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Ectopic *Engrailed 1* expression in the dorsal midline causes cell death, abnormal differentiation of circumventricular organs and errors in axonal pathfinding

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SUMMARY

A series of gain- or loss-of-function experiments performed in different vertebrate species have demonstrated that the *Engrailed* genes play multiple roles during brain development. In particular, they have been implicated in the determination of the mid/hindbrain domain, in cell proliferation and survival, in neurite formation, tissue polarization and axonal pathfinding. We have analyzed the consequences of a local gain of En function within or adjacent to the endogenous expression domain in mouse and chick embryos. In WEXPZ.En1 transgenic mice (Danielian, P. S. and McMahon, A. P. (1996) *Nature* 383, 332-334) several genes are induced as a consequence of ectopic expression of *En1* in the diencephalic roof (but in a pattern inconsistent with a local di- to mes-encephalon fate change). The development of several structures with secretory function, generated from the dorsal neuroepithelium, is severely compromised. The choroid plexus, subcommissural organ and pineal gland either fail to form or are atrophic. These defects are preceded by an increase in cell death at the dorsal midline. Comparison with the phenotype of *Wnt1*$^{sw/sw}$ (*swaying*) mutants suggests that subcommissural organ failure is the main cause of prenatal hydrocephalus observed in both strains. The formation of the posterior commissure is also delayed, and errors in axonal pathfinding are frequent. In chick, ectopic expression of En by in ovo electroporation, affects growth and differentiation of the choroid plexus.

Key words: Engrailed, Dorsal midline, Hydrocephalus, Choroid plexus, Pineal gland, Subcommissural organ, Axonal pathfinding, Posterior commissure, Cell death, Swaying, In ovo electroporation, Mouse

INTRODUCTION

The assembly of neural circuits in the vertebrate nervous system begins with the generation of functionally distinct neuronal cell types along the rostrocaudal and dorsoventral axes of the neural tube. Patterning of cellular identity is thought to depend on intrinsic differences in the character of neural progenitors as well as regional differences in environmental signals (Lee and Jessell, 1999). Among the developmental control genes that become expressed in a spatially restricted pattern during neural plate induction are the two *Engrailed* homeodomain-containing genes. In mouse, *En1* and *En2* are expressed from the 1- and 5-somite stages, respectively, in two dorsolateral patches of cells in the anterior neuroepithelium, which subsequently fuse ventrally to mark a broad band that will give rise to the mid-hindbrain junction. Following neural tube closure, En protein expression remains centered around the constriction (where it persists throughout development) and includes a large portion of the mesencephalon and most of rhombomere one. In the adult brain, En expression is limited to neuronal groups in the pons, the substantia nigra and to cells of the cerebellum (Davis and Joyner, 1988; Davis et al., 1991; McMahon et al., 1992). Gene inactivation experiments have demonstrated that *En1* has an early crucial role in the specification of the mes-metencephalic region (Wurst et al., 1994) and is an early target of *Wnt1* signaling (Danielian and McMahon, 1996) and that *En2* is involved in the patterning of cerebellar foliation (Joyner et al., 1991; Millen et al., 1994). The development of mes-metencephalic derivatives is controlled by a mechanism sensitive to the dosage of En proteins (reviewed by Wassef and Joyner, 1997). Finally, the two En proteins have extensive functional overlap (Hanks et al., 1995).

In chick, retrovirus-mediated misexpression of *En* genes (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996; Shigetani et al., 1997; Shamim et al., 1999) within the developing mesencephalon results in disruption of the gradient of cytoarchitectonic differentiation of the optic tectum, aberrant arborizations and perturbed targeting of the nasal axons and complete degeneration of the temporal axons. Misexpression of *En2* in the diencephalon by in ovo electroporation causes a rostral shift of the di/mesencephalic boundary and transformation of dorsal diencephalon into tectum (Araki and Nakamura, 1999). Similarly, ectopic

expression of *En2* in medaka fish leads to activation of mesencephalic markers in the diencephalon (Ristoratore et al., 1999). Thus, En misexpression within a domain of competence causes an identity change towards a more caudal phenotype.

To investigate whether ectopic and constitutive expression of En in a defined and restricted domain resulted in an identity change of the cells misexpressing the protein or whether the cells tolerated the presence of ectopic En and regulated according to their environment, we took advantage of a transgenic mouse line expressing *En1* in the dorsal midline from the diencephalon through the spinal cord (Danielian and McMahon, 1996). We show that the prenatal hydrocephalus reported in this line (Danielian and McMahon, 1996; Rowitch et al., 1999) is a consequence of the ectopic expression of *En1*, which affects the differentiation of dorsal circumventricular organs and the choroid plexus, the main site of production and secretion of the cerebrospinal fluid (CSF). To corroborate our findings, we analyzed the development of these organs in *Wnt1*$^{sw/sw}$ mutant embryos exhibiting documented abnormalities in the dorsal midline. Finally, to evaluate the effects of ectopic En expression in a different species, we misexpressed the *En2* gene in the developing chick embryo by in ovo electroporation and show that En misexpression in chick mimics some of the phenotypic manifestations of the murine transgene.

MATERIALS AND METHODS

Animals

Mouse lines used in this study have been previously described: WEXPZ.En1 (Danielian and McMahon, 1996), maintained on a wild-type outbred (OF1) background (the dosage of Wnt1 is normal); Wnt1$^{sw/sw}$ (purchased from the Jackson Labs); En1^{hd} (Wurst et al., 1994); En1^{Lki} (Hanks et al., 1995). The oligonucleotides 5′-TTT AAC GCC GTG CGC TGT TCG-3′ and 5′-GAT CCA GCG ATA CAG CGC GTC-3′ amplify a *lacZ*-specific product in WEXPZ.En1 animals. *Wnt1*$^{sw/sw}$ embryos were genotyped as described by Bally-Cuif et al. (1995).

Cell death assays

To detect dying cells, we injected Nile Blue Sulfate (NBS; 1:1000 w/v in ddH_2O) into the ventricles of E9.5-E15.5 embryos dissected in PBS. We also labeled dissected neural tubes as whole mounts using Terminal-UTP-Nick-End-Labeling (TUNEL) as described by Conlon et al. (1995).

Immunohistochemistry

Cryomicrotome sections were collected in PBT (PBS, 0.1% Tween 20), treated with 10% methanol, 3% H_2O_2 in 0.12 M phosphate buffer pH 7.2 for 20 minutes, rinsed in PBS, incubated overnight with the primary antibody diluted in PGT (PBS, 0.2% gelatin, 1% Triton X-100), washed in PBT, incubated with a biotinylated secondary antibody (1:200; Jackson), washed in PBT, incubated with ABC complex (1:400; Amersham), washed and revealed with DAB. Embryos at E9.5 and E10.5 were assayed as whole mounts. Antibodies used were: 2H3 (anti-neurofilament; DSHB), TuJ-1 (gift from A. Frankfurter; Moody et al., 1989; Lee et al., 1990), anti-EphA4 (Becker et al., 1995), anti-SCO spondin (gift from S. Gobron), 4D9 (Patel et al., 1989).

Whole-mount RNA in situ hybridization

Embryos were fixed and hybridized with one or two mRNA probes, as described by Bally-Cuif et al. (1995). Probes used for in situ were: *Bmp6* (Jones et al., 1991), *Bmp7* (Furuta et al., 1997), *Msx1* (Lyons et al., 1992), *cadherin 8* (Korematsu and Redies, 1997), *cadherin 11* (Hoffman and Balling, 1995), *ephrinA5* (Flenniken et al., 1996), *reelin* (Schiffmann et al., 1997), *Gdf7* (Lee et al., 1998), *Notch 2* (Mitsiadis et al., 1995), *Ttr* (gift from W. Blaner), *chickTtr* (Duan et al., 1991), *Igf2* (Soares et al., 1986), *Mf1* (Kume et al., 1998), *EphA4* (Nieto et al., 1992), *Pax6* (gift from S. Saule), *Tcf4*, *Pax5*, *Otx2*, *Fgf8* (Crossley and Martin, 1995).

Neuronal labeling

We used DiI (Molecular Probes) to trace axons on fixed embryos. A fine thread of DiI melted into paste was inserted into a lateral incision parallel to the di/mesencephalic midline. For observation and double labeling procedures, the fluorescence was photoconverted to a stable product. In brief, embryos were rinsed, treated with 0.1% H_2O_2, equilibrated in 0.1 M Tris, pH 8.2, transferred to the same solution containing 1.6 mg/ml DAB and incubated on ice for 30 minutes. Illumination of the embryos placed in a depression slide for 5-10 minutes converted the fluorescence into a brown color. In some cases, embryos were sectioned in a vibratome at 200 μm prior to photoconversion.

In ovo electroporation

We used White Leghorn chick embryos from local farms incubated at 38°C and staged according to Hamburger and Hamilton (1951). We injected the DNA solution (at 1 mg/ml) containing 0.25% Fast Green (Sigma) as a tracer, into the lumen of the neural tube and applied three 30-msecond 25 V pulses from an electroporator BTX-20 with electrodes CUY610 (ϕ 0.5 mm; gap 4 mm; TR Tech Co., Tokyo, Japan). Embryos were incubated for appropriate times and fixed in 4% paraformaldehyde. Typical survival rate was about 72% at 48 hours postelectroporation but declined thereafter.

Expression vectors

We used three plasmids: pTLmEn2m and pTLmEn2SRm (Joliot et al., 1998), respectively encoding myc-tagged versions of the wild-type and a mutant chick En2 protein and the unrelated pAdRSV GAL IX, encoding β-galactosidase (gift from P. Gilardi-Hebenstreit).

RESULTS

Expression pattern of the WEXPZ.En1 transgene

To investigate the effects of ectopic and constitutive expression of the *En1* gene in the dorsal midline, we took advantage of the WEXPZ.En1 transgenic line, which expresses the *En1* gene under the control of a 5.5 kb 3′ *Wnt1* enhancer element (Danielian and McMahon, 1996). This enhancer is activated at least as early as the 1-somite stage (Echerald et al., 1994); therefore, the onset of overexpression of *En1* in the transgenic embryos coincides with the earliest stage of endogenous *En1* expression in a broad region of the anterior neural plate. Cells with endogenous expression of *En1* also express the transgene; importantly, a rostral population of cells expresses *En1* ectopically, but only transiently. At the 3-somite stage the *En1* expression domain has extended so that its anterior limit approximately coincides with that of *Wnt1* at the anterior margins of the midbrain. By the 8-somite stage, most midbrain cells express elevated levels of *En1* and, in addition, scattered groups of cells at the choroid plexus/cerebellar boundary also express *En1* ectopically. At the 15-somite stage expression of the transgene in the midline has extended into the diencephalon and down to the spinal cord and, by E9.5, becomes refined to the dorsal midline (from the prosomere 2/3 boundary through the spinal cord; see Rubenstein et al., 1994 for nomenclature)

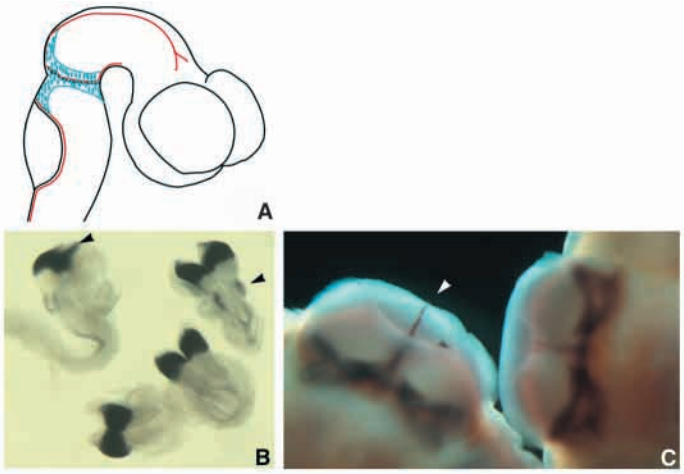

Fig. 1. Expression of the transgene. (A) Schematic representation of a E10.5 embryo, lateral view. Endogenous expression of *En1* (blue) is centered around the mid-hindbrain junction; ectopic expression of *En1* from the transgene (red) is restricted to the dorsal midline from the diencephalon to the spinal cord (except of the midline of the cerebellar plate) and to a ring just anterior to the isthmus. (B) Pattern of expression of *En1* in E8.5 wild-type (bottom) and transgenic (top) littermates. Arrowheads indicate the sites of ectopic expression of *En1*. (C) Ventral view of bisected brains of E15.5 wild-type (right) and transgenic (left) littermates. Ectopic expression of *En1* at the midline of the dorsal neural tube (arrowhead) persists in the transgenic embryos.

and to a narrow ring of expression just anterior to the isthmus, essentially recapitulating the expression pattern of *Wnt1*. Ectopic expression of *En1* along the dorsal midline persists throughout embryogenesis and into at least the first postnatal week (unpublished observations) as opposed to endogenous expression in the roof plate, which by E10.5 is confined to the posterior half of the mesencephalon, and becomes rapidly restricted to its caudal third (Fig. 1).

Embryos of the WEXPZ.En1 line exhibit prenatal hydrocephalus. In man, perinatal hydrocephalus results from an obstruction in CSF flow into the circulation, failure of CSF resorption through the arachnoid granulations or, occasionally, oversecretion of CSF (Rowland et al., 1991). In the transgenic embryos, aqueductal stenosis is an unlikely cause of hydrocephalus, because no obstruction in intraventricular communication between E10.5 and E15.5 was evident after tracer dye injections into the developing lateral ventricles (not shown). We hypothesized that ectopic expression of *En1* in the dorsal midline could affect the differentiation of dorsal structures with secretory function. The subcommissural organ (SCO) is a circumventricular organ located at the entrance of the aqueduct of Sylvius, and the choroid plexus represents the main site of synthesis and secretion of the cerebrospinal fluid (CSF). Ectopic *En1* expression in the dorsal midline could also affect the specification of premigratory neural crest cells, derivatives of which include the meninges covering the anterior brain and most of the cranial bones and corresponding sutures.

En expression in the dorsal diencephalic midline results in cell death and errors in axonal pathfinding

The first apparent morphological manifestation of the transgene was a shape change of the anterior diencephalon, where, starting at E10.5, two bilateral bulges formed in dorsal p2 (Fig. 2A,B). The dorsal midline of the diencephalon and anterior mesencephalon appeared wider and was frequently spotted with many large cell aggregates in the transgenic embryos (Fig. 2J inset, compared to wild-type littermates, I inset). To evaluate their nature, we analyzed cell death. In the transgenic embryos, Nile blue sulfate staining revealed necrotic cells at the roof and the border of the fourth ventricle at E9.5, and increased numbers of necrotic cells in the midline of the mid- and anterior mesencephalon and diencephalon at E10.5 and E11.5 (Fig. 2C,D; top). At E12.5, necrotic cells persisted in the midline, in particular at the site of the pineal anlage, where they remained evident at E13.5; no differences in cell death were detected among littermates at subsequent developmental stages (not shown). We confirmed that the aggregates were composed of apoptotic cells by the TUNEL assay (Fig. 2C,D; bottom).

The expression of several markers of the dorsal midline over its entire length (*Bmp6*, *Bmp7*, *Gdf7*, *Msx1*) was not affected in transgenic embryos. *EphrinA5*, known to be ectopically activated as a result of En protein misexpression (Logan et al., 1996), was detected rostral to its normal expression domain along the entire length of the diencephalic midline in E11.5-E16.5 transgenic embryos (Fig. 2E,F). Mesencephalic or isthmic markers, such as *Pax5* (our observations) and *Fgf8* (our observations and Danielian and McMahon, 1996) were not affected. In contrast, the expression of *cadherin 8* was downregulated in the midline cells of the rostral mesencephalon at E13.5, and expression of *cadherin 11* was abolished from the dorsal midline in transgenic neonates (not shown). In the diencephalon, expression of *Tcf4* was not affected and expression of *Pax6* in p1 and p2 was maintained in transgenic embryos at E10.5 and E11.5. Importantly, no induction of endogenous *En1*, detectable by ectopic X-gal staining, was observed adjacent to the diencephalic midline in WEXPZ.En1; $En1^{Lki/+}$ embryos (not shown). The pattern of activation and repression of different markers implied that, although misspecified, the diencephalic roof plate cells failed to adopt a well-defined alternative fate as a consequence of En1 misexpression.

The formation of the posterior commissure (pc) was delayed in the transgenic embryos. In wild-type embryos, the pc neurons project dorsally to cross the roof plate in the caudal diencephalon (p1) at E10.5 (Mastick and Easter, 1996). In transgenic littermates, dorsally projecting axons remained far from the midline as if they were repelled by it, as evidenced by immunohistochemistry with the anti-neurofilament antibody 2H3 and the neural-specific anti-class III β-tubulin antibody TUJ-1 (Fig. 2G,H). The pc axons still failed to reach the midline in the transgenic embryos at E11.5 (Fig. 2I,J), an observation consistent with the ectopic activation of *ephrinA5*, known to have repellent effects (see Discussion). Nevertheless, by E13.5, the pc axons succeeded in crossing the midline, albeit irregularly (Fig. 2K,L). They fasciculated forming tight bundles and exhibited navigation defects, frequently turning longitudinally and extending parallel to the midline (both in rostral and caudal directions), instead of proceeding to cross it (Fig. 2O,P). We analyzed the expression of markers that could account for the eventual crossing of the pc axons. EphA4, a receptor for ephrinA5, was undetectable in caudal p1 at E10.5 or E11.5, but clearly evident at E13.5, both in wild-type and

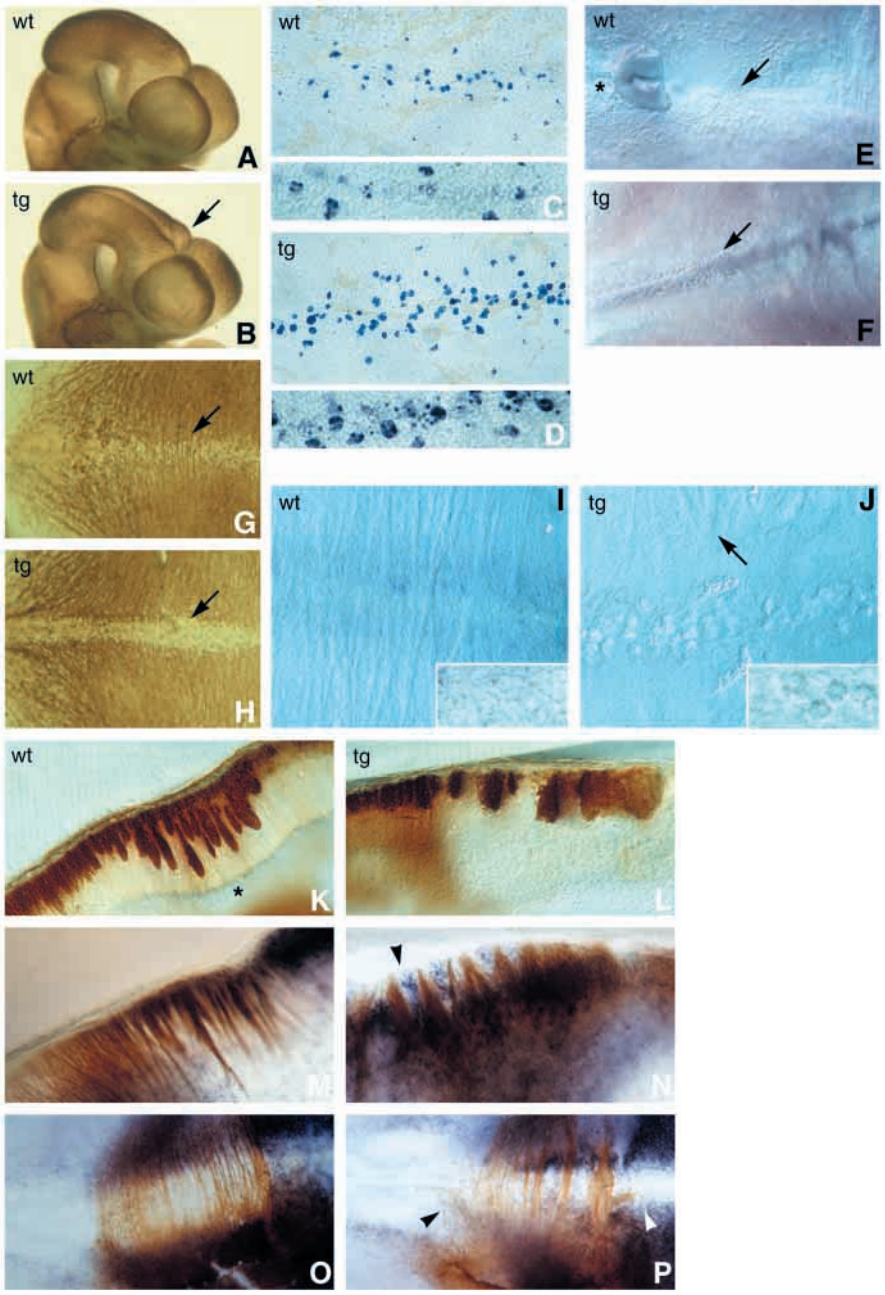

Fig. 2. Neural tube morphology and axonal pathfinding at the dorsal midline are altered in WEXPZ.En1 embryos (tg) compared to wild-type littermates (wt). In all panels, except A and B, anterior is to the left. (A,B) E10.5 embryos. The arrow points to the abnormal bulges forming at the anterior diencephalon in transgenic embryos. (C,D) E11.5. Top panels: Nile Blue Sulfate staining detects elevated numbers of necrotic cells in the diencephalic midline of transgenic (D) as compared to wild-type littermates (C). Bottom panels: TUNEL assay detects apoptotic cells within the large aggregates revealed by Nomarski optics; flat mount preparations. (E,F) E13.5. Ectopic En1 induces *ephrinA5* expression in the roof plate up to the anterior diencephalon (arrow in F); compare with wild-type (E). asterisk, pineal gland. (G-J) The axons of the posterior commissure are labeled with the TUJ-1 antibody at E10.5 (G,H) or detected using Nomarski optics at E11.5 (I,J). They cross the midline at the p1/mesencephalic border in wild type (arrow in G) but remain far from it in transgenic littermates (arrows in H and J). Insets in I, J: midline morphology. (K-P) E13.5 embryos. Mid- (K,L) and para- (M,N) sagittal vibratome sections and flat mount preparations (O,P); the axons of the posterior commissure are labeled with DiI and visualized in brown following photoconversion of DiI. The asterisk in K indicates the subcommissural organ, which forms an indentation in the ventricular surface. In wild-type embryos (K,M,O), the axons of the posterior commissure cross perpendicular to the midline in thin regular bundles. In transgenic embryos (L,N,P), pc axons cross the midline in thick irregular bundles and follow aberrantly oriented trajectories (arrowheads in P). *Reelin* mRNA (blue) is detected by in situ hybridization in M-P. *Reelin*-expressing cells (arrowhead in N) are found clustered around the tight axonal bundles in transgenic embryos.

transgenic embryos, coinciding with the crossing of the pc axons in the latter (not shown). *Reelin*, which encodes a secreted protein of the extracellular matrix, was induced at ectopic locations in the dorsal diencephalon of transgenic embryos, and appeared to contour the tight bundles of axons labeled by DiI (Fig. 2M,N,O,P).

Therefore, ectopic expression of *En1* in the dorsal midline resulted in increased cell death and mis-specification of midline cells, which displayed ectopic activation of a number of genes. As a consequence, the dorsally projecting axons of the pc fasciculated abnormally and displayed errors in pathfinding.

Ectopic *En1* interferes with differentiation of circumventricular organs

We examined whether the development of dorsal neural tube derivatives was affected by the ectopic expression of *En1* at the midline. The SCO primordium is closely associated with the posterior commissure at E10.5 (Rakic and Sidman, 1968) and appears as an indentation of the ventricle in wild-type embryos. This morphological landmark was absent from the transgenic embryos (see Fig. 2K,L). Early specific markers of the developing SCO are not available. Staining for *Gdf7* and *Notch 2*, which mark the SCO anlagen starting at E12.5 and E13.5, respectively (unpublished observations), demonstrated SCO failure in the transgenic embryos (Fig. 3A,B,D,E). At later stages, the ependymal cells of the SCO produce and secrete SCO-spondin (Gobron et al., 1996). This specific marker demonstrated near complete absence of the SCO in the transgenic embryos at E17.5 and in newborn pups (Fig. 3F,G).

The differentiation of the pineal primordium was

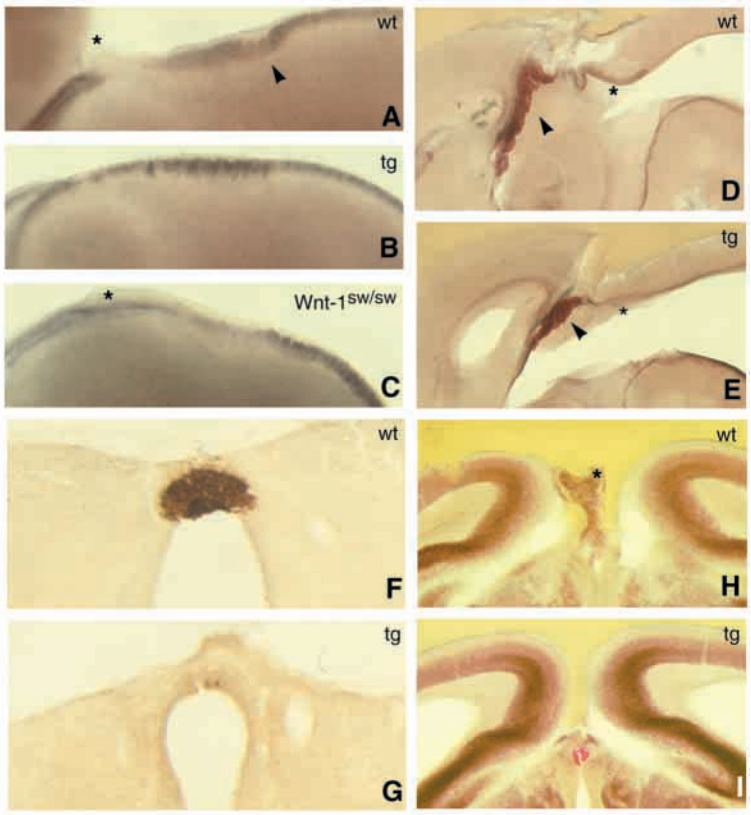

Fig. 3. The pineal gland and subcommissural organ fail to form in WEXPZ.En1 transgenic embryos. (A-C) E12.5. Lateral view (anterior to the left) of whole brains treated by in situ hybridization for *Gdf7*. In wild-type (A), *Gdf7* staining detects the SCO primordium (arrowhead), which is missing from WEXPZ.En1 transgenic (B) and *Wnt1*$^{sw/sw}$ mutant embryos (C). The pineal evagination (asterisk) is present in A, but is lacking in B and underdeveloped in C. (D,E) Double color in situ hybridization with *Notch 2* (light brown/purple) and *TTR* (red) on sagittal vibratome sections of P1 brains (anterior to the left). The SCO (asterisk) is well developed in wild-type (D) but lacking in transgenic pups (E). Note the underdevelopment of third ventricle choroid plexus (arrowheads in D and E). (F,G) Coronal sections of P0 brains, treated for the immunocytochemical detection of SCO-spondin, a specific marker of the secretory cells of the SCO. Very few cells are detected in transgenic (G) compared to wild-type brains (F). (H,I) In situ hybridization of *cadherin 11* in coronal sections of P1 brains. The pineal gland (asterisk) labeled in wild-type (H) is not detected in transgenic pups (I).

compromised in the transgenic embryos, as was indicated both by morphological criteria and expression data. Expression of *Otx2* failed to outline the pineal primordium in the transgenic embryos, already evaginating in the wild type at E11.5 (not shown). The pineal anlagen is marked by an interruption in *Gdf7* staining of the midline at E12.5 and E13.5 (our observations); in transgenic embryos, expression of *Gdf7* was continuous (Fig. 3A,B). The dorsalmost aspect of the evaginating pineal was marked by *cadherin 8* in the wild type; this staining was absent from transgenic embryos at E13.5 (not shown). Finally, the transgenic neonates were lacking pineal gland, as evidenced by in situ hybridization with *cadherin 11* (Fig. 3H,I). In contrast, the subfornical organ, located at the anterior dorsal wall of the third ventricle was found to be unaffected in the transgenic embryos and neonates, as demonstrated by in situ hybridization with *Notch 2* and *cadherin 11* (not shown).

Therefore, ectopic expression of *En1* in the dorsal midline caused impaired development of circumventricular organs.

Ectopic *En1* results in impaired development of the choroid plexus

Growth and patterning of the choroid plexus were affected in the transgenic embryos, as demonstrated by the expression of *Ttr* (a marker of choroid plexus epithelial cells (CPe) from the onset of their differentiation; Murakami et al., 1987; Thomas et al., 1989) and *Igf2* (an early marker of the invading mesenchymal cells, later also expressed by the gradually maturing CPe; Stylianopoulou et al., 1988; Cavallaro et al., 1993). In the fourth ventricle, the developing CPe was significantly reduced, starting at E10.5; in the caudal part, expression of *Ttr* was limited to a thin bilateral domain (Fig. 4A). At E11.5, the expression domain of *Ttr* remained smaller than wild type rostrally, and it was confined to the edges of the tela choroidea caudally (Fig. 4B,C). In addition, the vascularization of this structure was less extensive in the transgenic embryos compared to wild-type littermates, as visualised by intraventricular injection of India ink (not shown). At E12.5 the developing choroid plexus had started folding in wild type, but remained thin and stretched in transgenic embryos. At all subsequent stages, the choroid plexus of the fourth ventricle neither invaginated, nor arborized properly (Fig. 4G,H). Similarly, in the diencephalon, the CPe was reduced in transgenic embryos from the earliest stage of *Ttr* expression in this region (E11.5); the choroid plexus failed to fully expand and remained atrophic (Fig. 4E,F). In contrast, no differences between wild-type and transgenic littermates were observed in the expression domain of *Ttr* in the telencephalon, where the choroid plexus developed properly (not shown).

Choroid plexus and circumventricular organs are affected in *swaying* mutant embryos

In an attempt to understand the mechanism by which *En1* affected the differentiation of circumventricular organs and the choroid plexus, we investigated the development of these organs in mutant embryos exhibiting abnormalities in the dorsal midline. In the *Wnt1* mutant embryos, differentiation of the dorsal midline is affected (Shimamura et al., 1994); importantly, the dosage of En protein is altered (McMahon et al., 1992); and because of the deletion of both the mesencephalon and rhombomere 1 (McMahon and Bradley,

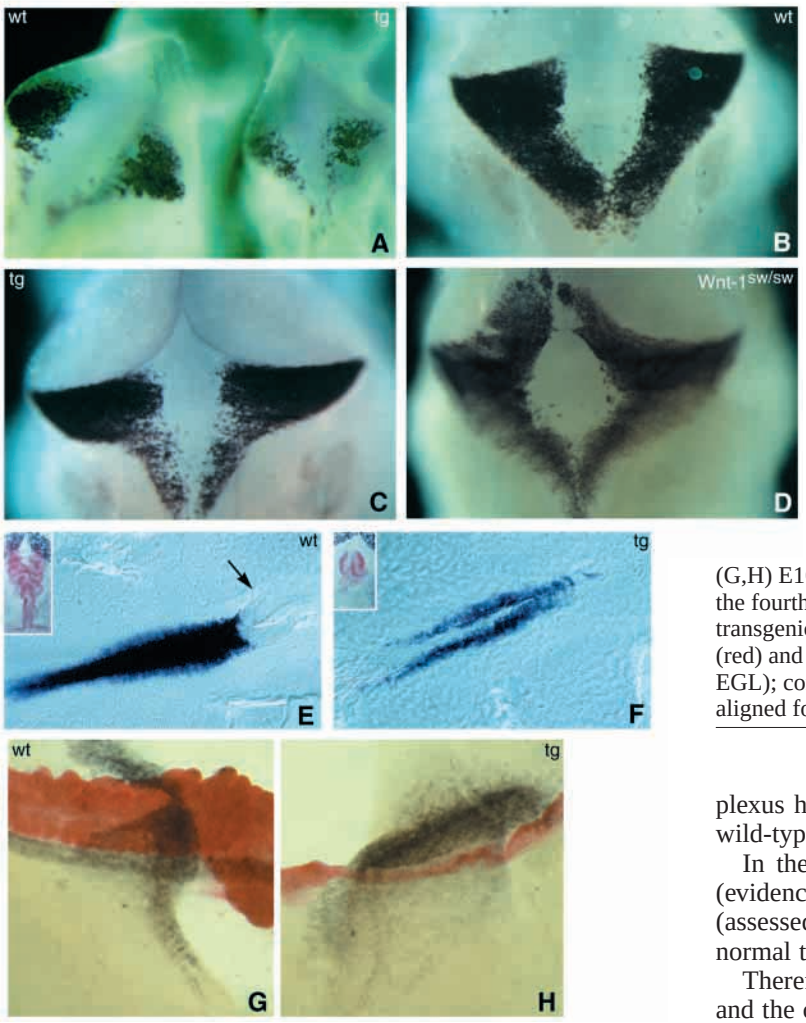

Fig. 4. Differentiation and growth of the choroid plexus are compromised in WEXPZ.En1 embryos. (A) E10.5. A *Ttr* probe stains two choroid plexus epithelial primordia differentiating laterally in the roof of the fourth ventricle in wild-type (left) and transgenic (right) embryos. There is a marked reduction in *Ttr* expression in the tela choroidea of transgenics. (B-D) E11.5. The two epithelial primordia fuse caudally in the midline in wild-type embryos (B). There are fewer *Ttr*-expressing cells in transgenic embryos (C) and growth of the choroid plexus is compromised. In *Wnt1*$^{sw/sw}$ mutants (D), *Ttr* identifies CPe cells at ectopic locations, rostral to the choroid plexus territory. Dorsal view. (E,F) E12.5. The choroid plexus primordium in the diencephalon of wild-type embryos (E) is labelled with *Ttr;* both its size and shape are affected in transgenic littermates (F). Flat-mount preparations of the rostral diencephalon; anterior is to the left. Arrow in E indicates the pineal evagination. Insets: at E14.5, growth of the choroid plexus of the diencephalon is reduced in transgenic embryos; coronal sections. (G,H) E16.5. The choroid plexus has expanded and invaginated into the fourth ventricle in wild-type (G), but remains atrophic in transgenic embryos (H). Double color in situ hybridization with *Ttr* (red) and *Math1* (purple; marking the migrating precursors of the EGL); coronal sections at the level of the fourth ventricle have been aligned for comparison. Only one-half of each section is shown.

1990; Mastick et al., 1996), the di/mesencephalic junction is modified.

We analyzed the differentiation of circumventricular organs in *Wnt1*$^{sw/sw}$ mutant embryos. We found that the SCO did not differentiate normally (as indicated by the expression pattern of *Gdf7* at E12.5; Fig. 3C). The pc appeared to cross the midline normally and expression of *reelin* was unaffected at E13.5. The pineal evagination was, at best, never as developed as in wild type (likely reflecting the phenotypic severity of the hypomorphic *swaying* mutation) and the pineal gland was atrophic at late stages of gestation.

We also investigated the development of the choroid plexus in *Wnt1*$^{sw/sw}$ embryos. At E10.5, the anterior part of the two CPe primordia was displaced rostromedially; scattered cells expressing *Ttr* were observed medially at the remnants of the mid-hindbrain. At E11.5, CPe tissue had extended into the area normally occupied by the cerebellar plate and, in addition, cells expressing *Ttr* ectopically persisted at the midline (Fig. 4D). In the rostral diencephalon at this stage, the medial expression domain of *Ttr* was absent from the *Wnt1*$^{sw/sw}$ embryos. At late embryonic stages, an ectopic expression domain of *Ttr* persisted rostral and medial to the infolded fourth ventricle choroid plexus, which, due to the mid/hindbrain deletion, was positioned on the surface. In the third ventricle, the choroid plexus had also developed, although it appeared smaller than wild-type and abnormally folded (not shown).

In the *En1* mutant embryos, choroid plexus development (evidenced by expression of *Ttr*) and pineal differentiation (assessed by morphological criteria and expression data) were normal throughout embryogenesis (not shown).

Therefore, the differentiation of circumventricular organs and the choroid plexus was affected in *swaying* embryos with a mutation interfering with correct development of the dorsal midline, but was normal in *En1* mutant embryos.

Neural crest derivatives in WEXPZ.En1 transgenic mice

We investigated whether ectopic *En1* expression affected derivatives of the cephalic neural crest implicated in the etiology of hydrocephalus, such as the meninges covering the anterior brain (Le Douarin and Kalcheim, 1999). We examined the development of the leptomeninges with three markers, *Notch 2* (Higuchi et al., 1995), *Mf1*, expressed in arachnoid cells (Kume et al., 1998), and *Igf2*, a marker of the developing pia and arachnoid (Stylianopoulou et al., 1988), but detected no obvious differences among littermates (not shown). Thus, by these criteria, the leptomeninges did not appear affected in the transgenic embryos and neonates.

Most of the cranial bones and corresponding sutures are also derivatives of the neural crest (Le Douarin and Kalcheim, 1999). To evaluate cranial bone development, we compared the skeletons of a total of nine wild-type and eight transgenic littermates postnatal weeks 2 and 3, after staining with Alcian Blue and Alizarin Red. Differences were observed in the shape and size of various bones. In the transgenic animals, the interparietal bone was larger, the parietal bone was shorter and wider and the supraoccipital bone was smaller than wild type. The lambdoid and sagittal sutures were wider in the transgenic

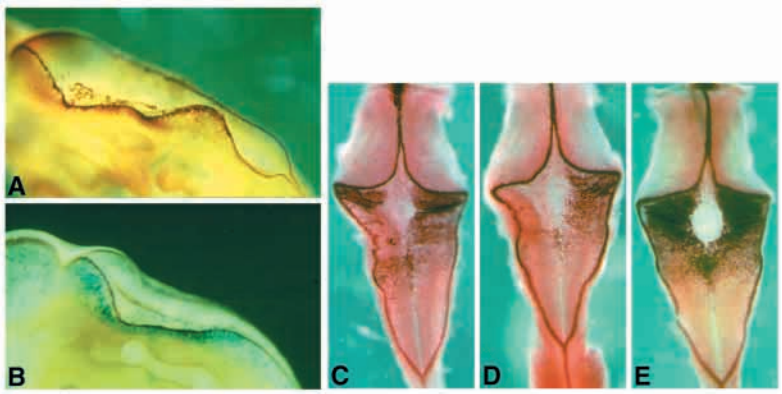

Fig. 5. Misexpression of *En2* in the developing chick embryo by in ovo electroporation. (A,B) Lateral view of the electroporated side 2 days postelectroporation at stage HH9-10. A *Gdf7* probe marks the border of the tela choroidea (purple) and the 4D9 antibody reveals the presence of ectopic En protein (brown). Forced expression of En2 (A) results in abnormal morphology of the choroid plexus border outlined with *Gdf7*, whereas β-galactosidase expression (B; revealed by X-gal histochemistry) does not interfere with its development. (C,D) Expression of En2 results in reduced growth at the border and interferes with differentiation of the choroid plexus epithelium. *Gdf7* outlines the border of the tela choroidea and *Ttr* marks the differentiating choroid plexus epithelial cells. There is reduced growth and differentiation on the electroporated side (left) compared to the control (right). (E) Example of an embryo electroporated with a control plasmid expressing β-galactosidase. There are no differences between the electroporated (left) and the control (right) sides. Embryos were assayed 4 days postelectroporation.

animals, the former being also altered in shape. In 50% of the transgenic animals, the frontal and nasal bones were also affected (not shown).

In ovo misexpression of *En2* interferes with choroid plexus development

To evaluate the effects of ectopic En protein in another vertebrate species, we misexpressed *En2* in the chick embryo by in ovo electroporation; the En proteins are functionally equivalent (Hanks et al., 1995) and a specific monoclonal antibody, 4D9 (Patel et al., 1989), permits the detection of ectopic En2 protein.

We determined that when the *En2*-expressing plasmid was injected into the neural tube at the level of the first (developmentally older) somite at the 7- to 11-somite stage, En2 protein was consistently detected at the border of the tela choroidea 2 days after electroporation. To outline the border, we used in situ hybridization with *Gdf7* prior to immunodetection of En2 with 4D9. Ectopic expression of *En2* was consistently accompanied by abnormal morphology of the border of the tela choroidea (Fig. 5A). Comparison of the electroporated to the non-electroporated side of the same embryo served as control. Moreover, misexpression of a mutated form of En2 (Joliot et al., 1998) or of β-galactosidase, did not result in abnormal border morphology (Fig. 5B). No obvious differences in cell death were observed.

We asked whether this ectopic *En2* expression affected not only border morphology, but the development of the fourth ventricle choroid plexus as well. In chick, a tela choroidea is already evident at E3, but *Ttr* expression is initiated only at E4 and the expanded *Ttr*-positive territory starts folding by E6. Contrary to what is observed in the mouse, the caudal part of the tela choroidea remains *Ttr*-negative (unpublished observations and Fig. 5E). Embryos were electroporated at the 7- to 8-somite stage, harvested 4 days postelectroporation (at E6) and assayed for *Ttr* and *Gdf7* expression by in situ hybridization. 86% (24 out of 28) of embryos electroporated with the En2 expression plasmid were affected at E6; 19 embryos had a wavy border to the tela choroidea and fewer *Ttr*-expressing cells on the electroporated side; the expression of *Gdf7* was interrupted at the caudal mesencephalic or the cerebellar midline in 3 embryos; the remaining two were only slightly affected. In contrast, only 11% (2 out of 18) and 10% (1 out of 10) of the embryos electroporated with the β-galactosidase or the mutated-En2 expression plasmids respectively, had a wavy border at E6; expression of *Ttr* was unaffected (Fig. 5C,D,E).

Therefore, ectopic expression of En in the developing chick embryo affected the growth at the border of the tela choroidea and the expression of markers of the differentiating choroid plexus, much as ectopic expression of En in the mouse embryo disrupted the development of the choroid plexus.

DISCUSSION

We have analyzed the effects of ectopic expression of Engrailed in the dorsal midline of the developing mouse embryo. We show that constitutive expression of Engrailed causes mis-specification of roof plate cells, which in turn perturb the navigation of commissural axons, and interferes with the development of structures deriving from the dorsal neuroepithelium. These defects are preceded by a localized increase in cell death. We propose that prenatal hydrocephalus develops in this transgenic line as a consequence of subcommissural organ failure.

Ectopic En1 in the dorsal midline of the diencephalon prevents differentiation of the SCO and pineal gland and results in hydrocephalus

Ectopic expression of En1 in the dorsal midline results in prenatal hydrocephalus, which gets worse and persists into adulthood. In available animal models of hydrocephalus, the disease develops postnatally and usually displays incomplete penetrance and variable expressivity. It has been attributed to increased secretory activity of the choroid plexus (*E2F-5* mutants; Lindeman et al., 1998) or abnormal differentiation of the meninges (*hy3*; Berry, 1961). Prenatal hydrocephalus in the *ch/ch* mutant (Gruneberg, 1943) results from an inhibition of CSF flow into the subarachnoid space caused by a mutation in the gene *Mf1* affecting arachnoid cell differentiation (Kume et al., 1998).

In the WEXPZ.En1 line, prenatal hydrocephalus is most likely caused by the failure of SCO differentiation and is possibly influenced by the abnormal differentiation of the choroid plexus, the main site of CSF synthesis and secretion

(see below). The SCO is located at the anterior end of the cerebral aqueduct in close association with the ventricular surface of the posterior commissure and secretes glycoproteins into the CSF (Rodriguez et al., 1998). Although its function remains enigmatic, several experimental paradigms support the notion that the absence of SCO may be a primary defect of congenital hydrocephalus. The *hyh* mouse (Bronson and Lane, 1990; Perez-Figares et al., 1998) and several spontaneous congenital hydrocephalic rats and mice have smaller than normal SCO, or even lack this structure completely (Takeuchi et al., 1987, 1988; Yamada et al., 1992). Moreover, a reduction in size of the SCO has been reported in two cases of human fetal hydrocephalus (Castaneyra-Perdomo et al., 1994). The SCO does not differentiate normally in $Wnt1^{sw/sw}$ embryos either (this study); indeed, the *Wnt1* mutant embryos (and the rare adult survivors) exhibit hydrocephalus in the caudal region of the cerebral hemispheres and the midbrain (Thomas and Capecchi, 1990). Our results provide evidence that *Wnt1* is required for the differentiation of the SCO. SCO agenesis in transgenic embryos could be a consequence either of increased cell death, or of a change in the developmental fate of cells destined to participate in SCO formation due to their maintaining En1 expression. The former is more probable, as increased cell death in the dorsal midline precedes the generation of SCO precursors in the primitive ependymal zone (E11; Rakic and Sidman, 1968).

The calvarial bone alterations in the transgenic animals are likely secondary to hydrocephalus. A dome-shaped head is associated with hydrocephalus in rats (Park and Nowosielski-Slepowron, 1979) and in various mutant mice. Much of the head skeleton is generated by cephalic neural crest (Le Douarin and Kalcheim, 1999) and *Wnt1* (and therefore ectopic *En* from the transgene) is expressed in progenitors of the neural crest (Echelard et al., 1994), and Wnt signaling is required for their expansion (Ikeya et al., 1997). The possibility exists therefore that at least some of the abnormalities in the calvarium reflect perturbations of the neural crest, despite the fact that neural crest formation does not appear affected in the transgenic embryos (not shown). Indeed, we did observe unilateral abnormalities in paired calvarial bones in 10 out of 16 chick embryos at E16-E18, following misexpression of *En2* at E2 (but also in 5 out of 17 embryos misexpressing β-galactosidase) (not shown); overexpression of En at early stages can therefore interfere with the development of some neural crest derivatives.

Differentiation of the pineal anlagen is compromised in the transgenic embryos and the newborn pups lack a pineal gland. The pineal primordium appears as a midline evagination of the diencephalic roof (Calvo and Boya, 1981). Evagination does not take place in the transgenic embryos, possibly because pineal precursors are eliminated by excessive cell death. Alternatively, the maintenance of En expression prior to and during the evagination process, may alter the adhesive properties of the precursor cells and interfere with pineal morphogenesis.

En1 ectopic expression causes errors in axonal pathfinding

The posterior commissure is affected in the WEXPZ.En1 embryos. Severe underdevelopment or loss of the pc has been reported in the *Small eye /Pax6* mutant embryos (Stoykova et al., 1996; Mastick et al., 1997; Grindley et al., 1997) and, interestingly, in rats and mice with spontaneous congenital hydrocephalus (Takeuchi et al., 1987; Yamada et al., 1992). In contrast, the pc is expanded caudally up to the fourth ventricle choroid plexus in the *Pax2/Pax5* double homozygous mutants, in a domain coinciding with the expansion of *Pax6* expression (Schawrz et al., 1999). In transgenic embryos, the pc axons form tight bundles and either cross the midline irregularly, or take a longitudinal turn and fail to cross altogether; *Pax6* expression in caudal p1 is not affected, but both *ephrinA5* (this study) and *ephrinA2* (Lee et al., 1997) are ectopically expressed in a narrow stripe of cells along the dorsal midline of the anterior mesencephalon; *ephrinA5* alone is also induced in the midline of the diencephalon (this study). It is well documented that ectopic Engrailed results in transcriptional activation of both genes in the chick (Logan et al., 1996; Shigetani et al., 1997). Both ligands repel retinal axons in vitro (Davenport et al., 1998; Gao et al., 1998) and exert an inhibitory effect on neurite outgrowth (Ohta et al., 1997; Yue et al., 1999); *ephrinA5* induces apoptotic cell death (Yue et al., 1999). Thus, activation of ephrinA ligands in the midline may indirectly lead to cell death and may also have a repellent effect on the dorsally projecting axons of the pc. EphA4, a receptor for ephrinA5, is expressed in the region of the pc at E13.5 but not at E10.5 (our observations) and could function to titrate out the initial inhibitory effect of the ligands. Reelin, an extracellular matrix protein involved in cell adhesion, is also upregulated in cells surrounding the tight axonal bundles of the posterior commissure revealed by DiI tracing. It is possible that the mere presence of reelin enables these tight bundles to cross the midline. Finally, it is intriguing to note that solubilized proteins secreted by the SCO inhibit the aggregation of cortical neurons in culture (Gobron et al., 1996). Failure of SCO differentiation in the transgenic embryos may therefore allow the pc axons to aggregate, since the secretory cells of the SCO project processes dorsally (Rodriguez et al., 1998), and are in close apposition with pc fibers (Rakic and Sidman, 1965).

Overexpression of En disrupts choroid plexus development

Ectopic expression of *En1* in the transgenic embryos interferes with choroid plexus differentiation and growth in the third and fourth ventricles. Not surprisingly, the choroid plexus of the lateral ventricles is not affected, as no ectopic *En1* expression occurs in the developing telencephalon. Likewise, ectopic expression of *En2* in the chick disrupts the normal morphology of the border of the tela choroidea and the differentiation of choroid plexus epithelial cells. In both cases, ectopic En protein likely interferes with the differentiation program of the border cells expressing it and leads to impaired growth and incorrect development of the mature choroid plexus. Because choroid plexus differentiation is affected, synthesis and/or secretion of proteins into the CSF may also be affected, thus indirectly contributing to hydrocephalus.

The events leading to the formation of the choroid plexus (CP) are not well understood. A transition in the arrangement of the neuroepithelial cells from pseudostratified to simple columnar is the first morphological evidence of choroid plexus differentiation (Sturrock, 1979) and epithelial-mesenchymal inductive interactions between the developing CPe and the

invading mesenchymal cells are thought to control its differentiation (Birge, 1961, 1962; Wilting and Christ, 1989). In both mouse and chick, differentiation of the CPe in the fourth ventricle starts laterally, adjacent to the *Wnt1*-expressing border, and caudal to the edge of the cerebellar plate, as indicated by the expression pattern of *Ttr*. Growth factors expressed in the invading mesenchymal cells (such as Igf2) could act as inducers and members of the Wnt family may be components of a signaling system operating at the border of the tela choroidea. In fact, Wnt factors are known to be involved in epithelial-mesenchymal interactions (Herzlinger et al., 1994; Kispert et al., 1998; Patapoutian et al., 1999) and expression of the *Wnt2b*, *Wnt3a* and *Wnt*5*a* genes is required at the hem of the embryonic cerebral cortex for the correct development of the telencephalic choroid plexus (Grove et al., 1998). Early differentiation of the choroid plexus is perturbed in the *Wnt1*$^{sw/sw}$ mutant diencephalon and metencephalon. This early phenotype could, in part, be explained by increased cell death, documented in *Wnt1*$^{neo/neo}$ embryos along the most anterior edge of the dorsal metencephalon at E9.5 (Serbedzija et al., 1996) and functional redundancy between Wnt family members expressed at the midline may account for the relatively mild phenotype observed at late developmental stages.

Taken together, these observations suggest that the dosage of Wnt1 and En proteins is important for the regionalization of the choroid plexus, by analogy to the well-established idea that they are crucial for the establishment of the cerebellar territory and the correct development of the cerebellum (reviewed by Wassef and Joyner, 1997).

Engrailed as a repressor of diencephalic fate

Ectopic expression of Engrailed in the diencephalon was recently shown to result in activation of mesencephalic markers in chick (Araki and Nakamura, 1999) and medaka fish (Ristoratore et al., 1999). In chick, ectopic *En2* caused an early repression of *Pax6*, subsequent activation of mesencephalic markers, and only transient activation of isthmic markers (Araki and Nakamura, 1999). Thus, it was proposed that En normally acts to repress the diencephalic fate in the mesencephalon. Ectopic expression of *En1* in the diencephalic midline of the WEXPZ.En1 embryos results in transcriptional activation of *ephrinA5*, but there is no ectopic expression of other mesencephalic or isthmic markers. In addition, there is no induction of endogenous *En1* next to the diencephalic midline and expression of *Tcf4* is normal. These observations suggest that although the roof plate cells no longer retain a diencephalic character, they do not adopt a mesencephalic fate either. It is worth mentioning that in chick, cells at the diencephalic midline also escaped re-programming following ectopic expression of *En2* (Araki and Nakamura, 1999).

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