

Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord

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SUMMARY

The precursors for oligodendrocytes, the myelinating cells of the vertebrate CNS, appear to be initially restricted to ventral regions of the embryonic rat spinal cord. These cells subsequently populate dorsal spinal cord regions where they acquire the mature characteristics of oligodendrocytes. To determine the location and timing of proliferation of oligodendrocyte precursors in the ventral spinal cord, and to map their pathways of migration *in vivo*, an assay that identifies mitotic cells was used in conjunction with antibodies that distinguish astrocytes, oligodendrocytes and their precursors.

Between E16.5 and E18.5, two hours after a maternal injection of BrdU, the majority of proliferating cells were located in a discrete cluster at the ventral ventricular zone dorsal to the ventral midline region of the developing spinal cord. By contrast, 12-24 hours following a BrdU injection at E16.5, increasing numbers of labeled cells were seen in the dorsal and more lateral locations of the spinal cord. These observations suggest that BrdU-labeled ventral ventricular cells, or their progeny migrate dorsally and laterally during subsequent spinal cord development. To determine the nature of these proliferating cells, cultures of dorsal and ventral

spinal cord from BrdU-labeled animals were double-labeled with antibodies that identify oligodendrocytes or astrocytes and anti-BrdU. In dorsal spinal cord cultures derived from animals that had received a single injection of BrdU at E16.5, the majority of proliferating cells differentiated into astrocytes while, in ventrally derived cultures from the same animals, the majority of proliferating cells differentiated into oligodendrocytes. In dorsal cultures prepared from animals that received multiple injections of BrdU between E16.5 and E18.5, many more cells were labeled with BrdU and approximately half of these differentiated into oligodendrocytes. These observations suggest that during embryonic development proliferating oligodendrocyte precursors are initially located at the ventral ventricular zone dorsal to the ventral midline region of the spinal cord and during subsequent maturation these cells or their progeny migrated dorsally in the ventricular region of the spinal cord, and laterally to reside in the developing white matter.

Key words: oligodendrocytes, spinal cord, glial proliferation, BrdU, rat nervous system

INTRODUCTION

As in other regions of the mammalian CNS, the two major classes of glial cells in the rat spinal cord are oligodendrocytes that are responsible for myelin formation (Bunge, 1968; Peters et al., 1990) and astrocytes that are responsible for a variety of functions including maintenance of CNS structure (Peters et al., 1990). The origin of these two major classes of glial cells has remained unresolved. While it seems likely that at least some astrocytes of the rat spinal cord are derived directly from the radial glial cells of the spinal cord (Levitt and Rakic, 1980; Liuzzi and Miller, 1987; Choi et al., 1983), the cellular source of oligodendrocytes is less well understood. Two distinct hypotheses have been proposed to account for the appearance of oligodendrocytes in the spinal cord. Spinal cord radial glial cells in the peripheral white matter have been suggested to give rise directly to oligodendrocytes (Choi and Kim, 1985; Choi et

al., 1983; Hirano and Goldman, 1988). Alternatively, it has been suggested that cells initially located around the central canal of the spinal cord become committed to either an astrocyte or oligodendrocyte fate and subsequently migrate to peripheral regions of the developing spinal cord where they undergo further proliferation and differentiation (Fujita, 1965; Gilmore, 1971).

Recent *in vitro* analysis suggested that during early embryonic development, the capacity to give rise to oligodendrocytes is restricted to ventral regions of the rat spinal cord (Warf et al., 1991). For example, when dorsal and ventral regions of the thoracolumbar E14 rat spinal cord were separated at the sulcus limitans and grown in isolated culture, oligodendrocytes developed in ventrally derived but not in dorsally derived cultures. These observations led to the proposal that oligodendrocyte precursors originate in ventral regions of the developing spinal cord and migrate dorsally during subsequent development (Warf et al., 1991).

Consistent with this hypothesis were the findings that in ventral spinal cord explant cultures oligodendrocyte precursors were highly motile, and that 18 hours after DiI labeling of the ventral aspect of cultured spinal cord segments, DiI-labeled oligodendrocytes were found in the developing dorsal columns (Warf et al., 1991). It remained unclear, however, if any specific region of the ventral spinal cord was the site of initial oligodendrocyte precursor development.

Because most spinal cord neuronal precursors complete cell proliferation before the majority of glial cells, assays for mitotic cells can be used to examine the location and subsequent migration of glioblasts at specific developmental stages. BrdU, a thymidine analogue, is useful in such an assay as it is incorporated in the DNA of actively proliferating cells (Gratzner, 1982; Nowakowski et al., 1989) and can be detected immunohistochemically using specific monoclonal antibodies. Since in the rat spinal cord neurogenesis is essentially complete by embryonic day E16 (Nornes and Das, 1974), while the majority of glial proliferation has yet to occur (Altman and Byer, 1974; Gilmore, 1971; Ling, 1976), BrdU labeling of dividing cells in animals older than E16 can be used to selectively identify glial precursors.

A disadvantage in using a BrdU-labeling approach to study the fate of CNS glial precursors is that these cells undergo considerable proliferation prior to differentiation and BrdU can be diluted by further cell division before the expression of differentiative characteristics. To circumvent this difficulty, cell cultures were prepared from embryonic spinal cords of animals injected with BrdU, and grown at low density under conditions that reduce glial precursor cell proliferation and promote cellular differentiation (Raff et al., 1984). Cultures were then double-labeled with antibodies to BrdU to identify the proliferating cells, in combination with antibodies A2B5 (Eisenbarth et al., 1979) to identify spinal cord oligodendrocyte precursors (Warf et al., 1991), O4 (Sommer and Schachner, 1981) to identify immature oligodendrocytes (Gard and Pfeiffer, 1989), anti-galactocerebroside (GC) to identify oligodendrocytes (Raff et al., 1978; Ranscht et al., 1982) or anti-glial fibrillary acidic protein (GFAP) to identify astrocytes (Bignami and Dahl, 1976; Bignami et al., 1972).

These studies demonstrate that, between E16.5 and E18.5, the majority of proliferating oligodendrocyte precursors are located in a distinct cluster of cells at the ventral ventricular zone dorsal to the ventral midline region of the spinal cord. During subsequent development, these cells migrate dorsally and laterally to populate developing spinal cord white matter. A smaller proportion of the actively proliferating spinal cord cells at this age also give rise to spinal cord astrocytes. These observations indicate that both astrocyte and oligodendrocyte precursors are proliferating as early as E16.5 in the rat spinal cord, and suggest that, in the embryonic rat spinal cord, oligodendrocytes develop from a distinct population of glial precursors specifically located at the ventral ventricular zone dorsal to the ventral midline region.

MATERIALS AND METHODS

Labeling of mitotic cells and quantitative analysis

To label proliferating cells in embryonic rat spinal cords, timed

pregnant rats were injected intraperitoneally with a 10 mg/ml aqueous solution of 5-bromodeoxyuridine (BrdU; Sigma). Depending on the specific experiment, animals received either a single injection of 3 ml or multiple doses of 3 ml/injection at least 12 hours apart. All injections of BrdU were delivered to anesthetized mothers, and animals were allowed to recover for the required survival time before being killed by ether inhalation and decapitation. Sibling embryos were decapitated and processed either for frozen sectioning or tissue culture. To quantify the number of proliferating cells in dorsal and ventral regions of the spinal cord at different embryonic ages, the total number of anti-BrdU-labeled cells in developing grey and white matter ventral to the sulcus limitans were counted separately and the results pooled to give the total number of ventral cells. To determine the proportion of proliferating cells in the ventral ventricular region of the spinal cord, the number of anti-BrdU-labeled cells located within 100 μm of the ventral ventricular zone were compared with the number of labeled cells in the remaining lateral regions of the ventral cord. Similarly, the number of dorsally located proliferating cells was determined by counting BrdU-labeled cells in the grey and white matter dorsal to the sulcus limitans. A minimum of fifty complete cross sections taken from twenty different animals from at least four separate litters were used for each experiment. In all cases, the data were pooled and the results expressed as the mean \pm the standard deviation. The age of the embryos in each litter was calculated using the morning following mating as the first day of gestation (E1). Animals were injected at 12(noon) for E16.5 etc and at 12(midnight) for E17.

Tissue preparation and immunocytochemistry

To prepare tissue for frozen sections, spinal columns containing both spinal cord and surrounding developing bone were removed from freshly dissected embryos. Tissue was fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour at room temperature and the spinal columns subsequently transferred to a solution of 30% sucrose in the same buffer and incubated for at least 24 hours at 4°C before sectioning. Spinal columns were then cut into short segments by hand, embedded in tissue-tek OCT compound (Miles Inc), frozen by immersion in liquid nitrogen and 4-6 μm thick transverse sections cut on a Zeiss Microm cryostat at -20°C. All sections were taken directly caudal to the 8th intercostal space, collected on gelatin-coated slides and stored at -20°C until use. In all preparations, care was taken to ensure that spinal cord sections were taken from the same levels in all injected animals.

To visualize those cells that had incorporated BrdU, an indirect immunofluorescence assay with anti-BrdU antibody was used (Gratzner, 1982; Nowakowski et al., 1989). Slides were defrosted, incubated in 2 N HCl for 35-40 minutes at room temperature, rinsed briefly in H₂O and twice for at least 10 minutes each in PBS containing 1% Tween 20. Sections were then incubated with mouse anti-BrdU antibody (Boehringer Mannheim) at a dilution of 1:100 in phosphate-buffered saline (PBS) containing 1% Tween 20 and 50% normal goat serum overnight at room temperature. The following day, sections were rinsed extensively in PBS and the binding of anti-BrdU antibody visualized by incubation in rhodamine-conjugated goat anti-mouse Ig (Cappel, 1:50) for 3 hours at room temperature. After further washing in PBS, sections were mounted in 80% glycerol in PBS containing 5% N-propyl gallate and examined on a Nikon Optiphot microscope equipped with epifluorescence. All photographs were taken at 20 \times magnification using Tri-X film rated at 400 ASA. In control experiments, the primary antibody was deleted from the staining procedure or replaced with normal serum from the same species and no specific staining was seen.

Preparation of low density spinal cord cultures

To determine if the cells incorporating BrdU following injections

from E16.5 to E18.5 represented oligodendrocyte or astrocyte precursors, cell cultures were prepared from BrdU-injected animals and labeled with cell-type-specific antibodies. Thoracic and lumbar regions of spinal cord were dissected from appropriate aged embryos following BrdU injections to the mother. Meninges were removed, and the dorsal and ventral regions of the spinal cord separated at the sulcus limitans. Minced spinal cord tissue was suspended in approximately 1 ml of calcium-/magnesium-free DMEM medium (CMF-DMEM), an equal volume of the same medium containing 0.1% trypsin added and the tissue incubated for 30 minutes at 37°C. 2 ml of CMF-DMEM containing 0.25% EDTA was added for a further 10 minutes at which point approximately 10 ml of DMEM containing 10% FBS was added and cell clumps dispersed by gentle trituration through a fire-polished Pasteur pipette, and gently pelleted. Dissociated cells were resuspended in DMEM+10% FBS and plated on poly-L-lysine-coated 12 mm coverslips at a density of approximately 2×10^4 viable cells/coverslip. After 12 hours, the medium was replaced with N2 (Bottenstein and Sato, 1979) containing 1% FBS and the cultures grown for 1, 2, 6 or 8 days before analysis.

Phenotypic analysis of BrdU-labeled cells

To identify the cell types that developed from proliferating precursors in the rat spinal cord, a double-label immunocytochemical analysis was performed, using antibodies against BrdU in combination with antibodies A2B5 to identify oligodendrocyte precursors (Eisenbarth et al., 1979), O4, to identify immature oligodendrocytes (Sommer and Schachner, 1981; Gard and Pfeiffer, 1989), anti-galactocerebroside to identify oligodendrocytes (Ranscht et al., 1982) and anti-GFAP (Accurate) to identify astrocytes.

Cultures were rinsed in serum-free DMEM and incubated in either A2B5 (Eisenbarth et al., 1979; supernatant 1:2), O4 (Sommer and Schachner 1981 supernatant 1:3) or anti-GC (Ranscht et al., 1982; ascites fluid 1:200) for 30 minutes at room temperature, rinsed and primary antibody labeling visualized by incubation in fluorescein-conjugated goat anti-mouse Ig (Cappel 1:100) for 30 minutes at room temperature. Cultures were then fixed in 4% paraformaldehyde in PBS for 10-12 minutes at room temperature and, following a brief rinse in PBS, treated with 2 N HCl for approximately 35 minutes at room temperature to denature DNA and allow anti-BrdU antibody binding. Following extensive rinsing in PBS+1% Tween 20 to establish neutral pH, cultures were incubated in rat anti-BrdU (Accurate) at a 1:5 dilution for 1 hour at room temperature. Binding of anti-BrdU antibody was visualized by labeling with rhodamine-conjugated goat anti-rat Ig (Cappel 1:50) for 45 minutes at room temperature. Cultures were then rinsed for a final time and mounted in 80% glycerol in PBS containing 5% N-propyl gallate. For visualization of BrdU-labeled astrocytes, a similar protocol was used with the modification that cultures were initially fixed in 5% acetic acid in methanol at -20°C for 10 minutes prior to HCl treatment, and rabbit anti-GFAP (1:100 Accurate), and fluorescein-conjugated goat anti-rabbit Ig (Cappel 1:50) used in the labeling procedure. All antibody incubations were carried out in buffer containing PBS with 1% Tween 20 and 50% normal goat serum (NGS) and rinses were in PBS and 1% Tween 20. In control experiments, primary antibodies were deleted from the staining protocol and no specific labeling was seen.

For quantitative analysis, a minimum of 200 BrdU+ cells were counted on at least 2 different coverslips taken from each of three separate experiments and the data pooled. In cultures derived from animals that had received a single injection of BrdU at E16.5, all the BrdU-labeled cells were counted on at least 2 different coverslips taken from each of three separate experiments. To determine if the culture conditions used in these studies supported the differential proliferation of either spinal cord oligodendrocyte or astrocyte precursors, parallel control cultures from non-BrdU-injected animals were incubated for 5 days with BrdU (10 μM) and

the proportion of O4 and GC immunoreactive oligodendrocytes and GFAP immunoreactive astrocytes that had incorporated BrdU during the culture period assayed on at least two different coverslips from three separate experiments using a similar quantitative assay to that described above. Less than 1% of the O4+ or GC+ cells incorporated BrdU while approximately 20% of the type-1-like GFAP+ astrocytes were BrdU labeled in such cultures.

RESULTS

Since the majority of neurogenesis is complete in the rat spinal cord by embryonic day 16 (E16), and the majority of glial cell proliferation has yet to occur, mitotically active spinal cord cells in animals older than E16 predominantly represent glioblasts (Nornes and Das, 1974; Altman and Bayer 1974). Using the thymidine analogue bromodeoxyuridine (BrdU) which is incorporated into the DNA of actively dividing cells, the location of proliferating glioblasts was examined in rat spinal cords between the ages of E16.5 and P3.

Localization of proliferating glioblasts in the embryonic rat spinal cord

To determine the location of proliferating glial precursors in the embryonic rat spinal cord, sections from animals labeled with BrdU were examined two hours after a single maternal injection at five separate time points during development (E16.5, E17, E17.5, E18.5 and E20.5). In animals that had received a single injection of BrdU at E16.5 and were killed two hours later, a cluster of BrdU-immunoreactive cells was seen at the ventral ventricular zone dorsal to the ventral midline region of the developing spinal cord (Figs 1, 2A,B). Quantitative analysis of the distribution of BrdU-labeled cells in these animals demonstrated that approximately 80% of the proliferating cells were located in the ventral region of the spinal cord (Table 1A). Of these ventrally located proliferating cells, the vast majority (approximately 82%) were located in the developing grey matter or around the ventral ventricular zone (Table 1B). The remaining BrdU-labeled cells in dorsal regions of the spinal cord 2 hours after an E16.5 injection of BrdU were predominantly located in dorso-lateral areas. These dorsally located BrdU-labeled cells may represent either the final stages of neurogenesis in the substantia gelatinosa (Nornes and Das, 1974) or glial precursors, since in vitro analysis suggests that E16 dorsal spinal cord contains both astrocyte precursors and a small proportion of oligodendrocyte precursors (Warf et al., 1991). These observations indicate that, at E16.5, the majority of mitotic cells are located at the ventral ventricular zone dorsal to the ventral midline region of the developing spinal cord.

Spinal cord glioblasts continue to proliferate after E16.5. In animals that received a single maternal injection of BrdU at E17 (Fig. 2C,D) or E17.5 (Fig. 2E,F) and were killed 2 hours later, a qualitatively similar distribution of proliferating cells was seen. At each age, a cluster of BrdU-labeled cells was localized at the ventral ventricular zone of the developing spinal cord and few proliferating cells were seen in other regions of the spinal cord. Quantitative analysis of the total number and location of proliferating cells in the

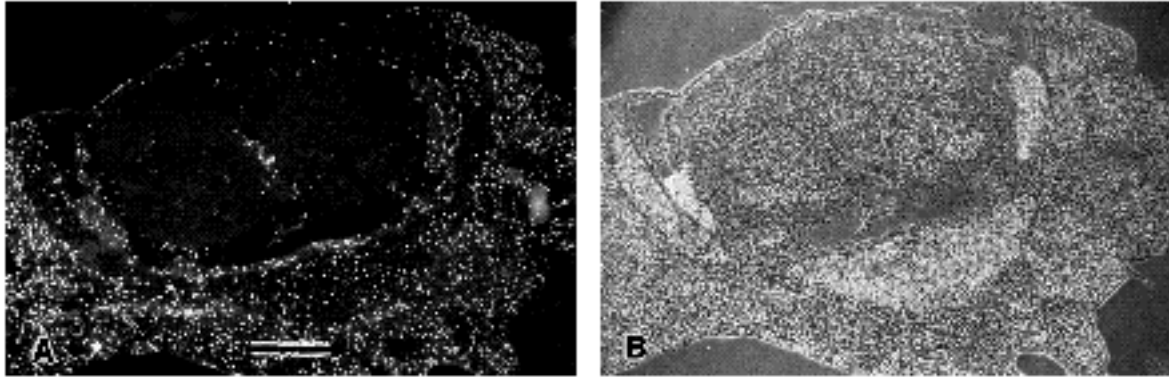


Fig. 1. In the E16.5 embryonic rat spinal cord, BrdU-labeled proliferating glial precursor cells are clustered at the ventral ventricular zone dorsal to the ventral midline region. Transverse frozen section of the whole spinal cord and surrounding tissue from an animal killed 2 hours after a single injection of BrdU labeled with anti-BrdU (A) and corresponding phase-contrast micrograph (B). Although extensive cell proliferation is apparent in the surrounding tissue, within the spinal cord the majority of proliferating cells are located in the region of the ventral ventricular zone dorsal to the ventral midline region. Bar, 250 μ m.

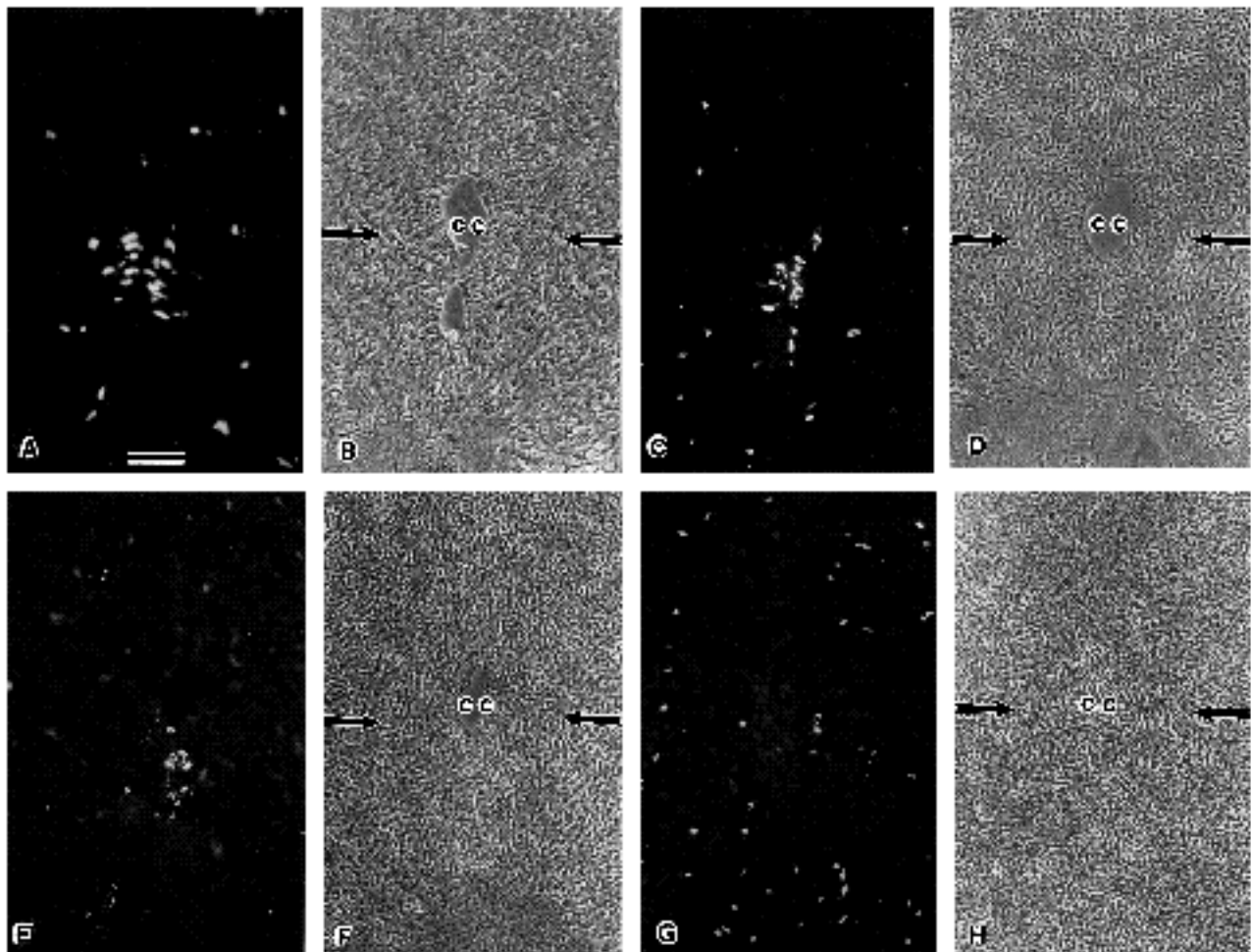


Fig. 2. Distribution of BrdU-labeled proliferating glial precursors in the embryonic rat spinal cord. Transverse frozen sections of the central portion of E16.5 (A,B), E17 (C,D), E17.5 (E,F) and E20.5 (G,H) rat spinal cords labeled with anti-BrdU antibodies (A,C,E,G) and corresponding phase-contrast micrographs (B,D,F,H). At each age animals were killed 2 hours after a single maternal injection of BrdU and sections taken from the same level of the spinal cord. Between E16.5 and E17.5 (A-F), approximately 80% of the proliferating cells are located ventrally and the majority of these ventrally located cell are concentrated at the ventricular zone dorsal to the ventral midline region of the spinal cord. At E20.5 (G,H) the proliferating cells are more uniformly distributed throughout the spinal cord and few proliferating cells are seen at the ventral ventricular zone. All sections were taken directly caudal to the 8th intercostal space in all animals. cc, central canal. The arrows indicate the interface between dorsal and ventral spinal cord at the level of the sulcus limitans. Bar, 50 μ m.

Table 1. Localization of proliferating cells during embryonic development of the rat spinal cord

A			
Age	Ventral BrdU+	Dorsal BrdU+	Total BrdU+
E16.5	49±12 (80%)	12±5 (20%)	61±15
E17	38±6 (84%)	7±4 (16%)	45±7
E17.5	30±8 (85%)	5±3 (15%)	35±8
E18.5	51±9 (73%)	19±7 (27%)	70±13
E20.5	55±10 (60%)	37±8 (40%)	92±15
B			Total
Age	Ventral/Vent	Ventral/Lat	Ventral BrdU+
E16.5	40±11 (82%)	9±3 (18%)	49±12
E17	27±4 (71%)	11±4 (29%)	38±6
E17.5	22±6 (73%)	8±4 (27%)	30±8
E18.5	34±7 (66%)	17±4 (33%)	51±9
E20.5	29±5 (53%)	26±6 (47%)	55±1

(A) The majority of proliferating glial precursors in the embryonic rat spinal cord are ventrally located between E16.5 and E18.5. By E20.5, the proliferating cells are more uniformly distributed between the ventral and dorsal regions of the cord.

(B) The majority of the ventrally located proliferating glial precursors are located in the ventricular zone of the spinal cord between E16.5 and E17.5. In older animals (E18.5 and E20.5), the proliferating cells are more uniformly distributed in the ventral spinal cord.

In all cases, animals were allowed to survive for 2 hours following a single maternal injection of BrdU at the age shown. The data represent the mean ± the standard deviation from a minimum of 50 complete spinal cord sections derived from analysis of at least five different animals from four separate litters. The numbers in brackets represent the calculated percentage of BrdU-labeled cells in each region of the spinal cord at each age assayed. In A, the distinction between dorsal and ventral spinal cord was made at the level of the sulcus limitans. In B, the distinction between the ventral ventricular region and the ventral lateral region was approximately 100 µm either side of the ventral ventricular space.

spinal cords of these animals indicated that the total number of proliferating cells per cross section decreased by approximately 42% between E16.5 and E17.5 (Table 1A). At both E17 and E17.5 approximately 85% of the BrdU-labeled cell were ventrally located (Table 1A), and the majority of the ventrally located cells were clustered in developing grey matter at the ventral ventricular zone dorsal to the ventral midline region (Table 1B).

In older embryos, the distinctive ventral ventricular zone pattern of glial cell proliferation was less apparent. In animals that received a single maternal injection of BrdU at E18.5 and were killed 2 hours later, there was a slight increase in the total number of proliferating cells (Table 1A). Although a cluster of BrdU-labeled cells was still apparent at the ventral ventricular zone, this cluster contained a smaller proportion of the BrdU+ cells than at younger ages. For example, approximately 33% of the ventral BrdU+ cells were located laterally at E18.5, compared with 18% at E16.5 (Table 1B). In animals that received a single maternal injection of BrdU at E20.5 and were killed 2 hours later, the BrdU-labeled cells were more uniformly distributed throughout the spinal cord with approximately 40% of BrdU-labeled cells being located dorsally (Table 1A) and approximately 47% of the ventral BrdU-labeled cells located laterally (Table 1B). There was no apparent cluster of cells at the ventral ventricular zone (Fig. 2G,H), and there was an increase in the total number of proliferating cells in the spinal cord (Table 1A).

Consistent with previous studies, (Gilmore, 1971; Sturrock, 1982; Ling, 1976), following a single BrdU injection into postnatal animals at P0 and P3, most BrdU-labeled cells were found in the peripheral developing white matter regions of the spinal cord. Little BrdU incorporation was seen in cells located at the ventral ventricular zone in these animals (data not shown), suggesting that proliferation of glial precursors at the ventral ventricular zone ceases, but continues in developing white matter during postnatal development.

Taken together these results suggest that between E16.5 and E17.5 the majority of proliferating glial precursor cells are located in the ventral ventricular zone dorsal to the ventral midline region of the spinal cord. At later embryonic ages (E20.5) the distribution of proliferating glial precursors is more uniform between dorsal and ventral spinal cord, and more BrdU+ cells are located in developing white matter.

Migration of glioblasts in the embryonic rat spinal cord

To follow the migration pathways of the glial precursor cells that were proliferating at E16.5 the distribution of BrdU-labeled cells was examined 2, 12 and 24 hours after a single maternal injection of BrdU at E16.5. Since the half-life of circulating BrdU is about 60-80 minutes in embryonic tissue (Packard et al., 1973), a single injection of BrdU pulse-labels a discrete population of cells dividing at E16.5. The subsequent location of the BrdU-labeled cells following increasing postinjection survival times can then be used to determine the pathway of migration of the labeled cells or their progeny.

In animals killed 2 hours after a single maternal injection of BrdU at E16.5, 80% of the BrdU-labeled cells were located in the ventral region of the spinal cord (Tables 1A, 2) and approximately 82% of these ventrally located cells were at the ventral ventricular zone or in developing grey matter (Table 1B) (Fig. 3A,B). In animals killed 12 hours after a single maternal injection of BrdU at E16.5, the total number of BrdU-labeled cells per cross section had increased slightly compared to that seen 2 hours after injection (Table 2) suggesting that some of the labeled cells

Table 2. Ventral and dorsal distribution of BrdU labeled cells at different survival times after a single E16.5 maternal BrdU injection

Age+survival time	Ventral BrdU+	Dorsal BrdU+	Total BrdU+
E16.5 +2 hours	49±12 (80%)	12±5 (20%)	61±15
E16.5 +12 hours	48±14 (66%)	24±9 (33%)	73±23
E16.5 +24 hours	54±14 (56%)	42±13 (44%)	96±22

The total number and proportion of dorsally located BrdU-labeled cells increases with survival time after a single maternal injection of BrdU at E16.5. Since the half life of available BrdU in the embryo is 60-90 minutes, a single injection represents a pulse of BrdU, and the fate of the labeled cells can be traced by increasing postinjection survival times. 2 hours after injection, only 20% of the labeled cells are dorsally located. By contrast, 24 hours after an injection, 44% of the BrdU-labeled cells are dorsally located. Note that the total number of BrdU-labeled cell/cross section has also increased as a result of the continued proliferation of the originally labeled cells. At each age, the counts represent the mean ± the standard deviation from a minimum of 50 complete cross sections taken from five different animals/litter derived from four different litters.

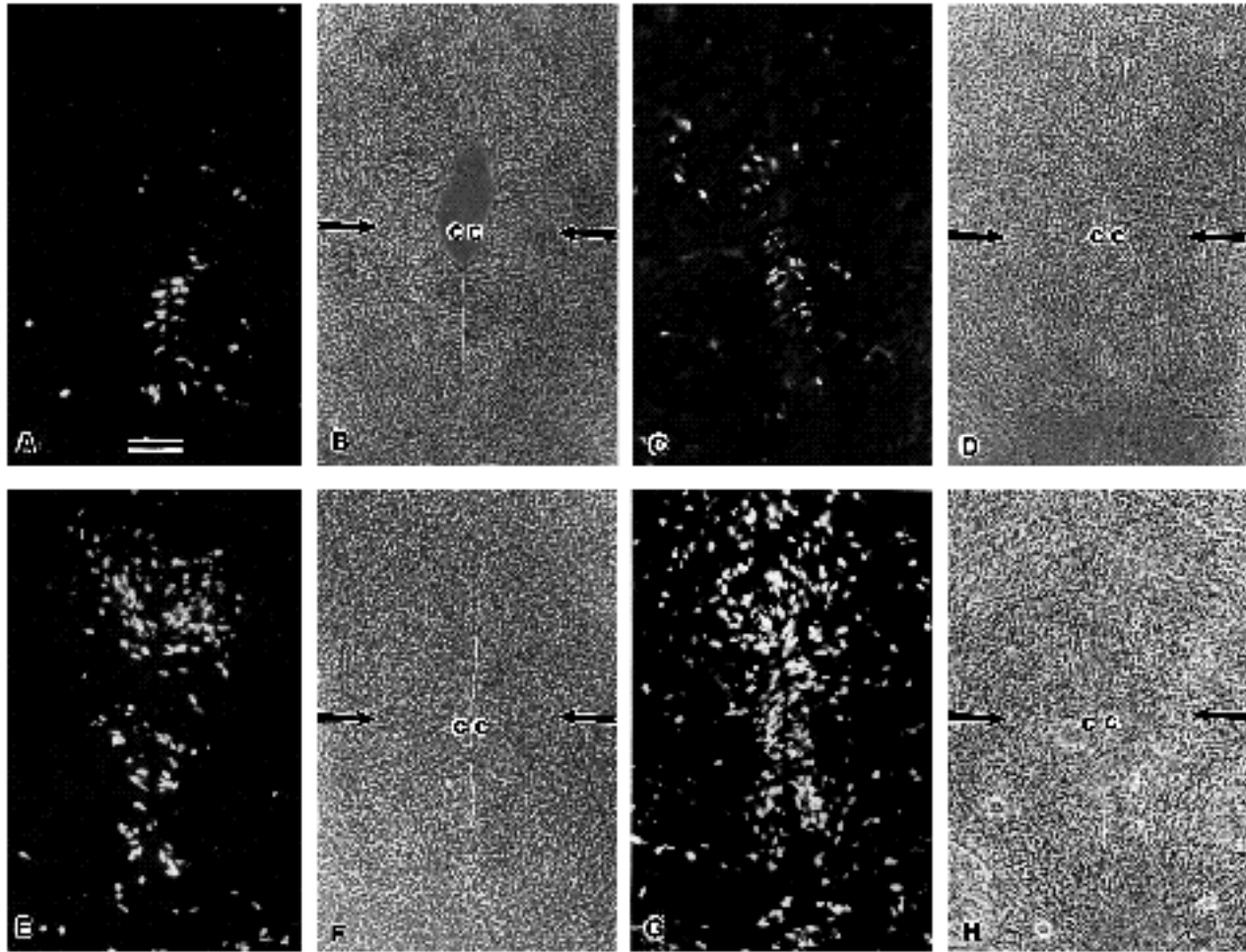


Fig. 3. Distribution of BrdU-labeled glial precursors in the rat spinal cord following increasing survival times after a single BrdU injection at E16.5 (A-F) or after multiple BrdU injections between E16.5 and E18.5 (G,H). (A,C,E,G) Anti-BrdU labeling; (B,D,F,H) corresponding phase-contrast micrographs. (A,B) Distribution of labeled cells 2 hours after a single injection of BrdU at E16.5. The majority of the proliferating cells are at the ventral ventricular zone dorsal to the ventral midline region. (C,D) Distribution of BrdU-labeled cells 12 hours after a single injection of BrdU at E16.5. Note, that the total number of BrdU-labeled cells has increased slightly, and approximately 33% of the labeled cells are located dorsally mainly in the ventricular region of the spinal cord. (E,F) Distribution of BrdU-labeled cells 24 hours after a single injection of BrdU at E16.5. Many more labeled cells were present in the dorsal region of the spinal cord, mainly clustered around the ventricular zone. The total number of labeled cells had also increased by approximately 50% over that seen at 2 hours as a result of the continued cellular proliferation. (G,H) Distribution of BrdU-labeled cells following four equally spaced maternal injections of BrdU between E16.5 and E18.5 and killed 3 hours after the final injection. Many BrdU-labeled cells are present in the ventral and dorsal regions of the spinal cord. While in the ventral region, labeled cells are closely associated with the ventricular zone, in dorsal regions they are more scattered, but have a similar general distribution to that seen in E. cc, central canal. Arrows indicate the interface between dorsal and ventral spinal cord at the level of sulcus limitans. Bar, 50 μ m.

had undergone cell division in the 12 hour survival period. Furthermore, the relative number of BrdU-labeled cells in the dorsal region of the spinal cord had also increased over that seen with a 2 hour survival time. For example, at 12 hours postinjection, 66% of the BrdU-labeled cells were ventrally located and 33% of the BrdU-labeled cells were dorsally located (Table 2). The majority (75%) of the dorsally located BrdU-labeled cells were seen in the region of the dorsal ventricular zone or in developing dorsal grey matter (Fig. 3C,D). In animals killed 24 hours after a single maternal injection of BrdU at E16.5, greater than 40% of the BrdU-labeled cells were dorsally located (Table 2; Fig. 3E,F). There was also an increase in the total number of

BrdU-labeled cells per cross section of approximately 50% (Table 2) suggesting that many of the cells labeled initially had divided during the 24 hour survival period. Quantitative analysis of the BrdU-labeled cells indicated that, while the number of cells in the ventral region of the spinal cord only increased by 12% over that seen in animals killed 2 hours after a single injection of BrdU at E16.5, the number of labeled cells in the dorsal region increased 3.5-fold over the same survival time. Thus, the greatest increase in BrdU-labeled cells seen in the longer term survival animals occurs in the dorsal region of the spinal cord.

Since the half-life of circulating BrdU is in the order of 90 minutes in embryonic tissues, and there is little or no pro-

liferation of intrinsic dorsally located cells during the 24 hour survival period between E16.5 and E17.5 (see Fig. 2 and Table 1A), the dorsally located cells seen 12 and 24 hour after a single injection of BrdU at E16.5 must have been derived from proliferating cells initially located in the ventral region of the spinal cord that subsequently migrated dorsally.

These observations suggest that between E16.5 and E17.5, the progeny of ventral ventricular zone proliferating glial precursors subsequently migrate dorsally. During the initial phase of the dorsal migration, cell proliferation is significantly reduced as shown by the absence of dorsally located BrdU-labeled cells in short-term survival animals (Table 1A). Between E18.5 and E20.5, however, cells in the developing white matter begin to proliferate locally.

To obtain a more complete picture of the distribution of proliferating cells during the period E16.5 to E18.5, the distribution of BrdU-labeled cells was examined following multiple injections of BrdU spaced 12 hours apart over the period E16.5 to E18.5 and killed 3 hours after the final injection. The distribution of BrdU-labeled cells in these animals was consistent with that seen 24 hours after a single injection at E16.5. However, many more spinal cord cells were labeled than in any single injection paradigm (Fig. 3G,H). The BrdU-labeled cells were distributed in the ventricular regions of both the ventral and dorsal spinal cord, as well as a few labeled cells in more lateral locations. While the majority of labeled cells in ventral regions of the spinal cord were concentrated at the ventral ventricular zone, in dorsal regions labeled cells were more diffusely distributed throughout the developing dorsal columns and dorso-lateral regions.

Characterization of the proliferating glial precursors in the embryonic rat spinal cord

To determine if the proliferating glial precursors represented oligodendrocyte or astrocyte precursors, cultures of dorsal or ventral spinal cord cells were prepared from animals two hours after either a single injection of BrdU at E16.5 (Fig. 2A,B) or multiple BrdU injections between E16.5 and E18.5 (Fig. 3G,H). The cells were then grown under conditions that reduce oligodendrocyte precursor proliferation and promote cellular differentiation in order to minimize dilution of the BrdU label. After 1, 2, 6 or 8 days in culture, cells were double labeled with anti-BrdU and either A2B5, O4, anti-GC or anti-GFAP antibodies and the proportion of BrdU-labeled cells of each particular phenotype determined. In spinal cord cultures, the expression of A2B5 immunoreactivity is not restricted to oligodendrocyte precursors but is also a characteristic of a distinct subpopulation of astrocyte precursors (Fok-Seang and Miller, 1992). Since, however, all spinal cord oligodendrocyte precursors transiently express A2B5 immunoreactivity (Fok-Seang and Miller, in preparation), labeling with this antibody provides a useful assay for the maximum number of immature oligodendrocyte precursors present in a culture of embryonic spinal cord.

Following an E16.5 maternal BrdU injection, only 1-2% of all cells in dorsal spinal cord cultures were BrdU labeled, consistent with the observed lack of proliferation in dorsal spinal cord at the time of injection (Figs 1, 2A,B). After 1 or 2 days in culture, approximately 40% of the BrdU+ cells

in dorsal cultures were A2B5+ precursors and 35-40% were GFAP+ astrocytes. After 8 days in culture approximately 75% of the BrdU+ cells had differentiated into astrocytes and 20% into O4+ or GC+ oligodendrocytes (Table 3), suggesting that the majority of dorsally located proliferating cells at E16.5 were astrocyte precursors. By contrast, in parallel cultures derived from ventral spinal cord regions, a larger proportion (5-10%) of the cells were BrdU labeled and consistent with the *in vivo* analysis, these cells constituted approximately 75% of all BrdU-labeled cells in the spinal cord. Of these ventral BrdU-labeled cells, greater than 70% were A2B5+ after 1-2 days in culture and after 8 days 60% had differentiated into O4+ or GC+ oligodendrocytes (Table 3), while only 34% had matured into GFAP astrocytes. Since the proportion of BrdU+ cells in both dorsally and ventrally derived cultures did not alter dramatically over the culture period, and parallel control cultures from unlabeled animals pulsed with BrdU demonstrated only limited proliferation of astrocytes (data not shown), these

Table 3. Antigenic characterization of the BrdU-labeled cells

Age	%BrdU Total	% BrdU +			
		A2B5	O4	GC	GFAP
(A) Dorsal spinal cord cultures					
E16.5 + 1	1.5 ± 0.8	49 ± 8	0	0	41 ± 7
E16.5 + 2	1.5 ± 1	39 ± 6	0	0	36 ± 3
E16.5 + 8	2/5 ± 1	13 ± 3	19 ± 8	7 ± 9	77 ± 16
E16.5-18.5 + 1	50 ± 10	51 ± 7	5 ± 2	0.3 ± 0.1	25 ± 2
E16.5-18.5 + 2	40 ± 8	50 ± 4	15 ± 5	0	21 ± 3
E16.5-18.5 + 6	37 ± 7	40 ± 5	49 ± 4	26 ± 7	42 ± 4
(B) Ventral spinal cord cultures					
E16.5 + 1	5 ± 0.1	69 ± 1	0	0	8 ± 2
E16.5 + 2	9 ± 1	74 ± 9	0.6 ± 0.1	0	19 ± 2
E16.5 + 8	6 ± 1	25 ± 6	55 ± 6	61 ± 7	34 ± 7
E16.5-18.5 + 1	48 ± 6	71 ± 14	10 ± 2	2 ± 2	16 ± 4
E16.5-18.5 + 2	45 ± 2	72 ± 3	31 ± 5	4 ± 2	23 ± 2
E16.5-18.5 + 6	38 ± 4	17 ± 4	43 ± 7	42 ± 7	27 ± 5

In dorsal spinal cord cultures established from animals after a single injection of BrdU at E16.5, less than 2% of the cells label with anti-BrdU. Of these cells, around 50% are initially A2B5+ and after 8 days in culture greater than 75% differentiate into astrocytes while less than 20% differentiate into oligodendrocytes. In parallel cultures derived from the ventral spinal cord, BrdU+ cells constituted approximately 5% of the total cells and around 70% were initially A2B5+. After 8 days in culture, 60% of the BrdU+ cells had differentiated into oligodendrocytes while only 34% were astrocytes.

In dorsal cultures derived from animals following multiple BrdU injections between E16.5 and E18.5, many more cells were BrdU labeled and approximately half of them differentiated into oligodendrocytes. Since the increase in dorsally located BrdU+ cells between E16.5 and E18.5 results from the ventral-to-dorsal migration of labeled cells, these results suggest that many of the migrating cells are oligodendrocyte precursors. Cultures were prepared as described in methods and the results represent the mean ± the standard deviation taken from at least two coverslips in three separate experiments. Note, that in some cases the percentages add up to greater than 100% due to overlapping expression of A2B5/O4, O4/GC and A2B5/GFAP. In every case non-overlapping phenotypes (GC/GFAP and O4/GFAP) add up to less than 100%. In all experiments a small proportion of BrdU-labeled cells were not labeled with any phenotypic marker and these cells may represent either neurons or early glial precursors.

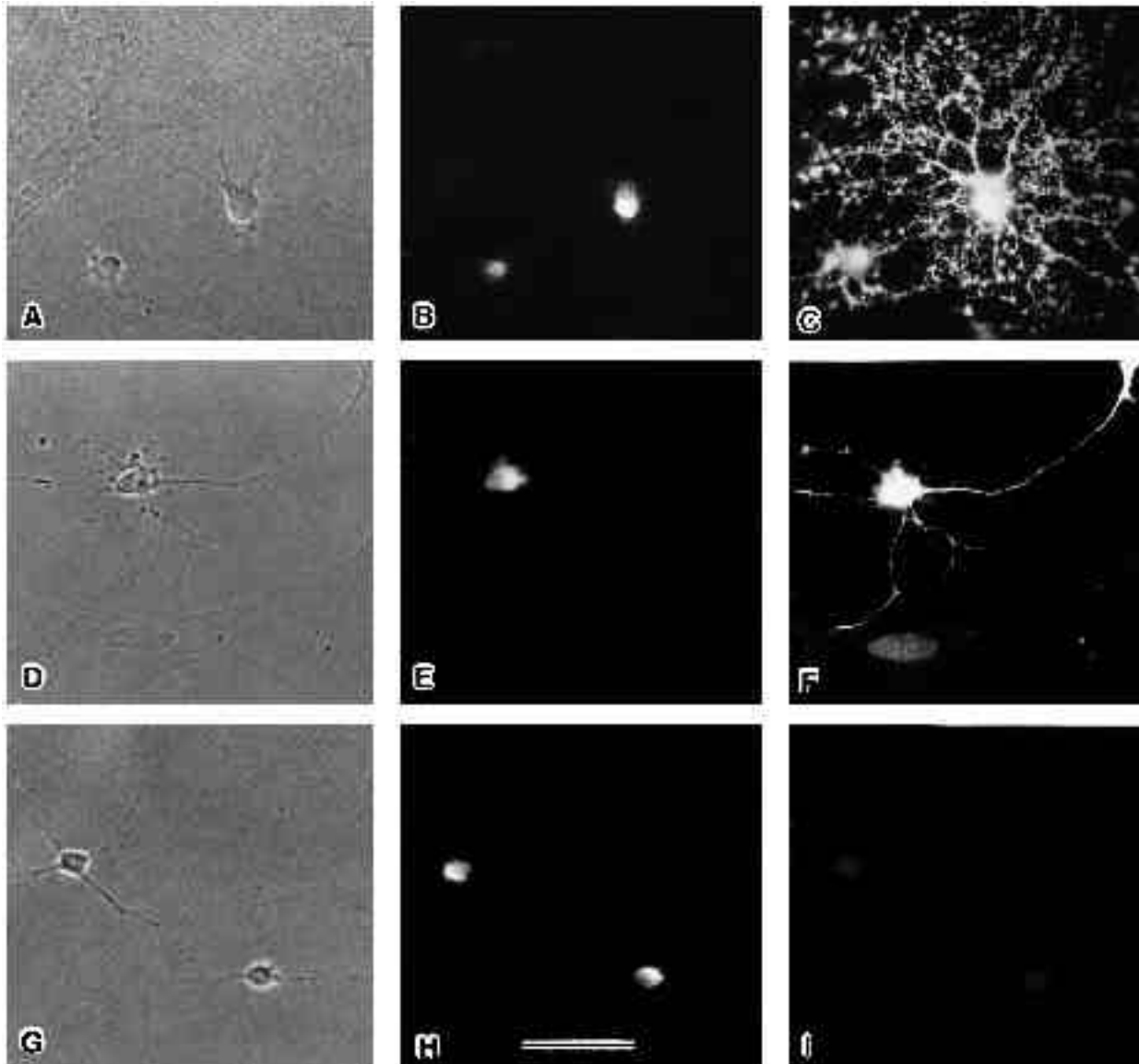


Fig. 4. Low-density spinal cord cultures established from animals after 4 equally spaced maternal injections of BrdU between E16.5 and E18.5. Cells were double labeled with anti-BrdU antibodies (B,E and H) and either anti-GC (C) or anti-GFAP (F and I) after 6 days in culture. Approximately 50% of the BrdU+ cells differentiated into either O4+ or GC+ oligodendrocytes in such cultures (A-C). Approximately 40% of dorsal and 27% of ventral BrdU+ cells differentiated into GFAP+ astrocytes and these were predominantly process bearing (D-F). A small proportion of BrdU-immunoreactive cells were unlabeled by any antibodies. These were small, bipolar or process-bearing cells (G-I) which may be either early glial progenitors or neurons. (A, D and G) Phase-contrast micrographs. Bar, 10 μ m.

results suggest that some of the BrdU+ cells in the ventral region of the spinal cord at E16.5 were oligodendrocyte precursors.

In all cultures, only a small proportion of cells of any phenotype carried the BrdU marker, consistent with labeling proliferating cells over only a short time period. In addition, a small proportion of BrdU-labeled cells were unlabeled by any of the antibodies. The majority of such cells had a small process-bearing morphology (Fig. 4) and could either be immature glial precursors or neuronal precursors.

Our previous studies suggested that oligodendrocyte precursors migrated from ventral to dorsal regions of the rat spinal cord during development. To assess whether the

BrdU+ cells that migrated to dorsal spinal cord 24 hours after a single E16.5 BrdU injection were oligodendrocyte precursors, isolated dorsal and ventral spinal cord cultures were prepared from such animals, and double labeled as described above. Unfortunately, the BrdU label was too weak to allow unambiguous identification of the cells in such cultures, probably due to dilution of the label by cell proliferation in the 24 hour period prior to establishing the culture. As an alternative approach, isolated dorsal and ventral spinal cord cultures were established following multiple BrdU injections between E16.5 and E18.5 and double labeled as described above. These cultures provided a larger number of BrdU-labeled cells for quantitative

analysis (Table 3). Furthermore, since *in vivo* assays demonstrated little proliferation of dorsal cells *in situ* between E16.5 and E18.5 (Fig. 2), the dorsally located BrdU-labeled cells must have been derived from cells initially located in the ventral spinal cord.

In dorsal cultures derived from animals that had received multiple injections of BrdU between E16.5 and E18.5, approximately half of the BrdU+ cells were A2B5+ and 25% were GFAP+ astrocytes after 1-2 days. After 6 days in culture, approximately 50% of the BrdU+ cells were O4 or GC+ oligodendrocytes and 40% were GFAP+ astrocytes (Table 3). When compared with dorsal cultures established from animals that received a single BrdU injection at E16.5, these results indicated a substantial increase in the total number of BrdU-labeled cells and in the proportion of BrdU-labeled cells that subsequently differentiated into oligodendrocytes. Since pulse-label studies indicated that the increase in dorsally located BrdU-labeled cells resulted from the dorsal migration of ventrally derived cells, these data suggest that many of the cells that migrated from ventral to dorsal regions of the spinal cord between E16.5 and E18.5 were oligodendrocyte precursors. In ventral cultures derived from animals that had received multiple injections of BrdU between E16.5 and E18.5, 70% of the BrdU-labeled cells were A2B5+ after 1-2 days and after 6 days 40% had differentiated into O4 or GC+ oligodendrocytes (Fig. 4). Only 27% of the ventral BrdU+ cells matured into GFAP+ astrocyte (Fig. 4) and 23% remained as A2B5+ precursors after 6 days *in vitro* (Table 3).

Taken together, these results suggest that between E16.5 and E18.5 proliferating oligodendrocyte precursors and possibly a subset of astrocyte precursors, are clustered at the ventral ventricular zone dorsal to the ventral midline region in the rat spinal cord. These cells or their labeled progeny then migrate both laterally and dorsally to the developing white matter. Throughout this migration period precursor proliferation is reduced.

DISCUSSION

Using a BrdU incorporation assay to identify mitotically active cells, we have examined the location of proliferating glial precursor cells in the embryonic rat thoracolumbar spinal cord. Between E16.5 and E18.5, the majority of proliferating glial precursors are located in a cluster of cells at the ventral ventricular zone dorsal to the ventral midline region. Pulse-labeling studies indicate that these ventral ventricular cells or their progeny migrate dorsally, around the central canal, and laterally to the developing white matter. During their migration, the proliferation of these glial precursors is considerably decreased. In late embryonic (E20.5), and early postnatal (P0-3) animals, the proliferation of cells at the ventral ventricular zone is decreased and consistent with previous studies (Gilmore, 1971; Ling, 1976), the majority of glial cell proliferation occurs predominantly in developing white matter.

The finding that glial precursors are initially located at the ventral ventricular zone of the spinal cord is consistent with a number of previous studies on spinal cord glial cell development. In both the chick and rat, glioblasts have been

proposed to develop from cells around the central canal that migrate to peripheral regions of the spinal cord (Fujita, 1965; Gilmore, 1971). In addition, proliferation of a ventrally located cluster of cells in the E17 rat spinal cord was suggested to give rise to non-neuronal cells (Altman and Byer, 1974). However, since a large proportion of glial cell proliferation in the spinal cord occurs during postnatal periods (Gilmore, 1971; Ling, 1976), the differentiated product of these early glioblasts has been difficult to determine by autoradiographic approaches due to dilution of the label.

By establishing low density cultures from embryonic rat spinal cords following BrdU injections, in combination with immunohistochemical characterization of the BrdU-labeled cells, we have been able to examine the phenotype and differentiative capacity of this population of ventrally derived migratory glioblasts. At E16.5, the ventrally located proliferating cells in the spinal cord were mainly composed of A2B5+ cells, a characteristic of oligodendrocyte precursors and a subpopulation of astrocyte precursors in the rat spinal cord culture (Warf et al., 1991; Fok-Seang and Miller, 1992). During the subsequent 8 day culture period approximately two thirds of the ventrally derived BrdU+ cells matured into O4+ or GC+ oligodendrocytes while one third matured into GFAP+ astrocytes.

At least two possibilities may explain the differentiation of the ventral ventricular zone cells into both oligodendrocyte and astrocytes. In other regions of the CNS, oligodendrocytes have been shown to develop from bipotential (O-2A) progenitor cells which have the potential to differentiate into both GC+ oligodendrocytes and a specific subpopulation of (type-2) astrocytes *in vitro* (Raff et al., 1983b; Raff, 1989; Miller et al., 1989). If the cells labeled by the BrdU injection paradigm represent the spinal cord equivalent of the O-2A progenitor cell, it would not be surprising that they give rise to both astrocytes and oligodendrocytes. Indeed, many of the BrdU-labeled astrocytes had a process-bearing morphology similar to type-2 astrocytes in cultures of other regions of the CNS (Behar et al., 1988; Levi et al., 1986a,b; Igraham and McCarthy, 1989). By contrast, an alternative explanation is that separate populations of astrocyte and oligodendrocyte precursors arise from this pool of glioblasts, and that the astrocyte precursors subsequently differentiate into a distinct class of spinal cord astrocytes. Recent studies indicate that there are multiple types of astrocytes in neonatal rat spinal cord cultures (Miller and Szigeti, 1991) some of which develop from distinct A2B5+ precursors (Fok-Seang and Miller, 1992).

Although these results clearly demonstrate that the BrdU-labeled glial precursors at the embryonic spinal cord ventral ventricular zone give rise to both oligodendrocytes and astrocytes, in cultures derived from BrdU injected animals, not all oligodendrocytes, astrocytes or their precursors were BrdU labeled. At least two possible explanations could account for these results. First, the proliferation of spinal cord glial precursors may occur over a longer time period than that examined during the BrdU pulse. Thus, only cells actively proliferating at the time of injection incorporate BrdU, while those cells that had proliferated before or after the BrdU pulse would not carry detectable BrdU. A number of observations support this hypothesis. For example, pre-

liminary studies using retroviral lineage analysis have demonstrated that proliferating spinal cord cells as early as E12 and E14 have the capacity to differentiate into oligodendrocytes in vitro (Zhang and Miller unpublished observation). In addition, the finding that in the developing chick spinal cord some glial cells share a common precursor with ventrally located motoneurons (Leber et al., 1990) suggests that some glial precursors may be proliferating prior to E16.5. Furthermore, glial proliferation has been shown to continue in the postnatal spinal cord (Gilmore, 1971; Ling 1976), well after the window of development covered by the BrdU pulse.

Alternatively, the non-BrdU-labeled astrocytes and oligodendrocytes may be directly derived from an different cellular pool of spinal cord glial precursors without further proliferation. Indeed, it has been proposed that oligodendrocytes and astrocytes develop directly from spinal cord radial glial cells (Choi et al., 1983; Choi and Kim, 1985; Hirano and Goldman, 1988). In other regions of the CNS, there is considerable evidence to support the concept of the radial glial origin of some astrocytes (Levitt and Rakic, 1980; Culican et al., 1990). If spinal cord oligodendrocytes also develop directly from radial glia, then the radial glial precursors for oligodendrocytes must also be restricted to the ventral region of the E14 spinal cord (Warf et al., 1991).

The observation that during embryological development, spinal cord oligodendrocyte precursors migrate from the ventral ventricular zone to dorsal and lateral locations provides further evidence in support of the hypothesis for the ventral origin of spinal cord oligodendrocytes (Warf et al., 1991) and demonstrates that spinal cord oligodendrocyte precursors are highly migratory. The migratory behavior of oligodendrocyte precursors is not restricted to the spinal cord. Transplantation of normal CNS tissue into the brain of shiverer mutant mice showed that oligodendrocytes or their precursors had the capacity to migrate long distances in the CNS (La Chapelle et al., 1984), while the migration of oligodendrocyte precursors has also been proposed during development of the cerebellum (Curtis et al., 1988), cerebral cortex (Levine and Goldman, 1988a,b) and optic nerve (Small et al., 1987).

Little is known of the mechanisms by which glial precursors initially located in the spinal cord ventricular zone migrate to the peripheral white matter. One attractive hypothesis is that these cells follow radial glial guides much like proliferating neuroblasts (Rakic, 1971, Hatten, 1990). Such a mechanism may account for the lateral migration of glial precursors through the developing grey matter. Once in developing white matter, oligodendrocyte precursors may follow axonal guides. Such axonal based migration could account for O-2A progenitor cell migration into the developing optic nerve (Small et al., 1987), and since the majority of oligodendrocytes ultimately become associated with axon tracts in the spinal cord, could account for the final stages of migration in the peripheral spinal cord.

It is less clear what directs the ventral-to-dorsal migration of oligodendrocyte precursors. The majority of BrdU-labeled precursors appear to migrate dorsally through the central region of the spinal cord, around the central canal and then fan out in dorsal regions. To our knowledge, there is no evidence of radial glial cells extending from the ventral

to the dorsal spinal cord in this pattern, suggesting that some other cellular mechanism may be mediating this migration. Recently it has been shown that O-2A progenitor cells, but not astrocyte precursors from other regions of the CNS migrate towards a source of PDGF under experimental conditions (Armstrong et al., 1990), and therefore an elevated level of PDGF may attract oligodendrocyte precursors to the dorsal part of the spinal cord. There is as yet no evidence for such a gradient of PDGF in the developing spinal cord; however, in primates, PDGF has been localized to the developing dorsal horns of the spinal cord (Sassahara et al., 1991).

Whatever the specific mechanisms involved in the subsequent migration of oligodendrocyte precursors, we propose that, in the embryonic rat spinal cord, a pool of oligodendrocyte precursors located at the ventral ventricular zone dorsal to the ventral midline region proliferate rapidly between E16.5 and E18.5. The proliferation of these cells or their progeny then significantly decreases as they migrate dorsally in the ventricular zone as well as laterally to the peripheral developing spinal cord white matter. During late prenatal and early postnatal stages glial cell proliferation continues in developing white matter and the cells undergo differentiation into oligodendrocytes and possibly astrocytes.

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