Nogo-A Regulates Neural Precursor Migration in the Embryonic Mouse Cortex

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Although Nogo-A has been intensively studied for its inhibitory effect on axonal regeneration in the adult central nervous system, little is known about its function during brain development. In the embryonic mouse cortex, Nogo-A is expressed by radial precursor/glial cells and by tangentially migrating as well as postmigratory neurons. We studied radially migrating neuroblasts in wild-type and Nogo-A knockout (KO) mouse embryos. In vitro analysis showed that Nogo-A and its receptor components NgR, Lingo-1, TROY, and p75 are expressed in cells emigrating from embryonic forebrain-derived neurospheres. Live imaging revealed an increased cell motility when Nogo-A was knocked out or blocked with antibodies. Antibodies blocking NgR or Lingo-1 showed the same motility-enhancing effect supporting a direct role of surface Nogo-A on migration. Bromodeoxyuridine (BrdU) labeling of embryonic day (E)15.5 embryos demonstrated that Nogo-A influences the radial migration of neuronal precursors. At E17.5, the normal transient accumulation of radially migrating precursors within the subventricular zone was not detectable in the Nogo-A KO mouse cortex. At E19, migration to the upper cortical layers was disturbed. These findings suggest that Nogo-A and its receptor complex play a role in the interplay of adhesive and repulsive cell interactions in radial migration during cortical development.

Keywords: corticogenesis, migration, neural precursor, Nogo-A

Introduction

Nogo-A was originally identified as a myelin protein that inhibits neurite outgrowth in the adult central nervous system (CNS) (Caroni and Schwab 1988; Spillmann et al. 1998; Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000). Its identification confirmed the concept that molecules expressed by oligodendrocytes inhibit axon regeneration in the adult brain and spinal cord (Schwab and Caroni 1988). This discovery was followed by intensive studies with the goal of developing new therapeutic strategies to enhance axon regeneration and functional recovery after CNS injury (Schwab 2004; Yiu and He 2006; Gonzenbach and Schwab 2008).

In addition to its oligodendrocyte expression, Nogo-A is also detected in neurons of both the peripheral nervous system and the CNS, in particular, during development (Josephson et al. 2001; Huber et al. 2002; Wang et al. 2002; Hunt et al. 2003; Mingorance et al. 2004; Dodd et al. 2005). Its neuronal functions remain largely elusive, however. During embryogenesis, a high expression of Nogo-A was reported in various species (Huber et al. 2002; O’Neill et al. 2004; Al Halabiah et al. 2005; Caltharp et al. 2007). Nogo-A is present in tangentially migrating cortical interneurons of mouse cortices (Tozaki et al. 2002; Richard et al. 2005; Mingorance-Le Meur et al. 2007). In chicken embryos, Nogo-A expression is observed in neural precursors during neural plate formation (Caltharp et al. 2007). Its localization in large projection neurons of the optic tectum and tectal-associated nuclei suggests a function of neuronal Nogo-A in neurite formation and synaptogenesis (Caltharp et al. 2007). So far, however, all these hypothetical roles of neuronal Nogo-A during development are based on correlations between its spatial and temporal expression pattern and corresponding developmental events. None of the proposed functions have been proven experimentally. The hypothesis that the growth inhibitory and growth cone repulsive membrane protein Nogo-A is involved in various developmental processes is plausible since such functions were found for many repulsive axonal guidance (Klein 2004; Martinez and Soriano 2005), extracellular matrix, and cell adhesion molecules (Hirano et al. 2003; Kadowaki et al. 2007). A recent study analyzing corticogenesis in Nogo-A -B, and -C knockout (KO) mutants revealed a disturbed migration of early tangentially migrating cortical interneurons (Mingorance-Le Meur et al. 2007). In vitro, the authors also found an increase in axon branching and early polarization of cortical neurons obtained from Nogo-deficient mouse cortices. These results support the idea that Nogo proteins play important roles during corticogenesis.

Here, we studied the developing mouse cortex and found Nogo-A expressed in radial glia, radially and tangentially migrating as well as postmigratory neurons. In embryonic forebrain-derived neurospheres, Nogo-A and some known Nogo receptor components were found on the precursor cell surface. Live imaging analysis of the movement of neurosphere-derived cells demonstrated that Nogo-A acts as a negative regulator of cell motility. In addition, we show that the adhesion and spreading of neural precursor cells were influenced by a Nogo-A substrate. Bromodeoxyuridine (BrdU)-labeling experiments demonstrated that in vivo Nogo-A plays a role in different phases of the radial migration process, probably controlling adhesive and repulsive interactions between migratory cells and their vicinity. Our data suggest a new regulatory mechanism mediated by the Nogo-A/Nogo receptor pathway influencing neuronal precursor cell motility during cortical development.

Materials and Methods

Animals

All animal procedures were in strict accordance with the guidelines of the Veterinary Department of the canton of Zurich. Experiments were performed with brains from embryonic and newborn wild-type (WT) and Nogo-A KO C57BL/6 mice (Simonen et al. 2003). The morning of plug detection in females was considered as embryonic day (E)0.5. Pregnant mice were sacrificed by cervical dislocation, and embryonic brains were harvested for further processing. Genotypes were controlled by polymerase chain reaction (PCR) analysis of genomic DNA (Simonen et al. 2003). Embryonic brains of WT and Nogo-A KO mice were dissected at E15.5, E17.5, or E19; fixed in 4%
paraformaldehyde (PFA); and cryoprotected in 30% sucrose in phosphate buffer before embedding in OCT (Tissue-Tek, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) at −40 °C. Brain coronal cryosections of 20 μm were cut and immunostained.

**Immunohistochemistry and Cytchemistry**

Primary antibodies: mouse, anti-APC (Anti-Adenomatus Polyposis Coli; 1:100; Calbiochem, Merck Chemicals Ltd., Nottingham, UK), anti-neulin (1:200; BD Pharmingen, BD Biosciences, CA), anti-tubulinβIII (TubIII, 1:1000; Promega, Dübendorf, Switzerland), anti-Satb2 (1:50; Abcam, Cambridge, UK); rabbit; anti-calbindin (1:2000; Swant, Bellinzona, Switzerland), anti-GFAP (glial fibrillary acidic protein; 1:2000; Dako, Glostrup, Denmark), anti-Lingo-1 (1:100; Abcam), anti-Nogo-A Rb173A (1:1000; Oertle et al. 2003; Dodd et al. 2005), anti-NgR (1:100; Alpha Diagnostic International, San Antonio, TX), anti-Pax6 (1:300; Covance), anti-p75 (1:1000; Promega), anti-Thr1 (1:1000; Millipore, Billerica, MA), anti-TROY (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA); rat, anti-BrdU (1:500; Biozol, Eching, Germany); and goat, anti-doublecortin (Dcx, 1:500; Santa Cruz). Secondary antibodies: species-specific Cy3 or Cy5 or biotin conjugated followed by incubation with streptavidin-dichlorotriazinyl amino fluorescein (all 1:50; Jackson ImmunoResearch, Suffolk, UK). Cell nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) nuclear stain (1:1000; Sigma-Aldrich, Buchs, Switzerland). Slides were coverslipped with Mowiol (Sigma-Aldrich).

Fluorescent images of embryonic cortices or immunostained cells were acquired using conventional fluorescence microscopy (Axioskop, Zeiss, Carl Zeiss AG, Feldbach, Switzerland; Leica AF6000) or on a confocal microscope (Leica DMB; Leica Microsystems, Wetzlar, Germany). Imaging was performed using Adobe Photoshop software.

**Neurosphere Cultures, Reverse Transcription Polymerase Chain Reaction, and Analysis of In Vitro Precursor Cell Migration**

For each experimental group, approximately 70-100 cells (from 3 independent experiments) were tracked during 7 h of video recording. Movies were further processed with the OpenLab and QuickTime software (Apple, Cork, Republic of Ireland). The distance covered by the imaged cells was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

**Cell Adhesion and Spreading Assay**

To test whether the highly inhibitory Nogo-A region, Nogo-delta20 (Oertle et al. 2003), affects neural precursor cell spreading, glass coverslips (1 cm²) were coated with poly-ω-lysine (5 μg/mL), washed 3 times, and subsequently coated with different concentrations of Nogo-A (50 and 100 pmol, diluted in PBS) for 1 h at 37 °C. Unbound Nogo-delta20 was removed by 3 washes with PBS. To evaluate whether spreading of neurosphere-derived cells is affected by fresh brain extract obtained from E18.5 WT and Nogo-A KO mouse embryos, glass coverslips were coated with different concentrations of fresh brain CHAPS-extracted material. Unbound coating material was removed by 3 washes with PBS. Forebrains of E15.5 WT and Nogo-A KO embryos were dissociated for isolation of neural precursor cells as described above. Cells were plated at a density of 8000 cells/cm² in DMEM/F12 supplemented with 2 μg/mL 20% FBS, cultured for 1 h, fixed with 4% PFA, and then scored. The average number of adhered and spread cells on the control substrates (PDL or glass), Nogo-delta20, or brain extract, respectively, was determined using a phase contrast Olympus IMT2 microscope and F-View camera (Soft Imaging Systems, Olympus Soft Imaging Solutions GmbH, Münster, Germany) at 20× by counting in 5 randomly chosen fields of view of the coverslips.
at cortical areas where no BrdU+ or Tbr1+ cells—depending on the kind of immunostaining—were detectable. After normalization, the mean of the densitometric values of each area was set to 1 for WT mice. All other values were calculated as ratio of the WT mice value.

Satb2+ cells were counted in a bin placed over a region in the upper CP of the cortex using the Leica AF6000 microscope (40× objective) and ImageJ software.

To assess possible influences of a changed tangential migration pattern, we quantified the number of Calbindin+ cells in the SVZ or CP for WT and Nogo-A KO cortices at E17.5 and E19.

For BrdU analysis, cortices of 5 WT and 5 Nogo-A KO embryos at E17.5 and 12 WT and 11 Nogo-A KO embryos at E19 were used; for analysis of the expression of the neuronal markers Tbr1 and SatB2, 6 WT and 6 Nogo-A KO embryos were used; and for the Calbindin quantification, cortices of 5 WT and 5 Nogo-A KO embryos were used; 3 sections per animal were analyzed.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using the appropriate test, unpaired Student’s t-test, or analysis of variance, followed by the Tukey’s test (GraphPad Prism, GraphPad Software Inc., CA). Significance was considered for P < 0.05.

Results

Nogo-A Is Expressed in Radial Glial Cells, Migrating Postmitotic as well as Postmigratory Neurons of the Embryonic Mouse Cortex

The Nogo-A expression pattern was assessed during forebrain development in mouse embryos. Nogo-A+ cells were detected in all cortical layers at E15.5 and E17.5 (Fig. 1A–C). Absence of staining in Nogo-A KO mice confirmed the specificity of the anti-Nogo-A antibody staining (Fig. 1D). To characterize Nogo-A+ cells, colabeling with specific lineage markers was performed (Fig. 1A–C).

Within the VZ, we observed Nogo-A labeling of low intensity in radially oriented processes. These Nogo-A+ processes were costained for nestin (Fig. 1C), showing that they are derived

Figure 1. Nogo-A is expressed in radial glial cells, migrating postmitotic as well as postmigratory neurons of the embryonic mouse cortex. Colocalization of Nogo-A (red), Dcx (blue), and TubβIII (green) in radially (arrowheads) and tangentially migrating postmitotic and in postmigratory neurons of the E15.5 (A) and E17.5 (B) mouse cortex. (C) Colocalization of Nogo-A (red) and nestin (green) in radial glial cells within the VZ and SVZ of the E15.5 mouse cortex. (D) Absence of anti-Nogo-A immunostaining in the E15.5 Nogo-A KO mouse cortex showing the specificity of the immunostaining procedure. Scale bars: A, B, and D = 100 μm; C = 50 μm.
from radial glial cells. To study whether Nogo-A is important for the morphology of radial glial cells, we compared the pattern of nestin+ radial processes in the developing cortex of WT and Nogo-A KO embryos. No major morphological differences of nestin+ structures were observed between the 2 genotypes, suggesting that Nogo-A is not essential for the formation of the radial glial network in the developing cortex (Supplementary Fig. S1).

At E15.5 and E17.5, we found Nogo-A, Dcx, and TubβIII triple-positive cells in the SVZ, IZ, SP, CP, and MZ (Fig. 1A,B). Dcx and TubβIII double-labeled radially and tangentially migrating postmitotic cells in the SVZ and IZ showed different intensities of Nogo-A staining. Many of the early neurons with a radial orientation expressed Nogo-A in the cell body and to a lower degree in the processes (Supplementary Fig. S2A). Tangentially migrating neurons were strongly Nogo-A+ in both the cell body and the processes (Supplementary Fig. S2B), an observation that confirms the finding by Mingorance-Le Meur et al. (2007). Prominent Nogo-A labeling was also detected in the majority of postmitotary neurons in the CP (Fig. 1A,B).

Nogo-A and the Nogo Receptor Components NgR, Lingo-1, TROY, and p75 Are Expressed in Embryonic Mouse Forebrain-Derived Neurospheres

Different studies suggest that neural stem cells isolated from embryonic forebrain–forming neurospheres in vitro correspond to precursor cells within the VZ (Jacques et al. 1998). We tested whether neurospheres express Nogo-A since they are derived from Nogo-A+ radial glial cells.

Nogo-A transcript was detected by RT-PCR in neurospheres derived from E15.5 mouse brain (Fig. 2A). Since the known inhibitory effects of Nogo-A on neurite growth involve a multi-protein receptor complex containing the membrane proteins NgR, Lingo-1, p75 (Fournier and Strittmatter 2001; Wang et al. 2002; Mi et al. 2004), and/or the p75-related molecule TROY (Mandemakers and Barres 2005; Park et al. 2005; Shao et al. 2005), we analyzed whether these molecules are present in cells forming neurospheres. As for Nogo-A, we also found transcripts for NgR, Lingo-1, TROY, and p75 (Fig. 2A).

When neurospheres were plated on coverslips in a medium without growth factors, cells started to migrate away from the neurospheres on PDL–coated dishes, the large majority of the migrating cells were nestin+ in both WT and Nogo-A KO cultures: The cultures generated approximately 65% astrocytes (GFAP+), 20% neurons (TubβIII+), and 15% oligodendrocytes (APC+). No major morphological differences of nestin+ were found in both WT and Nogo-A KO cultures: The cultures generated approximately 65% astrocytes (GFAP+), 20% neurons (TubβIII+), and 15% oligodendrocytes (APC+). The complete absence of staining with these 2 antibodies clearly demonstrated that only molecules localized on the cell surface were labeled with this procedure (data not shown).

Surface Nogo-A and Nogo-A Receptor Components Negatively Regulate the Motility of Migrating Neurosphere-Derived Cells

Twelve hours after plating the neurospheres on poly-d-lysine-coated coverslips, we recorded the migratory behavior of nestin+, Nogo-A+, and Nogo receptor+ cells using time-lapse imaging. Most cells migrated away from the neurospheres, but their movement was not continuous or linear over time. Pauses or migration in the reverse direction were frequently observed (Fig. 3B). Videos in the supplementary data (Supplementary Movies) show examples of such different behaviors. Because Nogo-A is present intracellularly as well as on the surface of neurosphere-derived cells (Fig. 2), we did not only compare the migration of cells derived from Nogo-A KO mice and WT cells but also used anti-Nogo-A function-blocking antibodies to assess whether the changes observed in the motility of Nogo-A-deficient cells were mediated by surface Nogo-A. The average distance covered by Nogo-A-deficient cells was significantly increased (76.47 μm ± 3.22/7 h, n = 70) compared with that of WT cells (64.45 μm ± 3.21/7 h, n = 81; Fig. 3C). When cells were treated with anti-Nogo-A antibody, the average distance covered by the migrating cells was also greater (95.22 μm ± 1.77/7 h, n = 74) compared with control antibody-treated WT cultures (64.66 μm ± 4.83/7 h, n = 104; Fig. 3F). Thus, the average distance covered was increased by 30% when surface Nogo-A was blocked by antibodies and at the same time higher than the distance covered by Nogo-A-deficient cells (Fig. 3C,F). This difference can be due to 2 reasons: Nogo-A-deficient cells migrate faster than WT cells or Nogo-A-deficient cells pause less or for a shorter time. We therefore measured the maximum speed of WT and Nogo-A-deficient cells and determined how frequently these cells were immobile for at least 500 s. No difference in the maximum speed was observed between WT cells (0.641 μm ± 0.041/min, n = 81) and Nogo-A-deficient cells (0.654 μm ± 0.036/min, n = 70; Fig. 3D), but the maximum speed of anti-Nogo-A antibody-treated cells was on average significantly higher (0.779 μm ± 0.036/min, n = 74) than that of cells with control antibody treatment (0.594 μm ± 0.033/min, n = 104). In addition, we found that Nogo-A-deficient cells paused less frequently (0.914 pauses ± 0.128/7 h, n = 70) than WT cells (1.346 pauses ± 0.161/7 h, n = 81; Fig. 3E) and also that anti-Nogo-A antibody–treated cells made fewer pauses than control antibody–treated cells (control: 1.269 pauses ± 0.115/7 h, n = 104; anti-Nogo-A: 0.892 pauses ± 0.106/7 h, n = 74; Fig. 3F). These data suggest that cessations of migratory movements are more frequent in the presence of Nogo-A. Taken together, the effect on the mobility of neurosphere-derived cells is likely mediated by cell surface Nogo-A.

At the time of the video analysis, that is, 12 h after plating of the neurospheres on PDL-coated dishes, the large majority of the migrating cells were nestin+. We then analyzed the differentiation of WT and Nogo-A KO cells after 7 days in differentiation medium by immunocytochemical stainings for neurons, astrocytes, and oligodendrocytes. The same proportions of all 3 CNS cell types were found in both WT and Nogo-A KO cultures: The cultures generated approximately 65% astrocytes (GFAP+), 20% neurons (TubβIII+), and 15% oligodendrocytes (APC+).
The same proportions of astrocytes, neurons, and oligodendrocytes were also found in cultures of WT cells grown in the presence of the anti-Nogo-A antibody 11C7 or of a control antibody. Since no significant difference in fate specification was found when Nogo-A was knocked out or neutralized, we assume that the effects of Nogo-A on cell locomotion are not due to a change in the proportion of differentiated cells with different mobility during the period of live imaging.

Figure 2. Nogo-A and the Nogo receptor components NgR, Lingo-1, TROY, and p75 are expressed by embryonic mouse forebrain-derived neurospheres. (A) Transcripts for Nogo-A, NgR, Lingo-1, TROY, and p75 are all detected by RT-PCR in E15.5 mouse forebrain-derived neurospheres (passage 5). Nogo-A is absent in Nogo-A KO mouse forebrain-derived neurospheres, but the receptor components persist. Total messenger RNA from neonatal mouse brain was used as a positive control. “+RT” and “–RT” indicate performance of reverse transcription with and without reverse transcriptase, respectively. (B) Nogo-A (red) immunoreactivity is found in precursor cells of a plated neurosphere cultured for 1 day without growth factors. Note the halo of migrating Nogo-A+ cells surrounding the sphere. (C) Localization of Nogo-A (red) on the cell surface of WT mouse neurosphere-derived cells. No immunostaining was observed on cells derived from Nogo-A KO mouse embryos. Nuclei are counterstained with green fluorescent Nissl stain. (D) Colocalization of NgR, Lingo-1, TROY, or p75 (red) and nestin (green) in migrating precursor cells emanating from plated neurospheres. (E) Colocalization of Nogo-A (red) and nestin (green) or TubβIII (green) in migrating precursor cells emanating from plated neurospheres. Approximately 1% of migrating cells are positive for TubβIII. (F) Expression of the radial cell glial marker Pax6 (green) in almost all neurosphere-derived cells. Scale bars: B = 100 µm; C = 10 µm; D = 10 µm; E = 50 µm; F = 100 µm, 50 µm.
As described above, NgR and Lingo-1, 2 Nogo receptor components known to be involved in Nogo-mediated growth cone collapse and nerve fiber growth arrest (GrandPre et al. 2000; Oertle et al. 2003; Mi et al. 2004), were present in the nestin+ neurosphere-derived cells (Fig. 2D). To test whether NgR and Lingo-1 play a role for the migration of precursor cells, we added function-blocking anti-NgR or anti-Lingo-1 antibodies to the neurosphere cultures. The total covered distance and the maximum speed of migration were increased, and the number of pauses was reduced with both antibodies, very similar to the results obtained with anti-Nogo-A antibodies (Fig. 3F-H). These data argue for a direct involvement of NgR and Lingo-1 in mediating the effects of Nogo-A on the migration of neurosphere-derived cells.

**Exogenous Nogo-A Negatively Regulates Adhesion and Spreading of Embryonic Neural Precursor Cells**

To study whether the effects of Nogo-A on cell motility are cell autonomous or substrate dependent, adhesion and spreading of neural precursor cells were evaluated on different substrates. Cell spreading was decreased by Nogo-delta20, the inhibitory region of Nogo-A, in contrast to the control substrate (Fig. 4A). We analyzed WT and Nogo-A KO precursor cells after they adhered on PDL-coated coverslips (control). Seventy-four percent of WT and 77% of Nogo-A KO precursor cells had spread after 1 h. Whereas a concentration of 50 pmol Nogo-delta20 did not decrease the number of spread cells significantly (74% of WT cells and 69% of Nogo-A KO cells), 100 pmol Nogo-delta20 inhibited the spreading of WT as well as Nogo-A KO.
neural precursor cells significantly (33% of WT cells and 41% of Nogo-A KO cells), with the majority of cells remaining round.

Adhesion of WT neurosphere-derived cells plated on a CHAPS protein extract of embryonic forebrain showed a difference depending on the genotype of the substrate/extract (Fig. 4B). Whereas an average number of 40 cells adhered on noncoated glass coverslips (control), the adherence on 10 μg/μL embryonic WT brain extract was decreased by approximately 35%. In contrast, the same protein concentration of brain extract derived from Nogo-A KO mouse embryos did not change the adhesion of neural precursor cells compared with the control situation. A higher protein concentration (40 μg/μL) of WT mouse brain extract had a very strong inhibitory effect on precursor cell adhesion for both genotypes, whereas Nogo-A KO mouse brain extract (Fig. 4B) showed a significant decrease in spreading. (A) A concentrations of 10 μg/μL WT brain extract caused a significant decrease in cell adhesion of WT and Nogo-A KO precursor cells compared with control substrate. Ten micrograms per milliliter Nogo-A KO brain extract, however, did not inhibit spreading of WT precursor cells. At 40 μg/μL, WT brain extract had a very strong inhibitory effect on precursor cell adhesion for both genotypes, whereas Nogo-A KO mouse brain extract impaired adhesion only mildly, probably through other repulsive molecules present in the extract. Bars represent mean ± SEM; ***P < 0.001 and **P < 0.01, unpaired Student’s t-test.

**Figure 4.** Nogo-A negatively regulates adhesion and spreading of embryonic neural precursor cells. Quantification of the proportions of WT and Nogo-A KO neural precursor cells plated on different concentrations of Nogo-delta20 or fresh brain extract from WT or Nogo-A KO mouse embryos. (A) Cells on control substrate (PDL) or on 50 pmol Nogo-delta20 showed normal spreading behavior after 1 h, while WT as well as Nogo-A KO precursor cells plated on a higher concentration of 100 pmol Nogo-delta20 showed a significant decrease in spreading. (B) A concentrations of 10 μg/μL WT brain extract caused a significant decrease in cell adhesion of WT and Nogo-A KO precursor cells compared with control substrate. Ten micrograms per milliliter Nogo-A KO brain extract, however, did not inhibit spreading of WT precursor cells. At 40 μg/μL, WT brain extract had a very strong inhibitory effect on precursor cell adhesion for both genotypes, whereas Nogo-A KO mouse brain extract impaired adhesion only mildly, probably through other repulsive molecules present in the extract. Bars represent mean ± SEM; ***P < 0.001 and **P < 0.01, unpaired Student’s t-test.

Discussion

Myelin-derived Nogo-A is one of the major inhibitory molecules for axon outgrowth in the adult CNS. While it has been intensively studied in this context, its function in neurons, where it is prominently expressed during development, still remains unclear. The present results suggest that Nogo-A plays a role for the radial migration of cortical precursor cells: In vitro, surface Nogo-A negatively modulated the locomotion of precursor cells via the Nogo receptor constituents NgR and Lingo-1, and in vivo, the radial migration of neuronal precursors in the E15–19 forebrain was disturbed in Nogo-A KO mice. During cortical development, Nogo-A is present in and on the surface of migrating and postmigratory neurons and in and on radial glial cells (Mingorance-Le Meur et al. 2007), a major source of neurons and glia (Gotz et al. 2002; Noctor et al. 2002; Rakic 2003) and an important guide for migrating cortical neurons (Rakic 1972; O’Rourke et al. 1992). The absence of Nogo-A in KO mice did not have a detectable effect on the overall architecture of the radial glial network. Radially and tangentially migrating postmitotic neurons located in the SVZ and IZ were Nogo-A positive, and it was present in high amounts in postmigratory neurons in the CP and MZ.
We studied the possible role of Nogo-A for the migration of nestin+ neural precursor cells. RT-PCR and immunofluorescence showed the presence of Nogo-A and the Nogo receptor components NgR, Lingo-1, TROY, and p75 in neurosphere-derived precursor cells. Live imaging revealed that Nogo-A–deficient cells migrated over a longer distance within a given time window compared with WT cells, mainly because they paused less. A similar but more explicit result was obtained by acute neutralization of Nogo-A by function-blocking antibodies. Importantly, anti-Nogo-A antibody–treated cells also showed a higher migration speed compared with control cells. Identical results were obtained with antibodies against the Nogo receptor components NgR or Lingo-1. Together, the data suggest that Nogo-A acts as a negative regulator or “brake” for migrating cortical precursors. This effect is mediated by surface Nogo-A via a receptor complex that includes the components NgR and p75.
Lingo-1. The antibody effects may be direct by steric hindrance of the Nogo-A-binding site or indirect by internalization and downregulation of Nogo-A or its receptor complex as it has been shown in vitro and in vivo (Weinmann et al. 2006). The more pronounced effects of the antibodies compared with the genetic ablation of Nogo-A may be due to the absence of compensatory reactions, for example, upregulation of other inhibitory factors, known to occur in many KO models.

The overall migration-enhancing effect by Nogo-A absence or blockade observed here could be due to a decrease in contact inhibition of locomotion (Abercrombie 1979). Recently, Nogo-A was shown to inhibit the migration of olfactory ensheathing cells through NgR and subsequent activation of RhoA (Su et al. 2007). RhoA is a crucial mediator of Nogo-A-induced growth cone collapse and neurite growth inhibition (Fournier et al. 2003; Schwab 2004). LimK-1, slingshot, and cofilin downstream of RhoA are important effectors leading to cytoskeletal reorganization and in particular to disassembly of the actin cytoskeleton (Hsieh et al. 2006, Montani et al. 2009). RhoA is known to be highly expressed during corticogenesis (Olenik et al. 1999) and to be involved in the regulation of cell adhesion and migration (Fukata et al. 2003). A significant increase in RhoA activity was also detected during collision of migrating neural crest cells (which express Nogo-A; unpublished observations); inhibition of Rock, a downstream target of RhoA, led to a loss of contact inhibition of locomotion of these cells, implicating RhoA as a downstream effector of contact inhibition in these peripheral neural precursors (Carmona-Fontaine et al. 2008).

To assess whether the effect of Nogo-A on cell migration was cell autonomous or substrate dependent, we plated WT and Nogo-A KO precursor cells on Nogo-delta20 or on brain extract derived from WT or Nogo-A-deficient embryonic forebrain. Adhesion as well as spreading were markedly reduced by Nogo-delta20 in all precursor cells, with WT cells reacting equally to Nogo-A KO precursors. WT brain extract inhibited the adhesions of precursor cells already at low concentrations, whereas Nogo-A KO brain extract exerted an anti-adhesive effect only at a high concentration and to a much lower extent than the WT substrate. We conclude from these results that the effects of Nogo-A on precursor cell migration are mainly exerted by the Nogo-containing substrate and less dependent on intracellular Nogo-A in the precursor cells themselves.

Our in vivo studies showed a disturbed migration pattern of radially migrating cortical neurons born at E15.5. This disturbance was seen at E17.5 and E19 but was no longer obvious in the adult. A recent report showed that in Nogo-A/B/C-deficient mice, the tangential migration of early-born interneurons derived from the ventrally located eminentia mediana into the cortex was delayed (Mingorance-Le Meur et al. 2007). The KO mice used, Nogo-A specific versus Nogo-A/B/C KO, and the developmental time points analyzed are different in the 2 reports. However, both studies show a role of Nogo for the migration of neuronal precursor cells in the developing mouse cortex. Corroborating the findings for late-born tangentially migrating interneurons (Mingorance-Le Meur et al. 2007), we did not detect any differences in the number of Calbindin+ cells between the WT and Nogo-A KO cortical areas we analyzed, indicating that the different distribution of BrdU+ cells within the cortex at E15.5-E19 was exclusively due to radially migrating neurons.

The disturbances of the in vivo radial migration pattern of neuronal precursor cells in the Nogo-A KO E15–19 mouse cortex are complex and difficult to understand in detail at present. We excluded an effect of Nogo-A on neural precursor cell proliferation by quantifying the total number of BrdU+ cells, which was not significantly different between WT and KO in the embryonic mouse cortex at E17.5. Cortical migration is a multistage process with 4 distinct phases of migration in which neurons change shape and direction of movement (Kriegstein 2005). After being born in the VZ, neurons move radially away from the ventricle to the SVZ and remain there for as long as 24 h. In fact, we detected an accumulation of BrdU+ cells at E17.5 in the form of a band between the VZ and IZ in the WT mouse cortex. The band corresponds to a zone that is enriched for Nogo-A expressed mainly by early neurons in the SVZ/IZ. In the Nogo-A KO mouse cortex, the newborn precursor cells were more dispersed over these layers, not forming such a distinct band. This could be due to the absence of a repulsive "boundary effect" normally exerted by Nogo-A in the WT SVZ. Subsequent to their pause in the SVZ, the newborn precursors often move back toward the ventricle (Kriegstein 2005). Finally, the cells associate with radial glial fibers to migrate to the outer layers of the CP, their final destination. At E19, cells labeled by BrdU at E15.5 in the Nogo-A KO mouse cortex had migrated less far into the CP than WT cells. Although we observed a higher motility of Nogo-A-deficient precursors in vitro, important directional cues required for radial in vivo migration may be disturbed in the Nogo-A KO mice. Thus, Nogo-A repulsive forces might initially help to restrict neurons to the SVZ and guide them back into the VZ, and Nogo-A expression in radial glial cells and neurons could play a role in the balance between adhesion and repulsion during directed movement of the cells along the radial glial fibers. Nogo-A deficiency could lead to over adhesive interactions, thereby slowing the radial migration.

Interestingly, in the postnatal cortex of the Nogo-A KO mice, we did not detect any major abnormalities, although a surprisingly high interindividual variability in the cell density of layers II–IV neurons was observed, perhaps pointing to incomplete compensatory mechanisms (data not shown).

In conclusion, we found that Nogo-A, well studied for its inhibitory function on axon growth in the adult CNS, plays a role in the regulation of the radial migration of cortical neuronal precursor cells in development. We did not find indications that processes such as precursor cell proliferation, differentiation, or survival are changed in absence of Nogo-A. Our data suggest that the effects of Nogo-A on migration are mediated by cell surface Nogo-A through a receptor complex that includes NgR and Lingo-1. Many mechanistic details remain to be studied, in particular, how and where these different players interact with each other in vivo during cortical development. The migration of newborn neurons is finely regulated by multiple adhesive/attractive molecules, for example, integrins, cell adhesion molecules, cadherins, and netrins as well as repulsive cues, for example, neuropilins/semaphorins, Slit/Robo, or ephrins (Marin and Rubenstein 2003; Chen et al. 2008). The present results show that Nogo-A can be included in the growing family of instructive cues regulating neuronal migration and axon growth during development and in the adult CNS.

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

Notes
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References

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