Neuronal Differentiation from Postmitotic Precursors in the Ciliary Ganglion

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In the chick ciliary ganglion, neuronal number is kept constant between St. 29 and St. 34 (E6–E8) despite a large amount of cell death. Here, we characterize the source of neurogenic cells in the ganglion as undifferentiated neural crest-derived cells. At St. 29, neurons and nonneuronal cells in the ciliary ganglion expressed the neural crest markers HNK-1 and p75NTR. Over 50% of the cells were neurons at St. 29; of the nonneuronal cells, a small population expressed glial markers, whereas the majority was undifferentiated. When placed in culture, nonneuronal cells acquired immunoreactivity for HuD, suggesting that they had commenced neuronal differentiation. The newly differentiated neurons arose from precursors that did not incorporate bromodeoxyuridine. To test whether these precursors could undergo neural differentiation in vivo, purified nonneuronal cells from St. 29 quail ganglia were transplanted into chick embryos at St. 9–14. Subsequently, quail cells expressing neuronal markers were found in the chick ciliary ganglion. The existence of this precursor pool was transient because nonneuronal cells isolated from St. 38 ganglia failed to form neurons. Since all ciliary ganglion neurons are born prior to St. 29, these results demonstrate that there are postmitotic neural crest-derived precursors in the developing ciliary ganglion that can differentiate into neurons in the appropriate environment. © 2002 Elsevier Science (USA)

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INTRODUCTION

During embryonic development, neural crest cells migrate along precise pathways and eventually differentiate into a wide variety of cell types, including autonomic and sensory neurons, Schwann cells, cartilage, melanocytes, and muscle. At the onset of migration, many neural crest cells are multipotent (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991). Lineage restriction is thought to occur during migration and/or after reaching their destinations, with the exception of neural crest-derived stem cells (Anderson, 1993a), which persist in some derivatives (Morrison et al., 1999). However, there is also evidence for early specification of some neural crest lineages; for example, a subpopulation of migrating mesencephalic neural crest cells, destined to form cholinergic neurons, already express a high affinity choline uptake transporter during their migration (Barald, 1988, 1989).

Little is known about the developmental potential or extent of lineage restriction in postmigratory neural crest cells. Although neurogenic genes such as neurogenins and mash-1 appear to influence the type of neurons produced by neural crest cells (Anderson, 1993b; Guillemot et al., 1993; Ma et al., 1998, 1999), the neuron vs glial lineage decision in nascent dorsal root ganglia appears to be regulated by cell–cell interactions (Wakamatsu et al., 2000). The developmental potentials of postmigratory neural crest cells have been examined previously by quail/chick chimera studies. Even at postmigratory stages, the fate of neural crest-derived cells can be altered, as demonstrated by back-transplantation studies. For example, when pieces of quail ciliary and nodose ganglia were back-transplanted onto pathways utilized by trunk neural crest cells of early chick host embryos, grafted cells differentiated into neurons in sympathetic ganglia (Ayer-Le Lievre and Le Douarin, 1982;
Dupin, 1984; Fontaine-Perus et al., 1988; Le Douarin et al., 1978). Results from these experiments also inferred that the undifferentiated neural crest cells residing in the peripheral ganglia could become neurons when challenged in a new environment.

Cells that form the ciliary ganglion condense by St. 18 (E2.5–E3), and the birth of all neurons is complete by St. 28 (E5.5, D’Amico-Martel, 1982; Dryer, 1994; Dupin, 1984; Rohrer and Thoenen, 1987)). Ciliary ganglion neurons are overproduced and subsequently pruned by naturally occurring cell death between St. 34 and St. 40, when the total number of ciliary ganglion neurons is reduced by 50% (Furber et al., 1987; Landmesser and Pilar, 1974). However, cell death is not limited to this period of time. There is an additional early wave of cell death at St. 29 (E6), but the number of neurons is rapidly restored to original levels, suggesting that a compensatory mechanism serves to stabilize neuronal number (Lee et al., 2001).

Because no ciliary ganglion neurons are born after St. 28 (E5.5), we hypothesize that there is a population of nonneuronal cells in the St. 29–31 ciliary ganglion that has the ability to differentiate into neurons. Here, we characterize the markers expressed by nonneuronal cells in the ciliary ganglion at St. 29 and test their ability to undergo neuronal differentiation in vitro and after transplantation in vivo. We find that neuronal differentiation is not preceded by DNA synthesis, suggesting that a subpopulation of postmitotic nonneuronal cells in the St. 29 ciliary ganglion can differentiate into neurons when they are placed in the appropriate environment. This precursor population is lost by St. 38 (E12), thus accounting for the subsequent drop in neuronal number in the ciliary ganglion observed by St. 40 (E14).

MATERIALS AND METHODS

Cell Culture

Ciliary ganglia were removed from Hamburger and Hamilton St. 29 (E6) (Hamburger and Hamilton, 1951) chicken embryos and dissociated as previously described (Nishi, 1996). Cells were plated on poly-lysine/laminin-coated glass tissue coverslips (Fisher) in Dulbecco’s Modified Eagles Medium (DMEM; Invitrogen), 10% heat-inactivated horse serum, 2% fetal calf serum, and 2% chick embryo extract. Recombinant chicken ciliary neurotrophic factor (chCNTF; produced in the Nishi lab) and recombinant fibroblast growth factor 2 (FGF-2; a gift from Dr. F. P. Eckenstein, University of Vermont) were used at 20 ng/ml.

Immunocytochemistry

Ciliary ganglion tissue sections or cell cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 150mM NaCl, 20mM Na phosphate, pH 7.4) and rinsed in PBS three times. They were then blocked in PBS with 0.2% Tween 20, 2% bovine serum albumin, and 2% normal goat serum for 1 h at room temperature. Slides or cultures were incubated in primary antibodies overnight at 4°C and immunoreactivity visualized by secondary antibodies purchased from Molecular Probes and Jackson Immunotech. Primary antibodies used were: HNK-1 (1:50), mouse anti-HuC/D (1:250; here referred to as anti-HuD; Molecular Probes; Marusich et al., 1994), mouse anti-HuA (1:2000; generous gift from Drs. J. Weston and M. Marusich, University of Oregon; Nakamura and Weston, 1997), 7B3 (1:500; generous gift from Dr. J. Weston, University of Oregon; Henion et al., 2000); mouse Islet-1 (1:100; Developmental Studies Hybridoma Bank, University of Iowa; Ericson et al., 1992), mouse anti-neurofilament (1:300; generous gift from Dr. Virginia Lee, University of Pennsylvania; Lee et al., 1987); rabbit anti-platelet derived growth factor receptor α (1:200; Santa Cruz Biotechnology; Winseck et al., 2002); rabbit anti-p75 neurotrophin receptor (p75NTR, 1:3000; generous gift from Dr. L. Reichardt, University of California, San Francisco; Weskamp and Reichardt, 1991), QCPN (1:10; developed by Drs. B. Carlson and J. Carlson, Developmental Studies Hybridoma Bank, University of Iowa; Selleck and Bronner-Fraser, 1995), and TuJ1 (1:500; Babcock; Lee et al., 1990).

Preparation and Immunostaining of Acutely Dissociated Cells

Ciliary ganglia were dissociated as described (Nishi, 1996) and cell suspensions centrifuged onto slides by using a Cytospin centrifuge (Shandon-Lipshaw). A total volume of 200 μl was loaded into each cytofunnel and spun at 1000g for 10 min. Cells were collected onto gelatin-subbed slides and air-dried 30 min to 1 h before postfixing with 4% paraformaldehyde. Immunostaining was performed by using the same protocol as described above.

Cell Counting of Dissociated Ciliary Ganglion Cultures

Dissociated ciliary ganglia that were centrifuged onto slides or cultured were both processed for immunocytochemistry and counterstained with a nuclear dye (Hoechst). Cell counting was done by using a Nikon Eclipse 800 Fluorescent microscope with a 60× oil objective. Ten nonoverlapping fields of view were counted; total cell number was obtained by counting Hoechst-positive nuclei. Percent of neuronal number was obtained by dividing total number of neurons (HuD+ cells) by the total cell number (Hoechst-labeled nuclei).

BrdU Labeling in Vitro

A total of 10 μM of BrdU (Roche) was applied to St. 29 ciliary ganglion cultures for 13–15 h. Ciliary ganglion cultures were fixed 3 days after BrdU addition and processed for anti-BrdU (1:500; SeraLab) and TuJ1 immunocytochemistry. Cultures were counterstained with a Hoechst dye (Sigma) to label all nuclei.

Enrichment of Nonneuronal Cells from Ciliary Ganglia

For injection experiments, we typically isolated 200–300 St. 29 and 120–150 St. 38 quail ciliary ganglia. Ganglia obtained from St. 29 quail embryos were incubated in 0.1% trypsin for 20–30 min, whereas ganglia from St. 38 embryos were incubated in trypsin for 45 min to 1 h. After triturating, cell suspensions from St. 38 ciliary ganglia were passed through a 70- or 100-μm nylon mesh to remove tissue clumps. Cells were plated onto poly-lysine/laminin-coated T-25 or T-75 tissue culture flasks (Nunc); about 45 St. 29 ciliary...
ganglia and 15 St. 38 ciliary ganglia were typically seeded onto a T-25 flask. After cells had adhered to the substratum, medium was replaced by 1× modified Puck’s glucose (MPG), and the flasks were manually shaken and monitored under an inverted microscope. During shaking, MPG was changed several times to rinse off neurons. Nonneuronal cells that remained in the flasks were recovered by incubating in 0.1–0.25% trypsin in MPG for 15–20 min and concentrated by centrifugation. Nonneuronal cell pellets were then resuspended in DMEM with 10% heat-inactivated horse serum, 2% fetal calf serum, and 2% chick embryo extract supplemented with 20 ng/ml recombinant chick CNTF and 20 ng/ml recombinant FGF-2.

Transplantation of Quail Cells into Chick Embryos and Tissue Processing

Adherent cells derived from St. 29 or St. 38 quail ciliary ganglia were purified as described above and injected into E2 (St. 9–15) chicken embryo hosts. Quail cell suspensions were concentrated in a microcentrifuge tube, loaded into a glass needle, and injected by pressure into the head mesenchyme of the embryo. After injections, embryos were returned to the incubator until the day of harvest (1–3 days after surgery), when they were fixed in 4% paraformaldehyde in PBS for 4 h at room temperature or overnight at 4°C. They were then rinsed three times in PBS and infiltrated with 5% and 15% sucrose (4–12 h each) sequentially. Finally, embryos were equilibrated for 2–4 h at 37°C in 7.5% gelatin for embedding. Embedded embryos were frozen in liquid nitrogen, and 10-μm serial cryostat sections were cut in a Microm cryostat. Immunohistochemistry for anti-QCPN (Developmental Studies Hybridoma Bank, University of Iowa), HNK-1, and anti-HuD was performed as described above. Fluorescent images were taken on a BioRad 1024 or Zeiss 510 laser scanning confocal microscope, and optical sections were collected at 2-μm intervals.

RESULTS

Many Nonneuronal Cells in St. 29 Ciliary Ganglia Are Undifferentiated

As a first step toward characterizing the nonneuronal cells in the early embryonic ciliary ganglion, we assessed the expression of various cellular markers by immunocytochemistry. Using antibodies against neural crest markers, p75 neurotrophin receptor (p75NTR; Heuer et al., 1990; Weskamp and Reichardt, 1991) and HNK-1, we found that neurons and nonneuronal cells in the ganglion were p75NTR+ and HNK-1+, consistent with the neural crest origin of this ganglion (Fig. 1A; Noden, 1978). When HuD immunoreactivity was examined simultaneously, we saw pockets of cells that were HuD+ but HNK-1−/p75NTR+, suggesting that they were crest-derived nonneuronal cells. In addition, neurons and nonneuronal cells in the ciliary ganglion expressed an undifferentiated cell marker, HuA. HuA belongs to the same RNA-binding protein family as HuD, but unlike HuD, HuA is highly expressed in undifferentiated cells of the nervous system (Wakamatsu and Weston, 1997). We have previously showed that HuA is expressed in all neurons (which are also HuD+) and as well as in a subset of nonneuronal cells in St. 29 ciliary ganglion in vivo (Lee et al., 2001). To identify glial cells in ciliary ganglion tissue sections, we used an antibody against the platelet-derived growth factor receptor α (PDGFRα; Winseck et al., 2002). At St. 29, anti-PDGFRα labeled a small population of cells that were HNK-1+ but not HuD+, suggesting that they were developing Schwann cells in the ganglion (Fig. 1B). In addition, we saw HNK-1+ cells that were neither HuD+ nor

FIG. 1. At St. 29, some nonneuronal cells in the ciliary ganglion express only neural crest markers in vivo. Embryos were staged and ciliary ganglia were processed for indirect immunofluorescence by using antibodies against neural crest markers p75NTR and HNK-1, a neuronal marker HuD, or a glial marker PDGFRα. (A) Section immunostained with anti-HuD (red), anti-p75NTR (green), and HNK-1 (blue). Many nonneuronal cells (HuD+) were p75NTR+ and HNK-1+ (arrow). (B) St. 29 ciliary ganglion immunostained with anti-HuD (red), anti-PDGFRα (green), and HNK-1 (blue). A subpopulation of nonneuronal cells expressed HNK-1 but did not express neuronal or glial markers (arrows).
PDGFRα⁺. These are likely to be neural crest-derived undifferentiated cells in St. 29 ciliary ganglion that have not expressed neuronal or glial markers.

St. 29 ciliary ganglia were dissociated in order to correlate marker expression with morphological subtype as well as to determine more definitively which markers colocalized with one another. Approximately one-half of the cells recovered in cell culture displayed morphology characteristic of neurons (phase bright cell body with neurites). These cells expressed neuronal-specific markers such as neurofilament, neuron-specific tubulin (βIII tubulin, TuJ1; Lee et al., 1990), HuD, and Islet-1 (Figs. 2A–2H). Immunoreactivity for neurofilament and tubulin were particularly strong in neurites, whereas HuD immunoreactivity was confined to the neuronal somata. Islet-1 staining was observed in neuronal nuclei. Consistent with our observations in vivo, anti-p75NTR labeled all neuronal and nonneuronal cells recovered from the St 29 ciliary ganglion (Figs. 2I–2P), and this expression overlapped with HNK-1 (Figs. 2M–2P).

Nonneuronal cells from St. 29 ciliary ganglia were flat, fibroblast-like in morphology, and expressed neural crest markers p75NTR and HNK-1. The markers O4 and 7B3, which are expressed when cells commit to a glial lineage (Henion et al., 2000; Rohrer and Sommer, 1983; Sommer and Schachner, 1981), were found in a small subset of nonneuronal cells (data not shown). Many more O4⁺ and

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7B3<sup>+</sup> cells were observed at St. 38 (data not shown), indicating that glial development commences much later than neural differentiation in the ganglion. Neither 7B3 nor O4 immunoreactivity colocalized with HuD.

Taking our in vivo and in vitro data together, St. 29 ciliary ganglion neurons expressed neuronal markers such as HuD and neurofilament as well as neural crest markers HNK-1 and p75<sup>NTR</sup>. Nonneuronal cells expressed p75<sup>NTR</sup> as well as HNK-1. A small subset of p75<sup>NTR</sup>+/HNK-1<sup>+</sup> nonneuronal cells was immunopositive for glial markers PDGFR<sub>α</sub>, O4, and 7B3. However, the majority of nonneuronal cells did not express either glial (PDGFR<sub>α</sub>, O4, 7B3) or neuronal (HuD, neurofilament) markers. These cells were HNK-1<sup>+</sup>, p75<sup>NTR</sup>+, and HuA<sup>+</sup>, suggesting that they were undifferentiated cells that were neural crest-derived.

Nonneuronal Cells from St. 29 Ciliary Ganglia Undergo Neuronal Differentiation in Culture

We tested whether cells isolated from St. 29 ciliary ganglia could differentiate into neurons in culture. All cells with neuronal morphology in freshly isolated dissociates of St. 29 ciliary ganglion expressed HuD and Islet-1 (Figs. 3A–3C) and represented about 51% of the cells in the ganglion. After 1 day in vitro, 55% of the cells still expressed HuD together with Islet-1 immunoreactivity (Figs. 3D–3F). These cells also expressed neurofilament and neuron-specific tubulin, confirming that they were mature neurons. However, an additional 18% of the cells had acquired HuD immunoreactivity. These cells were flat, had no neurites, and did not express Islet-1 (Fig. 3). These cells with a nonneuronal morphology that acquire HuD expres-
sion may be neuronal precursors that have commenced differentiation as neurons.

In experiments in which we quantified the number of cells in culture, we observed that the percent of differentiated neurons remained constant at 55% (184 ± 3 HuD^+ cells/333 ± 20 Hoechst-labeled cells in 10 fields of view at day 1; n = 3 cultures) vs 50% (235 ± 26 HuD^- cells out of 466 ± 35 Hoechst labeled cells in 10 fields of view at day 2;
n = 3 cultures) despite a 40% increase in the total number of cells. This suggested that new neurons were being generated. To determine whether these newly generated neurons arose from dividing nonneuronal cells, we treated St. 29 ciliary ganglion cultures with bromodeoxyuridine (BrdU, 13–15 h treatment). Three to four days after the BrdU pulse, no BrdU\(^+\) neurons were observed (Figs. 4A–4C), although many nonneuronal cells had BrdU\(^+\) nuclei \(n = 10\) cultures) and were p75\(^{NTR}\) (Figs. 4D–4F). Thus, when St. 29 ciliary ganglion cells were placed in culture, nonneuronal cells divided and contributed to the increase in total cell number. In addition, there appeared to be a concomitant increase in total neuronal number since the proportion of neurons remained unchanged amid an increase in total cell number. This is in agreement with the observation that ciliary ganglion neurons are born prior to St. 28 (D’Amico-Martel, 1982) and also suggests that there may be postmitotic neuronal precursors in St. 29 ciliary ganglion that can differentiate into neurons under certain conditions.

**Injection of Purified Nonneuronal Cells from Quail Ciliary Ganglia**

We tested the potential of the nonneuronal cells in St. 29 ciliary ganglion to form neurons by chick/ transferring The experiments described above suggested that nonneuronal cells in the ganglion could differentiate into neurons and were postmitotic. However, we neither had a marker for neuronal precursors nor understood the signals for neuronal differentiation in vitro. Therefore, we used the intact embryo as a “culture system.” By injecting quail nonneuronal cells from St. 29 ciliary ganglion to young (St. 9–14) chick hosts, we asked whether they could become neurons. We purified nonneuronal cells from ciliary ganglia based on the differential adhesive properties of neurons vs nonneuronal cells (Fig. 5). Ciliary ganglion cells were plated onto laminin/poly-lysine dishes and allowed to adhere for several hours, and then neurons were shaken off the culture dish. Nonneuronal cells produced in this way were 98–99% pure (Fig. 6) and were HuD\(^+\), HNK-1\(^-\) (Fig. 7).

Quail nonneuronal cells were injected in the head mesenchyme of HH St. 9–14 chick embryos (Sechrist et al., 1998). Donor quail cells were identified by an antibody, QCPN, that recognizes quail, but not chick nuclei (Le Douarin et al., 1996). Roughly 50–100 quail cells were injected into each chick embryo. To follow their migration and differentiation, embryos were collected 1, 2, or 3 days after surgery. Embryos that were harvested 3 days after transplantation were stained with QCPN and anti-HuD to assess neuronal differentiation.

When examined 6–9 h after operation, quail cells were observed around the injection sites with some cells starting to disperse (Fig. 8A). By the following day, QCPN\(^+\)/HNK-1\(^-\) cells could be observed migrating along with host neural crest cells (Fig. 8B). Two days after injection, quail cells had approached the vicinity of the developing ciliary ganglion and could be seen within a cluster of HNK-1\(^-\) cells that were in contact with the oculomotor nerve (Fig. 8C). When embryos were analyzed 3 days post-surgery, a small number of QCPN\(^+\) neurons were consistently observed in all chick ciliary ganglia that had quail cells \(n = 11\); Figs. 9B and 9C). QCPN\(^+\)/HuD\(^-\) cells also were within the ganglion, but we could not distinguish whether these would eventually differentiate into neurons or would become part of the glial population of the ciliary ganglion. Importantly, we never observed any QCPN\(^+\)/HuD\(^-\) cells outside the ciliary ganglion; this suggested that the quail cells differentiated only when they had reached the appropriate environment.

We next asked whether the developmental potential of nonneuronal cells from ciliary ganglia decreased with age. Nonneuronal cells were isolated from St. 38 quail ciliary ganglia and used as donors for transplantation (Figs. 9D and 9E). Similar to the St. 29 cells, St. 38 quail cells were found within and around the ciliary ganglion. Typically, more QCPN\(^+\) cells were observed in the host ciliary ganglion after transplantation of St. 38 than of St. 29 nonneuronal cells. This is likely due to the larger number of nonneuronal cells that can readily be isolated from older ganglia, or that nonneuronal cells from St. 38 may survive better than those from St. 29. Despite the greater numbers of transplanted cells, none of the St. 38 quail cells differentiated into neurons (Figs. 9D and 9E). These data suggest that the nonneuronal population from older ciliary ganglion have lost their neurogenic potential and/or have become committed to another lineage such that their fate cannot be changed even in when exposed to the environment of the ciliary ganglion.
The results of this study show that St. 29 ciliary ganglia contain nonneuronal cells that can undergo neuronal differentiation. First, a population of nonneuronal cells in the St. 29 ciliary ganglion expresses neither glial- nor neuronal-specific markers; rather, they express pan-neural crest markers such as p75NTR and HNK-1. Second, newly differentiated neurons appear in St. 29 ciliary ganglion cultures without cell division, suggesting that neuronal precursors in the ciliary ganglion are postmitotic. Third, nonneuronal cells isolated from St. 29 ciliary ganglion migrate and differentiate into neurons when grafted into younger hosts. This neurogenic potential appears to be restricted with time because nonneuronal cells from older quail ciliary ganglion (E10) failed to generate neurons under identical conditions. In the ciliary ganglion, neuroblasts complete their cell divisions by St. 28 (Dupin, 1984) and a peak of neuronal cell death occurs at St. 29 (Lee et al., 2001); thus, these results are consistent with the hypothesis that dying neurons are replaced by postmitotic precursor cells between St. 29–34.

The processes of neuronal differentiation and programmed cell death are typically considered to be uncoupled from one another. For example, even though programmed cell death is prominent during neurogenesis in the ventricular zone of the developing central nervous system (Blaschke et al., 1996), if cell death is blocked, then hypertrophy occurs in the central nervous system (Kuida et al., 1996). Similarly, in the peripheral nervous system, the prevailing view is that neurons are first overproduced, then eliminated by a process of target-dependent cell death. We have previously observed that programmed cell death is prominent in the developing ciliary ganglion after neuronal precursors have withdrawn from the cell cycle but before peripheral synaptogenesis (Lee et al., 2001). Despite the magnitude of this early ciliary ganglion neuron cell death at St. 29, total neuronal number in the ganglion remains constant prior to another period of cell death that occurs between St. 34 and St. 40 (E8–E14), suggesting that dying neurons are replaced between St. 29 (E6) and St. 34 (E8). Thus, during the development of the ciliary ganglion, cell death and replacement take place before final neuronal number is achieved. The cells that replace dying neurons at St. 29 are likely to be undifferentiated neural crest cells resident in this ganglion. In the vertebrate central nervous system, it has been shown that nonneuronal cells can differentiate into neurons after injury. For example, in the chick retina, glial cells can differentiate into neurons after damage induced by neurotoxin (Fischer and Reh, 2001). However, these glial cells reenter the cell cycle after neuronal cell death, whereas in the ciliary ganglion, neuronal precursors do not divide.

Two observations support the notion that neuronal differentiation occurred in St. 29 ciliary ganglion cultures. First, after 24 h in vitro, cells with a characteristic nonneuronal morphology (i.e., flat and lacking processes) acquired HuD immunoreactivity. These cells were unlikely to be neurons...
FIG. 8. Injected quail cells migrated in the host embryo and incorporated into the ciliary ganglion. (A) Six hours after injection, QCPN+/H11001 (green) and HNK-1+ (red) cells (arrows) could be observed in the midbrain level of the host. (B) One day after injection, a clump of QCPN+/HNK-1+ cells was migrating along with host neural crest cells (HNK-1+ only) in the midbrain level. (C) Two days after injection, some quail cells were approaching the area where the ciliary ganglion (CG) would be formed lateral to the developing forebrain (FB). The oculomotor nerve (OcN) originating from the midbrain (MB) had also arrived in the ciliary ganglion region.

FIG. 9. St. 29 quail ciliary ganglion nonneuronal cells differentiated into neurons, whereas St. 38 nonneuronal cells did not. (A) A low magnification image of the head region from a St. 25/26 chick embryo that was injected with St. 29 quail nonneuronal cells. The ciliary ganglion (CG) was located next to the eye (Eye) and adjacent to the forebrain (FB) and Rathke’s pouch (RP); the trigeminal ganglion (Trig) could also be seen in this section. (B, C) Quail cells (green) from St. 29 embryos migrated to the ciliary ganglion and some of them were neuronal (arrows). Other quail nonneuronal cells that were not HuD+ were also observed. Image in (B) is a higher magnification of the ciliary ganglion in (A), and (C) was taken from a different embryo. (D, E) Two examples of ciliary ganglion from St. 25/26 embryos that received St. 38 quail nonneuronal cells. Numerous quail nuclei could be seen throughout the ganglia but none of them were HuD+.
that have dedifferentiated because the proportion of neurons did not decrease. Such flat HuD⁺ cells have been described previously in other neural crest-derived ganglia and are indicative of commitment to neuronal differentiation (Marusich and Weston, 1992). When nascent dorsal root ganglia were dissociated, HuD⁺ cells were observed that were nonneuronal in morphology and also lacked other neuronal markers, such as neurofilament. Furthermore, the total number of cells increased with time in St. 29 ciliary ganglion culture, whereas the percent of neurons (HuD⁺) remained unchanged. Thus, new neurons were generated to sustain the overall percentage of neurons. The newly generated neurons were never observed to incorporate BrDU, although many nonneuronal cells were labeled. This is consistent with in vivo birthdating studies of the ciliary ganglion, indicating that no neurons are born by cell division after St. 28 (Dupin, 1984; Rohrer and Thoenen, 1987). Thus, the neurogenic precursors that gave rise to neurons are already postmitotic.

Evidence for the presence of neurogenic cells at St. 29 in the ciliary ganglion was also based on in vivo experiments in which purified nonneuronal cells from quail ganglia were injected onto cranial neural crest pathways. Prior to injection, purified nonneuronal cells were HNK-1⁻ and did not express neuronal markers such as HuD. After injection, cells that differentiated into neurons could be unambiguously identified by colocalization of the quail nuclear marker, QCPN, and the neuronal-specific marker, HuD. These cells were often observed within, but never outside, the ciliary ganglion. This suggests that the nonneuronal donor quail cells will only differentiate when they have migrated to a microenvironment that contains neuronal differentiation signals.

Our experiments studying the properties of purified nonneuronal cells from the ciliary ganglion extend studies examining the developmental potential of transplanted quail cells into chick hosts. Pieces of quail ciliary ganglion containing both neuronal and nonneuronal cells migrate and incorporate into sympathetic ganglia when back-transplanted into the trunk region of host chicks (Dupin, 1984; Le Douarin et al., 1978; Le Lievre et al., 1980). Transplanted neurons from dissociated ciliary ganglia were specifically identified as contributing to the catecholaminergic population in the sympathetic ganglia (Coulombe and Bronner-Fraser, 1986; Sechrist et al., 1998). To test whether nonneuronal cells of transplanted ganglia could differentiate into neurons, chimeric cranial sensory ganglia were created in which all of the nonneuronal cells were quail-derived. When these chimeric ganglia were grafted into the trunk, quail sympathetic neurons were observed (Ayer-Le Lievre and Le Douarin, 1982). However, unlike the ciliary ganglion, sensory ganglia undergo a prolonged period of cell division and neurogenesis (Hamburger and Levi-Montalcini, 1949). Thus, the nonneuronal cells of sensory ganglia likely contain a subpopulation of dividing precursors that give rise to the observed neurons. In support of this idea, when dorsal root ganglia were transplanted prior to the cessation of neurogenesis (<E7), they were able to give rise to neurons; on the other hand, donor dorsal root ganglia that were past that age failed to do so (Rohrer et al., 1985, 1986). In contrast, ciliary ganglion postmitotic non-neuronal cells seem to be able to differentiate into neurons under the appropriate conditions.

The cells that we observed expressing p75NTR and HNK-1 in the absence of glial- or neuronal-specific markers are likely to be multipotent neural crest cells. In mammals, p75NTR has been used to isolate neural crest cells from fetal sciatic nerve (Morrison et al., 1999), a structure previously thought to contain only glia and glial precursors. The p75NTR⁺ cells from the sciatic nerve appear to contain stem cell-like properties as they can self-renew as well as give rise to a variety of neural crest derivatives, including neurons, glia, and smooth muscle. In contrast, the neuronal precursors in St. 29 ciliary ganglion, by definition, cannot be stem cells since they neither self-renew nor divide. It is likely that nonneuronal cells in the ciliary ganglion are heterogeneous in nature, i.e., there are different pools of nonneuronal cells, but markers that are available do not allow further demarcation of different subtypes. Our studies suggest the existence of both dividing and nondividing precursors at St. 29. Indeed, glial cells in the ciliary ganglion expand and differentiate after the neuronal period of differentiation. It is probable that the proliferating precursors will become glia, whereas the nonproliferating precursors can become neurons and/or glia.

Our observation that nonneuronal cells from St. 38 ciliary ganglion fail to form neurons suggests that the precursor cells that are neurogenic are limited in number or that they have irreversibly committed to another lineage. This is consistent with observations that neurogenic precursors decrease with age (Marusich and Weston, 1992; Rohrer and Sommer, 1983). Similarly, transplantation studies showed that the ability of neural crest nonneuronal cells to repopulate neuronal population drops off as development progresses, as does the ability of ciliary ganglion neurons to switch transmitter phenotype (Sechrist et al., 1998). The disappearance of these neurogenic precursor cells is correlated with the time during which total neuron number in the ciliary ganglion is reduced by 50%. Our results demonstrate that neuronal birth can be temporally uncoupled from time of differentiation, such that the latter may occur many days after the last cell division. This may represent a mechanism for fine-tuning the numbers of neurons generated prior to subsequent readjustments that occur after target tissues are innervated.

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