

MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Integrin $\alpha 5 \beta 1$ is necessary for regulation of radial migration of cortical neurons during mouse brain development

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Keywords: cell adhesion, cerebral cortex, embryo, integrin, motility

Abstract

During cerebral cortex development, post-mitotic neurons interact with radial glial fibers and the extracellular environment to migrate away from the ventricular region and form a correct laminar structure. Integrin receptors are major mediators of cell–cell and cell–extracellular matrix interactions. Several integrin heterodimers are present during formation of the cortical layers. The $\alpha 5 \beta 1$ receptor is expressed in the neural progenitors of the ventricular zone during cerebral cortex formation. Using *in utero* electroporation to introduce short hairpin RNAs in the brain at embryonic day 15.5, we were able to inhibit acutely the expression of $\alpha 5$ integrin in the developing cortex. The knockdown of $\alpha 5$ integrin expression level in neural precursors resulted in an inhibition of radial migration, without perturbing the glial scaffold. Moreover, the same inhibitory effect on neuronal migration was observed after electroporation of a Cre recombinase expression plasmid into the neural progenitors of conditional knockout mice for $\alpha 5$ integrin. In both types of experiments, the electroporated cells expressing reduced levels of $\alpha 5$ integrin accumulated in the premigratory region with an abnormal morphology. At postnatal day 2, ectopic neurons were observed in cortical layer V, while a deficit of neurons was observed in cortical layer II–IV. We show that these neurons do not express a layer V-specific marker, suggesting that they have not undergone premature differentiation. Overall, these results indicate that $\alpha 5 \beta 1$ integrin functions in the regulation of neural morphology and migration during cortical development, playing a role in cortical lamination.

Introduction

In the developing cerebral cortex, a large number of neurons are generated in the ventricular (VZ) and sub-ventricular zone (SVZ) of the dorsal telencephalon and migrate radially along the glial fibers, to reach their final position in the cortical plate (CP) (Nadarajah *et al.*, 2003). The correct formation of the cortical layers requires that migrating neurons, from the premigratory zone to the CP, undergo a morphological transition from a multipolar to a bipolar shape (Kriegstein & Noctor, 2004; Noctor *et al.*, 2004; LoTurco & Bai, 2006). Adhesive interactions between the extracellular matrix (ECM), neurons and radial glia are likely to play important roles during this process of neuronal migration (Schmid & Anton, 2003; Sarkisian *et al.*, 2008). Major cell surface receptors for ECM are the integrins, which are heterodimers constituted by α and β subunits that bind specific ECM ligands [e.g. fibronectin (FN), laminin (LN) or collagen] (Hynes, 2002). Previous studies have suggested that $\alpha 3$ and αv integrin subunits may be important for interactions between migrating neurons and radial glia, whereas the integrin subunits $\alpha 6$ and $\beta 1$ regulate anchorage

of glial endfeet and meningeal basement membrane remodeling (Georges-Labouesse *et al.*, 1998; Anton *et al.*, 1999; Graus-Porta *et al.*, 2001; Belvindrah *et al.*, 2007). Regarding ECM ligands, the role of laminin in the organization of the mouse cortex has been demonstrated (Miner *et al.*, 1998; Halfter *et al.*, 2002; Chen *et al.*, 2009). And some evidence points to a role for FN as a potential ligand for guiding radial neuronal migration and regulating the interactions between neurons and radial glia. In particular, high levels of FN mRNA were found in the VZ during early corticogenesis and in the CP at later stages (Sheppard *et al.*, 1995). Stettler & Galileo (2004) observed that, in the chick optic tectum, FN was produced by radial glial fibers and was aligned along their surfaces during neuronal migration. Early embryonic lethality of FN-null embryos has so far precluded phenotypical studies in the developing nervous system. In addition, embryos lacking the $\alpha 5$ integrin subunit, which forms the $\alpha 5 \beta 1$ major FN receptor, also die at embryonic day (E)10.5 before cerebral cortex development (Yang *et al.*, 1993; Goh *et al.*, 1997). Recently, $\alpha 5 \beta 1$ has been implicated in regulating morphology of dendritic spines and formation of synapses in neurons (Webb *et al.*, 2007), as well as in mediating neurite outgrowth after injury (Gardiner *et al.*, 2007).

We developed two strategies to overcome the early embryonic lethality and analyse the potential roles of $\alpha 5 \beta 1$ integrin in neuronal migration during brain development. First, we introduced short hairpin

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Received 15 May 2009, revised 31 October 2009, accepted 22 November 2009

RNA (shRNA) against $\alpha 5$ integrin by *in utero* electroporation in embryonic brains to inhibit its expression in VZ and SVZ cells. Second, starting from $\alpha 5$ integrin floxed mice (A. van der Flier *et al.*, unpublished data), we introduced a Cre plasmid also by *in utero* electroporation to delete the $\alpha 5$ gene in VZ/SVZ cells (Calegari *et al.*, 2004; Chen *et al.*, 2008). Using the two approaches, we found that inhibition of $\alpha 5\beta 1$ expression in VZ and SVZ leads to defects in neuronal migration.

Materials and methods

Plasmids

$\alpha 5$ integrin target sequences were designed via the GenScript shRNA design tool and are as follows: shRNA-1 (R1), 5'-CCTGCTACCTCTCCACAGAAA-3'; shRNA-4 (R4), 5'-GCAGATCTCGGAGTCCTATTA-3'; shRNA-7 (R7), 5'-CTGCCTCAATGCCTCTGGAAA-3'; shRNA-9 (R9), 5'-ACTTTCAGATCCTCAGCAAGA-3'; control scrambled shRNA (Ctr), 5'-CACAATATCTGCCCGATCCA-3'. shRNA constructs were generated in the pRNAT-U6.1/Neo vector (GenScript, Paris, France), which allows expression of a coral green fluorescent protein (GFP) marker with the shRNA of interest. Full-length mouse $\alpha 5$ integrin (German Science Center for Genome Research RZPD, Berlin, Germany) and $\alpha 6$ integrin were cloned into pcDNA3.1 (Invitrogen, Cergy-Pontoise, France). For Cre-recombination experiments, we used the pCIG2 plasmid (kind gift of F. Polleux, University of North Carolina), encoding enhanced GFP (EGFP), and pxCANCre expressing Cre recombinase under the control of the CAG promoter (obtained from DNA Bank, Tsukuba Life Science Center, RIKEN, Japan).

Antibodies

Primary antibodies used in this study were against integrin $\alpha 5$ (rabbit, 1 : 500, Chemicon, Temecula, CA, USA; rat, 1 : 50, Pharmingen, Le Pont de Claix, France), integrin $\alpha 6$ (rat, 1 : 50, Pharmingen), bromodeoxyuridine (BrdU) (mouse, 1 : 100, Boehringer Mannheim), cleaved caspase 3 (Casp-3) (rabbit, 1 : 500, Cell Signalling, Saint Quentin en Yvelines, France), GFP (rabbit, 1 : 1000, Molecular Probes, Leiden, The Netherlands), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (mouse, 1 : 300, Chemicon), nestin/Rat 401 (mouse, 1 : 10, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2) (rat, 1 : 500, Abcam, Cambridge, UK), CCAAT-displacement protein (Cdp) (rabbit, 1 : 50, Santa Cruz Biotechnology) and integrin $\beta 1$ (rat, 1 : 100, Chemicon). Secondary antibodies used for immunocytochemistry or in Western blotting analyses were Alexa fluor 488 goat anti-rabbit IgG (Molecular Probes), Alexa fluor 594 goat anti-mouse IgG (Molecular Probes), horseradish peroxidase (HRP) goat anti-rabbit, anti-mouse and anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Cell lines and Western blot analysis

To evaluate the efficiency of shRNA constructs, HeLa cells were transiently transfected using Effectene reagent (Qiagen, Les Ulis, France). Two days after transfection, cells were lysed in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA and a protease inhibitor cocktail (Roche, Mannheim, Germany)]. The lysates were cleared by centrifugation at 17 000 g for

15 min at 4°C. The proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) for Western blotting.

Mice

All animal experiments were approved by the regional ethics committee for animal experimentation (CREMEAS) and in strict accordance with French regulations.

shRNA plasmid injections were performed in CD1 wild-type mice. For the production of integrin $\alpha 5$ floxed mice, a previously isolated genomic clone (Yang *et al.*, 1993) was used to clone the $\alpha 5$ integrin conditional targeting construct. The targeting vector contained the following elements: a CM1-TK cassette, followed by 2-kb 5'-genomic arm, an Frt-flanked PGF-neo cassette, the 1.5-kb genomic sequence containing the exon 1 (255 bp) was flanked by loxP sites, followed by a 4-kb 3'-genomic arm. R1 embryonic stem (ES) cells were electroporated, selected and screened for correct recombination and single integration by Southern blotting analysis. Subsequently, the PGK-neo cassette was removed *in vitro* by transient expression of Flip recombinase. Two karyotyped and correctly targeted ES clones gave germline transmission and gave identical experimental results. Cre-mediated excision of exon 1 has been confirmed by PCR and Southern blotting in $\alpha 5$ -floxed mice crossed to various transgenic Cre-strains as well as in derived cells. Loss of $\alpha 5$ protein after Cre-mediated recombination in several transgenic Cre-expressing strains could be detected by fluorescence-activated cell sorting, immunoblots and immunohistochemistry (A. van der Flier *et al.*, unpublished data).

In utero electroporation

shRNA constructs or the pxCANCre plasmid were transfected by *in utero* electroporation as previously described (Langevin *et al.*, 2007; S. Esquin and E. Georges-Labouesse, unpublished data). Briefly, pregnant CD1 or $\alpha 5^{f/f}$ mice and control mice were anesthetized by isoflurane inhalation and 1–3 μ L plasmids (3 μ g/ μ L) with 1% Fast Green was microinjected into the lateral ventricles of each embryonic brain at E15.5 using pulled glass capillaries. Electroporation was performed by delivering five 50-V pulses of 50 ms each at 950-ms intervals using the ECM electroporator (BTX, Holliston, MA, USA). The uterine horns were then placed back in the abdominal cavity to allow the embryos to continue development. The transfected brains were analyzed 2, 3 or 6 days after electroporation.

BrdU labeling

For proliferation studies, pregnant mice were injected intraperitoneally with 50 mg/kg body weight of BrdU (Boehringer Mannheim) 48 h after *in utero* electroporation. The electroporated brains were harvested 2 h after BrdU injection. In all experiments, mice were killed by cervical dislocation and anesthetized by isoflurane inhalation.

In situ hybridization

Frozen sections were processed for *in situ* hybridization with ³⁵S-labeled RNA probes as described in Georges-Labouesse *et al.* (1998). Fragments used as probes were a 5' fragment of $\alpha 5$ integrin cDNA (860 nt) or a 1-kb $\beta 1$ cDNA (gift from R. Fässler, Max Planck Institute, Martinsried, Germany).

X-Gal staining

Frozen sections were washed with phosphate-buffered saline (PBS) and stained with 1 mg/mL X-Gal (Promega, Paris, France) in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ overnight at 37°C. Sections were then rinsed with PBS and mounted in Eukitt (EMS, Harrisburg, PA, USA).

Immunohistochemistry and confocal microscopy

Embryonic brains were fixed in 4% paraformaldehyde in PBS, cryopreserved in 10% then in 20% sucrose/PBS and 10- μ m coronal sections were cut with a Leica cryostat (Wetzlar, Germany). Sections were permeabilized in PBS/0.1% Tween 20 and stained with primary antibodies overnight at 4°C. After washing, slides were incubated for 1 h at room temperature with secondary antibodies. Images were acquired on a Macroconfocal LSI developed by the imaging center of IGBMC (Leica Microsystems), a laser scanning confocal microscope (Leica) with a 40 \times /1.25 or 63 \times /1.40 oil-immersion objective, or a Leica DMRBP fluorescence microscope.

Statistical and quantification analyses

The dorsal–lateral region of the cerebral cortex was analysed for all electroporation experiments. To determine significant changes relative to control electroporations, in general, at least three independent electroporated brains were processed for each DNA condition. For each sample, three or four adjacent sections (300 cells) were analysed using a Macroconfocal LSI (Leica). Different subregions of the cerebral cortex were identified and visualized based on cell density using 4',6-diamidino-2-phenylindole (DAPI) staining (Molecular Probes) and GFP-positive cells were counted and assessed for their location. The fraction of GFP-positive cells from each brain compartment in the shRNA or Cre-electroporated brains were compared with the fraction of the equivalent compartment in the control conditions. Microsoft Excel software was used to analyse the data for statistical significance. Statistical analysis was performed using two-tailed Student's *t*-test between control and experimental conditions. Results are indicated as mean \pm SEM. The mean was calculated and set to 100%. *P* values below 0.02 were considered statistically significant and are indicated in each figure legend.

Results

It has been previously reported that the $\alpha 5$ integrin subunit is expressed in the ventricular region of the mouse developing cerebral cortex (Yoshida *et al.*, 2003). To confirm and refine this localization, we performed *in situ* hybridization with $\alpha 5$ or $\beta 1$ RNA probes on coronal sections of E14.5 mouse brains. In addition to a signal in blood capillaries, a strong signal for $\alpha 5$ integrin was detected in the ventricular zone, the region where neural cortical progenitors are located (Fig. 1A). The $\beta 1$ expression domain was broader (VZ/SVZ and CP), reflecting the expression of other $\alpha\beta 1$ integrins (Georges-Labouesse *et al.*, 1998). Staining experiments with antibodies at E14.5 confirmed the expression of $\alpha 5$ in neural progenitors and blood capillaries, but not in radial glial fibers or glial endfeet, while integrin $\alpha 6$ and $\beta 1$ showed broader expression domains including the VZ and the CP and the nestin-positive radial glial fibers (Fig. S1).

To investigate the functional role of $\alpha 5\beta 1$ integrin in neural progenitors, we used electroporation-mediated gene transfer to induce $\alpha 5$ integrin acute down-regulation by RNA interference. To reduce the

expression of $\alpha 5$ integrin, four shRNA constructs were designed, against different regions of $\alpha 5$ integrin mRNA. BLAST (NCBI) searches with the shRNA sequences revealed no significant homology with other integrin genes or other genes. To produce shRNA, we used the pRNAT-U6/neo vector (GenScript), which allows the expression of shRNA and coral (c)GFP. This vector has been used successfully to knockdown the *Lis1* gene (Tsai *et al.*, 2005). Effective suppression of $\alpha 5$ integrin expression was tested by cotransfecting mouse $\alpha 5$ integrin cDNA with each shRNA construct into HeLa cells. By Western blot analysis of cells lysed 48 h after transfection, we observed that $\alpha 5$ -shRNA 1 (R1) very efficiently decreased $\alpha 5$ integrin expression, while $\alpha 5$ shRNA 7 (R7) had an intermediate effect, when compared with $\alpha 5$ integrin protein levels in cells transfected with empty vector or control scrambled $\alpha 5$ -shRNA construct (Ctr) (Fig. 1B). Cotransfection with $\alpha 5$ -shRNA 4 and 9 (called R4 and R9) did not reduce $\alpha 5$ integrin expression, such that $\alpha 5$ integrin protein levels were similar to transfection with empty vector or Ctr construct (data not shown). To test the specificity of the R1 and R7 constructs for the $\alpha 5$ integrin chain, we transiently cotransfected HeLa cells with mouse $\alpha 6$ integrin cDNA and R1 or R7 $\alpha 5$ -shRNA constructs. Transfection of $\alpha 5$ -shRNA constructs did not affect the expression of the $\alpha 6$ integrin chain (Fig. 1C).

Constructs containing $\alpha 5$ -shRNAs (R1 and R7), or Ctr shRNA, or empty vector were introduced into neural progenitor cells by electroporation in the mouse neocortex at E15.5. This stage allowed us to target the waves of later-born post-mitotic neurons which will ultimately form the cortical layers II–IV at postnatal stages. Embryonic brains were harvested 3 days after electroporation at E18.5. First, we confirmed that the R1 construct was able to knockdown endogenous $\alpha 5$ integrin protein *in vivo* in the developing neocortex by performing staining for $\alpha 5$ integrin on coronal sections from R1 or control shRNA electroporated brains. In areas where GFP-positive cells were located, a decrease in the level of integrin $\alpha 5$ signal was observed in those brains electroporated with R1 but not in control brains (Fig. 1D).

It has been shown previously that $\alpha\beta 1$ receptors play a role in the regulation of neural progenitor cell proliferation, survival and migration in neurosphere cultures derived from postnatal day (P)1 mice (Leone *et al.*, 2005). To determine whether the down-regulation of $\alpha 5$ integrin perturbs cell proliferation of neural precursors at embryonic stages, BrdU incorporation experiments were performed. For this, BrdU was injected into experimental pregnant females at E17.5, 2 days after electroporation, and embryonic brains were collected 2 h after BrdU injection. After double immunostaining with antibodies against BrdU and GFP, we counted the number of BrdU and GFP double positive cells and compared this with the total number of GFP-positive cells in the VZ and SVZ of control (Ctr, *n* = 3) or R1 (*n* = 3) injected embryos. As shown in Fig. S2, A, there was no significant difference (*P* = 0.45) in the fraction of BrdU-positive cells between Ctr (11.5 \pm 0.6%) and R1 (11.4 \pm 1.5%), suggesting no impairment in proliferation rate. The same result was obtained with a phospho-histone H3 (pH3) staining at E18.5 (data not shown). A reduction of integrin could also affect cell survival. To assess cell death, activated caspase-3 labeling was performed on electroporated brain sections. At this stage, very few cells were positive for caspase-3 in both conditions. Thus, $\alpha 5$ integrin knockdown did not have significant effects on apoptotic cell death of cortical progenitors (Fig. S2B).

To analyse further the defects induced by the knockdown of $\alpha 5$, we investigated the distribution of electroporated cells in E18.5 brains. Electroporation of empty vector or control shRNA construct did not affect the distribution of electroporated cells which were found in the VZ/SVZ, intermediate zone (IZ) and CP. In contrast, in coronal sections of brains electroporated with the R1 construct, the majority of

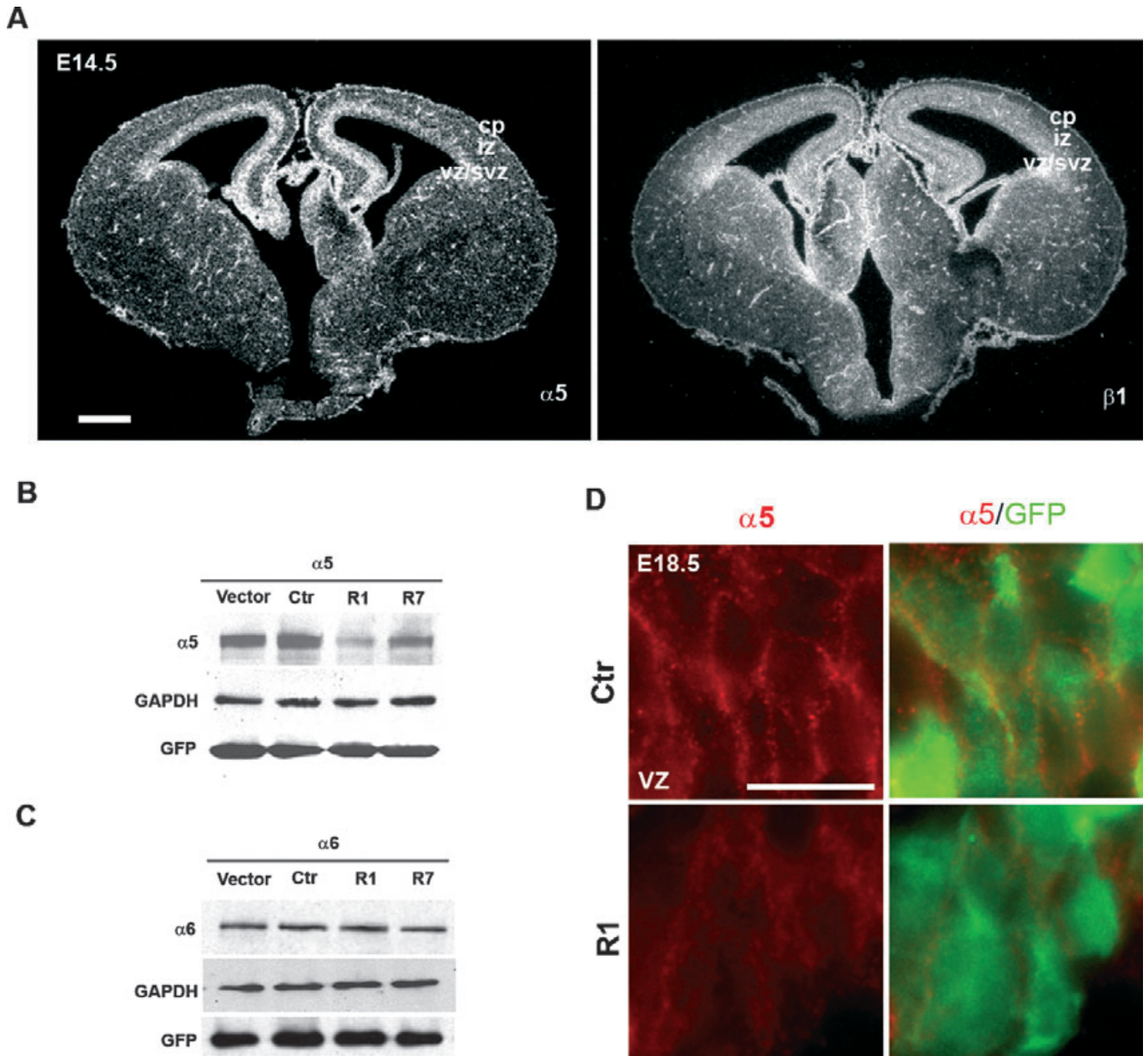
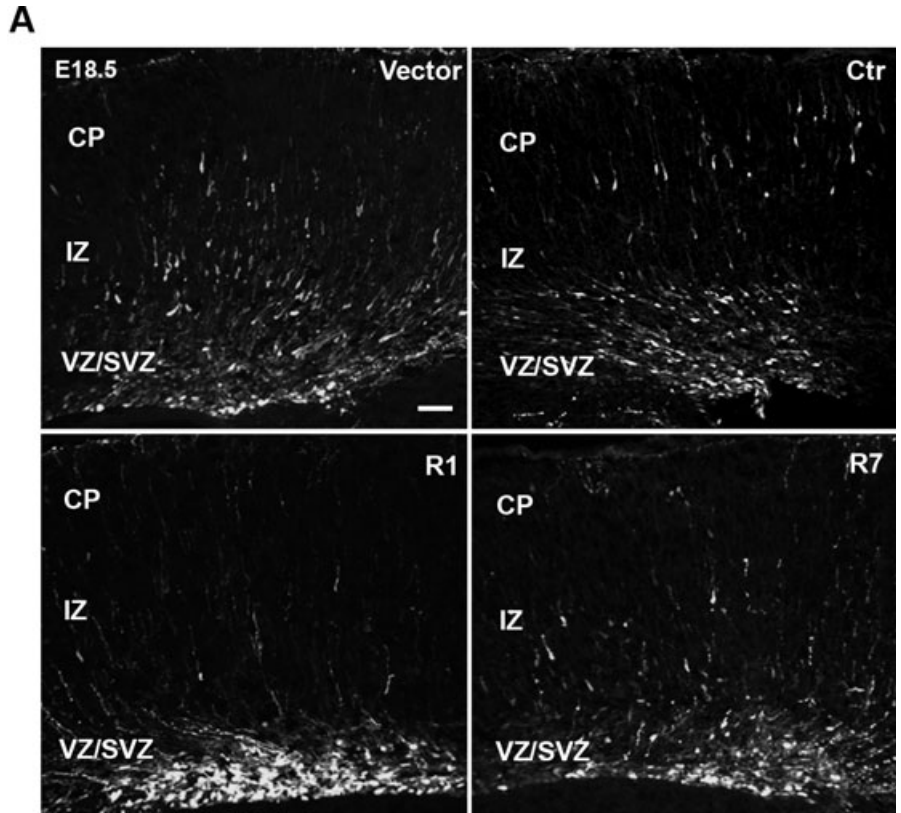


FIG. 1. Analysis of the expression and knockdown efficiency of $\alpha 5\beta 1$ integrin in the developing murine neocortex. (A) *In situ* hybridization of coronal brain sections at E14.5 revealed a signal in the VZ/SVZ for the $\alpha 5$ subunit; the signal for the $\beta 1$ subunit was widespread throughout the laminar structure of the cerebral cortex and visible in the VZ and SVZ in some areas. Scale bar, 500 μm . (B, C) pcDNA3.1 vector containing an $\alpha 5$ (B) or $\alpha 6$ (C) integrin murine cDNA was cotransfected into HeLa cells with pRNAT-U6.1/Neo empty vector, the control construct (Ctr), or two different shRNA constructs targeting mouse $\alpha 5$ integrin (R1 and R7). Forty-eight hours after transfection, cells were lysed and were subjected to immunoblotting for $\alpha 5$ integrin or $\alpha 6$ integrin. GAPDH and GFP are, respectively, loading and transfection efficiency controls. $\alpha 5$ -shRNA constructs were able to decrease $\alpha 5$ integrin expression by $\sim 80\%$ (R1) and $\sim 40\%$ (R7) but had no detectable effect on expression of the $\alpha 6$ integrin subunit. (D) High magnification of the VZ of E18.5 brain sections after electroporation with R1 $\alpha 5$ -shRNA. Immunostaining for $\alpha 5$ integrin (red) and GFP (green) showed a reduction of endogenous $\alpha 5$ integrin expression in the cortical neuronal progenitors compared with the control brain. Scale bar, 50 μm . VZ/SVZ, ventricular zone/subventricular zone; IZ, intermediate zone; CP, cortical plate.

GFP-positive cells were located in the VZ/SVZ with a limited number of cells in the IZ and CP (Fig. 2A). Electroporation of the R7 construct induced milder alterations as compared with the effects of the R1 construct, but still resulted in an abnormal distribution of GFP-positive cells (Fig. 2A).

To quantify the defects, we counted GFP-positive cells in the VZ/SVZ, IZ and CP regions of brains electroporated with the R1 construct ($n = 6$), with empty vector ($n = 4$) and with control construct ($n = 4$). In control brains, transfected with either the empty

vector or the Ctr construct, we observed the expected distribution of GFP-positive cells in the VZ/SVZ (empty vector: $59.8 \pm 1.0\%$; Ctr: $64.9 \pm 2.0\%$), in the IZ (empty vector: $33.2 \pm 0.8\%$; Ctr: $24.4 \pm 1.4\%$) and in the CP (empty vector: $6.9 \pm 1.2\%$; Ctr: $10.6 \pm 2.0\%$) (Fig. 2B). In contrast, in brains that were electroporated with the $\alpha 5$ -shRNA R1 construct, the majority of transfected cells were still located in the VZ/SVZ ($90.0 \pm 2.0\%$), with a small subset in the IZ ($7.6 \pm 1.4\%$) and CP ($2.2 \pm 1.0\%$) (Fig. 2B). These differences in the distribution of GFP-positive cells between the R1 and Ctr



B

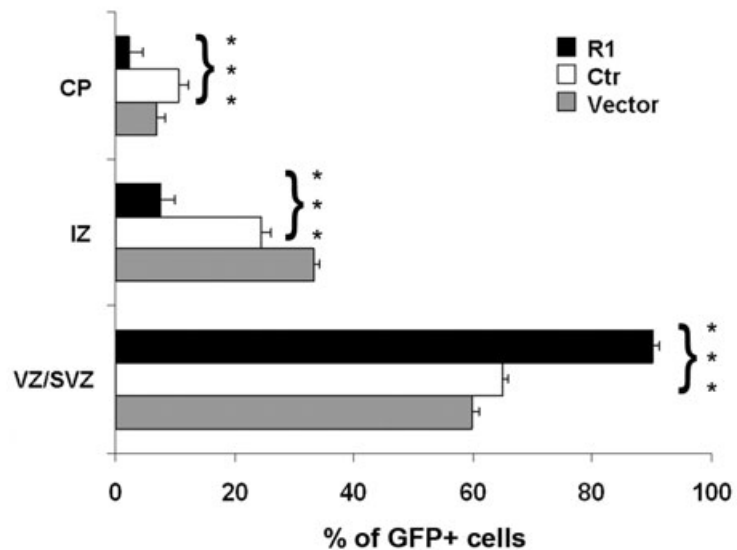


FIG. 2. Effect of $\alpha 5$ integrin knockdown in developing murine cerebral cortex. (A) Representative coronal sections of embryonic murine neocortex 3 days (E18.5) following electroporation of empty vector, Ctr, R1 and R7 $\alpha 5$ shRNAs together with coral GFP. Transfection of R1 and R7 shRNAs impaired radial migration. Scale bar, 100 μm .

(B) Quantitative analyses of the distribution of GFP-positive cells in the various cortical layers. The cortical wall was subdivided, and numbers of GFP-positive cells were counted in each layer and expressed as a percentage of the total. Statistical differences were seen for each layer, comparing the distribution of GFP-positive cells between Ctr and R1 $\alpha 5$ -shRNA. Asterisks indicate significant differences between the groups ($***P < 0.001$, using Student's *t*-test).

electroporated brains were statistically significant for each region ($P < 0.001$). An increase in the VZ/SVZ and a marked decrease in upper layers, without changes in proliferation or cell death, suggest that it is the migration process which is disturbed by the down-regulation of $\alpha 5$ integrin in neural progenitor cells.

Previous studies reported that deletion of the $\alpha 6$ or $\beta 1$ integrin subunits in the brain leads to defects in cortical laminar organization, associated with perturbations in the anchorage of radial glial fibers at the meningeal basement membrane (Georges-Labouesse *et al.*, 1998;

Graus-Porta *et al.*, 2001). To test whether the knockdown of $\alpha 5$ integrin in progenitors of the VZ at E15.5 perturbed the morphology of radial glial fibers or the formation of glial endfeet, coronal sections of brains electroporated with R1 and control constructs were immunostained with an anti-nestin antibody. GFP-nestin-positive radial glial fibers, containing the R1 construct, did not show any major morphological differences or glial endfeet defects compared with GFP-nestin-positive radial glia expressing a control construct (Fig. 3A). Thus, the reduction of integrin $\alpha 5\beta 1$ by electroporation at

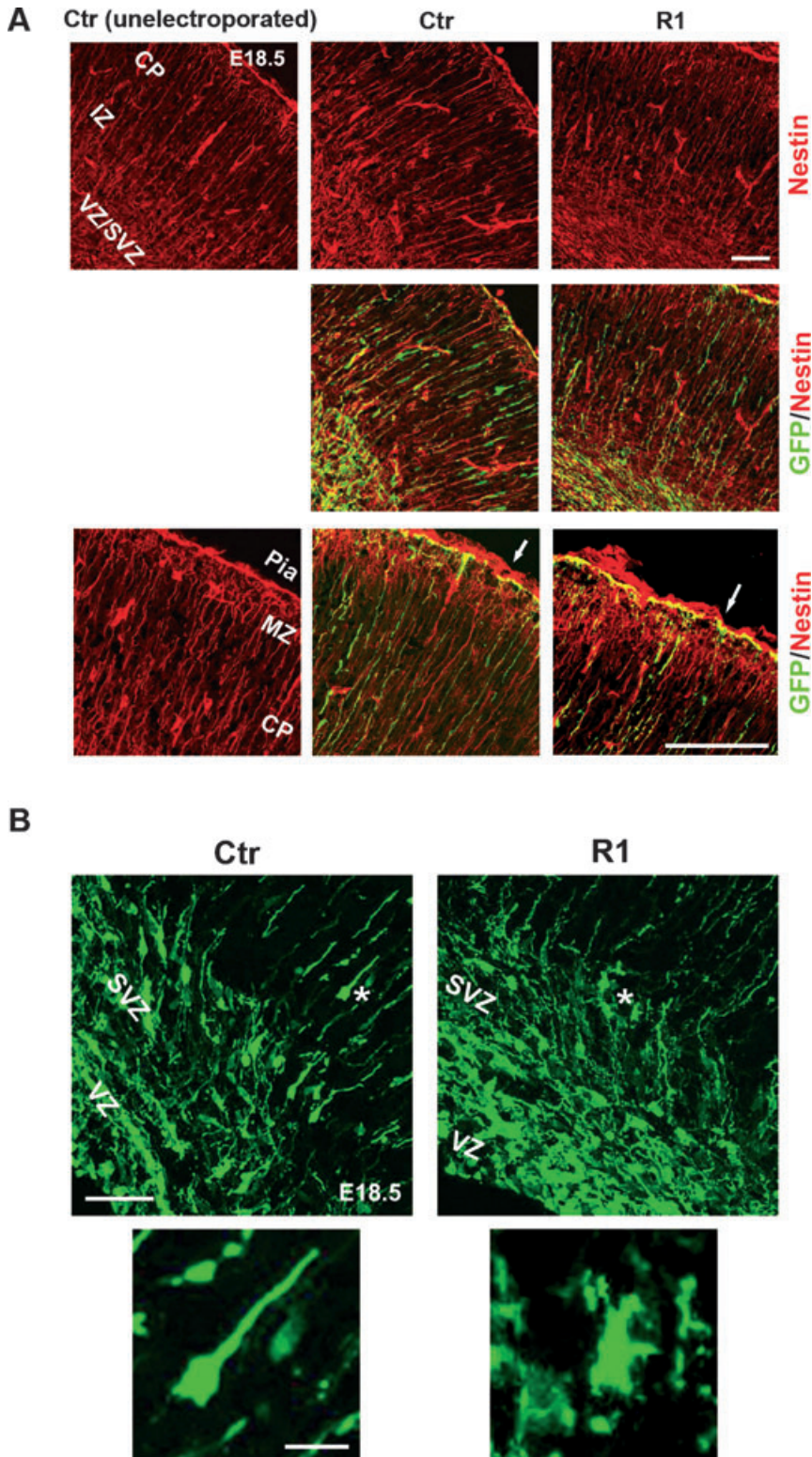


FIG. 3. Influence of $\alpha 5$ -shRNA on radial glial scaffold and neuronal morphology. (A) Coronal sections of unelectroporated hemisphere and of brains electroporated at E15.5 with Ctr and R1 $\alpha 5$ -shRNAs were stained with anti-Nestin (red) and GFP (green) antibodies. GFP-positive radial fibers transfected with R1 $\alpha 5$ -shRNA appeared normal as compared with unelectroporated and Ctr $\alpha 5$ -shRNA-transfected fibers. Scale bar, 100 μ m. Bottom panels are magnified views of the MZ. The white arrows indicate basal endfeet of radial fibers attached to the pia. Transfection of R1 $\alpha 5$ -shRNA does not seem to disturb formation of glial endfeet. MZ, marginal zone. Scale bar, 50 μ m. (B) Morphology of VZ/SVZ neurons transfected with Ctr or R1 $\alpha 5$ shRNAs. The electroporated cells with Ctr shRNA showed a normal bipolar morphology in the premigratory region (white asterisk, high magnification). In contrast, cells expressing shRNAs against $\alpha 5$ integrin are arrested within the VZ/SVZ with a multipolar shape (white asterisk, high magnification). Scale bars: top, 50 μ m; bottom, 10 μ m.

E15.5 did not induce major abnormalities in aspect or attachment of radial glial fibers.

It has been well documented that migration of cortical neurons is a complex process that includes radial glia-dependent and glia-independent steps (LoTurco & Bai, 2006). Recently, *in utero* electroporation of GFP plasmids followed by live imaging has allowed visualization of a population of multipolar cortical neurons which

seem to be transient intermediates during the migration process (Kriegstein & Noctor, 2004; LoTurco & Bai, 2006). Cells become multipolar when they exit the VZ and switch to a bipolar shape when they locate in the IZ. By examining the morphology of the $\alpha 5$ -shRNA R1-containing cells that accumulated in the premigratory zone, we found that many of them displayed a multipolar morphology, though abnormal, compared with the numerous bipolar cells transfected with

control shRNA (Fig. 3B). Many of the $\alpha 5$ -shRNA R1-containing multipolar cells presented a very irregular shape with several processes, as illustrated at high magnification in Fig. 3B.

To corroborate the results obtained with selective suppression of $\alpha 5$ integrin in the neuroepithelium by RNAi, we used a totally different approach, which was to induce the deletion of the $\alpha 5$ integrin gene by electroporating a vector encoding the Cre recombinase into cortical progenitor cells in the VZ of mice carrying an $\alpha 5$ integrin-floxed allele ($\alpha 5^{fl/fl}$, van der Flier *et al.*, unpublished data). To test for Cre activity of our Cre expression plasmid, we used *ROSA26* reporter mice (R26R), in which a stop cassette, flanked by loxP sites, inhibits *lacZ* gene expression (Soriano, 1999). We co-electroporated homozygous

R26R/R26R embryos with EGFP and the pxCANCre plasmid at E15.5, and examined the Cre recombination activity based on the expression of β -galactosidase resulting from Cre-mediated deletion of the floxed stop cassette after 3 days. X-gal staining showed specific *lacZ* activity in the EGFP + Cre electroporated region, indicating that Cre-mediated recombination had occurred (Fig. S3). We then co-electroporated EGFP and pxCAN-Cre plasmids into $\alpha 5^{fl/fl}$ mice ($n = 3$) and examined electroporated brains 3 days later. Migration defects were observed, comparable with those induced by $\alpha 5$ integrin silencing, with an accumulation of migrating neurons in the ventral region of the cortex (Fig. 4A). In contrast, in $\alpha 5^{fl/fl}$ mice electroporated with only EGFP ($n = 2$) or in wild-type mice co-electroporated with

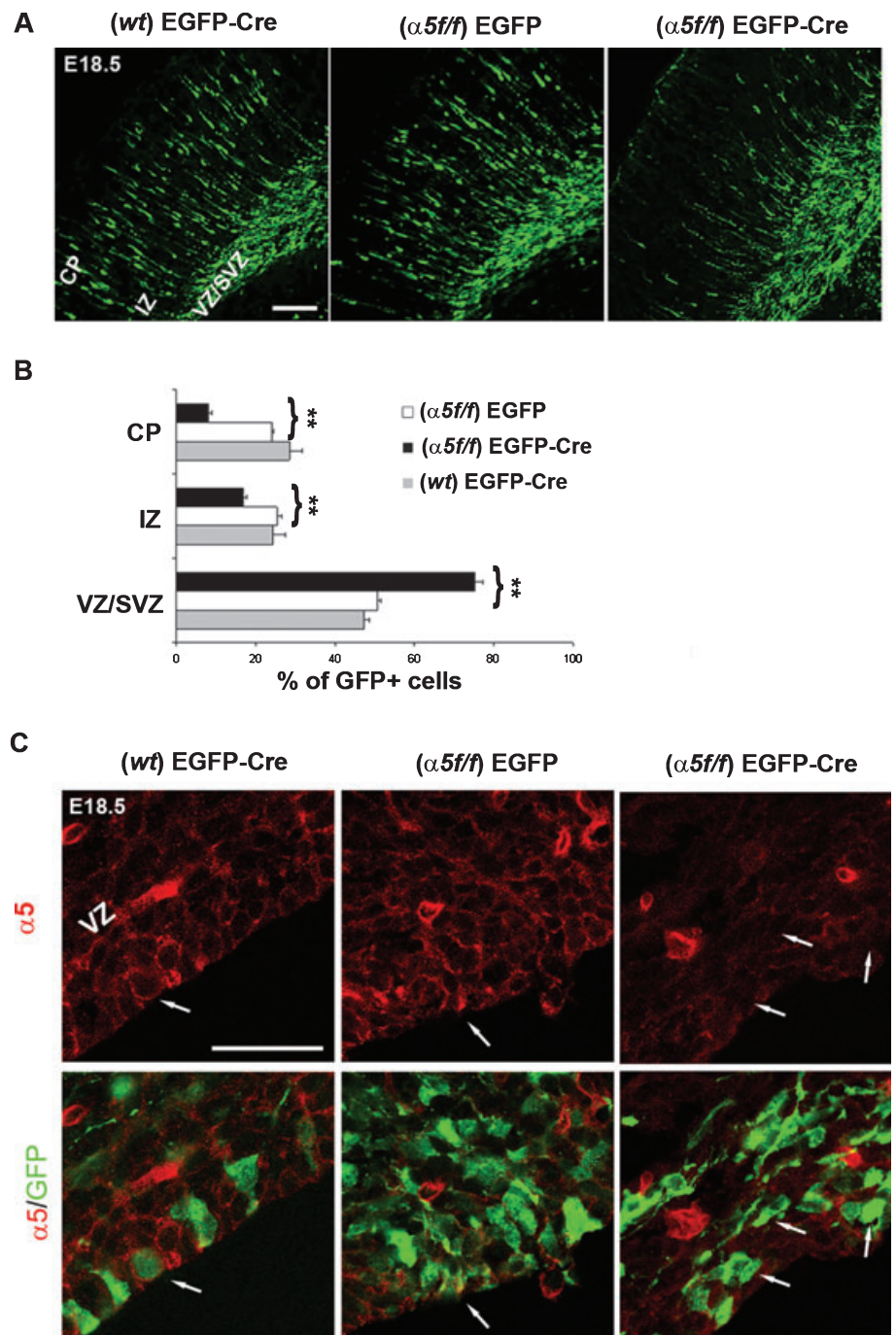


FIG. 4. Impaired radial migration after deletion of the $\alpha 5$ integrin gene. (A) Representative coronal sections of an E18.5 wild-type (*wt*) or floxed $\alpha 5$ ($\alpha 5^{fl/fl}$) mouse brain transfected at E15.5 with EGFP or EGFP + Cre expression plasmids. The expression of Cre recombinase in $\alpha 5^{fl/fl}$ mice inhibited radial migration. Scale bar, 100 μ m. (B) Quantitative analyses of the distribution of EGFP-positive cells in the various cortical layers. Statistical differences were seen for each layer, comparing the distribution of EGFP-positive cells in the $\alpha 5^{fl/fl}$ brain transfected with EGFP alone or EGFP + Cre plasmids. Asterisks indicate significant differences between the groups (** $P < 0.01$). (C) Reduction of $\alpha 5$ integrin expression in $\alpha 5^{fl/fl}$ neural progenitor cells electroporated with EGFP + Cre plasmids. The white arrows indicate the same cells immunostained for $\alpha 5$ integrin (red) and GFP (green). Scale bar, 20 μ m.

EGFP and Cre vectors ($n = 2$), the transfected cortical neurons migrated normally away from the VZ/SVZ to reach the CP (Fig. 4A). Quantification of EGFP-positive cells in the VZ/SVZ, IZ and CP regions of electroporated brains confirmed that Cre electroporation in $\alpha 5^{fl/fl}$ mice impaired radial migration. Over 75% of EGFP+Cre+ $\alpha 5^{fl/fl}$ cells remained in the VZ/SVZ, in contrast to 50% in control (EGFP only electroporations) ($P < 0.01$) (Fig. 4B). A fraction of EGFP+Cre+ $\alpha 5^{fl/fl}$ cells still initiated migration. By using markers of future layers V (Ctip2) (Leid *et al.*, 2004) or II/IV (Cux/Cdp) (Nieto *et al.*, 2004), a defect in migration was still present in these cells. As illustrated in Fig. S4, while control EGFP- $\alpha 5^{fl/fl}$ cells had reached and passed Ctip2 or Cux/Cdp-positive layers at E18.5, this was not the case for EGFP+Cre+ $\alpha 5^{fl/fl}$ cells, which were markedly delayed.

By staining coronal sections from $\alpha 5^{fl/fl}$ brains co-electroporated with EGFP + Cre vectors with an $\alpha 5$ integrin antibody, we were able to see a reduction of the $\alpha 5$ signal in EGFP-positive cells accumulated in the VZ/SVZ, indicating that the impairment of migration is indeed occurring in cells in which Cre recombinase had induced $\alpha 5$ integrin gene deletion (Fig. 4C).

As observed after electroporation of brains with siRNA R1 and R7, Cre-mediated deletion of $\alpha 5$ integrin gene in the cortical neural progenitors did not reveal gross morphological abnormalities in the glial processes or in the formation of glial endfeet (Fig. S5). In addition, morphological defects similar to those seen in the $\alpha 5$ -shRNA-R1 neurons were detected in the EGFP+Cre+ $\alpha 5^{fl/fl}$ migrating neurons (see multipolar cells with irregular shapes carrying several processes in Figs S4 and S5).

As mentioned, neuronal progenitors transfected at E15.5 should primarily produce neurons in postnatal layers II–IV (Langevin *et al.*, 2007). To investigate whether down-regulation of $\alpha 5$ integrin had an effect on formation of cortical layers II–IV, we performed *in utero* electroporation of shRNA R1 at E15.5 and examined the positions of transfected cells 6 days later (P2), a stage when targeted neurons have completed radial migration (Creppe *et al.*, 2009). A marked migration delay was observed in R1 electroporated brains compared with controls (Fig. 5A). In control transfected brains ($n = 3$) 49.3 \pm 0.8% of cGFP-positive cells reached layers II–IV. By contrast, in R1 electroporated brains ($n = 3$) only 20.3 \pm 1.6% of $\alpha 5$ -shRNA-express-

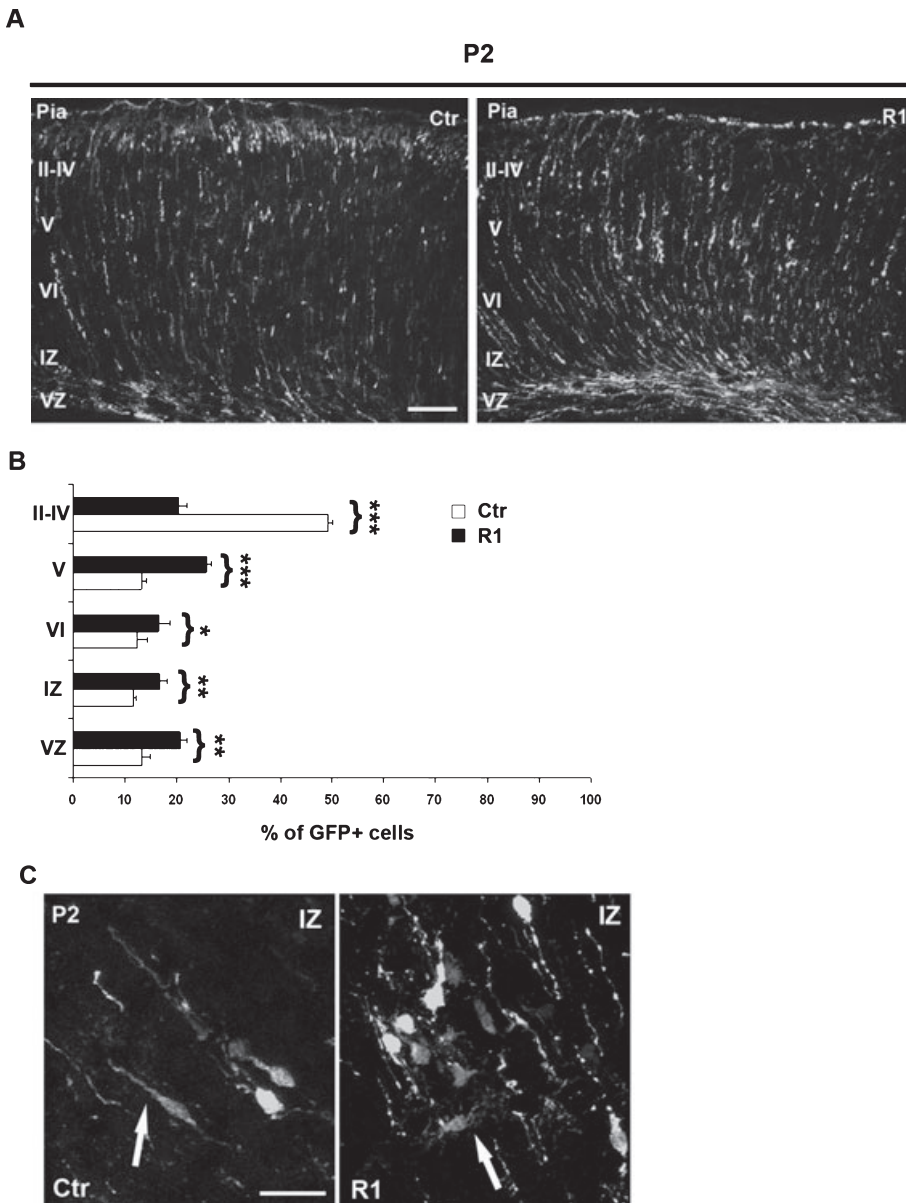


FIG. 5. Effect of $\alpha 5$ -shRNA on cerebral cortex laminar structure. (A) Representative coronal sections of embryonic murine neocortex 6 days (P2) following electroporation of Ctr and R1 constructs at E15.5. Transfection of R1 induces cortical lamination defects. Scale bar, 100 μm . (B) Quantitative analyses of the distribution of GFP-positive cells in the various cortical layers. Ctip-2 (red) labels layer V. Statistical differences were seen for each layer, comparing the distribution of GFP-positive cells between Ctr and R1 constructs. Asterisks indicate significant differences between the groups ($***P < 0.001$; $**P < 0.005$; $*P < 0.02$). (C) Morphology of neurons in IZ transfected with Ctr and R1 $\alpha 5$ shRNAs. Cells expressing shRNAs against $\alpha 5$ integrin showed atypical morphology compared with control transfected cells (white arrows). Scale bar, 50 μm .

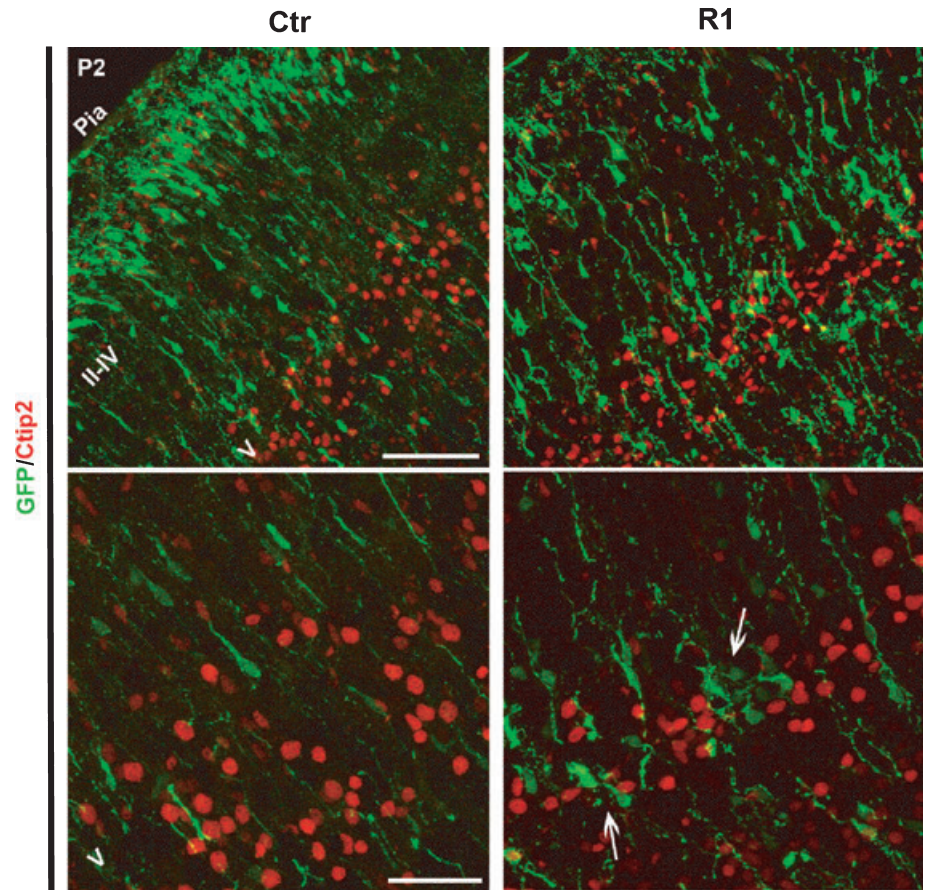


FIG. 6. Reduced expression of $\alpha 5$ integrin has no effect on neuronal differentiation. Electroporated brains were examined for the localization of the cortical marker Ctip2. In control and R1-transfected brains, neurons from layer V showed normal expression of Ctip2. Bottom panels are magnified views of layer V. The white arrows indicate neurons blocked in layer V but not expressing Ctip2. Scale bars: top, 50 μm ; bottom, 20 μm .

ing neurons were in layers II–IV ($P < 0.001$), whereas $58.9 \pm 5.3\%$ were located in cortical layers V and VI and in the IZ (Fig. 5B). Thus, the migration defects observed after 3 days still persist in the R1-transfected neurons after 6 days. In addition, in some regions along the cerebral cortex the $\alpha 5$ -shRNA-expressing neurons formed clusters and showed morphological abnormalities with formation of multiple neuronal protrusions (Fig. 5C). Similar morphological defects were already observed 3 days after electroporation in R1-transfected cells that accumulated in the ventricular region (Fig. 3B), and in the EGFP+Cre+ $\alpha 5^{fl/fl}$ migrating neurons (Figs S4 and S5), suggesting that $\alpha 5$ integrin could play a role in regulating neuronal polarity.

Interestingly, many $\alpha 5$ -shRNA-expressing neurons were located in layer V ($25.8 \pm 0.8\%$). This ectopic positioning could be due to defects in neuronal differentiation, as interactions of cells with the ECM are known to be critical for the establishment and maintenance of cell fate (Czyz & Wobus, 2001). To test this hypothesis, we performed immunostaining for Ctip2, a specific marker for layer V (Leid *et al.*, 2004). As illustrated in Fig. 6, the $\alpha 5$ -shRNA-expressing neurons accumulated in layer V did not express the Ctip2 transcription factor, suggesting that they were not typical deep layer V neurons (Fig. 6). In agreement with this result, staining at early stages of R1-electroporated brains with an antibody against β III tubulin (Tuj1) did not reveal obvious differences in the expression of this neuronal marker or ectopic labeling in the VZ, as compared with control brains (G. Marchetti *et al.*, unpublished observations). Together, our results indicate that the knockdown of $\alpha 5$ integrin in neural progenitors at E15.5 induces a delay in radial migration in the cerebral cortex associated with lamination defects at the level of cortical layers II–IV.

Discussion

This study provides evidence that down-regulation of the $\alpha 5 \beta 1$ integrin in the developing murine cerebral cortex impairs neuronal migration. We used an RNAi approach associated with *in utero* electroporation to reduce the expression level of $\alpha 5$ integrin in the neural progenitor cells in the VZ of E15.5 neocortex. By using two different shRNA sequences targeted against the $\alpha 5$ integrin gene, neuronal migration was inhibited. Both shRNA constructs reduced $\alpha 5$ integrin protein levels, whereas control shRNA constructs did not. We conclude that the impairment of neuronal migration was specifically due to the inhibition of $\alpha 5$ integrin expression. To exclude the possibility that the effect of $\alpha 5$ integrin shRNA on neuronal migration was dependent on potential off-target effects, we also used a different approach, which was to induce deletion of the $\alpha 5$ integrin gene in the cortical neural progenitors by Cre-mediated recombination. Migration defects were also observed with this approach. Therefore, by using two different approaches to inhibit expression of $\alpha 5$ integrin in the VZ of the developing neocortex, we were able to show that $\alpha 5$ integrin is required for the proper migration of cortical neurons. In agreement, it has been shown that tectal progenitors transfected with an antisense integrin $\beta 1$ RNA by retroviral infection failed to migrate into the chicken tectal plate (Galileo *et al.*, 1992).

The majority of the transfected cells that accumulated in the ventricular region of brains electroporated with R1 showed a multipolar morphology. Morphological defects were also observed in neurons in which the $\alpha 5$ integrin gene had been deleted. It has been proposed that several substages of multipolar migrating neurons exist, which differ by their migration rate, and may correspond to different

stages of migration (LoTurco & Bai, 2006). All of them switch to a bipolar shape when they exit the SVZ. This morphological transition requires a reorganization of the cytoskeleton. How these morphological changes are regulated remains to be elucidated. Molecules such as LIS1, doublecortin (DCX), Cdk5 and filamin A are necessary at different steps of this transition (LoTurco & Bai, 2006). Nagano *et al.* (2004) reported that FLNa, an actin-binding protein, acted on neuronal polarity and motility during mouse corticogenesis. They demonstrated that filamin A is involved in the multipolar-to-bipolar transition in migrating neocortical neurons. Indeed, filamin A reduction produces early arrest of multipolar cells in the SVZ similarly to integrin $\alpha 5$ knockdown while DCX RNAi induces 'late' arrest (LoTurco & Bai, 2006). As it has been shown that filamin A interacts with different integrin receptors and alterations of these interactions could affect cell migration and adhesion (Calderwood *et al.*, 2001), it is possible that $\alpha 5\beta 1$ integrin and FLNa play cooperative roles in neuronal migration and interact during cerebral cortex formation to regulate the transition from multipolar to bipolar morphology.

Several integrin mutations result in radial glial defects at the basement membrane, producing marginal zone heterotopia. Belvindrah *et al.* (2007) found that, in mice with a specific deletion of the $\beta 1$ integrin (*Itgb1*) gene in the post-mitotic neurons, neocortical development occurred normally. They concluded that $\beta 1$ integrins are essential in radial glia but not in neurons for the migration and proper development of the cerebral cortex. In contrast, in our studies, down-regulation of $\alpha 5$ integrin in the cortical neural precursors affects neuronal migration. These discrepancies could be due to acute knockdown of $\alpha 5$ integrin bypassing compensatory mechanisms that are implemented with genomic disruption. Indeed, acute loss-of-function approaches revealed functions in the case of the *doublecortin* or *connexin* genes (Bai *et al.*, 2003; Elias *et al.*, 2007). This down-regulation occurs in neural progenitors and could thus affect expression of $\alpha 5$ integrin in either or both neuroblasts and radial glia. However, under our conditions, radial glial fibers did not show marked defects, suggesting that $\alpha 5$ integrin does not play an essential role in glial morphology, consistent with the fact that we did not detect a clear $\alpha 5$ immunoreactivity in radial glial fibers. It should be kept in mind that at the stage that we performed *in utero* electroporation (E15.5), the glial scaffold could be already established. Indeed, electroporation at E11 and E13 of vectors which allow the expression of Cre recombinase into embryonic brains of $\beta 1$ integrin conditional mutant mice leads to stunted glial processes and lack of glial endfeet (Radakovits *et al.*, 2009). This phenotype is more severe than that observed in $\alpha 6$ integrin total knock-out mice, suggesting that other α subunits might be implicated (Georges-Labouesse *et al.*, 1998). To clarify if $\alpha 5\beta 1$ contributes to early stages of organization of glial fibers and formation of glial endfeet, it would be necessary to inhibit its expression at much earlier stages. In addition, although multipolar cells may not be always in contact with the glial scaffold, further analyses will be required to elucidate if subtle alterations of neuron/glia interactions contribute to the migration defects observed.

In contrast to experiments such as the knock-down of the elongator complex, in which delay in migration is temporary and no longer visible at P2 (Creppe *et al.*, 2009), we observed that the alterations of distribution of knocked-down $\alpha 5$ neurons persisted 6 days after electroporation at P2. Indeed, under control conditions, the majority of electroporated cells reached layer II–IV, while cells expressing $\alpha 5$ -shRNA R1 were located in layer V, which could have resulted from their altered fate or from defects in neuronal migration. Recent studies have revealed that regulation of integrin-mediated cell–matrix adhesions plays a role in differentiation of neuronal cells (Hajdo-Milasovic *et al.*, 2007). Interestingly, $\alpha 5$ -shRNA-R1-expressing

neurons accumulated in the cortical layer V were not positive for the layer V marker *Ctip2*. Therefore, reduction in $\alpha 5$ integrin expression did not affect differentiation of neuronal progenitors. Moreover, some $\alpha 5$ -shRNA-R1-expressing neurons showed an atypical morphology and an altered neuron polarity with misoriented neurites. These findings confirmed that the down-regulation of $\alpha 5$ integrin impairs radial migration, acting on neuronal morphology regulation.

In conclusion, our findings demonstrate that in early steps of neuronal migration in the cerebral cortex, the $\alpha 5\beta 1$ fibronectin receptor promotes migration away from the VZ/SVZ, and may also have a role in the regulation of neuronal morphology.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Expression of $\alpha 5$, $\alpha 6$ and $\beta 1$ integrin subunits in the developing cerebral cortex.

Fig. S2. Effects of $\alpha 5$ integrin down-regulation on cell proliferation and cell death of neural progenitors.

Fig. S3. Cre recombinase electroporation mediates efficient excision in the developing cerebral cortex.

Fig. S4. Impairment of cortical layering at E18.5 after deletion of the $\alpha 5$ integrin gene.

Fig. S5. Effects of $\alpha 5$ integrin gene deletion on radial glial scaffold and neuronal morphology.

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Acknowledgements

We thank W. Magnant and A. Vincent for their help with animals and L.-M. Langevin, C. Schuurmans and A. van Es for their advice during the setting up of *in utero* electroporation. We are grateful to V. Pfister for her expert technical assistance, to M. Koch for his help in confocal imaging, and to F. Polleux, R. Fässler, the DSHB, University of Iowa, and the Tsukuba Life Science Center, Riken, Japan, for reagents. This work was supported by funds from the CNRS, Inserm and Université de Strasbourg, as well as Association pour la recherche sur le cancer (ARC), Ligue régionale contre le cancer (LRC) and Association française contre les myopathies (AFM) to E.G.-L. R.O.H. is funded by the Howard Hughes Medical Institute and grants NHLBI PO1HL66105 and NIGMS GM064346. G.M. is a recipient of fellowships from the LRC and ARC. S.E. was supported by the French ministry of Research and ARC.

Abbreviations

BrdU, bromodeoxyuridine; Cdp, CCAAT-displacement protein; CP, cortical plate; *Ctip2*, chicken ovalbumin upstream promoter transcription factor-interacting protein 2; DAPI, 4',6-diamidino-2-phenylindole; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; IZ, intermediate zone; LN, laminin; MZ, marginal zone; PBS, phosphate-buffered saline; pH3, phospho-histone H3; R26R, *ROSA26* reporter mice; shRNA, short hairpin RNA; SVZ, subventricular zone; VZ, ventricular zone; $\alpha 5^{f/f}$, $\alpha 5$ integrin floxed mice.

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