

# Distinct Functions of $\alpha_3$ and $\alpha_v$ Integrin Receptors in Neuronal Migration and Laminar Organization of the Cerebral Cortex

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## Summary

Changes in specific cell–cell recognition and adhesion interactions between neurons and radial glial cells regulate neuronal migration as well as the establishment of distinct layers in the developing cerebral cortex. Here, we show that  $\alpha_3\beta_1$  integrin is necessary for neuron–glial recognition during neuronal migration and that  $\alpha_v$  integrins provide optimal levels of the basic neuron–glial adhesion needed to maintain neuronal migration on radial glial fibers. A gliophilic-to-neurophilic switch in the adhesive preference of developing cortical neurons occurs following the loss of  $\alpha_3\beta_1$  integrin function. Furthermore, the targeted mutation of the  $\alpha_3$  integrin gene results in abnormal layering of the cerebral cortex. These results suggest that  $\alpha_3\beta_1$  and  $\alpha_v$  integrins regulate distinct aspects of neuronal migration and neuron–glial interactions during corticogenesis.

## Introduction

The emergence of the functionally crucial laminar organization of the cerebral cortex depends on the appropriate migration and placement of neurons (Caviness and Rakic, 1978; Barth, 1987; Rakic, 1988a, 1988b, 1990; Caviness et al., 1989; Gray et al., 1990; Hatten, 1993; Reid et al., 1995; Volpe, 1995; Tan et al., 1998). In the developing cerebral cortex, the translocation of a neuron from the ventricular zone to its specific location involves initiation of cell movement, active migration along an appropriate pathway, and arrest of migration and deadhesion from radial glial guides as the neuron settles in the appropriate laminae of the cerebral cortex (Rakic, 1971, 1972). Specific cell–cell recognition, adhesion interactions between neurons, radial glia, and the surrounding extracellular matrix (ECM), are likely to instruct a neuron to begin, maintain, alter, or end its migration (Lindner et al., 1983; Grumet et al., 1985; Chuong et al., 1987; Edelman, 1988; Kunemund et al., 1988; Rutishauser and Jessell, 1988; Sanes, 1989; Chuong, 1990;

Fishell and Hatten, 1991; Takeichi, 1991; Galileo et al., 1992; Grumet, 1992; Fishman and Hatten, 1993; Tomasiwicz et al., 1993; Rakic et al., 1994; Stipp et al., 1994; Zheng et al., 1996; Baum and Garriga, 1997; Goldman and Luskin, 1998; Zhang and Galileo, 1998). A family of molecules that are capable of modulating such a phenomenon are integrins, the major cell surface receptors for the ECM that mediate cell–ECM and cell–cell adhesive interactions. Integrins are uniquely suitable for this purpose, since (1) different integrin receptors display different adhesive properties, (2) different integrins may regulate different intracellular signal transduction pathways and thus, different modes of adhesion-induced changes in cell physiology, and (3) integrins are capable of synergizing with other cell surface receptor systems to finely modulate a cell's behavior in response to multiple environmental cues (reviewed by Hynes and Lander, 1992; Vuori and Ruoslahti, 1994; Beauvais et al., 1995; Behrendtsen et al., 1995; Clark and Brugge, 1995; Cooper et al., 1995; Hadjiargyrou et al., 1996; Mannion et al., 1996; Stefansson and Lawrence, 1996; Wei et al., 1996; Banerjee et al., 1997; Palecek et al., 1997). Developmental changes in the cell surface integrin repertoire may thus modulate neuronal cell migratory behavior by altering the strength and ligand preferences of cell–cell adhesion during development.

Functional integrin receptors are heterodimers of  $\alpha$  and  $\beta$  subunits. There are 16  $\alpha$  subunits and 8  $\beta$  subunits, and they dimerize in multiple combinations to form over 20 different integrin receptors (Hynes, 1992). In general,  $\alpha$  subunits are thought to play a determinant role in the ligand specificity and thus, the biological response of individual integrin receptors. The binding of ECM or other cell surface molecules to the extracellular domains of integrins activates signal transduction cascades involving a diverse group of molecules, such as  $\text{Ca}^{2+}$ , protein kinases, phosphatases, SH2–SH3 adapter proteins, small GTPases, and phospholipid mediators, leading eventually to changes in the spatial localization of integrins on the cell surface and integrin–actin cytoskeleton interactions (Clark and Brugge, 1995; Hannigan et al., 1996). Developing neurons and glia in the CNS express a variety of integrin receptors and their ECM ligands (Galileo et al., 1992; Hirsch et al., 1994; Georges-Labouesse et al., 1998; Zhang and Galileo, 1998). Individual ECM components, including fibronectin, tenascin, thrombospondin, glycosaminoglycans, and laminin isoforms, as well as integrin-associating molecules such as CD9, are expressed in the developing cerebral cortex (O'Shea and Dixit, 1988; Liesi, 1990; O'Shea et al., 1990; Sheppard et al., 1991; DeFreitas et al., 1995).

The presence of a distinct set of integrins and their ligands in the developing cortical neurons and their associated radial glial cells suggests that they play a dynamic role in the emergence of the cerebral cortex (Galileo et al., 1992; Georges-Labouesse et al., 1998; Zhang and Galileo, 1998). To analyze the role of integrins in distinct aspects of neuronal migration and the resultant laminar organization of the cerebral cortex, we investigated the function of two major integrins,  $\alpha_3$  and  $\alpha_v$ , that

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are associated with developing neurons and radial glia (Hirsch et al., 1994; DeFreitas et al., 1995; Jacques et al., 1998) in cortical development. These studies suggest that the developmental regulation of  $\alpha_3$  and  $\alpha_v$  integrins on neuronal and glial cell surface modulates the active migration of undifferentiated neurons along radial glial pathways and the eventual aggregation of neurons into distinct layers in the cerebral cortex.

## Results

### Differential Distribution of $\alpha_3$ and $\alpha_v$ Integrin Receptors in the Developing Cerebral Wall

Changes in the adhesive behavior of neurons in different regions of the developing cerebral wall are reflected in distinct changes in cell function, shape, process extension, and cell-cell attachment (Rakic et al., 1974). To evaluate the paradigm that specific cell-cell recognition, adhesion interactions between neurons, radial glia, and the surrounding ECM, may instruct a neuron to begin, maintain, alter, or end its migration, we analyzed the expression patterns of  $\alpha_3$  and  $\alpha_v$  integrin receptors in the embryonic day 16 (E16) cerebral wall, when great numbers of neurons are in the process of migration.

$\alpha_3$  is expressed in a gradient-like fashion across the cerebral wall: highly in the ventricular zone cells and in the migratory neurons of the intermediate zone but downregulated in the postmigratory, upper cortical plate neurons (Figure 1A). In some brain sections, a small population of multipolar neurons in the intermediate zone was also immunolabeled with anti- $\alpha_3$  antibodies (data not shown).  $\alpha_v$  is present primarily in radial glial cells (Figure 1B). In vitro,  $\alpha_3$  and  $\alpha_v$  integrins are expressed in neurons and radial glia, respectively (Figures 1C–1E).

The distinctly different patterns of integrin receptor expression across the developing cerebral wall are indicative of the role these receptors may play in the adhesive properties, and thus the distinct phenotypic behavior of neurons as they traverse the embryonic cerebral wall from ventricular zone to their target layers. The complement of integrins expressed may enable developing neurons to choose selective pathways of migration in the developing cerebral cortex and modulate the rate at which they would migrate. We thus analyzed their function in neuronal migration and layer formation in the cerebral cortex.

### Distinct Effects of $\alpha_3\beta_1$ - and $\alpha_v$ -Containing Integrins in Neuronal Migration

The coordinated regulation of cell-cell recognition and adhesion and cell motility mechanisms underlies the migration of neurons from the ventricular zone to their respective layers in the developing cerebral cortex. To determine how  $\alpha_3\beta_1$  and other integrins might regulate distinct aspects of this dynamic process, we examined the consequences of inhibiting different neuronal and radial glial integrins during neuronal migration on radial glial substrates in vitro. The blockade of  $\alpha_3\beta_1$  receptors with Ralph3-1 monoclonal antibodies (mAbs) reduced the rate of migration by 36% but did not cause neuronal

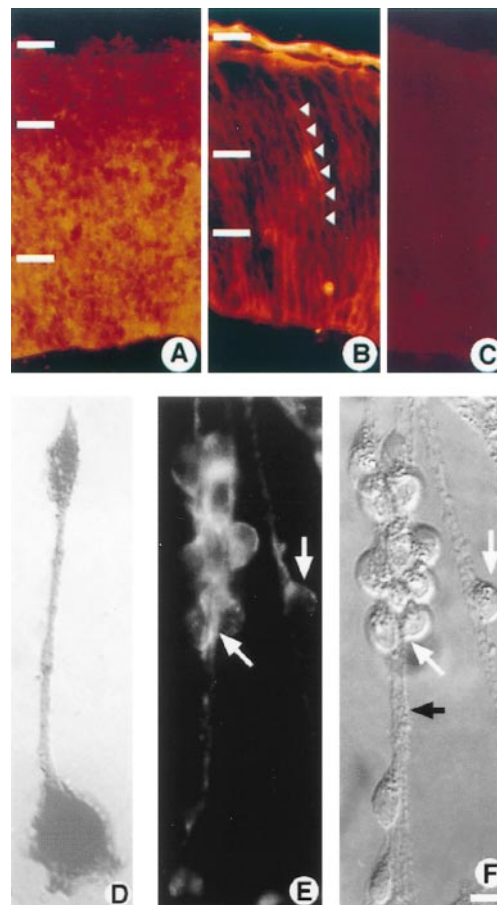
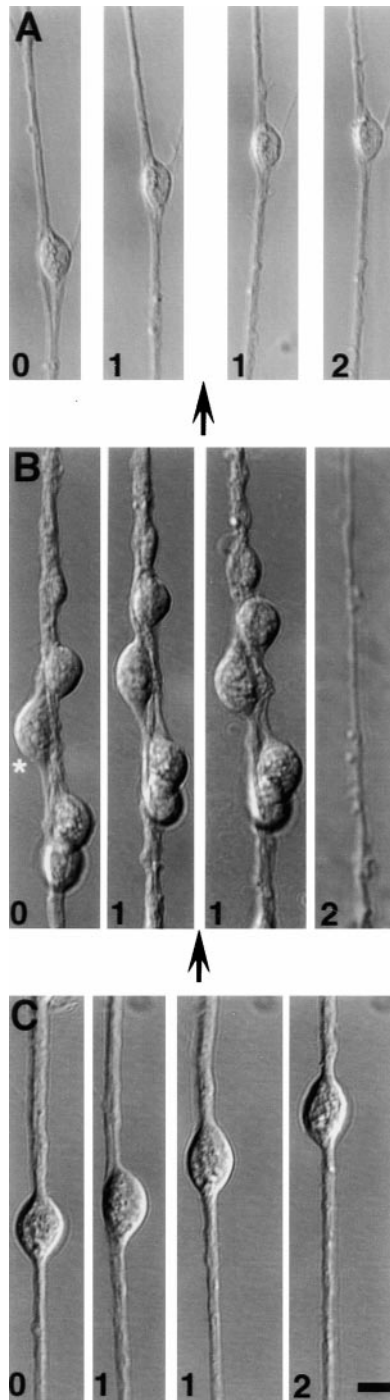


Figure 1. Differential Localization of Integrin Receptors in the Developing Cerebral Wall

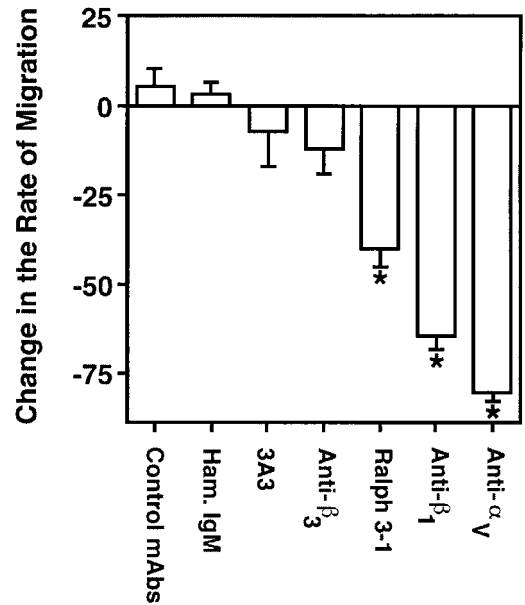
(A–C) Cryostat sections of E16 cerebral wall were immunolabeled with subunit-specific polyclonal anti- $\alpha_3$  (A),  $\alpha_v$  (B), and nonimmune rabbit serum (C).  $\alpha_3$  is expressed across the cerebral wall. However,  $\alpha_3$  expression is downregulated in the postmigratory, upper cortical plate neurons.  $\alpha_v$  is present primarily in radial glial cells. Arrowheads in (B) illustrate a radial glial process. Immunolabeling was visualized with Cy-3 immunofluorescence. Horizontal bars demarcate the different regions of the cerebral wall: top, cortical plate (CP); middle, intermediate zone (IZ); and bottom, ventricular zone (VZ).

(D–F) In cultured cortical neurons and radial glia,  $\alpha_v$  immunoreactivity is localized mainly to radial glial cells. A radial glial cell (D) was immunostained with a biotinylated anti- $\alpha_v$  hamster mAb and visualized with diaminobenzidine. In contrast,  $\alpha_3$  immunoreactivity is present primarily in migrating neurons and their leading and trailing processes (E and F). Migrating neurons and their radial glial guides were immunostained with anti- $\alpha_3$  Ralph3-1 mAbs and visualized with Cy-3 (E). (F) is a phase light image of (E) (migrating neurons, white arrow; radial glial process, black arrow). Immunoreactivity was absent when neurons and glia were stained with control mouse IgGs or hamster IgMs. Scale bar, 30  $\mu$ m (A–C) and 12.5  $\mu$ m (D–F).

detachment from their radial glial migratory guides (Figures 2A and 3). No changes in neuronal morphology other than the reduced rate of neuronal cell soma translocation were observed in the presence of Ralph3-1 mAbs. In contrast, the blockade of  $\alpha_v$  integrins that are present mainly in radial glial guides causes neurons to reduce their rate of migration (–79%), withdraw their leading and trailing processes, and eventually detach



**Figure 2.**  $\alpha_3$  and  $\alpha_v$  Integrins Modulate Cortical Neuronal Migration  
Cortical neuronal migration on radial glial processes was monitored before and after the addition of anti- $\alpha_3$  Ralph3-1 mAbs, anti- $\alpha_v$ , or control C1 mAbs. Images were taken at time 0 and 1 hr before the addition of antibodies (panels to the left of black arrow) and at 1 and 2 hr after antibody exposure (panels to the right of black arrow). Blocking  $\alpha_3$  receptors reduced the rate of neuronal migration ([A]; top four panels), whereas the inhibition of  $\alpha_v$  receptors led to a reduction in the rate of migration, the withdrawal of the leading and trailing processes of migrating neurons (see neurons next to and above the asterisk), and the eventual detachment of neurons from their radial glial guides ([B]; middle four panels). Exposure to control antibodies that recognize a ubiquitous neuronal and glial cell sur-



**Figure 3.** Effect of Anti-Integrin Antibodies on Cortical Neuronal Migration in Imprint Cultures

The rate of neuronal migration was measured before and after exposure to anti-integrin antibodies. Anti- $\alpha_3$ , - $\beta_1$ , and - $\alpha_v$  antibodies reduced the rate of migration by 40%, 63%, and 79%, respectively. Anti- $\alpha_1$ , - $\beta_3$ , control mouse mAbs, or hamster IgMs did not alter the rate of migration significantly. Anti- $\alpha_1$  (3A3), - $\alpha_3$  (Ralph3-1), and - $\beta_1$  (HA2/11) integrin antibodies and C1 control mAbs were added at 50  $\mu$ g/ml. Purified hamster monoclonal anti- $\alpha_v$  and - $\beta_3$  integrin antibodies and IgMs were added at 1  $\mu$ g/ml. The number of cells analyzed in each group are as follows:  $\alpha_1$ , 21;  $\alpha_3$ , 111;  $\beta_1$ , 55; C1, 73;  $\alpha_v$ , 129;  $\beta_3$ , 35; and hamster IgMs, 26. Data shown are the mean  $\pm$  SEM for each group; asterisk, statistically significant at  $p < 0.05$ .

from their radial glial guides (Figures 2B and 3). Antibodies specific to  $\beta_1$  integrins as well as pan  $\beta$  integrin polyclonal antiserum (Lenny) produced similar effects: an initial reduction in the rate of neuronal migration and the eventual detachment of neurons from their radial glial guides (Figure 3). The inhibition of  $\beta_3$  integrins, one of the  $\beta$  subunits known to associate with  $\alpha_v$  integrins, did not lead to any significant changes in the rate or pattern of neuronal migration. Similarly, the perturbation of  $\alpha_1\beta_1$  integrin with a 3A3 mAbs specific to that receptor did not affect neuronal migration. Furthermore, exposure to rabbit immunoglobulins, hamster IgMs, or control mAbs to a ubiquitous neuronal and glial cell surface molecule did not alter neuronal migration (Figures 2 and 3).

The above results suggest that different integrins control distinct aspects of neuronal migration during cortical development.  $\alpha_v$  integrins appear to control the basic cell-cell adhesion mechanisms needed to maintain optimal adhesive strength during neuronal migration, whereas  $\alpha_3\beta_1$  may modulate the necessary cell-cell recognition

face antigen did not affect neuronal migration ([C]; bottom four panels). Ralph3-1 and C1 mAbs were added at 50  $\mu$ g/ml. Anti- $\alpha_v$  antibodies were added at 1  $\mu$ g/ml. Scale bar, 12.5  $\mu$ m (A) and 10  $\mu$ m (B and C).

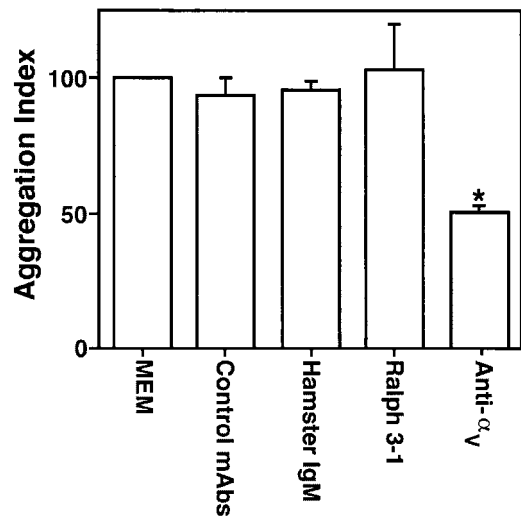


Figure 4. Effect of Anti-Integrin Antibodies on Neuron–Glial Adhesion

Dissociated cells from embryonic cerebral cortices were allowed to adhere to each other in unsupplemented MEM media or in media supplemented with purified monoclonal anti- $\alpha_3$  (Ralph3-1; 50  $\mu$ g/ml),  $\alpha_v$  (1  $\mu$ g/ml), C1 control mAbs (50  $\mu$ g/ml), and hamster IgMs (1  $\mu$ g/ml). The inhibition of  $\alpha_v$  integrins led to ~54% in neuron–glial adhesion, whereas the inhibition of  $\alpha_3$  integrins did not affect basic cell–cell adhesion. Results were normalized to aggregation in the absence of antibodies. Data shown are the mean  $\pm$  SEM; asterisk, statistically significant at  $p < 0.05$ .

and cell motility mechanisms during this process. Dynamic changes in these mechanisms, which are controlled by different integrins, are likely to instruct neurons to begin, maintain, alter, or end their migration during corticogenesis. In an attempt to decipher whether and how these integrins are involved in neuron–glial recognition, neuron–glial adhesion, neuronal motility, or a combination of these cellular processes, we tested the function of these integrins in neuron–glial reaggregation assays.

#### Functional Effect of $\alpha_3\beta_1$ - and $\alpha_v$ -Containing Integrins in Neuron–Glial Aggregation Assays

Dissociated neurons and glia from embryonic cerebral cortex were combined and allowed to adhere to each other under control conditions or in the presence of antibodies to  $\alpha_3\beta_1$  or  $\alpha_v$  integrins. The extent to which the anti-integrin antibodies perturbed the level and pattern of neuron–glial attachment and aggregation in these assays was compared with the extent and pattern of neuron–glial attachment obtained under control conditions (Figures 4 and 5). At the end of incubation under aggregation-promoting conditions, the extent of cell–cell aggregation and the cellular composition of the aggregates were analyzed. The inhibition of  $\alpha_v$  integrins leads to a significant reduction (54%) in neuron–glial adhesion (Figure 4). In contrast, the inhibition of  $\alpha_3$  integrins did not affect the extent of cell–cell adhesion (Figure 4). In the aggregates that formed under control conditions, neurons and astroglia adhered to each other

throughout the aggregates (Figures 5A and 5E). However, in the presence of  $\alpha_3$  integrin-blocking antibodies, neurons and glia tend to segregate from each other, either into pure aggregates in which only a few cells (1–10) in an aggregate are of the other cell type or into clustered aggregates in which neurons and glia are separated into distinct clusters within the same aggregate (Figure 5B). Under control conditions, 89% of all aggregates ( $n = 83$ ) were the mixed neuron–glial type, and the rest were clustered or pure aggregates. In contrast, when the  $\alpha_3$  integrin was blocked, 78% of all aggregates ( $n = 171$ ) were clustered or pure aggregates, and the rest were mixed neuron–glial aggregates. Similar results were obtained when embryonic neuronal and glial cells from  $\alpha_3^{-/-}$  and wild-type cerebral cortices were used in these aggregation assays (Figures 5C, 5D, and 5F). Neurons and glia from  $\alpha_3$  mutant brains tend to separate from each other, whereas those from wild-type brains adhered to each other freely.

In some cortical imprint neuronal migration assays, dense cohorts of neurons were found to migrate on radial glial fibers (Figure 5G). The inhibition of  $\alpha_3$  integrins in these assays caused neurons not only to stop their migration on radial glial strands but also to attach to each other rather than to their radial glial guides (Figures 5G and 5H). No such switch from gliophilic to neurophilic adhesive preference was noticed under control conditions.

Taken together, these results suggest that  $\alpha_v$  integrins are crucial for the maintenance of necessary levels of neuron–glial adhesive interactions during neuronal migration. In contrast,  $\alpha_3$  integrins appear to modulate neuron–glial recognition cues during neuronal migration and thus maintain neurons in a gliophilic mode until neuronal migration is over and layer formation begins. The gliophilic-to-neurophilic switch in the adhesive preference of developing neurons following the inhibition of  $\alpha_3$  integrins was reflected in the abnormal cortical organization of  $\alpha_3$  mutant mice.

#### Abnormal Cortical Laminar Organization in $\alpha_3$ Mutant Mice

The characteristic laminar architecture of the cerebral cortex is perturbed in  $\alpha_3$ -deficient mice when compared with that of wild types. Neurons in the cortical plate at postnatal day 0 (P0) are normally distributed in layers, with the early generated neurons localized in the deeper layers and the newly arrived ones distributed in the upper strata of the cortical plate. This characteristic laminar organization is absent in  $\alpha_3$  mutant mice (Figure 6). Furthermore, occasional, small focal accumulations of neurons (heterotopia) were also seen in the cerebral wall of mutant mice. These results indicate that cortical neurons do not migrate normally and thus fail to arrive and aggregate at their target layers in the absence of  $\alpha_3$  integrins.

To further investigate the pattern of cortical neuronal migration in  $\alpha_3$ -deficient mice, we labeled newly generated neurons with bromodeoxyuridine (BrdU) and analyzed the extent of their migration in the cerebral cortex at different time points after BrdU injection. When BrdU was injected at E13.5 and embryos were removed at

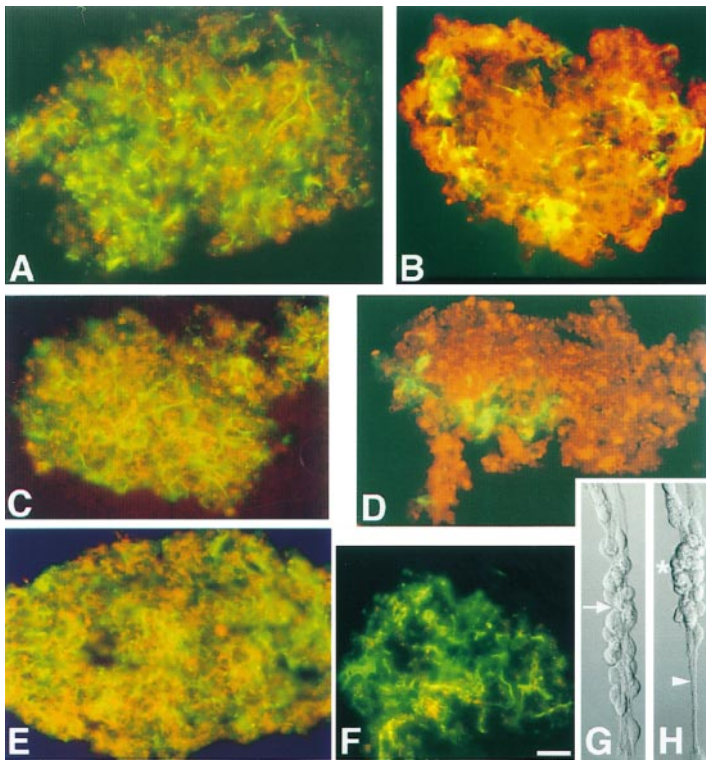


Figure 5.  $\alpha_3\beta_1$  Integrins Modulate Gliophilic Interactions of Cortical Neurons

The inhibition of  $\alpha_3\beta_1$  integrin causes developing cortical neurons to become less gliophilic and more neurophilic in their adhesive interactions. Cortical neurons and glial cells from developing cerebral cortex were dissociated and allowed to adhere to each other under control conditions (A and E) and in the presence of anti- $\alpha_3\beta_1$  integrin mAbs (Ralph3-1; [B]). Aggregates were also made from dissociated embryonic cortical cells from  $\alpha_3\beta_1$  integrin-deficient and wild-type mice (C, D, and F). Aggregates were then fixed, sectioned, and immunolabeled with neuron-specific anti-tubulin (Tuj-1) antibodies and astroglial-specific anti-GFAP antibodies. Tuj-1 immunolabeling was visualized with Cy-3 (red/orange), whereas GFAP immunoreactivity was visualized with Cy-2 (green). Images of neuronal and glial distribution in aggregates were obtained with a fluorescein/rhodamine filter. Under control conditions, in media devoid of antibodies (A) and in the presence of control mAbs (E), neurons (red/orange) and glia (green) intermingled and adhered to each other throughout the aggregates. However, when  $\alpha_3\beta_1$  integrin were blocked with Ralph3-1 mAbs, neurons and glia tended to segregate from each other and were found in separate domains within aggregates (B). Aggregates that are mainly of either neuronal or glial cell types were also found. A similar

pattern was also noticed when dissociated cells from  $\alpha_3\beta_1$  integrin-deficient (D and F) and wild-type (C) mice were used in these assays. Neurons and glia from wild-type cerebral cortex adhered freely to each other in aggregates (C). In contrast, when cells from  $\alpha_3\beta_1$  integrin-deficient cerebral cortex were used, neurons and glia separated from each other in the aggregates (D) or formed aggregates of mainly neuronal or glial (F) cell types. In neuronal migration assays, in which streams of migrating neurons (arrow, [G]) were observed on radial glial strands, the inhibition of  $\alpha_3\beta_1$  integrin with Ralph3-1 mAbs caused migrating neurons to adhere to each other in clusters (asterisk, [H]) rather than to the radial glial stand (arrowhead, [H]), indicating that the inhibition of  $\alpha_3\beta_1$  integrin results in a gliophilic-to-neurophilic switch in the adhesive preference of migrating neurons. Scale bar, 20  $\mu\text{m}$  (A–F) and 30  $\mu\text{m}$  (G–H).

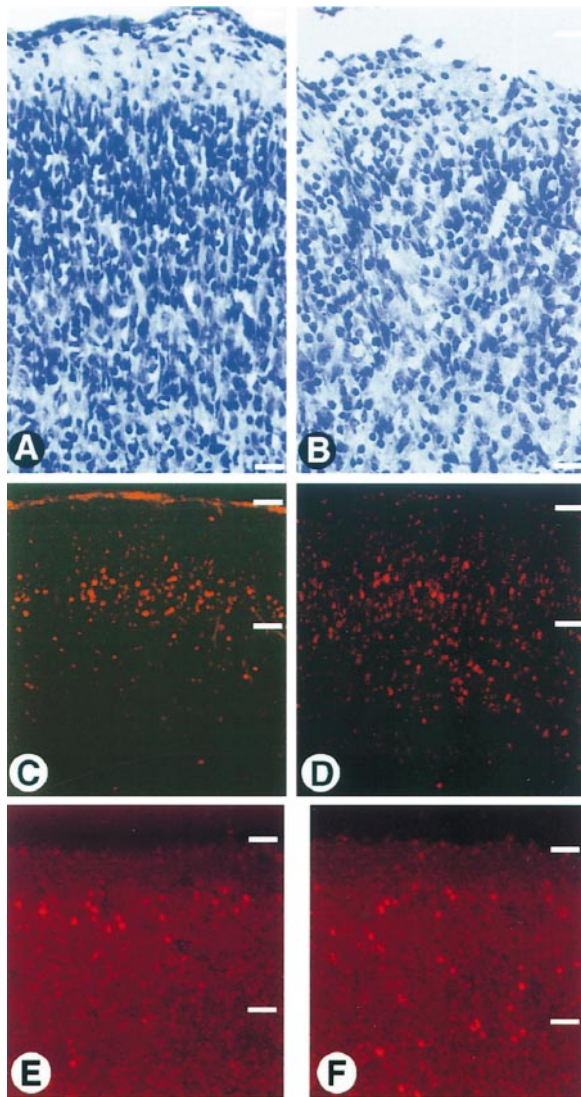
E19, we found that in wild-type mice, heavily labeled BrdU-positive neurons migrated normally and settled into their target layers in the deeper strata of the cortical plate. In contrast, in mutant embryos from the same litter, BrdU-positive neurons were found dispersed throughout the cortical plate and occasionally in the intermediate zone as well (Figures 6C and 6D). When BrdU was injected at E16 and embryos were removed at E19, BrdU-labeled cells were confined to the upper layers of the wild-type brains, whereas in mutant cerebral cortex, labeled cells were distributed mainly in the deeper layers (Figures 6E and 6F). Neurons that were born at the same time ended up in different cortical laminae in wild-type and mutant mice. Together, these results suggest that in  $\alpha_3$  mutant mice, cortical neurons do not migrate normally and thus, do not coalesce at the top of the cortical plate to form their destined cortical layers. In the absence of  $\alpha_3$  integrin-modulated bias toward gliophilic migratory mechanisms, cortical neurons may have been induced to utilize neurophilic migratory mechanisms (i.e., tangential) in the cerebral wall, thus leading to the observed abnormal placement of these neurons in the cortical plate of  $\alpha_3^{-/-}$  mice.

An outcome of the abnormal neuron-radial glial reciprocal interactions in the developing cerebral wall of  $\alpha_3^{-/-}$  mice is the premature appearance of astrocytes. Normally, as the period of neuronal migration reaches its

conclusion, radial glial cells transform into astrocytes (Schmechel and Rakic, 1979a, 1979b). The major thrust of this transformation occurs between E18 and P0 in murines (Misson et al., 1991). Immunolabeling of sections of E18 brains from wild-type and mutant embryos with astroglial cell-specific rat-401 mAbs indicates that significantly more astrocyte cells are present in the  $\alpha_3$  mutant cerebral wall compared with that of wild types (Figure 7). This is suggestive of the premature onset of the transformation of radial glial cells into astrocytes in the absence of  $\alpha_3$  integrins. The early onset of the phenotypic transformation of radial glial migratory guides into astrocytes, in turn, may affect subsequent phases of neuronal migration and thus further perturb the laminar formation in  $\alpha_3$  mutant cerebral cortex. Alternately,  $\alpha_3$  integrin's effects on the maturation, proliferation, and migration of astrocytes, independent of its effects on radial glial development or neuronal migration, may also have led to the premature appearance of astrocytes in  $\alpha_3^{-/-}$  brains.

## Discussion

Spatial and temporal changes in the expression of ECM molecules and integrin receptors in the developing cerebral wall parallel neuronal migration and the generation



**Figure 6. Disrupted Cortical Laminar Organization in  $\alpha_3$  Integrin-Deficient Mice**

Sections of the cerebral cortex from newborn (P0) wild-type (A), and  $\alpha_3$ -deficient (B) mice were stained with cresyl violet. Compared with the wild type (A), cortical neurons in  $\alpha_3$ -deficient brains (B) are distributed diffusely and are not organized into rudimentary layers at P0. Neurons that are destined for the deeper layers of cerebral cortex were labeled at their birth (E13.5) with BrdU, and their location was analyzed at E19, just prior to birth. Similarly, neurons that are destined for the upper layers of cerebral cortex were labeled at their birth (E16), and their distribution was analyzed at E19. BrdU immunoreactivity was visualized with Cy-3. When neurons were birthdated at E13.5, in wild-type mice, BrdU-labeled neurons were found in the deeper layers of the cerebral cortex (C). However, in  $\alpha_3$ -deficient cortex, BrdU-labeled neurons were present diffusely throughout the cortical plate and often in regions below the cortical plate as well (D). Similarly, when neurons were birthdated at E16, they arrived at their normal upper layer location in wild-type mice. In contrast, they were distributed primarily in deeper layers in mutant mice. Top and bottom bars of each panel indicate the top and bottom of the cortical plate. Scale bar, 15  $\mu\text{m}$  (A and B); 50  $\mu\text{m}$  (C and D); and 37.5  $\mu\text{m}$  (E and F).

of laminar organization in cerebral cortex. Here, we examined the distribution and function of the radial glial and neuronal integrins  $\alpha_V$  and  $\alpha_3$  during glial guided neuronal migration and cortical layer formation. Both  $\alpha_V$  and  $\alpha_3$  integrins are essential for the maintenance of normal neuronal migration on radial glial fibers. However,  $\alpha_V$  is essential for the basic neuron–glial adhesive interactions needed to maintain migration, whereas  $\alpha_3$  plays a more subtle role in maintaining proper neuron–glial recognition and the gliophilic bias of migrating neurons, the absence of which results in abnormal corticogenesis. These in vitro and in vivo observations indicate that the developmental function of these two integrins is instructive not only in different aspects of the migration of postmitotic cortical neurons but also in how they eventually coalesce into distinct layers in the cerebral cortex.

#### Function of $\alpha_V$ Integrins

$\alpha_V$  integrins expressed on radial glial cell surface can potentially associate with at least five different  $\beta$  subunits,  $\beta_1$ ,  $\beta_3$ ,  $\beta_5$ ,  $\beta_6$ , and  $\beta_8$ . Adhesive interactions involving fibronectin, vitronectin, tenascin, collagen, or laminin, ECM molecules that are highly expressed in the developing cerebral wall, are potentially mediated through these  $\alpha_V$ -containing integrins (Cheresh et al., 1989; Bodary and McLean, 1990; Moyle et al., 1991; Hirsch et al., 1994). In assays in which neuronal migration on radial glia was monitored, antibodies to  $\beta_1$  and  $\alpha_V$  integrins induced a reduction in the rate of the migration, roundup, and eventual detachment of neurons from their radial glial guides. Antibodies to  $\beta_3$  integrins did not affect neuronal migration. (The effect of  $\beta_5$  integrins was not investigated, owing to the lack of effective function-blocking antibodies to murine  $\beta_5$  integrins.) Together, these results suggest that  $\alpha_V\beta_1$  integrins play the more dominant role in neuron–glial attachment and anchoring during cortical neuronal migration than  $\alpha_V\beta_3$  integrins.  $\alpha_V\beta_1$  but not  $\alpha_V\beta_3$  or  $\alpha_V\beta_5$  integrins were also shown to be the primary modulators of cell migration in other cell types, such as O-2A oligodendrocyte precursor cells (Milner and Ffrench-Constant, 1994, 1996; Milner et al., 1996), embryonic stem cells (Fassler et al., 1995), and F9 teratocarcinoma cells (Stephens et al., 1993). Both transient cell–matrix interactions and cell anchoring mechanisms that are mediated by different  $\alpha_V$ -containing integrins are likely to modulate the process of neuronal translocation on radial glia in cerebral cortex. How these different  $\alpha_V$  integrins, in spite of similar ECM ligand specificities, impart distinctly different cellular effects remains unresolved. Differences in their modes of association with cytoskeleton (Wayner et al., 1991; Delannet et al., 1994), interactions with non-ECM neuronal cell surface molecules such as L1 (Montgomery et al., 1996), and the intracellular signaling cascades transduced in response to ligand binding may provide mechanisms for the functional hierarchy and diversity observed within  $\alpha_V$  integrins during neuronal migration in the developing cerebral cortex.

#### Function of $\alpha_3$ Integrins

Developing cortical neurons express  $\alpha_3\beta_1$  integrin. The ligands for  $\alpha_3\beta_1$  integrin include thrombospondin, LN-1

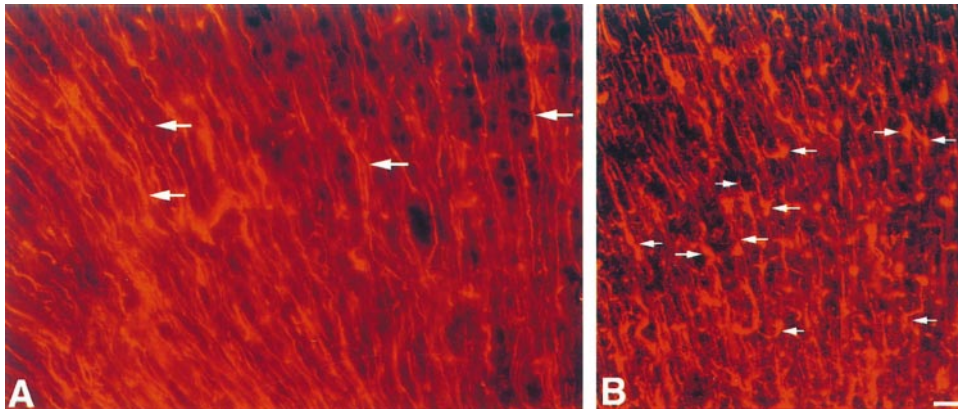


Figure 7. Premature Appearance of Astrocytes in  $\alpha_3$ -Deficient Mice

E18 brain sections were immunostained with astroglial-specific rat-401 mAbs to label both radial glia and astrocytes. In the midcerebral wall region of brains from wild-type mice, radial glial processes coursing through the cerebral wall were observed (arrows, [A]). In  $\alpha_3$ -deficient mice (B), in addition to some radial glial cells, many stellate, multipolar cells characteristic of type-1 astrocytes were seen (arrows, [B]). This is suggestive of the premature transformation of radial glial scaffold into astrocytes in the absence of  $\alpha_3$  integrins. Scale bar, 40  $\mu\text{m}$ .

(EHS-laminin), LN-2 (merosin), LN-5/6 (kalinin/epiligrin), fibronectin, collagen, entactin, and invasin (Wayner and Carter, 1987; Isberg and Leong, 1990; Carter et al., 1991; Elices et al., 1991; Dedhar et al., 1992; Tomaselli et al., 1993; Weitzman et al., 1993; Delannet et al., 1994; Miner et al., 1995; Fukushima et al., 1998; Kikkawa et al., 1998). Furthermore,  $\alpha_3\beta_1$  integrin has also been shown to homophilically interact with each other and heterophilically with other integrins, such as  $\alpha_2\beta_1$  (Sriramarao et al., 1993; Symington et al., 1993). All of the potential  $\alpha_3\beta_1$  integrin ligands have been found to be expressed in the developing cerebral wall (Liesi, 1990; Sheppard et al., 1991; Georges-Labouesse et al., 1998).

The expression of  $\alpha_3\beta_1$  integrin by the developing cortical neurons and the presence of its ligands in the cerebral wall, including the cell surface of radial glial migratory guides, support the hypothesis that  $\alpha_3\beta_1$  integrin may play a crucial role in cortical neuronal development. In assays for cortical neuronal migration, mAbs to  $\alpha_3\beta_1$  integrin inhibited the rate of neuronal migration by 40%. In the absence of  $\alpha_3\beta_1$  integrin, cortical neurons from  $\alpha_3^{-/-}$  mice prefer to adhere to other neurons rather than to their radial glial guides. Furthermore, mAbs to  $\alpha_3\beta_1$  integrin induced a similar switch from the gliophilic to neurophilic mode of neuronal interactions in neuron-glia adhesion assays *in vitro*. Adhesive interactions based on homophilic mechanisms appear to take precedence in the absence of functional  $\alpha_3\beta_1$  integrin. Similar effects on the pattern of neuron-glia adhesion were not observed when the function of  $\alpha_v$ ,  $\beta_1$ , or  $\beta_3$  integrins was blocked. Together, these results indicate that the primary function of  $\alpha_3\beta_1$  integrin during cortical development is not the maintenance of optimal levels of basic neuron-radial glial attachment and adhesion but the modulation of other adhesion-related interactions, such as neuron-glia recognition.

$\alpha_3\beta_1$  integrin on its own or in combination with other integrins may function to provide the necessary neuron-glia recognition interactions needed to promote neuronal translocation on radial glial processes across the cerebral wall and neuron-neuron interactions required

at the end of neuronal migration during the formation of cortical layers. The function of  $\alpha_3\beta_1$  integrin may depend on the activity of other integrins that are expressed (Tomaselli et al., 1990), cell phenotype, and the ECM and non-ECM ligands that are expressed in the immediate environment (Elices and Hemler, 1989; Kirchhofer et al., 1990; Chan and Hemler, 1993; Wayner et al., 1993; Yabkowitz et al., 1993; DeFreitas et al., 1995; Palecek et al., 1997).  $\alpha_3\beta_1$  integrin is highly expressed in the proliferating cells of the ventricular zone and in the migrating neurons of the intermediate zone. Thus, the expression of  $\alpha_3\beta_1$  integrin in the intermediate zone is consistent with its role in the initiation and maintenance of radial glial-based neuronal migration in the embryonic telencephalon. The level of  $\alpha_3\beta_1$  integrin expression decreases in the cortical plate region when compared with the other regions of the developing cerebral wall. Downmodulation of its expression in the cortical plate may trigger the decrease in a migrating neuron's bias for gliophilic adhesive interactions and thus promote the neurophilic recognition and adhesive interactions needed to organize neurons into distinct layers.

#### Mechanisms Underlying Laminar Organization of Cerebral Cortex

Neuronal migration on radial glia and other neural substrates enables neurons to organize themselves into distinct layers in cerebral cortex (Rakic, 1972, 1988, 1990; McConnell, 1988; O'Rourke et al., 1992). Neurons that are born early in development end up in the deeper layers of cerebral cortex, whereas neurons that are produced subsequently migrate through regions of older neurons to progressively superficial layers, thus resulting in the "inside-out" laminar organization of cerebral cortex (Angevine and Sidman, 1961; Rakic, 1974). This laminar organization of cerebral cortex is crucial for higher brain functions, such as cognition. Translocation of a neuron from the proliferative ventricular zone to its specific laminar location in the cortical plate involves the recognition of appropriate migratory guides,

initiation of cell movement, active migration on the selected pathway over a distance of several hundred cell lengths through a complex cellular environment, arrest of migration, deadhesion from the glial migratory substrate at the appropriate laminae, and an increased proclivity for neuron–neuron adhesive interactions with other layer-specific neurons (Hatten and Mason, 1990; Rakic et al., 1994; Rakic and Caviness, 1995; Anton et al., 1996). Thus, neuron–glial recognition, neuron–glial adhesion, and changes in the overall adhesive preference of developing neurons for radial glia or other neurons during migration are likely to be instructive in the eventual organization of neurons into distinct layers in the cerebral cortex.

Our results suggest that  $\alpha_V$  integrins on the radial glia provide the optimal levels of neuron–glial adhesion needed to maintain the migration of neurons to the cortical plate. The  $\alpha_3$  integrins, on the other hand, function initially to maintain the gliophilic bias of developing neurons during migration. Eventually, changes in the function, expression level, or ligand specificity of this receptor, as neurons reach the end of their migratory pathway, make neurons more attractive for other neurons than for radial glia. This switch from the gliophilic to neurophilic mode of neuronal interactions with other cells in the environment underlies the formation of neuronal layers in the cerebral cortex (Rakic, 1990). This is reflected in  $\alpha_3$  integrin mutant mice in whom cortical neurons, though capable of migration, do not reach their appropriate locations and aggregate into distinct cortical layers. The inability of neurons to arrive at their predestined layers at appropriate developmental stages may result from (1) the inability to recognize and adhere appropriately to specific radial glial pathways, (2) an abnormal preference for and dispersion along other nonradial glial migratory pathways in the developing cerebral cortex, and/or (3) the nonavailability of glial migratory substrates owing to the premature onset of radial glial transformation into astrocytes or abnormal astrocyte development. Furthermore, both  $\alpha_V$  and  $\alpha_3$  integrins have been shown to be necessary for ECM assembly and maintenance (Kreidberg et al., 1996; Yang and Hynes, 1996). Thus, the inability of cortical neurons to migrate and develop properly in the absence of these integrin receptors may also occur as a result of the disruption of ECM organization in these mutants. Integrins in migrating cells can sample and respond to ECM rigidity and composition and alter the strength of integrin–cytoskeletal linkage accordingly (Choquet et al., 1997). The integrin–cytoskeletal linkage serves to modulate the directed movement of integrin receptors on cell surface and thus generate force during oriented cell movement (Lawson and Maxfield, 1995; Schmidt et al., 1995; Felsenfeld et al., 1996; Palecek et al., 1997; Choquet et al., 1997). Therefore, abnormalities in ECM organization in integrin mutants could also result in abnormal patterns of oriented cell movement and behavior in the developing cerebral cortex.

Even though the laminar identity of a neuron seems to be determined at its birth (McConnell, 1988, 1989, 1995; McConnell and Kaznowski, 1991; Frantz and McConnell, 1996), acquisition of its other phenotypic traits (i.e., molecular identity, connectivity, and morphology) may depend on the environmental cues encountered by it during translocation from its site of birth in the ventricular

zone to its predetermined laminar position (Levitt et al., 1993, 1997). The changing patterns of adhesive interactions mediated by integrins during neuronal translocation across the cerebral wall may set in motion the developmental programs needed for the progressive acquisition of distinct neuronal phenotypes. Complements of integrins and ECM molecules that could serve as such inductive cues change in the cerebral cortex during development (Sheppard et al., 1991; Galileo et al., 1992; Hirsch et al., 1994; Georges-Labouesse et al., 1998; Zhang and Galileo, 1998). Thus, neurons that are destined to different cortical layers are likely to undergo distinctly different sets of adhesive interactions with their environment. This may serve as an essential mechanism for the acquisition of different cortical neuronal phenotypes. The determination of the relative contribution of different integrins during neuronal migration and of whether the pathway choice (e.g., radial versus tangential) of migrating neurons depends on the complement of integrins expressed will help to delineate such a mechanism.

Genetic analysis of mouse mutations and human neurologic disorders with disrupted cortical laminar organization has identified the ECM-like protein reelin (D'Arcangelo et al., 1995; Ogawa et al., 1995), cyclin-dependent kinase 5 (cdk5; Ohshima et al., 1996), p35 (a neuron-specific activator of cdk5; Chae et al., 1997), transcription factor pax-6 (Schmal et al., 1993; Caric et al., 1997), neurotrophin-4 (Brunstrom et al., 1997), signal transduction proteins mdab1 (Howell et al., 1997a, 1997b; Sheldon et al., 1997; Ware et al., 1997),  $\alpha_6$  integrins (Georges-Labouesse et al., 1998; Zhang and Galileo, 1998), and doublecortin (Gleeson et al., 1998) as regulators of cortical layer formation. Even though the disruption in cortical layering seen in  $\alpha_3$  mutants resembles that of *reeler*, reelin is distributed normally in its characteristic locale in the marginal zone Cajal-Retzius neurons in  $\alpha_3$  mutants. Reelin, which shows structural homology to ECM molecules such as tenascin (D'Arcangelo et al., 1995), is a potential  $\alpha_3$  integrin ligand. Furthermore, transient calcium fluxes that modulate neuronal migration in vitro can also regulate the polarized distribution and function of integrins in cells undergoing oriented migration (Lawson and Maxfield, 1995; Komuro and Rakic, 1996, 1998).

Further assessment of how integrin-mediated interactions relate to hitherto identified signals regulating neuronal migration and laminar organization in the cerebral cortex will serve to elucidate the diverse mechanisms involved in the generation of laminar organization in the cerebral cortex. The findings presented here indicate that  $\alpha_3$  and  $\alpha_V$  integrins, with their potential to sample and respond accordingly to their environment by inducing changes in cytoskeletal attachments and intracellular signaling cascades, are essential intermediaries in the processes of neuronal migration and laminar organization of the cerebral cortex.

#### Experimental Procedures

##### Antibodies to Integrins

Mouse monoclonal anti- $\alpha_3$  integrin antibodies (Ralph3-1; DeFreitas et al., 1995) and subunit-specific, affinity-purified polyclonal antisera to the cytoplasmic regions of  $\alpha_3$  and  $\alpha_V$  (Bossy and Reichardt, 1990;

Tomaselli et al., 1990) were the generous gifts of Dr. L. F. Reichardt (UCSF). Affinity-purified hamster mAbs to  $\alpha_v$  and  $\beta_6$  were obtained from Dr. D. Gerber (Gerber et al., 1996; MIT). Affinity-purified subunit-specific antibodies to  $\alpha_3$ ,  $\beta_1$  (Ha2/11), and nonimmune rabbit or hamster (IgM) immunoglobulins were obtained from Chemicon (Plantefaber and Hynes, 1989; Rossino et al., 1990; Mendrick and Kelly, 1993; DeFreitas et al., 1995). Ralph3-1 mAbs were purified by G Protein affinity chromatography (Mab trap kit, Pharmacia).

#### Mutant Mouse Strains

The generation and characterization of the targeted mutation in mouse  $\alpha_3$  integrins were described in Kreidberg et al. (1996). Genotypes of the embryos used were determined by PCR as described earlier (DiPersio et al., 1997).

#### Neural Cell Culture and Migration Assay

Dissociated neuron-glial cultures from embryonic brains and cortical imprint assays containing intact radial glial cells with migrating neurons attached to them were made as described previously (Anton et al., 1996, 1997). The embryonic cerebral wall imprinting procedure often results in radial glial cells with isolated or clusters of migrating neurons attached to them. Cortical neurons migrating on radial glial cells were monitored with a Zeiss Axiovert 135 microscope (GHS filter block, 460 nm excitation wavelengths) equipped with a Zeiss W63 objective lens. Images were recorded every 5–15 min with a Panasonic TQ-3031 optic disk recorder and the Attofluor Ratio Vision program (Atto Instruments). After 60–120 min of baseline recording, purified anti-integrin receptor antibodies (1–50  $\mu\text{g/ml}$ ) were added to the cultures, and monitoring continued for an additional 60–240 min. As controls, cultures were monitored unperturbed or after perfusing in media devoid of antibodies, media containing control mouse mAbs (50  $\mu\text{g/ml}$ ), or nonimmune hamster immunoglobulins (1  $\mu\text{g/ml}$ ). Changes in the rate of cell migration, morphological features of migrating neurons and glial cell substrates, and the extent of neuronal-glial cell contact were monitored before and after antibody perturbation. The extent of cell soma movement was divided by the time elapsed between observations to obtain the rate of cell migration for each neuron studied. Statistical differences between experimental groups were tested by a student's *t* test.

At the end of the observations, some cultures were fixed in 4% paraformaldehyde and processed for anti-GFAP (Datco), anti-neurofilament (Boehringer-Mannheim), Rat-401 mAb (a gift from Dr. S. Hockfield, Yale University; Hockfield and McKay, 1985), or anti-neuron-specific tubulin (TuJ-1 antibodies; a gift from Dr. A. Frankfurter, University of Virginia; Lee et al., 1991) immunohistochemistry. Glial fibrillary acidic protein (GFAP) and Rat-401 immunohistochemistry were used to analyze radial glial cells. Anti-neuron-specific tubulin or neurofilament labeling was used to identify migrating cells as neurons.

#### Aggregation Assays

Cerebral cortices from E16 rat (Sprague-Dawley) or mouse embryos were removed, cleared of pial membranes, cut into 300  $\mu\text{m}$  slices, collected in serum-free minimal essential medium (MEM) supplemented with 0.25% trypsin, and maintained at 37°C for 30 min. Following a few rounds (25) of gentle trituration, cells were filtered through a 35  $\mu\text{m}$  nylon mesh suspended in MEM/10% Horse serum supplemented with 80  $\mu\text{g/ml}$  DNase and 100  $\mu\text{g/ml}$  soybean trypsin inhibitor and centrifuged for 10 min at 500  $\times$  g. The cell pellet was washed twice with MEM/10% HS and dissociated mechanically with a fire-polished Pasteur pipette in the same medium. Cells were plated at 300,000 cells/2 ml in a 24 well dish and rotated at 90 rpm in a 37°C (95% O<sub>2</sub>/5% CO<sub>2</sub>) incubator for 24 hr. Some wells were supplemented with one of the following: Ralph3-1 mAbs (50  $\mu\text{g/ml}$ ), hamster anti- $\alpha_v$  mAbs (1  $\mu\text{g/ml}$ ), control mouse mAbs (C1; 50  $\mu\text{g/ml}$ ), or nonimmune hamster immunoglobulins (1  $\mu\text{g/ml}$ ). At the end of incubation, the number of unattached, viable, single cells in the suspension was counted. The aggregation index was calculated as follows: (number of single cells in unsupplemented media/number of single cells seeded originally)/(number of single cells in media supplemented with antibodies/number of single cells seeded originally)  $\times$  100. All of the aggregates were harvested, fixed in 4%

paraformaldehyde, cut into 10  $\mu\text{m}$  sections in a cryostat, and collected onto gelatin-coated glass slides. Cross sections of the aggregates were then labeled with glial-specific anti-GFAP antibodies and neuron-specific TUJ-1 or MAP-2 antibodies as described earlier (Anton et al., 1996, 1997). Changes in the pattern of neuron-glial attachment in these aggregates or their cellular composition were evaluated.

#### Histological and Immunohistochemical Procedures

Embryonic cerebral cortices or neuron-glial aggregates were fixed in 4% paraformaldehyde, cut into 10  $\mu\text{m}$  sections in a cryostat, and collected onto gelatin-coated glass slides. After air dried, sections were washed and blocked in Tris-buffered saline (TBS) containing 5% goat serum, 3% bovine serum albumin, and 0.01% Triton X-100 for 15 min before incubating in primary antibodies for 1 hr at room temperature. Following three rinses in blocking solution, sections were incubated with the appropriate Cy-3-, rhodamine-, or fluorescein-conjugated secondary antibodies (1:200 dilution; Jackson Immunochemicals) for 1 hr. Sections were then washed in TBS, counterstained with 10  $\mu\text{m}$  bisbenzimidazole, and mounted in mowiol (10% mowiol, 25% glycerol in 0.1 M Tris with *p*-phenylenediamine; Calbiochem) for observation in a Zeiss microscope equipped with catecholamine (excitation, 400–440 nm; barrier, LP 470 nm), rhodamine, and fluorescein filter sets. Cultures of embryonic cortical cells were processed identically, except the blocking buffer was devoid of Triton X-100 in some experiments. For integrin immunolocalization, some sections were fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 4–6 hr or in –20°C methanol for 10 min prior to immunostaining.

For Nissl staining, sections were incubated in cresyl violet solution (62.5 mM sodium acetate, 62.5 mM formic acid, 0.3125% aqueous cresyl violet) for 1–5 min, dehydrated through an ethanol series, cleared in xylene, and mounted in permount (Sigma). Some sections were stained with 0.1% basic fuschia (Sigma) for 20 min instead of cresyl violet. These sections were differentiated in 70% ethanol, glacial acetic acid (50  $\mu\text{l}/100$  ml) for 1 min prior to the aforementioned dehydration and clearance steps.

#### BrdU Birthdating Studies

In this assay, newly generated neurons were labeled with BrdU, and the extent of their migration and layer-specific localization was analyzed. Briefly, pregnant mice were injected intraperitoneally with BrdU (7.5 mg/kg body weight, dissolved in saline; Boehringer-Mannheim) on E13.5 and E16. Brains were removed at E19 just prior to birth ( $\alpha_3$  mutant mice do not generally survive beyond P0), fixed in 70% ethanol, embedded in paraffin, and cut into 10  $\mu\text{m}$  coronal sections. After deparaffinizing, sections were rinsed three times in PBS, fixed in 2 N HCl for 1 hr at room temperature, washed in PBS, and incubated for 4 hr with a monoclonal anti-BrdU antibody (1:75 dilution in PBS/0.5% Tween-20; Becton-Dickinson). Sections were then washed in PBS and incubated in 1:100-diluted anti-mouse IgG conjugated to Cy-3 (Jackson Immunochemicals) for 2 hr. Five minutes before the end of incubation with the secondary antibodies, bisbenzimidazole was added at a final concentration of 10  $\mu\text{M}$ . After three rinses in PBS, sections were mounted in mowiol/*p*-phenylenediamine and observed in a Zeiss microscope equipped with catecholamine (excitation, 400–440 nm; barrier, LP 470 nm) and rhodamine filter sets. Images of BrdU and bisbenzimidazole labeling in cortical sections were collected onto an optical disk by using the Image 1.5 (NIH) program. The comparisons between sections from different embryos were obtained from identical cortical regions corresponding approximately to posterior frontal, parietal, and anterior occipital areas.

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