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# Calcium-Dependent Adhesion Is Necessary for the Maintenance of Prosomeres

**Maria McCarthy, Erqian Na, Christine Neyt, Alex Langston, and Gord Fishell**[1]

*Developmental Genetics Program and Department of Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, New York 10016*

**Cell adhesion has been suggested to function in the establishment and maintenance of the segmental organization of the central nervous system. Here we tested the role of different classes of adhesion molecules in prosencephalic segmentation. Specifically, we examined the ability of progenitors from different prosomeres to reintegrate and differentiate within various brain regions after selective maintenance or removal of different classes of calcium-dependent versus -independent surface molecules. This analysis implicates calcium-dependent adhesion molecules as central to the maintenance of prosomeres. Only conditions that spared calcium-dependent adhesion systems but ablated more general (calcium-independent) adhesion systems resulted in prosomere-specific integration after transplantation. Among the members of this class of adhesion molecules, R-cadherin shows a striking pattern of prosomeric expression during development. To test whether expression of this molecule was sufficient to direct progenitor integration to prosomeres expressing R-cadherin, we used a retroviral-mediated gain-of-function approach. We found that progenitors originally isolated from prosomere P2 (a region which does not express R-cadherin), when forced to express this molecule, can now integrate more readily into R-cadherin-expressing regions, such as the cortex, the ventral thalamus, and the hypothalamus. Nonetheless, our analysis suggests that while calcium-dependent molecules are able to direct prosomere-specific integration, they are not sufficient to induce progenitors to change their regional identity. While diencephalic progenitors from R-cadherin-expressing regions of prosomere 5 could integrate into R-cadherin-expressing regions of the cortex, they did not express the cortex-specific gene *Emx1* or the telencephalic-specific gene *Bf-1*. Furthermore, diencephalic progenitors that integrate heterotopically into the cortex do not persist postnatally, whereas the same progenitors survive and differentiate when they integrate homotopically into the diencephalon. Together our results implicate calcium-dependent adhesion molecules as key mediators of prosomeric organization but suggest that they are not sufficient to bestow regional identities.** 

## INTRODUCTION

During development, the vertebrate embryonic neural tube progresses from a pseudostratified neuroepithelium into an exquisitely patterned mature CNS. This process requires the development of functionally and histologically distinct fates within the neuroepithelium, dependent on the anterior/posterior (A/P) and dorsal/ventral (D/V) position of the cells along the neuraxis. Segmentation appears critical to patterning of the anterior neural tube and occurs in two sequential stages. The first of these is marked by the emergence of the three characteristic morphological divisions of the anterior CNS, the prosencephalon, the mesencephalon, and the rhombencephalon. Within the prosencephalon and the rhombencephalon, a second set of more finely graded segmental subdivisions appears later in development. These secondary subdivisions are marked by the formation of morphological boundaries and segment-specific patterns of gene expression (reviewed in Lumsden, 1990; Rubenstein *et al.*, 1994, 1998).

The intradivisional segmentation of the anterior CNS has been best studied in the rhombencephalon of the chick (see Keynes and Lumsden, 1990). This work suggests that these secondary subdivisions result in the formation of neuromeric units, as a result of polyclonal restrictions. Cells within these restrictions develop homotypic adhesion characteristics that act to maintain the integrity of these neu-

[1] To whom correspondence should be addressed. Fax: (212) 263-2248. E-mail: fishell@saturn.med.nyu.edu.

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romeric boundaries (Wizenmann and Lumsden, 1997) and may be causally related to their formation (Inoue *et al.*, 1997; Xu *et al.*, 1999). This segmental organization is of interest as it is ultimately responsible for the development of the nuclear organization seen within the mature hindbrain, which among other things gives rise to the cranial nerves (Marin and Puelles, 1995).

Experiments in both the rodent and the chick suggest that the vertebrate diencephalon and telencephalon may also be segmentally divided into neuromeric compartments or prosomeres, analogous to the rhombomeric segments in the hindbrain. Observation of the changing morphology of the developing chick diencephalon has revealed that a series of segmental swellings transiently appear along the A/P axis (Vaage, 1969; Puelles *et al.*, 1987, 1991; Figdor and Stern, 1993).

Labeled progenitor cells within these segmental units undergo polyclonal expansion that respects the observed morphologic segment boundaries (Figdor and Stern, 1993). Furthermore, similar to the rhombomeric expression of Hox genes (reviewed in Wilkinson, 1993), the expression of homeodomain-containing genes, such as Dlx2 and Nkx2.1, respects these prosomeric divisions within the rodent forebrain (Bulfone *et al.*, 1993). Fate map analysis in chick by Puelles *et al.* (1991) suggests that a direct relationship between the prosomeric divisions and the nuclear structures that they ultimately give rise to may exist in this species. More recent experiments by this group have correlated specific cadherin expression in prosomeres and their divisions in the chick diencephalon with the morphologic nuclear fate of cells expressing those adhesion molecules (Yoon *et al.*, 2000; Redies *et al.*, 2000). The question of whether these "neuromeric" divisions ultimately give rise to discrete aspects of the mature diencephalon is at present unclear in mice, but based on the analysis in chicken it seems likely. Furthermore, mutagenesis that disrupts the prosomeric organization, such as seen in Pax6-null mice, results in abnormal diencephalic development (Stoykova *et al.*, 1996; Mastick *et al.*, 1997; Grindley *et al.*, 1997).

Recent experiments have asked whether the divisions within the prosencephalon possess homotypic adhesive properties (Krushel and van der Kooy, 1993; Götz *et al.*, 1996; Olsson *et al.*, 1998). Cell-mixing experiments of progenitors (E14 rat, E7/8 chick) from the regional divisions of both the rodent and the chick telencephalon suggest that progenitor cells possess region-specific adhesive properties at early but not late times during embryogenesis (Krushel and van der Kooy, 1993; Götz *et al.*, 1996). Furthermore it was noted that this sorting, like in the hindbrain (Wizenmann and Lumsden, 1997), is dependent on the expression of $Ca^{2+}$-dependent cellular adhesion molecules (CAMs) (Götz *et al.*, 1996).

To test if such a mechanism is utilized within the mouse diencephalon, we undertook a series of transplantation experiments to study whether isolated progenitors dissected from different prosomeres possess intrinsic prosomeric identity. We compared the patterns of integration of diencephalic progenitors isolated from different prosomeres, such that we could assess the role of calcium-dependent (CAD) (reviewed in Redies, 1995) or calcium-independent (CID) adhesion molecules (reviewed in Edelman and Jones, 1997). We found evidence for CAD adhesion molecules increasing the fidelity of progenitors to integrate in a prosomere-specific fashion. In addition, we show that forced expression of R-cadherin in P2-dorsal diencephalic progenitors (P2-Ddi) (a region normally devoid of R-cadherin expression) allowed these cells to more readily integrate into prosomeric regions where this molecule is expressed. However, integration aside, our data do not suggest that cadherin-mediated entry into heterotopic sites is sufficient to bestow altered regional identity on donor progenitors. Specifically, we found that diencephalic cells from R-cadherin-expressing regions that integrated into R-cadherin-expressing regions of the cortex initiated expression of neither the cortical-specific marker Emx-1 nor the telencephalic-specific marker BF-1. Furthermore we observed that diencephalic cells within cortical host regions are lost by early postnatal times, whereas those integrated within the diencephalic host regions differentiate normally. Together, our results suggest that while CAD adhesion molecules may act to maintain prosomeric integrity, they are not sufficient to allow prosomere-specific differentiation.

## MATERIALS AND METHODS

### *Isolation and Preparation of Progenitor Cells*

Donor tissue was obtained from either E12 or E14 timed pregnant CD1 (Charles River) *Emx1 tau-lacZ* knock-in (G.F. and A.L., unpublished) or *BF-1 LacZ* mice (gift from E.-S. Lai). The plug date was defined as embryonic day 1 (E1). Tissue from the ventricular zone of the dorsal region of prosomere 2 (P2-DDi) or from the ventralmost region of prosomere 5 (P5-VDi) was dissected in Leibowitz L-15 medium from the brains of two litters of mice (16–24 embryos) for each experiment. Tissue was rinsed in $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline (CMF-PBS) and dissociated by trituration in the presence of 0.05% DNase (Worthington) using one of three different conditions: (1) mechanical dissociation alone, (2) mechanical dissociation after a 30-min incubation at 37°C in 0.25% trypsin (Worthington) in Hepes-buffered saline (HBS) containing 0.02% EDTA (trypsinized cells), or (3) the same protease solution, without EDTA and supplemented with 10 mM $CaCl_2$ ($Ca^{2+}$-protected trypsinized cells). In the latter two cases, protease treatment was terminated by the addition of 10% FBS in DMEM, 0.05% DNase. Regardless of how progenitors were dissociated, cells were labeled with the lipophilic dye PKH-26-GL according to the manufacturer's instructions (Pharos for Sigma).

### *Transplantation*

E14 timed pregnant CD1 mice were used as recipients for transplantation. The animals were anesthetized with sodium pentobarbital (50 mg/kg) (Abbott Laboratories). A midline laparotomy was performed and the uterine horns were exposed. Each embryo was oriented in the embryonic sac by transillumination and the

cerebral vesicles were identified by the calvarian sutures. Using a micropipette attached to a Hamilton syringe, 50,000 cells in a volume of 0.75 $\mu$l was injected into the cerebral ventricle. After injection of all embryos, the uterine horns were placed back into the peritoneal cavity and the abdominal wall was sutured. The embryos were allowed to gestate *in utero* for 3 days and were sacrificed at E17.

Only brains in which clear signs of intraventricular injection were achieved (cells retained in the ventricle and distinct entry points of integration) were analyzed in this study. Furthermore, only cells which demonstrated a distinct soma and which had clearly migrated into the tissue were quantified, whereas brains with signs of intraparenchymal injection were excluded.

## Viral Misexpression of R-cadherin

***Replication-defective retrovirus preparation.*** Retroviruses were prepared as described previously (Gaiano *et al.*, 1999). Briefly, full-length (FL) R-cadherin constructs were made by blunt-end cloning a mouse R-cadherin cDNA (gift from Dr. M. Takeichi; Matsunami *et al.*, 1993) into the *Xho*I cloning site of the pCLC retroviral vector (Gaiano *et al.*, 1999). Thirty micrograms of viral construct plasmid was then cotransfected along with 35 $\mu$g of pHCMVG plasmid into 293-derived packaging line (293GP cells; Chiron, San Diego, CA) at 90% confluence on 150-mm tissue culture plates by calcium phosphate precipitation. Virus-containing supernatant was harvested at 24, 36, and 48 h after infection. The supernatants were concentrated by ultracentrifugation. Viral pellets were resuspended in 40 $\mu$l PBS and aliquots of virus were stored at −80°C.

***Viral infections and cell preparation.*** Tissue from the dorsal part of prosomere 2 (P2) from the brains of 12 E12 embryos was dissected and mechanically dissociated by trituration in the CMF-PBS − EDTA as described above. Half of the cells were infected with a 1:40 dilution of either CLC or FL R-cadherin pseudotyped virus (5–7 $\times$ $10^8$ cfu/ml) in a volume of 200 $\mu$l of neurosphere medium (2% B27, 1% N2, 1% 200 mM glutamine (Gibco)), 0.1% mitoC (Collaborative Research), 20 $\mu$g/ml bFGF (Gibco), 2 $\mu$g/ml heparin (in DMEM/F12 medium) containing 8 $\mu$g/ml Polybrene. Cells were infected for 2 h at 37°C in 5% $CO_2$. Cells were then washed free of exogenous virus and resuspended in 2 ml of neurosphere medium. Cells were grown as neurospheres for 7 days. On the day of transplantation, neurospheres were dissociated in the 0.25% trypsin in HBS containing 10 mM calcium for 30 min. Cells were dye-labeled and transplanted into E14.5 host embryos as described above. After 3 days survival, brains were harvested and processed as described above. Dissociated PKH-26 labeled cells were also cultured in differentiation medium (neurosphere medium without bFGF or heparin) for 5 days *in vitro* to assess viability, viral gene expression, and differentiation potential.

## 5-Bromo-2-deoxyuridine (BrdU) Labeling in Vivo

Timed pregnant E12 or E14 CD1 mice were injected with BrdU (50 mg/kg) (stock solution is 20 mg/ml BrdU, 2 mg/ml 5-fluorodeoxyuridine in 0.007 N NaOH; Sigma) every 6 h for 18 h before time of sacrifice. Tissue used for transplantation was prepared as described above and an aliquot of each cell suspension was pelleted onto poly-L-lysine-coated slides using a cytospin, for later immunostaining. Some brains were sacrificed immediately and fixed overnight in 70% EtOH and processed for paraffin embedding. Paraffin sections were cut at 7 $\mu$m on a microtome, mounted on charged slides, and used for immunohistochemistry.

## Tissue Preparation and Analysis

E17 mouse embryos were sacrificed (3 days after transplantation) and perfused transcardially with 2% paraformaldehyde in PBS. Brains were removed from the cranium and postfixed overnight in 2% paraformaldehyde, containing 30% sucrose in PBS. Brains were embedded in 3% agar in PBS and sectioned coronally at 80 $\mu$m on a Vibratome. PKH-26-GL labeled cells were imaged with a Zeiss Axioskop microscope equipped with cooled CCD digital camera (Micromax1300Y; Princeton Instruments), utilizing a Sony interline chip (Sony ICX061).

## Immunochemistry

***BrdU staining.*** Cytospin slides were fixed with acid–alcohol (90% EtOH:5% acetic acid:5% water) at room temperature for 30 min. Paraffin sections were dewaxed and rehydrated. Immunostaining for BrdU was performed using an Amersham cell proliferation kit as described by the manufacturer. Immunohistochemical product was visualized using the chromogenic substrate 3′,3′-diaminobenzidine (DAB) and nickel.

***X-gal staining.*** Cryosections were incubated overnight at room temperature in X-gal solution (0.87 g/L NaCl, 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal). The sections were washed free of excess substrate with PBS and mounted using Crystal-Mount (Fisher) and examined for LacZ-positive cells by light microscopy.

***Nestin immunocytochemistry.*** Cytospin slides were fixed with cold 4% paraformaldehyde in PBS, pH 7.4, for 10 min. Slides were washed with PBS and blocked with 10% normal goat serum, 0.1% Triton X-100 in PBS for 1 h at room temperature. The slides were incubated overnight at 4°C in a 1:5 dilution of mouse monoclonal anti-nestin (Rat-401; Developmental Studies Hybridoma Bank). A biotinylated secondary goat anti-mouse (Jackson) and ABC kit (Vector Laboratories) were used to form an immunohistochemical complex that was visualized using DAB.

***Immunohistochemistry.*** Immunolocalization of N-cadherin, R-cadherin,CD15 Lewis$^x$ antigen and $\beta$-1 integrin was performed on 20-$\mu$m fresh-frozen sections of E14 mouse brain. Tissue used for $\beta$-1 integrin staining was acetone fixed, whereas CD15, L1, and R- and N-cadherin localization was performed on 4% paraformaldehyde-fixed sections. Sections used for R- and N-cadherin staining were pretreated with 10 mM citrate buffer, pH 6.5, for 30 s, in a microwave at high power. Sections were blocked with 10% normal goat serum in PBS for 1 h at room temperature. The sections were incubated overnight at 4°C with primary antibody at the following dilutions: mouse monoclonal CD15 (AC4 monoclonal antibody) (gift from Drs. J. Dodd and T. Jessell (1986)) 1:1, rabbit polyclonal anti L1 1:1000 (gift from Dr. M. Grumet (1994)), rat monoclonal MNCD2 to N-cadherin (gift from Dr. M. Takeichi) 1:1000, rat monoclonal MRCD5 to mouse R-cadherin supernatant (1:1), and hamster anti-mouse CD29 (integrin $\beta$-1 chain) (Pharmingen) 1 $\mu$g/ml. The following fluorescent secondary antibodies were applied at room temperature for 1 h: FITC conjugated to (1) goat anti-mouse IgM (Jackson Immunoresearch), (2) goat anti-rabbit IgG (Jackson Immunoresearch), (3) goat anti-rat IgG (Jackson Immunoresearch), and mouse anti-hamster IgG (Pharmingen). Stained sections were mounted with Gel-Mount (Fisher) and

imaged with a Zeiss Axioskop microscope equipped with a digital camera (as described above).

### SDS–PAGE Electrophoresis and Western Blot Analysis

SDS–PAGE gels and immunoblots were performed as described by Sambrook *et al.* (1989). Briefly, dorsal diencephalic tissue was isolated and cells were prepared as described for transplantation. Dissociated cells were pelleted and pellets were normalized by wet weight quantitation. Cell pellets were resuspended in the appropriate normalized volume of suspension buffer (0.1 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 1 $\mu$g/ml aprotinin, 100 $\mu$g/ml PMSF) and homogenized on ice in a Dounce homogenizer. An equal volume of 2× SDS gel loading buffer (100 mM Tris, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added to the homogenate. Samples were boiled for 10 min and then sonicated on ice for 30 s. Equal volumes (10 $\mu$l) of the whole-cell lysates were loaded into wells of 10% SDS–PAGE gels. After electrophoretic separation the proteins were transferred to nitrocellulose by electrophoretic transfer.

Nitrocellulose blots were blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) overnight at 4°C. Blots were incubated with the appropriate primary antibody for 2 h at room temperature (MNCD2, rat anti-N cadherin 1:1000, gift from Dr. M. Takeichi (Hatta *et al.,* 1986) or rabbit anti-Trk B 1:4000; Santa Cruz). Blots were washed with PBS-T and then incubated for 1 h at room temperature with a 1:10,000 dilution of the appropriate HRP-conjugated secondary antibody (rabbit anti-rat IgG, Jackson ImmunoResearch, or donkey anti-rabbit IgG, Amersham Life Science). Finally, the nitrocellulose blots were washed extensively in PBS-T and developed using the ECL Plus luminescence Western blotting detection kit (Amersham Life Science).

## RESULTS

### Characterization of Proliferative Kinetics of Donor Tissue

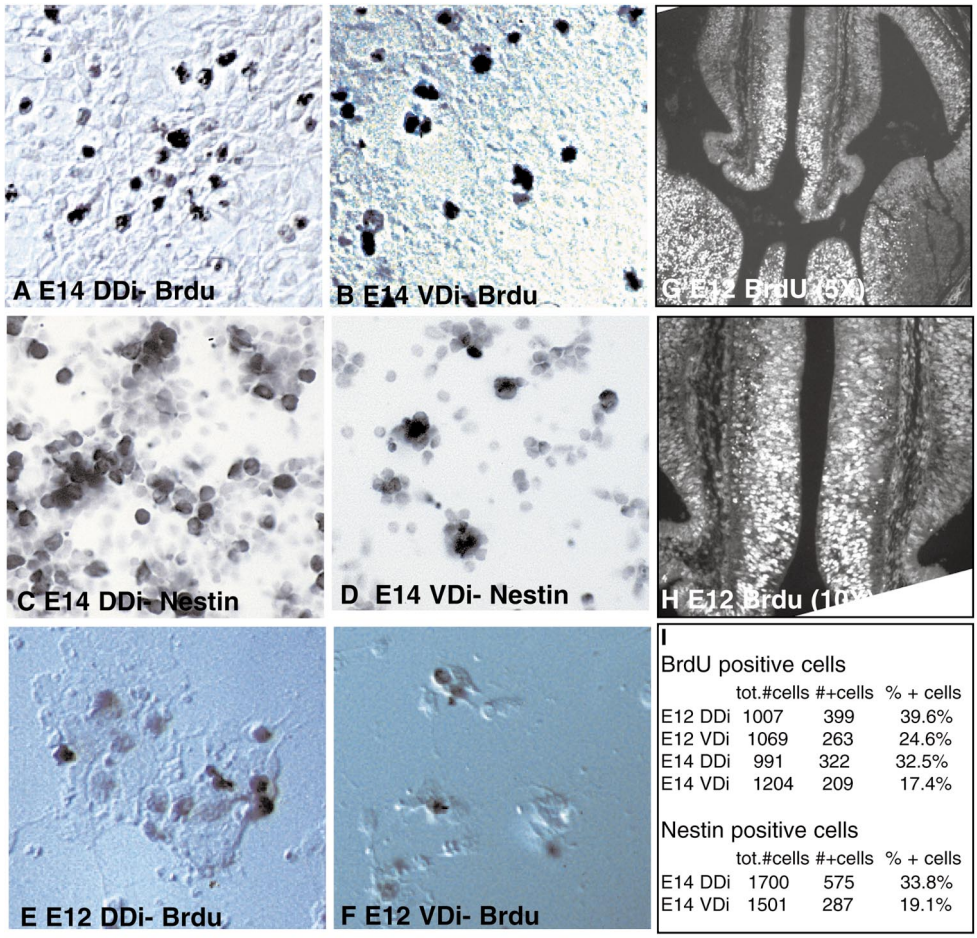
Tissue was isolated from two different stages of development, E12 and E14. This was done because diencephalic development precedes telencephalic development by approximately 24 h (Bayer, 1989). The diencephalic progenitor populations in this study were characterized by examining the proliferative state of the cells isolated for grafting. By injecting the mother carrying the donor embryos three times with BrdU over a 12-h period (with injections at 0, 6, and 12 h), we were able to label a substantial population of the dividing progenitors within forebrain proliferative zones (Takahashi *et al.,* 1992). E12 and E14 diencephalic progenitors were examined and were found to display nearly the same proliferative fraction and relative percentage of neuroepithelial progenitors by BrdU incorporation (Fig. 1I). The findings below therefore apply to either E12 or E14 progenitors. Examination of sections from brains of littermates of animals used for transplantation indicated that approximately 50% of the cells within the proliferative zone were labeled (Fig. 1H). Analysis of donor tissue that was dissociated, plated, and examined for BrdU incorporation showed that approximately 30% of the cells used for grafting were positive for BrdU staining (Figs. 1A, 1B, 1E, and 1F). Similarly, when these same cells were stained for the neural progenitor marker nestin (Lendahl *et al.,* 1990) approximately 30% of the cells were nestin-positive (Figs. 1C and 1D). Hence, a substantial number of each of the donor progenitor populations was actively undergoing proliferation at the time of their isolation. It has been demonstrated that postmitotic cells are incapable of integrating into host brains after intraventricular grafting (Cattaneo *et al.,* 1994). This suggests that it is the actively proliferating population that is able to integrate into the host tissues.

### Protease Treatment Affects the Pattern of Diencephalic Progenitor Integration after Transplantation

To examine the role of different classes of cellular adhesion molecules in the maintenance of neuromeric identity, neural progenitors from either E12 or E14 donor animals were dissected from either the P2-DDi or the P5-VDi (Figs. 2A and 2B). These are derivatives of the diencephalon and secondary prosencephalon, respectively (described in Bulfone *et al.,* 1993). Previous studies in the telencephalon have suggested that cell adhesion molecules might provide a mechanism for the maintenance of prosomeric identity (Krushel and van der Kooy, 1993; Götz *et al.,* 1996; Olsson *et al.,* 1998). To test this hypothesis *in vivo* we transplanted P5-VDi or P2-DDi progenitors using conditions that selectively remove or retain different classes of surface molecules. Specifically, progenitors were (1) mechanically dissociated or dissociated with trypsin in the (2) presence or (3) absence of 10 mM $Ca^{2+}$. The presence of calcium during trypsinization protects distinct classes of $Ca^{2+}$-dependent adhesion molecules such as N-cadherin and the selectin ligand CD15. Indeed, after trypsin digestion in the presence of calcium, immunoblot analysis demonstrated the selective retention of the calcium-dependent molecule N-cadherin (Fig. 3B), but not the neurotrophin receptor, Trk B (Fig. 3C).

All donor cells were labeled with the fluorescent lipophilic dye PKH-26 and transplanted into E14 recipients. Brains were collected after a 3-, 5-, or 15-day survival period ($E14_{inject}$–$E17_{sacrifice}$, $E14_{inject}$–$P0_{sacrifice,}$ $E14_{inject}$–$P10_{sacrifice}$) and analyzed by fluorescence microscopy. Figures 4A and 4C show schematically the areas in the E17 embryonic forebrain in which cells were scored. While more posterior regions of the CNS were examined (i.e., the mesencephalon and metencephalon), no integration was ever observed in these regions (data not shown). Unfortunately, since the prosomeric divisions are no longer apparent within the E17 diencephalon, it is only by extrapolation that inferences as to whether prosomeric-specific integration has occurred can be garnered.

Results from 3- ($E14_{inject}$–$E17_{sacrifice}$) or 5-day ($E14_{inject}$–$P0_{sacrifice}$) survival periods were similar. For both populations of diencephalic progenitors studied (E12 P2-DDi and P5-VDi), mechanical dissociation alone resulted in the widest




I

BrdU positive cells

| | tot.#cells | #+cells | % + cells |
|---|---|---|---|
| E12 DDi | 1007 | 399 | 39.6% |
| E12 VDi | 1069 | 263 | 24.6% |
| E14 DDi | 991 | 322 | 32.5% |
| E14 VDi | 1204 | 209 | 17.4% |

Nestin positive cells

| | tot.#cells | #+cells | % + cells |
|---|---|---|---|
| E14 DDi | 1700 | 575 | 33.8% |
| E14 VDi | 1501 | 287 | 19.1% |

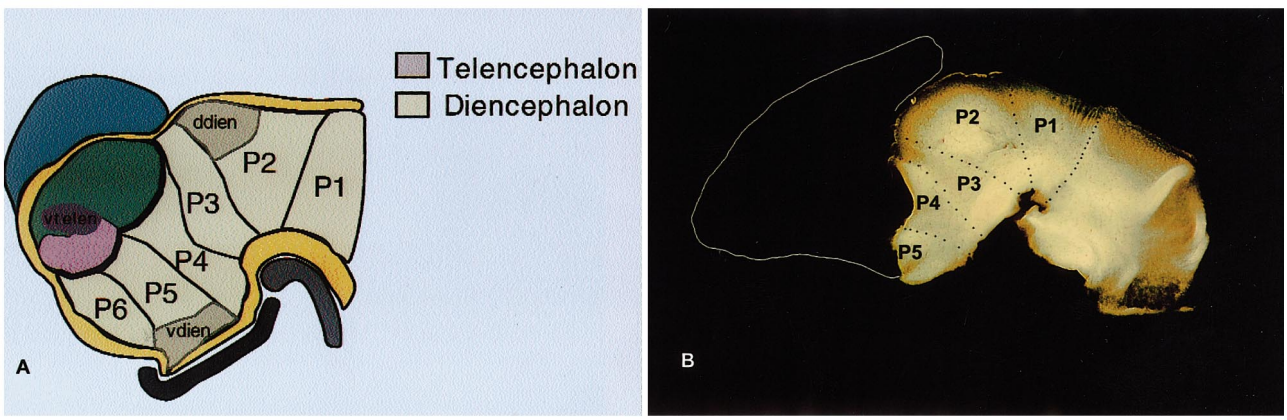



**FIG. 1.** Characterization of diencephalic progenitor cells. Diencephalic progenitor populations used for these transplantation studies were examined for their proliferation index and expression of the neural progenitor marker nestin. E12 and E14 timed pregnant CD1 female mice were injected three times with BrdU to label progenitors in the embryonic ventricular zone *in vivo* (see Materials and Methods for details). Aliquots of the single-cell suspensions used for the transplantations were fixed onto cytospin slides and were stained for BrdU incorporation and nestin expression by immunocytochemistry. (A) BrdU incorporation in E14 DDi and (B) VDi progenitors *in vitro* after labeling *in vivo*. (C and D) Nestin immunostaining of these same populations. (E and F) BrdU staining of E12 DDi and VDi progenitors *in vitro* after *in vivo* labeling. (G and H) *In vivo* BrdU staining in coronal sections within the forebrain of E12 embryos, from the same litters used for isolation of the E12 donor progenitors. (I) A table quantitating the percentages of BrdU- and nestin-positive cells in the populations used for transplantation.

**FIG. 2.** Schematic of the prosomeric divisions in the mouse prosencephalon. Tissue used for the isolation of progenitors in this study was dissected from prosomeres P2-DDi and P5-VDi. (A) A schematic of the prosomeres as they appear in the E10 mouse forebrain, as defined by Bulfone *et al.* (1993). The tissue dissected from prosomeres P2 and P5 used in this study have been highlighted in the schematic. Bulfone *et al.* have argued that the hypothalamus, which is derived from the ventral part of prosomere P5, is part of the secondary prosencephalon, while the P2 region used is part of the diencephalon. Nonetheless, classically the (P5-VDi) hypothalamus has been considered part of the diencephalon along with the thalamus and epithalamus (Altman and Bayer, 1986). (B) A whole-mounted explant showing the ventricular wall of the E12 mouse diencephalon with the location of the prosomeric divisions indicated. The forebrain of E12 mice has been isolated and the diencephalon bisected such that the medial wall of the third ventricle is visualized. Using oblique lighting on a dissection microscope prosomeres P1–P5 are visible in these explants when viewed from the ventricular surface. *Note.* The preoptic region, P6, was removed in the dissection.

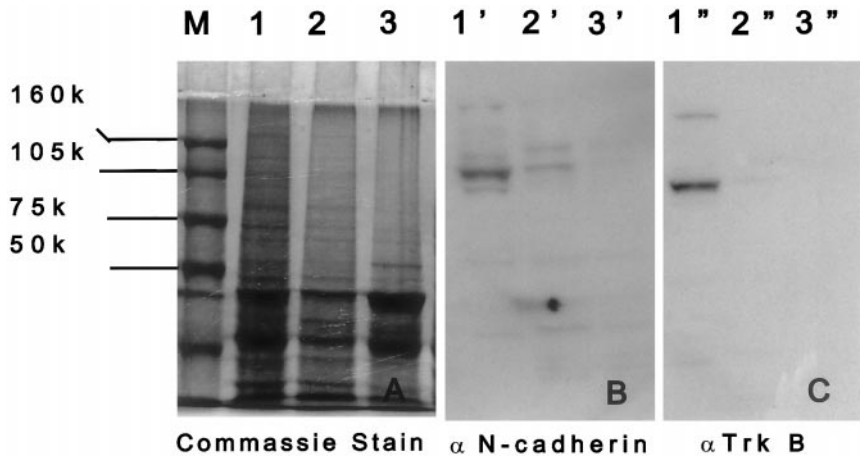



**FIG. 3.** Immunoblot analysis of proteins retained on progenitors, after dissociation by the three procedures used in this study: Whole-cell lysates of progenitor cells dissociated mechanically (lane 1) or with trypsin in the presence (lane 2) or absence (lane 3) of $Ca^{2+}$ were separated electrophoretically on 10% SDS–PAGE gels (A) and blotted onto nitrocellulose. Blots were probed with an antibody to N-cadherin (B) or Trk B (C). The Coomassie-stained gel in A shows a relative diminution of high-molecular-weight proteins in the trypsin-treated cells (lanes 2 and 3) compared to mechanically dissociated cells (lane 1). Calcium protects many high-molecular-weight bands from trypsinization as demonstrated by a comparison of lanes 2 and 3. Immunodetection with anti-N-cadherin antibody shows relative retention of a 130- (Linnemann *et al.*, 1994) and a 90-kDa (Paradies *et al.*, 1993) band in the trypsinized calcium-protected cell preparation (B, lane 2′) compared to cells trypsinized in the absence of calcium (B, lane 3′). Calcium provides no protection from trypsinization in the case of the 140-kDa transmembrane receptor Trk B (B, compare lanes 2″ and 3″).

spread pattern of integration (Figs. 4B, 4D, 5A, and 5C). Under these conditions, both P2-DDi and P5-VDi progenitors were capable of integrating and migrating within the telencephalon. Interestingly, P5-VDi progenitors integrate and migrate within the telencephalon better than P2-DDi progenitors, suggesting that this region may share progenitors with portions of the telencephalon (Golden and Cepko, 1996).

It is also interesting to note that when mechanically dissociated P2-DDi and P5-VDi progenitors integrated into the diencephalon they did not do so in a neuromere-specific manner. Both P2-DDi and P5-VDi mechanically dissociated progenitors showed roughly the same frequency of integration into different regions of the diencephalon (Figs. 4B and 4D). This implies either that these cells lack prosomeric identity or that the permissive integration of progenitors under these conditions masks their intrinsic prosomeric specificity (see below).

When $Ca^{2+}$-protected trypsinized progenitors were transplanted into the cerebral vesicles of E14 recipients, they too were capable of integrating into the telencephalon, although like the mechanically dissociated cells, diencephalic cells that integrated into the telencephalon are eliminated by P10 (Figs. 4B, 4D, 5B, and 5D). Interestingly, P2-DDi and P5-VDi progenitors that integrate into the diencephalon do so in a prosomere-specific manner, suggesting that $Ca^{2+}$-dependent adhesion molecules play a role in the maintenance of prosomeric identity.

Progenitors from both P2-DDi and P5-VDi trypsinized without $Ca^{2+}$ protection (from either developmental age) were incapable of significant integration and migration within the telencephalon (Figs. 6C, 6F,4B, and 4D). P2-DDi and P5-VDi donor cells formed tight clusters of cells adherent to the wall (Fig. 6F), but failed to migrate into the underlying subventricular and intermediate zones (Fig. 6C). In a small number of cases, cells did traverse the ventricular wall of the telencephalon (as in Fig. 6C) but even then remained in close proximity to it. Furthermore, these cells were round and undifferentiated and may have been in the process of dying. Consistent with this notion, even in the above experiments in which the diencephalic progenitors are dissociated mechanically or trypsinized using $Ca^{2+}$-protected circumstances (both of which resulted in far greater telencephalic integration), all cells that integrated within the telencephalon disappeared by P10.

In contrast, P2-DDi and P5-VDi cells dissociated after trypsinization without $Ca^{2+}$ protection integrated into the diencephalon, migrated extensively, and were able to differentiate appropriately (Figs. 6A, 6B, 6D, and 6E), as evidenced by their elaboration of axons and multiple dendritic processes. P2-DDi and P5-VDi progenitors, however, did not preferentially integrate into specific A/P regions of the diencephalon, showing no preference for the prosomeric region from which they were isolated. This pattern of integration suggests that both P2-DDi and P5-VDi progenitors possess trypsin-insensitive adhesion molecules that specify diencephalic but not prosomeric identity.

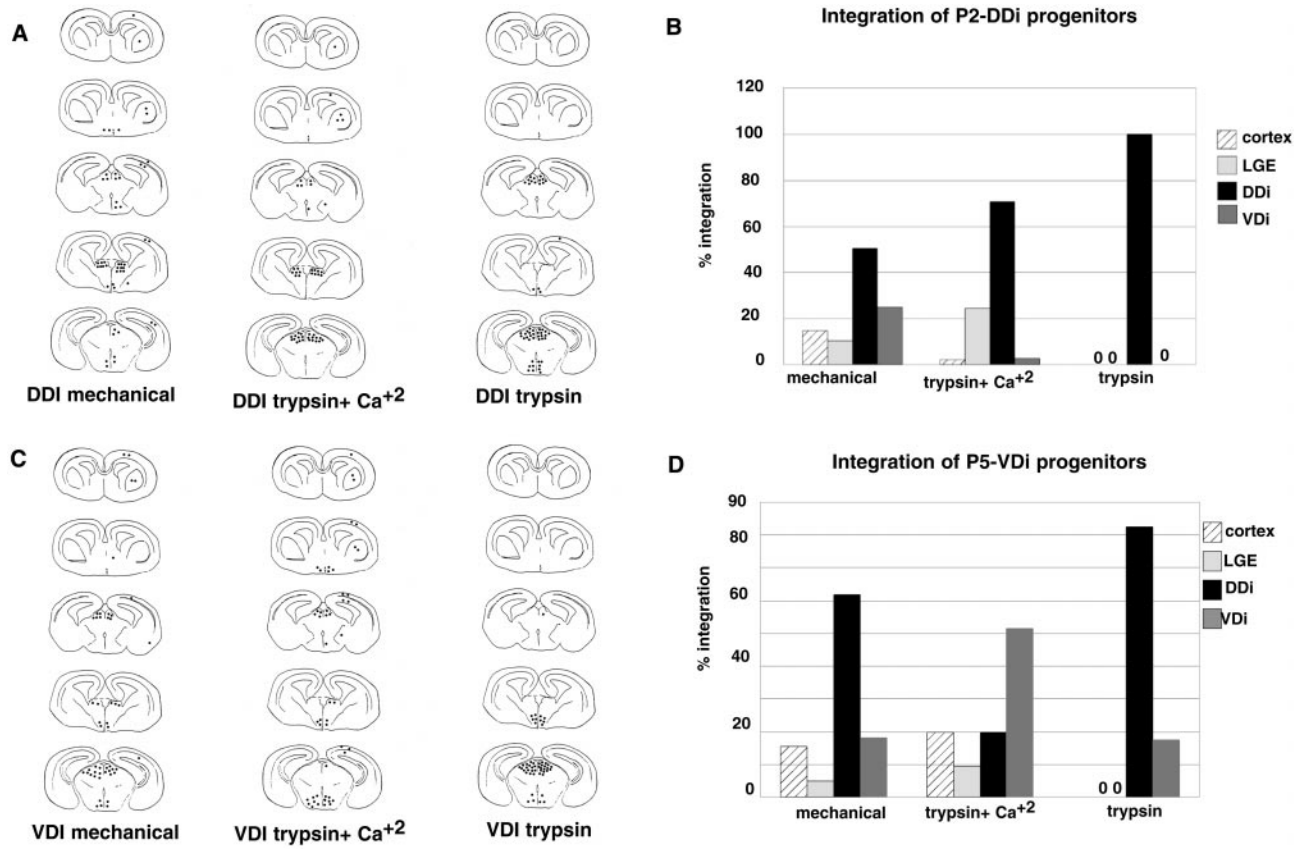



**FIG. 4.** Distribution of E12 diencephalic progenitor cells after transplantation and survival in host animals. (A and C) Schematics depicting the areas in the E17 embryonic forebrain into which different populations of E12 diencephalic donor cells integrated after E14 transplantation. Each dot represents 2% of the total number of PKH-labeled cells observed in the total number of brains examined for each treatment condition. Placement of the dots indicates the position of labeled cells along the anterior/posterior and dorsal/ventral axes. The three different conditions (mechanical dissociation or mechanical dissociation with trypsin with or without protection with calcium) used for cell dissociation at each time point (E12 and E14) are shown. Integration of E12 DDi and VDi progenitors (B and D). A summary of the tabulated data is represented graphically. Columns represent % DDi (B) or VDi (D) progenitor cell integration into the indicated forebrain areas.

### *The Distribution of Different Classes of Adhesion Molecules within the Prosencephalon*

Our studies implicated adhesion molecules as being mediators of migration, differentiation, and prosomeric identity. We therefore investigated the localization of a number of the molecules that are candidates for being involved in these processes within E14 forebrain. We examined by fluorescence immunohistochemistry the localization of N-cadherin, $\beta$-1 integrin, L1, the selectin ligand CD15 Lewis$^x$ antigen, and R-cadherin (Fig. 7). Notably, although many of these classes of molecules are not homogeneously distributed within the prosencephalon, only a few such as R-cadherin and the selectin ligand CD15 have distributions that truly respect at least some of the prosomeric boundaries.

At E14 in the mouse telencephalon, N-cadherin staining was throughout the forebrain but was strongest in the apical surface of the ventricular zone (Fig. 7A) (Packer *et al.*, 1997, mouse; Hatta *et al.* 1986, chick). $\beta$-1 integrin staining was homogeneous throughout the A/P and D/V axes of the forebrain (Fig. 7B). The calcium-independent trypsin-sensitive adhesion molecule L1 is localized in the differentiating zones of the forebrain (Fig. 7C). L1 antibody appears to stain axonal fibers of differentiating neurons in this zone.

In the mouse telencephalon, CD15 is localized to the dorsolateral walls of the cortex, but not the LGE (Fig. 7D), as well as in specific regions within the diencephalon (Fig. 7E). This staining pattern was consistent with the pattern previously described in the rat using the antibody to FORSE-1 that recognizes the CD15 Lewis$^x$ antigen (Tole *et al.*, 1995). FORSE-1 shows a banded expression pattern in the diencephalon, being highly expressed in both the dorsal and the ventral thalamus, but absent from the zona limitans intrathalamica (ZLI).

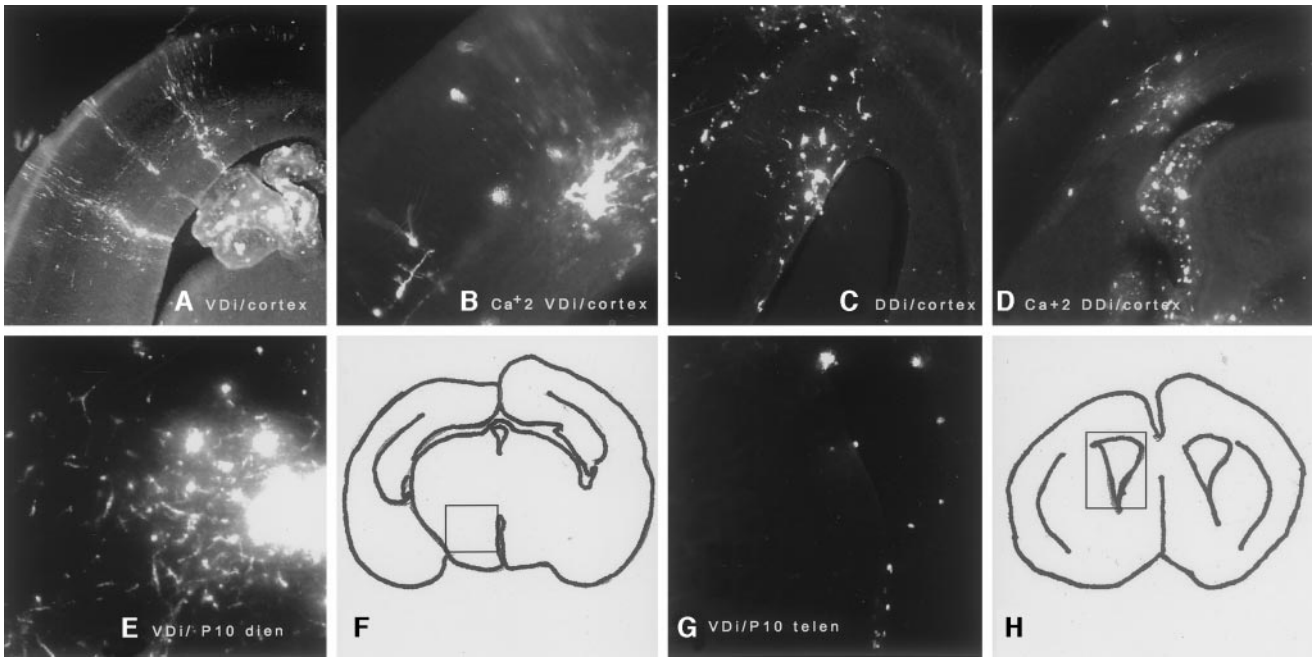



**FIG. 5.** Retention of cell adhesion molecules on the surface of both VDi and DDi progenitors allows integration and migration of progenitors into the telencephalon. In animals sacrificed at E17, E12 mechanically dissociated (A, VDi; B, DDi) or $Ca^{2+}$-protected trypsinized (C, VDi; D, DDi) progenitors integrate and migrate within the telencephalon (both cortex and LGE) after E14 intrathecal grafting. As with mechanically dissociated donor progenitors, extensive integration and migration was also observed within the diencephalon. While analysis of P0-sacrificed animals was indistinguishable from that of E17-sacrificed animals, in animals sacrificed at P10 (E–H), donor progenitors were no longer seen within the telencephalon. The faint signal seen in (G) is artifact also observable in the FITC channel. Quantitative analysis of the patterns of integration of mechanically dissociated or $Ca^{2+}$-protected trypsinized progenitors is depicted in Fig. 4.

In the E14.5 diencephalon, R-cadherin is expressed highly at ZLI, as well as at the junction of prosomeres P2/P3 (Fig. 7F). It is also present in the prospective ventral thalamus (P3) and the hypothalamus (P5) (Figs. 7F and 7G). Within the telencephalon, R-cadherin is expressed in lateral cortex and the cortical–striatal sulcus but is not expressed within the adjacent striatum (Figs. 7H and 7I).

While a plethora of other generic and region-specific adhesion molecules are no doubt present, this sampling demonstrates some of the diversity in expression patterns of this class of molecules. Together they suggest that determining the relationship between prosomeric identity and cell adhesion will be complex. Nonetheless, the approach of removing broad classes of adhesion molecules demonstrates that these surface proteins likely play an important role in both differentiation and the maintenance of regional identity (Takeichi, 1977; Götz *et al.*, 1996).

### *Retroviral Misexpression of R-cadherin Suggests It Plays a Role in Maintaining Prosomeres*

The immunolocalization studies discussed above implicated R-cadherin as a possible candidate to mediate the prosomere-specific calcium-dependent adhesion we observed in our transplantation assays. To test if R-cadherin expression is sufficient to direct the integration of progenitors after grafting into R-cadherin-expressing regions, we misexpressed FL R-cadherin using retroviral vectors in E12 mouse P2-Ddi progenitors. Retroviral constructs were based on the dicistronic pCLC vector (Gaiano *et al.*, 1999), which uses a hybrid CMV/$\beta$-actin internal promoter and carries the histochemical marker human placental alkaline phosphatase (PLAP) (Fig. 8A). P2-Ddi progenitors dissociated from the ventricular surface of E12 mouse diencephalon were infected with either control (CLC) or FL R-cadherin-containing virus *in vitro* at an m.o.i. of 3.5 for 2 h in neurosphere medium. Cells were then washed free of exogenous virus and grown as neurospheres for 7 days to allow for viral expression and progenitor expansion. Cells were then dissociated with trypsin in the presence of 10 mM calcium, PKH 26 labeled, and transplanted into E14 host embryos as described above. After a 3-day survival, E17 embryos were sacrificed and the forebrains examined by fluorescence microscopy to assess the number and location of labeled cells.

Both control-infected and FL R-cadherin-infected P2-Ddi progenitors were capable of integrating into both the telencephalon and the diencephalon, as had been previously

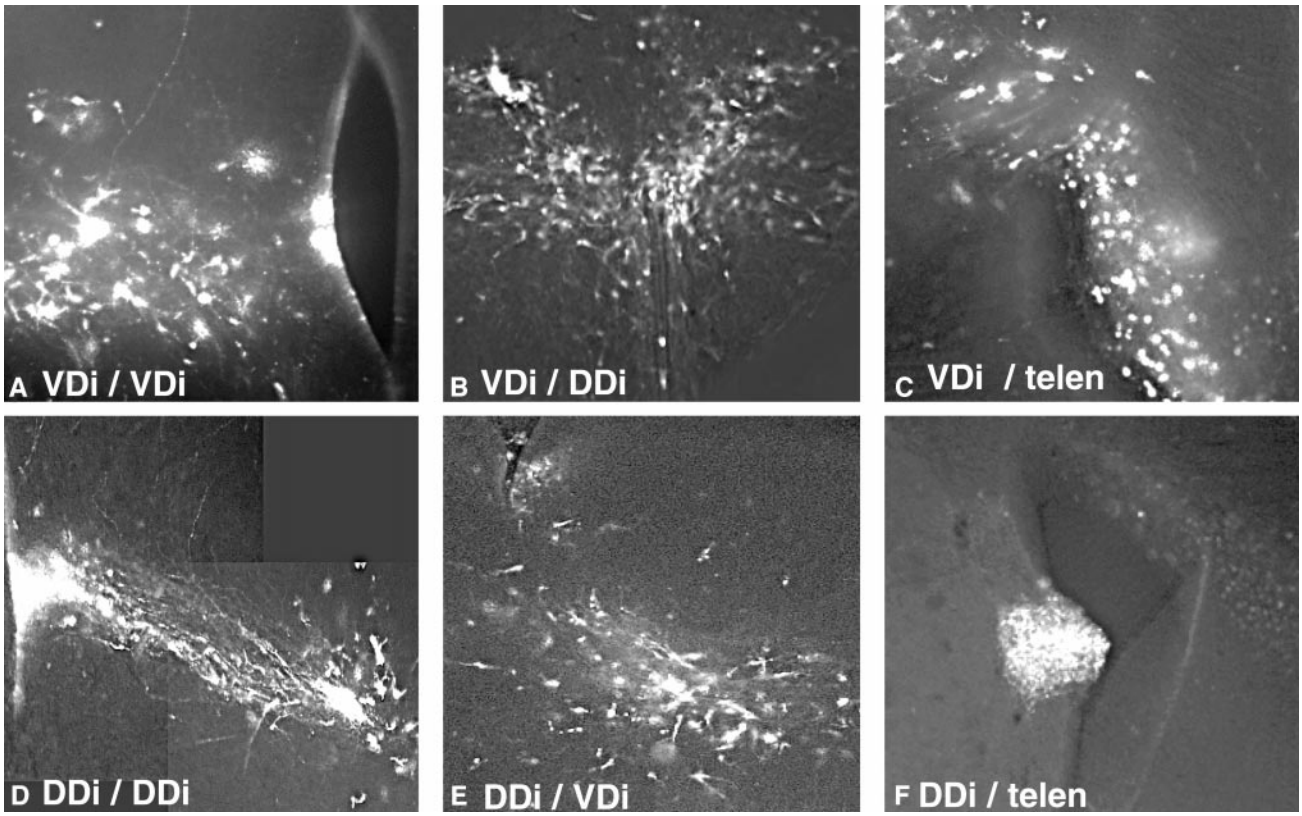



**FIG. 6.** Representative examples of integration of trypsinized diencephalic progenitors into the diencephalon and telencephalon. Both E14 VDi (A and B) and DDi (D and E) can integrate and migrate into the dorsal and ventral diencephalon of age-matched recipients, but neither cell type is capable of integration and migration into the telencephalon. Within the telencephalon, diencephalic cells form tight clusters adherent to the ventricular wall (F) or fail to migrate into the subventricular and intermediate zones (C). Cells that traverse the ventricle are small and rounded and may be dying. Figure 4 presents a quantitative analysis of these data.

shown for $Ca^{2+}$-protected trypsinized P2-Ddi cells (Figs. 8B–8D). However, ectopic expression of FL R-cadherin within this cell population skewed the integration pattern toward R-cadherin-expressing regions, such as the cortex and the ventral diencephalon, reminiscent of the integration pattern of P5-Vdi progenitors. These results suggest that R-cadherin plays a role in the maintenance of prosomeres.

### *Cellular Adhesion Molecules Are Not Sufficient to Induce Progenitors to Change Regional Identity*

As noted above, diencephalic cells, which retained calcium-dependent and or calcium-independent adhesion molecules, are capable of integrating within the telencephalon. Notably, however, diencephalic cells that integrated into the telencephalon differentiated with morphologies atypical of their ectopic location. For instance within the cortex, DDi cells with processes resembling either axons or dendrites did not have characteristic cortical morphology. These findings contrast with our previous studies using LGE (i.e., of telencephalic origin) progenitors from E12 and E14 mouse embryos (Fishell, 1995; Na *et al.*, 1998). In those studies, we routinely observed that progenitors that integrated within the telencephalon differentiated with morphologies appropriate to their host region. For instance, in those experiments many of the LGE progenitors that integrated within the cortex adopted the typical cytoarchitecture of pyramidal or stellate neurons. The inability of E12 diencephalic progenitors to adopt telencephalic morphologies suggests that they may be unable to respond to regional cues within the telencephalon. Further diencephalic progenitors that integrate into the telencephalon disappear after long-term survivals. By P10, although large numbers of transplanted cells persist within the diencephalon, those cells that integrated into the telencephalon have vanished (see Figs. 5E–5H).

To further test whether diencephalic progenitors that integrated within the telencephalon are induced to express telencephalic-specific markers, donor tissues were taken from transgenics in which LacZ expression is under the control of promoter elements of the telencephalic-specific

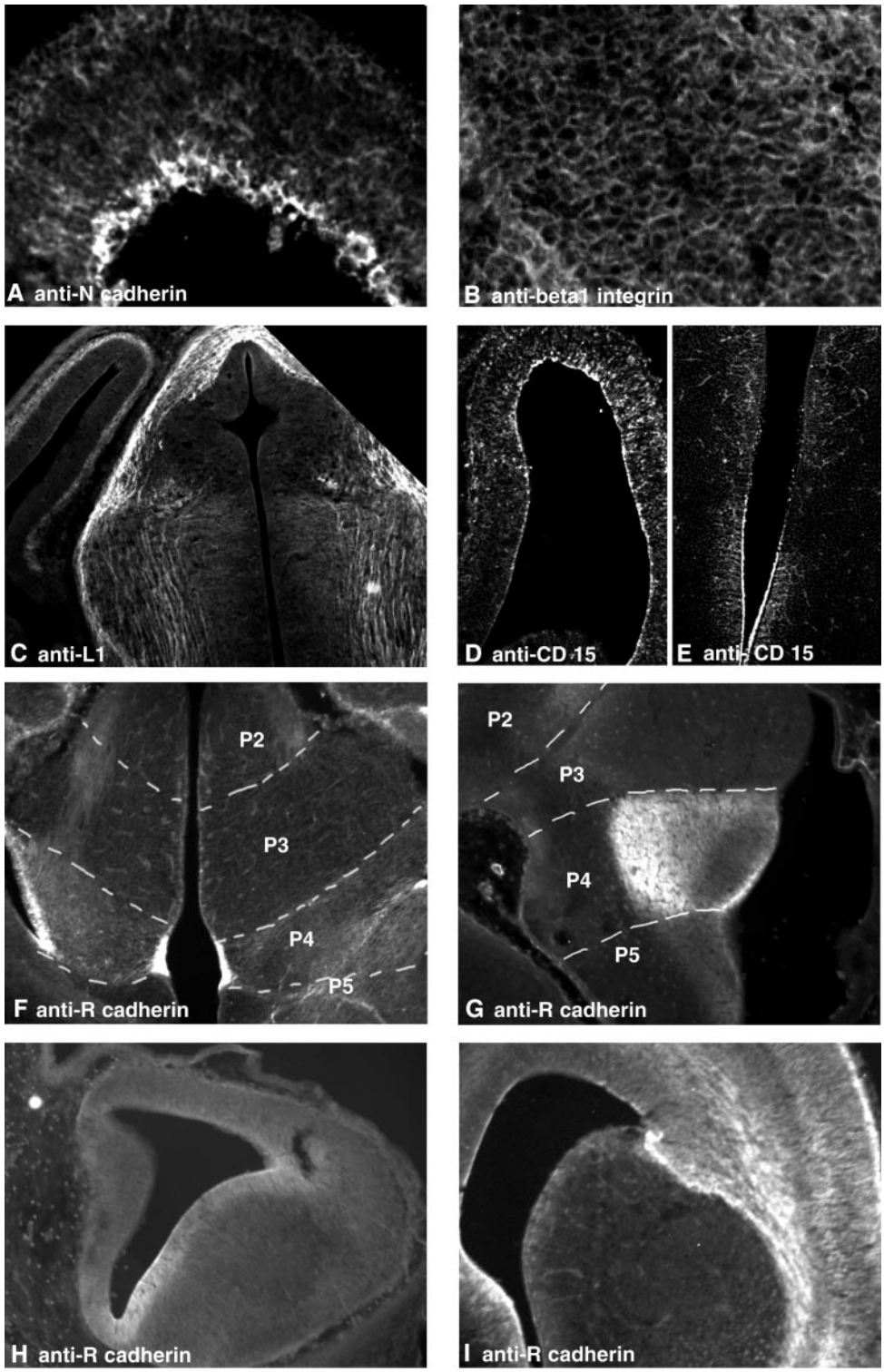



**FIG. 7.** Localization of adhesion molecules in the forebrain. $Ca^{2+}$-dependent adhesion molecules are preferentially located along the apical aspect of the ventricles of particular forebrain structures. (A) N-cadherin is expressed on the apical surface of the ventricular zone throughout the E14 cortex. Similar patterns of expression were observed in the striatum and diencephalon. (B) The CAD adhesion molecule $\beta$-1 integrin is homogeneously expressed on the plasma membrane of cells throughout the forebrain. (C) The Ig-domain-containing CAM, L1, is localized in the differentiating zones of the forebrain where it appears to be localized to axonal fibers. By fluorescence immunohistochemistry, CD15 $Lewis^x$ antigen is localized on the apical surface of neuroepithelial progenitor cells in E14 mouse cortex (D) and diencephalon (E). (F and G) R-cadherin staining is strong in the developing ventral thalamus (P4), less intense in the adjacent P5 prosomere, and not at all evident in P3 (F shows a coronal section, whereas G shows the same area in sagittal section). Coronal sections through the brain at E14 (H) show that R-cadherin expression is present in the dorsal and lateral cortex. (I) In a coronal section through the telencephalon at this age, R-cadherin staining is seen in the dorsal, medial, and lateral cortex and is highly expressed at the cortical–striatal sulcus, but not in the striatum.

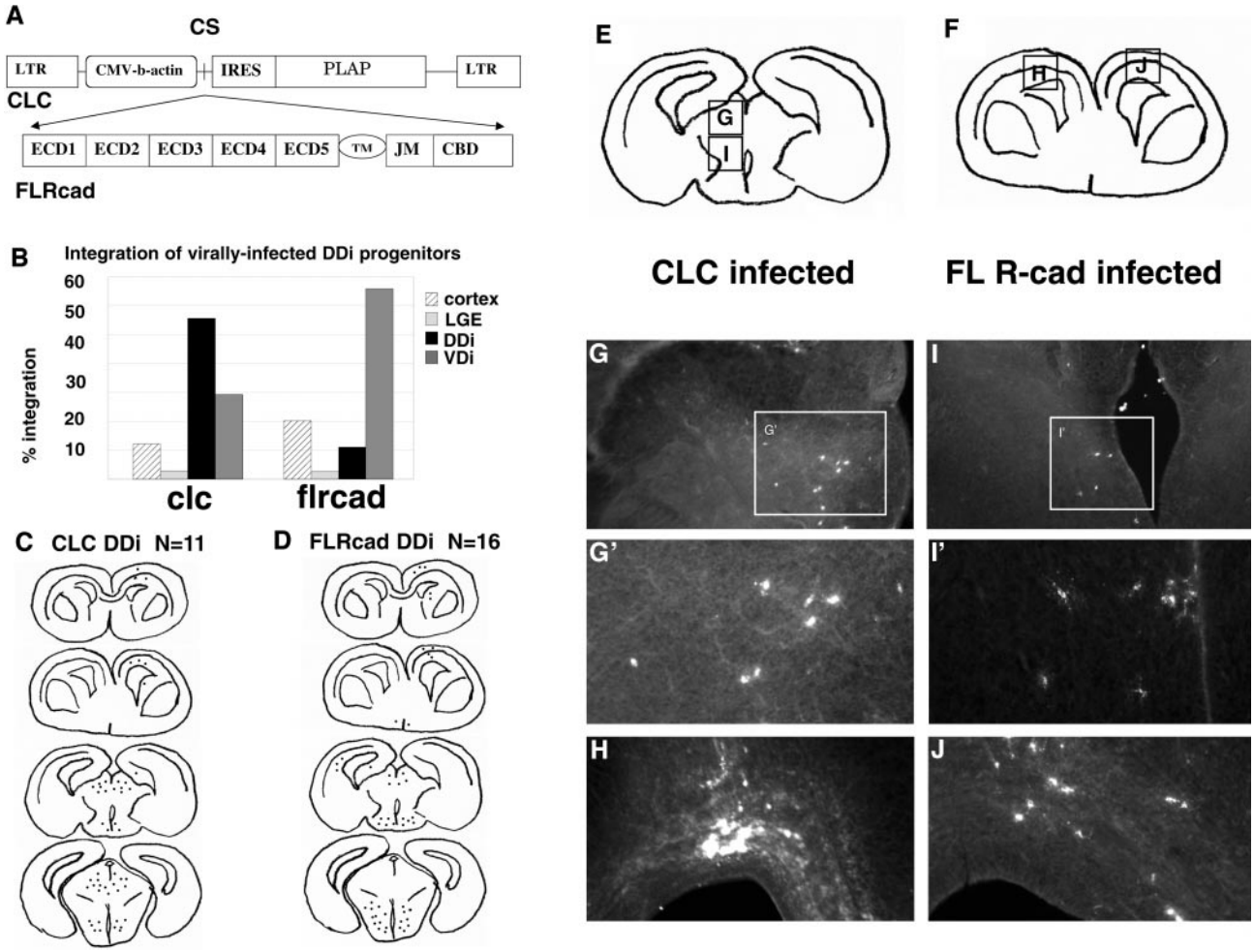



**FIG. 8.** Forced expression of FL R-cadherin in P2-Ddi cells alters their pattern of integration to regions where R-cadherin is abundant. (A) P2-Ddi progenitors were infected with either CLC control or FL R-cadherin-expressing virus *in vitro.* Control or R-cadherin virus-infected cells were then expanded *in vitro* for 7 days in the presence of bFGF. Control cells upon transplantation into host embryos preferentially integrate into their region of origin (Ddi) (B, C, G). To a lesser extent these cells also integrate into the cortex (C and H). In contrast, R-cadherin virus-expressing Ddi cells alter their pattern of integration and are predominantly found within the Vdi (B, D, I). In addition, R-cadherin-expressing Ddi cells show an increased propensity to integrate within the cortex (D and J). Notably, areas of increased integration of FL R-cadherin Ddi cells are predicted by the endogenous pattern of R-cadherin expression. (E and F) Schematics of the regions of integration shown at higher power in G–J.

gene *BF-1* (Xuan *et al.*, 1995) or the cortical-specific gene *Emx-1* (unpublished data). Mechanically dissociated P2-DDi or P5-VDi progenitors (from E12 heterozygous knock-in embryos for *BF-1-lacZ* or *Emx-1*) that integrated into the telencephalon did not express the lacZ transgene (data not shown). This suggests that these progenitors are not competent to respond to regional cues within the telencephalon. Unfortunately, diencephalic cells do not persist in the telencephalon long enough for us to assay mature neuronal markers such as region-specific neurotransmitters. Nonetheless their disappearance in and of itself suggests that they do not adopt a telencephalic phenotype.

## DISCUSSION

In this study, we found that removal, maintenance, or ectopic expression of cell surface adhesion molecules dramatically affects the pattern of integration of diencephalic progenitors upon intraventricular transplantation (Figs. 4B and 4D). Through this approach, we have been able to investigate the relationship between broad classes of cell surface molecules and their possible role in the maintenance of prosomeres. Our findings can be summarized by three observations. CAD molecules are required for transplanted progenitors to integrate appropriately into specific prosomeres. R-cadherin biases progenitors to integrate into

regions expressing this molecule. Diencephalic cells that integrate into the telencephalon are unable to express telencephalic markers and are ablated postnatally. In accordance with these findings, three general conclusions can be inferred: (1) CAD adhesion systems may be causally related to the maintenance of prosomere organization. (2) R-cadherin can mediate homotypic cell adhesion *in vivo*. (3) Diencephalic progenitors may possess cell autonomous regional character.

### *Role of Adhesion Molecules in the Developing Forebrain*

Our studies underscore the notion that cell adhesion molecules subserve multiple functions during development (Redies, 1995; Redies and Takeichi, 1996). While cellular adhesion molecules appear to function globally to promote the migration and differentiation of neurons, a subset of regionally expressed calcium-dependent adhesion molecules evidently assists in the maintenance of regional identity (Redies, 1995; Ganzler and Redies, 1995; Korematsu and Redies, 1997a,b; Redies and Takeichi, 1996). Our misexpression studies suggest that R-cadherin functions in the maintenance of the prosomeric organization of the ventral thalamus, hypothalamus, and cortex. In the following, we discuss our findings in light of previous studies that have identified specific adhesion molecules that have been proposed to function in the differentiation and regionalization of the prosencephalon.

### *Adhesion Molecules Promote Migration and Differentiation*

In addition to molecules with prosomere-specific patterns of expression, cell adhesion molecules shared between all regions of the prosencephalon must mediate the ectopic integration of diencephalic progenitors in the forebrain after transplantation. We demonstrate this by showing that removal of these molecules by protease treatment restricts progenitors to homotypic integration within the diencephalon. The molecules implicated by these experiments include both CAD and CID adhesion molecules. In accordance with this, many cellular adhesion molecules are shared between regions of the prosencephalon. For instance, a number of Ig-domain-containing molecules such as N-CAM, TAG-1, and L1 have been shown to participate in general mechanisms of neuronal migration and differentiation (Lindner *et al.*, 1983; Tomasiewicz *et al.*, 1993; Cremer *et al.*, 1994; Wolfer *et al.*, 1994; Redies *et al.*, 1997).

Specifically, we noted that the site of highest N-cadherin expression within the prosencephalon is at the apical surface of the ventricular zone. This is consistent with this molecule playing a role in the initial entry of progenitor cells from the ventricle into the parenchyma. Recent experiments in the chick suggest that N-cadherin has a role in allowing the emigration of neural crest cells from the neural tube (Nakagawa and Takeichi, 1998; Ganzler-Odenthal and Redies, 1998). We have found in preliminary studies that misexpression of a dominant negative N-cadherin virus in the E9.5 mouse forebrain results in the retention of infected progenitors in the ventricular zone at early times and biases their differentiation toward a glial fate by postnatal day 21 (data not shown). Consistent with this notion, Kwon *et al.* (2000) have reported a functional interaction between N-cadherin and p35–Cdk5 kinase complex. Notably, p35 (−/−) mice have a defect in migration of cortical neurons that results in the inability of cortical progenitors to migrate past their predecessors to form the normal inside-out layering of cortex. Clearly, an understanding of the functional interactions between adhesion molecules and other mechanisms affecting development is still in its early phases. Nonetheless, both our results and those of others suggest greater insight into this question will be forthcoming.

### *Calcium-Dependent Molecules Are Necessary for Maintenance of Prosomere-Specific Adhesion*

A number of CAD adhesion molecules, such as R- and E-cadherin, are expressed in complex patterns within specific regions of the diencephalon and telencephalon (the present results; Ganzler and Redies, 1995; Arndt and Redies, 1996; Korematsu and Redies, 1997a,b; also reviewed in Redies and Takeichi, 1996). Based on their patterns of expression, cadherin-6, -7, -8, -10, and -11, as well as E-, N-, and R-cadherin, have all been implicated in the regionalization of the forebrain (Redies and Takeichi, 1996). Another potential candidate that may play a role in this process is CD15 Lewis$^x$. CD15 Lewis$^x$ antigen has been shown to be identical to the FORSE-1 antigen (also called SSEA-1) and its presence has been demonstrated in many locations within the forebrain (Allendoefer *et al.*, 1995). Like many of the cadherins, CD15 has a banded pattern of expression within the diencephalon that respects the prosomeric boundaries. Furthermore, individuals with Rambam–Hasharon syndrome, in which proteins bearing the CD15 Lewis$^x$ antigen are lost, have severe mental retardation, cortical atrophy, and seizures (Frydmann *et al.*, 1992).

Our findings suggest that a subset of $Ca^{2+}$-dependent adhesion molecules may be required for the establishment and maintenance of prosomeres. This is evidenced by the increased fidelity of $Ca^{2+}$-protected trypsinized progenitors to integrate into the prosomeric region from which they were isolated. Cadherins have been shown to mediate homotypic cell adhesion and to be involved in mediating adhesion in multiple contexts during development (Knudsen *et al.*, 1998). Here we provide *in vivo* evidence that R-cadherin can directly bias progenitors expressing this molecule to integrate into R-cadherin-positive regions of the prosencephalon. While these data strongly support the notion that R-cadherin is required for the maintenance of forebrain prosomeres, direct proof of this hypothesis awaits loss-of-function data.

### *Individual Diencephalic Progenitors Retain Intrinsic Diencephalic Character*

The finding that trypsinized E12 diencephalic progenitors preferentially integrate into the diencephalon suggests that, by this point in development, these progenitors may be intrinsically specified to a diencephalic fate. The molecular basis of this specificity could be mediated by adhesion mechanisms dependent on specific glycolipids or trypsin-resistant cell adhesion molecules synthesized in diencephalic, but not telencephalic, progenitors. However, this specification is not mediated by cell adhesion mechanisms alone, since diencephalic progenitors that ectopically integrate into the telencephalon rarely take on morphologies consistent with their ectopic location. Rather, in most cases, these cells maintain an immature cellular morphology in the telencephalon. In addition, diencephalic cells that ectopically integrate into the telencephalon fail to express the telencephalic marker *BF-1* or the cortical marker *Emx-1* (results not shown). Furthermore diencephalic cells that integrate into the cortex disappear postnatally. Together these findings are consistent with the idea that by E12.5 these cells may already have a specified regional identity.

In conclusion, our findings suggest that a variety of adhesion systems contribute globally to differentiation within the forebrain but are not sufficient to specify their regional phenotype. The global importance of CAMs for integration implicates a role for adhesion systems in the promotion of early steps in neuronal migration and differentiation. In parallel the actions of prosomere-specific adhesion molecules likely act to maintain segmental identity. Finally, regardless of which adhesion molecules were preserved, diencephalic cells that integrate into the telencephalon fail to express telencephalic markers and are ablated postnatally. Hence it seems that while adhesion molecules are active in controlling progenitor interactions, diencephalic cells may be autonomously specified in forebrain from the onset of neurogenesis.

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