INTRODUCTION

Over chordate evolution, the anterior brain and its surrounding head expanded spectacularly in vertebrates. This development coincided with the appearance within the subphylum of a pluripotent embryonic cell population called the neural crest. Evidence that the neural crest was instrumental in shaping the head began to accrue from fate-mapping experiments that showed the distribution of neural crest cells (NCCs) in the avian embryo (Johnston, 1966). The use of a stable selective cell-marking technique, based on the construction of quail-chick chimeras allowed the definitive demonstration that NCCs give rise to most connective components of the head, including dermis, tendons and intercalating membranes of cephalic muscles (Le Lièvre and Le Douarin, 1975; Noden, 1983; Couly et al., 1993; Couly et al., 1996; Köntges and Lumsden, 1996). All blood vessels are composed of an inner layer of endothelial cells and an immediately adjacent layer of pericytes. Pericytes are indispensable for the formation of mature blood vessels (reviewed in Doherty and Canfield, 1999). They also mediate capillary vasoconstriction and secrete specialised extracellular matrices for microvessels within the neuroepithelium (Balabanov and Dore-Duffy, 1998), kidney (Schlondorff, 1987), liver (Kawada, 1997) and bone (Doherty and Canfield, 1999). In addition to endothelial and pericytic components, larger blood vessels also possess one or more concentric layers of connective and smooth muscle cells, constituting the elastic tunica media and the fibrous tunica externa.

Endothelial cells are derived from mesodermal precursors able to yield both the angiogenic and haematopoietic lineages, the most primitive of which are characterised by the expression of the vascular endothelial growth factor receptor 2 (Vegfr2) (Eichmann et al., 1993; Couly et al., 1995).

Most connective tissues in the head develop from neural crest cells (NCCs), an embryonic cell population present only in vertebrates. We show that NCC-derived pericytes and smooth muscle cells are distributed in a sharply circumscribed sector of the vasculature of the avian embryo. As NCCs detach from the neural folds that correspond to the future posterior diencephalon, mesencephalon and rhombencephalon, they migrate between the ectoderm and the neuroepithelium into the anterior/ventral head, encounterig mesoderm-derived endothelial precursors. Together, these two cell populations build a vascular tree rooted at the departure of the aorta from the heart and ramified into the capillary plexi that irrigate the forebrain meninges, retina choroids and all facial structures, before returning to the heart. NCCs ensheathe each aortic arch-derived vessel, providing every component except the endothelial cells. Within the meninges, capillaries with pericytes of diencephalic and mesencephalic neural fold origin supply the forebrain, while capillaries with pericytes of mesodermal origin supply the rest of the central nervous system, in a mutually exclusive manner. The two types of head vasculature contact at a few defined points, including the anastomotic vessels of the circle of Willis, immediately ventral to the forebrain/midbrain boundary. Over the course of evolution, the vertebrate subphylum may have exploited the exceptionally broad range of developmental potentials and the plasticity of NCCs in head remodelling that resulted in the growth of the forebrain.

Key words: Pericyte, Vascular, Carotid, Branchial, Quail-chick chimera, Evolution, Neural crest, Meninges, Forebrain, Head

SUMMARY

INTRODUCTION

The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain

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In contrast, the outer wall layers, while originating from the mesoderm in the body, can be made by NCCs in the head (reviewed in Le Douarin and Kalcheim, 1999). Cephalic NCC contribute to the muscular and connective wall of large arteries derived from the branchial arches (Le Lièvre and Le Douarin, 1975), including the cardiac septum that separates the aorta from the pulmonary artery trunk (Waldo et al., 1998). Continued ramifications of the branchial arteries define a distinct anatomical sector of the ventral and anterior head. A second vascular sector of non-branchial arteries irrigates the dorsal, posterior head (Fig. 1). These two domains meet behind the optic chiasm in an anastomotic structure known as the circle of Willis. Strikingly, the branchial sector overlaps that part of the head to which the NCC mesenchyme makes major structural contributions.

In the present work we have analysed the precise contribution of NCCs that originate from successive anteroposterior levels of the neural folds to cephalic blood vessels. We find that the dorsal/posterior vascular compartment depends upon the cephalic paraxial mesoderm for all components of the blood vessel wall, while the ventral/anterior compartment derives its pericytes and musculo-connective wall entirely from NCCs. The two domains are delimited distally within the meninges by a sharp boundary between the forebrain and the midbrain.

The forebrain and the retinae are thus irrigated by capillaries of composite mesoderm and neural crest origin, consistent with their support by other neural crest-derived connective tissues. In addition to the trophic role we have previously demonstrated for cephalic NCCs in early forebrain development, it appears plausible that there was a causal relationship between the construction of a NCC-dependent vascular domain and the continued expansion of the anterior brain and head over the course of evolution.

MATERIALS AND METHODS

Isotopic grafts of the right neural fold were performed from quail donors into stage-matched chick hosts at the three to five somite stage (ss), on embryonic day (E) 2. The length of the neural fold taken corresponded to the posterior half of the diencephalon (PD), the anterior half of the mesencephalon (AM) or the posterior half of the mesencephalon (PM) regions, approximately 100 μm each, or the AM and PM together. Stage- and position-specific micrometry was performed as outlined previously (Grapin-Botton et al., 1995).

Additional chicken embryos were examined in which an individual rhombomeric neural fold had been replaced by its isotopic, isochronic quail counterpart, as described previously (Couly et al., 1996). Neural folds from given rhombomeric levels are referred to as r1-r8. The neural folds of r8 were mapped over the length of each of the first three somites, but the results, being similar, were grouped. All rhombencephalic-grafted embryos were fixed in Zenker’s solution and treated with the Feulgen-Rossenbeck stain procedure, described previously (Le Douarin, 1969), as an alternative method to localise quail cells in older embryos.

ISH of Vegfr2 (Eichmann et al., 1993), and the premigratory and

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**Fig. 1.** The dorsal cephalic vascular tree, excluding the jaws, ventral view. Six pairs of aortic arch arteries form during amniote embryogenesis, looping from the ventral to the dorsal aorta. Three of them persist in the adult: the third pair, as a segment of the common carotid arteries; one of the fourth pair, as the aorta; and the sixth, as a segment of the pulmonary arteries. The common carotid arteries give rise to a ventral arterial pathway, of which the internal carotid (C. i.) and derived arteries (pink) target the upper face, eyes, and forebrain. The distal part of this route is made of vascular elements derived from the first three aortic arches. The other arterial pathway in the head (green) targets the midbrain and hindbrain, and arises from the vertebral arteries (V.). Both trees start from the brachiocephalic artery trunks, which diverge from the ventral (ascending) aorta. The common carotid arteries immediately course into the neck and face. The vertebral arteries project into the head along the underside of the hindbrain after fusing to form the basilar artery. The two systems contact at the circle of Willis (box), which surrounds the optic chiasm ventral to the diencephalon. The posterior sides of this famous vascular polygon are made by the bifurcation of the basilar artery, while derivative branches of the internal carotid arteries constitute the anterior sides. An anastomotic artery, the posterior communicante (P. c.), connects them; in humans, a median fusion between the anterior cerebral arteries completes the ‘circle’. The circle of Willis represents the anatomical interface between the ventral and dorsal vascular trees. B., basilaris; C. c. a., carotis cerebri anterior; C. c. m., carotis cerebri mediales; C. c. p., carotis cerebri posterior; Ce. d., cerebellaris dorsalis; Ce. v., cerebellaris ventralis; C. i., carotis interna; Eth., ethmoidalis; T. m. d., teci mesencephalici dorsalis; T. m. v., teci mesencephalici ventralis; V., vertebralis. Adapted, with permission, from Baumel (Baumel, 1979).
domains come to directly underlie the ectoderm.

During HH8, the presumptive telencephalic primordia occupy dorsolateral domains within the anterior neural fold (Fig. 2A). As the neural folds of the anterior neural tube approach each other, the presumptive telencephalic domains come to directly underlie the ectoderm.

RESULTS

The prosencephalic vascular plexus forms concomitantly with the arrival of NCCs

Cephalic mesenchyme may come from either NCCs or cephalic mesoderm. In order to recognise the distinct origins of the cells that give rise to the early vascular system of the anterior head, the relative positions of its neural crest and endothelial components over time were examined by in situ hybridisation. Vegfr2 is expressed in endothelial cells and their precursors (Eichmann et al., 1993). Sox10 is a homeobox-containing gene that is specifically expressed in all early migrating cephalic NCC, later functioning in both central and peripheral nervous system glial lineages (Cheng et al., 2000).

These epithelia are subsequently separated by NCCs between HH9 and HH115. At HH9 (7-9ss, E2), NCCs emigrate from the diencephalic and mesencephalic neural folds, spreading both toward the pharynx and rostrally toward the anterior neural fold (Fig. 2B), but they do not encounter endothelial cells before HH10.

At HH14 (Fig. 2C,D), mesoderm-derived endothelial precursors have mixed with the NCCs. Together, they intervene between the ectoderm and the caudal telencephalon, but have not yet spread over the rostral telencephalon. Chimeric embryos made with unilateral neural fold grafts at the mesencephalic level show that anterior NCCs fan out bilaterally, whereas those that migrate towards the pharynx remain essentially unilateral (Fig. 2E). The rostral-moving NCCs continue to lead the invasive front, closely followed by capillaries that are organising adjacent to the neuroepithelium (Fig. 2D,F).

Two bilateral eminences evade from the prosencephalon during this period. The first, the optic vesicles, appear in the ventrolateral prosencephalon at HH9. The second, the telencephalic vesicles, only emerge from the dorsolateral prosencephalon at HH19 (E3). Capillaries begin to penetrate the chicken ventral rhombencephalic and mesencephalic neuroepithelium on HH18 (late E2), but the telencephalon is invaded by blood vessels ventrally at HH24 (E4) and dorsally after HH26 (E5; not shown). In chimeras, quail NCCs are first seen within the host telencephalon associated with the first capillaries on HH24 (Fig. 2G).

Anterior cephalic NCCs participate in all forebrain vasculature

After isotopic grafts of neural folds at the levels of the posterior diencephalon or the mesencephalon, quail NCCs are abundant within the meninges of the forebrain, to the exclusion of the rest of the central nervous system (Fig. 3A). The greatest contribution to the telencephalic meninges is from mesencephalic NCCs, while posterior diencephalic NCCs favour the ventral diencephalic meninges, although both regions of the neural folds give rise to cells in all parts of the forebrain meninges. There is no apparent difference in distribution within the meninges between NCCs from the anterior or posterior mesencephalon.

Avian meninges are made of two layers. The outer dura mater is continuous with the condensing periosteum of the underlying NCC-derived skull (Coulby et al., 1993), and is also NCC-derived (data not shown). The inner arachnoid, rich in

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Table 1. Chimeric embryos used to construct fate map of cephalic vascular walls

<table>
<thead>
<tr>
<th>Graft NF</th>
<th>Di</th>
<th>Ant mes</th>
<th>Post mes</th>
<th>Mes</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
<th>R8 (s1-3)</th>
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<tr>
<td>HH stage at harvest (number of samples)</td>
<td>16 (6)</td>
<td>10 (2)</td>
<td>26 (1)</td>
<td>10 (1)</td>
<td>29 (2)</td>
<td>35 (2)</td>
<td>33 (2)</td>
<td>18 (1)</td>
<td>28 (1)</td>
<td>26 (1)</td>
<td>29 (2)</td>
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<td>33 (1)</td>
<td>14 (1)</td>
<td>12 (1)</td>
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<tr>
<td>38 (1)</td>
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<td>32 (1)</td>
<td>35 (1)</td>
<td>24 (2)</td>
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<td>Total (59)</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>15</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td>3</td>
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Ant mes, anterior mesencephalic; Di, diencephalic; Mes, mesencephalic; NF, neural folds; Post mes, posterior mesencephalic; R, rhombomere; s1-3, at the level of somites 1 to 3.

early migratory neural crest marker Sox10 (Cheng et al., 2000) was performed directly on 5 µm paraffin sections of embryos fixed in 11% formaldehyde, 60% ethanol, 10% acetic acid. Digoxigenin-UTP-labelled Vegfr2 probe and fluorescein-labelled Sox10 probe were diluted in hybridisation mix to approximately 1 ng/ml each. The probes were applied to sections, which had been deparaffinized, rehydrated, digested with 10 µg/ml proteinase K for 7 minutes, postfixed in 4% paraformaldehyde and rinsed. After hybridisation overnight at 60°C, slides were washed twice in 50% formamide, rehydrated, digested with 10 µg/ml proteinase K for 7 minutes, postfixed in 4% paraformaldehyde, 60% ethanol, 10% acetic acid. Digoxigenin-UTP-labelled Vegfr2 probe and fluorescein-labelled Sox10 probe were diluted in hybridisation mix to approximately 1 ng/ml each in NTMT. The slides were then treated with 0.1 M glycine, pH 2.2 for 15 minutes, washed twice in 50% formamide, postfixed in 4% paraformaldehyde and rinsed. After hybridisation overnight at 60°C, slides were washed twice in 50% formamide, rehydrated, digested with 10 µg/ml proteinase K for 7 minutes, postfixed in 4% paraformaldehyde, 60% ethanol, 10% acetic acid. Digoxigenin-UTP-labelled Vegfr2 probe and fluorescein-labelled Sox10 probe were diluted in hybridisation mix to approximately 1 ng/ml each in NTMT. The slides were then treated with 0.1 M glycine, pH 2.2 for 15 minutes, equilibrated in MABT and AP-conjugated anti-fluorescein antibody (Roche) applied at 1:8000 for IHC. For a red precipitate, 2-(4-iodophenyl)-3-(4-nitrophyenyl)-5-phenyl tetrazolium chloride (INT) and BCIP were used in NTMT. The slides were then treated with 0.1 M glycine, pH 2.2 for 15 minutes, equilibrated in MABT and AP-conjugated anti-fluorescein antibody (Roche) applied at 1:8000 for IHC. For a red precipitate, 2-(4-iodophenyl)-3-(4-nitrophyenyl)-5-phenyl tetrazolium chloride (INT) and BCIP were used at 248 mg/ml each in NTMT.
blood vessels, is inseparable from the pia mater, which constitutes a component of the blood-brain barrier; pial pericytes are closely associated with the outer aspect of the vascular endothelium that penetrates the neuroepithelium. The arachnoid/pia mater, known collectively as the leptomeninges, include numerous NCC located in the walls of parenchymal blood vessels (Fig. 3B, HH32).

Grafted cells found in the forebrain are not labelled after IHC with the QH-1/MB-1 antibody, and hence are not endothelial (data not shown). Quail NCC reside on the outside of the capillaries and co-localise with alpha smooth muscle actin, seen via 1A4 IHC (Fig. 3B,D). These observations are consistent with pericytic identity. In larger arteries, such as the internal carotid, NCCs are located within the 1A4-immunolabelled smooth muscular tunica media (Fig. 3C).

Caudal NCCs map to proximal vessels, rostral NCCs map to distal vessels

Given a number of shared characteristics between pericytes and smooth muscle cells (Alliot et al., 1999), we examined other blood vessels derived from the aortic arches for the presence of NCC. All arteries of the face and jaw that we examined, which branch off from the common carotid arteries, have tunica media of NCC origin.

The more distal an artery from the heart, the further rostral the origin of the NCCs that give rise to the pericytes and/or smooth muscle cells of that part of the vessel (Fig. 4A). Examples of distalmost blood vessels include the meningeal capillaries of the prosencephalon (Fig. 3B), and the pituitary and ophthalmic arteries. The non-endothelial components of these vessels come from posterior diencephalic and mesencephalic NCCs. As shown in Fig. 4, cells from the posterior diencephalon also contribute to part of the wall of the internal carotid artery (Fig. 4C), r2 NCCs to the proximal maxillary artery (Fig. 4D), r4 NCCs to the stapedian artery (Fig. 4E) and r5 NCCs to the common carotid artery (Fig. 4F). Grafts from adjacent levels of the neural folds gave rise to cells in overlapping domains of the same complement of blood vessels, indicating a gradual transition within the vascular wall from NCCs of one origin to NCCs of a neighbouring origin. The tunica media of long arteries such as the internal carotids spans multiple subdivisions of the neural folds (in this example, from PD to r4 included). The same holds true for the ventral and anterior cephalic veins, such as the jugulars, to which NCC from r4 to r6 contribute (not shown).

Likewise, the more proximal an artery to the heart, the further caudal the origin of the NCC along the neuraxis. The distribution of NCCs derived from r6, r7 and r8 within the musculo-connective wall of the large arteries overlapped greatly (Fig. 5A). All three contributed to the ventral aorta as

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**Fig. 2.** Cephalic NCCs and mesodermal cells intermingle by way of opposing dispersion patterns. (A) Section through a HH7 (4ss, early E2) embryo at level of the presumptive diencephalon. No mesodermal cells, in particular endothelial precursors (blue, Vegfr2 expression), intervene between the ectoderm and the prosencephalic alar plate. (B) Distribution of rostral-spreading NCC (red, Sox10 expression) and endothelial cells (blue, Vegfr2 expression) at early HH10 (10ss, E2) in a transverse section at the level of the anterior prosencephalon. (C) Parasagittal section of HH14 (E2) embryo, anterior right. Endothelial cells (blue, Vegfr2 expression) and NCCs (red, Sox10 expression) occupy the mesenchyme intervening between the neuroepithelium and ectoderm except around the rostral telencephalon. Sox10 is also expressed in the ventral diencephalon (see Cheng et al., 2000). Boxed region is magnified in D. (D) NCCs precede endothelial tubes as they insinuate together between the ectoderm and neuroepithelium of the telencephalon. (E) Distribution of NCCs (brown, QCPN IHC) at HH14 after a unilateral graft of an anterior mesencephalic neural fold, in transverse section at the level of the ventral diencephalon. NCCs disperse bilaterally rostral to the graft but remain unilateral near the pharynx. (F) On a slightly more anterior section of the same embryo, grafted NCCs can be seen to separate neuroepithelium from ectoderm and to be accompanied by capillaries (asterisk) as in D. (G) NCCs (arrows) from a grafted mesencephalic neural fold begin to penetrate the telencephalic neuroepithelium from the surrounding mesenchyme at HH24 (E4). Scale bars: 100 μm in A-C,E,G: 50 μm in D,F.
Origin of cephalic vascular sectors

seen by E5 and later (Fig. 5B,C,F,H,J), as well as to the thin tunica media of the cardinal veins and the sinus venosus (Fig. 5I). NCCs from r6 were found in the wall of the proximal common carotid arteries, relaying the r5 NCCs. NCCs from r7 and r8 participated in the brachiocephalic arteries (Fig. 5D-G,J-K), sigmoid valves of both the aorta (Fig. 5J,L) and pulmonary artery trunk (Fig. 5F,G,J), and the distal conotruncus. The deeper part of the conotruncus contained NCC of solely r8 origin at HH29 (E6.5) and HH33 (E8.5) (not shown).

No NCCs from any level of the cephalic neural folds were found in the media of the cerebellar or occipital arteries at the ages examined (not shown). These vessels are directly connected to the vertebral arteries. The head is thus divided into two vascular domains that meet at the forebrain/midbrain boundary both within the meninges and in the larger vessels, at the circle of Willis (Figs 1, 4). They occupy distinct ventral and dorsal domains from the heart to the brain.

**Anterior cephalic NCCs occupy overlapping but distinct niches in connective tissues**

We examined the distribution of cephalic NCC in other soft connective tissues in further detail. In these derivatives as well, a similar logic was maintained in the fate map.

Grafted NCC from PD are apparent in the ventrolateral periocular structures (sclera, choroid and interstitial cells of the ventral oculomotor muscles and lacrimal glands) (Fig. 6A-C). PD quail cells also surround and infiltrate the developing pituitary and salivary glands.

NCCs from the AM region incorporate largely into dorsomedial periocular structures, the scleral papillae and dermis, the nasal septum and the telencephalic choroid plexus. Examples of their presence as pericytes within oculomotor muscles, the optic chiasm, the neurohypophysis and the adenohypophysis are shown in Fig. 6E-H, respectively. Most or all NCCs in these locations can be double-labelled with α smooth muscle actin (not shown). However, many NCC in the adenohypophysis did not, representing interstitial cells.

NCCs from the PM region migrate largely into the first branchial arch maxillary processes. They also participate in the ciliary ganglia, optic nerves and coalescing ventromedial periocular structures (choroid pericytes, sclera, oculomotor muscle interstitial cells) as observed at HH26 (E5).

**DISCUSSION**

**NCCs are distributed along the proximal-distal axis of cephalic vascular media**

The frontier of the NC- and mesoderm-derived meninges surrounding the brain coincides with a classically described anatomical interface in the head between two distinct vasculatures. We have shown here that the forebrain (telencephalon and diencephalon) is the only part of the central nervous system into which NCCs penetrate. Arteries with walls composed of NCC derivatives also supply the entire ventral (facial) and anterior head, the connective tissues of which are likewise of NCC origin. In contrast, the fully mesodermal vertebral arteries supply the dorsal/posterior part of the head and neck, caudal to the diencephalon (Couly et al., 1992; Couly et al., 1993). The two vascular trees join and re-diverge at the level of the optic chiasm, within the circle of Willis (Fig. 1).

This striking demarcation of vascular domains reveals the point from which a new part of the head expanded in vertebrates with respect to the chordate phylum as a whole. Gans and Northcutt proposed that the neural crest played a key role in the evolution of the face, in particular for the skeletal and muscular elements of the jaws (Gans and Northcutt, 1983). The essence of their theory is that these novel NCC-derived...
Fig. 4. Sequential distribution of NCC from successive neural fold origins in the walls of cephalic arteries. (A) Cephalic NCCs (colours, this study) and mesoderm (greys; Le Lièvre, 1976; Couly et al., 1987) contribute to the musculo-connective wall of separate arterial trees in the head. Red corresponds to cells derived from posterior diencephalic (PD), anterior and posterior mesencephalic (AM, PM) neural folds; orange corresponds to rhombomere (r)1; yellow to r2; green to r4; turquoise to r5; and blue to r6 (in the vascular media of a schematic E7.5 chicken head). Boundaries overlap between domains ensured by NCCs of given origins in vessel walls. Within the meninges of the central nervous system, pink denotes those derived from PD, AM and PM NCCs; grey denotes those of mesodermal origin, with a sharp boundary between the two at the diencephalon/mesencephalon junction. Levels of sections shown in C-F are indicated, where lower panel is a magnification of the artery indicated in the upper panel. Levels of Fig. 3B and 3C are also shown. (B) Ink-injected E8 quail, showing both branchial and vertebral artery ramifications. (C-F) The lower panels show the enlargement of the areas boxed in the upper panels. (C) E8 chimera after graft of PD neural fold, in transverse section – internal carotid artery. (D) E8 chimera after graft of r2 neural fold, in transverse section – maxillary artery. (E) E8 chimera after graft of r4 neural fold, in transverse section – stapedia artery. Quail cells revealed by Feulgen-Rossenbeck stain (lower panel inset) are false-coloured in brown in the lower panel. (F) E8 chimera after graft of r5 neural fold, in transverse section – common carotid artery. B., basilaris; C. c. a., carotis cerebralis anterior; C. c. m., carotis cerebralis medialis; C. c. p., carotis cerebralis posterior; Ce. v., cerebellaris ventralis; C. i., carotis interna; Eth., ethmoidalis; L., lingualis; Md., mandibularis; Mx., maxillaris; Occ., occipitalis; Oph., ophthalmica interna; P. c., posterior communicante (circle of Willis); St., stapedia; St. te., stapedia temporalis; St. sup., stapedia supraorbitalis; T. m. v., tecti mesencephalis ventralis; V., vertebralis. Adapted, with permission, from Hughes (Hughes, 1934) and Baumel (Baumel, 1979). Scale bars: 0.5 cm in B; 250 μm in C-F (top); 50 μm in C-F (bottom).
Origin of cephalic vascular sectors

Cephalic structures may have permitted vertebrates to adopt an advantageously active feeding lifestyle. Supporting evidence comes from demonstrations that the face, jaws and skull are derived from the neural crest in modern-day vertebrates (see, for example, Couly et al., 1993; Imai et al., 1996).

The forebrain, in particular the telencephalon, embodies an evolutionarily recent morphological change in the central nervous system, not a novel structure altogether. The faceless and jawless cephalochordate *Amphioxus* presents sensory and endocrine functions in the anterior end of its nerve cord (Lacalli et al., 1994; Lacalli and Kelly, 2000). Gene expression patterns in this region recall those present in the vertebrate...
forebrain (reviewed by Zimmer, 2000). The common chordate ancestor to vertebrates and cephalochordates probably had a similarly rudimentary forebrain, whose functions were amplified and developed in the vertebrate diencephalon (photosensation via the eyes and pineal gland, hormone secretion from the ventral diencephalon and pituitary) and telencephalon (chemosensation).

Within vertebrates, the telencephalon assumed integrative functions and importance over time, possibly owing to the presence of a blood supply consecrated directly to its growing needs for oxygen and nutrition. Our current observations demonstrate that a NCC-supported vasculature developed to irrigate both the NCC-derived jaws, already present in lower vertebrates, and the forebrain, greatly expanded in higher vertebrates. We have previously shown that NCC mesenchyme has a trophic effect on the early forebrain, namely on the cerebral hemispheres. In the absence of this mesenchyme, achieved by ablation of the posterior diencephalic and mesencephalic neural folds, the forebrain neuroepithelium undergoes massive cell death preceding its normal period of vascularisation (Etchevers et al., 1999). Thus, the neural crest has three distinct roles in the development of the forebrain: an antiapoptotic effect at an early stage of neurogenesis, a second trophic role via its contribution to the leptomeninges and cephalic vasculature, and a third role, in the protection of the forebrain by means of the dura mater and the skull.

**Initial dispersion of anterior cephalic NCCs accounts for their final distribution**

The lack of cell emigration from the neural folds of the prosencephalon, anterior to the burgeoning eyes (Couly and Le Douarin, 1988), creates a rostral niche that is filled by NCCs spreading forward, fan-like, from the posterior diencephalic neural folds. In the posterior head, NCCs from any given rhombomere colonise more than one branchial arch to surround its aortic artery (Birgbauer et al., 1995; Köntges and Lumsden, 1996; Couly et al., 1996). When part of the cephalic neural folds are removed by surgical ablation, NCC from regions rostral and/or caudal to the excision disperse to ectopic compensatory locations in the head (Couly et al., 1996; Salvidar et al., 1997; Etchevers et al., 1999; Kulesa et al., 2000). In this way, cephalic NCCs radiate from their dorsal points of origin into wider ventral swathes, with some limited mixing among cells of neighbouring origins.

The patterns of dispersion that establish the anterior mesenchyme early on presage the final distribution of NCCs in the head mesectoderm. Fate-mapping small domains of the anterior neural folds shows that NCC from the posterior

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**Fig. 6.** NCCs integrate into periocular and secretory tissues. (A) Parasagittal section of E12 embryo grafted with posterior diencephalic neural folds, in area of ventral eye. (B) Area enlarged shows the palatine artery with NCC-derived smooth muscle cells and a portion of the palatine membrane bone. (C) Part of a lachrymal gland, showing interstitial (arrowheads) and pericytic (arrows) participation of NCCs. (D) Lateral parasagittal section of E12 embryo grafted with anterior mesencephalic neural folds, indicating regions magnified in E-H. (E) NCC-derived pericytes (arrows) accompany the capillaries of the dorsal rectus oculomotor muscle. (F) Both glia (arrowheads) and capillary pericytes (arrows) within the optic chiasm are derived from NCCs. (G) Pericytes (arrows) are the only cells of graft origin within the neurohypophysis. (H) Both interstitial cells (arrowheads) and pericytes (arrows) in the adenohypophysis come from NCCs. Scale bars: 250 μm in A; 100 μm in B,C,E-H; 1 mm in D.
diencephalic or anterior mesencephalic neural folds occupy the ventral and dorsal anterior heads respectively, the posterior mesencephalic NCCs remaining essentially lateral to their original rostrocaudal level in the jaws.

In particular, we have observed that posterior diencephalic NCCs are found within the walls of the pituitary vascular plexus (Fig. 6G,H and Etchevers et al., 2001), adjacent to the first branchial arch with its complement of anterior rhombencephalic NCC. This surprising distribution reflects the topological deformation of the longitudinal axis of the head around the end of the notochord, which brings the anterior transverse neural fold ventral and caudal to the future hypothalamus (Coulby et al., 1987). The mesencephalic NCC participate in the meninges of the forebrain (this study) as well as its overlying dermis and skull (Coulby et al., 1993). Mesencephalic NCCs actually constitute the most anterior mesectoderm, by occupying a rostrally expanding area dorsal to that populated by diencephalic NCC.

There are two conclusions to be drawn from these results. First, adjacent points in the neural folds map to contiguous areas of the head along its original rostrocaudal axis, reflecting the dispersion of cephalic NCC during migration. Second, cooperation between the cephalic mesoderm and the neural crest is necessary to build a vascular tree in that part of the head that is constructed predominantly by NCCs. Mesodermal cells initially located in a ventral position migrate dorsally and mix with the ectomesenchyme of neural fold origin. Cephalic mesodermal mesenchyme is the site of two successive waves of cell determination and differentiation. Cells expressing VEGF-R2 (Eichmann et al., 1993) become endothelial cells of the developing blood vessels. From this stage onwards, they become associated with NCCs that differentiate into the pericytes and musculo-connective tissue of the outer blood vessel walls. The second wave of commitment affecting mesodermal cells concerns the head muscles, for which ectomesenchymal cells form connective components such as membranes and tendons (Noden, 1983; Coulby et al., 1996; Köntges and Lumsden, 1996).

NCC may be involved in human vascular pathologies

Recent fate maps of NCC in the mouse (Imai et al., 1996; Jiang et al., 2000) confirm the importance of avian studies to interpreting mammalian vascular remodelling. Although we have observed NCC from r8 in the proximal portion of the pulmonary arteries, the posterior limit of r8 was not mapped in this study. Waldo and Kirby have also found that the NCCs of rostral r8 do not participate in the distal pulmonary arteries but rather continue in the media of the transient ductus arteriosus (sixth aortic arch), connecting their proximal portion to the dorsal aorta (Waldo and Kirby, 1993). At this intersection, vascular media no longer contain NCCs. According to the logic of the vascular fate map we establish in this paper, it appears likely that caudal r8 cells temporarily contribute to the distal ductus arteriosus, rather than the distal pulmonary arteries. These latter vessels are probably the product of remodelling between two initially distinct parts of the vascular tree. The pulmonary arteries thus would recombine a proximal, NCC-ensheathed portion with a distal, mesoderm-ensheathed segment. This situation resembles the late anastomosis that occurs to establish the circle of Willis. Waldo and Kirby also proved the necessity for NCCs in the septation of the aorta from the pulmonary artery trunk within the mesodermal context of the heart (Waldo and Kirby, 1993). Septation is compromised in a number of congenital conditions that affect other derivatives of the neural crest (reviewed in Le Douarin and Kalcheim, 1999). As is the case for forebrain vessels, cephalic NCCs make the musculo-connective wall of large vessels near the heart, suggesting that vascular remodelling in the head, neck and heart is dependent on NCC participation.

The fact that the branchial arteries give rise to a distinct vascular domain is pertinent to phakomatoses such as Sturge-Weber syndrome (reviewed by Masson, 1970) or meningioangiomatosis (reviewed by Chakrabarty and Franks, 1999). These diseases involve calcification of forebrain capillary pericytes in the cerebral hemispheres; Sturge-Weber syndrome is also associated with ipsilateral facial angiomata. It is striking that NCCs from the same source as the forebrain meninges and pericytes normally differentiate into membrane bones when they are located in a subectodermal position (Coulby et al., 1993).

Pericytes and smooth muscle cells share a common lineage

Pericytes, immediately adjacent to the vascular endothelium of both arteries and veins, are not in themselves smooth muscle cells. They do, however, share some properties and markers, of which one interesting representative is nestin (Alliot et al., 1999). Like other nestin-expressing cell types, vascular pericytes seem to retain a certain context-dependent flexibility in their differentiation, acquiring characteristics suggestive of smooth muscle, fibroblasts, osteoblasts, adipocytes or chondrocytes in vitro (reviewed in Doherty and Canfield, 1999). Our results demonstrate unequivocally and for the first time that in an entire vascular circuit, from the heart to capillaries and back, there can be one common source of precursor cells for both the smooth muscle walls and the pericytes.

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