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Rac1-Dependent Cell Cycle Exit of MGE Precursors and GABAergic Interneuron Migration to the Cortex

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Cortical γ-aminobutyric acid (GABA)ergic interneurons are characterized by extraordinary neurochemical and functional diversity. Although recent studies have uncovered some of the molecular components underlying interneuron development, including the cellular and molecular mechanisms guiding their migration to the cortex, the intracellular components involved are still unknown. Rac1, a member of the Rac subfamily of Rho-GTPases, has been implicated in various cellular processes such as cell cycle dynamics, axonogenesis, and migration. In this study, we have addressed the specific role of Rac1 in interneuron progenitors originating in the medial ganglionic eminence, via Cre/loxP technology. We show that ablation of Rac1 from Nkx2.1-positive progenitors, results in a migratory impairment. As a consequence, only half of GABAergic interneurons are found in the postnatal cortex. The rest remain aggregated in the ventral telencephalon and show morphological defects in their growing processes in vitro. Ablation of Rac1 from postmitotic progenitors does not result in similar defects, thus underlying a novel cell autonomous and stage-specific requirement for Rac1 activity, within proliferating progenitors of cortical interneurons. Rac1 is necessary for their transition from G1 to S phase, at least in part by regulating cyclin D levels and retinoblastoma protein phosphorylation.

Keywords: cortical development, inhibitory interneurons, Rho-GTPases

Introduction

γ-Aminobutyric acid (GABA)ergic interneurons of the cerebral cortex, comprising ~20% of cortical neurons, play important roles in cortical function and have been implicated in severe disorders such as schizophrenia and epilepsy. These cells are characterized by a remarkable morphological, molecular, and functional diversity, and recent studies have uncovered some of the molecular components underlying the generation of this diversity (Marin and Rubenstein 2003; Metin et al. 2006). All cortical interneurons originate from the ganglionic eminences, which are well-defined domains of the subpallial ventricular area, and migrate tangentially to populate the developing cortex (Marin and Rubenstein 2001; Marin and Rubenstein 2003). The cellular and molecular mechanisms guiding inteneurons from their subpallial origins to the cortex have only recently started to be elucidated. The long list of cues involved includes long-range attractive and repulsive factors, surface-bound permissive and instructive molecules, and motogenic factors (Marin and Rubenstein 2003; Metin et al. 2006; Butt et al. 2007). In contrast to the multiplicity of extracellular signals, the intracellular proteins that mediate the response to these cues are unknown (Martini et al. 2009).

Proteins of the Rac subfamily of Rho-GTPases are involved in many cellular functions, such as regulation of actin dynamics, cell cycle progression, establishment of polarity, and axonogenesis (Jaffe and Hall 2005; Watabe-Uchida et al. 2006; Koh 2007). This subfamily consists of 3 members: Rac1, a ubiquitously expressed protein; Rac2, expressed mostly in the hematopoietic system; and Rac3, which is highly enriched in the nervous system (Malosio et al. 1997).

Specifically, within the context of neuronal migration, experimental studies have shown that the migration defect in Lis1-deficient neurons correlates with disregulated Rho-GTPases and actin cytoskeleton (Kholmanskikh et al. 2003) and that Rac1 is involved in the interkinetic nuclear migration of cortical progenitor cells (Minobe et al. 2009). Specific inactivation of Rac1 from the ventricular zone (VZ) of the telencephalon ($Rac1^{fl/fl}$;$Foxg1^{Tg(Cre)}$) shows a wide spectrum of defects that range from severe defects in axonogenesis to perturbation in radial migration of cortical neurons as well as increased cell death of nascent neurons (Chen et al. 2007, 2009). In addition, interneurons derived from the medial ganglionic eminence (MGE) are absent from the cortex (Chen et al. 2007). In this mouse line, however, it is hard to distinguish cell autonomous from non–cell autonomous defects. An increase in cell cycle exit in the lateral ganglionic eminence (LGE) in $Rac1^{fl/fl}$;$Foxg1^{Tg(Cre)}$ embryos has also been observed (Chen et al. 2009). Therefore, Rac1 appears to play a crucial role in different cell biological processes in distinct neuronal populations and, possibly, distinct developmental time windows. This is not surprising, given the fact that small Rho-GTPases play various roles in the development of different cell types and tissues (Jaffe and Hall 2005).

The precise role of Rac1 in the development of cortical interneurons remains unclear. To restrict ablation of Rac1 in the MGE and examine the specific role of Rac1 in cortical interneurons originating in this region, we combined an Nkx2.1-Cre transgenic mouse (Fogarty et al. 2007) with a conditional allele of Rac1 (Walmsley et al. 2003), thus deleting the Rac1 gene in the MGE. Our analysis indicates that 50% of Rac1-deficient GABAergic interneurons originating in the MGE fail to migrate toward the cortex due to an intrinsic defect. This is likely due to their inability to progress through the restriction point within the G1 phase of the cell cycle.

Specific deletion of Rac1 in postmitotic interneurons resulted in no discernible phenotype, confirming the specific requirement for this protein in Nkx2.1-positive progenitors rather than postmitotic migratory MGE-derived neurons. These findings suggest a novel cell autonomous and stage-specific requirement of Rac1 activity within proliferating progenitors of cortical interneurons.

Materials and Methods

Generation of Nkx2.1-Cre- and Lhx6-Cre-Directed Conditional Rac1-Deficient Mice

Animals carrying a floxed allele of Rac1 ($Rac1^{fl/fl}$) (the fourth and fifth exon of the Rac1 gene are flanked with loxP sites, Walmsley et al. 2003) were crossed to $Nkx2.1^{Tg(Cre)}$ mice (Nkx2.1 transgenic Cre, Fogarty et al. 2007), in order to generate the $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$ genotype, and to $Lhx6^{Tg(Cre)}$ mice (Fogarty et al. 2007), in order to generate the $Rac1^{fl/fl}$;$Lhx6^{Tg(Cre)}$ genotype. The $ROSA26^{fl\text{-}STOP\text{-}fl\text{-}YFP}$ allele was also inserted as an independent marker (Srinivas et al. 2001) to allow visualization of the Rac1-mutant neurons, via yellow fluorescent protein (YFP) expression. $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;R26R-$YFP^{+/-}$ and $Rac1^{+/fl}$;$Nkx2.1^{Tg(Cre)}$;R26R-$YFP^{+/-}$ animals will be referred to as $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$ and $Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$, respectively, in Materials and Methods, and Figures and similarly, $Rac1^{fl/fl}$;$Lhx6^{Tg(Cre)}$;R26R-$YFP^{+/-}$ and $Rac1^{+/fl}$;$Lhx6^{Tg(Cre)}$;R26R-$YFP^{+/-}$animals will be written as $Rac1^{+/fl}$;$Lhx6^{+/Cre}$ and $Rac1^{fl/fl}$;$Lhx6^{+/Cre}$, respectively, in Materials and Methods, and Figures. The genotyping was performed by polymerase chain reaction (shown in Supplementary Fig. 1*E*), using specific primers for:

Rac1
1-GTTGAAGGTGCTAGCTTGGGAACTG
2-GAAGGAAGAAGAAGCTGACTCCCATC 3-CAGCCACAGGCAATGACAGATGTTC
YFP
1-GCTCTGAGTTGTTATCAGTAAGG
2-GCGAAGAGTTTGTCCTCAACC
3-GGAGCGGGAGAAATGGATATG
Nkx2.1-Cre
1-GTCCACCATGGTGCCCAAGAAGAAG
2-GCCTGAATTCTCAGTCCCCATCTTCGAGC
Lhx6-Cre
1-GAGGGACTACCTCCTGTATC
2-TGCCCAGAGTCATCCTTGGC

Additional details for the genotyping of animals are available on request.

For timed pregnancies, the day of the vaginal plug was designated as embryonic day E0.5 and the day of birth was considered as P0.

Immunohistochemistry

For immunohistochemistry, embryonic brains were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) overnight, whereas adult animals were first perfused with 4% PFA and then the brains were dissected and immersed in the same fixative for 3 h. Following fixation, the tissues were cryoprotected by infiltration in 30% sucrose in PBS and 10 μm cryostat sections were collected on superfrost slides. For immunostaining, the slides were washed in 0.1%Triton X-100 in PBS (PBT), then blocked in 1% fetal bovine serum and 0.1% BSA in PBT for 1 h at room temperature (RT), and subsequently incubated with primary antibodies, diluted in blocking solution, for 18 h at 4 °C. Following this, the slides were washed in PBS (3 washes, 10 min each) and incubated with the secondary antibody, diluted in blocking solution, for 2 h at RT. After another 3 washes (10 min each), the slides were mounted, using MOWIOL (Calbiochem, Darmstadt, Germany). Primary antibodies used: mouse anti-Rac1 (1:20; Cytoskeleton, Denver, CO), mouse monoclonal anti-GFP (1:500; Invitrogen, San Diego, CA), rabbit polyclonal anti-GFP (1:5000; Minotech biotechnology, Heraklion, Greece), rat monoclonal anti-GFP (1:500; Nacalai Tesque, Kyoto, Japan), rabbit polyclonal anti-Lhx6 (1:200; Lavdas et al. 1999), rabbit polyclonal anti-GABA (1:1000; Sigma, Saint Louis, MI), mouse monoclonal anti-parvalbumin (PV) (1:1000; Chemicon, Temecula, CA), rabbit polyclonal anti-calretinin (CR) (1:1000; Swant, Bellinzona, Switzerland), rat monoclonal anti-NPY (Sigma, 1:500), rabbit polyclonal anti-somatostatin (Som) (1:500; Abcam, Cambridge, MA), rat polyclonal anti-BrdU (1:1000; Oxford Biotech, Oxford, UK), rabbit polyclonal anti-Ki67 (1:1000; Vector Laboratories, Burlingame, CA), rabbit anti-cleaved caspase 3 (1:200; Cell signaling, Beverly, MA), rabbit anti-PH3 (1:200; Upstate, Temecula, CA), mouse anti-Tuj1 (1:500; Covance, Berkeley, CA), rabbit anti-neurofilament (1:500; NF-M, Covance), mouse monoclonal anti-cortactin (1:200; Upstate, NY). Secondary antibodies used: goat anti-mouse-Alexa Fluor-488, -555, or -633, goat anti-rabbitAlexa Fluor-488, -555, or -633 and goat anti-rat-Alexa Fluor-488, -555, or -633 (all from Molecular Probes, Eugene, OR, all 1:800). Alexa 555-phalloidin was used for F-actin staining (Invitrogen).

BrdU Incorporation and Staining

Pregnant females of designated embryonic stages were injected intraperitoneally with BrdU (50 μg/animal gr) and sacrificed at appropriate stages to collect the embryos. The brains were processed for immunohistochemistry, with GFP, Ki67, or PH3 antibody, and then, they were treated with 2 N HCl to expose the BrdU antigen, so that BrdU antibody staining could follow.

Western Blot

MGE and LGE from E13.5 forebrain tissue was isolated and lysed in 85 mM Tris pH7.5, 30 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 120 mM glucose, 1% Triton X-100, 60 mM octyl β-D glucopyranoside (Sigma), and 1 mM phenylmethylsulfonyl fluoride. Lysates were run on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred into nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Membranes were subsequently blocked with 5% milk in PBS, 0.1% Tween-20, and immunoblotted with mouse anti-Rac1 (1:500, Cytoskeleton), rabbit anti-Phospho-Rb (1:1000, Cell Signaling), rabbit anti-cyclin D2 (1:500, Santa-Cruz Biotechnology, Santa-Cruz, CA, recognizing also cyclin D1), and mouse anti-actin (1:4000, Chemicon) diluted in blocking solution. Secondary antibodies used: anti-mouse-IgG-Horseradish Peroxidase and anti-rabbit-Horseradish Peroxidase (1:4000; GE Healthcare, Buckinghamshire, UK).

RNA In Situ Hybridization

Nonradioactive in situ hybridization on fixed cryostat sections was performed as described previously (Denaxa et al. 2001; Kyriakopoulou et al. 2002). Riboprobes used were specific for *Gad67* (kindly provided by Dr F. Guillemot, National Institute for Medical Research, Medical Research Council, Mill Hill) *Lhx6* (Grigoriou et al. 1998), *NPY*, and *Somatostatin* (Liodis et al. 2007).

MGE Matrigel Explants

MGE explants were prepared from forebrains derived from E13.5 mouse embryos. Small tissue fragments corresponding to the subventricular zone (SVZ) of the MGE were incubated for 1 h in L15 + 10% fetal calf serum at 37 °C in 5% CO_2. Subsequently, explants were placed in a 3D Matrigel gel matrix (BD Biosciences, San Jose, CA) and cultured for 24 or 48 h in Neurobasal medium in 4-well plates (Nunc, Naperville, IL) as described previously (Metin et al. 1997). Explants were then fixed in 4% PFA and analyzed using an epifluorescence microscope (Axiophot/Zeiss, Oberkochen, Germany).

MGE Dissociated Cell Culture

MGEs derived from forebrains of E13.5 mouse embryos were treated with trypsin (0.2%, Gibco) 10 min at 37 °C in 5% CO2. Subsequently, the cells were plated on coverslips coated with collagen (5 mg/mL) (Gibco) or Matrigel gel matrix using Neurobasal medium for 48 h.

Quantification and Statistical Analysis

For quantification of GABAergic interneurons at P0 or for assessing the different interneuronal subpopulations in P15 brains, at least 3 pairs of

littermate animals were used ($Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$ vs. $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$). For each pair, 4 sections corresponding to distinct bregmata along the rostrocaudal axis (−1.94 and 2) were selected, all including the barrel cortex field. Using appropriate molecular markers (PV, CR, somatostatin, NPY, YFP, or GABA), images were obtained with a confocal microscope (Leica TCS SP2, Leica, Nussloch, Germany), cells in the entire barrel field were counted and an average rostrocaudal number was calculated for the interneuron subpopulations of $Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$ and $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$ animals. In all cases, data are presented as mean ± standard error of mean (SEM). The effect of the genotype on each subpopulation was assessed using ANOVA for repeated measurements and Student's *t*-test.

For quantification on embryonic sections, at least 3 pairs of littermates were used in each case ($Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$ vs. $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$). For each pair, 4 sections were used for confocal microscopy, all containing the region corresponding to the MGE, from rostral to caudal levels. From these 4 sections, an average number of cells was calculated for comparison of the 2 genotypes. The effect was assessed using ANOVA for repeated measurements, and Student's *t*-test, and all data are presented as mean ± SEM. The ImageJ program was used for the measurements of the leading process length and angles in vivo. Thirty cells were randomly picked on each of 4 consecutive sections per animal, and the statistical analysis was performed using ANOVA for repeated measurements and Student's *t*-test.

In order to quantify the Rac1 defect on MGE matrigel explants, 4 pairs of E13.5 littermate embryos were used ($Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$ and $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$). From each embryo, the MGE was isolated and 5 explants were cultured from each MGE. After 48 h in culture, the 10 further away positioned neurons were selected from each explant, and their distance from the explant perimeter was measured. An average migrating distance was obtained for each MGE, and the effect of the genotype was assessed using ANOVA for repeated measurements and Student's *t*-test. The data represent mean ± SEM.

Quantification of axon lengths in vitro, on matrigel or collagen, was performed using the ImageJ program, and statistical significance was assessed using Student's *t*-test. At least 100 cells were measured of each genotype in 3 different experiments.

Results

Conditional Ablation of Rac1 from MGE-Derived Interneurons by Nkx2.1-Cre: Defects during Embryogenesis

To examine the role of Rac1 in the development of the subpopulation of cortical interneurons originating in the MGE, we generated mice homozygous for a floxed allele of Rac1 ($Rac1^{fl}$) (Walmsley et al. 2003) in the presence of an Nkx2.1-Cre transgene ($Nkx2.1^{Tg(Cre)}$) (Kessaris et al. 2006; Fogarty et al. 2007). Expression of this transgene recapitulates the pattern of expression of the endogenous Nkx2.1 gene and has been previously used to lineally mark MGE-derived cortical interneurons, which can be identified by the expression of calbindin (CB), PV, and somatostatin (Som) (Sussel et al. 1999; Fogarty et al. 2007; Butt et al. 2008; Wonders et al. 2008). To be able to identify and follow the cells in which Rac1 has been deleted, we also introduced the reporter Rosa26StopYFP which expresses YFP in a Cre-dependent manner (Srinivas et al. 2001). $Rac1^{+/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ animals were used as controls, while $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ were used as Rac1 mutants. Ablation of the Rac1 protein from the MGE of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ animals occurs at E12 (Supplementary Fig. 1*A*,*B*) and by E13.5 (Supplementary Fig. 1c1, c2, *C*, d1, d2, *D*), no YFP^+ MGE-derived cells coexpress Rac1 and YFP, as assessed by immunohistochemistry. In addition, by western blotting, we do not recognize the Rac1-specific signal in E13.5 lysates from the MGE (Fig. 5*C* and Supplementary Fig. 1*F*).

In E13.5 control embryos, YFP-expressing interneurons are migrating tangentially forming the characteristic 2 cellular streams in the marginal (MZ) and the intermediate (IZ)/SVZ (Marin and Rubenstein 2003; Metin et al. 2006). In contrast, in $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ embryos, the migration of MGE-derived interneurons is disrupted since YFP^+ cells are found only ventrally with respect to the pallial–subpallial boundary (cf. Fig. 1*A*,*D*). Consistently, analysis of the expression of MGE-specific interneuronal markers, such as Lhx6 (Lavdas et al. 1999; Cobos et al. 2005, 2006; Liodis et al. 2007) and Som, showed absence of positive cells within the developing neocortex of E13.5 embryos (Fig. 1*A*–*D*). Similarly, the expression of *gad67* mRNA reveals fewer migrating cells in the cortex of E14.5 $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ embryos (Fig. 1*H*) compared with the cortex of control littermates (Fig. 1*G*).

At E16.5 (Fig. 1*I*–*P*), we observe that YFP^+ interneurons manage to reach the developing cortex of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ embryos, but their number is significantly reduced compared with their control littermates. However, relative to control embryos, the vast majority of YFP^+ cells in the forebrain of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ embryos aggregate ventrally in their place of birth (asterisks in Fig. 1*I*,*M* and big white arrows in Fig. 1*J*,*K*,*N*,*O*). The ventral aggregation of YFP^+ cells in the brains of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ animals can be seen even at P0 (Supplementary Fig. 2), resulting in a significant reduction of $GABA^+YFP^+$ interneurons that are present in the mutant cortex at P0 (arrows in Fig. 1*S*,*T*), compared with the controls (arrows in Fig. 1*Q*,*R*). Almost half of the MGE-derived cells are absent from the mutant brains (Fig. 1*U*). Taken together, these developmental studies suggest that the specific deletion of Rac1 in the VZ of the MGE results in a dramatic reduction in the number of interneurons that enter the pallium.

$Nkx2.1^+$ MGE-resident progenitors contribute to other cell types in addition to the cortical GABAergic interneurons, such as oligodendrocytes and striatal interneurons (Kessaris et al. 2006). We investigated whether ablation of Rac1 affects these cells, as well. Indeed, using immunohistochemistry against YFP, we observed that the total number of YFP cells in the striatum of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ animals were significantly reduced compared with control brains (P15, Supplementary Fig. 3*A*′, *A*, a). Nevertheless and in agreement with our data on the cortical subpopulations (see below, Fig. 2 and Supplementary Fig. 5), the percentage of $ChAT^+$;YFP^+ cells to the total YFP^+ population in the striatum remained unaffected (Supplementary Fig. 3*B*,*B*′, b).

Similar analysis for the expression of Olig2 and YFP showed that the number of $Olig2^+$ MGE-derived oligodendrocytes (P0, Supplementary Fig. 3*C*, *C*′, c; E14.5 and 16.5, data not shown) was significantly reduced in the cortex of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ animals, while its ratio to the total YFP^+ cells remained unaffected. Therefore, Rac1 ablation from the MGE affects all cell populations originating there.

Delayed Onset of Migration of Rac1-Deficient MGE-Derived Interneurons

To further examine the migratory behavior of Rac1-deficient MGE-derived interneurons, we placed fragments of MGE dissected from E13.5 control and $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ embryos in explant cultures for 24 or 48 h in a 3D Matrigel matrix (Supplementary Fig. 4*A*,*B*). At the end of the

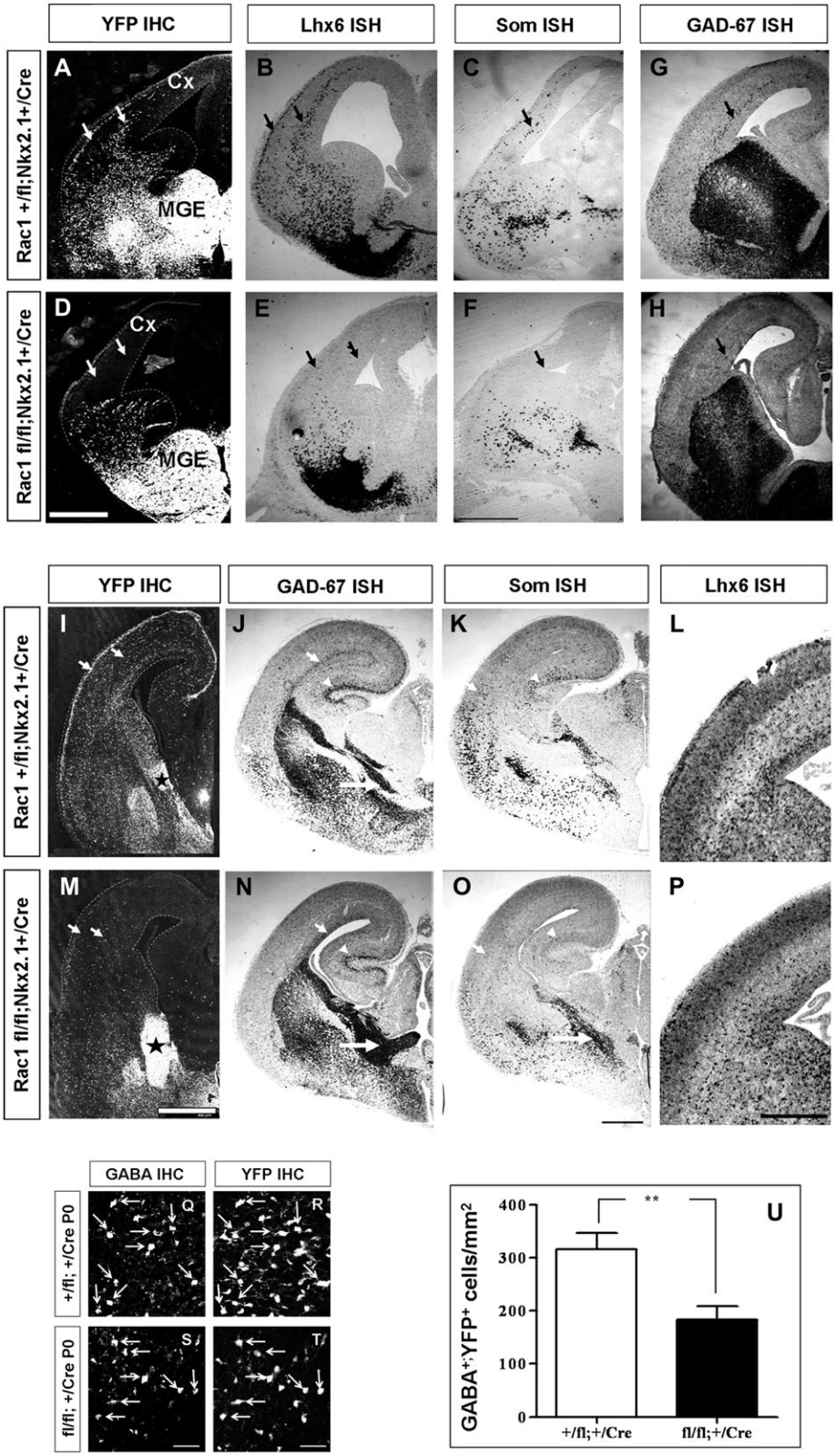

Figure 1. Ablation of Rac1 from GABAergic interneurons leads to a delayed migration toward the developing neocortex. (*A*–*P*) Coronal sections from the forebrain of developing embryos. At E13.5 (*A*–*F*) and E14.5 (*G*–*H*), Rac1-deficient interneurons (*D*, *E*, *F*, *H*: $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$) fail to migrate toward the developing neorcortex, compared with the control cells (arrows in *A*, *B*, *C*, *G*: $Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$), as indicated by specific markers of GABAergic interneurons. Later on, at E16.5 (*I*–*P*), some of the Rac1-deficient cells manage to reach the cortex (arrows and arrowheads in *M*, *N*, *O*, *P*), although most of the cells remain aggregated at the ventral telencephalon (black asterisks in *M* and big white arrows in *N*, *O*), as revealed by staining for YFP and other interneuronal markers, in contrast to the control interneurons that have almost completed their tangential migration (black asterisk in *I* and big white arrows in *J*, *K*). As a result, significantly reduced $GABA^{+}$–YFP^{+} interneurons are present in the P0 mutant brains (arrows in *Q*, *R*) compared with the control (arrows in *S*, *T*). (*U*) Statistical analysis was performed using Student's *t*-test (**P value $<<$ 0.05) (for details on statistical analysis, see Materials and Methods). Arrows and arrowheads indicate the migratory streams of the marginal zone (MZ) and intermediate zone (IZ). Scale bars: A, D, I, M, 300 μm; B, C, G, E, F, H, J, K, L, N, O, P, 100 μm; Q, R, S, T, 75 μm.

culture period, we measured the longest distance traveled by interneurons exiting the explant. At 24 h, a relatively large number of cells had emigrated from control explants (Supplementary Fig. 4*A*), but no cells had emerged from Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ MGE explants (Supplementary Fig. 4*B*). At 48 h, cells invading the surrounding matrigel matrix were observed in both the control and Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ explants. However, the longest distance traveled by cells in mutant explants after 48 h was approximately half to that observed in control explants (Supplementary Fig. 4*E*). Nevertheless, the distance traveled by the mutant cells during the second 24 h in culture was not significantly different from the distance traveled by control cells during the same period (Supplementary Fig 4*F*), suggesting that the speed of the migrating cells is not altered when Rac1 is ablated.

These findings indicate that the interneuron defect observed in Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ animals is intrinsic to the MGE-derived cells and not due to a secondary defect in the cellular environment. In addition, they suggest that upon deletion of Rac1, the initiation of migration of MGE cells occurs later than in control cases. Importantly, these ex vivo observations correlate with the observed in vivo phenotype, where mutant cells start their migration later than the control ones.

Rac1 Ablation from MGE-Derived Interneurons by Nkx2.1-Cre: Defects in Postnatal Brains

To examine the long-term consequences of Rac1 ablation in the development of GABAergic neurons in the cortex, we also analyzed the mice postnatally. Since the vast majority of these animals die at around weaning time, our analysis was carried out at P15. At this stage, we observed no differences in the overall structure of the brain between control and mutant animals, as assessed by histological staining with cresyl violet (data not shown). The number and distribution of interneurons in the neocortex were examined either by expression of the lineage marker YFP (Fig 2*A* and Supplementary Fig. 5*A*,*A*′) and the general marker GABA (at P0, Fig. 1*Q*,*R*) or markers that are specifically expressed in MGE-derived interneuronal subpopulations such as Lhx6, PV, and Som (Fig. 2*B*,*B*′,*C*,*C*′,*D*,*D*′, respectively, and Supplementary Fig. 5*B*,*B*′,*C*,*C*′*D*,*D*′, respectively). We have also examined calretinin (CR) and NPY (Fig. 2*E*,*E*′,*F*, respectively, and Supplementary Fig. 5*E*,*E*′*F*,*F*′, respectively), which are primarily expressed by cortical interneurons derived from the caudal ganglionic eminence (CGE) (Fogarty et al. 2007; Butt et al. 2008). Although many areas of the cortex were examined, we focused primarily in the barrel cortex (Fig. 2*G*) from where images were obtained and counts were performed (see Materials and Methods). A significant reduction of the number of YFP cells in Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ cortices is shown in Figure 2*A*. A similar reduction was observed in the number of cells expressing markers appropriate for MGE-derived interneuron subpopulations, namely Lhx6, PV, and Som (Fig. 2*B*,*C*,*D*, respectively). However, the percentage of YFP^{+} neurons that co-express Lhx6, PV, and Som was not significantly different between control and Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$(Fig. 2*B*′,*C*′,*D*′, respectively), indicating that the ability of Rac1-deficient precursors to differentiate into different mature interneuron subtypes is not compromised, despite their reduced numbers. CGE-derived interneurons, expressing

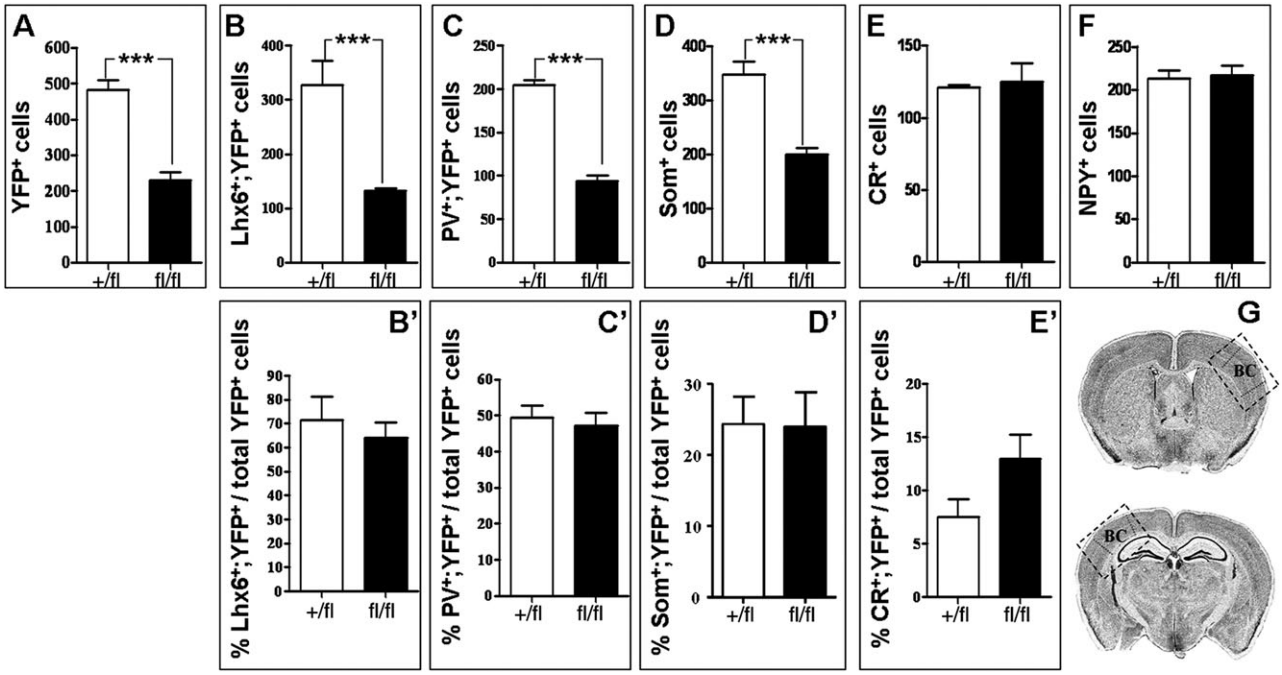

Figure 2. The number of MGE-derived interneurons subtypes is highly reduced in the adult Rac1 mutant brains. On coronal sections from P15 Rac1-deficient brains, the number of YFP^{+} interneurons is reduced to half compared with the control (*A*). In agreement with this, Lhx6^{+} cells are also severely reduced in the mutants (*B*) and so are most of the subpopulations that derive from Lhx6 precursors, like PV (*C*), and somatostatin (*D*). However, the percentage of Lhx6;YFP, PV;YFP and Som;YFP double-positive cells over the total number of YFP cells in the cortex remains unaffected, indicating a migration rather than a differentiation defect of these cells (*B*′, *C*′, *D*′). The number of calretinin^{+} (*E*, *E*′), and NPY^{+} (*F*) interneurons is not altered in the Rac1 conditional KO brains. These subpopulations are mainly derived from the CGE, where Nkx2.1 is not expressed. Even the small percentage of CR^{+} cells that derives from the MGE and expresses YFP is not significantly altered. (*G*) Representation of the brain sections that were used for counting. The dashed boxes indicate the area counted on each bregma, which includes the barrel cortex field (BC) between the lines. Statistical significance was assessed, using ANOVA for repeated measurements and Student's *t*-test (**P value $<<$ 0.05) (for details on statistical analysis, see Materials and Methods).

calretinin and NPY, show no significant change in their numbers or distribution in the adult cortex (Fig. 2*E*,*E*′,*F* and Supplementary Fig. 5*E*,*E*′*F*,*F*′). Together, our results show that the neocortex of Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ P15 animals has a dramatic reduction in the number of MGE-derived interneurons.

Rac1 Ablation in Postmitotic Interneurons Does Not Influence Their Development

In order to verify the necessity of Rac1 in the Nkx2.1-positive progenitors of the MGE and to exclude an additional essential role for Rac1 in postmitotic cells, we deleted Rac1 specifically in postmitotic MGE-derived interneurons expressing Lhx6 using Lhx6-Cre transgenic mice (Fogarty et al. 2007). Ablation was confirmed by western blot analysis using protein extracts from E13.5 MGE tissue (Supplementary Fig. 1*F*). Rac1$^{fl/fl}$;Lhx6$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ mice die soon after birth, rendering analysis of the adult brains impossible. This could be a result of defects in other systems where Lhx6 is expressed (i.e., oral ectomesenchyme, Grigoriou et al. 1998; Denaxa et al. 2009). Therefore, we restricted our experiments to embryonic and early postnatal stages. Control and Rac1$^{fl/fl}$;Lhx6$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ cortices from E13.5 embryos (Fig. 3*A*, a, *D*, d) and newborn mice (Fig. 3*B*,*C*,*E*,*F*) were analyzed using a similar set of markers employed for the analysis of Rac1$^{fl/fl}$;-Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ animals, such as Lhx6 and somatostatin. As expected, approximately 97% of YFP cells were also expressing Lhx6 (Fig. 3*H*). No difference is observed in cortical interneurons between the 2 genotypes, either in their number or distribution, as indicated by immunohistochemistry for YFP and Lhx6 at E13.5 (Fig. 3*G*,*H*) or in situ hybridization for Lhx6 and Som mRNA at P0 (Fig. 3*I*). These findings contrast those obtained from Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ mice and indicate that the requirement of Rac1 activity for the development of cortical interneurons is restricted to the progenitors that reside in the VZ of the MGE.

Rac1 Ablation Does Not Affect the Proliferation or Survival of MGE-Derived Progenitors

In order to study whether the reduction in the number of cortical interneurons is due to perturbed proliferation in the VZ, we assessed the pool size of proliferating

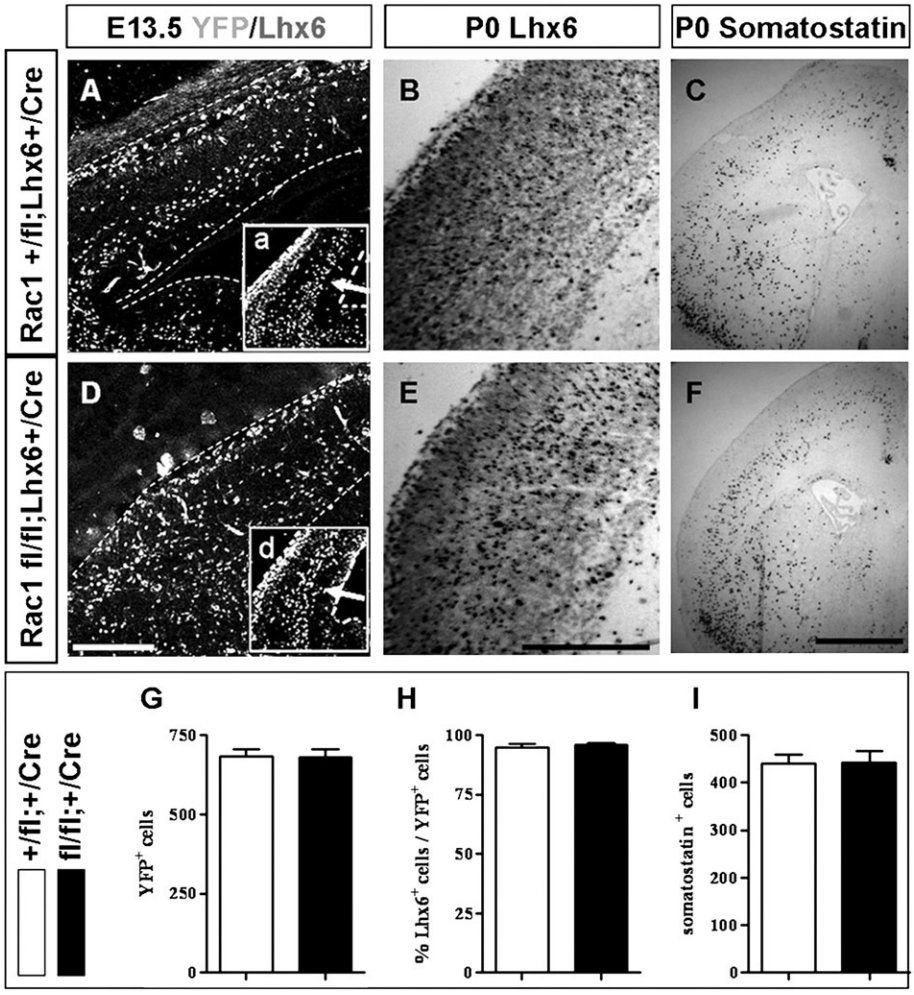

Figure 3. Ablation of Rac1 from postmitotic GABAergic interneurons, using the Lhx6-Cre line, does not affect the development of the neocortex. Migration of interneurons occurs normally in E13.5 Rac1$^{fl/fl}$;Lhx6$^{+/Cre}$ embryos (*D*), compared with their control Rac1$^{+/fl}$;Lhx6$^{+/Cre}$ littermates (*A*), as revealed by staining for YFP and Lhx6 (white arrows in a, d). Additionally, the postnatal brain of Rac1$^{fl/fl}$;Lhx6$^{+/Cre}$ animals (*E*, *F*) has normal distribution and number of GABAergic interneurons, compared with Rac1$^{+/fl}$;Lhx6$^{+/Cre}$ animals (*B*, *C*), as indicated by several markers. All Lhx6^{+} cells are YFP expressing (*H*) and there are no differences in the YFP^{+} (*G*) or som^{+} cells (*I*). White arrows in *A* and *D* indicate the migrating cells. Scale bars: A, D, 150 μm/B, E, C, F, 100 μm.

progenitors and their mitotic potential in the MGE of control and Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ embryos. For this, we pulse-labeled neural progenitors with BrdU at E12.5 and E13.5, collected the embryos half an hour later, and immunostained for BrdU and phospho-histone-3 (PH3; Fig. 4*A*,*B*). This analysis reveals no differences in the number and distribution of mitotic cells in Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ embryos compared with their control littermates (Fig. 4*C*). Thus, the reduced number of GABAergic cells in the cortex of mutant mice is not due to defective proliferation or aberrant mitotic activity of the Rac1-deficient cells.

To further explore the cellular mechanisms leading to the observed phenotype of Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ mice, we examined apoptotic cell death at several embryonic stages of these animals. Immunostaining of coronal sections at E12.5, E13.5, E14.5, and E16.5 control and Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ embryos for activated caspase 3 reveals no difference in apoptotic cell death between the 2 groups (Supplementary Fig. 6 and data not shown). Therefore, it is unlikely that apoptotic cell death can account for the reduced number of cortical interneurons present in the Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ brains by the end of embryogenesis.

Inactivation of Rac1 Perturbs G1 Phase Progression and Reduces Cell Cycle Exit of MGE-Derived Progenitors

We show that Rac1-deficient cortices are missing half of their MGE-derived interneurons at P15. Our in vivo and explant culture studies thus far are consistent with the idea that deletion of Rac1 in the MGE results in a defect in the initiation of migration by interneuron precursors. To further investigate the cause of this defect, we compared the cell cycle dynamics of MGE progenitors between control and Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$embryos. For this, we pulse-labeled neural progenitors with BrdU at E12.5 and collected the embryos 24 h later for anti-BrdU and anti-Ki67 double-labeling (Supplementary Fig. 7) Cells expressing Ki67 are considered to be in the cell cycle, whereas Ki67-negative cells have exited the cell cycle. The fraction of BrdU^{+} cells that do not express Ki67 (Ki67^{-}) was estimated in the MGE of control and mutant embryos, as well as in the LGE which served as an internal control, as the Nkx2.1-Cre transgene is not expressed in this region (Supplementary Fig. 7). This analysis revealed a specific decrease of BrdU^{+}; Ki67^{-} progenitors from the MGE in mutant embryos, indicating that fewer progenitors had exited the cell cycle in the absence of Rac1. The respective percentage of LGE-derived progenitors is not altered between mutant and control littermates (Fig. 5*A*). Similar analysis was performed on E14, E15, and E16 brains, 24 h after BrdU injection, in order to examine whether Rac1-deficient cells of the MGE exhibit a delayed cell cycle exit even at later developmental stages (Fig. 5*B*). We observed a similar reduction in the cell cycle exit index of mutants versus control animals confirming that even at later developmental stages fewer progenitors exit the cell cycle in the absence of Rac1.

Our data so far suggest a defect in the cell cycle of Rac1-deficient MGE-derived progenitors. However, PH3 and BrdU detection indicate normal G2-M and S phases of the cell cycle, respectively. To further characterize the observed defect, we performed western blot analysis on MGE tissue from E13.5 embryos, in order to detect the protein levels of phosphorylated retinoblastoma tumor suppressor protein (Rb), as a marker for G1 phase of the cell cycle. We found that Rac1$^{fl/fl}$;Nkx2.1$^{Tg(Cre)}$;R26R-YFP$^{+/-}$ embryos have greatly diminished amounts of phosphorylated Rb in their MGE cells, compared with control littermates (Fig. 5*C*). We additionally analyzed the levels of cyclin D proteins since cyclins are crucial for phosphorylation of Rb and progression of the cell cycle through the G1 phase. We find a 2-fold reduction in the levels of cyclin D proteins in the MGE of

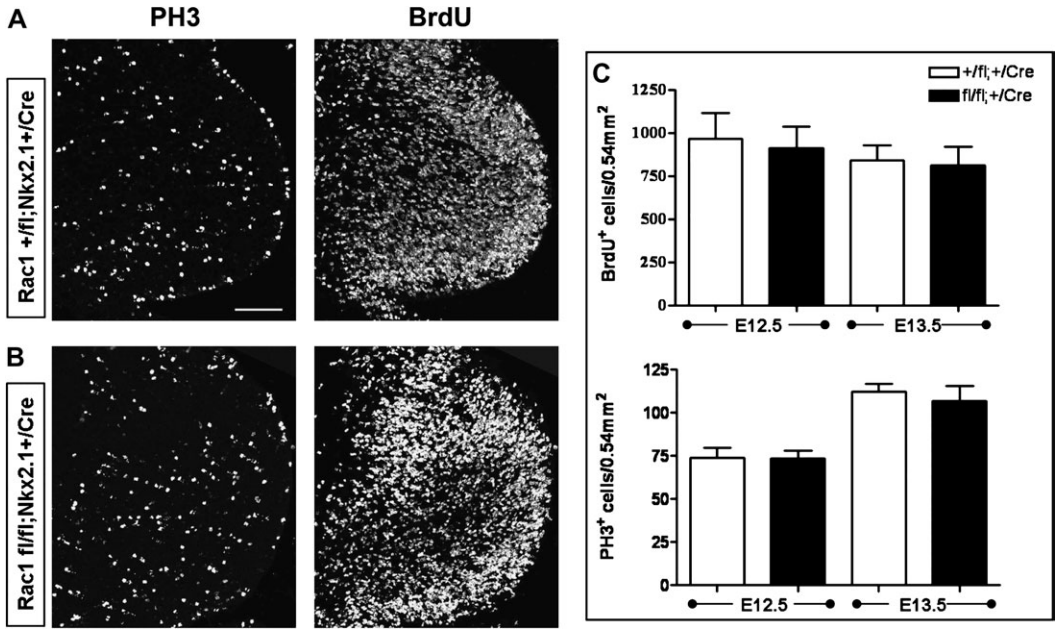

Figure 4. Ablation of Rac1 does not affect the proliferation of MGE-derived progenitors. The mitotic activity of MGE-derived progenitors was assessed using immunohistohemistry for PH3 and BrdU on E13.5 brains were BrdU was incorporated for 30 min (*A*, *B*). The number of total BrdU^{+} cells is not altered between mutant and control embryos (*C*), and no differences were observed in the number or distribution of PH3^{+} mitotic cells in the mutant embryos compared with the controls (*D*). Four consecutive sections from each embryo were used for quantification, and statistical analysis was performed using ANOVA for repeated measurements and Student's *t*-test. Error bars represent the standard error of mean. Scale bars: 75 μm.

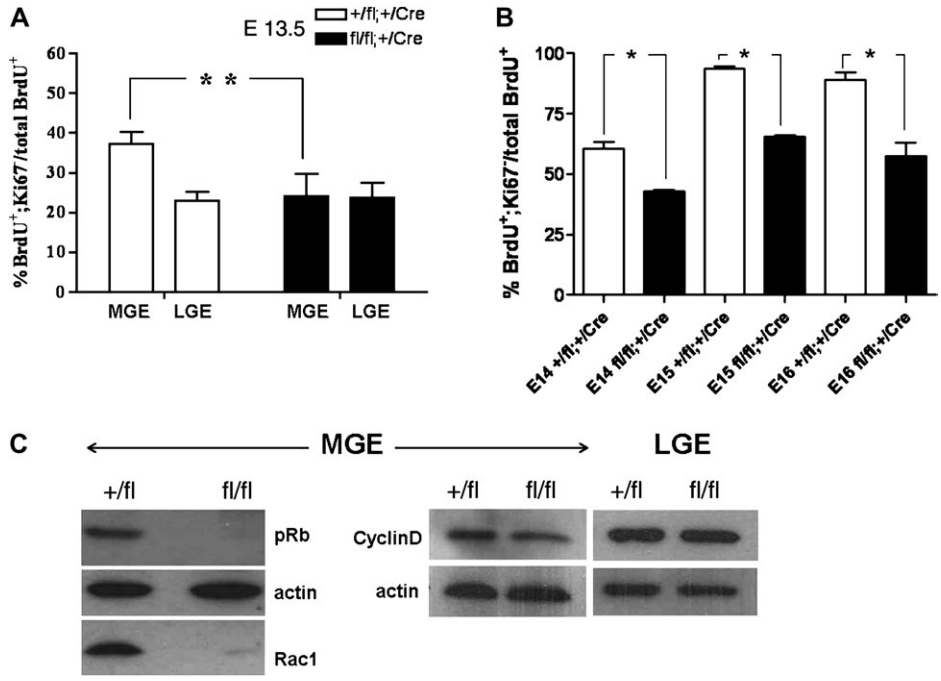

Figure 5. Rac1 absence leads to a reduced exit of GABAergic interneurons from the cell cycle and an imbalance in cyclin D expression. Cell cycle exit was assessed, by calculating the percentage of $BrdU^+$; $Ki67^-$ cells to the total number of $BrdU^+$ cells in the SVZ of the MGE and LGE in 4 sets of embryos (*A*). This percentage is significantly decreased in the MGE of Rac1-deficient embryos, compared with their control littermates. The respective percentage of the LGE is not altered in mutant embryos. (*B*) The same cell cycle exit index was calculated for embryos at E14, E15, and E16, showing that the Rac1-deficient cells are not able to exit the cell cycle even at later developmental stages. Four consecutive sections from each embryo were used for quantification and statistical analysis was performed using ANOVA for repeated measurements and Student's *t*-test. Error bars represent the standard error of mean. (*C*) Phosphorylated Rb is highly diminished from Rac1-deficient MGE-derived cells. Additionally, cyclin D levels are decreased, as indicated by western blot analysis of MGE-derived tissue, whereas in the LGE, similar levels of cyclin D expression are observed between Rac1-deficient and control cells.

$Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;R26R-$YFP^{+/-}$ embryos compared with control littermates (Fig. 5*C*). The defect is specific to MGE-derived cells since similar analysis, using tissue from the LGE at E13.5, revealed no differences in the levels of cyclin D proteins. Altogether, these data indicate a specific role for Rac1 in the progression of the MGE-derived cells through the cell cycle, possibly during the G1 phase.

The Majority of Mature Interneurons in $Rac1^{fl/fl}$; $Nkx2.1^{Tg(Cre)}$;R26R-$YFP^{+/-}$ Brains Derive from Early Born MGE-Derived Progenitors

Given the fact that ablation of Rac1 occurs at E12 and that cells are found in dramatically reduced numbers in the mature brain, we wanted to examine the birth date of the cells present in the mutant cortices at P0. For this, we pulse-labeled pregnant females with BrdU at 2 developmental stages, E11.5, when Rac1 is still present in the progenitors, and E14.5, when Rac1 is completely absent from MGE-derived cells. P0 brains were processed for BrdU and YFP immunolabeling (Fig. 6). The percentage of early-born cells (BrdU at E11.5, white arrows in Fig. 6*A*,*B*) to the total YFP^+ population is not significantly altered in the mutant P0 brains (Fig. 6*B*,*E*) compared with P0 control brains (Fig. 6*A*,*E*). In contrast, the percentage of late born cells (BrdU at E14.5, white arrows in Fig. 6*C*,*D*), present in the mutant P0 brains (Fig. 6*D*,*E*), is significantly reduced compared with the control brains (Fig. 6*C*,*E*). These results indicate that the majority of MGE-derived interneurons, found in the postnatal cortex, descend from early progenitors, whereby Rac1 is still active and fewer numbers derive from late-born progenitors.

Morphology of Rac1-Deficient Interneurons

Due to the known role of Rac1 in modulating cytoskeletal processes, we proceeded to assess whether there were any defects in the morphology and migrating processes of Rac1-deficient interneurons. Using YFP immunostaining on E13.5 brain sections, we examined the morphology of the cells in vivo in order to detect possible differences in the number of their processes, their length, and their branching during migration. We observe no morphological differences in the control and mutant YFP^+ interneurons that have left the MGE and are found close to the pallial–subpallial boundary (cf. Fig. 7*F*,*G*). Consistently, the length of the leading processes (Fig. 7*A*), their number (Fig. 7*B*), and the number of primary branches (Fig. 7*C*) remain unaffected.

In addition, we examined the ability of Rac1-deficient cells to position themselves toward the expected direction during migration by measuring the percentage of cells with orientation opposite to the migration route and the angle formed between their leading process and the presumptive migration axis. Our measurements reveal no significant differences between the Rac1-deficient and control cells, regarding their angles during migration or the orientation of their leading process toward the cortex (Fig. 7*D*,*E*).

Although the morphology of migrating interneurons in vivo appears to be normal, we wanted to gain better information on the general morphology of Rac1-deficient cells, including those that do not move and remain aggregated ventrally. To achieve this, we dissociated cells from E13.5 MGE tissue and cultured them on collagen (Fig. 7*I*,*J*) or matrigel-coated

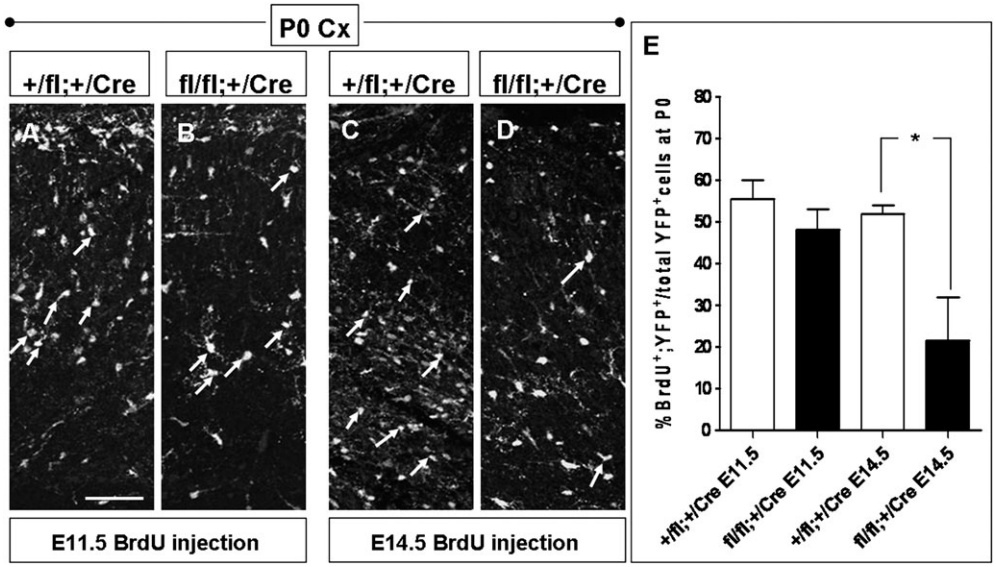

Figure 6. The majority of MGE-derived cells found in P0 mutant brains descend from early-born progenitors. Immunohistochemistry at P0 sections for YFP and BrdU, after incorporation either at E11.5 (*A*, *B*) or E14.5 (*C*, *D*). (*E*) The percentage of early-born MGE-derived cells in the cortex of mutant mice (white arrows in *B*) is not significantly different from the controls (white arrows in *A*). The respective number of late-born cells, however, is reduced in mutant brains (white arrows in *D*) compared with controls (white arrows in *C*), highlighting that the majority of MGE-derived cells found in the mature cortex of mutant mice, descend from early-born progenitors, before Rac1 ablation. Scalebar: 75 μm.

coverslips (data not shown). After 48 h in culture, the cells were fixed and stained for YFP and cytoskeletal markers such as F-actin and cortactin (Fig. 7*I*,*J*). A statistically significant number of Rac1-deficient neurons appear to have defects with respect to the actin cytoskeleton of their growth cone (Fig. 7*K*). In addition, a large percentage of the Rac1-deficient cells cultured in vitro fail to exhibit normal growth cone morphology, while the length of their neurites is significantly shorter compared with control cells (Fig. 7*K*).

Taken together, these data suggest that despite their migratory impairment, Rac1-deficient cells that migrate toward the cortex do so in a similar fashion to control cells. However, a large percentage of Rac1-deficient neurons, most likely the population that remains aggregated in the ventral telencephalon, appear to have affected growth cones and abnormal axon extension, which is consistent with previously reported roles of Rac1 in cytoskeletal dynamics (Jaffe and Hall 2005; Watabe-Uchida et al. 2006; Koh 2007; Tahirovic et al. 2010).

Discussion

In this manuscript, we show that the specific ablation of Rac1 function from Nkx2.1-expressing MGE-derived progenitors leads to a perturbation of their cell cycle exit accompanied by decreased levels of cyclin D proteins and reduced phosphorylation of Rb, a critical checkpoint of the cell cycle. Our results suggest that cells do not progress normally through cell cycle and a large number of them fail to migrate out of the MGE, thus aggregating in the ventral telencephalon. As a consequence, we observe decreased migration of cortical interneurons from the MGE toward the developing cortex, which results in a severely reduced number of all MGE-derived neurons in the adult brain. In contrast, ablation of Rac1 from Lhx6-positive interneurons that have already become postmitotic does not affect their development, further confirming the necessity of Rac1 in Nkx2.1-positive progenitors in order for them to complete their cell cycle.

Rac1 Deficiency Results in Reduced Numbers of MGE-Derived GABAergic Interneurons in the Cortex

Although many cues have been implicated in cortical interneuron development and migration, little information exists about the intracellular mechanisms involved. Since Rac1 is known to be downstream of several guidance cues, like ephrins/Eph, netrin1/DCC, Robo/Slit, and Semaphorins/Plexins (Jaffe and Hall 2005; Lowery and Van Vactor 2009), we reasoned that it may play a role in the signal transduction events that allow GABAergic interneurons to respond to guidance cues. However, we found that Rac1 activity is dispensable in postmitotic interneurons that are undergoing migration. Instead, its function is required in the mitotic progenitors of interneurons, still resident in the VZ of the MGE.

We have shown that interneurons populating the cortex are half in number and mainly derive from early-born MGE progenitors, before Rac1 ablation. These cells differentiate normally into the different subpopulations of interneurons as assessed by the expression of GABA and specific calcium-binding proteins and neuropeptides. In addition, the proportions of the different interneuron subpopulations relative to the total YFP-positive MGE-derived population are maintained. More specifically, like in control animals, ~80% of Rac1-deficient cells in the cortex visualized by YFP, express Lhx6, which is known to regulate the PV^+ and Som^+ fate of MGE-derived interneurons of the Nkx2.1 lineage (Fogarty et al. 2007; Liodis et al. 2007), and the percentage of Lhx6-expressing and PV-expressing cells is not significantly altered in mutant compared with control cortices. As expected, subpopulations of interneurons expressing CR and NPY that derive primarily from the CGE, which is a territory negative for Nkx2.1 expression (Fogarty et al. 2007; Butt et al. 2008), are not affected in number or distribution in the $Rac1^{fl/fl}$; $Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ brains.

The 50% reduction we observe in $Lhx6^+$ GABAergic interneurons is in disagreement with Chen et al. (2007), who

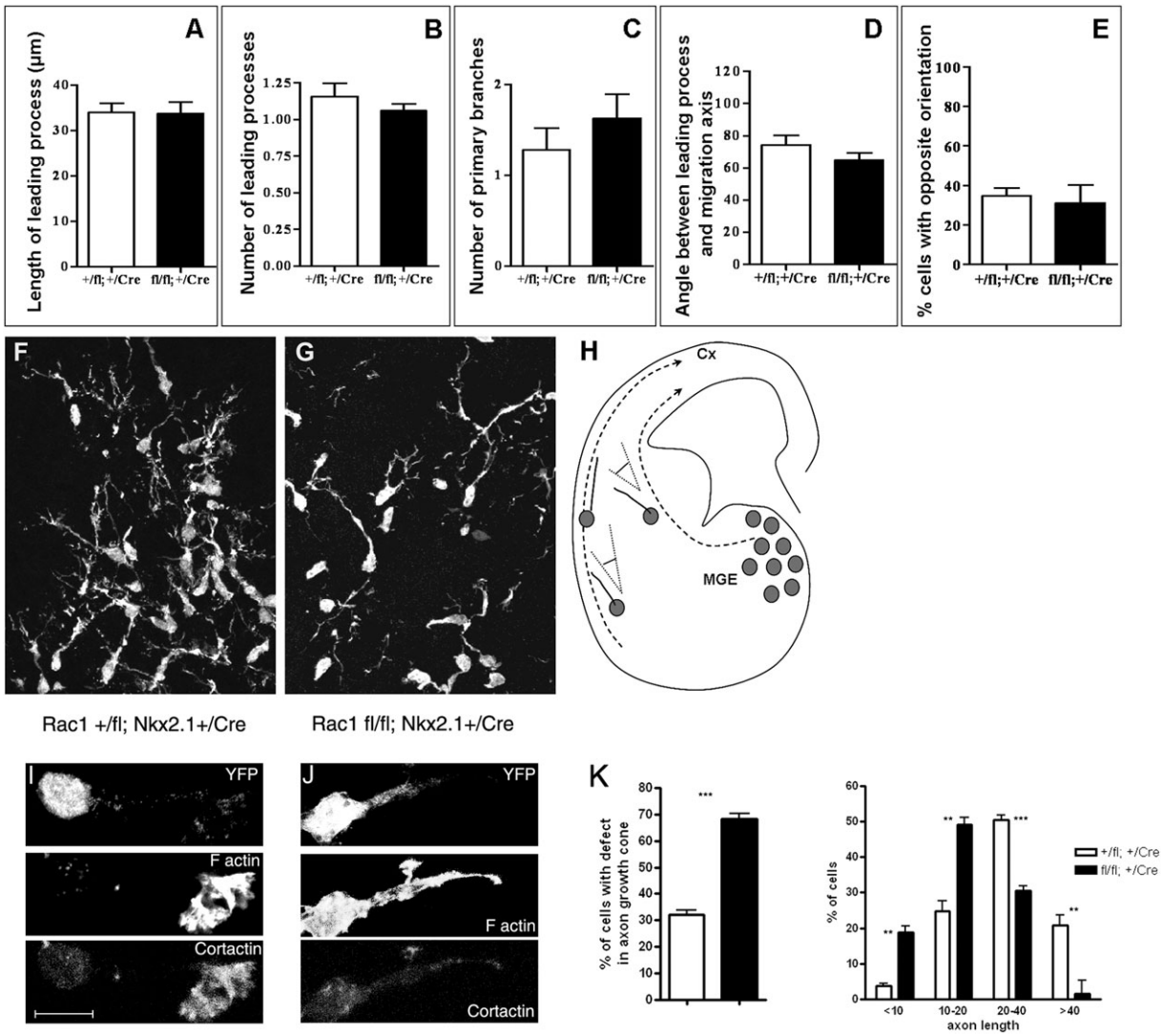

Figure 7. The morphology of Rac1-deficient interneurons. In vivo morphology of $Rac1^{+/fl}$;$Nkx2.1^{+/Cre}$ (*F*) and $Rac1^{fl/fl}$;$Nkx2.1^{+/Cre}$ (*G*) interneurons at E13.5 after they have left the MGE and are found close to the pallium/subpallium boundary, visualized by YFP expression. Rac1-deficient cells that manage to migrate have no differences compared with the control interneurons, in terms of length of their leading processes (*A*), number of processes (*B*), and number of primary branches (*C*). The angle formed between the leading process and the presumptive migratory axis, as well as the percentage of cells with orientation opposite to the expected, is not significantly altered in Rac1-deficient cells, compared with controls (*D* and *E*). (*H*) Cartoon depicting the migration axis (dashed lines) and the angle formed between the leading processes of migrating cells and the migratory route. (*I*–*J*) In vitro morphology of interneurons that have remained in the MGE at E13.5 after 2DIV on collagen is visualized by YFP, F-actin (phalloidin), and cortactin expression. Rac1-deficient cells (*J*) do not have normal growth cones compared with the control cells (*I*). The difference observed in the growth cones is statistically significant and a large percentage of Rac1-deficient cells fail to extend long leading processes compared with control cells. (*K*; ***P value $<<<$ 0.05). Measurements were performed using ImageJ and analyzed by the Student's *t*-test. Error bars represent the standard error of mean. Cx: cortex. Scale bars F–G, 75 μm; I–J 7 μm.

showed a complete absence of $Lhx6^+$ cells in the cortex at E16.5 and absence of $GAD67^+$ cells at E18.5. This disagreement may originate in the fact that Chen et al. are using a different Cre line (namely, the Foxg1 Cre) to ablate Rac1 from the VZ of the entire telencephalon. Therefore, it is likely that defects outside the MGE may contribute to the reported phenotype.

Rac1-Deficient Interneurons: Morphology and Migration

Previous studies have demonstrated that Rac1 affects migration in several cellular populations. More particularly, Rac1 acting through WAVE and Arp2/3 regulates neuronal polarization (Tahirovic et al. 2010) and is required to promote actin polymerization at the front of migrating cells, thus pushing forward the leading edge membrane (Gardiner et al. 2002; Itoh et al. 2002). Rac1 is also required for microtubule assembly during cell movement, acting via IQGAP1/CLIP170 (Fukata et al. 2002). The dynamic interplay between microtubule and actin elements is crucial to the response of neuronal growth cones to guidance cues (Dent and Kalil 2001; Zhou and Cohan 2004). Therefore, one could hypothesize that in our study, Rac1-deficient MGE-derived interneurons might have defects in actin/microtubule dynamics, leading to their inability to migrate according to the appropriate spatiotemporal context. Our in vitro data from explant cultures of the mutant cells reveal a significant impairment in migration in agreement with our in vivo data.

Both our in vivo analysis and in vitro assays allowed us to follow the migrating cells. However, a large number of Rac1-deficient neurons remain in their place of origin. Dissociation

of the ventral telencephalic tissue and in vitro cultures on collagen permitted us to visualize their morphology and cytoskeletal aspects. We observed that a significant number of these YFP+ cells fail to extend their axons beyond a certain length and their growth cones lack normal F-actin distribution. Additionally, cortactin, an important nucleation-promoting factor of Arp2/3 that has an essential role in many actin-remodeling processes such as migration and axon guidance (Ammer and Weed 2008), appears to be extensively reduced in the mutant neurons.

Taken together, our data suggest that Rac1 plays a role in the cytoskeletal reorganization of interneurons during the extension of their leading processes. Nevertheless, a large number of cells manage to migrate toward the cortex with normal morphology in terms of their leading processes, underlying a compensating mechanism, possibly involving other Rho-GTPases that share functional similarity with Rac1, like Cdc42, that could take over the role of Rac1 in migrating mutant cells (Fukata et al. 2002; Jaffe and Hall 2005). Such a possibility cannot be excluded and could hinder the elucidation of the unique role of Rac1. Future studies with combinatorial depletion of potentially compensating GTPases could provide more precise information on their functions.

Rac1 Regulates Progression of the Cell Cycle in MGE-Derived Progenitor Cells

It is known that the function of Rac1 may be different, depending on the specific cell type and tissue (Jaffe and Hall 2005). Previous studies have reported that Rac1 affects neuronal development in several aspects. It has been demonstrated that Rac1 along with Cdc42 play crucial roles in the proliferation of neural crest stem cells (Fuchs et al. 2009). In addition, ablating its function from the VZ of the entire telencephalon by the Foxg1 Cre line leads to severe axonal malformations (Chen et al. 2007) and results in a reduced pyramidal progenitor pool and microcephaly (Chen et al. 2009; Leone et al. 2010). The reduced size of the brain reported in Chen et al. (2009) is caused by accelerated cell cycle exit and increased apoptosis. Our results are consistent with the hypothesis that ablation of Rac1 in the MGE (using the Nkx2.1 Cre line) is causing progenitors to remain longer in the cell cycle with no accompanying effect on apoptosis.

In our study, we specifically ablated Rac1 from MGE-derived cells, either from the progenitor stage (with Nkx2.1-Cre) or postmitotically (with Lhx6-Cre). In the former case, in the absence of Rac1, reduced numbers of MGE-derived progenitors exit the cell cycle. Instead, they retain Ki67 expression, which characterizes cells actively cycling. It is known that Rho-GTPases influence the activity of cyclin-dependent kinases during G1 phase and the organization of microtubules and actin during M phase (Jaffe and Hall 2005). Our data suggest that Rac1-deficient cells are most likely not halted during the S, G2 or the M phase, as we do not observe any differences in the incorporation of BrdU (S phase) and the expression of PH3 (late G2-M phase), respectively. Therefore, the observed difference could be due to a percentage of cells that remain in the G1 phase and do not migrate toward the cortex.

Cell cycle progression is accomplished via tightly regulated expression of Rho, Rac1, and ERK, which exert an important role in the balanced expression of cyclin-dependent kinases (Welsh et al. 2001). Rac1-deficient MGE precursors display an imbalance in cyclin expression, as we observe reduced levels of cyclin D proteins. Additionally, we find that Rac1-deficient cells have barely detectable levels of phosphorylated Rb. This protein regulates the cell cycle by controlling progression through the restriction point within the G1 phase of the cell cycle (Sherr 1996). Cyclins of the D family and their downstream kinases are necessary for the phosphorylation of Rb (reviewed in Jaffe and Hall 2005). Once active CDK4/6–cyclin D complexes form, they phosphorylate Rb, and allow the progression of the cell cycle through the G1 checkpoint. In our study, the ablation of Rac1 in MGE-derived cells could result in the 2-fold decrease in cyclin D expression observed, through a yet unknown cascade. This could in turn lead to a severely reduced phosphorylation of Rb. Such an impediment could result in a decrease in the number of progenitors that exit the cell cycle to start migrating on time. The mutant cells remain aggregated ventrally, thus resulting in the phenotype observed in the mature brain of Rac1 mutant mice. In contrast to previous studies, whereby ablation of Rac1 from the entire forebrain caused decreased proliferation and premature cell cycle exit for pyramidal neurons (Chen et al. 2009; Leone et al. 2010), we show that ablation of the protein specifically from MGE-derived interneurons does not affect proliferation but leads to a decreased cell cycle exit. This finding underlies the differential role of Rac1 in distinct neuronal populations.

The specificity of the defect in the cell cycle is even more evident, when taking into account that no defect is observed in interneuron development if Rac1 is ablated postmitotically in vivo. The lack of phenotype in these Rac1fl/fl;Lhx6 Cre mice highlights the fact that Rac1 is indispensable for interneurons when they are still mitotic and nicely correlates with the cytoskeletal phenotype observed in the mutant neurons. By exiting the cell cycle late, it is possible that MGE-derived cells of $Rac1^{fl/fl}$;$Nkx2.1^{Tg(Cre)}$;$R26R\text{-}YFP^{+/-}$ mice fail to receive crucial cues, acting at the subventricular zone in a strict spatiotemporal manner, which would allow the cells to extend their axons and respond to important guidance molecules that would direct them to the cortex. In support of this, progenitors born after the ablation of Rac1 at E12 are found in reduced numbers in the mature cortex compared with early-born cells (i.e., E11.5). It is also possible that a percentage of the aggregated cells found ventrally in the brain even postnatally are cells that failed to exit the cell cycle, thus remaining in their place of origin. Consequently, they do not migrate and interneurons are found in reduced numbers in the mature cortex.

Although several studies have been carried out to reveal the role of Rho-GTPases in multiple systems, the molecular details of their role in the development of the brain is largely unknown, mainly because of the differences, these proteins exhibit regarding their function, in different cellular populations. Our study is the first one to demonstrate a novel intrinsic role of Rac1 specifically in MGE-derived neuronal progenitors. Through potential regulation of cyclin D protein levels and Rb phosphorylation Rac1 could facilitate the transition from the G1 phase of an actively cycling condition to the postmitotic/migrating phase.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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References

Ammer AG, Weed SA. 2008. Cortactin branches out: roles in regulating protrusive actin dynamics. Cell Motil Cytoskeleton. 65:687–707.

Butt SJ, Cobos I, Golden J, Kessaris N, Pachnis V, Anderson S. 2007. Transcriptional regulation of cortical interneuron development. J Neurosci. 27:11847–11850.

Butt SJ, Sousa VH, Fuccillo MV, Hjerling-Leffler J, Miyoshi G, Kimura S, Fishell G. 2008. The requirement of Nkx2-1 in the temporal specification of cortical interneuron subtypes. Neuron. 59:722–732.

Chen L, Liao G, Waclaw RR, Burns KA, Linquist D, Campbell K, Zheng Y, Kuan CY. 2007. Rac1 controls the formation of midline commissures and the competency of tangential migration in ventral telencephalic neurons. J Neurosci. 27:3884–3893.

Chen L, Melendez J, Campbell K, Kuan CY, Zheng Y. 2009. Rac1 deficiency in the forebrain results in neural progenitor reduction and microcephaly. Dev Biol. 325:162–170.

Cobos I, Calcagnotto ME, Vilaythong AJ, Thwin MT, Noebels JL, Baraban SC, Rubenstein JL. 2005. Mice lacking Dlx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy. Nat Neurosci. 8:1059–1068.

Cobos I, Long JE, Thwin MT, Rubenstein JL. 2006. Cellular patterns of transcription factor expression in developing cortical interneurons. Cereb Cortex. 16(Suppl 1):182–188.

Denaxa M, Chan CH, Schachner M, Parnavelas JG, Karagogeos D. 2001. The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. Development. 128:4635–4644.

Denaxa M, Sharpe PT, Pachnis V. 2009. The LIM homeodomain transcription factors Lhx6 and Lhx7 are key regulators of mammalian dentition. Dev Biol. 333:324–336.

Dent EW, Kalil K. 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. J Neurosci. 21:9757–9769.

Fogarty M, Grist M, Gelman D, Marin O, Pachnis V, Kessaris N. 2007. Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. J Neurosci. 27:10935–10946.

Fuchs S, Herzog D, Sumara G, Buchmann-Moller S, Civenni G, Wu X, Chrostek-Grashoff A, Suter U, Ricci R, Relvas JB, et al. 2009. Stage-specific control of neural crest stem cell proliferation by the small rho GTPases Cdc42 and Rac1. Cell Stem Cell. 4:236–247.

Fukata Y, Kimura T, Kaibuchi K. 2002. Axon specification in hippocampal neurons. Neurosci Res. 43:305–315.

Gardiner EM, Pestonjamasp KN, Bohl BP, Chamberlain C, Hahn KM, Bokoch GM. 2002. Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. Curr Biol. 12:2029–2034.

Grigoriou M, Tucker AS, Sharpe PT, Pachnis V. 1998. Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. Development. 125:2063–2074.

Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. 2002. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. Mol Cell Biol. 22:6582–6591.

Jaffe AB, Hall A. 2005. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 21:247–269.

Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. 2006. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nat Neurosci. 9:173–179.

Kholmanskikh SS, Dobrin JS, Wynshaw-Boris A, Letourneau PC, Ross ME. 2003. Disregulated RhoGTPases and actin cytoskeleton contribute to the migration defect in Lis1-deficient neurons. J Neurosci. 23:8673–8681.

Koh CG. 2007. Rho GTPases and their regulators in neuronal functions and development. Neurosignals. 15:228–237.

Kyriakopoulou K, de Diego I, Wassef M, Karagogeos D. 2002. A combination of chain and neurophilic migration involving the adhesion molecule TAG-1 in the caudal medulla. Development. 129:287–296.

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. 1999. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci. 19:7881–7888.

Leone DP, Srinivasan K, Brakebusch C, McConnell SK. 2010. The Rho-GTPase Rac1 is required for proliferation and survival of progenitors in the developing forebrain. Dev Neurobiol. 70:659–678.

Liodis P, Denaxa M, Grigoriou M, Akufo-Addo C, Yanagawa Y, Pachnis V. 2007. Lhx6 activity is required for the normal migration and specification of cortical interneuron subtypes. J Neurosci. 27:3078–3089.

Lowery LA, Van Vactor D. 2009. The trip of the tip: understanding the growth cone machinery. Nat Rev Mol Cell Biol. 10:332–343.

Malosio ML, Gilardelli D, Paris S, Albertinazzi C, de Curtis I. 1997. Differential expression of distinct members of Rho family GTP-binding proteins during neuronal development: identification of Rac1B, a new neural-specific member of the family. J Neurosci. 17:6717–6728.

Marin O, Rubenstein JL. 2001. A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci. 2:780–790.

Marin O, Rubenstein JL. 2003. Cell migration in the forebrain. Annu Rev Neurosci. 26:441–483.

Martini FJ, Valiente M, Lopez Bendito G, Szabo G, Moya F, Valdeolmillos M, Marin O. 2009. Biased selection of leading process branches mediates chemotaxis during tangential neuronal migration. Development. 136:41–50.

Metin C, Baudoin JP, Rakic S, Parnavelas JG. 2006. Cell and molecular mechanisms involved in the migration of cortical interneurons. Eur J Neurosci. 23:894–900.

Metin C, Deleglise D, Serafini T, Kennedy TE, Tessier-Lavigne M. 1997. A role for netrin-1 in the guidance of cortical efferents. Development. 124:5063–5074.

Minobe S, Sakakibara A, Ohdachi T, Kanda R, Kimura M, Nakatani S, Tadokoro R, Ochiai W, Nishizawa Y, Mizoguchi A, et al. 2009. Rac is involved in the interkinetic nuclear migration of cortical progenitor cells. Neurosci Res. 63:294–301.

Sherr CJ. 1996. Cancer cell cycles. Science. 274:1672–1677.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 1:4.

Sussel L, Marin O, Kimura S, Rubenstein JL. 1999. Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development. 126:3359–3370.

Tahirovic S, Hellal F, Neukirchen D, Hindges R, Garvalov BK, Flynn KC, Stradal TE, Chrostek-Grashoff A, Brakebusch C, Bradke F. 2010. Rac1 regulates neuronal polarization through the WAVE complex. J Neurosci. 30:6930–6943.

Walmsley MJ, Ooi SK, Reynolds LF, Smith SH, Ruf S, Mathiot A, Vanes L, Williams DA, Cancro MP, Tybulewicz VL. 2003. Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. Science. 302:459–462.

Watabe-Uchida M, Govek EE, Van Aelst L. 2006. Regulators of Rho GTPases in neuronal development. J Neurosci. 26:10633–10635.
Welsh CF, Roovers K, Villanueva J, Liu Y, Schwartz MA, Assoian RK. 2001. Timing of cyclin D1 expression within G1 phase is controlled by Rho. Nat Cell Biol. 3:950–957.
Wonders CP, Taylor L, Welagen J, Mbata IC, Xiang JZ, Anderson SA. 2008. A spatial bias for the origins of interneuron subgroups within the medial ganglionic eminence. Dev Biol. 314:127–136.
Zhou FQ, Cohan CS. 2004. How actin filaments and microtubules steer growth cones to their targets. J Neurobiol. 58:84–91.