

## Anterior cephalic neural crest is required for forebrain viability

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Accepted 25 May; published on WWW 19 July 1999

### SUMMARY

The prosencephalon, or embryonic forebrain, grows within a mesenchymal matrix of local paraxial mesoderm and of neural crest cells (NCC) derived from the posterior diencephalon and mesencephalon. Part of this NCC population forms the outer wall of capillaries within the prosencephalic leptomeninges and neuroepithelium itself. The surgical removal of NCC from the anterior head of chick embryos leads to massive cell death within the forebrain neuroepithelium during an interval that precedes its vascularization by at least 36 hours. During this critical period, a mesenchymal layer made up of intermingled mesodermal cells and NCC surround the neuroepithelium. This layer is not formed after anterior cephalic NCC ablation. The neuroepithelium then undergoes massive apoptosis. Cyclopia ensues after forebrain deterioration and absence of intervening frontonasal bud derivatives. The deleterious effect of ablation of the anterior NC cannot

be interpreted as a deficit in vascularization because it takes place well before the time when blood vessels start to invade the neuroepithelium. Thus the mesenchymal layer itself exerts a trophic effect on the prosencephalic neuroepithelium. In an assay to rescue the operated phenotype, we found that the rhombencephalic but not the truncal NC can successfully replace the diencephalic and mesencephalic NC. Moreover, any region of the paraxial cephalic mesoderm can replace NCC in their dual function: in their early trophic effect and in providing pericytes to the forebrain meningeal blood vessels. The assumption of these roles by the cephalic neural crest may have been instrumental in the rostral expansion of the vertebrate forebrain over the course of evolution.

Key words: Pericyte, Telencephalon, Vascularization, Neural crest, Meninges, Forebrain, Chimera, Chick, Quail

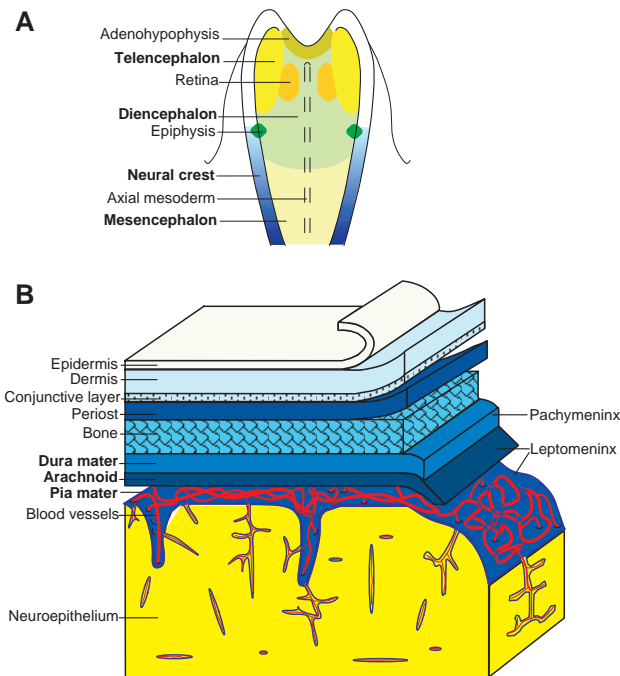
### INTRODUCTION

After neurulation, the cells of the vertebrate central nervous system proliferate extensively as they organize into functionally and morphologically distinct regions. The forebrain, derived from the embryonic prosencephalic vesicle, is structurally subdivided into (1) the diencephalon, yielding the eyes, optic nerves and chiasm, thalamus, hypothalamus and neurohypophysis, and (2) the telencephalon, essentially composed of the olfactory bulbs and cerebral hemispheres. Among chordates, the paired cerebral hemispheres are a vertebrate-specific evolutionary development. A fate map of the early anterior neural plate, constructed by the quail-chick chimera technique (Couly and Le Douarin, 1985, 1987), shows that, in birds, the presumptive territories of the cerebral hemispheres are located in two anterolateral areas adjacent to the neural folds. In contrast, the ventral diencephalic areas destined to yield the unique hypothalamus, posthypophysis and optic chiasm are found medially, separating the anlagen of the eyes; the territory of the adenohypophysis is located in the rostral transverse neural fold. The dorsal diencephalon, including the epiphysis, arises from the caudally adjacent region. Cephalic neural crest cells (NCC) emigrate from the fused neural folds caudal to the epiphysis, from neural plate

levels corresponding to the presumptive regions of the posterior diencephalon, mesencephalon and rhombencephalon (Fig. 1A; c.f. Couly and Le Douarin, 1987). Similar organization of the anterior neural plate appears to be conserved, with minor variations, in other vertebrate classes (reviewed in Rubenstein et al., 1998).

Following the mediodorsal closure of the neural tube, the eyes and telencephalon are sites of particularly intense growth. This cell proliferation leads to the rostral protrusion of first the eyes and then the cerebral hemispheres beyond the anterior end of the notochord and prechordal plate (e.g. Couly and Le Douarin, 1988; Shimamura et al., 1995).

Forebrain growth is accompanied by the concomitant development of the meninges, membranes surrounding the central nervous system, which comprise an outer dura mater and an inner leptomeninx (*lepto-*, thin). The leptomeningeal matrix is initially composed of mesenchymal NCC and a primitive vascular net of endothelial cells. Johnston (1966) first observed a potential contribution of NCC to the forebrain meninges, using a short-lived radioactive tracer. Subsequently, systematic labeling of defined regions of the cephalic neural folds, using the quail-chick chimera system, demonstrated that not only each layer of the forebrain meninges (Fig. 1B) but also the facial skeleton, frontal and parietal bones, and overlying



**Fig. 1.** (A) Fate map of the anterior neural plate. Adapted from Couly et al. (1987, 1988). The paired territories of the presumptive telencephalon lie in the lateral neural plate, dorsal to the future anterior diencephalon and rostral to neural crest-producing neural folds (blue). (B) Anatomy of the layers of the late embryonic head. Blue layers correspond to derivatives of the neural crest surrounding the anterior brain. The meninges (dura mater, arachnoid and pia mater) enclose the entire central nervous system (CNS). The pia mater continuously surrounds all of the circumconvolutions of the CNS and isolates ingressing arachnoidal blood vessels from the neuroepithelium. It is a constituent of the blood-brain barrier. The subarachnoid space, well-defined in humans, is obscured in the chicken. The term 'leptomeninx', employed in the text, thus refers to both the arachnoid and pial layers of the meninges.

dermis originate from the cephalic neural crest (Le Lièvre and Le Douarin, 1975; Couly and Le Douarin, 1987; Couly et al., 1993). The NC contribution to the leptomeninx includes pericytes and connective tissue cells (H. C. E., G. C. and N. M. Le D., unpublished data), but excludes the endothelial cells, which arise from a small territory of the anterior paraxial mesoderm within the head fold, adjacent to the prosencephalon (Couly et al., 1995).

Thus, the cephalic NCC penetrate the forebrain neuroepithelium together with vascular buds made up of endothelial cells. This double origin of the leptomeninx from the NC and paraxial mesoderm is exclusively found in the forebrain; in the rest of the central nervous system (CNS), the meninges are entirely of mesodermal origin (Le Lièvre, 1976; Couly et al., 1992).

Unlike pericytes, which can be either of neural crest or mesodermal origin, blood vessel endothelial cells of both the head and body are strictly mesodermal in origin (reviewed in Le Douarin, 1982). The blood vessel endothelium segregates from other cephalic mesodermal derivatives as early as the head-fold stage, as seen by the precocious expression of a tyrosine kinase receptor to the vascular endothelium growth

factor (VEGF), designated as VEGFR2 (Eichmann et al., 1993). VEGFR2-expressing cells give rise to the endothelial walls of blood vessels, which form the perineural vascular plexus of the developing leptomeninx in the head (Eichmann et al., 1993).

The coincidence of meningeal construction with the initial phase of forebrain growth led to the present investigation, aimed at exploring how the anterior cephalic NCC might participate in the development of the forebrain. Strikingly, after removal of the posterior diencephalic and mesencephalic neural folds, the neuroepithelium of the entire future forebrain underwent apoptosis within 1 day following closure of the anterior neural tube, although the telencephalic neural folds had been left intact. Cell death occurred well before the onset of budding of the bulbs destined to form the telencephalic cerebral hemispheres. The ventral diencephalon and retinae were not included in this degenerative process because ectopic migration and proliferation of NCC populations caudal to the excised territory partially compensated for the rostral deficiency. Embryos subjected to neural fold ablation became cyclopic, while the mesencephalon and a variable extent of the caudal and ventral diencephalon became in turn the rostralmost portion of the brain.

The timing of forebrain apoptosis led to the conclusion that the presence of NC-derived mesenchyme is necessary for the survival and growth of the prosencephalic neuroepithelium during a phase preceding the onset of its vascularization by at least 36 hours. NCC transplanted heterotopically from the level of the rhombencephalon, but not the trunk, can compensate for the effects of anterior neural fold ablations. The grafted compensatory cells later differentiate into rostral head-specific derivatives such as meningeal and intraencephalic pericytes. The entire cephalic NC is therefore capable of participating together with endothelial cells to build the meninges. In normal development, however, only the rostral population does so. This emphasizes that the paraxial mesodermal population near the prosencephalon behaves differently from that of the rest of the head, since on its own it is not capable of constructing forebrain meninges.

In all other parts of the CNS, the paraxial mesoderm ensures the construction of the leptomeninx. The reason for which it is not so for the anterior brain may be because the amount of paraproencephalic mesoderm is scanty during forebrain expansion. To compensate this deficit of mesodermal cells, NCC take over the production of pericytes and other connective tissue in this area. In fact, NCC have already been shown to play this role in the construction of the skull around the forebrain and in forming the dermis in the facial and forehead areas.

We have thus tested the capacity of various types of mesoderm to replace the anterior NC and to form a complete leptomeninx, which permits the survival and development of the cerebral hemispheres. When added to neural fold-ablated embryos, any level of the paraxial mesoderm (but not trunk lateral plate mesoderm) can replace the NCC to partially or fully maintain viability in the forebrain neuroepithelium. Grafted paraxial mesoderm differentiates into both endothelial cells and pericytes under these circumstances. Our results thus indicate that it is the presence of a primitive leptomeninx that is needed for the survival and subsequent growth of the developing prosencephalon.

## MATERIALS AND METHODS

### Operations

Chimeras were constructed between quail and chick embryos by grafting either mesoderm or neural folds from the rhombencephalic or truncal levels of stage-matched quail donors to the prosencephalic area of chick hosts. These are described below and schematized in Fig. 2. *Gallus gallus* (JA57 line, Institut de Sélection Animale, Lyon, France) and *Coturnix coturnix japonica* eggs were incubated for approximately 30 hours to obtain embryos from between the 2- and 5-somite stage, stage 7 to 8 of Hamburger and Hamilton (1951, HH7-8), or between the 7- and 10-somite stage, HH9-10 for late controls.

The fate maps of the neural folds constructed by Couly et al. (1993) and Grapin-Botton et al. (1995) were used to define the regions corresponding respectively to the telencephalon, diencephalon, mesencephalon and first rhombomere (r1) at the stage of the operation. The length of each presumptive area was then determined in a case-specific manner by using an ocular micrometer; the anterior extremity of the embryo and the anterior limit of the first somite pair were chosen as fixed reference points. Host embryos were visualized by injection under the blastoderm of 5% India ink in PBS, or in Tyrode's solution, supplemented with antibiotics (Gibco). Embryos were subsequently fixed at a range of times from 6 hours after the operation to embryonic day 9 (E9).

### Experiment 1: neural fold ablations

The cephalic neural folds release NCC from the mid-diencephalic level caudally (Couly and Le Douarin, 1987). Anterior to this level, which corresponds to the site of the prospective epiphysis, the neural folds remain epithelial. The effects of the removal of the entire pool of NCC that invest the anterior head were examined in this group of experiments. The neural folds of the posterior diencephalon, mesencephalon and rhombomere 1 (r1) (Experiment 1a, 1d), or the posterior diencephalic and mesencephalic neural folds alone (Experiment 1b, 1c), were ablated by extirpation with tungsten scalpels. Neural folds were removed either bilaterally (Experiments 1a, 1b, 1d) or unilaterally (Experiment 1c). To confirm that none of these ablations included the presumptive territory of the telencephalon, a length of neural folds including the levels of posterior diencephalon, mesencephalon and r1 was replaced bilaterally with the equivalent tissue from stage-matched quail donors (Experiment 1d). In Experiment 1e, similar bilateral neural fold ablations to Experiment 1a were made at HH9-10, after NCC emigration had begun.

### Experiment 2: capacity of r1 and posterior neural folds to compensate for removal of the anterior source of NCC

This experimental series was aimed at testing the capacity of NCC from posterior axial levels to replace the rostral cephalic NCC. Three types of experiments were performed, in all of which the neural folds from the posterior diencephalon, mesencephalon and r1 were removed bilaterally. For Experiment 2a, the r1 segment was replaced bilaterally by its quail counterpart, leaving the posterior diencephalon and mesencephalon without neural folds. In Experiment 2b, the entire length of the ablation was replaced by unilateral or bilateral grafts of neural folds corresponding to r4 through r8 (limit between somite pairs 4 and 5). The same length of neural folds from the unsegmented trunk level of quail donors, at stages ranging from 10 to 17 somites, was grafted bilaterally in Experiment 2c.

### Experiment 3: cephalic mesoderm grafts

The paraxial mesoderm adjacent to the presumptive ventrolateral diencephalon (area C of Couly et al., 1995; area 5 of Couly et al., 1992) was exposed by surgical removal of the ectoderm from quail donors at 2- to 5-somite stages. In order to test its endogenous capacity to give rise to pericytes, this 'paraprosencephalic' mesodermal population was removed by means of a Pasteur pipette pulled to a diameter of approximately 80  $\mu\text{m}$ , and transferred to stage-matched

chick hosts in which the homotopic mesenchyme had been disrupted (Experiment 3a). In another experiment, the same graft was placed heterotopically at the level of the mesencephalon (Experiment 3b).

### Experiment 4: mesodermal grafts after neural fold ablations

Bilateral neural fold ablations were performed as described in Experiment 1a on chick hosts. Next, paraxial mesoderm from adjacent to the ventrolateral diencephalon (Experiment 4a, 'paraprosencephalic' mesoderm) or lateral mesencephalon (Experiment 4b, 'paramesencephalic' mesoderm) was surgically removed from quail donors and grafted into the chick host dorsally, and secured under adjacent rostral ectoderm. Paraprosencephalic and paramesencephalic mesoderm correspond respectively to areas 5 and 3 of Couly et al. (1992) or areas C and D of Couly et al. (1995). In Experiments 4c and 4d, large pieces of trunk mesoderm were isolated from 10- to 17-somite-stage quail donors in the non-segmented region and cleaned of surrounding tissues (including ectoderm) by means of 1 $\times$  pancreatin (Gibco) in PBS. Then grafts of approximately 150 $\times$ 300  $\mu\text{m}$  were cut from paraxial (Experiment 4c) or lateral plate (Experiment 4d) mesoderm for transfer to hosts as above.

### Immunohistochemistry

Three monoclonal antibodies (mAb) were used: QCPN (anti-quail, Developmental Studies Hybridoma Bank; undiluted hybridoma supernatant of IgG1 isotype), MB1/QH1 (anti-quail endothelium and white blood cells, Péault et al., 1983, and Pardanaud et al., 1987; 1:1500 dilution of ascites fluid of IgM isotype), and 1A4 (anti-smooth muscle actin, Sigma; 1:400 dilution of ascites fluid of IgG2a isotype). Embryos were processed as described by Catala et al. (1996), using appropriate goat secondary antibodies conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP). The chromogenic reaction for AP was performed using the Vector AP substrate kit III according to manufacturer's instructions; for HRP, 0.1 mg/ml diaminobenzidine (Sigma) and 0.005% H<sub>2</sub>O<sub>2</sub> in PBS were used. In triple-stained sections, slides were bathed in 0.1 M glycine, pH 2.2 after the first AP reaction, before applying the next primary antibody and another AP-conjugated secondary antibody. Under these circumstances, the second AP-conjugated immunocomplex was revealed with Fast Red substrate tablets (Sigma). Sections were generally counterstained in Gill's hematoxylin solution and observed under a Leica light microscope.

### Cell death and proliferation

29 embryos from Experiment 3a were harvested at HH19. All were soaked in Nile blue-containing Pannett and Compton's solution (Jeffs and Osmond, 1992) to examine cell death in toto, and photographed while the embryos were still alive. After paraffin sections were cut at 5  $\mu\text{m}$  and rehydrated, the TUNEL reaction was carried out on 23 embryos according to the instructions in the kit by Boehringer Mannheim, using 0.1 mg/ml diaminobenzidine, 0.005% H<sub>2</sub>O<sub>2</sub> in PBS as the chromogenic substrate for localization of cell death.

### In situ hybridization

Probes were synthesized from linearized template plasmid for chicken *Hoxa3* (a kind gift of Dr R. Krumlauf). *Hoxa3* riboprobe incorporating <sup>35</sup>S-UTP (Amersham) were applied to sections of embryos fixed in Carnoy's solution, pretreated as described by Eichmann et al. (1993). Rinses were performed according to Wilkinson and Nieto (1993). Slides were dipped in 1:1 water/Kodak NTB-2 photographic emulsion, developed 10 days later in Kodak D-19 and counterstained with Gill's hematoxylin.

## RESULTS

### Gross anatomy of the head following the partial ablation of cephalic neural folds

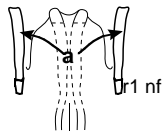
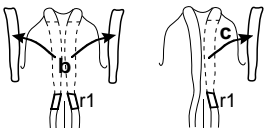
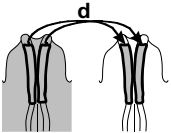
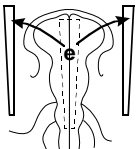
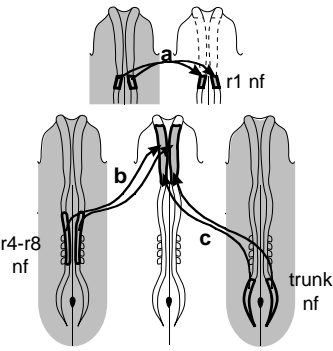
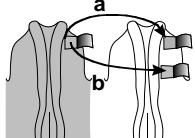
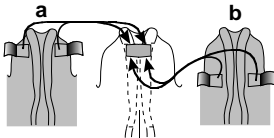
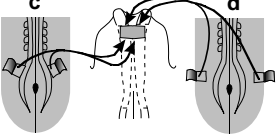
We first examined the effects of ablation of the neural folds from which NCC emigrate to participate in the telencephalic

and diencephalic meninges (Experiment 1). These correspond to the neural folds of the future diencephalon, caudal to the level of the epiphysis, and the mesencephalon (Couly et al., 1987).

In Experiment 1a (Fig. 2), a length of neural folds including those of the posterior diencephalon, mesencephalon and the first rhombomere (r1) was removed bilaterally (Fig. 3A-C). As

a result, the telencephalon and dorsal diencephalon of operated embryos were strikingly absent in 37/37 cases observed from embryonic day (E)3 on (E3,  $n=26$ ; E4,  $n=1$ ; E5,  $n=2$ ; E6,  $n=1$ ; E7,  $n=3$ ; E8,  $n=4$ ). The interocular distance was visibly reduced in 14/24 embryos at E2 (HH15-HH18). This phenotype was always accompanied by the severe reduction or absence of the frontonasal bud, resulting in various degrees of fusion of the optic cups going from synophthalmia with associated hypotelorism (Fig. 3H-J) to complete cyclopia (Fig. 3D-G). In operated embryos, the two independent retinal domains had converged after formation, but the eyes shared a medial lens and orbit (Fig. 3F). Oculomotor muscles of the rectus medialis were equally absent in cyclopic embryos. While the cerebral hemispheres were never present, the size of the ventral prosencephalon varied. In many animals, the optic chiasm, hypothalamus and neurohypophysis were present. In extreme cases ( $n=6$  out of 37 embryos at E3-E8), the diencephalon was reduced to a stub with a recognizable neurohypophysis (Fig. 3F). The adenohypophysis was always formed as well. The volume of the mesencephalic ventricle varied, but the morphology and neuronal lamination of the optic tecta appeared essentially normal (Fig. 3F).

Experiment 1b consisted of a similar ablation that did not include the neural folds of r1. In 4 out of 7 cases, we saw the same severe deficiencies as when the ablation included the neural folds of r1. However, normal external head morphology was observed in 3 out of 7 embryos between E4 and E7 (not

Exp't	n (stages)	
1a	60 (E2-E8)	
1b	7 (E4-E7)	
1c	5 (E5, E6)	
1d	3 (E4-E8)	
1e	8 (E4-E5)	
2a	6 (E3-E8)	
2b	31 (E2-E8)	
2c	3 (E4, E5)	
3a	2 (E6)	
3b	3 (E5-E9)	
4a	8 (E5)	
4b	21 (E3-E6)	
4c	6 (E3-E8)	
4d	5 (E3, E8)	

**Fig. 2.** Schematic representation of the experimental strategy. The number of cases for each type of experiment, and their ages at harvest, are indicated next to a diagram illustrating the manipulation or graft. Host embryos were operated at HH7-8 unless otherwise indicated. Experiment 1a: ablation of posterior diencephalic, mesencephalic and first rhombomeric (r1) neural folds (nf). Experiment 1b: ablation of posterior di- and mesencephalic neural folds alone. Experiment 1c: unilateral ablation of posterior di- and mesencephalic neural fold. Experiment 1d: homotopic, bilateral replacement of posterior di- and mesencephalic neural folds of chick with quail equivalent. Experiment 1e: ablation of posterior di-, mesencephalic and r1 neural folds in HH9-10 embryos, after NCC migration had begun. Experiment 2a: homotopic, bilateral replacement of r1 neural folds of chick with quail equivalent, after ablation of posterior diencephalic, mesencephalic and r1 neural folds in host. Experiment 2b: heterotopic, bilateral replacement of posterior di-, mesencephalic and r1 neural folds of chick with a length of quail neural folds corresponding to r4-r8. Experiment 2c: heterotopic, bilateral replacement of posterior di-, mesencephalic and r1 neural folds of chick with a length of quail neural folds corresponding to unsegmented somitic levels of donors at HH10-12. Experiment 3a: homotopic graft of parapsencephalic mesoderm from quail to chick. Experiment 3b: heterotopic graft of parapsencephalic mesoderm from quail to chick at level of mesencephalon. Experiment 4a: addition of parapsencephalic mesoderm from quail to chick in which posterior diencephalic, mesencephalic and r1 neural folds had been previously ablated. Experiment 4b: addition of parapsencephalic mesoderm from quail to chick in which posterior di-, mesencephalic and r1 neural folds had been previously ablated. Experiment 4c: addition of trunk-level paraxial mesoderm from HH10-12 quail to chick in which posterior di-, mesencephalic and r1 neural folds had been previously ablated. Experiment 4d: addition of trunk-level lateral plate mesoderm from HH10-12 quail to chick in which posterior di-, mesencephalic and r1 neural folds had been previously ablated.



shown). In these cases, the telencephalon, though present, was reduced in size compared to stage-matched control embryos.

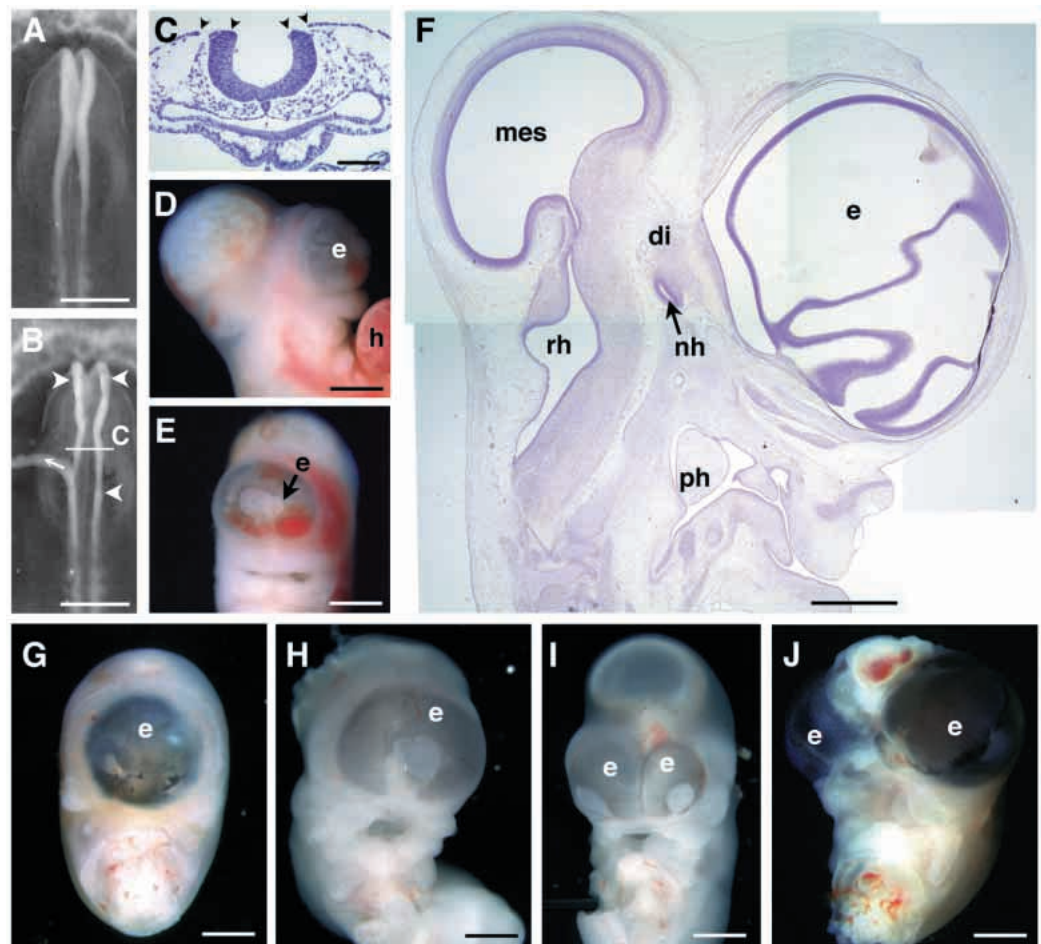
In order to see that the effects of neural fold ablation were indeed due to the NCC, three control experiments were performed (not shown). First, unilateral neural fold ablations (Experiment 1c, Fig. 2) did not lead to significant morphological consequences in any of 5 operated embryos, through contralateral NC compensation. Second, bilateral replacement of the ablated tissue in 3 embryos (Experiment 1d, Fig. 2) with similarly excised neural folds from stage-matched quails, confirmed that the normally formed telencephalon and anterior diencephalon were of host origin when examined at E4 and E8, although the meninges were graft-derived. Third, bilateral ablation of the dorsal neural tube, including the neural folds, from embryos in which NCC migration had previously begun (Experiment 1e, Fig. 2) led to the maintenance of telencephalic cerebral hemispheres in all 8 embryos examined at E4 and E5.

Removal of the anterior neural folds prior to NCC emigration thus severely hampers the development of the telencephalon, the dorsal diencephalon, the frontonasal bud and the anterior part of the ventral diencephalon, while not affecting that of the eyes. As a consequence of the defect in telencephalic and frontonasal development, the two eye fields tend to fuse at the midline, generating various degrees of cyclopia.

### Defective forebrain development is a result of massive cell death in the telencephalic and diencephalic neuroepithelium

Cell death was examined in toto, in control embryos and after bilateral neural fold ablations (Experiment 1a), prior to fixation at HH15 (E2), HH18 (late E2) and HH19 (early E3). Control embryos ( $n=8$ ) took up Nile blue sulfate, showing cell death concentrated in the ventral optic lens (Fig. 4A,G, arrows) and nasal epithelium (Fig. 4G, arrowhead). However, a prominent blue zone of cell death was apparent in the prosencephalon at HH15 (Fig. 4D, arrow) or localized in its protuberant remnant in its protuberant remnant below the eyes at HH18-19 (Fig. 4J, arrow). The TUNEL

technique confirmed numerous apoptotic figures in sections through the same areas (HH15,  $n=6/6$ ; HH18-19,  $n=15/17$ ). In unoperated embryos, apoptosis within the prosencephalon was sparse at all ages with the exception of a dense, localized region of the future olfactory neuroepithelium (Fig. 4C at HH15; Fig. 4I at HH18). Stage-matched operated embryos had a zone of apoptosis which included and extended beyond these areas to comprise most of the prosencephalic vesicle (Fig. 4F). The telencephalon is substantially reduced by the third embryonic day (Fig. 4J,K), and what is left is undergoing apoptosis (Fig. 4L). In unoperated embryos, NCC and mesodermal cells finish surrounding the anterior encephalic vesicle and form the leptomeninges at HH15, while vascular invasion of the telencephalic neuroepithelium by NC-supported capillaries normally begins at HH24 (H. C. E., G. C. and N. M. Le D., unpublished data). In the absence of the anterior NC population, prosencephalic cell death is underway on HH15 (Fig. 4E) and the prospective telencephalon already eliminated



**Fig. 3.** Ablation of cephalic NC causes the subsequent absence of forebrain territories (Experiment 1). Embryo before (A) and after operation (B, between arrowheads) at 5-somite stage. The right neural fold has been ablated but is still being detached on the left (arrow). (C) Transverse section taken through the level of operation of same embryo, fixed 2 hours after neural fold ablation (arrowheads). (D,E) A synophthalmic embryo at E8 in side and frontal view (h, heart). (F) In a parasagittal section from the same embryo, the fused eyes (e) are directly apposed to the remnant of the diencephalon (di), and the entire telencephalon is missing. The retinal fusion interface is apparent; part of the neurohypophysis (nh) is visible (mes, mesencephalon, rh, rhombencephalon, ph, pharynx). (G-J) Examples of facial malformations range from complete cyclopia and reduction of naso-fronto-maxillary structures (G) to synophthalmia (H, I) to hypotelorism (J). Bars: A,B,F, 0.5 mm; C, 100 μm; D,E,G-J, 1 mm.

by HH19. Apoptosis thus begins up to 58 hours before normal vascularization of the forebrain and is complete 22 hours later.

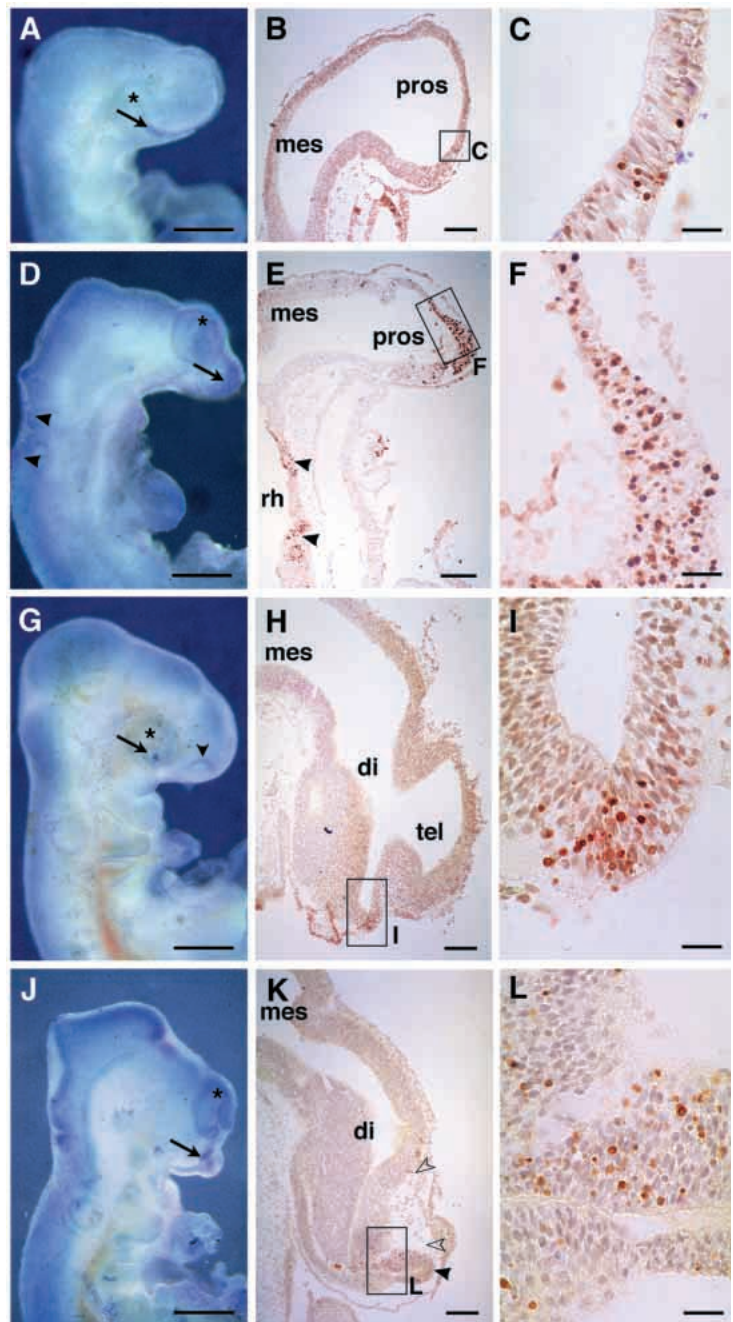
Because the presumptive telencephalic territory itself is not included in the neural fold ablations of Experiment 1, damage to it cannot be responsible for the telencephalic deficiency and cell death observed. One hypothesis to explain the elimination of the forebrain in absence of NCC is that their proximity is critical for survival and further development of the forebrain. We then explored whether other cell populations also had the capacity to restore telencephalic growth in place of the endogenous NC.

#### NCC from r1 can migrate rostrally and partly rescue the survival of the telencephalon

When only the diencephalic and mesencephalic neural folds were removed in Experiment 1b, nearly half of the embryos retained diencephalic and some telencephalic tissue. One would expect that these regions would be absent, since the diencephalic and mesencephalic neural crest give rise to meninges that cover the entire diencephalon and telencephalon. Based on previous results (see Fig. 7 of Couly et al., 1996), we postulated that the host NC could be responsible for a partial compensatory effect through rostral migration. Experiment 2a was designed to see whether NCC from r1, which does not normally make meningeal pericytes, would be able to migrate rostrally in the absence of anterior NCC. A length

of neural folds including those of the posterior diencephalon, mesencephalon and r1 was ablated as in Experiment 1a, and the neural folds of r1 were replaced bilaterally by their quail equivalent (Figs 2, 5A). Out of the 6 embryos observed, 3 embryos retained some telencephalic neuroepithelium (E3,  $n=1$ ; E6,  $n=1$ ; E8,  $n=1$ ; Fig. 5D), while 3 embryos lacked the entire telencephalon (E3,  $n=1$ ; E6,  $n=2$ ; Fig. 5B). In the embryos examined at E3, quail cells were found to have migrated into the mesenchyme ventral and anterior to the eyes (not shown). At E6 and E8, grafted cells from r1 became pericytes within either the residual diencephalon (Fig. 5C) or the diencephalon and telencephalon (Fig. 5E,F). These cells did not express the antigen for MB1/QH1, a mAb recognizing quail endothelial and blood cells (Fig. 5C), but did contain

**Fig. 4.** The prosencephalon undergoes progressive apoptosis after removal of the diencephalic and mesencephalic NC (Experiment 1). (A) Unoperated embryo stained with Nile blue, HH15. A stripe of normal cell death is indicated in the ventral eye and prosencephalon (arrow). (B) Parasagittal section through the embryo in A, after the TUNEL reaction. The region magnified in C is indicated. (C) Natural apoptosis occurs in a restricted part of the ventral prosencephalon. (D) Operated embryo at HH15, Nile blue. The eyes are closer together and a large zone of apoptosis is visible (arrow). Two normal domains of cell death in the rhombencephalon are indicated with arrowheads. (E) Parasagittal section through the embryo in D, after the TUNEL reaction. The region magnified in F is indicated, and the two zones of normal hindbrain apoptosis shown with arrowheads. (F) Most of the prosencephalon is undergoing vigorous apoptosis at this stage, although dying cells have not yet been cleared (compare brain profiles in B and E). (G) Unoperated embryo stained with Nile blue, HH18. Normal cell death is present in the optic lens (arrow) and nasal epithelium (arrowhead), as well as in scattered ectodermal cells. (H) Parasagittal section through the embryo in G, after the TUNEL reaction. The region magnified in I is indicated. (I) A restricted zone of the prospective olfactory neuroepithelium is undergoing apoptosis, but cell death is sparse elsewhere in the forebrain. (J) Operated embryo at HH18, Nile blue. The eyes are nearly synophthalmic in this case, and a dying remnant of the forebrain is indicated with an arrow. (K) Parasagittal section through the embryo in J, after the TUNEL reaction. The region magnified in L is indicated. Note that the caudal diencephalon is mostly intact in this embryo, but the telencephalon is severely reduced (arrowhead). Many cells have already been cleared dorsally (between open arrowheads; compare profiles in H and K). (L) The remnant of telencephalon is undergoing widespread cell death. Pros, prosencephalon; tel, telencephalon; di, diencephalon; mes, mesencephalon; rh, rhombencephalon; asterisk, eye. Bars: A,D,G,J, 0.5 mm; B,E,H,K, 100  $\mu$ m; C,F,I,L, 20  $\mu$ m.





alpha smooth muscle actin, characteristic of pericytes (Fig. 5E,F). The majority of NCC from r1 remained ventrolateral to the eyes, participating in the first branchial arch, mesenchymal cells of the adenohypophysis and some ventrolateral periocular mesenchyme.

NCC from r1 normally migrate into the first branchial arch and give rise to mandibular and hyoid components (Couly et al., 1996; Köntges and Lumsden, 1996). We show here that when the neural folds anterior to r1 are removed, NCC from r1 are capable of a significant rostral migratory diversion, into areas normally colonized by NCC from diencephalic and mesencephalic neural folds, where they partially compensate for the ablation and rescue forebrain tissue. Such compensation attenuates to varying degrees the phenotype observed after removal of the anterior cephalic neural crest.

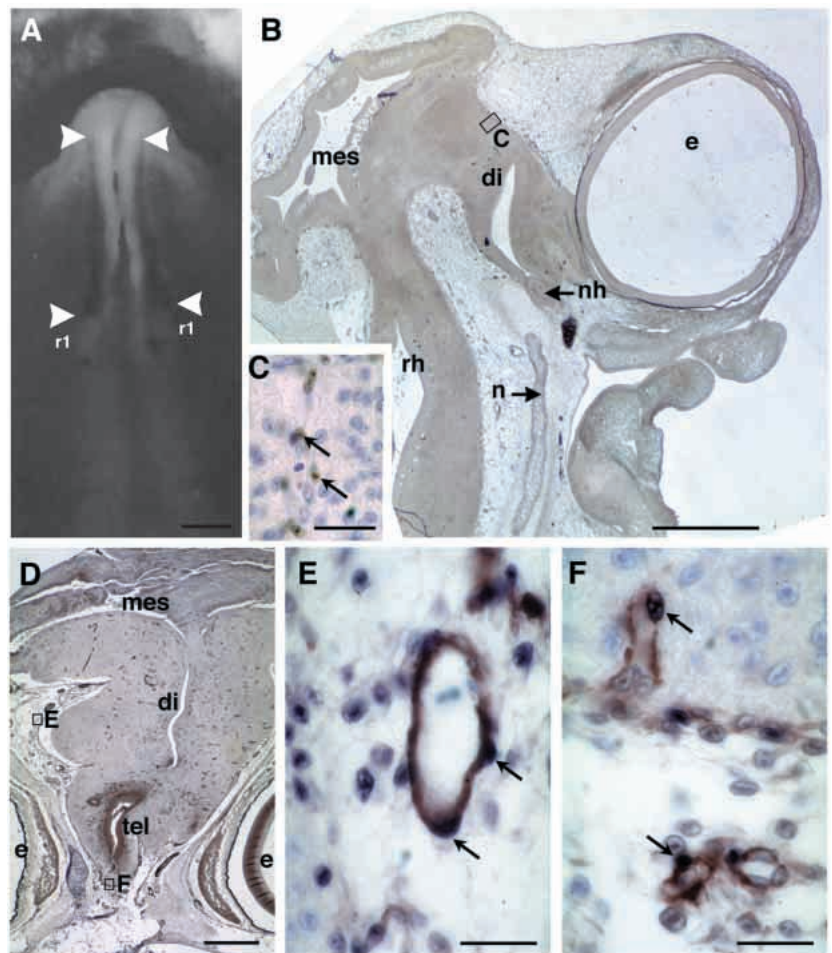
### NCC from the posterior rhombencephalon can substitute for anterior populations and rescue telencephalic survival

In a second step, we tested the capacity of posterior rhombencephalic NCC to prevent forebrain cell death. NCC from rhombomeres 4 to 8, like the neuroepithelium from which they derive, express *Hox* genes of the first four paralogue groups when migrating in situ. They normally do not participate in the meninges of the hindbrain or any other region, but do migrate into all branchial arches except for the first arch (Couly et al., 1996). In Experiment 2b (Fig. 2), the neural fold corresponding to r4 to r8 included (i.e. from the r3/r4 limit to the level of the fourth somite), was transplanted unilaterally (E2,  $n=9$ ; E3,  $n=2$ ; E5,  $n=2$ ; E6,  $n=8$ ; E8,  $n=1$ ) or bilaterally (E2,  $n=3$ ; E3,  $n=1$ ; E5,  $n=2$ ; E6,  $n=2$ ; E7,  $n=1$ ) from quail donors into chick hosts after an ablation including the neural folds of the posterior diencephalon, mesencephalon and r1 (Fig. 6A). The grafted neural fold integrated seamlessly into the host, and embryos formed normally (Fig. 6B). The heterotopic NCC contributed to each of the derivative cell types described for the homotopic anterior NCC population: meningeal pericytes (Fig. 6C), frontonasal bud cartilage, dermis, periocular structures and connective tissue of the oculomotor muscles and adenohypophysis. Pericytes derived from the grafted NCC were found uniquely within the prosencephalon and its meninges. Nonetheless, some of the posterior rhombomeric crest population retained the expression of *Hoxa3* in ectopic locations such as the mesenchyme surrounding Rathke's pouch and the ventral diencephalon as well as the first branchial arch (Fig. 6D,E). Interestingly, *Hoxa3* was not visible in the meninges or pericytes that colonized the forebrain region (not shown). These grafted cells apparently downregulated *Hoxa3* expression after having reached the anterior neuroepithelium.

In Experiment 2c, a length of approximately 450  $\mu\text{m}$  of neural folds from the unsegmented trunk level of 10- to 17-somite-stage quail donors was grafted bilaterally into the di-/mesencephalic region (E4,  $n=1$ ; E5,  $n=2$ ). Embryos presented defects identical to those in which the ablation of the endogenous neural folds was not followed by any graft (c.f. Le Douarin et al., 1977). The few migratory cells observed were sometimes associated with cranial nerve IV (data not shown).

### Restoration of prosencephalic development by mesoderm addition

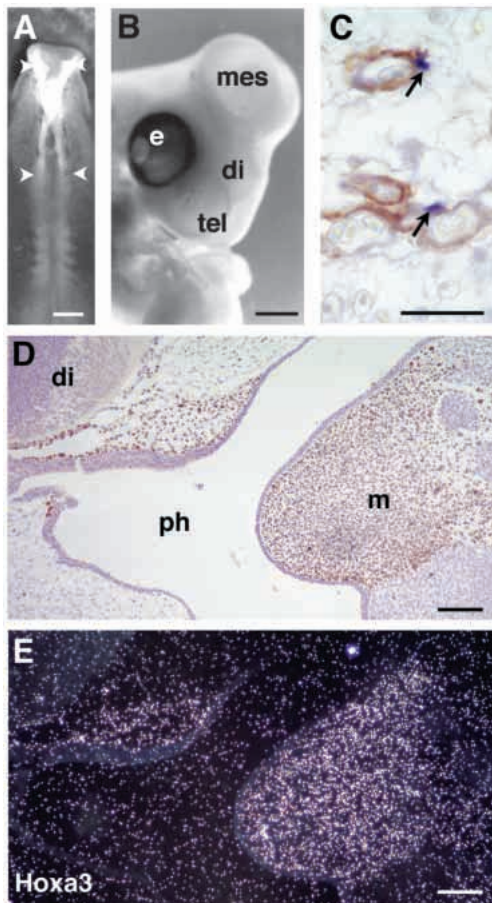
After bilateral ablation of a length of neural folds



**Fig. 5.** Partial compensation for the NC deficiency after ablation of diencephalic and mesencephalic neural folds comes from NCC of the first rhombomere (Experiment 2a). (A) Immediately after operation, at 4-somite stage. The length of the neural fold ablation is indicated between the arrowheads and bilaterally grafted r1 neural folds are visible below the ablation. (B) The range of external defects in the 6 embryos examined resembled that observed after Experiment 1b. This case, shown in slightly parasagittal section at E6, lacked a telencephalon and was synophthalmic (di, diencephalon; mes, mesencephalon; rh, rhombencephalon; n, notochord; nh, neurohypophysis). (C) Grafted cells are found within the diencephalic meninges and penetrating the neuroepithelium (arrows), in association with blood vessels. (D) Another case, shown in transverse section at E6, had some telencephalic tissue (tel) and normally spaced eyes (e). Areas enlarged in E and F are indicated. (E) r1 NCC (blue), when associated with blood vessels, colocalize with alpha-smooth muscle actin (brown), confirming that they are pericytes. (F) Graft-derived pericytes are found in the telencephalic meninges and neuroepithelium, although r1 NCC do not normally participate in meninges. Bars: A, 200  $\mu\text{m}$ ; B,D, 0.5 mm; C,E,F, 20  $\mu\text{m}$ .

corresponding to the posterior diencephalon, mesencephalon and r1, the endogenous paraxial mesoderm, adjacent to the ventrolateral prosencephalon at HH7-8 (termed 'paraprosencephalic'), is not sufficient to support prosencephalic viability and growth. This might be due either to the incapacity of this mesodermal area to yield pericytes in the brain, or to the fact that the number and placement of mesodermal cells in the paraprosencephalic area do not allow the production of both endothelial and pericytic cell populations for the dorsal prosencephalon.

First, to establish if the paraprosencephalic mesoderm normally differentiates into pericytes as well as endothelial cells (Couly et al., 1995), we grafted it from quail donors into chicken hosts at the prosencephalic level near the neural tube



**Fig. 6.** Heterotopic substitution of the diencephalic and mesencephalic neural folds by those of the posterior rhombencephalon leads to respecification of the fate of the grafted NCC (Experiment 2b). (A) Operated embryo, with neural folds from the level of r4 to r8 grafted between the arrowheads (corresponding to the level of the posterior diencephalon, mesencephalon and r1). (B) The same embryo at E6, with normal forebrain vesicles. (C) Grafted NCC within the forebrain meninges (blue, arrows) also make alpha-smooth muscle actin (brown). (D) The ectopic NCC (brown, QCPN; parasagittal section of embryo in B) retain expression of the anteroposterior position gene *Hoxa3* in inappropriate locations. (E) In an adjacent section, *Hoxa3* expression in the first branchial arch and around the ventral diencephalon. Di, diencephalon; m, maxillary arch; ph, pharynx. Bars: A, 200  $\mu$ m; B, 1 mm; C-E, 50  $\mu$ m.

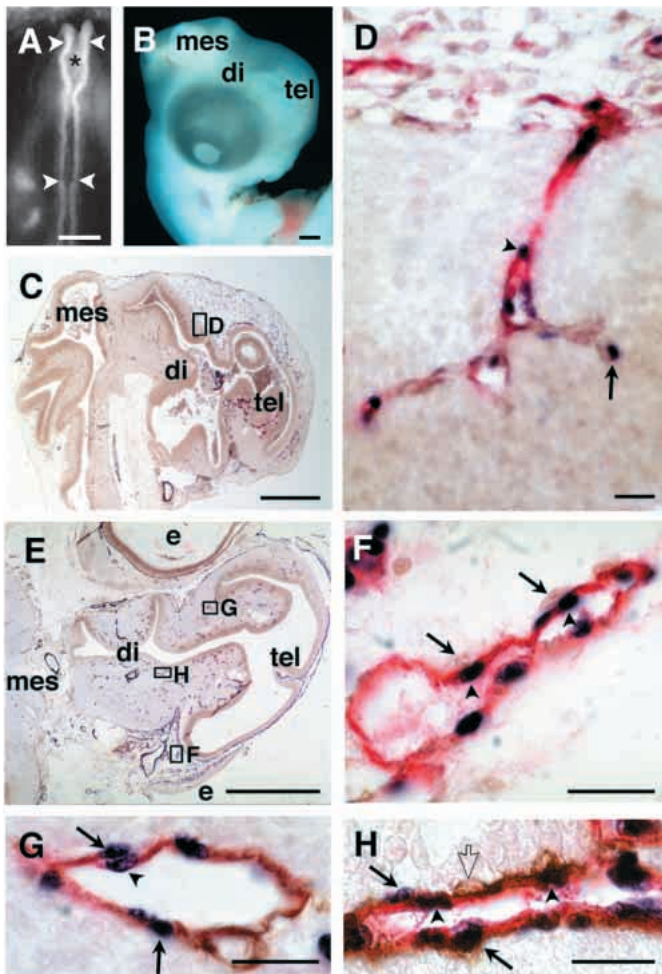
in addition to the endogenous paraprosencephalic mesoderm (Experiment 3a, Fig. 2). At HH29 (E6, not shown), grafted cells participated in the endothelial wall of blood vessels both in and around the forebrain ( $n=2$ ). While most quail cells were MB1/QH1 positive, a few were identified by the mAb 1A4 as pericytes in blood vessels near the eyes (not shown), although grafted pericytes were not observed in the brain. When placed lateral to the mesencephalon (Experiment 3b, Fig. 2), the same population of cells differentiated only into endothelium, but not into pericytes, in blood vessels external to the brain (E5,  $n=1$ ; E6,  $n=1$ ; E9,  $n=1$ ). In conclusion, the paraprosencephalic mesoderm yields mostly endothelial cells of blood vessels within and outside of the brain, and virtually no pericytes, although it does possess the capacity to differentiate into this cell type. However, it never contributes to the pericytes of the prosencephalic meningeal or intraencephalic vessels, nor to pericytes of the mesencephalon when grafted ectopically (also see Couly et al., 1995). Endogenous pericyte-forming populations in either location contribute to the blood vessels of the brain and its meninges, at the expense of the grafted paraprosencephalic mesoderm.

To remove this competition, in Experiment 4a (Fig. 2), quail paraprosencephalic mesoderm was grafted above the dorsal prosencephalon after bilateral ablation of posterior diencephalic, mesencephalic and r1 neural folds (Fig. 7A). In 5 out of 8 embryos observed at E5, the telencephalon was restored (Fig. 7B,C). The grafted tissue, localized by QCPN mAb immunoreactivity, was found adjacent to the neuroepithelium, and quail cells were visible within the forebrain tissue and meninges (Fig. 7D) as 1A4 mAb-positive pericytes and MB1/QH1-positive endothelial cells. In the 3 other embryos, the forebrain had not developed; while the grafted cells were present within the head mesenchyme, they were not adjacent to the neuroepithelium. The capacity of the paraprosencephalic mesoderm to yield pericytes within the forebrain meninges or parenchyme is thus restricted to experimental situations where the normal source of forebrain pericytes (the NC) is lacking and where this mesodermal population is grafted in a dorsal position over the neuroepithelium to support its host counterpart.

Paramesencephalic mesoderm was similarly grafted above the dorsal prosencephalon (Experiment 4b, Fig. 2). During normal development, this mesodermal population yields all components of the midbrain leptomeninx, including blood vessel endothelial cells and pericytes (Couly et al., 1995). It was therefore interesting to test the capacity of the paramesencephalic mesoderm to rescue neuroepithelial survival in the forebrain region and to examine the subsequent differentiation of the grafted cells. In 14/21 embryos, both telencephalon and diencephalon developed (Fig. 7E). Localized in the host by the QCPN mAb, the grafted paramesencephalic mesoderm gave rise to both intraencephalic endothelial cells and pericytes (Fig. 7F-H). Vessels were generally chimeric, with host pericytes and donor endothelial cells (Fig. 7F), donor pericytes and host endothelial cells, or combinations of both (Fig. 7G,H). Here too, in successful rescues, the grafted cells were located dorsally and in contact with the telencephalon. NC-derived mesenchyme is therefore not the only tissue able to promote forebrain development.

Lastly, embryos received grafts of the paraxial mesoderm of





**Fig. 7.** The addition of paraxial mesoderm from lateral to the prosencephalon or mesencephalon to anterior NC-ablated embryos rescues the forebrain (Experiment 4). (A) Operated embryo from Experiment 4a, from which posterior diencephalic, mesencephalic and r1 neural folds were removed (between arrowheads) and a graft of paraproencephalic mesoderm placed over the prosencephalon (asterisk). (B) The same embryo at E5, with normal forebrain vesicles. (C) Parasagittal section of the same embryo, with region enlarged in D indicated. (D) Triple stain of grafted mesoderm (QCPN, blue nuclei), pericytes (1A4, brown) and grafted endothelial cells (MB1/QH1, red) shows that chimeric blood vessels penetrate the forebrain neuroepithelium, and that grafted cells may become both endothelial cells and pericytes. (E) Parasagittal section of an E5 embryo from Experiment 4b, grafted with paramesencephalic mesoderm as above. Vessels magnified in F-H are indicated. (F) Meningeal blood vessel, with endothelial cells of graft origin (arrowheads) and pericytes of host origin (arrows). (G) Telencephalic blood vessel, with endothelial cells of host origin (arrowhead) and pericytes of graft origin (arrows). (H) Diencephalic blood vessel, with graft-derived pericytes (arrows) and endothelial cells (arrowhead) but also host-derived pericytes (open arrow). Tel, telencephalon; di, diencephalon; mes, mesencephalon; e, eye. Bars: A, 200  $\mu$ m; B,C,E, 1 mm; D,F-H, 20  $\mu$ m.

the trunk after NC ablation (Experiment 4c). We observed a delay in the degeneration of the forebrain neuroepithelium with respect to embryos having undergone NC ablation without an additional graft. Both of the 2 cases examined at E3 had normal

prosencephalic morphology (not shown). In contrast, in 4 other embryos at E5 to E8, the same experiment led to the absence of telencephalon and dorsal diencephalon, despite the presence of quail endothelial cells in the head.

The lateral plate mesoderm of the trunk (Experiment 4d) was unable to rescue telencephalic growth at all at E3 ( $n=2$ ), E6 ( $n=1$ ) or E8 ( $n=1$ ), nor did it differentiate into endothelial cells or pericytes in this context. Among mesodermal populations, the mesenchymal paraxial mesoderm of the head is best capable of replacing the anterior cephalic NCC in order to maintain viability in the forebrain.

## DISCUSSION

The major finding of this work is that after formation of the primitive encephalic vesicles, in order to survive and grow, the prosencephalon needs to be surrounded by mesenchymal cells that are normally derived from the neural folds of the prospective diencephalon and mesencephalon. These cells yield the leptomeninges, in cooperation with the anteriormost region of the cephalic paraxial mesoderm (paraproencephalic mesoderm).

The mesodermal component of the forebrain leptomeninx normally provides the endothelial walls of blood vessels that penetrate the neuroepithelium (Couly et al., 1995), while the neural crest component yields accompanying pericytes plus connective tissue cells. We have shown here that, when the anterior neural folds are removed from the presumptive level of the epiphysis down to r2, most of the forebrain undergoes cell death during a period that precedes the onset of vascularization by 36 to 58 hours, but which coincides with the assembly of the perineural vascular network of the forebrain during normal development. Progressive cell death in the prospective telencephalon and dorsal diencephalon takes place during the late second and early third embryonic days (HH15-19); vascularization would begin within the neuroepithelium of these areas only on the fourth embryonic day (HH24; unpublished results). The deleterious effect of ablation of the anterior NC cannot be interpreted as a deficit in vascularization because it takes place well before the time when blood vessels start to invade the neuroepithelium.

The outcome of this early cell death is the elimination of neuroepithelial territory destined to become the cerebral hemispheres and rhinencephalon, as well as a variable amount of the diencephalon, resulting in fusion of the two eyes over the area normally occupied by the telencephalon. This phenotype is reminiscent of the organization of the rostral head of *Amphioxus*, an invertebrate chordate considered to be the nearest evolutionary relative to vertebrates. Like the residual forebrain of the chicken embryo without anterior NCC, the cerebral vesicle of larval *Amphioxus* terminates in a number of unpaired, median structures associated with the vertebrate diencephalon: an infundibular organ, an epiphysis and a frontal 'eye' (Lacalli et al., 1994). Confirming its similarity to the diencephalon, the anterior end of the *Amphioxus* cerebral vesicle expresses a unique *Distal-less* gene homologue, reminiscent of the forebrain-restricted neuroepithelial expression of related vertebrate *Dlx* genes (Holland et al., 1996). A homologue to the *Drosophila Orthodenticle* gene is also expressed in the cerebral vesicle and frontal eye (Williams

and Holland, 1996). In both agnathan and gnathostome vertebrates, NCC that express the related *Otx* gene(s), derived from *Otx*-expressing diencephalon and mesencephalon, fill a head region that contains the forebrain as well as the first pharyngeal arch (Tomsa and Langeland, 1999). The removal of these NCC from the anterior head of the chicken embryo phenocopies some aspects of a putative ancestral brain organization. The expansion of the prosencephalic vesicle in vertebrates is likely to be a direct consequence of the appearance of the NC cell type and its extension of the vascular system of the brain, as has been proposed for the skeletogenic components of the head (Gans and Northcutt, 1983; reviewed in Kuratani et al., 1997).

When the anterior neural folds have not been replaced, the ventral and caudal diencephalon nonetheless survives and develops to a variable extent. The anterior rhombencephalic NC (r1) can partially compensate for the ablation of the diencephalic and mesencephalic NC by extending its migration cranially. We attempted to rescue the cyclopic phenotype induced by NC ablation by grafting either rhombencephalic or truncal NC heterotopically to the site of ablation, or by adding mesoderm of cephalic and trunk origin to the presumptive forebrain level. When the rhombencephalic NC is substituted for the diencephalic and mesencephalic NC, at least one *Hox* gene continues to be expressed in some NCC derivatives. Nonetheless, posterior cephalic NCC have the developmental potential to maintain forebrain viability like anterior cephalic NCC, although only the anterior population normally contributes pericytes to brain meninges and intraencephalic blood vessels. In contrast to cephalic NCC, the trunk NCC cannot participate in the formation of the anterior meninges, nor does it prevent cell death in the developing forebrain. This population, unlike the cephalic NC, does not normally participate in the smooth muscle walls of blood vessels or spinal cord meninges in the body. Its incapacity for alternative differentiation in the environment of the head (Le Douarin et al., 1977) extends to its lack of a trophic effect for the forebrain neuroepithelium.

When cephalic or somitic paraxial mesoderm is grafted over the dorsal prosencephalon of neural fold-deprived embryos, cell death is prevented in the forebrain. This demonstrates that the environment of the anterior head does not in itself prevent mesodermal differentiation into pericytes. Under experimental circumstances, even the paraproencephalic mesoderm can provide the meningeal and parenchymal blood vessels with pericytes, although it does not do so during normal development. It should be noted that the paraproencephalic mesoderm also does not participate in the construction of meninges when the NC is simply eliminated. Its incapacity might therefore lie either in cell quantity or in localization. Endogenous paraproencephalic mesoderm is located adjacent to the ventral part of the neuroepithelium (Couly et al., 1993); in the absence of NCC it fails to spread dorsally to cover the developing forebrain (H. C. E., G. C. and N. M. Le D., unpublished data). A perineural capillary plexus forms only once endothelial cells derived from the paraproencephalic mesoderm surround the prosencephalon after migration in normal development, or through substitution of the endogenous anterior NC by dorsal grafts of extra paraxial mesoderm or any other cephalic NC. In contrast, the local paramesencephalic mesoderm ensures all meningeal and blood

vessel formation of the midbrain (Couly et al., 1992). During normal development, diencephalic and mesencephalic NCC might act as a scaffold to promote the dorsal migration of paraproencephalic mesodermal cells so that the expanding neuroepithelium becomes fully surrounded by the meningeal anlage.

Our work identifies an important step in neurogenesis: it follows neural induction and precedes vascularization, and requires the presence of 'paraneural' mesenchymal cells for growth to proceed. Are the NCC-derived pericytes and connective cells, or the mesodermally derived endothelial cells, the necessary component of the forming meninges for the forebrain? In the absence of NCC, the prosencephalon perishes, despite the presence of endothelial cells near its ventral aspect. Pericytes are not necessary for the construction of vascular plexi from endothelial precursors in vitro (reviewed in Pepper and Montesano, 1990) or in vivo (Benjamin et al., 1998). If a proximate vascular plexus was sufficient to maintain forebrain viability, one would predict that, after anterior NCC ablations, the ventral telencephalon would persist. It does not. However, cephalic NCC can be replaced in their trophic capacity by mesodermal cells. The mixture of mesenchymal cells, of both mesodermal and NCC origin, of the future leptomeninges may thus secrete trophic factor(s) necessary for neuroepithelial viability before the blood supply has been ensured. Such factor(s) remain to be identified.

The authors gratefully acknowledge the technical assistance of Louis Addade and Fabrice Chatonnet, the aid of Françoise Viala, Francis Beaujean and Sophie Gournet in preparing the figures, and extensive and helpful commentary from Dr Anne Eichmann and Dr Marie-Aimée Teillet. We would also like to thank Dr Robb Krumlauf for the chicken *Hoxa3* probe. The QCPN hybridoma was obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, under contract NO1-HD-6-2915 from the National Institute of Child Health and Human Development. This work was supported by the Centre National de la Recherche Scientifique and the Collège de France. H. C. E. was a Predoctoral Fellow in the Biological Sciences of the Howard Hughes Medical Institute.

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