showed that the expression of either Six3 anteriorly or Irx3 posteriorly differentially primes anterior and posterior forebrain tissue to respond to Shh and Fgf signals, and results in the induction of either anterior forebrain or posterior forebrain-specific genes, respectively.

The Wnt inhibitor Dkk1 is produced in the prechordal plate and is present at the right time and place to block a tonic Wnt signal that confers posterior identity on the forebrain at neural-plate stages (Glinka et al., 1998). Moreover, Wnt family members are expressed in the posterior neural plate by HH stage 4-5 (Nordstrom et al., 2002), which indicates that Wnt signaling could be involved in early regionalization events of the developing forebrain. Our headfold culture experiments implicate differential Wnt signaling as the mechanism by which the forebrain determinants Six3 and Irx3 are induced in the neural plate. Ectopically supplied Wnt3a was sufficient to inhibit the expression of the anterior forebrain determinant Six3, whereas inhibition of the Wnt pathway eliminated the expression of the posterior forebrain determinant Irx3. These experiments support a model whereby Dkk1 from the prechordal plate inhibits a Wnt signal in the most anterior neural tissue, thus causing a switch from a Wnt-induced, Irx3-positive posterior forebrain fate to a Six3-positive, anterior forebrain fate (Fig. 7).

Prospective dT/vT explant culture experiments showed that these explants are not irreversibly committed to their appropriate anterior or posterior forebrain fates. Culture of headfold explants for 24 hours in the presence of Wnt-response inhibitors was insufficient to elicit a complete change from posterior to anterior identity, as measured by the expansion of Six3 expression and the concomitant repression of Irx3. This indicates that respecification is a two-step process at least, in which the loss of Irx3 expression precedes the possible expansion of Six3. It might be predicted that extended culture in the presence of Wnt or Wnt-response inhibitors allows complete reprogramming of these explants to an alternate fate, but in these cases morphological changes in the explants precluded unambiguous interpretation of the in situ results. Nevertheless, our findings support a model whereby inhibition of the Wnt pathway in anterior tissue that overlies the prechordal plate represses posterior forebrain identity, and sets up an anterior fate on which later inductive cues can act (Fig. 7).

A Wnt signal is necessary and sufficient for specifying dT identity

Although the influence of Wnt signaling on Irx3 expression indicates an early role for Wnts in forebrain patterning, the subsequent expression of Wnt3 and Wnt3a indicate a subsequent role for Wnts in dT specification. An exogenous Wnt3a signal appears to be capable of acting as a posteriorizing agent that specifies the dT fate, as measured by Gbx2 induction in our prospective forebrain-explant system. The observation that not all explants respond to Wnt3a-conditioned medium by expressing Gbx2 indicates differences in these explants that might be caused by small variations between dissections and the embryonic stages from which these explants are derived. In addition, it remains to be determined whether higher doses of Wnt3a result in a higher percentage of Gbx2-expressing explants.

The ability of endogenous zli-derived signals to induce either vT or dT markers in prospective vT and dT explants was assessed using a heterochronic co-culture system. Because the expression patterns of inductive molecules at the zli are well-described at HH stage 17, zli tissue from these older embryos was used as a source of inductive molecules. Culture of zli tissue adjacent to prospective vT or dT explants is capable of inducing vT- and dT-specific gene induction. Because zli explants are not homogenous sources of inductive signals, we expect that the relative position of the prospective dT/vT explants to zli explants affects the induction of Gbx2 and Dlx2,
which might explain why just over 50% of the explants do not respond under these co-culture conditions.

The induction of Gbx2 in prospective dT explants by zli tissue is mediated through a transforming Wnt signal, which is likely to be Wnt3 or Wnt3a. Inhibiting the Wnt response in co-cultures causes prospective dT tissue to acquire a VT-specific fate. Therefore, a Wnt signal that is either from the zli or present in the prospective dT is instructive in specifying the posterior/dT tissue in two ways. First, a Wnt signal induces the dT markers Irx3 and Gbx2. Second, a Wnt signal inhibits the induction of the VT markers Six3 and Dlx2 (see Fig. 7). In turn, Six3 inhibits the expression of Gbx2 and Wnt3 and promotes the expression of Dlx2.

Our observation that the VT is seemingly insensitive to Wnt signals could be caused by the lack of a crucial component of the Wnt signaling pathway in the VT. Interestingly, Tcf4 is expressed in the prospective dT but not the VT (Galceran et al., 2000) and might be an important mediator of the differences in competency to respond to Wnt signaling on either side of the zli. Alternatively, Wnt inhibitors expressed in the prospective VT could prevent Wnt-receptor activation. SFRP-2, a known Wnt antagonist (Ladher et al., 2000), is expressed in the developing VT with a sharp posterior boundary of expression at the zli.

Although the in vitro studies presented in this work indicate a role for Wnt signaling in diencephalic development, they also demonstrate the existence of an undefined factor or factors that induce the VT fate. Possible candidates for VT inducers include Shh and Fgf8. Both are expressed at the zli and we are currently examining their roles in Dlx2 induction.

Our results agree fundamentally with the Nieuwkoop model of neural induction. However, the loss of anterior forebrain in Dkk1 mutants indicates that active suppression of Wnt signaling is required for the formation of anterior neural tissue, which implies the presence of tonic Wnt signals in the developing forebrain. It appears that the presence of Dkk1 is required for the expression of Six3, but it is unknown if Six3 expression, in turn, requires a distinct inducer. Expression of Irx3 in the posterior forebrain is likely to be induced by a Wnt signal, consistent with our observation that Wnt5a can induce Irx3 in forebrain explants.

The expression of Six3 and Irx3 are crucial for the subsequent distinct differentiation of tissue in the anterior and posterior forebrain. Our observation that misexpression of Six3 in the Irx3 domain causes the repression of dT-specific and the activation of VT-specific gene expression demonstrates the key role of Six3 in the induction of anterior forebrain fate. The normal pathway for VT specification, in which expression of Six3 predicates that of Dlx2, involves continual inhibition of the Wnt response, whereas the inductive steps that allow Gbx2 expression in Irx3-positive cells are mediated by Wnts, presumably Wnt3 and Wnt3a. The mechanism by which the interface of Irx3 and Six3 domains becomes the zli is unclear, but indicates the presence of signaling events at this border.

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