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Embryonic Origin of Avian Corneal Sensory Nerves

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Sensory nerves play a vital role in maintaining corneal transparency. They originate in the trigeminal ganglion, which is derived from two embryonic cell populations (cranial neural crest and ectodermal placode). Nonetheless, it is unclear whether corneal nerves arise from neural crest, from placode, or from both. Quail–chick chimeras and species-specific antibodies allowed tracing quail-derived neural crest or placode cells during trigeminal ganglion and corneal development, and after ablation of either neural crest or placode. Neural crest chimeras showed quail nuclei in the proximal part of the trigeminal ganglion, and quail nerves in the pericorneal nerve ring and in the cornea. In sharp contrast, placode chimeras showed quail nuclei in the distal part of the trigeminal ganglion, but no quail nerves in the cornea or in the pericorneal nerve ring. Quail placode-derived nerves were present, however, in the eyelids. Neural crest ablation between stages 8 and 9 resulted in diminished trigeminal ganglia and absence of corneal innervation. Ablation of placode after stage 11 resulted in loss of the ophthalmic branch of the trigeminal ganglion and reduced corneal innervation. Noninnervated corneas still became transparent. These results indicate for the first time that although both neural crest and placode contribute to the trigeminal ganglion, corneal innervation is entirely neural crest-derived. Nonetheless, proper corneal innervation requires presence of both cell types in the embryonic trigeminal ganglion. Also, complete lack of innervation has no discernible effect on development of corneal transparency or cell densities.

Key Words: **neural crest; placode; trigeminal ganglion; nerves; cornea; quail–chick chimera.**

INTRODUCTION

The cornea is a transparent tissue at the anterior-most surface of the eye, refracting and transmitting light to the retina without image distortion. It is surrounded by a transition region known as the limbus, which in turn is surrounded by the conjunctiva. The cornea is comprised of three cellular layers: anterior-most is the epithelium, which consists of five to seven layers of cells; the middle layer, or stroma, contains keratocytes, whose extracellular matrix comprises the bulk of the cornea; posterior-most is a monolayer known as the endothelium, although it is not thought to be related to blood vessel endothelium developmentally. Both the keratocytes and endothelium are derived from neural crest.

The vertebrate cornea is one of the most highly innervated tissues on the surface of the body (Rozsa and Beuerman, 1982; Beuerman and Pedroza, 1996). Transparency of the cornea, as well as its ability to refract and transmit light to the retina, are necessary for the proper function of the eye. Corneal nerves in adults play a major role in maintaining the transparency of this tissue in a variety of ways, including secretion of neurotransmitters, such as acetylcholine (Mittag *et al.*, 1974), and perhaps by anterograde transport of trophic substances that are necessary for the maintenance of the corneal epithelium (Mishima, 1957). Conversely, lack of innervation results in corneal anesthesia and keratitis (Beuerman and Schimmelpfennig, 1980).

During embryonic development of the chick, prospective corneal nerves arrive at the ventro-temporal peripheral region of the developing cornea before embryonic day 5 (E5). Instead of entering the cornea directly, these nerves then extend dorsally and ventrally to form a ring around the cornea. Upon completion of the nerve ring at about E10, numerous fascicles begin to invade the corneal stroma radially (Bee, 1982), where they branch and bifurcate in an orderly manner to innervate the overlaying epithelium (Bee *et al.*, 1986; Müller *et al.*, 1996).

Corneal nerves derive from the trigeminal ganglion, a cranial sensory ganglion that derives from two distinct cell populations: the cranial neural crest and placode. During embryonic development, neural crest and placode cells bilaterally migrate underneath the head ectoderm

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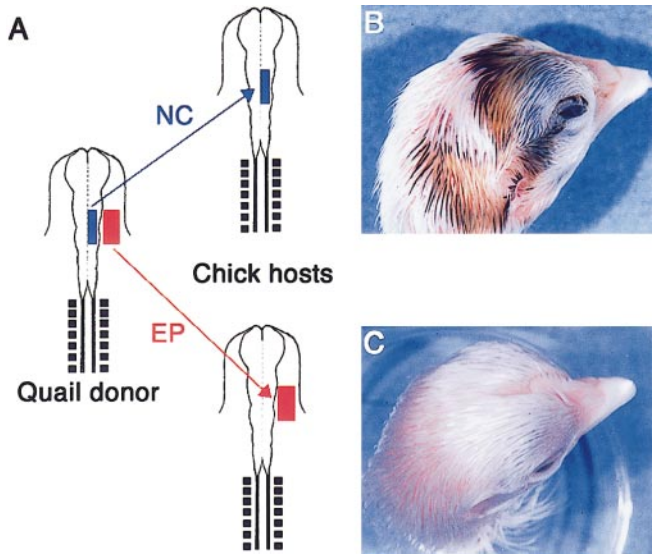

FIG. 1. Neural crest and placode transplantation and the subsequent E18 quail–chick chimeras. (A) Schematic showing midbrain region isotopic transplantation of stage-9 quail neural crest (NC) or placode (EP) cells into stage-matched chick hosts. (B) Neural crest chimera showing pigmentation of some feathers on operated side. (C) Placode chimera showing (due to the absence of neural crest-derived quail melanocytes) that there were no contaminating quail neural crest cells in the graft.

and condense into various cranial ganglia on both sides of the body, with the neural crest located proximal to and placode distal to the developing central nervous system (Johnston, 1966). Among these cranial ganglia are the trigeminal ganglia of the fifth (V) cranial nerve (Yntema, 1944; Hamburger, 1961). Afferent axons from the trigeminal ganglia innervate most of the face, both corneas, eyelids, and the mouth (Watanabe and Yasuda, 1970; Noden, 1980a,b).

Neural crest cells are a migratory cell population that differentiates into a wide variety of cell types in vertebrates (reviewed by Hall, 2000). In the cranial region of a developing embryo, neural crest cells give rise to melanocytes, sensory neurons, and glia, as well as facial bones and cartilage (Le Lièvre and Le Douarin, 1975; Noden, 1978a,b; Le Douarin, 1982). Placode cells, on the other hand, are thickened regions of head ectoderm (Le Douarin *et al.*, 1986; Noden, 1991) that are induced to form in different cranial regions by the endoderm and mesoderm (Jacobson, 1963a,b), as well as by the neural tube (Jacobson, 1963c; Stark *et al.*, 1997). Placode cells that arise from prominent thickenings of the ectoderm contribute to the eyes, nose, ears, and lateral line organs, whereas less prominent (trigeminal and epibranchial) placodes give rise to cranial sensory ganglia. Together, placode cells give rise to ciliated sensory receptors, sensory neurons, endocrine and neuroendocrine cells, glia, and other supporting cells (see reviews by Noden, 1991; Web and Noden, 1993; Baker and Bronner-Fraser, 2001).

Studies using anterograde transport of horseradish peroxidase from scarified corneal surfaces of cats (Morgan *et al.*, 1978; Marfurt, 1981), mice (Arvidson, 1977), monkeys (Morgan *et al.*, 1978), rabbits (Marfurt *et al.*, 1989), and rats (Marfurt, 1988) have shown that corneal sensory innervation is derived mainly from the ophthalmic branch of the trigeminal ganglion. Nonetheless, these dye-tracing experiments have failed to determine whether corneal nerves derive from neural crest, placode, or both. Lack of molecular markers that can distinguish between neural crest and placode-derived nerves has made it impossible to determine their origin at the cellular level. Here, the quail–chick chimera technique was utilized, together with species-specific antibodies to sensory nerves, to show that corneal sensory innervation is derived solely from neural crest. In addition, the results indicate that ablation of neural crest results in the loss of corneal innervation, whereas ablation of placode results in loss of corneal nerves by a mechanism that appears to be indirect, but required nonetheless. Such noninnervated corneas appeared to develop normal transparency and cellular density in the major layers, suggesting that cornea nerves do not play a major role in these processes.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chick (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*) eggs were purchased from Nelson Hatchery (Manhattan, KS) and B&D Game farm (Harrah, OK), respectively. Eggs were incubated in a forced-draft incubator at 38°C and approximately 70% relative humidity for 26–48 h to obtain embryos with 4–16 somites (HH stages 8–12; Hamburger and Hamilton, 1951). Chick embryos were lowered within the eggs by withdrawing 2 ml of albumin using an 18-gauge needle. Each egg was windowed just above the embryo and Pelikan Fountain India ink diluted at 1:10 in phosphate-buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HP0_4{\cdot}H_2O$, 1.4 mM KH_2PO_4, 0.9 mM $CaCl_2{\cdot}2H_2O$, 0.49 mM $MgCl_2{\cdot}6H_2O$, pH 7.4) was injected under the blastoderm to increase visibility of the embryo. The vitelline membrane covering the head region of the embryo was peeled away by using a pulled glass needle. Quail embryos were lifted from the eggs by using filter paper rings (Flamme, 1987), rinsed, staged, and kept at room temperature in PBS until needed.

Quail–Chick Grafts

Stage-matched chick and quail embryos between stages 8^+ and 9 (6–7 somites) were prepared as described above. Appropriate regions to be operated on were determined based on the fate map of D'Amico-Martel and Noden (1983). For neural crest chimeras (n = 39), a segment of the dorsal neural tube between the mesencephalon and metencephalon regions of the midbrain was unilaterally removed from the chick host and replaced with a corresponding

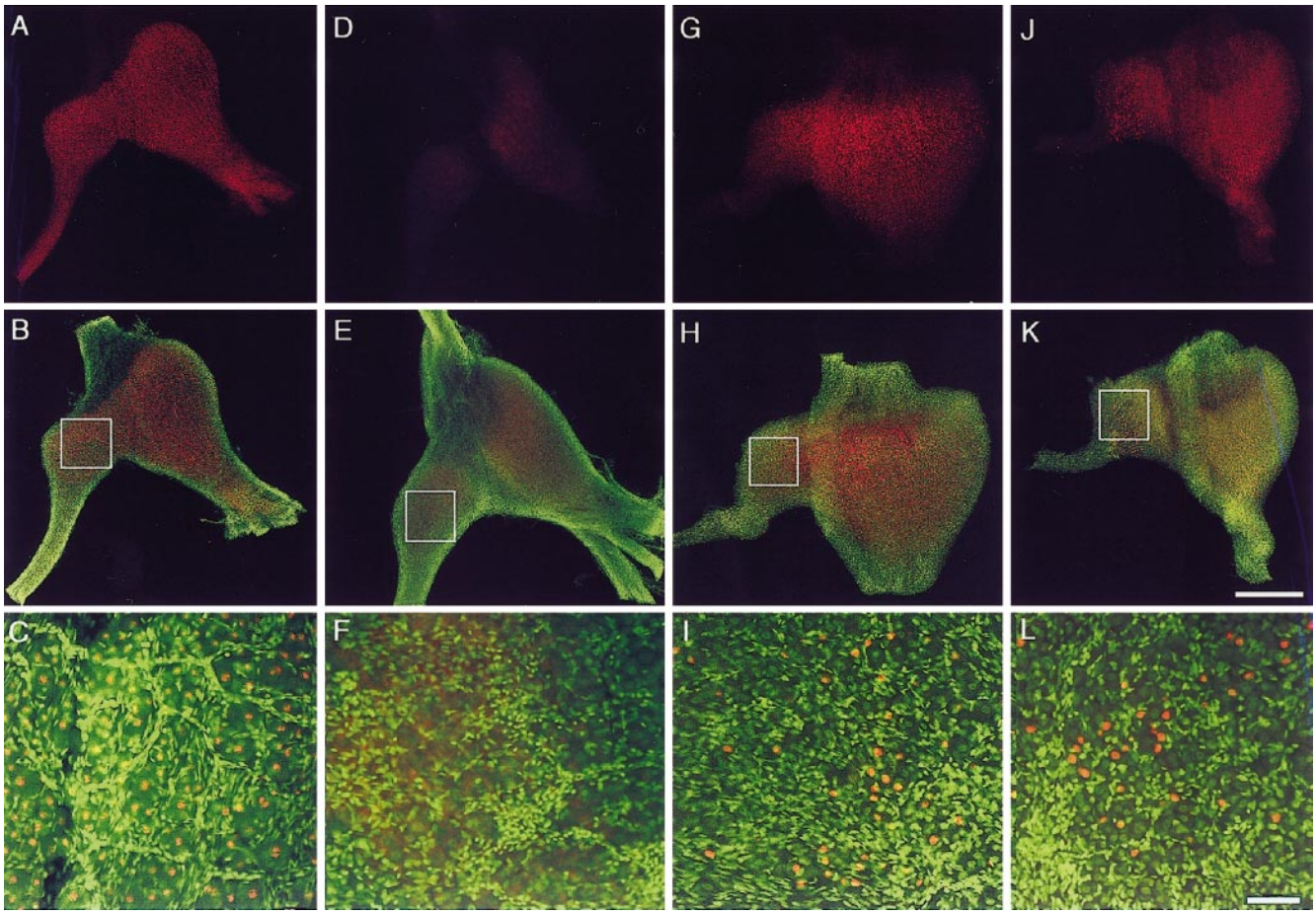

FIG. 2. Confocal images of whole-mount control and chimeric trigeminal ganglia showing QCPN-positive nuclei. (A–L) Trigeminal ganglia were immunostained with QCPN (red) then counterstained with PicoGreen (green). White boxes (B–K) are selected regions that are enlarged in (C–L), respectively. (A-C) Quail control. (D–F) Chick control. (G–I) Neural crest chimera. (J–L) Placode chimera. Bars in (A), (B), (D), (E), (G), (H), (J), (K), 0.5 mm; in (C), (F), (I), (L), 50 μm.

region from a quail donor (Fig. 1A). For placode chimeras (n = 23), ectoderm was removed from the midbrain region of the chick host and replaced with quail ectoderm from a similar region (Fig. 1A). Eggs were sealed with Scotch tape and chimeric embryos were reincubated for an additional 5–19 days at 38°C and approximately 70% relative humidity, with eggs carefully turned two to five times a day.

Ablations

Neural crest ablations were done as previously described by Yntema (1944) and based on fate maps of Couly and Le Douarin (1987) and D'Amico-Martel and Noden (1983). Briefly, neural crest (dorsal part of the neural fold including a small strip of ectoderm immediately lateral to it) was removed bilaterally from the region between the mesencephalon and first pair of somites of stages 8–9 (4–7 somites) or in the midbrain region of stage 10–12 (10–16 somites) chick embryos. Ectoderm ablations were done as previously described by Hamburger (1961) and based on the fate map of D'Amico-Martel and Noden (1983). Ectoderm adjacent to the neural tube was unilaterally removed from the midbrain region to the level of either the myelencephalon (stage 8–10) or the auditory placode in stage 11–12 (13–16 somites) chick embryos. Operated embryos were reincubated for an additional 14 days.

Antibodies and Immunostaining

All antibodies were diluted in CMF–PBS (154 mM NaCl, 2.97 mM $Na_2HPO_4{\cdot}7H_2O$, 1.06 mM KH_2PO_4, pH 7.4) containing 0.2% (w/v) bovine serum albumin, 0.2% (v/v) Triton X-100, and 5% (v/v) heat-inactivated goat serum (PBT). Quail-specific neural antibody QN (mouse IgG1; Tanaka *et al.*, 1990) and quail-specific nuclear marker antibody QCPN (mouse IgG1, developed by Bruce and Jean Carlson and available from the Developmental

Studies Hybridoma Bank, University of Iowa) were used diluted 1:1 in PBT. Mouse monoclonal anti-neuron-specific β-tubulin antibody TuJ1 (IgG2a; BABCO) was used diluted 1:500 in PBT to visualize all nerves. Fluorochrome-conjugated goat secondary antibodies were purchased from Molecular Probes [Alexa Fluor 488 anti-mouse IgG1, Alexa Fluor 594 anti-mouse IgG1, and Alexa Fluor 594 anti-mouse $F(ab')_2$ fragments] and used diluted 1:200 in PBT.

After appropriate periods of incubation at 38°C, embryos were collected in cold 4% paraformaldehyde (in Na-K-PO_4 buffer [0.062 M KH_2PO_4, 0.109 M Na_2HPO_4], pH 7.2; Barrett *et al.*, 2000) fixative. Corneas and trigeminal ganglia were removed from embryos and fixed further at room temperature for 2 h with mild agitation, then rinsed twice in CMF–PBS. Corneas were processed for whole-mount immunostaining as described by Barrett *et al.* (1999). Briefly, fixed corneas were trimmed, digested in an enzyme cocktail consisting of collagenase and hyaluronidase, then rinsed in CMF–PBS. Corneas and trigeminal ganglia were washed four times in PBT before overnight incubation in primary antibodies. Chimeric corneas were first labeled with QN, washed extensively in PBT, and then counterstained with TuJ1. Trigeminal ganglia labeled with QCPN were briefly counterstained with PicoGreen (a double-stranded DNA marker; Molecular Probes) to label all nuclei. Chimeric corneas, trigeminal ganglia, and eyelids were immunostained, together with corresponding quail and chick control tissues.

Transparency Test and Sectioning

Corneal transparency test was performed as previously described by Coulombre and Coulombre (1958), with minor changes, and comprises ability to photograph a mesh when viewed through the cornea. Briefly, fixed corneas were laid convex side up over a 200-mesh grid (Electron Microscopy Sciences, Fort Washington, Philadelphia) in a polystyrene dish filled with CMF–PBS. Photographs of the grid then were taken through the cornea using the same light, exposure, and magnification. After photography, each cornea was marked with small incisions, then immunostained for innervation. After imaging, noninnervated and control corneas were processed and sectioned at 10 μm as previously described (Wiens *et al.*, 1995), then stained with PicoGreen as described by Lwigale (1999).

Imaging

Stained corneas and ganglia were rinsed in CMF–PBS and mounted on slides using 50% (v/v) glycerol in CMF–PBS. Immunofluorescence staining of nuclei in the trigeminal ganglia and nerves innervating the corneas and eyelids was viewed in whole-mounts by using appropriate filters and images captured using a laser confocal microscope (Zeiss LSM 410, Oberkochen, Germany). After fixation, trigeminal ganglia and unstained corneas were photographed with a Leica MPS 52 camera (Heerbrugg, Switzerland) mounted on a Wild M5 stereo dissecting microscope (Heerbrugg). Contrast and brightness of some fluorescent images were enhanced by using Adobe Photoshop version 5.5 (Adobe Systems Incorporated, San Jose, CA).

RESULTS

Corneal Nerves Are Derived Entirely from Neural Crest Cells

In the chick embryo, cornea innervation begins at about E10 and continues for the next 8 days, until the cornea is fully innervated (Bee, 1982). To establish the cellular identity of corneal nerves, neural crest and placode chimeras at E18–E20 were first visually assessed for successful incorporation of the grafted tissues. Neural crest chimeras showed dark pigmentation of feathers on the operated side (Fig. 1B), indicating that melanocytes differentiated from some of the grafted quail neural crest. Placode chimeras lacked such pigmentation (Fig. 1C), as expected, indicating that there were no contaminating quail neural crest cells in the graft.

The cell bodies of the neurons that aggregate to give rise to the nerves that innervate the cornea are located in the trigeminal ganglion. These neurons arise from both small (neural crest) and large (placode) cell bodies (Gaik and Farbman, 1973). To ensure that the grafts of neural crest and placode tissues gave rise to trigeminal cells, ganglia from chimeras, together with controls from chick and quail embryos, were immunostained with an antibody to a quail-specific perinuclear marker, QCPN. All nuclei were labeled in the quail control trigeminal ganglia (Figs. 2A–2C), whereas the chick control trigeminal ganglia (Figs. 2D–2F) showed no staining for QCPN. Neural crest chimeras showed abundant quail nuclei located in the dorsal (proximal) part of the trigeminal ganglion (Figs. 2G–2I). In contrast, placode chimeras showed quail nuclei in the ventral (distal) parts of both the ophthalmic and maxillomandibular branches of the trigeminal ganglion (Figs. 2J–2L). Overlays of QCPN images with those derived from PicoGreen staining (e.g., Figs. 2B, 2E, 2H, 2K and 2C, 2F, 2I, 2L) often do not generate yellow pseudo-color because QCPN labels the perinuclear membrane, whereas PicoGreen labels double-stranded DNA within nuclei; i.e., in each doubly labeled cell, the two images are not expected to overlap extensively.

Given that both quail-derived neural crest and placode cell bodies were present in the trigeminal ganglia, their afferent nerves were revealed in the cornea using a quail-specific neural maker, QN. All nerves in the quail control cornea were stained by the QN antibody (Fig. 3A) and by the anti-neuron-specific β-tubulin antibody (TuJ1) used as a counterstain (Figs. 3B–3D). No QN-positive nerves were present in the chick control cornea (Fig. 3E), although it is fully innervated, as shown by the TuJ1-positive nerves (Figs. 3F–3H). All neural crest chimeras analyzed ($n = 39$) showed quail neural crest-derived (QN-positive) nerves in their corneas (Figs. 3I–3L). The number of QN-positive nerves was highest when neural crest was grafted between stages 8 and 9, and decreased with older embryos up to

about stage 12 (data not shown), at which time very few nerves were seen in the chimeric corneas, suggesting that trigeminal neural crest cells migrate from the neural tube between stages 8 and 12. All placode chimeras analyzed ($n = 23$) showed no quail placode-derived (QN-positive) nerves in the corneas (Fig. 3M), although the corneas were fully innervated (Figs. 3N–3P).

Some chimeras were sacrificed at E10 when corneal nerves begin to emerge from the pericorneal nerve ring and radially invade the cornea (Bee, 1982). At this stage, no quail placode-derived nerves were seen in the nerve ring (Fig. 4A), although the nerve ring was present and the cornea was partially innervated (Fig. 4B). In contrast, quail neural crest-derived nerves were seen in the surrounding nerve ring, as well as inside the developing cornea (Figs. 4C and 4D). These results demonstrate that only neural crest-derived nerves are located in the nerve ring surrounding the developing cornea and subsequently innervate the entire cornea. The data suggest that, although both neural crest and placode cells populate the trigeminal ganglion, corneal nerves are derived solely from neural crest cells.

Trigeminal Placode- and Neural Crest-Derived Nerves Innervate the Eyelids

The data above suggest that placode-derived nerves do not contribute to the nerve ring or to corneal innervation. To investigate whether such nerves innervate other tissues within the vicinity of the eye, some neural crest and placode chimeras were fixed between E9 and E10 and quail-derived nerves were traced from the trigeminal ganglion (using QCPN, not shown) to the pericorneal nerve ring and cornea and to the eyelids. Because of the difficulty involved in staining multiple tissue layers, eyelids were dissected from the corneas before immunostaining. Nerves derived from the transplanted quail neural crest or placode cells were identified utilizing the same methods used earlier to demonstrate corneal nerves (see Fig. 3). Chimeric corneas yielded staining similar to that of the pericorneal nerve ring and corneal nerves shown in Fig. 4. Quail-control eyelids showed positive staining for both QN (Fig. 5A) and TuJ1 (Figs. 5B and 5C). Chick-control eyelids showed no staining for QN (Fig. 5D) but showed positive staining for TuJ1 (Figs. 5E and 5F). Chimeric eyelids showed quail neural crest-derived nerves (Figs. 5G–5I), as well as quail placode-derived nerves (Figs. 5J–5L) in the eyelids. These results indicate that, although placode-derived nerves do not contribute to the pericorneal nerve ring and cornea, they (together with neural crest-derived nerves) innervate the eyelids.

Ablation of either Trigeminal Neural Crest or Placode Prevents Corneal Innervation

Ablation of cranial neural crest has been shown to result in diminished ganglia comprised entirely of placode cells, whereas ablation of placode leads to development of only the maxillomandibular branch of trigeminal ganglia comprised entirely of neural crest cells (Yntema, 1944; Hamburger, 1961). Nonetheless, these ablations do not cause substantial deformations to the embryos. Here, either neural crest or placode was ablated at various stages of development, with embryos incubated for an additional 14 days, then assessed for effects on the trigeminal ganglion and corneal innervation.

Neural Crest Ablations

Given that the neural crest that gives rise to corneal nerves emigrated from the neural tube between stages 8 and 12, neural crest was bilaterally ablated from chick embryos at these stages of development. Embryos operated on between stages 8 and 9 failed to heal completely and part of the brain remained exposed. The midbrain region of these embryos was greatly reduced and some of them were cross-beaked (not shown). Nonetheless, the eyes and other facial structures (such as the eyelids and external auditory meatus) of these embryos appeared normal morphologically. Embryos operated on between stages 10 and 12 looked normal at E14 when they were euthanized and fixed. Table 1 (A) summarizes results from 34 sides of 17 experimental embryos between stages 8 and 12.

Embryos whose neural crest was ablated between stages 8 and 9 (Fig. 6A, $n = 11$) showed a drastic reduction in the size of trigeminal ganglia (Fig. 6B) compared with corresponding controls (Fig. 6C). Trigeminal ganglia could not be located in seven embryos, probably because they were too diminished to be differentiated from the dura mater covering them and the petrous bone in the middle cranial fosa that would normally cradle the ganglia. Four corneas from both stages 8 and 9 showed complete lack of innervation (Fig. 6D). Few nerves were seen in the limbus surrounding the cornea (arrow). Some of the corneas had a few nerve bundles that branched and bifurcated extensively to innervate much of the cornea (Fig. 6E), compared with a control cornea nerve pattern (Fig. 6F). Previous studies have shown that juxtaposition of ectoderm and neural tube allows regeneration of neural crest cells (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995) that are capable of forming cranial ganglia (Moury and Jacobson, 1990). Thus, it is possible that after neural crest ablation at stages 8 and 9, the ectoderm and neural tube were able to regenerate, become juxtaposed, and generate new neural crest cells that then migrated into the trigeminal ganglion. Some of the nerves derived from such late-forming neural crest were able to reach and innervate the cornea (Fig. 6E), whereas in some cases they did not enter the cornea (Fig. 6D). These data suggest that corneal nerves are neural crest-derived and that there is only a short developmental time period during which the cornea is receptive to pathfinding by trigeminal axonal growth cones.

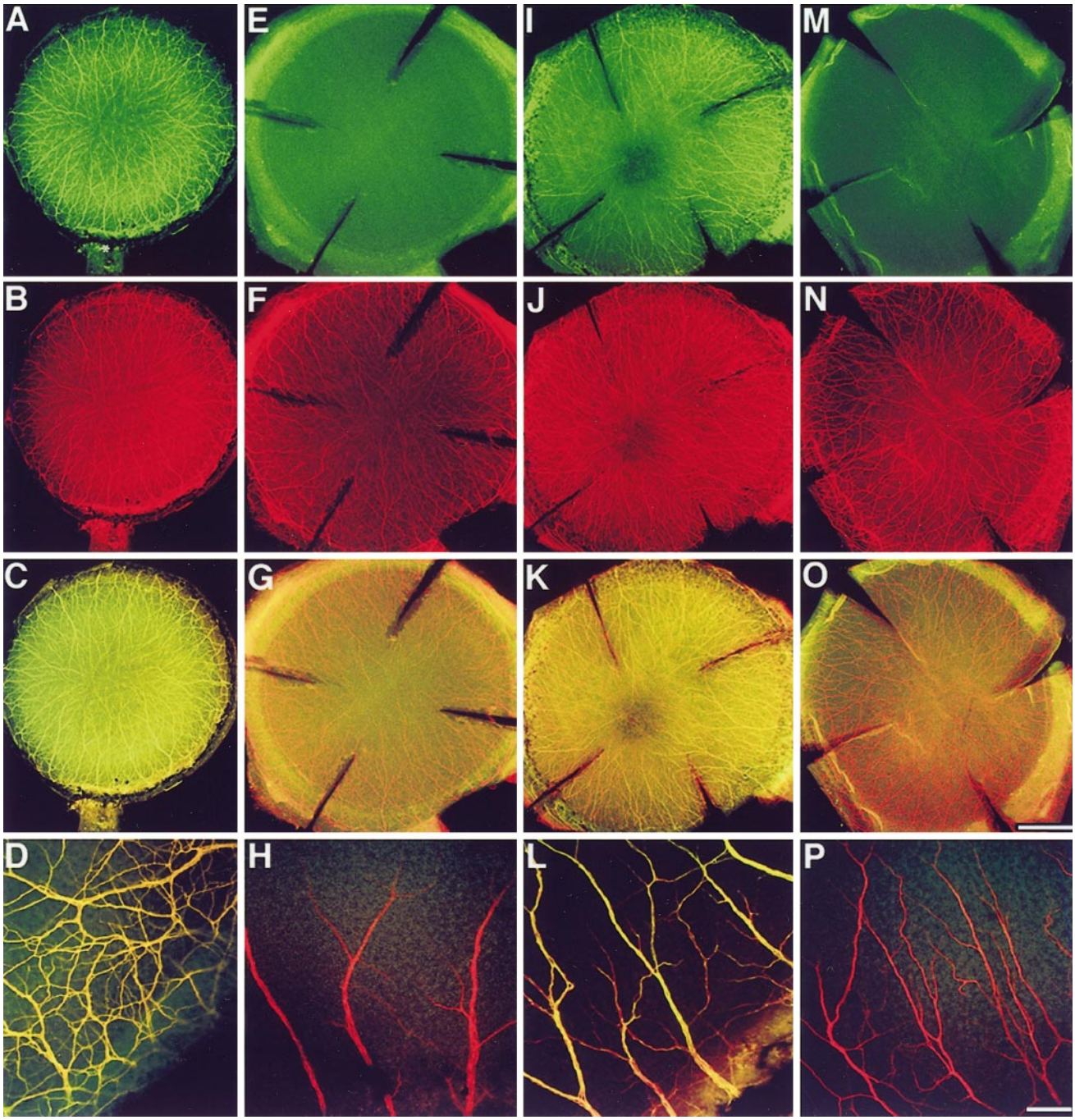

FIG. 3. Confocal images of whole-mount control and chimeric corneas showing quail-derived (QN-positive, green) nerves and all (TuJ1 positive, red) nerves. Digital overlay of the two (C, G, K, O) shows yellow quail-derived nerves. (A–D) Quail control. (E–H) Chick control. (I–L) Neural crest chimera. (M–P) Placode chimera. (D, H, L, P) Higher magnifications of control and chimeric embryos distinct from those shown at lower magnification. Bars in (A–C), (E–G), (I–K), (M–O), 0.5 mm; in (D), (H), (L), (P, 100 μm.

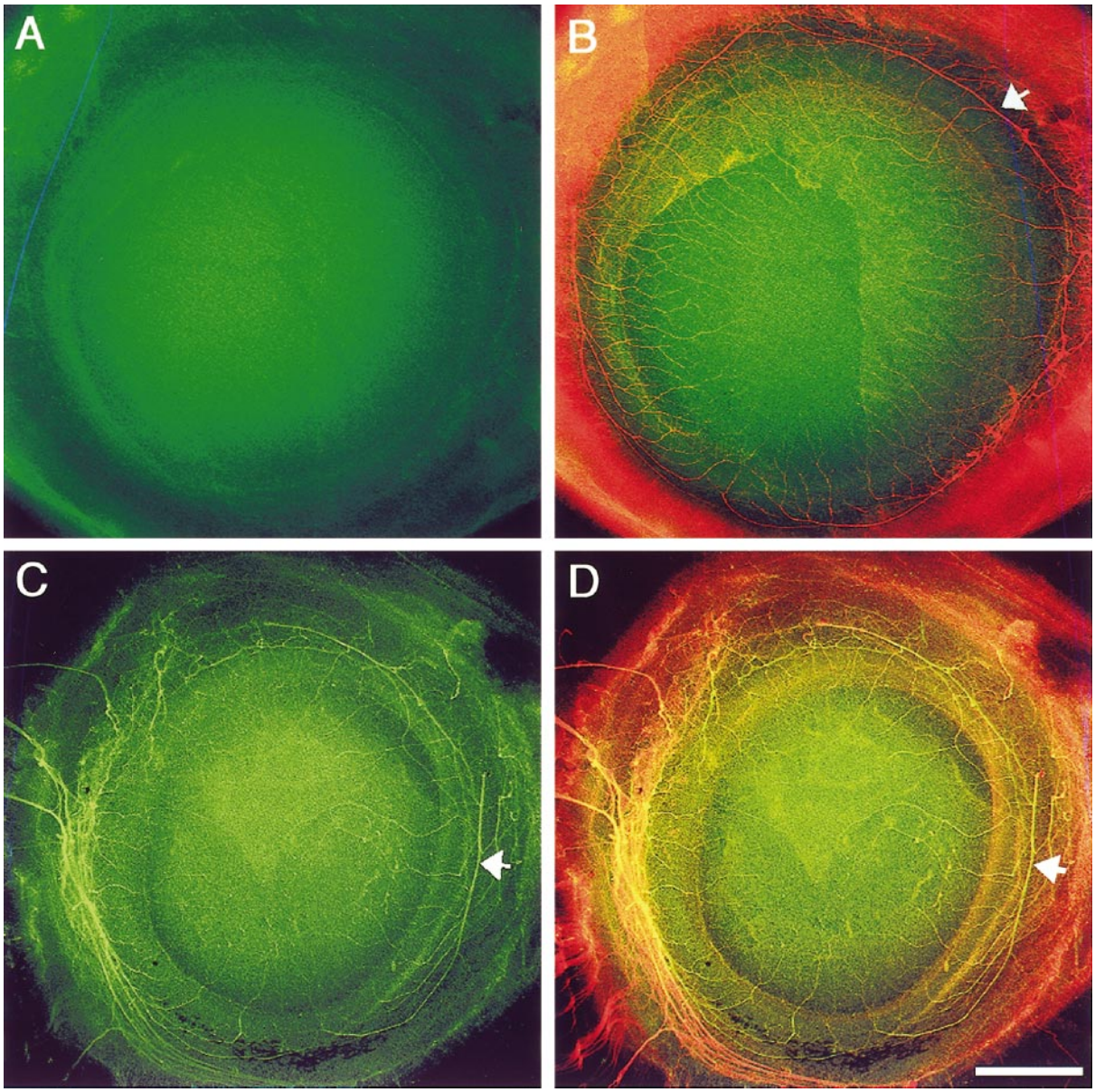

FIG. 4. Whole-mount immunostaining of E10 chimeric corneas showing nerves in the cornea and surrounding nerve ring. (A, B) Placode chimera. (C, D) Neural crest chimera. (A, C) Quail-derived (QN-positive, green) nerves. (B, D) Double-labeling with TuJ1 to show quail-derived (yellow) and all other corneal nerves (red). The arrowheads indicate the nerve ring. Bar, 0.5 mm.

Some of the embryos whose neural crest was ablated at stage 9 and all experimental embryos operated on between stages 10 and 12 had normal trigeminal ganglia (Fig. 6C) and corneal innervation similar to that of controls (Fig. 6F). This suggests that most of the trigeminal neural crest has migrated away from the neural tube after stage 9. Since neural crest cells are mitotic after they condense to form the trigeminal ganglia (D'Amico-Martel and Noden, 1980), the few cells ablated after stage 9 may easily have been replaced to form normal ganglia and corneal innervation.

The above results indicate that ablation of neural crest cells prior to their migration drastically decreases the size of the trigeminal ganglion (which, in this case, is probably comprised of mostly or, only placode cells). As a consequence of this ablation, complete lack of corneal

TABLE 1
Effects of Neural Crest or Placode Ablation on Trigeminal Ganglion Morphology and Corneal Innervation

Tissue ablation	HH stage	*n*	Trigeminal ganglia	Corneal innervation
(A) Neural crest	8–9	18[a]	Reduced in size (n = 11, Fig. 6B)	Complete loss of innervation (n = 1, Fig. 6D)
			ND[b] (n = 7)	Very few nerves (n = 12, Fig. 6E)
				Almost normal innervation (n = 2)
	9–12	16[a]	Normal	Normal
(B) Placode	8–10	5	Normal	Normal
	11	3	Ophthalmic branch reduced (Fig. 7C)	Normal
	12	11	Ophthalmic branch absent; only maxillomandibular branch present (Fig. 7B)	Very few nerves (Fig. 7E)

[a] Because neural crest ablation was done bilaterally, each trigeminal ganglion and cornea on that side of the embryo were treated as one data set, separate from the corresponding tissues from the other side of the embryo.
[b] Could not locate trigeminal ganglia (in usual location), but all corneas from these cases had a few nerves.

innervation can occur (Table 1A), further suggesting that corneal nerves are derived entirely from neural crest cells and that placode cells cannot regulate to form corneal nerves in the absence of neural crest cells.

Placode Ablations

Ectoderm was ablated from the right sides of embryos between stages 8 and 12. Embryos ablated of ectoderm at all stages looked normal at the time of fixation. The data from placode ablations are summarized in Table 1 (B). Embryos operated on between stages 8 and 10 (not shown, n = 5) had normal trigeminal ganglia and corneal innervation similar to those of left side controls (Figs. 7D and 7F). Ablations at stage 11 (not shown, n = 3) resulted in trigeminal ganglia with a smaller ophthalmic branch (Fig. 7C), but normal corneal innervation. Ablations at stage 12 (Fig. 7A, n = 11) resulted in the complete disappearance of the ophthalmic branch of the trigeminal ganglion, and the maxillomandibular branch which formed was reduced in size (Fig. 7B). The corneas from the right sides of these embryos showed a drastic reduction in corneal innervation (Fig. 7E). These results confirm what has been shown previously: placode cells are required for the proper formation of the trigeminal ganglion (Yntema, 1944; Hamburger, 1961; Noden, 1978b), and what has been proposed previously: in the absence of placode cells, neural crest-derived nerves fail to properly innervate their normal peripheral targets (Hamburger, 1961; Noden, 1978b). Corneal innervation, when any occurred at all, despite the complete lack of the ophthalmic branch, was probably derived from the maxillomandibular neural crest, as previously observed by Marfurt *et al.* (1989).

Complete Lack of Innervation Has No Detectable Effect on the Development of Corneal Transparency or on the Cell Densities of Its Cellular Layers

In the chick embryo, corneal transparency increases dramatically at about E14 (Coulombre and Coulombre, 1958), shortly after the initiation of corneal epithelial innervation at about E12 (Bee, 1982). Since these two events happen during the same developmental period, the role of corneal nerves during the development of transparency was assessed by comparing some of the corneas from E14 experimental embryos (whose neural crest was ablated) with stage-matched controls. Corneas lacking innervation (n = 4, Figs. 8A and 8C) did not show detectable differences in transparency as compared with controls (Figs. 8B and 8D), as assessed by the ability to visualize a mesh grid as viewed through the entire cornea. Following this transparency test, some of the noninnervated and control corneas were sectioned and stained with PicoGreen to label all nuclei of the different cell layers (Lwigale, 1999). There was no obvious difference in cellular density between noninnervated (Fig. 8E) and fully innervated control corneas (Fig. 8F). These results suggest that innervation of the cornea does not play a major role during development of avian corneal transparency and infer that it is not involved in determining corneal cell densities during embryogenesis.

DISCUSSION

It is generally accepted that cranial ganglia of dual origin send both neural crest- and placode-derived afferent nerves to the tissues they innervate, but the data presented here represent an exception to that generalization. Dye-tracing studies using a variety of organisms (Arvidson, 1977; Morgan *et al.*, 1978; Marfurt, 1981, 1988; Marfurt *et al.*, 1989)

have indicated repeatedly that cell bodies of corneal nerves are located in the ophthalmic branch of the trigeminal ganglion. Nonetheless, such methods have not allowed discrimination between neural crest- and placode-derived projections. Here, the quail–chick chimera technique was used, together with species-specific monoclonal antibodies to nerves, to demonstrate for the first time that corneal sensory innervation (from the trigeminal ganglion of dual origin) is entirely derived from neural crest cells. In addition, it was demonstrated that ablation of either neural crest or placode cells at a critical period during embryonic development results in deformed trigeminal ganglia and lack of corneal innervation, but with no apparent effect on corneal cellular density or on development of transparency. These results reveal an heretofore undetected developmental requirement for the presence of placode-derived cells in the trigeminal ganglion by neural crest-derived nerves to allow corneal innervation.

Origin of Corneal Nerves

Neural crest cells that contribute to the trigeminal ganglion arise following the fusion of the neural folds of the midbrain region (between the mesencephalon and metencephalon) of approximately stage-9 chick embryos (Noden, 1975). The neural crest cells then migrate ventro-laterally on both sides of the embryo, condense in the dorsal region of the forming trigeminal ganglion, and continue to divide. At about stage 11, placode cells, which by then are mostly postmitotic (D'Amico-Martel and Noden, 1980), delaminate from the ectoderm lateral to the midbrain region, migrate, and condense caudal and lateral to the dividing neural crest. At about E2, differentiated placode cells begin to send out afferent neurons (from the trigeminal ganglion), later followed by neural crest-derived neurons beginning at about E4 (D'Amico-Martel and Noden, 1980; Covel and Noden, 1989). The dual origin of the trigeminal ganglion was demonstrated earlier by using autoradiographic (D'Amico-Martel and Noden, 1980) and the quail–chick chimera technique (D'Amico-Martel and Noden, 1983; Couly and Le Douarin, 1985, 1987). Here, the dual origin of the trigeminal ganglion has been confirmed using the quail–chick chimera technique, together with a quail-specific-QCPN monoclonal antibody. Further, using a quail-specific-QN monoclonal antibody, it has been demonstrated that part of the neural crest cell population of the trigeminal ganglion sends out afferent nerves through the ophthalmic branch, which initially form a nerve ring around, and later innervate, the cornea. In contrast to neural crest cells, placode cells do not send afferent nerves to the pericorneal nerve ring or into the cornea. However, both neural crest and placode cells send out afferent sensory nerves to the eyelids, an observation supported by dye-tracing experiments carried out in chick embryos (Noden, 1980a; Covel and Noden, 1989).

Neural Crest Ablation

Previously, it was suggested that neural crest formation in the midbrain region commences shortly after stage 8 (Lumsden *et al.*, 1991) and goes on until shortly after stage 10 (Barembaum *et al.*, 2000). In the present study, the number of quail neural crest-derived nerves was highest in the chimeric corneas when isotopic transplantations of midbrain neural crest cells were performed at about stages 8+ and 9, and decreased in subsequent stages until about stage 12, when very few quail nerves could be identified in the cornea. These data suggest that trigeminal neural crest cells emigrate from the neural tube between stages 8 and 12.

Following this observation, neural crest from the midbrain level was ablated bilaterally in stages 8–9 chick embryos to confirm the above observation that corneal nerves were derived entirely from neural crest. Secondly, it was also determined whether, in the absence of trigeminal neural crest cells, placode cells could undergo a regulative transformation and functionally replace neural crest. Only embryos operated on between stages 8 and 9 showed greatly reduced midbrain regions that remained exposed. Nonetheless, these and embryos operated on between stages 10 and 12 displayed normal development of the eyes and facial structures, confirming earlier observations (Yntema, 1944; Hamburger, 1961; McKee and Ferguson, 1984; Scherson *et al.*, 1993).

Some of the embryos operated on at stage 9 and all embryos operated on between stages 10 and 12 formed normal trigeminal ganglia and corneal innervation. Trigeminal ganglia from embryos operated on between stage 8 and some stage 9 were generally smaller than controls, but all the major branches were present. In some cases, no trigeminal ganglia were found in their usual location, but no instances were seen of two separated ganglia as reported earlier by Yntema (1944) and Hamburger (1961). Some of these embryos showed complete lack of corneal innervation, whereas some showed substantial reduction in corneal innervation. However, nerves were seen in the limbus surrounding the completely noninnervated corneas but none entered, suggesting that neural crest cells were able to regulate after ablation, contribute to the trigeminal ganglion, but could not enter the cornea. This suggests that there is a narrow time window of opportunity for neural crest-derived pathfinding axonal growth cones to enter the corneal stroma. However, the possibility that in the absence of neural crest, placode-derived nerves can innervate the limbus but fail to enter the cornea cannot be ruled out. Nonetheless, these data confirm my initial observation that corneal nerves are derived entirely from neural crest cells and also suggest that placode cells cannot regulate to form corneal nerves in the absence of neural crest cells.

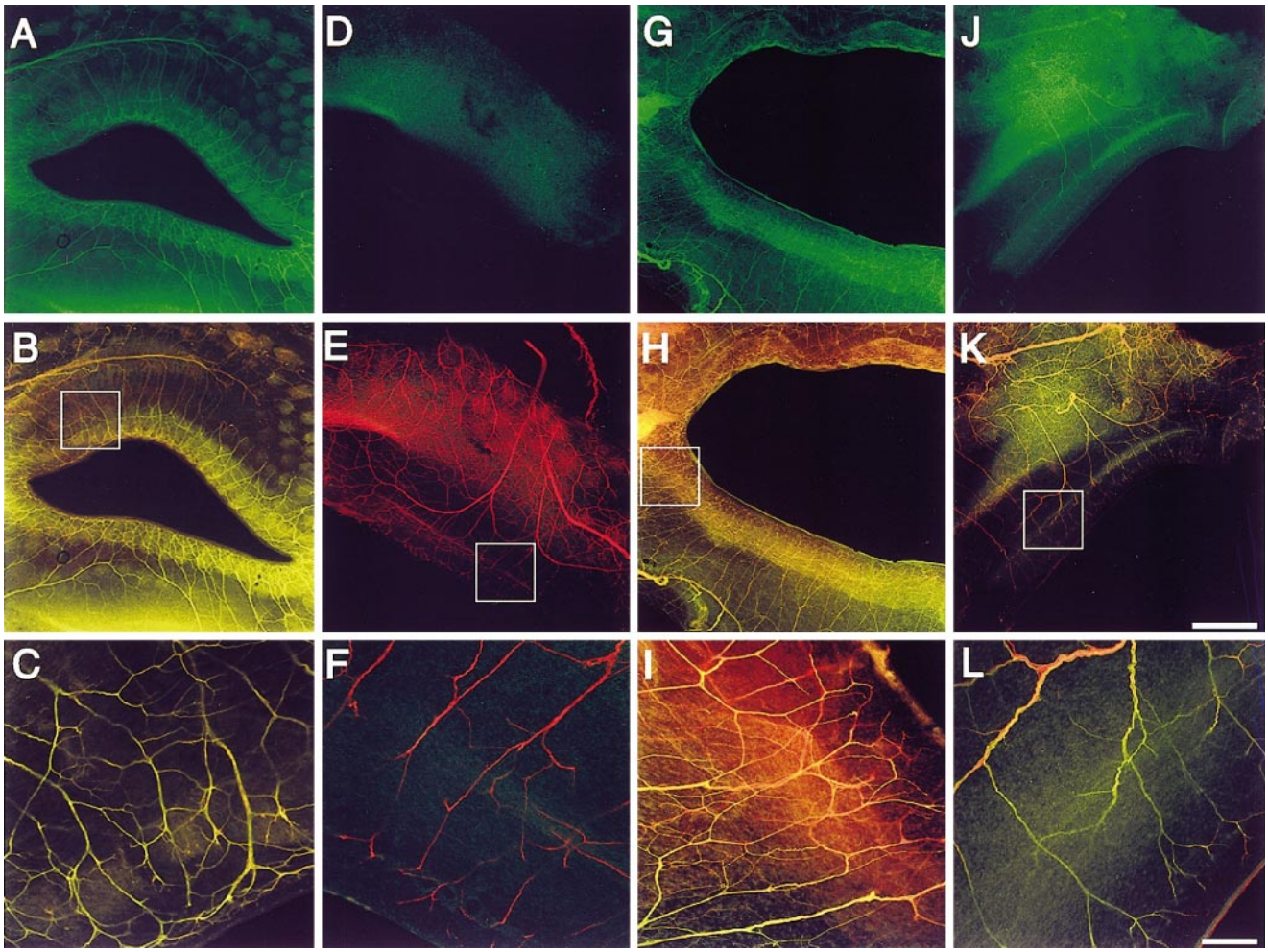

FIG. 5. Whole-mount control and chimeric eyelids showing quail-derived (QN-positive, green) and TuJ1-positive (red) nerves. Overlay of the two (B, E, H, K) shows yellow quail-derived nerves where they are present. (C, F, I, L) Magnifications of selected regions (white boxes) from (B), (E), (H), and (K), respectively. (A–C) Quail control. (D–F) Chick control. (G–I) Neural crest chimera. (J–L) Placode chimera. Bars in (A), (B), (D), (E), (G), (H), (J), (K), 0.5 mm; in (C), (F), (I), (L), 100 μm.

Placode Ablation

The experiments above clearly indicate that corneal sensory innervation is derived solely from neural crest cells. Previously, it was shown that ablation of trigeminal placode results in absence of the ophthalmic branch and formation of only the maxillomandibular lobe of the trigeminal ganglion (Yntema, 1944; Hamburger, 1961; Noden, 1978b). This suggests that interaction between neural crest and placode cells is necessary for the proper formation of the trigeminal ganglion and projection of afferent nerves to their peripheral targets (reviewed by Noden, 1991).

To explore whether ablation of the trigeminal placode has an impact on corneal innervation, appropriate cranial ectoderm was ablated from embryos between stages 8 and 12. Embryos operated on between stages 8 and 10 formed morphologically normal trigeminal ganglia and normal patterns of corneal innervation, possibly due to reconstitution of ectoderm in the ablated region before stage 12, the time at which it becomes specified to form trigeminal precursors (Baker and Bronner-Fraser, 2000). Placode ablation at stage 11 resulted in somewhat smaller trigeminal ganglia that nevertheless had all three major branches; although the ophthalmic branch was greatly reduced, corneal innervation was normal. This result suggests that ablation at stage 11 allows a few placode cells to populate the trigeminal ganglion, thus enabling the neural crest cells to aggregate in the ophthalmic branch and innervate the cornea. In con-

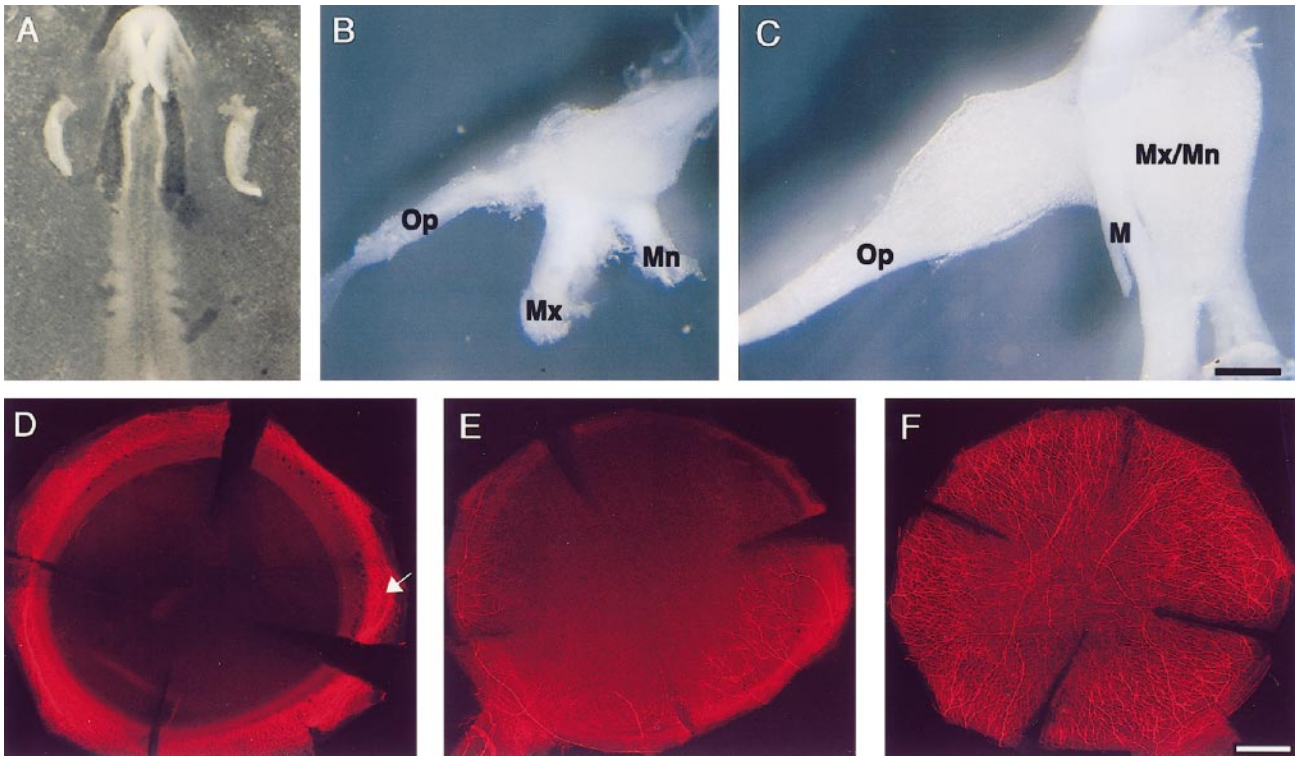

FIG. 6. Bilateral ablation of neural crest in the midbrain region of chick embryos results in diminished trigeminal ganglia and prevention of corneal innervation. (A) Stage-8 embryo showing the region of ablated neural folds, including adjacent ectoderm and the excised tissues. (B) Embryonic day-14 trigeminal ganglion from operated embryo. (C) Embryonic day-14 control trigeminal ganglion showing the two major lobes and the motor nerve. (D–F) Embryonic day-14 corneas immunostained with TuJ1 showing: (D) a noninnervated cornea with nerves in the ring that failed to enter the stroma (arrow) and remained in the limbus, (E) a partially innervated cornea with few nerves that branch extensively, and (F) a control cornea showing all nerves. Op, ophthalmic branch; M, trigeminal motor nerve; Mn, mandibullar branch; Mx, maxillary branch. Bars, 0.5 mm.

trast, ablation at stage 12 resulted in absence of the ophthalmic branch and the formation of only the maxillomandibular branch of the trigeminal ganglion; corneal innervation was greatly reduced. These results suggest that ablation at stage 12 removes ectoderm that has already been specified to form placode and does not allow time for its regeneration. Hence, trigeminal ganglia form that are devoid of those placode cells which play a major role in the formation of at least one of its major branches, the ophthalmic branch. The maxillomandibular branch forms anyway, due to the presence of the trigeminal motor nerve (reviewed by Noden, 1991). Dye-tracing experiments have shown that few cell bodies are labeled in the maxillomandibular branch when horseradish peroxidase is applied to scratched corneas (Marfurt *et al.*, 1989). Therefore, given the complete lack of the ophthalmic branch of the trigeminal ganglion, it is not surprising that few corneal nerves formed. In addition to confirming that placode cells are necessary for the proper formation of the trigeminal ganglion in general (Yntema, 1944; Hamburger, 1961; Noden, 1978b), this result suggests that neural crest-derived corneal innervation specifically is dependent upon the presence of placode cells at least within the ganglion. Thus, although placode cells do not form corneal nerves *per se*, they appear to be necessary within the ganglion to support the neural crest cells in their direct formation of corneal nerves. Crucial interactions between these two cell types therefore appear to occur within the trigeminal ganglion and will be the subject of future studies.

Complete Lack of Corneal Innervation and Its Effect on the Development of Transparency and Cell Densities

Corneal transparency is maintained via proper dehydration of the stroma by Na^+/K^+ ATPase pumps located on the basolateral side of cells of the endothelium (see reviews by Gordon, 1994; Stiemke *et al.*, 1995; Edelhauser, 2000), as

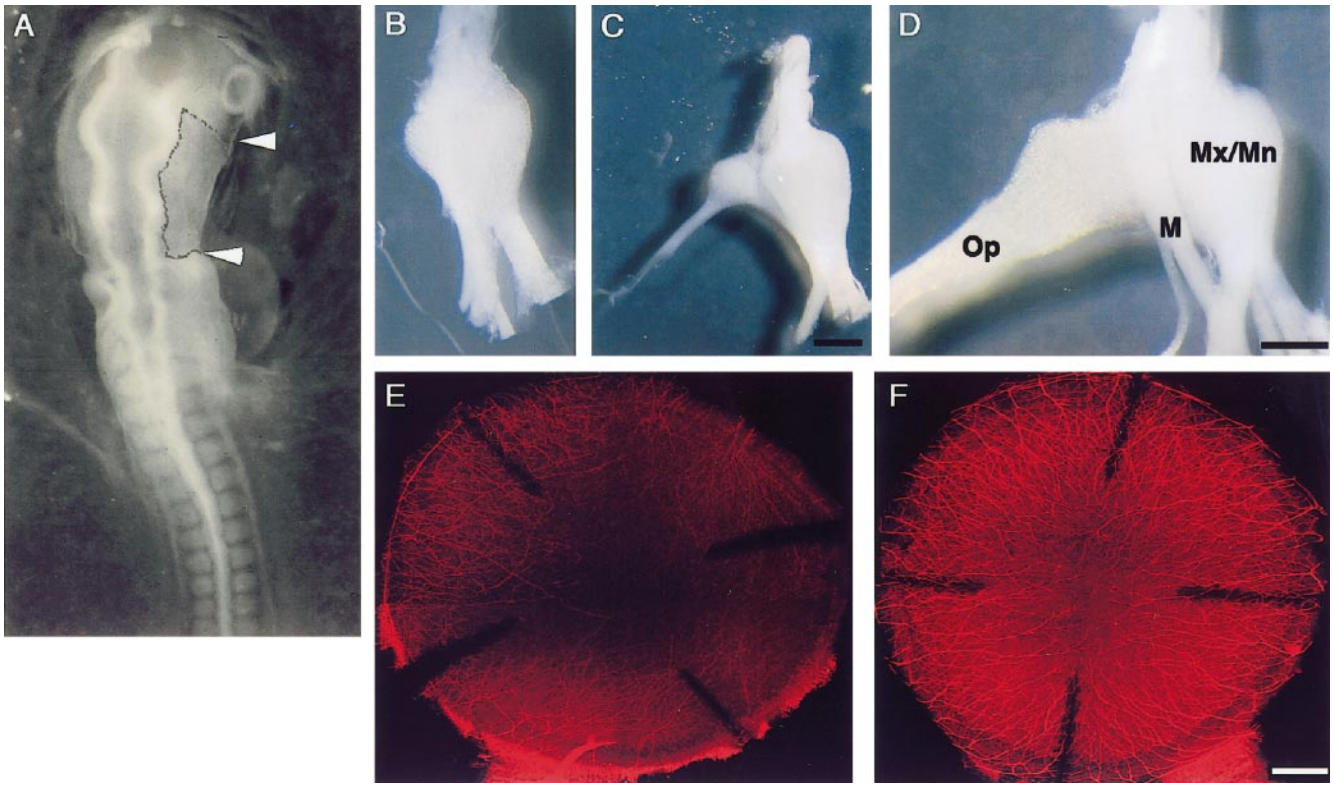

FIG. 7. Unilateral ablation of ectoderm from a stage-12 chick embryo results in the absence or reduction of the ophthalmic trigeminal branch and a reduction in the number of corneal nerves. (A) Stage-12 chick embryo showing the region of ablated ectoderm (traced out between arrowheads). (B–D) Embryonic day-14 trigeminal ganglia showing: (B) absence, (C) reduction of ophthalmic branch of trigeminal ganglion, and (D) normal trigeminal ganglion. (E, F) Embryonic day 14 corneas immunostained with TuJ1 showing: (E) partial innervation due to ablation of ectoderm at stage 12, and (F) complete innervation shown by ablation of ectoderm at stage 11 or earlier, and controls. Op, ophthalmic branch; M, trigeminal motor nerve; Mn, mandibullar branch; Mx, maxillary branch. Bars, 0.5 mm.

well as the epithelium, which acts as a barrier to fluid loss. It has been shown that denervation of the cornea in normal adult eyes results in substantial loss of transparency (Beuerman and Schimmelpfennig, 1980) due to decreased mitotic activity and hence, disruption of the epithelial barrier (Mishima, 1957), clinical conditions known as exposure and neurotrophic keratitis (Foster, 1994). Nevertheless, at embryonic stages, it has been very challenging to produce corneas similarly lacking innervation, apparently because of the great ability of the trigeminal neural crest cells to regenerate after ablations.

Here, for the first time, it is shown that it is possible to produce virgin noninnervated corneas by ablating neural crest cells between stages 8 and 9 of chick embryonic development. Since development of corneal transparency begins (day 13–14) shortly after its innervation (day 10–11), the possible role of corneal nerves in the development of its transparency was investigated. No differences were observed between noninnervated and innervated control corneas at the same developmental stage. Although chick corneal transparency has classically been monitored by this technique in the past (Coulombre and Coulombre, 1958), the method has been questioned recently (Maurice, 2001), suggesting that possible neural dependence of transparency might not have been detected by the grid-imaging method used here.

Because the constituent cells of each cellular layer of the cornea contain nuclei with characteristic morphologies (Lwigale, 1999), noninnervated corneas were sectioned and stained to assess whether corneal nerves were involved in maintaining the mitotic activities of developing corneal cells. There was no discernible difference in cell densities in any cellular layer of the cornea between normal and noninnervated corneas. This suggests that, although corneal nerves are critical to the maintenance of transparency in adult corneas, they do not play a major role during the development of its transparency, nor in the processes involved in determining its cellular densities during embryogenesis.

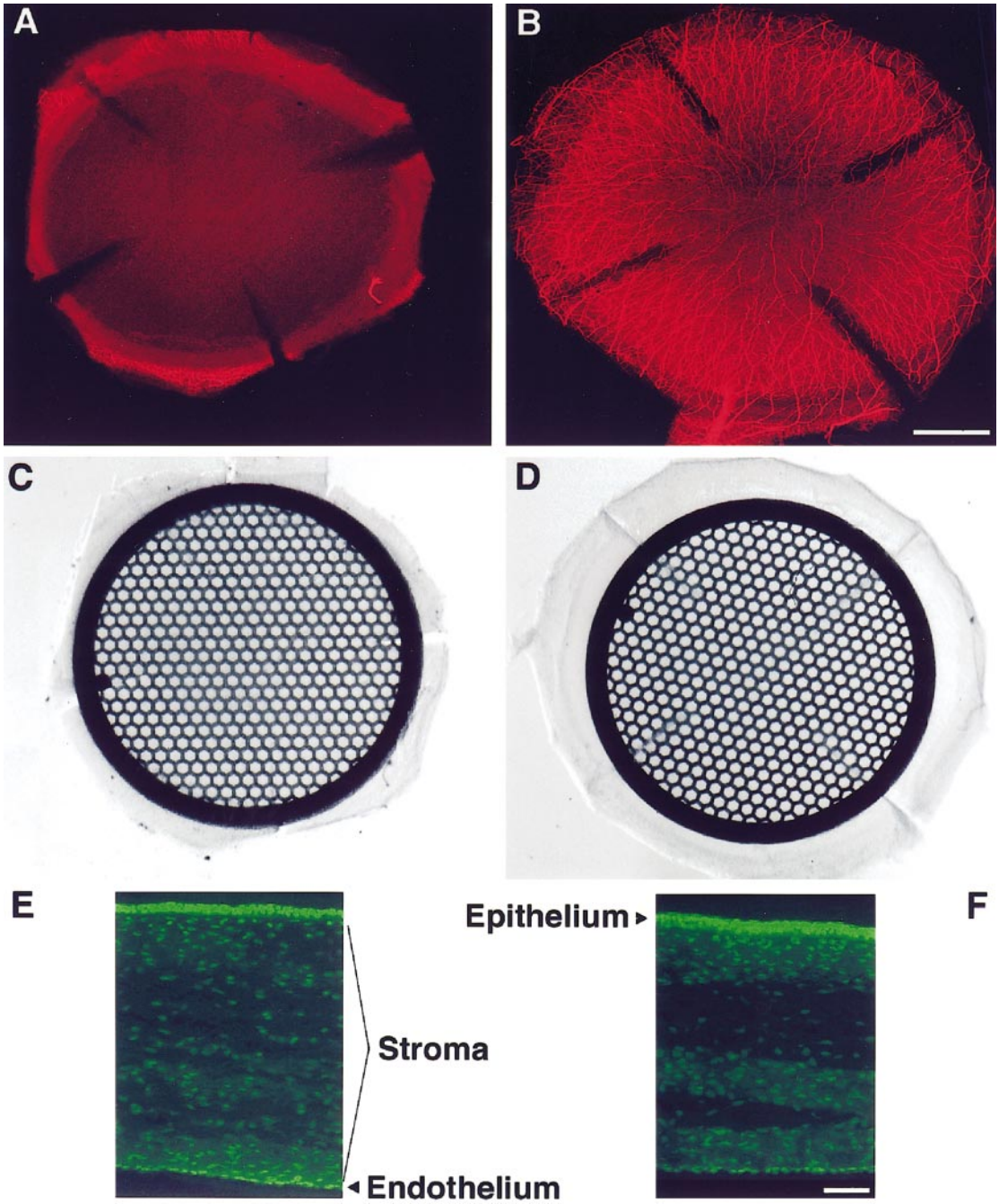

FIG. 8. Complete lack of innervation has no apparent effect on corneal transparency or on cell density. Corneas were assessed for transparency before immunostaining and only data from noninnervated and control corneas were used. (A, B) Corneas immunostained with TuJ1. (C, D) Transparency test of corneas in (A) and (B) using a 200-mesh grid. (E, F) Cross-sections of (A) and (B) stained with PicoGreen to show all nuclei of cellular layers of the cornea. Bars in (A–D), 0.5 mm; in (E), (F), 50 μm.

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