Involvement of the Myelin-Associated Inhibitor Nogo-A in Early Cortical Development and Neuronal Maturation

Nogo-A is a myelin-associated protein expressed by neurons and myelinating mature oligodendrocytes in the central nervous system. Although most research has focused on the participation of Nogo-A in the prevention of axonal regeneration and plasticity in the adult, little attention has been paid to the putative functions of Nogo-A during embryonic development. Here we examined the general pattern and cell-specific distribution of Nogo-A in the prenatal mouse telencephalon. In addition, we studied the development of the major axon tracts and radial and tangential migration in Nogo-A/B/C knockout mice. The pattern of Nogo-A showed distinct distribution in radial glia and postmitotic neurons, in which it is particularly enriched in developing axons. Similarly, Nogo-A was enriched at the leading process of tangentially migrating interneurons but not detectable in radial migrating neurons. Although a low level of Nogo-A appears to be on the surface of many cortical neurons but not detectable in radial migrating neurons. Although a low level of Nogo-A appears to be on the surface of many cortical neurons, most proteins have intracellular localization. In Nogo-deficient background, neurons displayed early polarization and increased branching in vitro, probably reflecting a cell-intrinsic role of Nogo proteins in branching reduction, and early tangential migration was delayed. On the basis of these observations, we propose that Nogo proteins, particularly Nogo-A, are involved in multiple processes during cortical development.

Keywords: axon tract, Nogo, radial glia, tangential migration

Introduction

Nogo-A, or RTN4A, belongs to the reticulon (RTN) family, comprising over 250 proteins whose functions are starting to be elucidated (Oertle and Schwab 2003). In addition to being classified as an RTN, Nogo-A was identified as an antigen for the monoclonal antibody IN-1 directed against central nervous system (CNS) myelin proteins (Spillmann et al. 1998; Chen et al. 2000). IN-1 treatment enhances axonal regeneration and sprouting in lesioned and healthy CNS neurons (Buffo et al. 2000; Fouad et al. 2001), and it has been argued that part of this activity is due to blockade of Nogo-A exposed on the cell surface (Chen et al. 2000). However, the surface localization of Nogo proteins remains controversial (Dodd et al. 2005; Miao et al. 2006; Voeltz et al. 2006). Nogo proteins share an RTN homology domain, which contains 2 transmembrane regions separated by a 66 amino acid loop found in all 3 Nogo isoforms (Nogo-A, -B and -C), which bind to Nogo receptor (NgR) and inhibit axonal growth (Oertle et al. 2003). In addition, Nogo-A contains a large N-terminal region that also shows inhibitory properties to several cell types, including neurons (Oertle et al. 2003; Schweigreiter et al. 2004).

Nogo-A expression has been reported in perinatal and adult CNS (e.g., Hunt et al. 2002a) and precedes NgR expression (e.g., Mingorance et al. 2004). Interestingly, Nogo-A expression is particularly enriched in some telencephalic axonal tracts during early embryonic development (Tozaki et al. 2002; Mingorance et al. 2004; Richard et al. 2005), indicating that Nogo-A functions independently of NgR, and probably participates in axonal tract formation or neurite growth. In addition to a putative extracellular function, Nogo-A may have an intracellular function because it is present in the tubular endoplasmic reticulum (ER) and contributes to the formation of the ER tubular network in nonneuronal cells (Voeltz et al. 2006). Similarly, several intracellular functions have been reported for other RTN members in nonneuronal cells, such as vesicle trafficking and endocytosis (Iwahashi and Hamada 2003; Steiner et al. 2004; Wakana et al. 2005). However, a description of the putative roles of neuronal Nogo-A during cortical development is not available and only the expression pattern at perinatal stages has been reported (Josephson et al. 2001; Tozaki et al. 2002; Wang et al. 2002; Mingorance et al. 2004; Richard et al. 2005). Nogo-A has become a major target for therapeutic intervention to facilitate axonal regeneration after CNS lesion (Schweigreiter and Bandtlow 2006). It is therefore of major importance to clarify the function of this protein in neurons, in particular during the prolonged period of cortical neuron migration, neuritogenesis, and differentiation.

With this in mind, here we conducted a detailed analysis of Nogo-A messenger RNA (mRNA) and protein expression pattern in the embryonic mouse forebrain, paying particular attention to the cerebral cortex. During embryonic development, Nogo-A was expressed by radial glia through corticogenesis. In addition, neuronal Nogo-A protein was expressed in postmitatory cortical neurons, in which it preferentially localized to the growing axon. Similarly, γ-aminobutyric acidergic (GABAergic) tangentially migrating neurons from the ganglionic eminence expressed Nogo-A, targeting the protein to their leading processes. Although Nogo-mutant mice showed no relevant changes in axonal tracts, absence of Nogo resulted in alterations in the migration of early cohorts of GABA-ergic neurons during corticogenesis. We further determined an increase in axon branching and early polarization in vitro in Nogo-deficient neurons. Taken together, our data indicate that Nogo proteins are involved in a number of functions during corticogenesis, which include modulation of tangential neuronal migration and neurite formation and maturation, thus reflecting a cell-intrinsic role of these proteins in neurons.

Materials and Methods

Animals and Tissue Collection

All procedures were performed in accordance with the guidelines approved by the Spanish Ministry of Science and Technology, following...
European standards. A total of 10 pregnant OF1 mice (Iffa Credo, Lyon, France) were used. The morning of plug detection was considered as embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0). Fetuses were removed by cesarean section after deep anesthesia of the mother with chloral hydrate (3.5 mg/kg, i.p. injection) and transcardially perfused with 4% paraformaldehyde dissolved in phosphate buffer saline (PBS) 0.1 M. After perfusion, brains were removed and postfixed in the same solution for 12 h (immunohistochemistry [IHC]) or 2 days (in situ hybridization [ISH]) at 4°C. Immunoreagents were diluted in PBS containing 0.1% Triton X-100, 0.2% gelatin, and 5% preimmune serum. Alternatively, to preserve plasma membrane integrity, Triton X-100 was not used for ICC. Slides were viewed and analyzed with an Olympus, Barcelona, Spain, FV500 confocal microscope using sequential scanning to prevent crosstalk. Only brightness and/or contrast in figures were adjusted using Adobe, Barcelona, Spain, Photoshop CS.

**Immunohistochemical and Immunocytochemical Methods**

IHC of free-floating sections was performed as described (Mingorance et al. 2004). Controls, including omission of the primary antibody or its substitution by normal serum, prevented immunostaining. For immunocytochemistry (ICC), primary cultures were permeabilized with PBS containing 0.1% Triton X-100 and blocked with 10% fetal bovine serum. Incubation with primary and secondary antibodies was performed at 4°C for 1 h. Immunoreagents were diluted in PBS containing 0.1% Triton X-100, 0.2% gelatin, and 5% preimmune serum. Alternatively, to preserve plasma membrane integrity, Triton X-100 was not used for ICC. Slides were viewed and analyzed with an Olympus, Barcelona, Spain, FV500 confocal microscope using sequential scanning to prevent crosstalk. Only brightness and/or contrast in figures were adjusted using Adobe, Barcelona, Spain, Photoshop CS.

Primary antibodies were used at the following concentrations: anti-Nogo-A rabbit polyclonal (Liu et al. 2002) at 1:400-1:1500; anti-Calbindin mouse monoclonal (DSHB, Iowa City, IA) at 1:500; anti-Beta III tubulin mouse monoclonal (Dako) at 1:500; anti-nestin mouse monoclonal (Tozaki et al. 2002) at 1:5; anti-5-bromodeoxyuridine (BrdU) mouse monoclonal (Dako, Barcelona, Spain) and rat monoclonal (Immunologicals Direct Oxfordshire, UK) at 1:75; anti-Vimentin goat polyclonal (Santa Cruz Biot, Santa Cruz, CA) at 1:350 and mouse monoclonal (Dako) at 1:500; anti-nestin mouse monoclonal (Becton Dickinson, Franklin Lakes, NJ) at 1:350; anti-Beta III tubulin mouse monoclonal (Sigma-Aldrich, Dorset, UK) at 1:1000; anti-calbindin mouse monoclonal (BD Biosciences, Madrid, Spain) at 1:500; anti-TAG-1 mouse monoclonal (DSHB, Iowa City, IA) at 1:500; anti-Calbindin rabbit polyclonal (Swant, Switzerland) at 1:500; and anti L1 rabbit polyclonal at 1:3000 (Dr Rathjen’s gift). Phalloidin-tetramethyl rhodamine isothiocyanate (Sigma-Aldrich) at 1:5000 was used to identify actin and the cortical depth of the somatosensory area (400-800 µm thick). Nogo-A/B/C (Nogo) mutant mice and wild-type littermates were generated from heterozygous intercrosses. The distribution of Nogo-A was examined using a sense mRNA probe, which produces the same cellular labeling (oligodendrocyte and neurons) as the Nogo-A specific probe at the developmental stages analyzed in the present study (Hunt et al. 2003). In addition, Huber et al. (2002) also demonstrated using other ISH probes that both “common” Nogo and Nogo-A specific probes give similar results during embryonic development in neocortex and hippocampus. Glutamic acid decarboxylase 65 and 67 (GAD 65/67) mRNA probes were kindly provided by Dr Allan Tobin (University of California, Los Angeles, CA) and have also been previously characterized (Erlander et al. 1991; Pascual et al. 2004).

**Results**

**General Pattern of Embryonic Expression of Nogo-A during Forebrain Development**

The distribution of Nogo-A was examined using a Nogo antisense mRNA probe, which produces the same cellular labeling as the Nogo-A antisense probe during embryonic development (see Huber et al. 2002; Hunt et al. 2002b; Mingorance et al. 2004 for details) and a polyclonal antibody specifically recognizing Nogo-A (Liu et al. 2002; Mingorance et al. 2004). Nogo mRNA expression was observed as early as E12.5 in a pattern that closely matched Nogo-A immunostaining (Fig. 1). At E14.5, Nogo-A staining was found throughout the entire rostrocaudal extent of the telencephalon (Fig. 1A). High Nogo-A labeling was observed.
in the olfactory bulb, tectum, cerebellum, and spinal cord, with lower levels in the cerebral cortex and the diencephalon. In the hippocampus, Nogo-A was already detected at E12.5 and became enriched in the CA3–CA1 regions by E14.5 (Fig. 1B,C). In addition, Nogo-A antibody labeled cortical afferents and efferents (such as the corticothalamic and thalamocortical tracts; Fig. 1D–F), the hippocampal fimbria (Fig. 1F), the corpus callosum (Fig. 1E), the anterior commissure (Fig. 1D–F), and the lateral olfactory tract (Fig. 1F).

**Presence of Nogo-A in Radial Glial Cells through Cortical Ontogenesis**

At E12.5, Nogo-A was detected in radially oriented processes spanning the cortical anlage and resembling radial glia (Fig. 2A). This staining was specific because the polyclonal antibody used in the study failed to label brain sections from Nogo-A-deficient mice and a different antibody rendered similar radial labeling (Supplementary Fig. 1). Double immunostaining with Nestin or Vimentin and a different antibody rendered similar radial labeling (Supplementary Fig. 1). Notably, Nogo-A was expressed by radial glial cells from both the ventral (Fig. 2D) and the dorsal telencephalon (Fig. 2A–C).

**Nogo-A Expression in Cortical Neurons during Development**

During early cortical development, the first generated postmitotic neurons in the cortical anlage are located in the preplate (De Carlos and O’Leary 1992; Valverde et al. 1995; Del Rio et al. 2000). Around E13, the successive cohorts of postmitotic neurons arising from the ventricular zone settle in the developing cortical plate and split the preplate into the marginal zone and the subplate (Marín-Padilla 1988; McConnell 1995; Hatten 1999). We studied the pattern of Nogo-A expression in developing neurons in the presumptive somatosensory SS cortex along development. At E12.5, TUJ-1-positive preplate cells were also labeled with Nogo-A antibodies over the profuse labeling of Nogo-A-positive radial glia (Fig. 3A,B). The further expression of Nogo-A by preplate-derived neurons at E13.5 onwards in the marginal zone and the subplate was also confirmed by colocalization with MAP2 (Fig. 3C). At E15.5–E16.5, neurons in the upper portion of the cortical plate and the intermediate zone (IZ) were double labeled with TUJ-1 or MAP2 and Nogo-A antibodies (Fig. 3C and not shown). However, cells located in the lower portion of the cortical plate were not labeled with anti-NogoA antibodies (Fig. 3C,D). BrdU labeling of postmitotic neurons at E15.5 (destined to cortical layers V–IV) and analysis at E18.5 demonstrated that BrdU-positive cells during migration aposed radial glia in the lower cortical plate did not express Nogo-A (Fig. 3D), which indicates that Nogo-A labeling in the perikaryon of radially migrating neurons occurs at late stages of migration.

Furthermore, in corticogenesis subsets of radially migrating neurons in the lower cortical plate begin to extend their axons contralaterally and toward subcortical structures after crossing the subplate (McConnell 1995; Metin and Godement 1996; Auladell et al. 2000). Some of these neurons, such as those pro-

**Figure 1.** Expression of Nogo-A in the embryonic mouse forebrain. (A) Low-power photomicrographs illustrating the distribution of Nogo-A in a sagittal section of an E14.5 telencephalon. Nogo-A labeling was very intense in the olfactory bulb. In dorsal telencephalic regions, Nogo-A labeling follows a rostral-caudal gradient in the cerebral cortex and the medial ganglionic eminence displayed low levels of Nogo-A compared with neocortex and hippocampus. Cortical layers can be easily delineated. (B, C) In the developing hippocampus, Nogo-A protein and mRNA are relevant in the hippocampal preplate at lower levels in the remaining layers at E14.5. (D–F) Paired sections at different rostrocaudal levels at E15.5 and E18.5 processed for Nogo-A ICC and Nogo-ISH. (D) At E15.5, in medial telencephalic regions, Nogo-A immunoreactivity is particularly intense in cortical axonal tracts (arrowheads) and the anterior commissure (open arrowhead). In parallel sections, Nogo mRNA levels are high in the neocortex or hippocampus and thalamic nuclei (E). At E18.5, Nogo-A is enriched in the cortico-cortical connections at the corpus callosum (F), the anterior commissure (F), and the lateral olfactory tract (F). Nogo mRNA is specially enriched in the cerebral cortex and subcortical regions (e.g., striatum). In the developing cortex, Nogo mRNA labels the lower portion of the cortical plate (layer VI–V) and the subplate-layer VIb (F). Abbreviations: AC, anterior commissure; CB, cerebellum; CC, corpus callosum; cp, cortical plate; f, hippocampal fimbria; MGE, medial ganglionic eminence; H, hypothalamus; HI, hippocampus; IC, internal capsule; LOT, lateral olfactory tract; NC, neocortex; SC, spinal cord; mz, marginal zone; OB, olfactory bulb; PPL, preplate; PTEC, pretectum; S, septum; ST, striatum; sp, subplate; T, thalamus; TEC tectum; vz, ventricular zone. Scale bars: A = 500 μm; B, C = 100 μm; D–F = 500 μm.
jecting the corticofugal tract, express TAG-1 as a characteristic marker (Fig. 3E). At E15.5, Nogo-A protein was absent from the perikaryon of these TAG-1--positive neurons located in the lower cortical plate but present in corticofugal axons (Fig. 3E,F). This preferential targeting of Nogo-A to the growing axon was confirmed in vitro using E15.5 cortical explants (Fig. 3G). After 3 days in vitro, TAG-1--positive axons also expressed Nogo-A, which was also enriched in the distal portion of the growing axons but much lower in the region close to the perikaryon (Fig. 3G). These results were further confirmed in dissociated cortical embryonic neurons in vitro (Fig. 5). At following stages (E17.5--E18.5), cortical plate neurons displayed Nogo-A labeling in their soma and apical dendrite, which correlates with the end of migration and is also time coincident with the innervation of the subcortical targets, as described in other studies (e.g., Metin and Godement 1996; Del Rio et al. 2000).

**Nogo-A is Targeted to the Leading Process of Tangentially Migrating Interneurons**

During development, cortical GABA-ergic interneurons generate from the ganglionic eminences and migrate through the IZ and subventricular zone (SVZ) before integrating into the cortical plate (De Carlos et al. 1996; Parnavelas 2000; Marin and Rubenstein 2003; Kriegstein and Noctor 2004). Between E13.5 and E16.5, a band of tangential processes immunoreactive for Nogo-A was prominent in the lower IZ and SVZ (Fig. 4A). The timing, location, and waved morphology of these Nogo-A--positive structures suggested that they represented neurites of tangentially migrating neurons (Fig. 4B). This was confirmed by double-labeling experiments using TUJ-1 and Nogo-A antibodies (Fig. 4C). Interestingly, Nogo-A--positive structures corresponded to the whole leading process of TUJ-1--positive migrating interneurons, whereas Nogo-A was selectively absent from their perikaryon (Fig. 4C, compare with insert in 4B). We estimated that most (95%) TUJ-1--positive tangentially migrating neurons displayed Nogo-A labeling targeted to their leading processes. In addition, we observed that approximately 50% of the TUJ-1--positive cells were also positive for Calbindin (Fig. 4D) (Anderson et al. 1997; Del Rio et al. 2000). The polarized morphology and migration of GABA-ergic neurons is influenced by numerous extracellular signals (Marin et al. 2003; Flames and Marin 2005; Lopez-Bendito et al. 2006). This morphology reversed to multipolar when cells from the medial ganglionic eminence were cultured without extrinsic influences, which induced them to stop migrating and start differentiating (Fig. 5A). In these culture conditions, Nogo-A labeled equally all radially oriented neurites and the cell soma of medial ganglionic interneurons identified with double labeling with Calbindin (Fig. 5B). In addition, we detected high expression levels of Nogo-A in most but not all the cortical interneurons in the adult brain, after migration is naturally concluded (Fig. 4E-H) (Mingorance et al. 2004). However, in agreement with the in vitro observations, the different subsets of interneurons analyzed (e.g., Calbindin and Parvalbumin positive) in the neocortex and hippocampus in vivo also display Nogo-A
ubiquitously distributed in the whole cell (Fig. 4E–H). These data show that intracellular distribution of Nogo-A changes depending on the morphology and characteristics (e.g., from migratory to resting cellular phenotype) of GABA-ergic cells, and indicate that Nogo-A distribution is associated with intracellular compartments (e.g., ER) or cytoskeletal proteins.

Analysis of the Subcellular Distribution of Nogo-A in Cortical Neurons In Vitro
To define the subcellular localization of Nogo-A protein in developing neurons, we first assessed the presence of Nogo-A at the neuronal plasma membrane. For this purpose, we used nonpermeabilized neurons and double immunolabeling of Nogo-A with Oregon green-tagged WGA, which stains membrane glycans (see Methods for details). As a control, the monoclonal TUJ-1 antibody was used to label microtubules in occasionally permeabilized neurons during the experimental procedure (Fig. 6A,B). The polyclonal antibody against Nogo-A used in this study detects only the amino-terminal portion of Nogo-A (Liu et al. 2002). In our experiments, Nogo-A colocalized with WGA labeling in most TUJ-1-negative nonpermeabilized neurons (Fig. 6A), which indicates that the N-terminus of Nogo-A is present at the neuronal surface, and corroborates results by Dodd et al. (2005) and Jin et al. (2003). Further control experiments revealed the specificity of the procedure because the same Nogo-A antibody failed to stain Nogo-deficient neurons cultured for 2 days under permeabilizing and nonpermeabilizing conditions (Fig. 6C,D).

Nogo-A labeling in growth cones was restricted to the central region and matched microtubule distribution (Fig. 6E). This pattern switched from widespread distribution of Nogo-A throughout the soma and neurites in permeabilized cortical neurons after 2 days in culture, to become preferentially targeted to growing axons after 8 days in culture, as evidenced by dendritic labeling with MAP2 (Fig. 5C). Nogo-A localization was affected by the microtubule depolymerizing drug Nocodazole,
which largely reduced the presence of Nogo-A in neurites (Fig. 6F,G), indicating that the intracellular distribution of this protein in neurons is dependent on microtubule stability or cellular elements associated with microtubules. Indeed, microtubules are required for the formation and movement of the ER network (Terasaki et al. 1986), and colocalization of Nogo-A with the ER marker Calnexin was confirmed in neurons (Supplementary Fig. 2). In addition, both Nogo-A and Calnexin labeling were eliminated from neurites when cells were treated with Triton X-100 to remove membranes but not following mild membrane extraction with Saponin, in which organelle membranes interacting with cytoskeleton are not extracted (Supplementary Fig. 2) (Nakata and Hirokawa 1987; Pigino et al. 1997, 2001). Taken together, our results indicate that although low levels of Nogo-A are detected on the surface of many cortical neurons in vitro, the highest levels are associated with the ER, in agreement with previous reports (Voeltz et al. 2006).

**Nogo proteins Modulate Axonal Branching In Vitro**

The distinctive localization of Nogo-A in neurons suggests its participation in neurite or axon development. To test this hypothesis, we compared the development of cortical neurons from E15.5 Nogo-A/B/C (Nogo) mutant mice (Zheng et al. 2003) and wild-type littermates in vitro (Fig. 7A,B). Our results indicate that, although the total number of neurites did not differ between knockout and control cells (Fig. 7E), the percentage of neurons displaying a major neurite at 15 h in vitro (i.e., being polarized, see Materials and Methods) was significantly
Figure 6. Subcellular localization of Nogo-A in cortical neurons in vitro. (A, B) WGA-positive Tubulin-negative neurons were analyzed for detection of Nogo-A in nonpermeabilized neuronal cultures (see Methods for details). Neurons I (in A) and III-IV (in B) display Nogo-A labeling revealing the extracellular exposure of the N-terminal domain of Nogo-A at their plasma membrane. Neurons II and III in (A) and neuron V in (B) showed Tubulin staining, which indicates undesired permeabilization. (C, D) The Nogo-A antibody used in the experiments of panels A, B does not label permeabilized neurons from Nogo-deficient mice (D) in contrast to wild-type neurons (C). (E) Nogo-A staining in growth cones occupies the central domain in close colocalization with Tubulin (arrowheads) but does not enter the peripheral actin-rich domain. (F) Cell treatment with Nocodazole causes loss of lamella from growth cones and redistribution of Nogo-A from neurites (arrowheads) to the cell soma. The fluorochrome used in each double immunofluorescence is indicated as above. Scale bars A-F = 10 μm.
higher in knockout neurons than in controls but not significantly different after 30 h in vitro (Fig. 7F). These observations indicate faster polarization in the absence of Nogo proteins. Interestingly, a 2-fold increase in the number of branches in the major neurite was observed in knockout cells compared with controls at both 15 and 30 h in vitro (Fig. 7C,D,H). Thus, although approximately 60% of knockout polarized neurons had more than 2 branches, almost 55% of controls had no branches. Taken together, these data suggest that the absence of Nogo resulted in faster polarization and an increase in neurite branch- ing at least in vitro.

**Nogo-Deficient Mice Showed Deficits in Tangential Migration**

Our results on Nogo-A expression, together with the in vitro studies, indicate that Nogo-A potentially affects tract development or neuronal migration. To address this possibility, we studied the phenotype of Nogo-deficient mice during cortical development. We initially determined the effects of the absence of Nogo on radial and tangential neuronal migration. BrdU pulse labeling experiments revealed no differences in radial migration between mutant and wild-type mice (not shown). In addition, L1 immunostaining revealed that Nogo-deficient mice displayed normal cortical tracts (Supplementary Fig. 3). Next, we examined the distribution of several cohorts of GABA-ergic neurons in the neocortex of Nogo-mutant mice (Fig. 8). Early interneurons are generated at E12-E14 from the medial ganglionic eminence, and migrate toward the neocortex through the IZ in close association with corticofugal fibers, whereas late interneurons are generated at E14-E16 from both the medial and the lateral ganglionic eminences and migrate toward the cortex following the SVZ (Fig. 8) (e.g., Parnavelas 2000; Marin and Rubenstein 2001). We followed separately the positioning of early- and late-generated interneurons in mutant and control mice by analyzing the distribution of double-labeled BrdU/Calbindin cells in the SS cortex of P6 mice following BrdU injection at E12.5 or E15.5 (Fig. 8C,D). Interestingly, a statistically significant reduction of about 25% in the number of early interneurons (generated at E12.5) in P6 Nogo-deficient mice was detected (Fig. 8C,E). In contrast, interneurons generated around E15.5 displayed no significant difference in number in either mouse model (Fig. 8D,E). Finally, we found that the total number of interneurons in the SS cortex at P15, P21 and adult mutant mice was not affected, as assessed by GAD ISH (Fig. 8F), nor were particular interneuronal populations such as those expressing Parvalbumin or Calbindin (Fig. 8G–I). Therefore, Nogo may participate in the migration process of early cohorts of GABA-ergic neurons to the cortex and its absence delays the migration of E12.5-generated interneurons toward the neocortex.

**Discussion**

The developmental expression of the myelin-associated inhibitor Nogo-A in neurons at perinatal stages has been reported previously (Tozaki et al. 2002; Mingorance et al. 2004; Richard et al. 2005). However, few data are available about the putative role of neuronal Nogo-A during early stages of corticogenesis (E11-E16). Here we provide evidence that Nogo proteins play distinct roles during cortical development and neuronal maturation (e.g., participating in tangential migration of the first cohorts of GABA-ergic neurons or modulating neurite formation). In addition, the relevant expression of Nogo-A in radial glial cells, considered to be the major source of neurons and glial cells during development (Gotz et al. 2002; Gotz and Barde 2005), opens up the attractive possibility that Nogo-A may also participate in the physiology of this cell type.

**Nogo-A Expression Correlates with Neurogenesis and Maturation of Cortical Neurons during Embryonic Development**

Our results indicate that Nogo-A expression by cortical neurons is developmentally regulated and that it follows their gradient of positioning and maturation of the cerebral cortex. Indeed, neuronal Nogo-A expression is postmitotic, first appearing in...
neurons populating the preplate (E11–E12) to further split into their derivatives (subplate and marginal zones), and appearing in postmitotic cells lying in the upper portion of the emerging cortical plate at E15–E16. The progression of Nogo-A protein expression in the cortical plate also follows the "inside-out" pattern of positioning and maturation (e.g., Rakic 1974; Bayer et al. 1991).

Furthermore, it is remarkable to notice that Nogo-A staining in postmitotic cortical plate neurons is enriched in the distal portion of the growing corticofugal axon, appearing only in the perikaryon in later stages of radial migration. Nogo-A protein is enriched in growth cones of embryonic olfactory axons and in the distal tip of elongating neurites (Tozaki et al. 2002; Richard et al. 2005). Moreover, the appearance of Nogo-A labeling in the soma of cortical neurons is time coincident with the innervation of subcortical regions (e.g., internal capsule), as described in other studies (e.g., Del Rio et al. 2000). Taken together, the onset and evolution of Nogo-A expression in projecting neurons progresses from the axon to the neuronal soma as indicative of neuronal maturation, which may be modulated by the establishment of axonal connections with cell targets.

Figure 8. Absence of Nogo alters the migration of early cohorts of cortical interneurons. (A, B) Scheme of the migratory routes followed by early and late-generated interneurons. Although early (E12; A) generated interneurons from the MGE preferentially follow the IZ, in close contact with corticofugal axons (green), later generated interneurons (E15; B) from the lateral ganglionic eminence use a different route. (C, D) BrdU/Calbindin colocalization following BrdU injection at E12.5 in the SS cortex of P6 mice. (E) Nogo-deficient mice display a 25% reduction in the number of E12.5-generated interneurons compared with control littersmates (\( P < 0.05 \) in a 2-tailed \( t \)-test) but not in the number of E15-generated interneurons. This reduction cannot be further detected at P15 (I–I) by counting total GABA-ergic neurons (V–V), total Parvalbumin- (G–G), or total Calbindin-positive interneurons (H, I). The fluorochrome used in each double immunofluorescence is indicated as above. Abbreviations as in Figures 1 and 2. I–VI; neocortical layers. Scale bars: F–H = 200 \( \mu \)m.
Functions of Nogo-A during Cortical Development

with this, the genetic ablation of Nogo-A expression during cell migration. Therefore, although we were unable to detect Nogo-A in the perikaryon of radial migrating neurons using several antibodies, we observed that tangential neurons displayed high levels of Nogo-A during migration, mainly targeted to their leading processes. In agreement with this, the genetic ablation of Nogo induced a delay in the tangential migration of the early cohorts of GABA-ergic interneurons into the cerebral cortex, in contrast with undetectable layering defects in principal neurons.

What is the function/s for the enriched Nogo-A distribution in the leading process of migrating interneurons and corticofugal axons? Although these functions remain to be determined, several hypotheses can be postulated. Nogo proteins form membrane channels in the cellular ER, which may control ER physiology, including the control of calcium stores (Dodd et al. 2005). The overexpression of Nogo-B, for example, modifies ER homeostasis by increasing cytoplasmic calcium, which may lead to cell death (Kuang et al. 2006). Remarkably, in nonpathological conditions, local calcium transients are present in the leading process of tangentially migrating cells and modulate their migration (Moya and Valdeolmillos 2005), and calcium dynamics also regulate axon extension and guidance at the growth cone (Gomez and Spitzer 1999; Robles et al. 2003). Therefore, Nogo proteins may also modulate ER dynamics and local calcium concentration in the leading process during tangential migration and neurite growth. In addition, Nogo proteins could also be involved in the regulation of membrane dynamics required for membrane extension in axons and leading processes. Indeed, similarity between the leading processes of nonradial migratory neurons and growing axons has been reported (Yee et al. 1999).

Another tentative hypothesis is that Nogo-A also exerts extracellular functions. Indeed, our in vitro data indicate that low levels of Nogo-A also occur on the surface of cortical axons in vitro. This observation complements the studies which indicated that a percentage of Nogo-A is present at the plasma membrane of neurons (Jin et al. 2003; Dodd et al. 2005). Studies by Metin and Godement demonstrated that early-generated interneurons (E11–E13) in the medial ganglionic eminence use the corticofugal tract to reach the dorsal pallium by migrating in close contact with corticofugal fibers (Metin and Godement 1996). In our study, we found a specific decrease in the number of early-generated interneurons (E12.5 cohort) that populate the SS cortex, which indicates the participation of Nogo in this process. Although warranting further study, these data suggest that Nogo-A may also regulate tangential migration by acting as an adhesion molecule in the corticofugal tract. In this regard, previous studies by our group have demonstrated that Nogo-A is involved in cell adhesion because it induces axonal fasciculation (Mingorance et al. 2004). Moreover, amino-blocking of Nogo-A with specific antibodies induces axon sprouting of healthy adult neurons (Buffo et al. 2000) and mature hippocampal slices (Cavreiro et al. 2004). On the basis of all these observations, it is tempting to propose that in addition to other intracellular functions, Nogo-A has extracellular functions by interacting with unknown membrane molecules. However, with the present data we cannot rule out additional roles of Nogo-A in other processes that produce a delay in the migration of the early-generated GABA-ergic interneurons (e.g., controlling the cell proliferation or survival for these particular cohorts of cortical interneurons).

Interestingly, neuronal NgR expression in the neocortex does not start until late prenatal and early postnatal stages (Josephson et al. 2002; Wang et al. 2002; Mingorance et al. 2004), indicating that during most embryonic development the functions of cell-surface Nogo-A are not mediated by NgR. NgR-related receptors, such as NgR2 and 3, are also unlikely to mediate Nogo-A functions because they do not bind to Nogo-A (Barton et al. 2003; Venkatesh et al. 2005). However, in a recent study a receptor for the amino portion of Nogo-B (NgBR) has been identified (Miao et al. 2006). Because Nogo-B N-terminal region is also present in Nogo-A, the authors cannot completely rule out the possibility that NgBR also serves as a receptor of Nogo-A, although this hypothesis requires further studies.

Nogo-A Expression by Radial Glia Cells during Development

One of the most unexpected results of this study is the observation of Nogo-A expression by radial glial cells. As in neurons, Nogo-A is not restricted to a specific radial glial population in the developing telencephalon, and both radial glia of the dorsal and ventral telencephalon express this protein, although they exhibit intrinsic differences in the neurogenic potential and cell cycle duration (Gotz et al. 1998; Malatesta et al. 2003).

Radial glial cells are transient proliferative cells that are the source of most neurons and astroglial cells of the cerebral cortex (Levitt and Rakic 1980; Gotz et al. 2002; Rakic 2003). Interestingly, although mature cortical astrocytes lack Nogo-A expression shortly after their differentiation, they re-express it when they become reactive after injury (Mingorance et al. 2004; Marklund et al. 2006). Indeed, radial glial cells and reactive astrocytes share the expression of several molecules that are not usually present in resting astrocytes, such as Vimentin and RC2 (Pixley and de Vellis 1984; Ridet et al. 1996; Doetsch et al. 1997). In addition, high Nogo-A levels are also detected in high proliferative glial cell lines (Mingorance et al. 2004), and other RTN proteins not detected in resting astrocytes, such as RTN3, are overexpressed by highly invasive human astrocytoma tumor cells (Huang et al. 2004). Therefore, expression of Nogo-A may be added to the list of events, characteristic of proliferative state, that are shared between radial glia and reactive astrocytes.

Intracellular Nogo-A May also Regulate Cell Polarization and Neurite Growth

A study by Richard et al. (2005) raised the possibility that Nogo-A regulates axonal growth and dendritic remodeling through modulating microtubule dynamics. Here we describe the preferential targeting of Nogo-A to the end portion of growing neurites in vitro. In addition, the enriched presence of Nogo-A in the growth cone and distal portions of neurites has been also reported (Tozaki et al. 2002; Richard et al. 2005). In agreement with this, our results indicate alterations in cell polarization (defined as transition from stage 2 to stage 3) and neurite branching of cultured cortical neurons from Nogo-deficient mice. These effects might be difficult to observe in vivo because multiple environment-specific mechanisms may compensate single gene deficiencies. These findings indicate that Nogo proteins are required for appropriate branching pattern in cultured...
neurons and that the absence of these proteins leads to early neuronal polarization. Although the participation of Nogo-B or C in these processes cannot be ruled out, our data points to Nogo-A as the main protein responsible for these effects, which are likely to be cell autonomous. This conclusion complements the findings of Voelz et al. (2006) who described that overexpression of Nogo-A leads to the formation of neurite-like processes in nonneuronal cells. In conclusion, on the basis of our data, we propose that Nogo proteins, particularly Nogo-A, are involved in multiple processes during cortical development. This would open an avenue of research that may reveal the details of the functions of Nogo-A in neurons and radial glial cells.

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

Notes
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Address correspondence to Ana Mingorance-Le Meur, PhD, Department of Cellular and Physiological Sciences, University of British Columbia—Life Sciences Institute, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Email: alemeur@interchange.ubc.ca or to José A. del Río, PhD, Neurobiology of Development and Regeneration, Institute for Research in Biomedicine and Department of Cell Biology, Barcelona Science Park, University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain. Email: jadelrio@pcb.ub.es.

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