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Pax3-Expressing Trigeminal Placode Cells Can Localize to Trunk Neural Crest Sites but Are Committed to a Cutaneous Sensory Neuron Fate

Clare V. H. Baker,[1,2] Michael R. Stark,[3] and Marianne Bronner-Fraser

Division of Biology, Beckman Institute 139-74, California Institute of Technology, Pasadena, California 91125

The cutaneous sensory neurons of the ophthalmic lobe of the trigeminal ganglion are derived from two embryonic cell populations, the neural crest and the paired ophthalmic trigeminal (opV) placodes. Pax3 is the earliest known marker of opV placode ectoderm in the chick. Pax3 is also expressed transiently by neural crest cells as they emigrate from the neural tube, and it is reexpressed in neural crest cells as they condense to form dorsal root ganglia and certain cranial ganglia, including the trigeminal ganglion. Here, we examined whether Pax3+ opV placode-derived cells behave like Pax3+ neural crest cells when they are grafted into the trunk. Pax3+ quail opV ectoderm cells associate with host neural crest migratory streams and form Pax3+ neurons that populate the dorsal root and sympathetic ganglia and several ectopic sites, including the ventral root. Pax3 expression is subsequently downregulated, and at E8, all opV ectoderm-derived neurons in all locations are large in diameter, and virtually all express TrkB. At least some of these neurons project to the lateral region of the dorsal horn, and peripheral quail neurites are seen in the dermis, suggesting that they are cutaneous sensory neurons. Hence, although they are able to incorporate into neural crest-derived ganglia in the trunk, Pax3+ opV ectoderm cells are committed to forming cutaneous sensory neurons, their normal fate in the trigeminal ganglion. In contrast, Pax3 is not expressed in neural crest-derived neurons in the dorsal root and trigeminal ganglia at any stage, suggesting either that Pax3 is expressed in glial cells or that it is completely downregulated before neuronal differentiation. Since Pax3 is maintained in opV placode-derived neurons for some considerable time after neuronal differentiation, these data suggest that Pax3 may play different roles in opV placode cells and neural crest cells.

INTRODUCTION

Neurons in cranial sensory ganglia in vertebrates are derived from two embryonic cell populations, the neural crest (reviewed in Wakamatsu and Weston, 1997) and neurogenic ectodermal placodes (reviewed in Baker and Bronner-Fraser, 2001; Graham and Begbie, 2000; Webb and Noden, 1993). Neurogenic placodes and neural crest cells share many similarities: both cell populations arise from the ectoderm at the border between the neural plate and epidermis, and both undergo an epithelial–mesenchymal transition and migrate. Each placode forms different derivatives; similarly, neural crest cells differ in fate along the neural axis (Le Douarin and Kalcheim, 1999; Hall, 1999). Taken as a whole, both placodes and neural crest are able to form cutaneous and visceral sensory neurons, neuroendocrine cells, glia, and cells secreting a biomineralized matrix (see Baker and Bronner-Fraser, 1997, 2001). Unlike neural crest cells, however, neurogenic placodes are confined to the head, and they do not form autonomic or enteric neurons, melanocytes, smooth muscle, cartilage, or bone.

Given the relationship between neural crest cells and neurogenic placodes, we have explored whether placode-derived cells have the potential to form neural crest cell derivatives. We showed previously that the transcription factor Pax3 is the earliest known marker for ophthalmic trigeminal (opV) placode ectoderm in the chick (Stark *et al.*, 1997). In amniotes, only the trigeminal (Vth) ganglion contains neurons derived both from placodes and neural crest cells (Hamburger, 1961; D'Amico-Martel and Noden,

[1] To whom correspondence should be addressed. Fax: +44 1223 333786. E-mail: cvhb1@cam.ac.uk.

[2] Present address: Department of Anatomy, Downing Street, Cambridge CB2 3DY, England.

[3] Present address: Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 84602.

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1983; Ma *et al*., 1998). The trigeminal ganglion is formed by the fusion of two separate ganglia, the ophthalmic (opV) and maxillomandibular (mmV) trigeminal ganglia (see Schlosser and Northcutt, 2000; Baker and Bronner-Fraser, 2001). The neural crest contributes small-diameter, proprioceptive and cutaneous sensory neurons to the proximal region of the ganglion, while separate opV and mmV placodes contribute large-diameter, cutaneous sensory neurons to the distal regions of their respective ganglionic lobes (Hamburger, 1961; Johnston, 1966; Noden, 1975, 1978, 1980).

In addition to its expression in opV placode-derived cells, Pax3 is also expressed transiently by both trunk and cranial neural crest cells as they emigrate from the neural tube, and it is reexpressed in neural crest cells as they condense to form dorsal root ganglia and some cranial sensory ganglia, including the trigeminal ganglion (Goulding *et al*., 1991; Stark *et al*., 1997). Trunk neural crest cells give rise to cutaneous, visceral, and proprioceptive sensory neurons in the dorsal root ganglia, and to autonomic neurons in the sympathetic ganglia; they also form adrenal medullary cells, Schwann cells, and melanocytes (Le Douarin and Kalcheim, 1999; Hall, 1999). Pax3 function is required cell-autonomously in neural crest cells for dorsal root ganglion formation (Li *et al*., 1999), and there is some evidence for Pax3 involvement in regulating sensory neurogenesis in trunk neural crest cells (Koblar *et al*., 1999). The joint expression of Pax3 by neural crest cells and opV placode-derived cells raised some interesting questions about the similarities between these two cell populations.

Previously, we showed that, when substituted for the nodose placode, which forms visceral sensory neurons in the nodose ganglion, Pax3+ opV placode cells are unable either to express the early nodose placode marker Pax2 or to form *Phox2a*+, nodose-type neurons; instead, they form Pax3+ neurons that differentiate on a trigeminal-like schedule and do not incorporate into the nodose ganglion (Baker and Bronner-Fraser, 2000). However, the commitment of these cells to a trigeminal-like fate was tested in a placodal environment, which might provide differentiation cues that are also present in the normal trigeminal environment. Furthermore, the precise neuronal phenotype of the resultant neurons was not examined.

Here, we investigated whether Pax3+ opV ectoderm cells behave like Pax3+ neural crest cells when they are grafted into the trunk of the embryo, where no placodes form. We found that Pax3+ opV ectoderm cells incorporate into host neural crest cell migratory streams and contribute Pax3+ neurons to both dorsal root and sympathetic ganglia. However, they are committed to forming large-diameter, cutaneous sensory neurons, consistent with their normal trigeminal fate. While Pax3+ neural crest-derived cells in both the dorsal root and trigeminal ganglia are primarily found at the edges of the ganglia and do not express the neuronal marker Hu, Pax3+ opV placode-derived neurons maintain Pax3 expression for a considerable time after neuronal differentiation, both in the trigeminal ganglion during normal development and when grafted into the trunk. These data may imply that Pax3 plays different roles in neural crest cells and opV placode cells. We conclude that Pax3+ opV placode-derived cells are committed to forming cutaneous sensory neurons and are consequently unable to adopt any other fates typical of the neural crest.

MATERIALS AND METHODS

Quail–Chick Grafts

Fertilized quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus domesticus*, White Leghorn and Rhode Island Red) eggs were obtained from commercial sources and incubated at 38°C in a humidified atmosphere. A window was cut in the quail shell and a 1:25 mixture of India ink and Ringer's solution injected into the sub-blastodermal cavity to reveal the embryo. The vitelline membrane in the region of the surgery and the ectodermal region to be grafted were removed by using a pulled glass needle. The chick embryo host was similarly prepared, and the ectoderm from the region of the graft site was removed immediately before transfer of the donor ectoderm. The window was sealed with tape (Scotch Nr. 3841) and the host egg replaced in the incubator.

Immunohistochemistry

Embryos collected after 13 h to 2 days of incubation were fixed in 4% paraformaldehyde for 1–2 h and processed for cryostat sectioning as described (Sechrist *et al*., 1995). Embryos collected after 3–6 days of incubation were fixed in 4% paraformaldehyde for 4–6 h at room temperature or overnight at 4°C and similarly processed. Cryosections (8–14 μm) of embryos were mounted on Superfrost Plus glass slides (Fisher) and the gelatin removed by treating the slides in phosphate-buffered saline (PBS) at 42°C for 5 min.

Antibodies (see below and Table 1) were diluted in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100. For the anti-quail neurite (QN) antibody and anti-Trk antibodies only, slides were blocked for 1 h in 10% heat-treated sheep serum in the same solution and 5% sheep serum was included in the primary antibody solution. Slides were generally incubated overnight at 4°C in primary antibody solution, and for 1–2 h in secondary antibody solution, with three 5-min washes in PBS between each step. Where biotinylated secondary antibodies were used, slides were further incubated for 1 h in avidin-NeutrAvidin Alexa 350 (Molecular Probes) at 1:100 in PBS, followed by three 5-min washes in PBS. Slides were washed in distilled water, mounted in Fluoromount-G (Southern Biotechnology) and viewed by fluorescent microscopy. Only those chimeras that had successfully incorporated the donor graft were scored.

Table 1 lists the primary antibodies used in this study, the dilutions used, their sources, and appropriate references. Subtype-specific FITC-, TRITC-, biotinylated-, horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies were obtained from Southern Biotechnology, Zymed, and Amersham. Subtype-specific Alexa488-, Alexa568-, and Alexa594-conjugated secondary antibodies were obtained from Molecular Probes.

Some embryos collected after 2–4 days of incubation (E3.5–E6) were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid), dehydrated in 100% ethanol, changed into Histosol (National Diagnostics), embedded in Paraplast (Oxford Labware) under reduced air pressure, and sectioned at 5–6 μm. The

TABLE 1
Antibodies Used in the Course of this Work

Antibody	Antigen	Subtype	Used at:	Source	Reference
Hu	human neuronal protein HuC/D	mouse IgG2b	1:500	Molecular Probes	Wakamatsu and Weston (1997)
MF20	myosin heavy chain	mouse IgG2b	1:3	DSHB	Bader *et al.* (1982)
NF-M (RMO 270.3)	neurofilament	mouse IgG2a	1:300	Dr. Virginia Lee	Lee *et al.* (1987)
Pax3	Pax3	mouse IgG2a	1:5000	Dr. Charles Ordahl	see Baker *et al.* (1999)
QCPN	quail	mouse IgG1	1:1	DSHB (B. Carlson and J. Carlson)	see Selleck and Bronner-Fraser (1995)
QN	quail neurites	mouse IgG1	1:3	Dr. Hideaki Tanaka	Tanaka *et al.* (1990)
TH	tyrosine hydroxylase	mouse IgG2a	1:1	DSHB	Fauquet and Ziller (1989)
TrkB	TrkB	rabbit IgG	1:1000	Dr. Louis Reichardt	von Bartheld *et al.* (1996)
TuJ1	neuronal class III β-tubulin	mouse IgG2a	1:500	BAbCO (CRP Inc.)	Lee *et al.* (1990)

Note. DSHB, Developmental Studies Hybridoma Bank. The Developmental Studies Hybridoma Bank was developed under the auspices of the NICHD and is maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

sections were dried overnight at 37°C and stored at room temperature. Endogenous peroxidase was blocked by incubating sections for 30 min in 0.015% hydrogen peroxide in PBS. The sections were then processed for QCPN staining as described above, but using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (Zymed) at 1:200 as the secondary antibody. The color reaction was performed in 0.05 mg/ml diaminobenzidene 3–3′ tetrahydrochloride (DAB; Sigma), 0.0045% hydrogen peroxide in PBS. The sections were then incubated overnight at 4°C with anti-neurofilament antibody, followed by 2 h at room temperature in alkaline phosphatase-conjugated secondary antibody at 1:300 in PBS. The AP color reaction was performed by using AP substrate kit I (red) (Vector Laboratories), according to the manufacturer's instructions. Gill's hematoxylin (Fisher) was used as a background nuclear stain. The slides were dehydrated and mounted in Permount (Fisher).

RESULTS

We wished to test whether Pax3+ ophthalmic trigeminal (opV) placode cells behave like Pax3+ neural crest cells when grafted into the trunk, where no placodes form and where all peripheral neurons are derived from the neural crest. In order to do this, we grafted opV ectoderm from quail donors into the trunk of chick embryo hosts.

During normal development, Pax3 is induced in opV ectoderm by a diffusible signal from the midbrain (Stark *et al.*, 1997; Baker *et al.*, 1999). Robust Pax3 expression is only seen in opV ectoderm explants after the seven-somite stage (Baker *et al.*, 1999); however, mesencephalic neural crest cells also migrate beneath opV ectoderm from the eight-somite stage onwards (see Baker *et al.*, 1997). These con-

FIG. 1. Three- to six-somite-stage opV ectoderm is induced to express Pax3 and form neurons when grafted adjacent to the trunk neural tube, over the segmental plate mesoderm. (A) Schematic showing the graft procedure. ss, somite-stage. (B) Transverse section through the graft region of a chick host collected 23.5 h after receiving a graft of 5-somite stage quail opV ectoderm over the segmental plate at the 17-somite stage. QCPN+ quail nuclei are shown in red, Pax3 in green, and the neuronal marker Hu in blue. Pax3+ quail cells (yellow nuclei; arrow) are seen in the ectoderm and in the dermomyotome (dm), which is itself Pax3+. One of the Pax3+ quail cells in the dermomyotome is a neuron (arrowhead). Quail endothelial and other mesenchymal nuclei are derived from contaminating cephalic mesoderm. (C) Transverse section through the graft region of a chick host collected 25 h after receiving a graft of 4-somite stage quail opV ectoderm over the segmental plate at the 10-somite stage. QCPN+ quail nuclei are shown in red, Pax3 in green, and the neuronal marker Hu in blue. Pax3+ quail neurons (yellow nuclei; arrowheads) are seen in the ectoderm and beneath the dermomyotome (dm). (D) Histogram showing the percentage of chimeras with Pax3+ and/or neuronal quail cells 18–28 h (1 day) and 2 days after receiving grafts of opV ectoderm from 3- to 6-somite-stage quail donors. Neuronal cells were identified by using antibodies against either Hu, neurofilament (NF-M) or neuron-specific β-tubulin. The number of grafts (n) is listed at the top of each column. dpg, days postgraft. (E) Histogram showing the distribution of Pax+Hu−, Pax3+Hu+, and Pax3-Hu+ cells in those chimeras analyzed with antibodies against both Pax3 and Hu. A single embryo can contain representatives of all three cell classes. The percentage of chimeras containing Pax3+Hu− cells drops with time, while the percentage containing Pax3−Hu+ cells increases, suggesting that Pax3 is downregulated after Pax3+ neurons differentiate. The number of grafts (n) is listed at the top of each column. dm, dermomyotome; dpg, days postgraft; ect, ectoderm; nt, neural tube; ss, somite-stage.

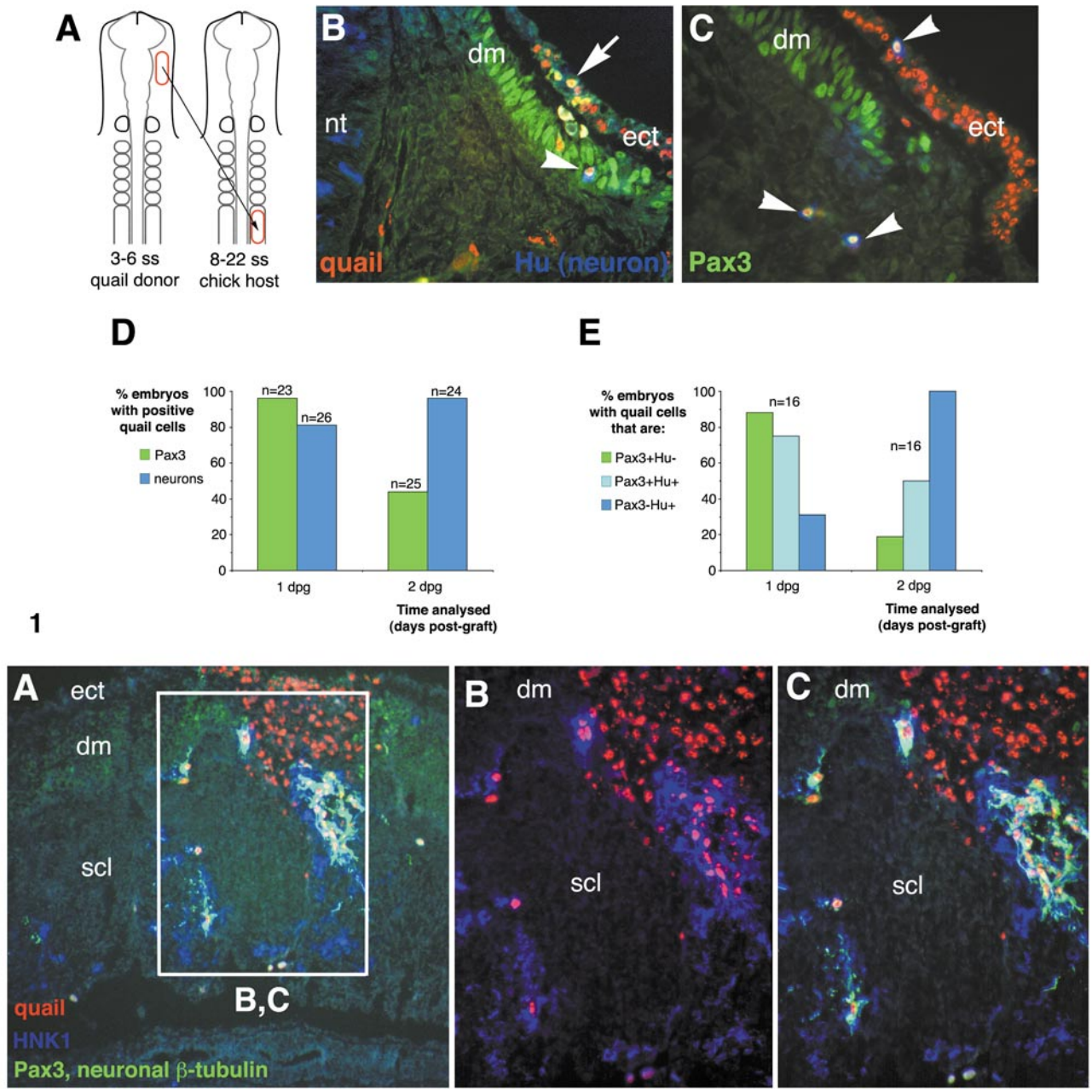

FIG. 2. OpV ectoderm-derived neurons colocalize with migrating host neural crest cell streams in the rostral somites. (A) Parasagittal section through the graft region of a chick host collected 28 h after receiving a graft of 5-somite-stage quail opV ectoderm over the segmental plate at the 16-somite stage. Migrating neural crest streams through the rostral somites can be seen by HNK1 staining (blue). Nuclear Pax3 expression in the dermomyotome (dm) and cytoplasmic neuron-specific β-tubulin staining are both in green. Quail cell nuclei are red. (B) Higher power view of the boxed region in (A), encompassing two migrating neural crest streams (blue) through the rostral somites. Quail nuclei (red) are primarily localized within the host neural crest streams (blue). Like the host neural crest cells, at least some of the quail cells express HNK1. (C) Same view as in (B), now also showing neuron-specific β-tubulin staining in green. Many of the quail cells have already differentiated as neurons. dm, dermomyotome; ect, ectoderm; scl, sclerotome.

taminating neural crest cells are hard to remove even with enzyme treatment. However, a signal from the trunk neural tube, to which trunk ectoderm is not competent to respond, induces Pax3 in opV ectoderm when it is grafted over the paraxial mesoderm (Baker *et al*., 1999) (see schematic in Fig. 1A). Thus, we were able to graft two- to six-somite-stage

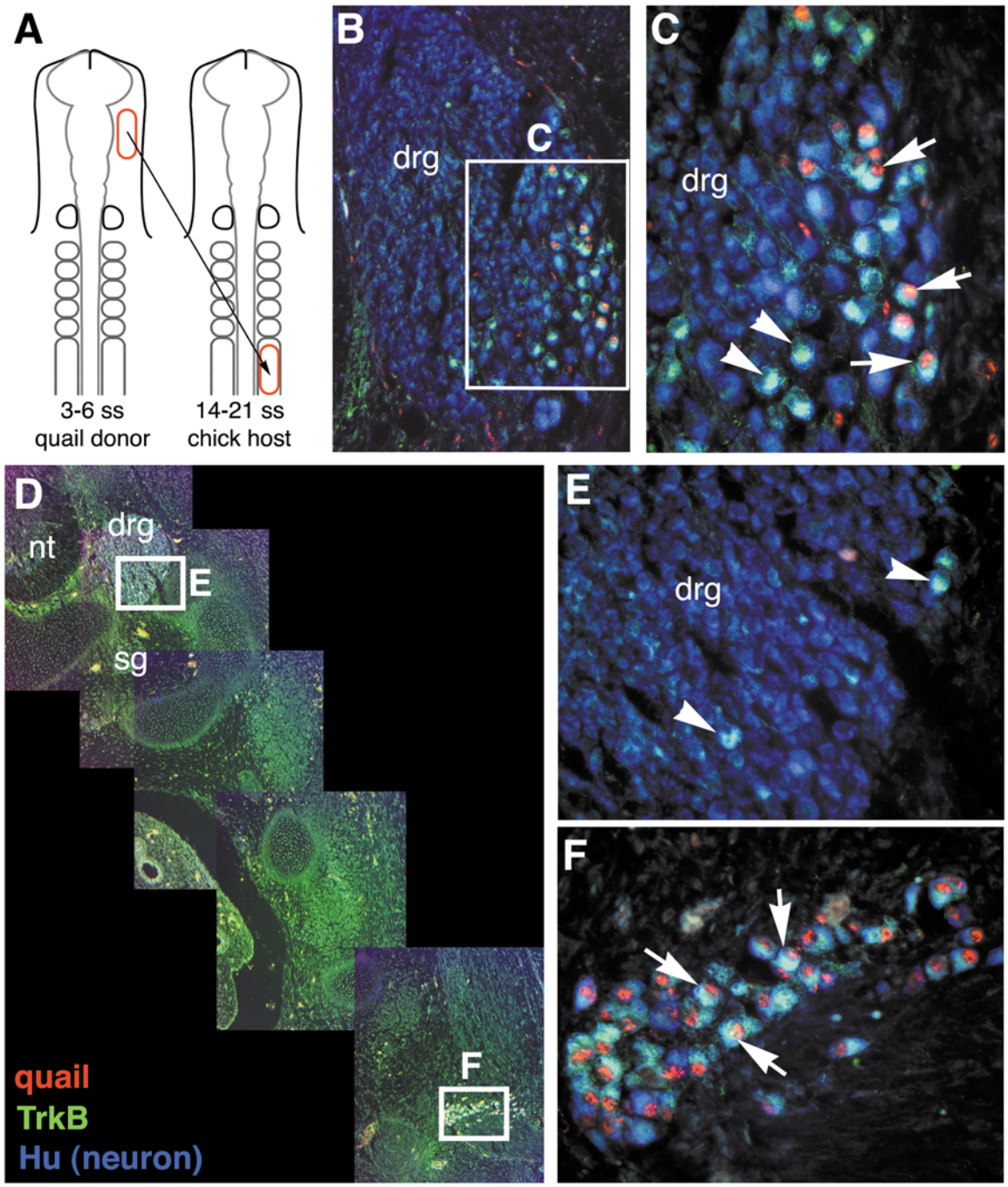

FIG. 3. Neurons derived from 3- to 6-somite-stage opV ectoderm grafted adjacent to the trunk neural tube are large in diameter and express TrkB. (A) Schematic showing the graft procedure. ss, somite-stage. (B) Transverse section through a dorsal root ganglion (drg) of a chick host collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 17-somite stage. Hu staining (neurons) is seen in blue, TrkB in green, and quail nuclei in red. (C) Higher-power view of the boxed area in (B), showing large TrkB+ quail neurons (arrows) together with large TrkB+ host neurons (arrowheads) in the ventrolateral region of the dorsal root ganglion (drg). (D) Composite showing a transverse section through the graft region of a chick host collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 18-somite stage. (E) Higher power view of the dorsal root ganglion (drg) in (E), showing large TrkB+ host neurons (arrowheads) in the ventrolateral region of the ganglion. (F) Higher power view of the ectopic quail ganglion associated with the ventral root (not visible in this section) in (D), showing it to be composed of large TrkB+ quail neurons (arrows). drg, dorsal root ganglion; nt, neural tube; sg, sympathetic ganglion; ss, somite-stage.

opV ectoderm into the trunk over the paraxial mesoderm, where it is induced to express Pax3. Pax3+ cells delaminating from the grafted ectoderm are then exposed both to migrating trunk neural crest cells and to the multiple differentiation cues that operate on these cells.

Three- to Six-Somite-Stage opV Ectoderm Is Induced to Express Pax3 and Form Pax3+ Neurons When Grafted Adjacent to the Trunk Neural Tube

We had previously found that Pax3 expression correlated with neurogenesis in opV placode ectoderm (Baker and Bronner-Fraser, 2000). Here, we analyzed neurogenesis and Pax3 expression in 3- to 6-somite-stage opV ectoderm a day after grafting it adjacent to the trunk neural tube, over the segmental plate mesoderm of 8- to 22-somite-stage chick hosts (E2.5–E3) (see Fig. 1A for a schematic of the graft). In our current experiments, we identified neurons primarily using an antibody against the neuron-specific RNA-binding protein Hu (Wakamatsu and Weston, 1997). However, some embryos were analyzed instead with the TuJ1 antibody, which binds neuron-specific β-tubulin (Lee *et al*., 1990). We found that 96% of embryos analyzed for Pax3 ($n = 23$) contained Pax3+ quail cells, either in the ectoderm or mesenchyme (usually in or near the dermomyotome) or both, while 81% of embryos analyzed for neurons contained Hu+ or TuJ1+ quail cells, also in or near the dermomyotome ($n = 26$) (Figs. 1B–1D). Indeed, Pax3+Hu+ quail cells were occasionally seen in the ectoderm, showing that neurogenesis can sometimes occur even before the epithelial–mesenchymal transition (Fig. 1C). This is consistent with occasional neurofilament expression in opV placode ectoderm as early as the 13- to 14-somite stage (J. Sechrist and M.B.-F., unpublished observations).

We also analyzed some embryos for Pax3 and Hu or TuJ1 expression at 2 days postgraft (E3.5–E4). These embryos had received grafts of 2- to 6-somite-stage opV quail ectoderm over the segmental plate mesoderm at the 10- to 20-somite stage. Fewer embryos contained Pax3+ quail cells (44%; n = 25), but almost all (96%) contained Hu+ or TuJ1+ quail cells ($n = 24$) (Fig. 1E).

Pax3 Is Downregulated after Neuronal Differentiation

The increase in the percentage of embryos containing quail neurons with time, and corresponding decrease in the percentage of embryos with Pax3+ quail cells (Fig. 1D), suggested that Pax3 expression is downregulated some time after neuronal differentiation. To analyze this further, we directly compared Pax3 versus Hu expression in those embryos scored both for Pax3 and Hu expression at 1 and 2 days postgraft (Figs. 1B, 1C, and 1E). One day after grafting two- to six-somite-stage opV ectoderm over the segmental plate mesoderm, Pax3+Hu− and Pax3+Hu+ quail cells were primarily seen, in 88 and 75% of chimeras, respectively; only 31% of chimeras contained Pax3−Hu+ quail cells ($n = 16$) (Fig. 1E). Again, all three cell classes were sometimes seen in the same embryo. However, the proportions changed at 2 days postgraft, with only a minority of chimeras (19%) containing Pax3+Hu− quail cells, 50% containing Pax3+Hu+ quail cells, and all containing Pax3−Hu+ cells ($n = 16$) (Fig. 1E).

These results suggest that the sequence of events at the cellular level is: (1) Pax3 expression, (2) Hu expression in Pax3-expressing cells, and (3) down-regulation of Pax3. The few Pax3−Hu+ cells seen 1 day postgraft (see Fig. 1E) are thus suggested to represent cells that have already down-regulated Pax3 expression.

Survival and Distribution of Quail Neurons Derived from opV Ectoderm Grafted Adjacent to the Trunk Neural Tube

We next analyzed the long-term fate of the Pax3+ opV ectoderm-derived neurons in the trunk. A total of 83 embryos, ranging from the 9- to 21-somite-stage at the time of the graft, received grafts of two- to six-somite-stage opV ectoderm over the segmental plate mesoderm. They were sectioned and analyzed at 2–7 days postgraft (E3.5–E9) for the presence and location of neurons, using either Hu, TuJ1, or the anti-neurofilament antibody. The results at 2–5 days postgraft, 6 days postgraft, and 7 days postgraft are summarized in Table 2.

There was a clear reduction with increasing age in the percentage of embryos with surviving quail neurons: 96% of embryos ($n = 53$) contained quail neurons at 2–5 days postgraft; this dropped to 76% at 6 days postgraft ($n = 21$), and still further, to 50%, at 7 days postgraft ($n = 8$). The neurons were found in a wide variety of locations at varying frequencies: in the dorsal root ganglia, sympathetic ganglia, scattered along the ventral root (motor nerve) where they were sometimes collected into ganglia, and even in or near the myotome and in other ectopic sites (see Table 2). The presence of quail neurons along the ventral root is reminiscent of the presence of opV placode-derived neurons along the ophthalmic trigeminal nerve (see Baker *et al*., 1997). The formation in some cases of ectopic ganglia on the ventral root resembles trigeminal ganglion formation by trigeminal placode-derived cells after neural crest ablation (Hamburger, 1961; Stark *et al*., 1997).

Interestingly, host neurons were sometimes observed in ectopic locations in chimeric embryos at all stages. They were found in or near the myotome and along the ventral root (data not shown), on both operated and unoperated sides, although this was not quantified.

OpV Ectoderm-Derived Neurons in the Trunk Colocalize with Migrating Host Neural Crest Cells

The contribution of opV ectoderm-derived neurons to neural crest-derived ganglia after grafts over the segmental plate mesoderm suggested that opV ectoderm-derived cells might incorporate into the migrating streams of host neural

TABLE 2

Number and Percentage of Embryos Containing Quail Neurons and Their Location in Specific Sites within the Embryo after Grafts of 2- to 6-Somite-Stage opV Ectoderm over the Segmental Plate Mesoderm (adjacent to the trunk neural tube) at the 9- to 21-Somite-Stage (total $n = 83$)

				% of embryos with neurons in:					
Dpg	Total *n*	Number with neurons	% with neurons	DRG	SG	Near aorta	VR	VR ganglion	Myotome & other
2–5	53	51	96	47	22	29	55	16	49
6	21	16	76	44	25	25	63	13	81
7	8	4	50	25	0	50	75	0	25

Note. Dpg, days post-graft; DRG, dorsal root ganglion; SG, sympathetic ganglion; VR, ventral root.

crest in the rostral somites. In order to test this hypothesis, we used the HNK1 antibody (which labels neural crest cells and other cell types; Tucker *et al.*, 1984) to analyze parasagittal sections of three chimeric embryos 26–28 h postgraft (at E3). These embryos had received grafts of 5-somite stage opV ectoderm over the segmental plate mesoderm at the 16- to 17-somite-stage. Pax3+ quail cells in these embryos were found to be colocalized with migrating host neural crest cells in the rostral somite (Fig. 2). Interestingly, the quail cells also expressed HNK1 (Figs. 2B and 2E), and many of them had already differentiated as neurons, as seen by neuron-specific β-tubulin expression (Figs. 2A and 2C). It is not possible to conclude from these data that the quail neurons are following neural crest migration cues, as they may simply be swept along by the surrounding host neural crest cells. Nonetheless, their colocalization with host neural crest cells in these migration streams is likely to explain the incorporation of opV ectoderm-derived neurons in neural crest-derived ganglia when the ectoderm is grafted over the segmental plate mesoderm.

By E8, opV Ectoderm-Derived Neurons in All Locations Are Large in Diameter and Express TrkB

Six days after grafting 3- to 6-somite-stage opV ectoderm over the segmental plate of 14- to 21-somite-stage hosts (at E7.5–E8), quail neurons were found in 76% of the embryos ($n = 21$). In those embryos with neurons ($n = 16$), all of the neurons were large in diameter and were found in a variety of locations (see Table 2). A total of 81% of embryos with neurons contained a few scattered neurons in or near the myotome; 63% contained neurons scattered along the ventral root, 44% in the dorsal root ganglia (exclusively in the ventrolateral region of the ganglia), and 25% in the sympathetic ganglia. An ectopic ganglion on the ventral root was found in 2 embryos (13% of those with neurons).

We stained representative slides from 15 of these embryos with an antibody against the neurotrophin receptor TrkB, which is expressed by many placode-derived (distal) cutaneous neurons in the trigeminal ganglion at E7–E8 (Dechant *et al.*, 1993; Williams *et al.*, 1995). TrkB is also expressed by some of the large-diameter neural crest-derived neurons in the ventrolateral region of the dorsal root ganglia: in the mouse, these are probably mechanoreceptive (Klein *et al.*, 1993; Jones *et al.*, 1994; Liebl *et al.*, 2000; Rifkin *et al.*, 2000). In all of the TrkB-stained slides from 14 of the embryos (93%), all quail neurons in all locations were TrkB+ (Figs. 3B–3D and 3F). In slides from the remaining embryo (7%), 4 TrkB-negative quail neurons were found on the ventral root; however, all other quail neurons analyzed in this embryo were TrkB+, including all the neurons in an ectopic ganglion on the ventral root.

Interestingly, most of the host neural crest-derived neurons found ectopically on the ventral root or in the myotome were large-diameter and TrkB-negative at E8 (data not shown), although this was not quantified. In one chimeric embryo analyzed at E8, many large-diameter host neurons were seen on the ventral root on the operated side, although these were not collected into a separate ganglion (data not shown).

These results clearly show that by E8, all of the neurons derived from Pax3+ opV ectoderm cells in the trunk are large in diameter, and virtually all of them express TrkB, regardless of their location within the embryo.

OpV Ectoderm-Derived Neurons in Sympathetic Ganglia Are Not Sympathetic Neurons

For 11 embryos with quail neurons in and/or near the sympathetic ganglia at 4–6 days postgraft (E5.5–E8), we used an anti-tyrosine hydroxylase antibody together with the anti-Hu antibody to determine whether these quail neurons were catecholaminergic (Fig. 4). No tyrosine hydroxylase-positive quail neurons were found in any of these embryos (Figs. 4A and 4B). Furthermore, the quail neurons in sympathetic ganglia at E8 (6 days postgraft) were clearly much larger in diameter than the surrounding host neurons (Figs. 4A and 4B), comparable to large-diameter neurons in the dorsal root ganglia (Figs. 4G and 4H). In contrast, control isotopic, isochronic grafts of quail trunk neural tube into 24-somite-stage chick hosts at the level of the last 6 somites clearly showed tyrosine hydroxylase expression in small-diameter quail neural crest-derived

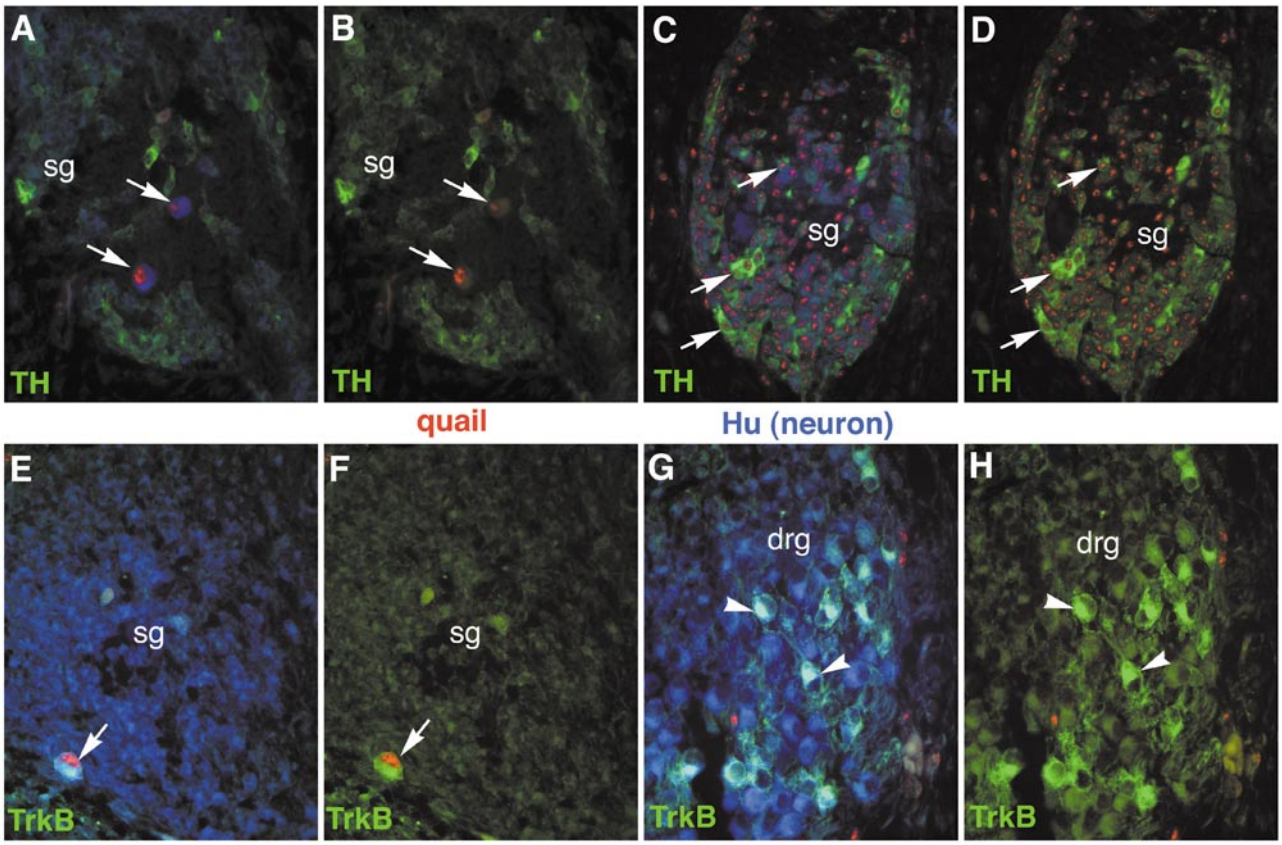

FIG. 4. OpV ectoderm-derived neurons in sympathetic ganglia do not express the catecholaminergic marker tyrosine hydroxylase. At E8, they are large in diameter and express TrkB. For all panels, Hu staining (neurons) is seen in blue and QCPN staining (quail nuclei) in red. All panels are at the same magnification. (A, B) Transverse section through the sympathetic ganglion (sg) of a chick host collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 17-somite stage. Two quail neurons (arrows) can be seen within the ganglion; unlike surrounding host neurons, they are large in diameter and do not express tyrosine hydroxylase (green). (B) Same section as (A), showing red (quail nuclei) and green (tyrosine hydroxylase) channels only. (C, D) Transverse section through the sympathetic ganglion (sg) of a chick host collected 6 days after receiving an isotopic, isochronic graft of quail trunk neural tube at the level of somites 18–24 at the 24-somite stage. The ganglion is almost entirely made up of quail neural crest-derived neurons, most of which clearly express tyrosine hydroxylase (green; arrows) and are small in diameter (compare with large-diameter quail neurons in A and B: all panels are at the same magnification). (D) Same section as (C), showing red (quail nuclei) and green (tyrosine hydroxylase) channels only. (E, F) Transverse section through the sympathetic ganglion (sg) of a chick host collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 18-somite stage. One quail neuron (arrow) can be seen at the edge of the ganglion; unlike the surrounding small host neurons, it is large in diameter and expresses TrkB (green). (F) Same section as (E), showing red (quail nuclei) and green (TrkB) channels only. (G, H) For comparison with (E) and (F), a view of the host dorsal root ganglion (drg) from the same section as in (E) and (F), showing large-diameter TrkB+ (green) host neurons (arrowheads) in the ventrolateral region of the ganglion (all panels are at the same magnification). Quail nuclei here are endothelial cells. (H) Same section as (G), showing red (quail nuclei) and green (TrkB) channels only. drg, dorsal root ganglion; sg, sympathetic ganglion; TH, tyrosine hydroxylase.

neurons in the sympathetic ganglia at 5–6 days postgraft (E7–E8; $n = 3$) (Figs. 4C and 4D).

We also analyzed TrkB expression in sections from 2 of the 11 embryos containing quail opV ectoderm-derived neurons in the sympathetic ganglia. We found that only the quail neurons in the sympathetic ganglia in these embryos were TrkB+; all the host sympathetic neurons were TrkB-negative (Figs. 4E and 4F).

Clearly, therefore, both by morphological (large-diameter) and molecular criteria (tyrosine hydroxylase-negative, TrkB+), opV ectoderm-derived neurons located in and near the sympathetic ganglia are not sympathetic neurons.

TrkB+ opV Ectoderm-Derived Neurons in Dorsal Root Ganglia Are Likely to Be Cutaneous

By E8 in the chick, cutaneous and proprioceptive afferents from dorsal root ganglia in the lumbar region show

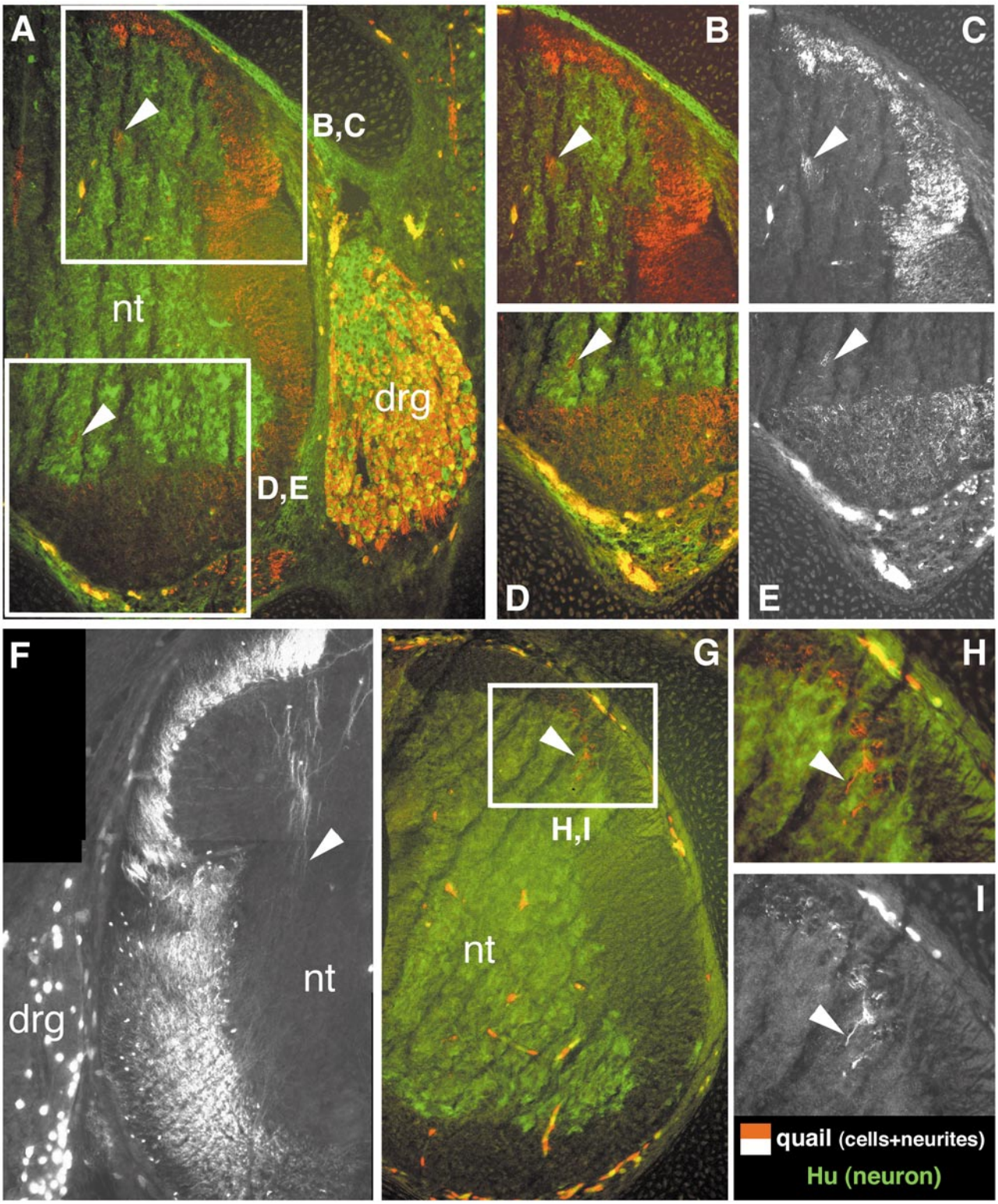

FIG. 5. OpV ectoderm-derived neurons in dorsal root ganglia project to the lateral region of the dorsal horn at E8, suggesting they are cutaneous sensory neurons. (A) Transverse section through an E8 control embryo, collected 6 days after receiving an isotopic graft of quail neural tube at the level of the last 6 somites at the 21-somite stage. Peripheral ganglia are composed of quail neurons for some level beyond the grafted neural tube, enabling analysis of quail neurites (using the QN antibody) in regions where the neural tube (nt) is entirely host-derived. In this section, the dorsal root ganglion is made up almost entirely of quail neural crest cells (QCPN staining; red nuclei). Neurons

striking differences in their central projections. Cutaneous afferents project to the lateral laminae of the dorsal horn, while proprioceptive afferents project much further ventrally, even extending to the ventral horn (Eide and Glover, 1997). We used the quail neurite-specific antibody QN (Tanaka *et al*., 1990) to analyze the central and peripheral projections of quail neurons within chick host embryos after control grafts of neural tube (trunk neural crest) and grafts of opV ectoderm.

In control isotopic grafts of the entire neural tube at the level of the last-formed 6 somites (17- to 24-somite-stage donors, 16- to 22-somite-stage hosts) analyzed at 5–7 days postgraft (E7–E9; $n = 7$), all the peripheral ganglia were composed of quail neurons in the region of the grafted neural tube itself and also for some distance beyond it. This enabled us to analyze the central projections of quail dorsal root ganglion neurons in regions where the neural tube itself was entirely host-derived. In 1 embryo analyzed 6 days postgraft (E8), quail neurites were visible within the lateral dorsal horn (presumably cutaneous) (Figs. 5A–5C) and also in the ventral neural tube, near the lateral motor column (presumably proprioceptive) (Figs. 5A, 5D, and 5E) (see Eide and Glover, 1997). In 2 embryos analyzed 7 days postgraft (E9), quail neurites were visible both within the dorsal horn (presumably cutaneous) and in the central neural tube, clearly projecting beyond the dorsal horn and thus presumably proprioceptive (Fig. 5F) (see Eide and Glover, 1997). Thus, we felt confident of being able to find cutaneous and proprioceptive central projections of quail neurites after grafts of opV ectoderm adjacent to the trunk neural tube, at least when numerous quail neurons were present. Peripheral quail neurites were also seen in the dermis (and sometimes epidermis) and muscle of most of the embryos analyzed for QN staining at 4–7 days postgraft.

Four embryos analyzed at E8 (6 days postgraft; total $n = 21$) contained many (20 or more) TrkB+ quail neurons in the ventrolateral region of a single dorsal root ganglion. These embryos had received grafts of 4-somite-stage opV ectoderm over the segmental plate at the 17- to 18-somite stage. For 3 of these embryos, slides in the region of the quail dorsal root ganglion neurons were stained with the QN antibody. Quail neurites were seen in the dorsal column of all three embryos, while in one embryo, quail neurites were clearly visible within the lateral dorsal horn (Figs. 5G–5I and 6A–6C). Peripheral quail neurites were also seen in nerves in the dermis of this embryo (Figs. 6A, 6D, and 6E). No QN staining was seen more ventrally in the spinal cord in any of these embryos. Interestingly, peripheral quail neurites were also seen in nerves in the dermis of an embryo which lacked quail neurons in the dorsal root ganglion, but had numerous large-diameter TrkB+ neurons along the ventral root, suggesting that these neurons also project to the skin (Figs. 6F and 6G).

The large TrkB+ neural crest-derived neurons in the ventrolateral dorsal root ganglion are thought to be mechanoreceptive, i.e., cutaneous sensory neurons (Klein *et al*., 1993; Jones *et al*., 1994; Liebl *et al*., 2000; Rifkin *et al*., 2000). OpV placode-derived neurons in the opV ganglion are large in diameter; many are TrkB+ (Dechant *et al*., 1993; Williams *et al*., 1995), and all are cutaneous (Hamburger, 1961; Noden, 1980). The opV ectoderm-derived neurons in the dorsal root ganglion are all in the ventrolateral region of the ganglion; all are large in diameter and TrkB+, and at least some of these neurons send projections to the dorsal horn and to the dermis at E8. Taken together, these results suggest that the opV ectoderm-derived neurons in the ventrolateral dorsal root ganglion are cutaneous in nature, consistent with their normal fate in the trigeminal ganglion.

Pax3+ Neural Crest Cells in Dorsal Root Ganglia Are Not Neurons

The above data suggest that Pax3+ opV ectoderm-derived cells are committed to their normal fate, forming apparently cutaneous, large-diameter, TrkB+ neurons. How does this compare with the fate of Pax3+ neural crest cells? Neural crest cells transiently express Pax3 as they emigrate from the neural tube, and reexpress Pax3 as they condense to form the dorsal root and trigeminal ganglia (Goulding *et*

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are shown in green (Hu staining). (B) Higher power view of boxed region in (A), showing QN+ quail neurites (red; arrowhead) within the dorsal horn. Neurons in the dorsal horn are green (Hu staining). Quail neurites are also seen outside the dorsal horn in the dorsal column, projecting along the anteroposterior axis. (C) Same view as in (B), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowhead) are white. (D) Higher power view of boxed region in (A), showing QN+ quail neurites (red; arrowhead) ventrally within the neural tube. Neurons are green (Hu staining). (E) Same view as in (D), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowhead) are white.(F) QN (quail neurites) and QCPN (quail nuclei) staining in a transverse section through a control E9 embryo, collected 7 days after receiving an isotopic graft of quail neural tube at the level of the last 6 somites at the 19-somite stage. In this monochrome view, many quail neural crest cells (white nuclei) are present in the dorsal root ganglion (drg) and quail neurites can be seen within the central neural tube (nt) (arrowhead). (G) Transverse section through an E8 chick host, collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 17-somite stage. Neurons are shown in green (Hu staining). (H) Higher power view of the lateral region of the dorsal horn in (G), showing QN+ quail neurites (red; arrowheads) within the dorsal horn itself. No QN+ quail neurites were seen more ventrally within the neural tube (nt). The bright cells and nuclei at the edge of the neural tube are blood cells and quail endothelial cells. (I) Same view as in (H), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowhead) are white. drg, dorsal root ganglion; nt, neural tube.

al., 1991; Stark *et al*., 1997). Does the second phase of Pax3 expression, in neural crest cells in coalescing sensory ganglia, correlate with adoption of a cutaneous sensory neuron fate?

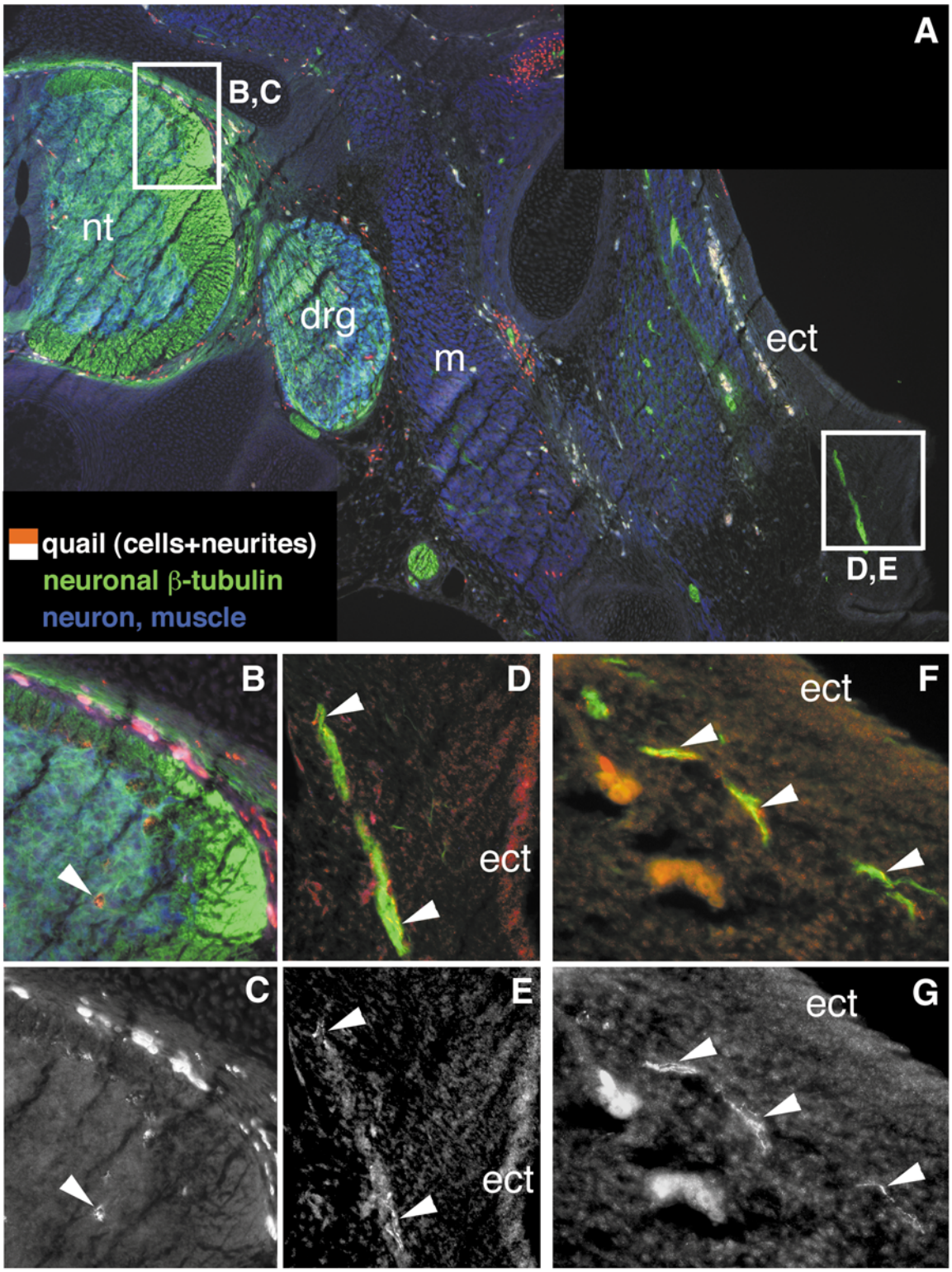
We analyzed sections of unmanipulated chick embryo trunks at different stages from stage 18 (E3) to stage 34 (E8) for expression of Pax3 and the neuronal marker Hu in the dorsal root ganglia (Fig. 7). Unexpectedly, we found that Pax3 and Hu were not coexpressed in cells in the dorsal root ganglia at any stage. Instead, Pax3+Hu− cells were primarily seen at the edges of the ganglia, particularly at the dorsomedial edge, surrounding Pax3-Hu+ dorsal root ganglion neurons (Figs. 7A–7F). By stage 34 (E8), Pax3 expression seemed to be lost within the dorsal root ganglion (Fig. 7G). These results may suggest that Pax3 expression correlates with adoption of a non-neuronal, i.e., glial fate. A role for Pax3 in glial cells is supported by the involvement of Pax3 in the differentiation of myelinating Schwann cells (Kioussi *et al*., 1995; Blanchard *et al*., 1996). Alternatively, neural crest cells in dorsal root ganglia downregulate Pax3 before they express neuronal markers.

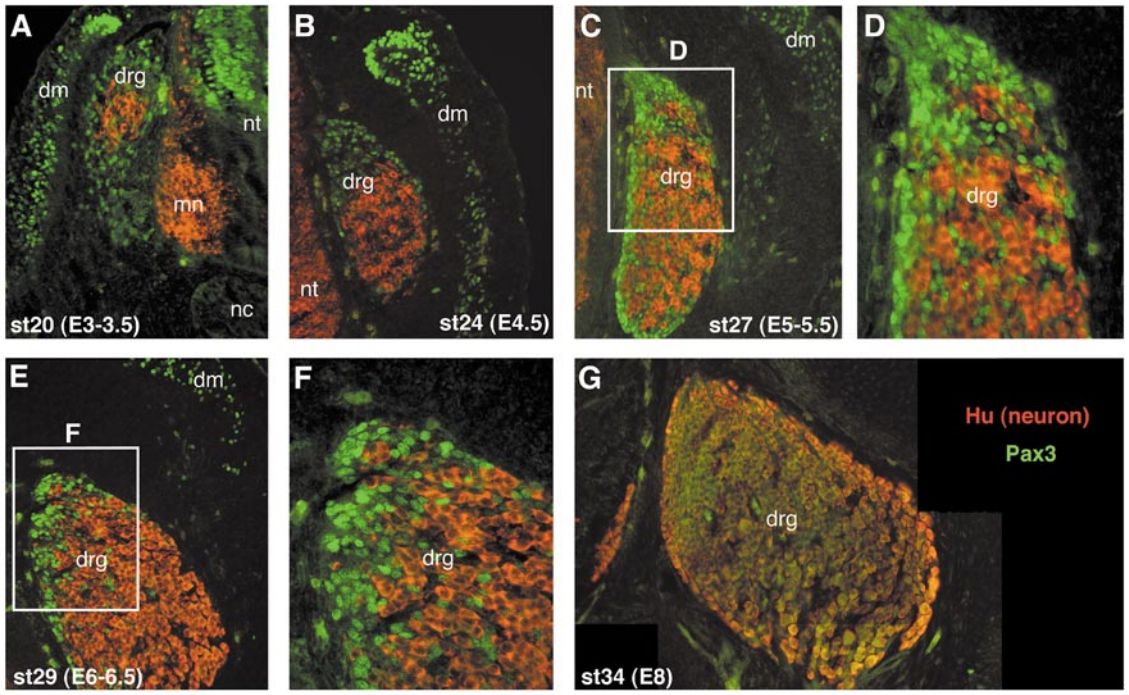
We also analyzed Pax3 versus Hu expression in sections of the trigeminal ganglia of the same embryos (Fig. 8). Our results confirm our previous in situ hybridization-based data, showing that Pax3 is initially expressed at high levels in opV placode-derived neurons, and at much lower levels in the proximal (neural crest-derived) portion of the opV lobe and also within the mmV lobe (Stark *et al*., 1997). We show here that Pax3 is not expressed in mmV placode-derived neurons (Figs. 8A and 8B). These are clearly distinguishable from neural crest-derived neurons before E4, as the latter have not yet differentiated (D'Amico-Martel, 1982). Once they do differentiate, neural crest-derived trigeminal neurons, which are located proximally within the ganglion and are smaller in diameter than placode-derived neurons, are also Pax3-negative (Fig. 8C). By E5.5, opV placode-derived neurons are also Pax3-negative. Therefore, it seems likely that, as in dorsal root ganglia, Pax3 expression in neural crest cells in the trigeminal ganglion correlates with adoption of a non-neuronal fate. Alternatively, Pax3 is downregulated as soon as neural crest-derived neurons differentiate. In either case, this is very different from Pax3 expression in opV placode-derived neurons, which persists for a substantial period after neuronal differentiation (Figs. 8A and 8B), although it is eventually downregulated (see Fig. 1E).

DISCUSSION

Cranial neurogenic placodes and neural crest cells both originate from ectoderm at the border of the neural plate and epidermis (see Baker and Bronner-Fraser, 2001). They form some overlapping derivatives, including cutaneous sensory neurons in the trigeminal ganglion (Hamburger, 1961; Johnston, 1966; Noden, 1975, 1978, 1980). The transcription factor Pax3, which is the earliest known marker for the ophthalmic trigeminal (opV) placode (Stark *et al*., 1997), is also expressed transiently in emigrating neural crest cells. Later, Pax3 is reexpressed in neural crest cells in sensory ganglia, including both dorsal root ganglia and trigeminal ganglia (Goulding *et al*., 1991; Stark *et al*., 1997). We wished to discover whether Pax3+ opV placode cells behave like Pax3+ neural crest cells when removed from their normal environment and placed in the trunk, where no placodes form. We found that although Pax3+ opV placode cells in the trunk contribute neurons to dorsal root and sympathetic ganglia, they are committed to their normal fate: in all locations, they form large-diameter, TrkB+ neurons that are apparently cutaneous in nature. Pax3 is maintained in opV placode-derived neurons after they differentiate, although it is eventually downregulated. In contrast, Pax3 is not expressed in differentiated neural crest-derived neurons in either the dorsal root or trigeminal ganglia; instead, it is expressed in non-neuronal cells located primarily at the edges of the ganglia. These data may suggest that Pax3 plays different roles in the opV placode and the neural crest.

FIG. 6. OpV ectoderm-derived neurons in dorsal root ganglia project to the lateral region of the dorsal horn at E8, while peripheral quail neurites are also seen in the dermis. (A) Composite showing a transverse section through an E8 chick host, collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 17-somite stage. drg, dorsal root ganglion; ect, ectoderm; m, myotome; nt, neural tube. Quail nuclei (QCPN) and neurites (QN) are shown in red; neuronal β-tubulin (TuJ1) staining is shown in green; neurons (Hu) and muscle (MF20) are in blue. (B) Higher power view of boxed region in (A), showing quail neurites (red; arrowheads) within the dorsal horn. The bright cells and nuclei at the edge of the neural tube are blood cells and quail endothelial cells. (C) Same view as in (B), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowheads) are white. (D) Higher power view of boxed region in (A), showing QN+ quail neurites (red; arrowheads) in nerves (green TuJ1 staining) in the dermis. ect, ectoderm. (E) Same view as in (D), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowheads) are white. (F) Higher power view of similar region of a different E8 embryo, collected 6 days after receiving a graft of 4-somite-stage quail opV ectoderm over the segmental plate at the 14-somite stage. It contained quail neurons along the ventral root and in the myotome, but not in the dorsal root ganglia. QN+ quail neurites (red; arrowheads) are visible in nerves (green TuJ1 staining) in the dermis. The bright cells are blood cells. ect, ectoderm. (G) Same view as in (F), showing the red channel only in monochrome, i.e., QN+ quail neurites (arrowhead) are white.





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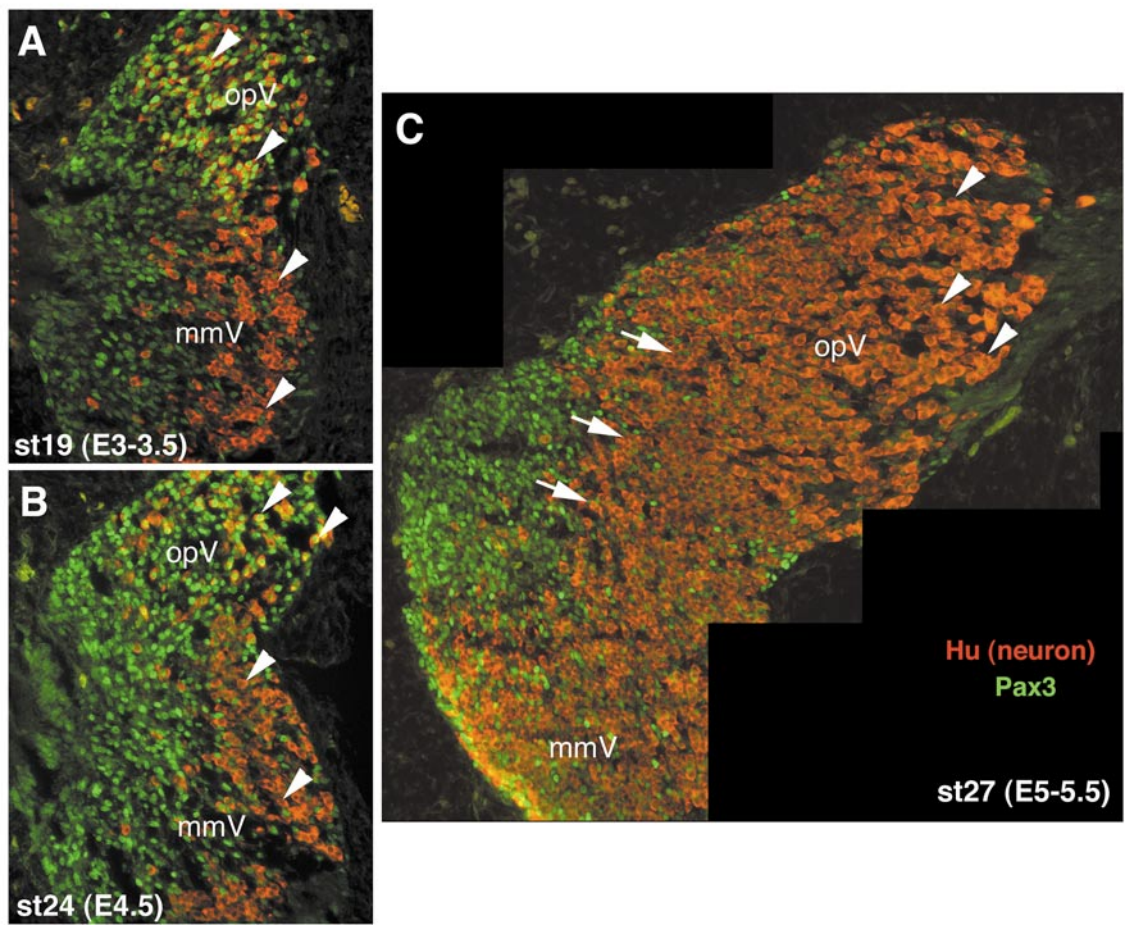

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Pax3+ opV Ectoderm Cells Incorporate into Neural Crest-Derived Ganglia but Are Committed to Forming Large-Diameter, TrkB+ Neurons

We grafted two- to six-somite-stage opV ectoderm from quail donors over the segmental plate mesoderm of chick hosts, where it is induced to express Pax3 by the adjacent trunk neural tube (trunk ectoderm is not competent to respond to this signal; Baker *et al*., 1999). It gave rise within 24 h to Pax3+ neurons that incorporated into host neural crest migration streams within the rostral somite. Pax3 was maintained for a considerable time after neuronal differentiation but was subsequently downregulated. At 6 days postgraft (E7.5–E8), most embryos still contained quail neurons. Some of these neurons were found within dorsal root ganglia (always in the ventrolateral region) and, less often, in sympathetic ganglia. However, quail neurons were also found in several ectopic sites, primarily scattered on the ventral root, where they were occasionally collected into ectopic ganglia, but also scattered in or near the myotome.

Despite the incorporation of quail neurons into neural crest-derived ganglia, all quail neurons in all locations at E8 were large in diameter and, where analyzed, virtually all expressed the neurotrophin receptor TrkB. Where quail neurons were present in or near the sympathetic ganglia, they were tyrosine hydroxylase-negative at E5.5–E8, even when they were closely associated with host tyrosine hydroxylase+ cells. Indeed, where analyzed at E8, quail neurons in sympathetic ganglia were large in diameter and expressed TrkB. Sympathetic neurons, in contrast, are small in diameter and express TrkA (see Reichardt and Fariñas, 1997).

In the avian trigeminal ganglion, all placode-derived neurons, and most neural crest-derived neurons, are cutaneous (Hamburger, 1961; Noden, 1980). Although both TrkC and TrkB are expressed in placode-derived (distal) neurons in the avian trigeminal ganglion, TrkB is expressed in the most distal neurons within the placode-derived portion of the ganglion (Dechant *et al*., 1993; Williams *et al*., 1995). Interestingly, trigeminal ganglion neurons are born in a distal-to-proximal wave, with distal TrkB+ and TrkC+ placode-derived neurons generated before proximal neural crest-derived TrkA+ neurons (D'Amico-Martel, 1982; Williams *et al*., 1995; Huang *et al*., 1999). Hence, TrkB+ neurons may well represent the first-born neurons within the ganglion. Similarly, within the dorsal root ganglion, ventrolateral TrkB+ and TrkC+ neurons are generated earlier than dorsomedial TrkA+ neurons (Fariñas *et al*., 1998).

Differences in Trk expression between neuronal subpopulations in the dorsal root ganglion appear at the time of initial neurogenesis (Fariñas *et al*., 1998; Rifkin *et al*., 2000), suggesting that specification toward a particular neuronal subtype is tightly linked with neuronal differentiation (although environmental cues are able to modify neuronal phenotypes; see, e.g., Smith and Frank, 1987; Davies, 1994, Davies, 1997). Our results are entirely consistent with this. Pax3+ opV ectoderm-derived cells form large-diameter, TrkB+ neurons that maintain this phenotype not only in the ventrolateral dorsal root ganglion, where apparently identical neural crest-derived neurons are found, but also in the sympathetic ganglia, where completely different neurons are found. Their survival in multiple ectopic sites, for example, in the myotome, is probably owing to trophic support from peripheral projections (see Reichardt and Fariñas, 1997).

Large-Diameter, TrkB+ opV Ectoderm-Derived Neurons in Dorsal Root Ganglia Are Likely to Be Cutaneous

When quail opV ectoderm-derived neurons were present in the dorsal root ganglia at E7.5–E8 (after grafts of 2- to

FIG. 7. Neural crest-derived neurons in the dorsal root ganglion do not express Pax3. (A) Transverse section through the trunk of a stage 20 (E3–E3.5) chick embryo, showing Pax3+ cells (green nuclei) in the dorsal neural tube (nt), dermomyotome (dm) and coalescing dorsal root ganglion (drg). Pax3-negative neurons (red, Hu staining) are present in the center of the ganglion. mn, motor neurons. (B) Transverse trunk-level section of a stage 24 (E4.5) chick embryo, showing Pax3-negative neurons in the dorsal root ganglion (drg) surrounded by Pax3+ cells. (C) Transverse trunk-level section of a stage 27 (E5–E5.5) chick embryo, showing Pax3-negative neurons in the dorsal root ganglion (drg) surrounded by Pax3+ cells. (D) Higher power view of boxed region in (C). (E) Transverse section through a stage 29 (E6–E6.5) chick embryo, showing Pax3-negative dorsal root ganglion neurons with Pax3+ cells concentrated dorsomedially and at the edge of the ganglion. (F) Higher power view of boxed region in (E). (G) Transverse trunk-level section through a stage 34 (E8) chick embryo, showing apparent loss of Pax3 staining in the dorsal root ganglion. dm, dermomyotome; drg, dorsal root ganglion; mn, motor neurons; nc, notochord; nt, neural tube.

FIG. 8. Neural crest-derived neurons in the trigeminal ganglion do not express Pax3. All panels are at the same magnification. (A) Parasagittal section through the trigeminal (V) ganglion of a stage 19 (E3–E3.5) chick embryo, showing Pax3+ cells (green nuclei) in both the ophthalmic (opV) and maxillomandibular (mmV) lobes). Neurons are shown in red (Hu staining). Neural crest-derived neurons have not yet differentiated. Only the placode-derived neurons in the opV lobe are Pax3+: mmV placode-derived neurons are Pax3-negative. Arrowheads, placode-derived neurons. (B) Parasagittal section through the trigeminal ganglion of a stage 24 (E4.5) embryo. Neural crest-derived neurons are just beginning to differentiate at this stage. Only opV placode-derived neurons (distal) are Pax3+. Arrowheads, placode-derived neurons. (C) Parasagittal section through the trigeminal ganglion of a stage 27 (E5–E5.5) embryo. Placode-derived neurons (arrowheads) are located distally and are large in diameter; neural crest-derived neurons (arrows) are proximal and small in diameter. Pax3 expression has been lost from opV placode-derived neurons by this stage; neural crest-derived neurons are also Pax3-negative. mmV, maxillomandibular trigeminal; opV, ophthalmic trigeminal.

6-somite-stage opV ectoderm over the segmental plate mesoderm), they were all large in diameter, TrkB+, and located in the ventrolateral region of the ganglia. They were indistinguishable from nearby large-diameter, TrkB+ neural crest-derived host neurons that are also present in this region of the ganglion. In the mouse, large-diameter TrkB+ neurons in this region are probably mechanoreceptive (Klein *et al*., 1993; Jones *et al*., 1994; Liebl *et al*., 2000; Rifkin *et al*., 2000). In contrast, the majority of large-diameter TrkC+ neural crest-derived neurons in the ventrolateral region of the dorsal root ganglia are proprioceptive in the mouse and chick (Klein *et al*., 1994; Smeyne *et al*., 1994; Rifkin *et al*., 2000), although a limited subpopulation of these neurons is cutaneous in the chick (Oakley *et al*., 2000). TrkB expression alone is not indicative of a cutaneous phenotype, however: the primary neurons of the mesencephalic trigeminal nucleus in the midbrain, which are thought to be neural crest-derived (Narayanan and Narayanan, 1978), also express TrkB (and TrkC) and are dependent on the TrkB ligand brain-derived neurotrophic factor, but are proprioceptive (Williams *et al*., 1995; Noden, 1980; Davies, 1987).

By E8, cutaneous afferents from dorsal root ganglia in the lumbar region of the chick project to the lateral region of the dorsal horn, while proprioceptive afferents project much further ventrally in the spinal cord (Eide and Glover, 1997). Neural crest-derived large-diameter TrkB+ dorsal root ganglion neurons also project to the dorsal horn in the chick (Rifkin *et al*., 2000). We used a quail neurite-specific antibody (Tanaka *et al*., 1990) to confirm that we could detect quail neurites both dorsally and ventrally within the neural tube at E8 after control isotopic grafts of quail neural tubes at caudal cervical/rostral thoracic levels. After grafts of opV ectoderm to similar levels, and where many quail neurons were present in the ventrolateral dorsal root ganglion at E7.5–E8, we found quail neurites within the lateral region of the dorsal horn, but not further ventrally. Furthermore, peripheral quail neurites were also seen in the dermis. Taken together, these results suggest that TrkB+ opV ectoderm-derived neurons are cutaneous in nature. This implies that Pax3+ opV ectoderm-derived cells are committed to following their normal trigeminal fate, even in the trunk environment, where no placodes form. Hence, they are unable to adopt any other fates characteristic of the neural crest.

OpV Ectoderm-Derived Neurons Often Associate with the Ventral Root

OpV ectoderm-derived neurons in the trunk were often found in association with the ventral root, occasionally forming ectopic ganglia. Similarly, in the trigeminal system in the chick embryo, opV placode-derived neurons are often found scattered along the ophthalmic trigeminal nerve (see Baker *et al*., 1997). Sensory neurons have also been described along the oculomotor nerve in some mammals (Bortolami *et al*., 1977, 1990). When the olfactory nerve is disrupted in chick embryos, gonadotropin-releasing hormone+ neurons from the olfactory placode, which normally migrate into the brain along the olfactory nerve, instead migrate on the ophthalmic trigeminal nerve (Murakami *et al*., 1998).

Interestingly, host neurons, which must be neural crest-derived, were frequently observed on the ventral root on both operated and unoperated sides of chimeric embryos, and also occasionally scattered within the myotome. Occasional host neurons on the ventral root were seen to express TrkB, but for the most part they were TrkB-negative. Multipotent neural crest stem cells that can differentiate into neurons or glia are known to be present on peripheral nerves in fetal rats (Morrison *et al*., 1999). In the chick, overexpression of the basic helix–loop–helix transcription factors Neurogenin1 and Neurogenin2 leads to the formation of sensory neurons on peripheral nerves in the trunk (Perez *et al*., 1999). Similarly, overexpression of Phox2a leads to the formation of sympathetic neurons on peripheral nerves in the trunk (Stanke *et al*., 1999). These results presumably reflect forced neuronal differentiation of neural crest stem cells present on peripheral nerves. The host neurons that we have seen on the peripheral nerves in the trunk are probably the result of aberrant neuronal pathway choice by neural crest stem cells in what is a predominantly glial environment.

Pax3 Is Not Expressed in Neural Crest-Derived Neurons in the Trigeminal or Dorsal Root Ganglia

Pax3 has previously been shown to be expressed transiently in emigrating neural crest cells and also, later, in neural crest cells in the dorsal root ganglia and certain cranial sensory ganglia, including the trigeminal (Goulding *et al*., 1991; Stark *et al*., 1997). In the *splotch* mutant mouse, which lacks a functional *Pax3* gene, neural crest cells migrate but dorsal root and cranial sensory ganglia are much reduced (Tremblay *et al*., 1995; Li *et al*., 1999; Epstein *et al*., 2000). The requirement for Pax3 in neural crest cells is cell-autonomous (Li *et al*., 1999).

We show here that neural crest-derived neurons in the trigeminal and dorsal root ganglia, detected using the Hu antibody, do not express Pax3. In the dorsal root ganglia, Pax3 is expressed in Hu-negative cells at the edges of the ganglia, particularly concentrated at the dorsomedial edge. Since Pax3 is involved in the differentiation of myelinating Schwann cells (Kioussi *et al*., 1995; Blanchard *et al*., 1996), this may suggest that Pax3 is expressed in glial precursors within the ganglion, and/or that it is downregulated immediately upon neuronal differentiation. In either case, this is very different from the situation in opV placode cells, which retain Pax3 expression for some time after neuronal differentiation (although Pax3 is eventually downregulated). It is possible, therefore, that Pax3 functions differently in the opV placode and in neural crest cells.

Can Placodes Form Neural Crest-Like Derivatives?

We have found that Pax3+ opV ectoderm-derived cells are committed to a cutaneous sensory neuron fate, consistent with their normal fate as trigeminal neurons, even in the trunk environment where no placodes form. This is in agreement with and extends the conclusions of our previous experiments, in which we substituted opV ectoderm for the nodose placode and found that Pax3+ opV cells formed Pax3+ neurons that did not express the nodose neuron marker *Phox2a* and were excluded from the nodose ganglion (Baker and Bronner-Fraser, 2000).

Although our current data show that Pax3+ opV ectoderm cells are unable to adopt any other fates characteristic of the neural crest, it is possible that Pax3-negative opV ectoderm cells can do so. These cells are not yet committed to a trigeminal fate, as they are able to form *Phox2a*+ neurons in the nodose ganglion (Baker and Bronner-Fraser, 2000). Furthermore, dissociated opV ectoderm cells injected directly into the neural crest cell migration pathway might behave differently from opV ectoderm grafted above the segmental plate mesoderm as an epithelial sheet. We are currently investigating these ideas.

There is some evidence that nodose placode-derived cells are able to compensate to a certain extent for the ablation of nearby cardiac neural crest cells (Kirby, 1988a,b). The nodose placode normally gives rise to visceral sensory neurons in the nodose ganglion that innervate the heart and other visceral organs (reviewed in Zhuo *et al*., 1997). The "cardiac" neural crest, which originates from the same axial level as the nodose placode (D'Amico-Martel and Noden, 1983), gives rise both to parasympathetic cardiac ganglia and to ectomesenchymal cells that are essential for septation of the outflow tract (Kirby *et al*., 1983; Kirby and Stewart, 1983). After ablation of the premigratory cardiac neural crest, the cardiac ganglia form, but the choline acetyl transferase (ChAT)-immunoreactive neurons within them are mainly derived from the nodose placode (Kirby, 1988a). Although the function of these ChAT+ nodose placode-derived neurons was not tested, ChAT is not normally expressed in nodose ganglion neurons in the chick (Kirby, 1988a) (although it is present in the nodose ganglion of at least some mammals; Zhuo *et al*., 1997). Furthermore, the nodose placode also contributes ectomesenchyme to the cardiac outflow tract in the absence of the cardiac neural crest, although this cannot rescue the septation defect (Kirby, 1988b). Hence, nodose placode-derived cells apparently have some ability to form cardiac neural crest-like derivatives.

Interestingly, cardiac ganglion neurons, like nodose placode-derived neurons, express Phox2a (Valarché *et al*., 1993; Tiveron *et al*., 1996; Morin *et al*., 1997). Phox2a expression in the chick is sufficient to elicit expression of catecholaminergic and cholinergic marker genes, including ChAT, in multipotent neural crest precursor cells (Stanke *et al*., 1999). The cholinergic differentiation factor, leukemia inhibitory factor, which can direct a noradrenergic to cholinergic switch in sympathetic neurons, is present in the heart (Yamamori *et al*., 1989). It is possible, therefore, that the expression of ChAT in nodose placode-derived neurons in cardiac ganglia does not in fact reflect their adoption of an autonomic fate. Instead, it may represent the expression of a cholinergic marker gene in nodose-type sensory neurons that form ectopic ganglia on the heart in the absence of cardiac neural crest cells. However, this cannot explain the formation of ectomesenchyme by the nodose placode after cardiac neural crest ablation (Kirby, 1988b).

It is clear from the wide spectrum of placodal derivatives that, taken as a whole, placodes are a highly multipotent cell population. Nonetheless, despite their common origin with neural crest cells from ectoderm at the neural plate border, different placodes are induced at different times, by different signals from different tissues (Graham and Begbie, 2000; Baker and Bronner-Fraser, 2001). Each placode has its own unique characteristics and it may be misleading to consider them as a group (Graham and Begbie, 2000), even though at very early stages there is evidence for a common preplacodal primordium (see Baker and Bronner-Fraser, 2001). Some placodes, therefore, may be much more similar to the neural crest than are others. For example, the olfactory placode, which originates from the anterior neural plate border where no neural crest cells form, gives rise to glia, supporting cells and neuroendocrine cells as well as to olfactory receptor neurons (see Baker and Bronner-Fraser, 2001). Its capacity to form neural crest-like derivatives may therefore be greater than that of the opV placode, whose only derivatives are cutaneous sensory neurons.

In conclusion, we have shown that Pax3+ opV ectoderm-derived cells in the trunk form Pax3+ neurons that incorporate into host neural crest migration streams and contribute to neural crest-derived ganglia. However, in all locations, they form large-diameter, TrkB+ neurons that appear to be cutaneous in nature. Hence, Pax3+ opV ectoderm-derived cells are committed to their normal trigeminal fate, and are unable to adopt any other fates characteristic of the neural crest. Furthermore, neural crest-derived neurons in the trigeminal and dorsal root ganglia do not express Pax3, suggesting that Pax3 function in neural crest cells may differ from its function in opV placode cells.

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