Cell migration in the developing chick diencephalon

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SUMMARY

We previously reported that retrovirally marked clones in the mature chick diencephalon were widely dispersed in the mediolateral, dorsoventral and rostrocaudal planes. The current study was undertaken to define the migration routes that led to the dispersion. Embryos were infected between stages 10 and 14 with a retroviral stock encoding alkaline phosphatase and a library of molecular tags. Embryos were harvested 2.5-5.5 days later and the brains were fixed and serially sectioned. Sibling relationships were determined following PCR amplification and sequencing of the molecular tag. On embryonic day 4, all clones were organized in radial columns spanning the neuroepithelium, which was composed primarily of a ventricular zone at this age. No tangential migration was seen in the ventricular zone. On embryonic day 5, most clones remained radial with many cells located in the ventricular zone; however, a few clones had cells migrating perpendicular to the radial column, in either a rostrocaudal or dorsoventral direction. The tangential migration began just beyond the basal limit of the ventricular zone. On embryonic days 6 and 7, many clones had cells migrating perpendicular to the radial column, which spanned from the ventricular to the pial surface. The migrating cells appeared to be aligned along axes that were perpendicular to the radial column. Using a combination of DiI tracing, immunohistochemistry and electron microscopy, we have determined that axonal tracts are present and are aligned with the migrating cells, suggesting that they support the non-radial cell migration. These data indicate that migration along pathways independent of radial glia occur outside of the ventricular zone in more than 50% of the clones in the chick diencephalon.

Key words: cell migration, lineage, brain, diencephalon, chick

INTRODUCTION

Among the features that make the developing nervous system challenging to study is the fact that neurons are frequently located some distance from their birthplace. Migration, sometimes along circuitous routes, is a prominent feature of the developing nervous system. Nonetheless, the mechanisms underlying cell migration in the vertebrate nervous system remain largely unknown. The most extensively studied pathway for migration is a radial one, in which cells move from their birthplace with the aid of a specialized glial cell, the radial glial cell (Rakic, 1990). Lineage analysis using retroviral vectors has confirmed the occurance of radial migration, but also has led to the conclusion that at least some clonal dispersion cannot be explained by guidance along radial glial cells (Gray et al., 1988; Austin and Cepko, 1990; Walsh and Cepko, 1992; Reid et al., 1995; Arnold-Aldea and Cepko, 1996; Golden and Cepko, 1996; Szele and Cepko, 1996). In keeping with these data, imaging of live cells has revealed non-radial cell migration in the ferret cerebral cortex (O'Rourke et al., 1992) and murine and ferret telencephalic ventricular zone (VZ) (Fishell et al., 1993; O'Rourke et al., 1997). In addition, migration in the telencephalon can occur from the anterior lateral ventricle into a more rostral structure, the olfactory bulb,

using a novel mechanism dependent upon psa-NCAM (for a review see O'Rourke, 1996).

The location and timing of non-radial cell migration appear to vary among the different regions of the CNS. For example, in the rodent cerebral cortex, clones initially form radial columns (Walsh and Cepko, 1988, 1993; Austin and Cepko, 1990). However, once in the intermediate zone (IZ) or cortical plate (CP), a subset of cells in a clone appear to turn and migrate orthogonal to the radial array. This type of migration was frequently in the dorsal to ventral direction (Austin and Cepko, 1990). Clonal analysis in the chick tectum has also revealed that a subset of cells migrate perpendicular to the radial array that comprises the majority of cells in most clones (Gray and Sanes, 1991). These cells also show a dorsal to ventral bias with little or no anterior to posterior dispersion. The non-radial cell migration in the chick tectum was characterized by migration occuring in only two laminae and was limited to specific cell types. In the rat striatum, some cells leaving the VZ and other cells originating in the subventricular zone (SVZ) had a strong bias to migrate in the dorsoventral direction; almost no migration was seen in the rostrocaudal direction (Halliday and Cepko, 1992). A common feature of these reports is that initial migration was radial, while subsequent, non-radial cell migration appeared to occur after cells

left the VZ. It is not clear why there is frequent and extensive non-radial cell migration within the developing CNS. Furthermore, since much of the non-radial cell migration appears directed, it is unlikely that cells randomly disperse once they leave the VZ. Equally obscure are the mechanisms employed for non-radial cell migration.

Classical studies using light and electron microscopy have led to a description of the radial glial fibers that provide one pathway for radial migration. Radial glia are bipolar cells that have their nucleus and perikaryon in the VZ. A short process attaches the cell to the ventricular surface and a long process extends out of the VZ, through the SVZ and IZ, to the pial surface, where an end foot attaches the cell to the outer limit of the nervous system, the outer limiting membrane (Rakic, 1990). Radial glia have been recognized along the entire neuroaxis from the prosencephalon to the spinal cord (Hatten, 1990). In the cerebellum, a specialized glial cell called the Bergmann glial cell functions as the radial guide, supporting the inward migration of granule cells from the external granular cell layer to the internal granular cell layer (Hatten, 1990; Hatten and Mason, 1990).

Lineage analysis conducted in the mature chick diencephalon revealed that clones were dispersed in the mediolateral, dorsoventral and rostrocaudal planes (Golden and Cepko, 1996). In the present study, the timing and location of clonal dispersion was investigated using a library of retroviral vectors (Golden et al., 1995). Labelled, clonally related cells were analyzed on consecutive embryonic days during the period of clonal dispersion. The data indicate that initial migration is strictly radial, with cells forming radial columns similar to those found in other regions of the nervous system. Beginning on E5, clonal dispersion orthogonal to the radial column, in both the rostrocaudal and dorsoventral directions, was observed. The non-radial cell migration was virtually always outside of the VZ. It began at the basal margin of the VZ and then occurred at multiple levels within the maturing mantle zone (i.e. the region of the diencephalon located outside the VZ). Furthermore, the migration appearred random in that cells had oriented processes and appeared to migrate in specific directions. This observation suggested that cells migrated along cellular processes. We found that axonal processes were present at the time and place of the non-radial migration and that their orientation was consistant with a role in supporting the non-radial cell migration.

MATERIALS AND METHODS

In vivo infection

The neural tube of fertilized virus-free White Leghorn chicken embryos was injected with a library of retroviral vectors as previously described (Fekete and Cepko, 1993; Golden and Cepko, 1996). The retroviral vector, CHAPOL, was constructed from a replication-defective avian retrovirus, CHAP (Ryder and Cepko, 1994), which encodes the human placental alkaline phosphatase gene and a degenerate oligonucleotide tag (Golden et al., 1995). Approximately 0.3-0.5 µl of CHAPOL stock was injected into the neural tube at stages 10-14 (Hamburger and Hamilton, 1951). Infected embryos were incubated in a humidified, 37°C chamber for 1.5-5.5 days, at which time the embryos were harvested in PBS. After fixation overnight in 4% paraformaldehyde (in PBS, pH 7.4) at 4°C, brains were washed with three changes of PBS and whole-mount stained for alkaline phos-

phatase (AP) activity (Ryder and Cepko, 1994; Golden et al., 1995). Brains with AP+ cells were either cryoprotected overnight in 30% sucrose and embedded in OCT medium for cyrosections, or dehydrated and infiltrated with paraffin. Brains were oriented to section in either the coronal or horizontal plane. OCT-mounted brains were cut on a Reichart-Jung CM3000 cryostat at 30 or 60 µm and paraffin sections were cut on a Zeiss microtome at 30 µm. Sections were collected serially and mounted sequentially on Superfrost/Plus microscopic slides (Fisher Scientific). Cells infected with the retrovirus were identified by the purple formazan precipitate.

Sections that included the diencephalon and AP+ cells were photographed with a Nikon SMZ-U stereomicroscope equipped with a Nikon DX FX-35WA Camera, or on a Zeiss Axiophot microscope using Kodak Elite 100 film. Kodachromes were scanned into a Macintosh Quadra 650 using a Kodak 2035 plus slide scanner and Adobe Photoshop software. The location and cell type of AP+ cell and/or cluster of cells was given a unique identification based upon the section number. Once documented, a subset of AP+ cell clusters (with a small group of surrounding white AP– cells) were removed using heat-pulled glass micropipettes and the sequence of the degenerate oligonucleotide determined using cycle sequencing following PCR (Golden et al., 1995; Golden and Cepko, 1996). Brains embedded in paraffin were treated in the same fashion, except that coverslips were removed in 100% xylene followed by hydration of the tissue through graded ethanol concentrations with final washing in PBS.

DiI labeling

Radial glia were labeled as previously described (Gray and Sanes, 1992; Golden and Cepko, 1996). Briefly, brains from uninfected E7 chicks were dissected in PBS and fixed overnight in 4% paraformaldehyde. After washing in PBS, approximately 0.1 ml of a 2.5 mg/ml solution of DiI (Molecular Probes, Inc.) in 100% ethanol was injected into the right tectum using a 30-gauge needle. The DiI solution was allowed to passively fill the entire ventricular system. The brains were then placed in a 60 mm Petri dish filled with sterile water and 0.03% sodium azide and incubated for 1 week at 37°C. After incubation the brains were imbedded in 5% agar (in H$_2$O) and sectioned on a vibratome at 200 µm. The sections were mounted on Superfrost/Plus microscopic slides, coverslips secured with gelvatol and viewed on a Biorad confocal microscope. Photos were taken with Kodak Elite 100 film.

Electron microscopy

Embyros were incubated in a humidified chamber until the appropriate day of gestation, at which time the embyros were dissected and fixed in 2.5% glutaraldehyde and 0.8% paraformaldehyde in 0.1 M PBS at 4°C overnight. The diencephalon of each embryo was then dissected out, washed in 0.1 M PBS, and then post-fixed in 1.0% osmium tetroxide and 1.0% potassium ferrocyanide in 0.1 M PBS in the dark for 2 hours. Tissues were washed in distilled water and stained with 2.0% uranyl acetate for 30 minutes followed by dehydration through grades of acetone at room temperature (RT). Infiltration was carried out by sequentially submerging tissue pieces in 1:1 100% acetone:embed-812-araldite for 1 hour, 1:2 for 1 hour, 1:3 for 3 hour, and 100% embed-812-araldite for 2 hours on a rotator at RT. Tissues were then oriented in capsule molds filled with embed-812-araldite and cured at 60°C for 48 hours. Blocks were trimmed and 1 µm sections stained with Toluidine Blue and examined for orientation and preservation. Regions of interest were sectioned at 80-90.5 nm on an LKB V ultramicrotome and the sections transfered to an EM grid. The sections were then viewed and photograhed using a Philips 201 transmission electron microscope.
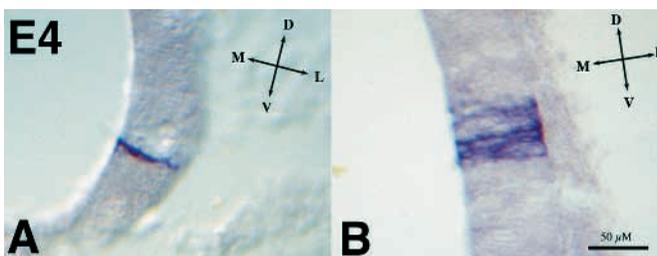
Immunohistochemistry

After fixation with 4% paraformaldehyde, chick brains were washed in 0.1 M PBS and cryoprotected with 30% sucrose. The brains were
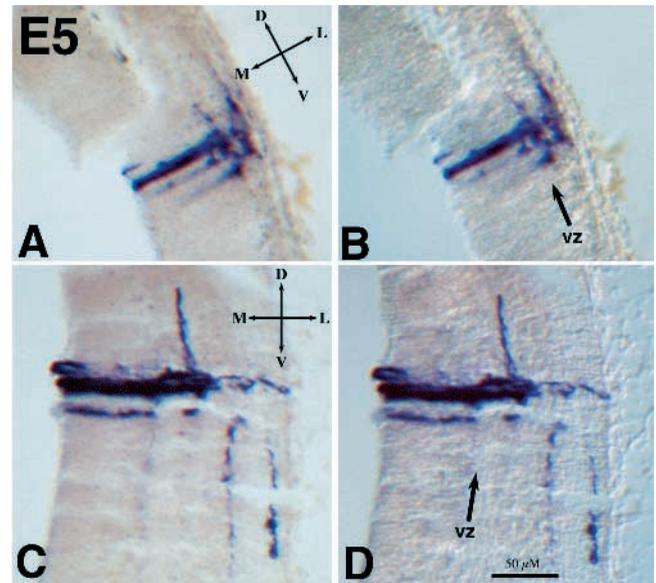
then frozen in OCT medium and sectioned coronally or sagittally on a cryostat as described above. Sections were baked overnight at 37°C and stored at −70°C. At the time of staining, sections were brought to room temperature and blocked in 0.1 M PBS, 10% fetal calf serum, 2% donkey serum and 0.1% Tween-20. Sections were then incubated in primary antibodies overnight. Primary antibodies and dilutions used were as follows: 8A1, recognizing low molecular weight neurofilament (Barnstable, 1987), 1:100; 2H3, recognizing intermediate weight neurofilament (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA), 1:10; 3A10 and E/C8 are both directed against neurofilament-associated proteins (also obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA), 1:10; Tuj1, recognizing a neuron-specific class III tubulin (Geisert and Frankfurter, 1989), 1:100; and 8D9, directed against Ng-CAM (Lemmon and McLoon, 1986), 1:10. After washing in 0.1 M PBS, sections were incubated for 2 hours with the appropriate secondary antibody conjugated to either Texas Red or fluorescein isothiocyanate (donkey anti-mouse IgG, Jackson Immunologicals). Finally, sections were washed in 0.1 M PBS and coverslips secured using Vectoshield (Vector Labs). Sections were observed under UV illumination on a Zeiss Axioplan microscope.

RESULTS

Chick embryos infected with CHAPOL between stages 10 and 14 (Hamburger and Hamilton, 1951) were killed on embryonic days 4 (E4; $n=8$), 5 ($n=10$), 6 ($n=9$) and 7 ($n=7$), and the patterns of clonal dispersion were examined in the diencephalon. At E4, the earliest time point studied, virtually all (96%) of the clusters of cells were organized in radial columns (Table 1). A radial column is defined as a tight cluster of AP+ cells that spans from the ventricle to the pial surface of the brain (Fig. 1A). Clones formed either a single radial column or clustered radial columns separated by unlabelled cells (Fig. 1B). These columns were perpendicular to the ventricular surface and resembled the radial columns identified in other regions of the developing CNS (Turner and Cepko, 1987; Gray et al., 1988; Austin and Cepko, 1990; Turner et al., 1990; Gray and Sanes, 1991; Hemond and Glover, 1993; Fekete et al., 1994). The recovery and sequencing of the unique oligonucleotide sequence carried by each retrovirus (Golden et al., 1995) from six of the 12 single radial columns at E4, and an additional five single radial columns from later ages, confirmed that each radial column was a clone. In the E4 to E6 embryos

Fig. 2. Non-radial cell migration was first observed at E5. Non-radial cell migration began at the basal limit of the VZ (A, bright field optics and B, Nomarski optics; VZ to the left). In some clones, non-radial cell migration occurred in multiple directions (C, bright field optics and D, Nomarski optics; VZ to the left). In this example, dispersion is dorsal (up) and ventral (down). Furthermore, migration was found at multiple levels in the developing mantle zone (C,D), not just at the basal limit of the VZ where it was first noted (A,B).

there were 45 groups of clustered columns. The molecular tag was recovered and sequenced from cells within individual columns of 11 such groups. In each case, the cells in all of the columns of a particular cluster were derived from a single progenitor. In order to determine if there were infected cells that were migrating in the VZ, but were missed due to the fact that they did not express detectable AP activity, AP− groups of cells were picked from around the AP+ radial columns. No retroviral genomes were recovered from the areas around AP+ clones at E4, E5 or E6. In E7 brains, genomes were occasionally recovered. This analysis indicates that a subset of early migrating cells was not missed because they were not expressing AP.

At E4, only one radial column had an associated cell exhibiting non-radial migration (see below). This clone was located in the middle of the lateral wall of the diencephalon. A single cell was adjacent to the radial column but directed ventrally. At E5 the majority of clones (73.5%) were arranged as single or clustered radial columns. However, 24% of clones had cells that had begun to migrate perpendicular to the radial column (Fig. 2). The start of non-radial cell migration appeared to be coincident with the formation of the diencephalon mantle zone, located basally to the VZ. Similar numbers of clones with non-radial cell migration were found on E6 and E7 (Table 1). With few exceptions, non-radially oriented cells were located outside the VZ (Figs 2, 3 and 4). Non-radial cell migration was first found to occur just beyond the basal margin of the VZ; however, with expansion of the diencephalon mantle, migration was found at multiple levels within the mantle zone (Figs 3A, 4).

17 of 53 presumptive clones that showed non-radial disper-

Fig. 1. Representative clones observed at E4. Clones spanned the mediolateral extent of the VZ, as a single radial column (A) or as a group of radial columns (B). Analysis of cells in the single columns and those in multiple radial columns revealed that both patterns were clonally derived. Multiple columns were interpreted as arising from the mixing of labeled and unlabeled progenitor cells in the VZ and not migration in the VZ (see text). The ventricle is to the left and the pial surface to the right in each figure.

Table 1. Summary of clones

Cell	Day	Radial clones[a]	non-radial[b]	vz spread[c]	VZ mixing[d]	nvz spread[e]	RC[f]	DV[g]
43c	E4	1	0	0	0	0	0	0
43d	E4	3	0	0	2	0	0	0
43e	E4	2	0	0	1	0	0	0
43f	E4	3	0	0	2	0	0	0
43g	E4	9	0	0	4	0	0	0
43j	E4	3	0	0	0	1*	0	1
43m	E4	2	0	0	1	0	0	0
43n	E4	2	0	0	2	0	0	0
Total (%)		25	0	0	12 (48%)	1 (4%)	0	1 (4%)
16A‡	E5	7	0	0	0	1	0	1
16B‡	E5	9	1	0	2	3	3	0
16E‡	E5	6	0	0	0	0	0	0
16F‡	E5	3	0	0	0	1	1*	0
16G‡	E5	4	0	0	1	0	0	0
16H‡	E5	5	0	0	0	0	0	0
16J‡	E5	14	0	0	5	3	3	0
12.4	E5	4	0	0	2	2*	0	2
12.14	E5	10	1	0	5	4	1	3
12.23	E5	6	0	0	3	2*	0	2
Total (%)		68	2	0	18 (26%)	16 (24%)	8 (12%)	8 (12%)
11.2	E6	7	0	0	4	1	0	2
11.4	E6	10	0	1†	4	3	0	4
11.1‡	E6	7	0	0	4	4	2	1
11.13	E6	4	0	0	2	2	1	2
11.15‡	E6	5	0	1†	5	4	2	4
2.11	E6	2	0	0	1	1	0	1
2.21	E6	1	0	0	0	0	0	0
2.12	E6	2	0	0	1	0	0	0
2.4	E6	4	0	0	2	1	0	1
Total (%)		42	0	0	23 (55%)	16 (38%)	5 (12%)	15 (36%)
26.12‡	E7	9	1	0	0	2	2	0
26.2	E7	1	0	0	0	0	0	0
26.3	E7	3	0	0	0	1	0	1
7.19	E7	3	0	0	2	0	0	0
26.17	E7	11	2	0	2	4	1	3
26.8	E7	>10	>10	0	>10	>10	>10	>10
26.16	E7	>10	>10	0	>10	>10	>10	>10
Total (%)		>47	>33	0	>24	>27	>23	>24

*Early migration.
†Each clone with one cell showing tangential migration in vz.
‡Cut horizontally.
[a]Clones with a radial organization.
[b]Clones with a non-radial organization only, no radial component.
[c]Clones showing dispersion within the VZ.
[d]Clones showing mixing in the VZ (see Fig. 1B).
[e]Clones showing dispersion outside the VZ only +/− radial column.
[f]Rostocaudal cell dispersion.
[g]Dorsoventral cell dispersion.

sion were analyzed by PCR and sequencing of their molecular tags. In each case, this analysis confirmed that they were clones, by establishing that the sequence of the degenerate oligonucleotide was the same in the cells within the radial column and those located some distance from the radial column.

Non-radial cell migration occured in both the dorsoventral and the rostrocaudal directions. Furthermore, cells from some clones migrated either dorsally or ventrally (Figs 2, 4A), while cells in other clones migrated only dorsally, and in other clones, only ventrally. In an attempt to determine if members of a clone chose dorsal versus ventral migration based upon their relative positions within the diencephalon, the diencephalon was divided into thirds along the dorsal-ventral axis. Five clones showed non-radial cell migration in the dorsal third of the diencephalon. Four of the five clones had ventrally
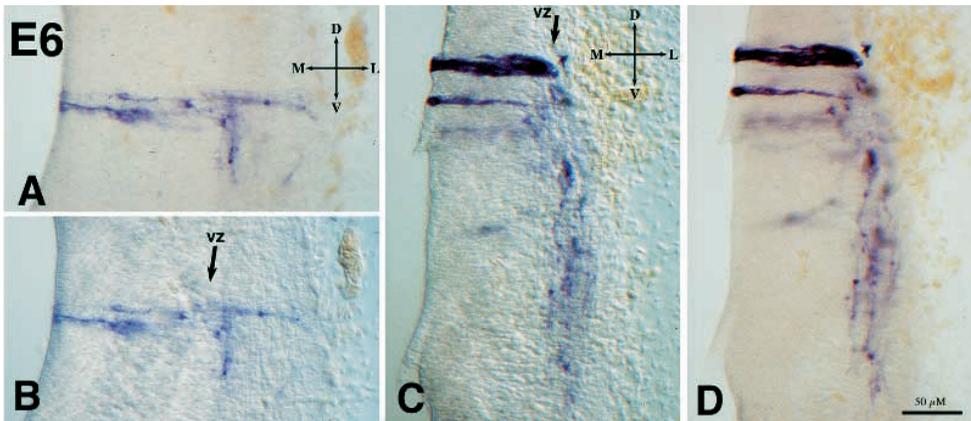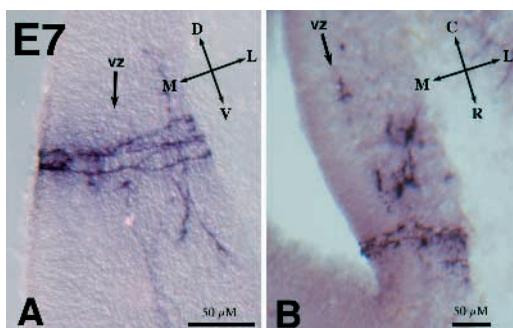
Fig. 3. Non-radial cell migration at E6. A single radial column extends from the VZ (left in photomicrograph) to the pial surface (A, bright field optics and B, Nomarski optics). A migrating cell with a trailing process is seen making a right angled turn from the radial column at the margin of the VZ (A). A clone showing directed migration from the radial column (C, Nomarski optics and D, bright field optics). All cells in this clone were migrating from dorsal to ventral and the cells were found migrating at multiple levels of the mantle zone, not just at the basal limit of the VZ.

directed migration only and one clone had cells migrating in both dorsal and ventral directions. No clones had cells migrating in the dorsal direction alone. 14 clones in the middle third of the diencephalon had non-radial cell migration, with one half (7/14) being ventrally directed. Four had bidirectional migration and the remaining three were directed only dorsally. Seven clones were located in the ventral third of the diencephalon. Three of these seven clones had cells migrating only ventrally, two had cells migrating both dorsally and ventrally, and two showed migration in the dorsal direction only.

The observations discussed above on dorsoventral migration were made using brains sectioned in the coronal plane. To observe migration in the rostrocaudal direction, a series of brains were cut in the horizontal plane; E5 (*n*=7), E6 (*n*=2) and E7 (*n*=1). In these brains, cells dispersed in the rostrocaudal direction were observed (compare 16 A, B, E-H and J, cut in the horizontal plane, to 12.4, 12.14 and 12.23, cut in the coronal plane, Table 1 and Fig. 4B). Several clones had cells migrating in both the rostral and caudal directions. As with dorsoventral migration, rostrocaudal migration was not seen at E4, although all brains examined at E4 were cut in the coronal plane and thus it is possible we did not recognize a small number of cells dispersing rostrocaudally; at E5, it was as frequent as DV migration. The rostrocaudal migration observed at E5 appeared to take place just beyond the basal

limit of the VZ, similar to that seen with dorsoventral migration. As the mantle zone increased in thickness, migration was observed at multiple levels, again similar to that observed in the dorsoventral direction. Since clones were found to be dispersed in both the rostrocaudal direction and the dorsoventral direction, it is possible that cells were migrating away from the radial column at various angles relative to these two directions. Following the migration of live cells will be required to determine the exact pathways taken by these cells during clonal dispersion. At later ages not as many brains were cut in the horizontal plane as in the coronal plane, and therefore the data at these ages shown in Table 1 are skewed to observations of dorsoventral migration.

Radial glial fibers are a probable substrate for the early radial migration of clones out of the VZ. To investigate whether radial

Fig. 4. Non-radial cell migration at E7. Multiple cells are noted migrating both dorsally (up) and ventrally (down) from a radial column (A, Nomarski optics). Trailing processes indicate a 90° turn was made from the radial column. A horizontally sectioned brain shows rostral to caudal migration (B). All migration was seen to take place outside the VZ.
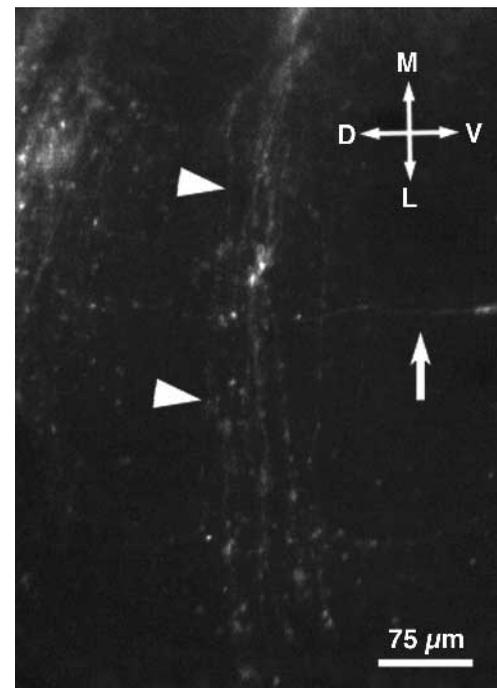
Fig. 5. DiI labeling of a non-radially oriented cell process and radial glia. Radial glial fibers (arrowheads) course from the ventricular surface (top) to the pial surface (bottom). A single process (arrow) is seen coursing perpendicular to the radial glia.

glia also provide non-radial pathways for guidance, radial glial fibers were visualized by labeling the fibers with DiI. Classic radial glia were identified in E7 brains extending from the ventricular surface to the lateral margin (pial surface) of the brain. However, in addition to labeling radial glia, the DiI labelling revealed the presence of long processes perpendicular to the radial glial fibers (Fig. 5). No obvious connections were found between radial glia and these fibers running parallel to the surface of the brain. Furthermore, the fibers running perpendicular to the radial glia could be morphologically distinguished from radial glia by the absence of small beads along the fibers and a slightly thinner diameter. These data suggested that the non-radially oriented processes may be axons.

To further characterize the nature of the non-radially oriented cellular processes, transmission electron microscopy and immunohistochemistry were performed on E4-E6 chick brains. By electron microscopy, cellular processes were predominantly oriented radially on E4 (Fig. 6A), although occasional cell processes appeared to be turning from a radial orientation (data not shown). Non-radially directed cell processes could not be traced more than several μm in the tangential direction on E4.

In contrast, on E5 numerous non-radially oriented cell processes were found just beyond the basal limit of the VZ (Fig. 6B,C). Clusters of multiple, parallel cell processes were usually found together, forming tight bundles (Fig. 6D,E). At the ultrastructural level these cell processes possessed parallel intermediate filaments, suggestive of neurofilaments and microtubules. These two features support the hypothesis that the clusters of cellular processes oriented perpendicular to radially oriented fibers are axons. Additional evidence that these presumed axons support non-radial cell migration came from the observation that non-radially oriented cells appeared to be either coursing through these non-radially oriented fibers (Fig. 6E,F) or attached in other ways to the surface of the bundles of cell processes. Just as migration was found to occur outside the VZ, the bundles of non-radially oriented cell processes were always located beyond the VZ in the developing mantle layer and were present in the dorsoventral and rostrocaudal planes of all embryos examined on E5 (n=6) and E6 (n=4). These non-radially oriented cell processes were not present on E4 (n=4), again paralleling the finding that non-radial migration did not occur on E4.

The axonal nature of these cell processes was also supported by an immunohistochemical analysis.

Antibodies to various neurofilaments and neurofilament-associated proteins (monoclonal antibodies 8A1, 3A10, 2H3 and E/C8), microtubules (monoclonal antibody Tuj1) and a chick axon surface marker (monoclonal antibody 8D9) were applied to E4-E6 coronal sections of chick embryos. Monoclonal antibody 8D9 showed staining outside the VZ on E5 and E6, but no staining on E4 (Fig. 7A-D). This pattern of staining was as predicted by the finding of non-radially oriented cell processes with the morphology of axons, as shown in Fig. 6. Monoclonal antibody 8A1 was also found to immunolabel cell processes located outside the VZ and running perpendicular to the course of radial glia on E5 (Fig. 7E), but not on E4. The pattern of staining by Tuj1 was similar to that of 8D9 (data not shown). 3A10, 2H3 and E/C8 showed no staining. Together, data from DiI tracing, electron microscopy and immunohistochemistry bolster the hypothesis that non-radial cell migration is supported by axons.

DISCUSSION

Cell migration is an integral component of nervous system development. Understanding the pathways and control of cell
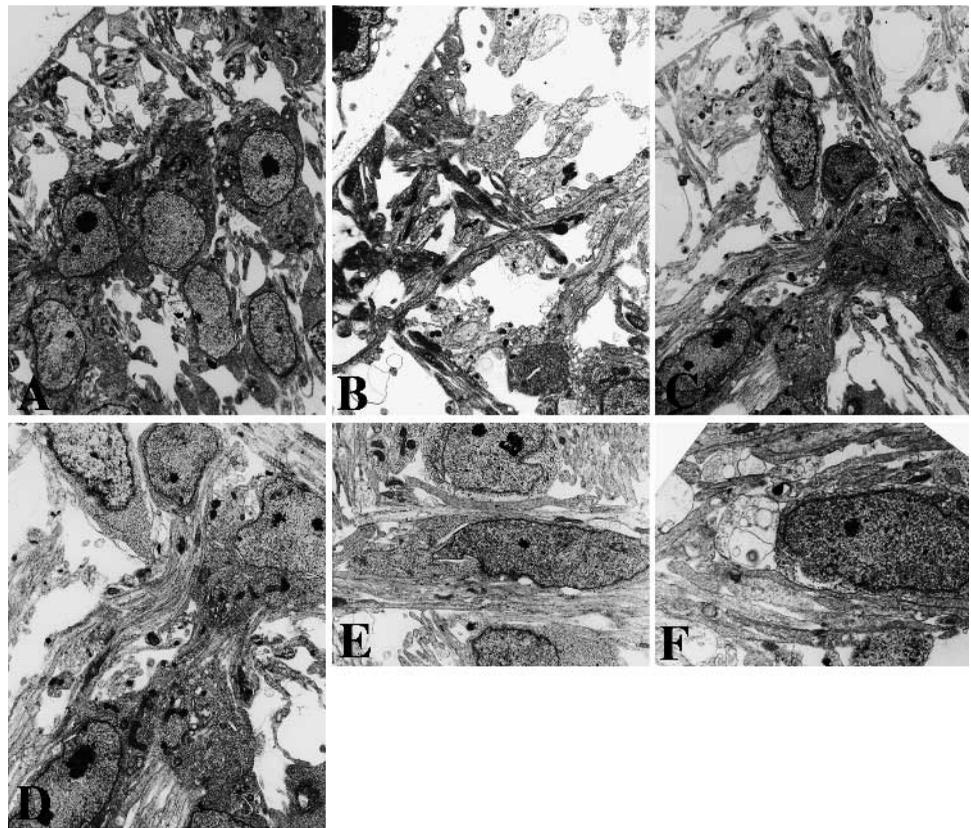
Fig. 6. Electron micrographs of the developing chick diencephalon. (A) On E4 the VZ spanned virtually the entire thickness of the diencephalic wall. All processes were radial and no non-radially oriented cell processes were found. (B,C) On E5, multiple cellular processes were organized into bundles that are oriented perpendicular to the radial fibers and parallel to the pial surface. (D) A high power image of C shows a non-radially migrating cell (lower left) migrating among multiple fiber bundles. (E) A cell with the characteristic morphology of a migrating cell (center), including a leading process extending to the left and a notched nucleus in the direction of the leading process, is shown. The cell appears to migrate through bundles of axons. (F) Another example of a cell migrating among fibers from a single bundle. *, pial surface; A, 3000×; B, 3000×; C, 3000×; D, 4500×; E, 4500×; F, 7000×.

Fig. 7. Immunohistochemical detection of Ng-CAM and neurofilament protein. No staining was seen on E4. (A) DAPI and (B) monoclonal antibody 8D9 detected with a secondary antibody linked to rhodamine; A and B are the same field. On E5 (C), strong axonal staining was seen only outside the VZ (VZ on right; combined DAPI and monoclonal antibody 8D9 detected with a secondary antibody linked to rhodamine). (D) High power image of labeled axon on E5 (monoclonal antibody 8D9). (E) Low molecular weight neurofilament protein
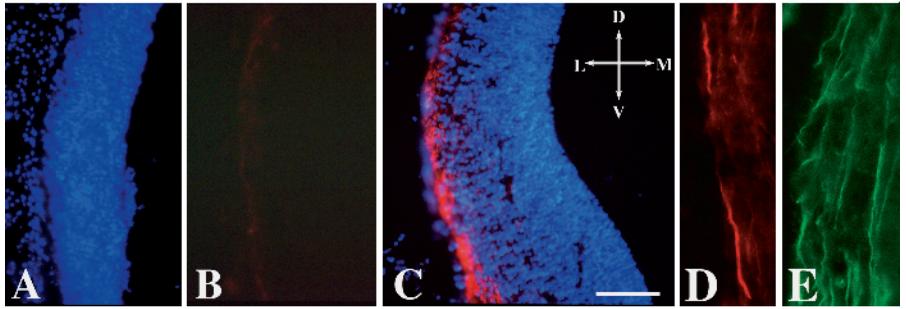
(monoclonal antibody 8A1 detected with a secondary antibody linked to fluorescein) was also present within non-radially oriented axonal processes outside the ventricular zone on E5, but not on E4 (data not shown). Scale bars 50 μm (A-C); 12 μm (D and E).

migration should provide insights into how cells form the proper structures and find the proper synaptic partners. While radial migration along glial guides is a well established pathway of migration, recent studies have begun to elucidate non-radial pathways of cell migration in multiple regions of the nervous system. Non-radial cell migration is a prominent feature of development in the chick telencephalon (Szele and Cepko, 1996), diencephalon (Golden and Cepko, 1996), tectum (Gray and Sanes, 1991), cerebellum (Ryder and Cepko, 1994), spinal cord (Leber and Sanes, 1995; Phelps et al., 1996) and the mammalian cerebral cortex (Austin and Cepko, 1990; O'Rourke et al., 1992; Walsh and Cepko, 1993; O'Rourke et al., 1995) and striatum (Halliday and Cepko, 1992). In some of these cases, a high percentage of cells show non-radial cell migration, suggesting that this type of migration plays an important role in the shaping of the tissue (Walsh and Cepko, 1992; O'Rourke et al., 1995; Reid et al., 1995; Golden and Cepko, 1996).

The data presented here and in our earlier report (Golden and Cepko, 1996) are supported by previous findings derived from the quail-chick chimera system (Balaban et al., 1988). Transplantation of prosencephalic tissue from quail to chick and vice versa revealed that dorsal to ventral migration of cells occurred after E4, and that mixing of cells did not occur in the VZ, indicating that migration takes place after cells have emerged from the VZ into the developing mantle zone (Fig. 8). Rostrocaudal dispersion was not investigated in the the quail-chick chimera studies.

The mechanisms governing radial cell migration have begun to be defined. Neuronal migration is supported by astroglial membranes that require several factors, including astrotactin (Zheng et al., 1996), a presumptive ligand for radial migration. Furthermore, radial cell migration has been blocked by antibodies to astrotactin (Zheng et al., 1996), L1 (Asou et al., 1992), tenascin (Husmann et al., 1992), and as yet undefined epitopes, 1A1 (Mittal and David, 1994), D4, NJPA-1 (Anton et al., 1996) and T61 (Meyer and Henke-Fahle, 1995). In addition, when an antisense RNA complementary to β1-integrin was expressed using a retrovirus in the chick optic tectum, infected cells failed to migrate out of the VZ, possibly reflecting failure to attach to radial glial fibers (Galileo et al., 1992). β1-integrin is believed to interact with laminin, an extra-cellular matrix molecule with a putative role in cell migration. Taken together, these studies indicate that radial migration is a complex process that requires cells to attach to a glial fiber,

migrate along the glial fiber, and eventually get off the glial fiber. Each step is likely to involve multiple components.

In addition to radial migration, several novel pathways of migration have recently been identified. One example is the rostral migratory stream, a specialized pathway for neuronal migration in postnatal rats (Hinds, 1988; Luskin, 1993). Investigation of this non-radial pathway has revealed a glial meshwork surrounding chains of neuroblasts migrating from the SVZ to the olfactory bulb (Lois et al., 1996). Migration along this path requires the polysialic acid found on the 180 kDa form of NCAM (Tomasiewicz et al., 1993). Axons have been shown to provide support for neuronal migration in several regions of the brain. A population of hypothalamic neurons that secrete GnRH are born in the olfactory placode and subsequently migrate into the hypothalamus via the vomeronasal nerve (Fueshko and Wray, 1993; Wray et al., 1993; Yoshida et al., 1995). This unique path does not appear to require radial glial support. A population of neurons have also been shown to migrate along the commissural axons in the developing spinal cord (Phelps et al., 1996).

The non-radial cell migration that occurs in the diencephalon also appears to employ axons for support. The spatial and temporal appeerence of the cellular processes we have identified in this study exactly parallel the location and timing of non-radial cell migration by virally marked cells. The cellular
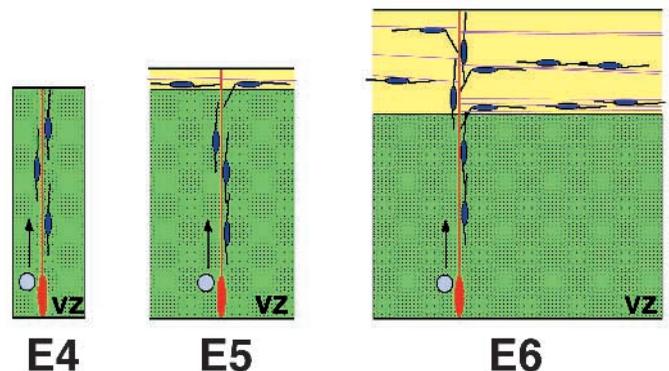
Fig. 8. A model summarizing the timing and location of non-radial cell migration in the diencephalon. All clones on E4 show a purely radial arrangement. Beginning on E5, non-radial cell migration begins just beyond the basal limit of theVZ in the developing mantle zone. On E5 and later, migration occurs in multiple directions and in multiple levels outside the VZ.

processes have the ultrastructural and immunohistochemical features of axons. We are currently investigating the locations of the cell bodies that elaborate these axons. Furthermore, we are exploring the molecular interactions between these axons and non-radially migrating cells in an attempt to begin to elucidate the interactions that govern non-radial migration.

The current study and the data cited above indicate that migration orthogonal to radial columns occurs in virtually every region of the CNS. One region that is conspicuously absent from this list, however, is the retina. Retinal clones are almost uniformly radial, although there can be some mixing in the VZ (Fekete et al., 1995; Reese et al., 1995) and relatively minor lateral displacement of several cell types that form lateral connections (Turner et al., 1990). It is possible that the retina does not require non-radial cell migration for development, as the cells travel a very short distance from their site of origin to their final location, and/or because it is fairly uniform in its cellular composition, in comparison to some of the more complex areas of the CNS.

One of the striking features of non-radial cell migration is that cells of some clones appear to have a clearly defined direction in which they migrate (see Fig. 3C,D). Moreover, cells within other clones can migrate in different directions, some migrating dorsally, some ventrally, some rostrally, some caudally and some at less well-defined angles. At least some cells appear to 'choose' a particular path, as opposed to simply meandering through the tissue, exhibiting a behavior similar to that of growth cones at a choice point. Perhaps some of the molecules and mechanisms used for axonal guidance will be important for cellular migration. Indeed, migration of neurons from the rhombic lip to the basis pontis may be disrupted in a netrin mutant mouse (Serafini et al., 1996). If cells actively choose a particular path, it appears that the choice is exercised soon after a cell is generated. Cells appear to migrate radially through the VZ and then, immediately upon exiting the VZ, exercise their choice. The results of such choices are that siblings end up occupying different functional domains.

Although mature diencephalic clones generally occupy multiple functional domains, clones of approximately the same size, occupying roughly the same amount of territory, can vary a great deal in the final pattern of distribution of cells, i.e. some clones span great distances rostrocaudally, but not dorsoventrally, and others show the opposite pattern (Golden and Cepko, 1996). It is possible that this reflects heterogeneity in the progenitor pool residing in the early embryonic VZ. Lineally based information could also account for the observation reported here that some clones can have cells that migrate dorsally but not ventrally, ventrally but not dorsally, or in both of these directions. In addition, distinctive migration patterns were seen in a small subset of clones in the hypothalamus (Arnold-Aldea and Cepko, 1996) and in clones of the telencephalon (Szele and Cepko, 1996). As the clones that differ in migration patterns are fairly large and originate from approximately the same region of the VZ, lineally based information originating from a heterogeneous pool of progenitors could account for the differences in choice of migration paths. An understanding of the mechanisms that support and guide migration, as well as the basis for a cell's choice of migration paths, will allow an assessment of whether lineage plays a role in these processes.

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