

# Dorsal Spinal Cord Neuroepithelium Generates Astrocytes but Not Oligodendrocytes

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## Summary

There is evidence that oligodendrocytes in the spinal cord are derived from a restricted part of the ventricular zone near the floor plate. An alternative view is that oligodendrocytes are generated from all parts of the ventricular zone. We reinvestigated glial origins by constructing chick–quail chimeras in which dorsal or ventral segments of the embryonic chick neural tube were replaced with equivalent segments of quail neural tube. Ventral grafts gave rise to both oligodendrocytes and astrocytes. In contrast, dorsal grafts produced astrocytes but not oligodendrocytes. In mixed cultures of ventral and dorsal cells, only ventral cells generated oligodendrocytes, whereas both ventral and dorsal cells generated astrocytes. Therefore, oligodendrocytes are derived specifically from ventral neuroepithelium, and astrocytes from both dorsal and ventral.

## Introduction

During development, different classes of neurons and glia are generated from the neuroepithelial precursors that line the ventricles of the brain and the lumen of the spinal cord, the so-called ventricular zone (VZ). In general, neurons are produced before glial cells; neuron production is mostly complete before birth, whereas gliogenesis starts before birth and continues during early postnatal life in mammals (post-hatch in birds). It is not known what causes neuroepithelial cells to switch from neuronal to glial cell production, nor is it clear whether all neuroepithelial precursors contribute to gliogenesis or only specific subsets. In order to understand the mechanisms of cell fate specification and switching by multipotential precursors, we need to know where in the neuroepithelium various types of glia are generated.

We and others have taken a combined cell culture and immunohistochemical approach to search for the developmental origins of oligodendrocytes, the myelin-forming glia of the CNS. For example, Ono et al. (1997)

used monoclonal antibody O4, which recognizes sulfatide and other antigens on oligodendrocyte precursors (Sommer and Schachner, 1981; Bansal et al., 1989), to show that chick optic nerve oligodendrocytes develop from migratory progenitor cells that originate in a specialized subdomain of the VZ in the ventral diencephalon. There is a persuasive body of evidence that spinal cord oligodendrocytes also are derived from a specialized ventral domain of the neural tube neuroepithelium, summarized as follows. (1) Cells derived from the ventral but not the dorsal half of the embryonic day 7 (E7) chick or E14 rat spinal cord can give rise to oligodendrocytes when cultured in chemically defined medium (Warf et al., 1991; Trousse et al., 1995; Hall et al., 1996; Pringle et al., 1996; this paper). (2) Several gene products that are known to be expressed in oligodendrocyte precursors are localized in the VZ near the floor plate of the embryonic spinal cord. These markers include the alpha subunit of the platelet-derived growth factor receptor (PDGFR $\alpha$ ) (Pringle and Richardson, 1993; Nishiyama et al., 1996; Pringle et al., 1996; Calver et al., 1998), the enzyme 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) (Yu et al., 1994), O4 antigens (Ono et al., 1995), the NG2 proteoglycan (Nishiyama et al., 1996), and myelin proteolipid protein PLP/DM-20 (Timsit et al., 1995). After they first appear in the ventral VZ, these putative precursors proliferate rapidly and migrate throughout the cord, including the dorsal-most regions, before the appearance of differentiated oligodendrocytes (Noll and Miller, 1993; Pringle and Richardson, 1993; this paper). (3) When PDGFR $\alpha$ <sup>+</sup> cells in late embryonic rat spinal cords were purified by immunoselection and cultured in defined, low serum medium, they all gave rise to oligodendrocytes (Hall et al., 1996). Conversely, very few oligodendrocytes developed in cultures of spinal cord cells that had been depleted of PDGFR $\alpha$ <sup>+</sup> cells by antibody-mediated complement lysis (Hall et al., 1996). This implies that all PDGFR $\alpha$ <sup>+</sup> cells in the embryonic cord are oligodendrocyte progenitors and that most or all oligodendrocytes develop from these ventrally derived progenitors. Similar experiments in chicks using antibody O4 to mark and manipulate progenitor cells in culture have led to similar conclusions (Miller et al., 1997).

Cameron-Curry and Le Douarin (1995) used a different approach to map oligodendrocyte origins and came to a different conclusion: oligodendrocytes are derived from all parts of the spinal cord neuroepithelium. They generated chick–quail chimeras (Le Douarin, 1993) in which ventral segments of embryonic chick neural tube were replaced with equivalent tissue from quail neural tube and vice versa. Using an *in situ* hybridization probe specific for quail oligodendrocytes, they found that both ventral and dorsal neural tube gave rise to oligodendrocytes (Cameron-Curry and Le Douarin, 1995).

We have reinvestigated the origins of oligodendrocytes using chick–quail chimeras. We found that oligodendrocytes develop *in vivo* only from ventral neural tube grafts. We also cultured chick ventral or dorsal

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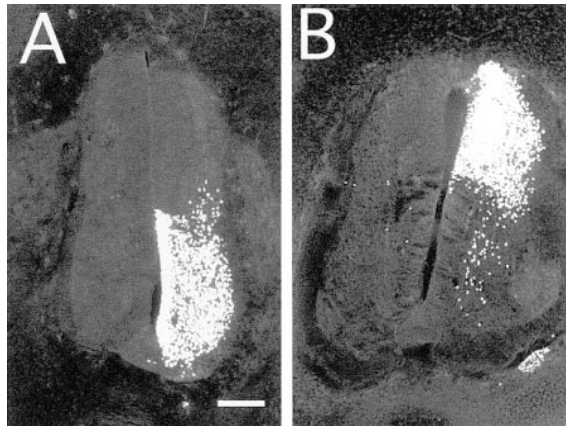


Figure 1. Chick-Quail Chimeras Fixed and Labeled with Antibody QCPN at E5 to Visualize Quail Cells

Quail tissue grafted on E1.5 into the ventral (A) or dorsal (B) chick neural tube incorporated seamlessly into the chimeric cord, and by E5 gross morphology was normal. There was little mixing of graft and host cells at this age, although there appeared to be directed migration of cells from the dorsal graft into ventral territory (B). Scale bar = 200  $\mu$ m.

neural tube cells either alone or in coculture with quail dorsal or ventral cells. Oligodendrocytes always developed from ventral, but not dorsal, neural tube cells. Our data therefore support the bulk of other evidence for a localized ventral source of oligodendrocytes.

Our grafting experiments also provide evidence that dorsal neuroepithelium gives rise to astrocytes in the dorsal white matter but to no cells, glial or otherwise, in ventral white matter. Astrocytes in ventral white matter must therefore be derived from ventral neuroepithelium. In support of this, astrocytes developed in cultures of both dorsal and ventral neural tube cells. Therefore, there are at least two (and possibly many) sites in the spinal cord neuroepithelium that generate astrocytes, but there is only one source of oligodendrocytes.

## Results

### Ventral Neural Tube Grafts

We prepared donor tissue for grafting by microdissecting the ventral half of the neural tube from E1.5 quail embryos (stage 7–8; Zacchei, 1961), cutting along the floor plate, and dividing the left and right neural tube fragments into approximately 0.2 mm lengths. We prepared chick recipients by excising ventral fragments from E1.5 chick embryos (stage 9–10; equivalent to quail stage 7–8) below the ninth somite, inserting the quail donor tissue, and incubating the chimeras for varying periods of time at 38°C. We call these ventral grafts.

Some chimeras were fixed at the equivalent of E5 (chick stage 27), and serial 15  $\mu$ m transverse sections were cut through the upper thoracic spinal cord. Every tenth section was labeled with monoclonal antibody QCPN, which labels quail but not chicken cell nuclei, in order to locate the graft and to assess the contribution it made to the chimeric spinal cord. An example of a ventral graft is shown in Figure 1A. In general, the grafts

integrated well and the chimeric cords were morphologically normal. The boundary between graft and host was usually close to the dorsoventral midpoint at E5. There was always a sharp boundary between graft and host, including the VZ, and little or no contribution to the contralateral side of the cord (Figure 1A). The length of the grafts ranged from 0.9 mm to 1.5 mm in the rostrocaudal direction. Four of the five grafts that we examined occupied half or less of the dorsoventral axis of the cord all along their lengths, while the fifth graft extended into dorsal territory for a short distance at its anterior end.

### Oligodendrocytes Develop from Ventral Grafts

We allowed some chimeras to develop until E15–E18 in order to investigate oligodendrocyte development. We cut serial transverse sections through the spinal cord from the rostral toward the caudal end, labeling every tenth section with monoclonal QCPN to locate the graft. Only chimeras with a normal spinal cord morphology were analyzed further. An example of a chimera labeled on E18 is shown in Figure 2A. At this age, QCPN-labeled quail cells were present in all parts of the operated half of the cord, including the dorsal-most regions. The majority of quail cells could be classified as either cells with small nuclei, which were concentrated in white matter, or cells with larger nuclei, which were mainly restricted to ventral gray matter (Figure 2A). We presume that the former are glial cells and the latter, ventral neurons. A small number of quail cells had crossed into the contralateral side in the vicinity of the commissural tract beneath the central canal and more into the contralateral dorsal funiculus. It is clear that the QCPN-positive cells in the dorsal parts of the cord must have migrated there from the ventral graft through the host tissue.

In addition, all the ependymal cells lining the lumen of the cord on the operated side were QCPN positive. There were many chick (i.e., QCPN negative) neurons in the dorsal gray matter of the chimeric cord that must have developed earlier from chick precursors in the VZ of the host, yet there were no QCPN-negative chick ependymal cells on the operated side of the central canal at E18 (Figure 2A, inset). This implies that the ependymal cells of the E18 cord are derived from a ventral subset of VZ cells present at earlier ages and that the dorsal VZ does not contribute to the ependymal lining of the canal. This conclusion is strongly supported by the results of our dorsal grafting experiments, described in a later section.

We established the time course of myelination in normal chicks and quails by labeling spinal cord sections with anti-myelin basic protein (MBP). In chick, small numbers of MBP<sup>+</sup> oligodendrocytes appear in ventral axon tracts by E15 (stage 41; Hamburger and Hamilton, 1951; data not shown). Subsequently, MBP immunoreactivity increases and spreads through the lateral and dorsal fiber tracts, so that the mature distribution of myelin in the white matter is observed by E18 (stage 44; hatching is on E21). MBP labeling followed a similar course in quails, but it commenced 3 days earlier (quails hatch around E18). To discover whether oligodendrocytes developed from ventral quail grafts, we labeled

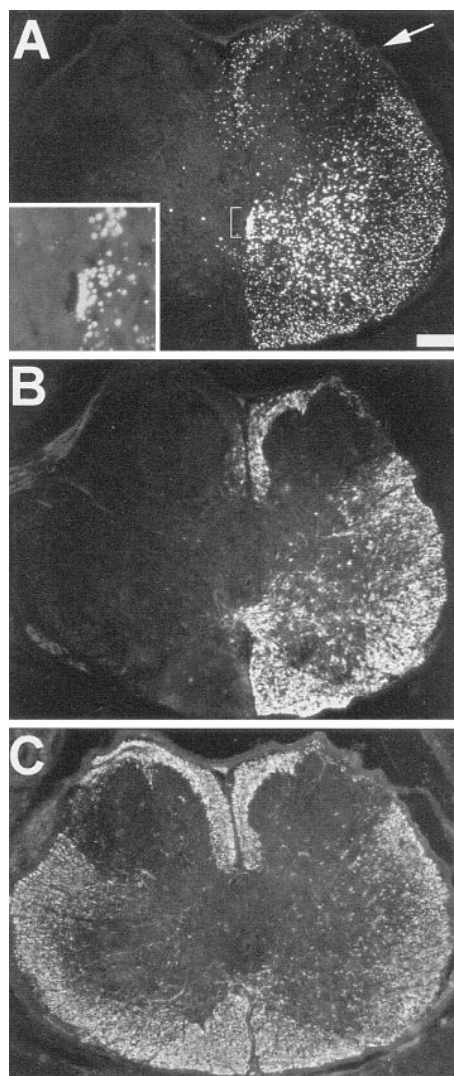


Figure 2. A Quail Ventral Graft Analyzed on E18

Three consecutive sections through the chimeric region of the cord were labeled with antibody QCPN (A) to visualize quail cells, anti-SMP (B) to visualize quail myelin, and anti-MBP (C) to visualize both chick and quail myelin. The ventral graft has given rise to large, brightly QCPN-labeled ventral neurons, ependymal cells around the central canal ([A], inset), and many cells with small nuclei in the ventral, lateral, and dorsal white matter (A). Many of these latter cells colabel with anti-SMP, identifying them as quail oligodendrocytes ([B]; see also Figure 7E). Note that anti-SMP labels only the operated side of the chimeric cord (B). This graft demonstrates that oligodendrocyte progenitors migrate from the ventral neural tube into all parts of the dorsal and lateral cord. Scale bar = 100  $\mu\text{m}$ .

serial transverse sections of E18 chimeric spinal cords with anti-MBP or anti-Schwann cell myelin protein (SMP), which specifically labels quail myelin. In every chimera that we examined ( $n = 5$ ), anti-MBP labeled the white matter on both sides of the cord, while anti-SMP labeled only the operated half (compare Figures 2B and 2C). This confirms the specificity of anti-SMP for quail myelin and demonstrates that quail ventral grafts can generate oligodendrocytes. Double labeling with anti-SMP and QCPN confirmed that the SMP-positive oligodendrocytes were of quail origin (Figure 7E, inset). Therefore,

oligodendrocytes develop from ventral neural tube, in agreement with previous evidence (see Introduction) and consistent with the experiments of Cameron-Curry and Le Douarin (1995). The patterns of SMP and MBP labeling on the operated side were very similar even in the dorsal-most white matter, indicating that ventrally derived quail oligodendrocytes have no preference for specific axon tracts. Their presence in the dorsal funiculus (Figures 2B and 3E–3H) is a clear demonstration that oligodendrocytes or, more likely, their progenitors can migrate from ventral to dorsal.

#### Long-Range Longitudinal Migration of Oligodendrocyte Progenitors

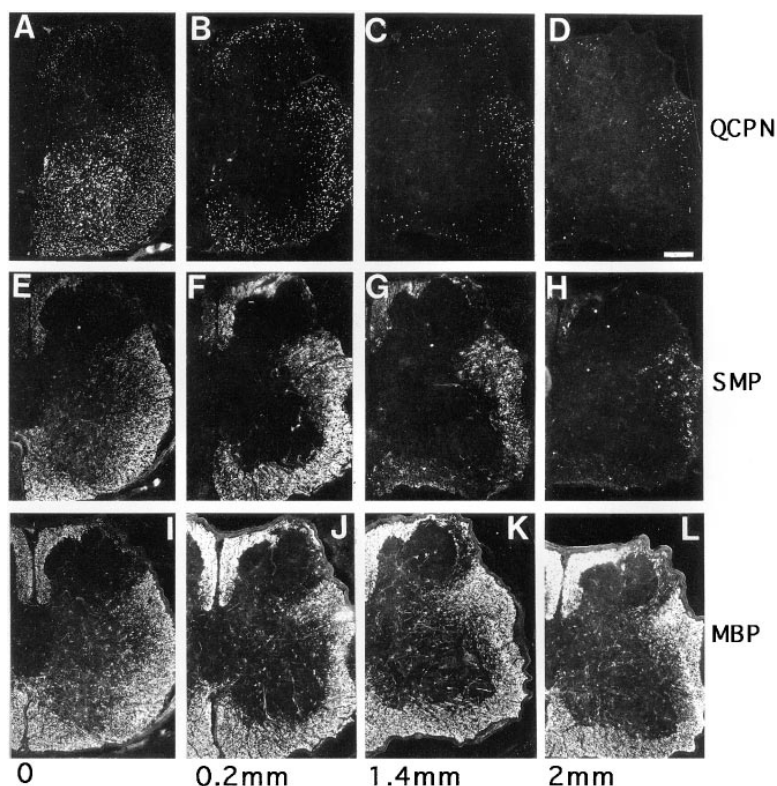
To assess the ability of oligodendrocyte lineage cells to migrate in the longitudinal direction, we examined sections outside of the body of the ventral quail grafts. We defined the ends of the graft as the last sections to contain QCPN-labeled neurons and ependymal cells, which did not seem to spread significantly into host tissue as they were lost abruptly over a distance of about 150  $\mu\text{m}$  at both the rostral and caudal ends of the graft (Figures 3A and 3B). However, quail oligodendrocytes were present in the white matter more than 2 mm beyond the ends of the graft (Figures 3D and 3H). This shows that oligodendrocyte lineage cells (presumably progenitors) can migrate long distances along axon tracts during normal development. The furthest-migrating cells were preferentially located in the dorsal and lateral fiber tracts (Figures 3D and 3H).

#### Dorsal Neural Tube Grafts

We also grafted segments of dorsal E1.5 quail neural tube into equivalent regions of E1.5 chick embryos (dorsal grafts) below the ninth somite. We fixed and examined some of the grafts at E5–E7.5 (chick stages 27–32). We cut serial 15  $\mu\text{m}$  transverse sections through the chimeric spinal cords and labeled every tenth section with QCPN to reveal quail cells. Only grafts that had integrated well into the host, giving rise to a morphologically normal spinal cord, were examined further. As for the ventral grafts, the boundary between chick and quail tissue was clear with little or no intermixing of chick and quail cells within the VZ (Figure 1B). However, there did appear to be some migration of dorsally derived quail cells, presumably neurons, into the ventral gray matter of the host (Figure 1B). In addition, the quail grafts contributed strongly on the operated side (and sometimes also on the contralateral side) to neural crest-derived tissues such as the dorsal root ganglia, peripheral nerves, enteric ganglia, and presumptive melanocytes in the skin (Figures 1B, 4E, and 5A; data not shown). Of the five dorsal grafts examined at E5–E7.5, two remained within dorsal territory all along their lengths, while the remaining three ventured deep into ventral territory over at least part of their lengths. We ascribe these differences to variations in the microsurgical procedure and the wound-healing process.

#### Oligodendrocytes Do Not Develop from Dorsal Grafts

We allowed some chimeras to develop to E15–E18 to allow oligodendrocytes to develop. At these ages the



**Figure 3. Longitudinal Migration of Oligodendrocyte Lineage Cells from a Ventral Quail Graft**

The rostral and caudal ends of a ventral graft are sharply defined at E18 by the loss of graft-derived ependymal cells and neurons as one leaves the graft (compare [A] and [B]). Quail oligodendrocytes are found at least 2 mm away from the ends of the graft in both the rostral and caudal directions (D and H). Anti-MBP labeling is shown for comparison (I–L). Scale bar = 100  $\mu$ m.

approximate position of the dorsal quail graft could be visually determined by the presence of a patch of dark quail feathers, generated from the neural crest, on the back of the otherwise white embryo. We serially sectioned through the region of the graft and labeled every tenth section with antibody QCPN to locate quail cells. The grafts ranged from 1 mm to 7.2 mm in length, depending on the original size of the grafted tissue.

We assessed the dorsoventral extent of the grafts by the distribution of quail neurons, cells with relatively large, brightly labeling nuclei in the gray matter of the cord. For analysis of oligodendrocyte development, we selected only those grafts ( $n = 4$ ) that did not descend deep into ventral territory at any point along their length, as judged by the absence of quail motor neurons—easily recognized by their particularly large cell bodies and their location in the ventral horns. Two different chimeric spinal cords are shown in Figures 4A–4C and Figures 4D–4F, respectively. We labeled neighboring sections with antibody QCPN to visualize quail nuclei, anti-SMP to visualize quail myelin, or anti-MBP to visualize both chick and quail myelin. While anti-MBP labeled both operated and unoperated sides of the cord equally (Figures 4C and 4F), showing that oligodendrocytes were present in all parts of the white matter, there was no signal with anti-SMP anywhere in the spinal cord (Figures 4B and 4E), indicating that quail oligodendrocytes were not generated from the dorsal grafts up to E18. Anti-SMP did label quail-derived myelin in the PNS (e.g., spinal roots; see Figure 4E), providing a positive control for the antibody labeling and showing that dorsal grafts contributed to the migratory neural crest as expected. We also analyzed the grafts by in situ hybridization with

$^{35}$ S-labeled RNA probes against myelin proteolipid protein (*PLP*) transcripts (both chick and quail) and *SMP* transcripts (quail specific) in case *SMP* mRNA was present but not translated into protein in the chimeric spinal cords. However, we were unable to detect any *SMP* transcripts in the chimeric cords (Figure 5C), although *PLP* transcripts (presumably chick derived) were present on both the operated and unoperated sides (Figure 5B). *SMP* transcripts were readily detected in peripheral nerves of the chimera on the operated side (data not shown) as well as in quail spinal cords that were processed in parallel (Figure 5D). These experiments provide compelling evidence that dorsal neuroepithelium does not generate oligodendrocytes. The failure of dorsal grafts to generate oligodendrocytes in our hands contrasts with the experiments reported by Cameron-Curry and Le Douarin (1995).

#### **The Dorsal Ventricular Zone Does Not Contribute Ependymal Cells to the Mature Spinal Cord**

It was noteworthy that despite the large number of dorsal quail neurons generated by our dorsal grafts, there were no quail ependymal cells around the lumen of the spinal cord at E15–E18, although in some parts of some chimeras there were quail-derived cells at the midline just dorsal to the central canal (Figure 4A, inset). This is consistent with our observation that ventral grafts seem to contribute all of the ependymal cells present at E15–E18 (Figure 2A, inset) and reinforces our opinion that the dorsal VZ of the early neural tube does not contribute to the ependymal layer of the late embryonic or adult spinal cord. This highlights a likely reason for the disparity between our conclusions and those of

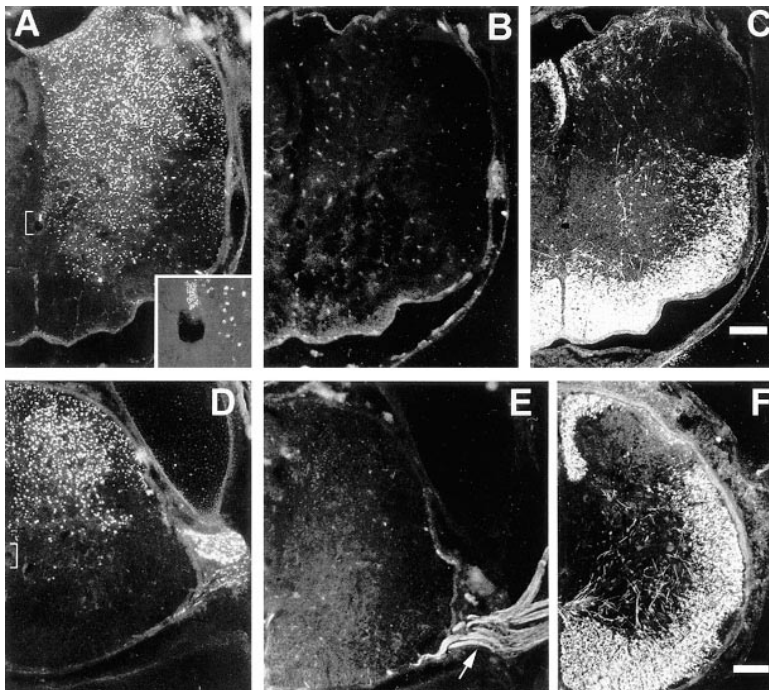


Figure 4. Dorsal Quail Grafts Analyzed on E18

Two different chimeric spinal cords are shown ([A–C] and [D–F], respectively). Consecutive or nearby sections (not more than 100  $\mu\text{m}$  apart) were labeled with antibody QCPN (A and D), anti-SMP (B and E), or anti-MBP (C and F). Both dorsal grafts gave rise to many quail neurons in the dorsal region of the cord (A and D). The position of the spinal cord lumen is indicated by a square bracket in (A) and (D). The graft shown in (A), which descended further ventral than that in (D), gave rise to a small number of neuroepithelial precursors at the midline above the lumen ([A], inset), unlike the graft of (D), which did not generate any midline cells. Neither graft contributed to ependymal cells around the open lumen. Unlike ventral grafts, no SMP-positive (quail) oligodendrocytes were generated by dorsal grafts (B and E). Note that neural crest-derived peripheral quail myelin labeled with anti-SMP, providing a control for antibody labeling ([E], arrow). Anti-MBP labeled chick myelin in all parts of the white matter (C and F). We conclude that oligodendrocytes do not arise from dorsal neuroepithelium. Scale bars = 100  $\mu\text{m}$ .

Cameron-Curry and Le Douarin (1995): the criterion they used to define a “dorsal” quail graft—i.e., the presence of quail ependymal cells around the dorsal part of the lumen at E15—indicates that the dorsal grafts they describe did in fact descend deep into ventral territory at

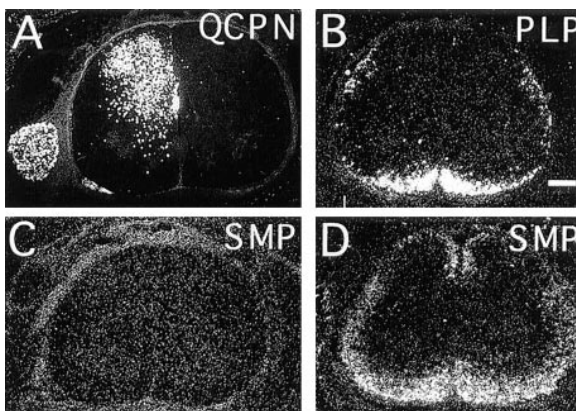


Figure 5. Dorsal Quail Graft Analyzed on E15

The sections shown (A–C) were all taken from within 100  $\mu\text{m}$  of each other in the region containing the graft. They were labeled with antibody QCPN (A) or subjected to in situ hybridization with  $^{35}\text{S}$ -labeled cRNA probes for *PLP* (recognizes both chick and quail oligodendrocytes) (B) or *SMP* (recognizes only quail oligodendrocytes) (C). This particular dorsal graft gave rise to many dorsal neurons and some midline precursors but not yet to many white matter cells ([A]; note this chimera is earlier than those in Figure 4). The grafted dorsal tissue also gave rise to dorsal root ganglion neurons and peripheral glia (A). *PLP* transcripts (presumably chick derived) were present on both operated and unoperated sides of the cord (B), but no quail *SMP* transcripts were visible anywhere in the spinal cord (C). The *SMP* probe did label graft-derived peripheral nerves and dorsal root ganglion in this experiment (data not shown) and also white matter in an E12 quail spinal cord that was processed in parallel on the same glass slide (D). Scale bar = 100  $\mu\text{m}$ .

earlier ages when precursor cells were being specified (see Discussion).

Further compelling evidence that the ependymal lining of the mature spinal cord lumen is derived from only the ventral-most part of the VZ of the early neural tube comes from examining chimeric cords at an intermediate age, E9 (Figure 6). At that age, the opposing faces of the spinal cord lumen are juxtaposed in the dorsal region, and only the ventral-most region near the floor plate remains in its original open configuration (Figure 6). This is the start of the process of “obliteration” of the lumen and its associated VZ (Waldeyer, 1876; Wechsler, 1966; Böhme, 1988; Richardson et al., 1997; see Discussion), which results in the formation of the much-reduced central canal of the mature cord. Although the graft shown in Figure 6A occupies the full dorsal half of the VZ on the operated side, it contributes nothing to the VZ around the open lumen. In order to contribute any cells to the VZ around the open lumen (future central canal), the graft has to occupy the major part of the spinal cord and cannot, we think, be fairly described as dorsal (Figure 6B). This also raises the interesting question of what happens to the neuroepithelial cells of the dorsal VZ during later development (e.g., compare Figure 6A and Figures 4A and 4D). Note that the sections shown in Figures 6A and 6B are from different rostrocaudal levels of the same chimeric spinal cord. Cameron-Curry and Le Douarin (1995) also acknowledge that their dorsal grafts could have a variable ventral border—the different types of grafts that they describe (e.g., A1, A2, and A3; their Figure 1) resulted from this sort of variability within individual chimeras.

#### Dorsal Neuroepithelium Generates Astrocytes in Dorsal White Matter

Despite the fact that our dorsal quail grafts did not generate oligodendrocytes, they did give rise to numerous

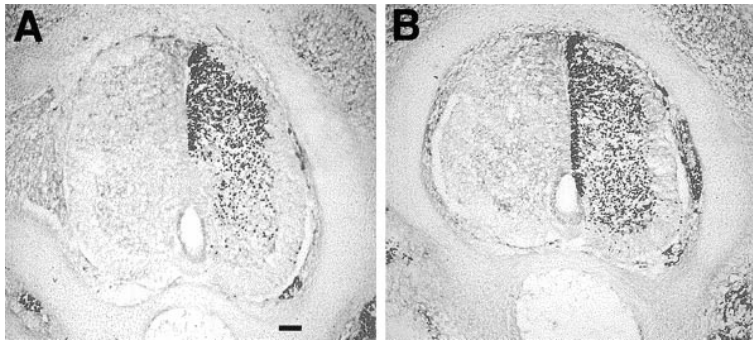


Figure 6. Dorsal Quail Graft Analyzed on E9 (A and B) illustrate different parts of the same dorsal quail graft. The sections are immunolabeled with antibody QCPN to visualize quail cells. This was a wedge-shaped graft that descended deep into ventral territory at one point along its length (B). Note the densely labeling VZ cells at the midline (diaminobenzidine reaction product). In (A) the graft covers around half of the VZ but does not come close to the open lumen; in (B) the graft covers about nine-tenths of the VZ, including the part that surrounds the dorsal half of the open lumen. By reference to the open lumen, therefore, the graft in (B) could perhaps be de-

scribed as “dorsal” (Cameron-Curry and Le Douarin, 1995), although in our opinion only grafts such as that in (A) are truly dorsal. This distinction probably underlies the different conclusions drawn by ourselves (this paper) and Cameron-Curry and Le Douarin (1995) regarding the oligodendrogenic fate of the dorsal VZ. Scale bar = 100  $\mu$ m.

QCPN-positive cells in the dorsal white matter (see Figures 4A, 4D, and 7D). These cells had small nuclei and this together with their location strongly suggested that they were glial cells, presumably astrocytes. We labeled chimeric spinal cord sections with QCPN to mark graft-derived (quail) cells and an antibody against glial fibrillary acidic protein (GFAP) to identify differentiated astrocytes. Many of the QCPN-labeled nuclei were surrounded by GFAP-positive processes, indicative of quail astrocytes (Figure 7D). However, because the anti-GFAP labels cell processes rather than cell soma, it was often not possible to assign confidently GFAP immunoreactivity to a particular nucleus. Because of this and of the fact that the anti-GFAP antibody is not quail specific, we cannot rule out the possibility that some or many of the GFAP-positive cells in the dorsal white matter of the chimeric cords were derived from chick ventral cells and not from the quail dorsal graft. However, because the dorsal grafts did not generate any cells in ventral white matter (Figures 4A and 4D), we can deduce with certainty that ventral white matter astrocytes must be derived from the ventral VZ.

To obtain complementary data on the developmental origins of astrocytes and oligodendrocytes, we turned to cell culture. We dissected spinal cords from E6 (stage 29) chick embryos or E6 (stage 32) quail embryos into dorsal and ventral halves, dissociated the cells, and plated them on glass coverslips in defined medium containing 0.5% fetal calf serum (see Experimental Procedures). After culturing for 7–9 days to the equivalent of E13–E15, we fixed the cultures and labeled with monoclonal anti-galactocerebroside (GC) to identify oligodendrocytes. The great majority of oligodendrocytes were found in cultures of ventral cells. In eight separate experiments with chick cells, we counted a total of 10,436 cells (identified by nuclear staining with bisbenzimidazole) in ventral cultures, of which 1,075 (10.3%) were GC<sup>+</sup> oligodendrocytes; in the corresponding dorsal cultures, we counted a total of 7,228 cells, of which 11 (0.15%) were oligodendrocytes. Similar data were obtained with quail cultures; in eight experiments, a total of 657 out of 7,152 (9.2%) ventral cells but only 26 out of 8,615 (0.3%) dorsal cells were oligodendrocytes. This was as expected from previous culture experiments (Warf et

al., 1991; Trousse et al., 1995; Hall et al., 1996) and is consistent with the results of our grafting experiments, described above. We also labeled parallel cultures at the equivalent of E15 with anti-GFAP and found that astrocytes developed in both ventral and dorsal cultures of both chick and quail cells.

In addition, we mixed equal numbers of cells from quail ventral and chick dorsal spinal cords and cultured them together to investigate the possibility that loss of interactions between dorsal and ventral cells when they are cultured separately can alter their fates and produce misleading results. We incubated the mixed cultures until the equivalent of E13–E15, fixed the cells, and double labeled with QCPN and anti-GC. We found that the great majority (93%  $\pm$  4%,  $n$  = 8) of the oligodendrocytes that developed in these cultures were QCPN positive and therefore derived from quail ventral spinal cord (e.g., Figure 7A), reinforcing our conclusion from separate dorsal and ventral cell cultures. Reciprocal experiments in which chick ventral and quail dorsal cells were cultured together led to similar conclusions; the great majority of oligodendrocytes (96%  $\pm$  4%,  $n$  = 9) was QCPN negative and therefore derived exclusively from chick ventral cells (e.g., Figure 7B). Representative data from three experiments are shown in Table 1.

When parallel mixed cultures of quail ventral and chick dorsal cells were labeled with QCPN and anti-GFAP, we found both QCPN-positive (quail) and QCPN-negative (chick) astrocytes. It was not possible to count the astrocytes in these cultures because many of them were tightly aggregated and could not be distinguished one from another. However, it was our impression that similar numbers of quail and chick astrocytes were present in the cocultures. The same result was obtained from cocultures of chick ventral and quail dorsal cells—similar numbers of QCPN-positive and QCPN-negative astrocytes were generated. A field of quail astrocytes in a coculture of quail dorsal and chick ventral cells is shown in Figure 7C.

Taken together, our *in vivo* and *in vitro* experiments strongly support the view that astrocytes come from both dorsal and ventral neuroepithelium, while oligodendrocytes come only from ventral neuroepithelium.



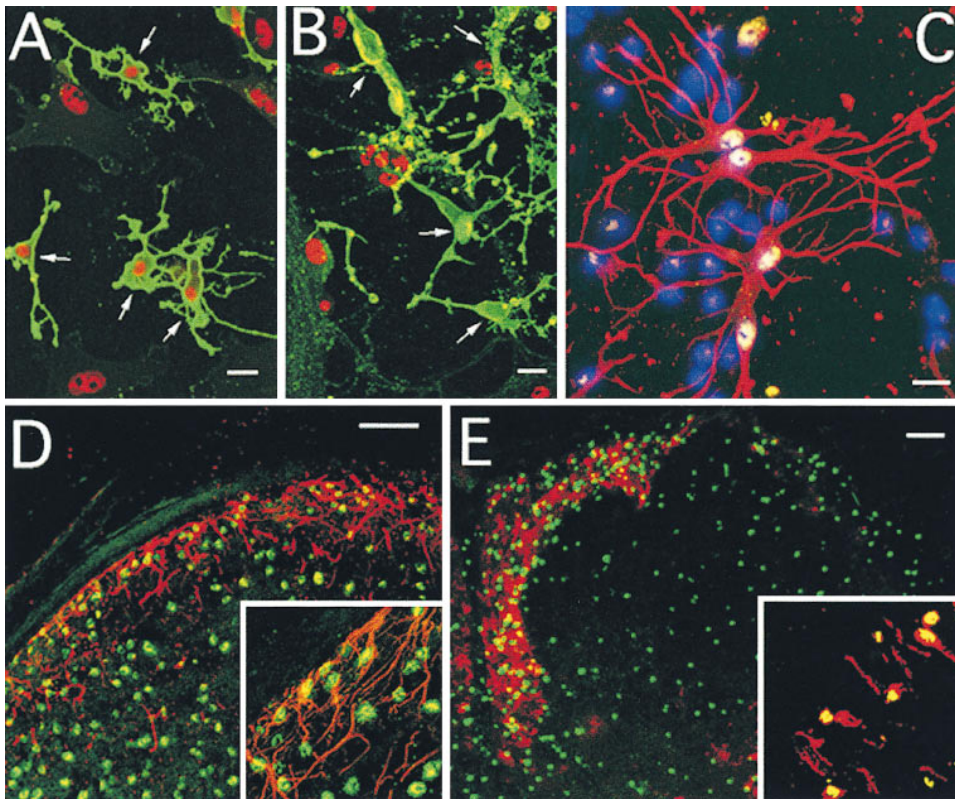


Figure 7. Quail Glial Cells in Culture and in Chimeric Spinal Cords In Vivo

(A) Mixed culture of quail ventral cells and chick dorsal cells, double immunolabeled with antibody QCPN to label quail nuclei (red) and anti-GC to label oligodendrocytes (green). The vast majority of oligodendrocytes in such cultures are of quail (ventral) origin (e.g., arrows).  
(B) A reciprocal culture—mixed chick ventral and quail dorsal cells. Again, the vast majority of oligodendrocytes are ventrally derived (i.e., chick) (arrows).  
(C) Mixed culture of quail dorsal cells and chick ventral cells, triple labeled with antibody QCPN to label quail nuclei (pale yellow), anti-GFAP to identify astrocytes (red), and bisbenzimidazole to mark all cell nuclei (dark blue). The field contains four quail astrocytes overlying a group of unlabeled chick cells. Quail astrocytes were also found in mixed cultures of chick dorsal and quail ventral cells, indicating that astrocyte precursors originate in both dorsal and ventral spinal cord neuroepithelium.  
(D) Part of an E18 chimeric spinal cord in the region of a dorsal quail graft, double labeled with antibody QCPN (green) and anti-GFAP (red). There are many quail nuclei in both the gray matter and the peripheral white matter, together with GFAP-labeled astrocyte processes in the white matter. At higher magnification (inset), many of the quail nuclei in white matter are closely associated with astrocyte processes, suggesting that the quail dorsal graft has given rise to astrocytes.  
(E) The dorsal region of an E18 chimeric spinal cord containing a ventral quail graft, double labeled with QCPN (green; yellow in inset) and anti-SMP (red). Many quail cells have migrated from the ventral graft into the dorsal gray and white matter. At higher magnification (inset), it is apparent that the majority of quail cells in dorsal white matter are SMP-positive oligodendrocytes.  
All images are confocal micrographs except (E). Scale bars = 10  $\mu\text{m}$  (A–C) or 100  $\mu\text{m}$  (D–E).

## Discussion

### Ventral Origin of Oligodendrocytes

The developmental origins of glial cells have been difficult to define for several reasons. First, the tritiated thymidine pulse-labeling approach that has been successful for mapping the birth dates and sites of origin of many neuronal populations is less useful for mapping glial origins. This is because glial precursors, unlike neuronal precursors, continue to divide extensively after they move away from the VZ, eventually diluting the label beyond the limits of detection (Altman, 1966). Second, the precursors of at least some glia, including oligodendrocytes, migrate far and wide from their origins in the VZ, so that their final resting places provide little

clue to their source (Levine and Goldman, 1988; Reynolds and Wilkin, 1988; Leber et al., 1990; Levison et al., 1993; Leber and Sanes, 1995). Because mature glial cells are spread widely through the CNS, there has been a natural tendency to suppose that their precursors must also be distributed widely in the VZ. However, there is now a persuasive body of histochemical and other types of evidence (see Introduction) suggesting that spinal cord oligodendrocyte precursors originate exclusively in a ventral domain of the VZ near the floor plate. The experiments reported in our present paper support this view. Our chick–quail chimera experiments demonstrate that ventral neuroepithelium generates oligodendrocytes in vivo whereas dorsal neuroepithelium does not. Our findings differ from those of Cameron-Curry and Le

Table 1. Cocultures of Chick and Quail Spinal Cord Cells

Experiment	Culture Type	Total Cells Counted	Quail Cells	Chick OLs	Quail OLs	Ventral OLs % of Total
1	CV + QD	720	328 (45%)	223	1	>99%
	QV + CD	1918	930 (48%)	31	243	89%
2	CV + QD	1494	593 (40%)	73	2	97%
	QV + CD	2659	810 (30%)	1	99	99%
3	CV + QD	430	236 (55%)	240	0	100%
	QV + CD	498	275 (55%)	5	80	94%

Representative data from 3 of 9 mixed chick/quail cell culture experiments. We determined the proportion of chick to quail cells in the mixed cultures by counting the total number of cells (bisbenzimidazole-stained nuclei) and the number of quail (QCPN<sup>+</sup>) cells in twenty random fields of view over two coverslips for each experiment (typically 40%–60%). We counted the number of GC<sup>+</sup> oligodendrocytes (OLs) and determined whether they were chick (QCPN<sup>-</sup>) or quail (QCPN<sup>+</sup>) in origin. In all experiments the great majority of oligodendrocytes were derived from ventral cells. C, chick; Q, quail; V, ventral; D dorsal (e.g., CV + QD, chick ventral and quail dorsal).

Douarin (1995), who reported that oligodendrocytes are generated from both dorsal and ventral neuroepithelium. As discussed below, it seems clear that this discrepancy results from our different definitions of what constitutes a dorsal graft.

#### Longitudinal Migration of Oligodendrocyte Progenitors

We found graft-derived oligodendrocytes in longitudinal axon tracts more than 2 mm away from the ends of our ventral grafts. This confirms previous evidence that oligodendrocyte progenitors can migrate relatively long distances during normal development (Small et al., 1987; Leber and Sanes, 1995; Ono et al., 1997; this paper) and suggests that axons are a preferred substrate for their migration. Because of this, migratory progenitors originating at any particular rostrocaudal level of the spinal cord will give rise to oligodendrocytes over an extended region of the cord.

#### Dorsal Precursors Have the Potential to Generate Oligodendrocytes If Placed in a Ventral Context

At the time we and Cameron-Curry and Le Douarin (1995) performed our grafts (E2) and for some time afterward, precursors in the VZ are not yet committed to their final fates and can be respecified by appropriate signals. For example, E2–E4 dorsal neural tube explants do not normally give rise to oligodendrocytes when cultured on their own in defined medium but can be induced to do so by coculturing with fragments of notochord or in the presence of purified Sonic hedgehog protein (Shh) (Trousse et al., 1995; Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996). The fate of dorsal neuroepithelial precursors can also be switched in vivo by transplanting notochord fragments into an ectopic dorsal or dorsolateral position (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996). This is analogous to induction of other ventral cell types, such as motor neurons, by ventral midline cells (reviewed by Tanabe and Jessell, 1996). Therefore, dorsal neuroepithelium retains the potential to generate oligodendrocytes up to a certain age, even if it does not normally realize this potential in vivo because of its position. In grafting experiments such as we describe here, it is not so important where the donor tissue is taken from as where it ends up in the chimeric spinal cord at the time precursor cell specification is going on (sometime between E2 and

E6). Where it ends up is not entirely predictable as it depends on how the grafted tissue incorporates into the host following surgery and wound repair. In preparing a dorsal graft, if the gap in the host should be made too large, then the transplanted dorsal tissue might expand to fill the space available. If it expands into ventral territory, then it will come under the influence of the floor plate and be respecified as bona fide ventral neuroepithelium.

We frequently found that our nominally dorsal grafts came to occupy more than the dorsal half of the spinal cord, and sometimes they occupied almost the entire operated side of the chimeric cord along at least part of their length (e.g., Figure 6B). These latter grafts, like genuine ventral grafts, gave rise to large numbers of oligodendrocytes throughout the white matter on that side of the cord (data not shown), as predicted from the discussion above. Moreover, because of longitudinal cell migration, grafts that approached the floor plate along only a fraction of their length gave rise to oligodendrocytes over an extended region of the cord, including the entire chimeric region, and even beyond the ends of the graft.

#### Obliteration of the Spinal Cord Lumen

The lumen of the spinal cord and the VZ that surrounds it do not remain static during development. During early embryogenesis, when neuroepithelial cells are still proliferating and before their fates are specified, the neural tube grows in both the anterior–posterior and dorsal–ventral directions. At a certain point, between E4 and E6 in the chick, the neuroepithelium stops expanding and neuronal production commences, first in the ventral cord and then progressing dorsally. During the latter stages of neuronogenesis, the lumen of the spinal cord starts to shrink again in a process known as “obliteration” (Waldeyer, 1876; Wechsler, 1966; Böhme, 1988). This involves the opposing faces of the dorsal VZ coming into contact and “zipping together” in the dorsal-to-ventral direction (Wechsler, 1966; Böhme, 1988; Richardson et al., 1997). At the end of this process, which happens between E8 and E12 in the chick (Richardson et al., 1997), the open lumen of the spinal cord is reduced to about one-fifth of its former size and is positioned in the ventral part of the spinal cord. Initially, the dorsal neuroepithelial cells remain closely apposed at the midline of the cord and form a visible line of cells that is



sometimes called the dorsal glial septum (Böhme, 1988). Later, they disappear altogether, possibly by programmed cell death (Isomura et al., 1986). In any case, the central canal and its associated VZ at ages after E12 are only ventral remnants of what was present earlier, before E8.

Cameron-Curry and Le Douarin (1995) defined a dorsal graft as one that gives rise to ependymal cells around the dorsal part of the lumen at E12–E15 (see their Figures 3E, 3F, and 4A–4D); however, in view of the discussion above (also see our Figure 6), it seems clear that their dorsal grafts must have expanded into ventral territory at earlier ages when neuroepithelial cell fates were being decided, and they became ventralized by the floor plate. A truly dorsal neural tube graft should not contribute at all to the ependymal lining of the cord at E15, and we disregarded grafts that did. It seems probable that this is the main reason why our conclusions differ from those of Cameron-Curry and Le Douarin (1995).

Although the dorsal grafts that we examined at E15–E18 did not contribute to the ependyma around the canal, in some places they gave rise to cells at the midline just dorsal to the central canal. This region had the appearance of a residual germinal zone but we do not know whether it was still active at these ages. In some regions, our grafts gave rise to the majority of neurons on the operated side, except the most ventral neurons such as motor neurons, but still did not generate oligodendrocytes. This indicates that the majority of the VZ does not generate oligodendrocytes and is consistent with the idea that oligodendrocyte progenitors are derived from a small part of the ventral neuroepithelium. It seems increasingly likely that the ventral focus of PDGFR $\alpha^+$ , which we described previously (Pringle and Richardson, 1993; Yu et al., 1994; see Introduction), is the sole source of oligodendrocytes in the cord.

#### Dorsal Precursors Generate Astrocytes

Although our dorsal grafts did not generate oligodendrocytes, they did give rise to glial cells in the dorsal white matter. It seems likely that at least some of these must be white matter astrocytes, and indeed some of the graft-derived cell nuclei were closely associated with bundles of GFAP filaments. However, it is often difficult to relate GFAP immunoreactivity to particular cell nuclei because of their different subcellular locations; additionally, our antibody does not distinguish chick and quail GFAP, so this conclusion cannot be regarded as definitive. Nevertheless, we can state with confidence that dorsal grafts do not contribute any cells to ventral white matter, implying that both astrocytes and oligodendrocytes in ventral white matter must originate in the ventral VZ. Ventral quail grafts give rise to many cells in dorsal gray and white matter, many of which are oligodendrocytes or their precursors, but we cannot tell whether astrocytes also migrate dorsally from the ventral grafts. Nevertheless, our combined *in vivo/in vitro* data strongly suggest that dorsal astrocytes are produced from dorsal neuroepithelium and ventral astrocytes are produced from ventral neuroepithelium. Whether this means that astrocytes are generated equally from all parts of the VZ or, alternatively, that there are two or more foci of astrocyte precursors located at discrete

sites within the VZ remains to be seen. Miller and Szigeti (1991) have described several different morphological varieties of astrocytes in spinal cord cultures, but it is not known whether these represent different lineages, possibly specified in different parts of the VZ, or whether they have distinct functions or locations in the intact spinal cord. We also observed a range of astrocyte morphologies in our cultures ranging from flat epithelioid to stellate process-bearing cells, but we did not detect any obvious tendency for these different types to arise specifically in dorsal or ventral cultures.

In summary, we have presented evidence that supports the view that different glial cell types are generated in different parts of the VZ and that reinforces previous evidence that spinal cord oligodendrocytes are derived from a specialized region of the VZ near the floor plate.

#### Experimental Procedures

##### Chick–Quail Chimeras

Fertilized white Leghorn chicken eggs (Needle Farm, Kent, UK) and Japanese quail eggs (Rosedean, Cambridgeshire, UK) were incubated at 38°C for approximately 36 hr until embryos reached stage 9 (chick) (Hamburger and Hamilton, 1951) or stage 9–10 (quail) (Zacchei, 1961). Donor quail tissue was prepared by transferring the embryo into dispase (Boehringer, 1 mg/ml) in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) and removing the neural tube with notochord attached (to mark the ventral side) using a flame-sharpened tungsten needle. The neural tube was subdivided longitudinally into dorsal and ventral quadrants and then transversely into suitable lengths for transplantation. The notochord was removed prior to transplanting the ventral tissue.

The recipient chick embryos were prepared as follows. The eggs were washed with 70% ethanol and 1 ml of albumin was removed with a hypodermic needle inserted through the shell. A window was cut in the shell above the embryo and India ink (Pelican drawing ink number 17 black, Hanover, FGR) diluted 1:30 in Howard's Ringer solution was injected into yolk beneath the embryo. The vitelline membrane was pierced and peeled back with a tungsten needle and 100  $\mu$ l of Howard's Ringer containing antibiotics added to the embryo surface. Ventral grafts were performed by removing the ventral half of the chick neural plate on one side over a length of approximately 3 somites in the upper thoracic region and replacing this with a ventral fragment of quail neural tube. Dorsal grafts were performed in a similar manner, removing a dorsal fragment of the host neural plate and grafting into this location. Following surgery, the eggs were resealed with adhesive tape (Tesa Band, Bierdorf, FGR) and incubated at 38°C. At various ages (E5–E18) the embryos were removed, killed, and fixed for histochemistry.

##### Histochemistry

Embryos were killed by decapitation and immersion fixed in cold 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hr, then cryoprotected by immersion in cold 20% (w/v) sucrose in PBS for 24 hr. Tissues were immersed in OCT embedding compound (BDH), frozen on solid CO<sub>2</sub>, and stored at –70°C before sectioning. Frozen sections (15  $\mu$ m) were cut on a cryostat and collected on 3-aminopropyltriethoxysilane (APES)-coated glass microscope slides. Sections were dried in air for 2 hr before storing at –70°C.

Monoclonal antibody QCPN specifically labels a quail perinuclear antigen (Sharma et al., 1995) and can be used to distinguish quail from chick cells in chimeras. Monoclonal anti-SMP (Dulac et al., 1988; Dulac et al., 1992) recognizes a protein present in quail but not chick Schwann cells and oligodendrocytes, at least not at the ages studied here (e.g., see our Figure 2B). Both antibodies were obtained from the Developmental Studies Hybridoma Bank (see Acknowledgments) and were used as undiluted hybridoma supernatants for immunolabeling. Anti-MBP rabbit serum was a gift from D. Colman (Mount Sinai School of Medicine, New York); it recognizes MBP from a range of species including both chick and

quail. For immunolabeling, it was diluted 1000-fold in PBS containing 1% (v/v) Triton X-100 and 10% (v/v) normal goat serum. Anti-GFAP was a mouse monoclonal ascites (Sigma), diluted 100-fold as above. Secondary antibodies were rhodamine- or fluorescein-conjugated goat-anti-rabbit or goat-anti-mouse immunoglobulin (all from Pierce). Sometimes diaminobenzidine labeling (ABC kit, Vector Laboratories) was used instead. After labeling, slides were washed in PBS, dehydrated in ethanol, cleared in xylene, and mounted in XAM (BDH).

Our *in situ* hybridization procedure has been described (Pringle and Richardson, 1993; Pringle et al., 1996). <sup>35</sup>S-labeled RNA probes were transcribed *in vitro* from cloned cDNAs. The quail *SMP* cDNA (a gift from Nicole Le Douarin) was a 400 bp partial cDNA in Bluescript (Promega); antisense probes were made by linearizing with HindIII and transcribing with T3 RNA polymerase (T3pol), and sense (control) probes were made with EcoRI and T7pol. The chick *PLP* probe was made from a cloned partial cDNA generated by RT-PCR (N. P. P., unpublished data); the antisense probe was generated with NotI and T7pol, the sense probe with AatII and SP6pol.

### Spinal Cord Cultures

Spinal cords from E6 chicks or quails were dissected into dorsal and ventral halves using flame-sharpened tungsten needles. The tissue was digested in 0.25% (w/v) trypsin in Earle's buffered saline (calcium and magnesium-free; GIBCO) for 15 min at 37°C, then fetal calf serum was added to a final concentration of 10% and the tissue physically dissociated by trituration through a 1 ml Gilson pipet tip. Cells were washed by centrifugation and resuspended in Bottenstein and Sato (1979) defined medium modified by addition of conalbumin instead of transferrin (chick Sato's; Pringle et al., 1996) and plated in a 50  $\mu$ l droplet on poly-D-lysine-coated glass coverslips (5  $\times$  10<sup>4</sup> cells/coverslip). After allowing the cells to attach, medium was added to 200  $\mu$ l. Some cultures contained only dorsal or ventral cells derived from either chick or quail, others a mixture of equal numbers of quail dorsal plus chick ventral cells or vice versa. Incubation was for 8 or 9 days at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. After incubation, cells were fixed in 4% (w/v) paraformaldehyde for 5 min at room temperature and immunolabeled with antibody QCPN together with anti-GC or anti-GFAP. Prior to mounting in Citi-fluor, some coverslips were stained with 0.1  $\mu$ g/ml bisbenzimidazole (Hoechst) in PBS for 5 min at room temperature to label cell nuclei.

Numbers of QCPN-positive and -negative cells in mixed chick/quail cultures were counted (ten random fields of view, at least 400 cells total) to calculate the proportions of chick and quail cells. Only cultures that contained similar proportions of chick and quail cells (no less than 40% of either) were analyzed further. Then QCPN-positive and -negative oligodendrocytes were counted (at least 75 and up to 277 GC<sup>+</sup> oligodendrocytes per experiment). The total number of experiments (n) was 9 for chick ventral plus quail dorsal cultures, and 8 for quail ventral plus chick dorsal cultures.

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