Neuronal Nogo-A Modulates Growth Cone Motility via Rho-GTP/LIMK1/Cofilin in the Unlesioned Adult Nervous System

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Nogo-A has been extensively studied as a myelin-associated neurite outgrowth inhibitor in the lesioned adult central nervous system. However, its role in the intact central nervous system has not yet been clarified. Analysis of the intact adult nervous system of C57BL/6 Nogo-A knock-out (KO) versus wild-type (WT) mice by a combined two-dimensional gel electrophoresis and isotope-coded affinity tagging approach revealed regulation of cytoskeleton-, transport-, and signaling growth-related proteins, pointing to regulation of the actin cytoskeleton, the neuronal growth machinery, and in particular the Rho-GTPase/LIMK1/cofilin pathway. Nogo-A KO adult neurons showed enlarged, more motile growth cones compared with WT neurons. The phenotype was reproduced by acute in vitro neutralization of neuronal Nogo-A. LIMK1 phosphorylation was increased in Nogo-A KO growth cones, and its reduction caused the decrease of KO growth cone motility to WT levels. Our study suggests that in the unlesioned adult nervous system, neuronal Nogo-A can restrict neuronal growth through negative modulation of growth cone motility.

During postnatal development, central nervous system (CNS) neurons lose their ability to regenerate in part due to the presence of neurite outgrowth inhibitors, e.g. in myelin or in the glial scar (1, 2). A number of molecules with suggested growth inhibitory activity are present in the adult CNS: Nogo-A, oligodendrocyte myelin glycoprotein, myelin-associated glycoprotein, repulsive guidance molecule (2–4), and certain ephrins (5, 6), semaphorins (7–9) and proteoglycans (1). Nogo-A has been extensively studied for its role in inhibiting axonal regeneration as well as compensatory fiber growth in the injured adult CNS in vivo (2, 4, 10). Neutralization of Nogo-A through in vivo application of function-blocking antibodies induces neuronal growth after spinal cord or brain injury in adult rodents as well as primates (2, 11). A moderate increase in regeneration and compensatory fiber growth after lesion has been also observed in Nogo-A knock-out (KO) mice at different degrees depending on intrinsic genetic differences in the different mouse strains analyzed (10, 12–15). In the adult CNS, Nogo-A is found predominantly in the innermost adaxonal and outermost myelin membranes and in oligodendrocytes (16, 17). During development, Nogo-A is expressed in neurons, where it has been suggested to play a role in neuronal migration and cortical development (18) and to regulate synaptic plasticity (19–21). Neuronal expression of Nogo-A is down-regulated following the onset of myelination but persists at higher levels in particular neuronal populations such as in the hippocampus (16, 22), olfactory bulb, deep cerebellar nuclei, spinal motor neurons (23), and dorsal root ganglia (DRG) (24). The possible roles of neuronal Nogo-A in the intact adult CNS have not been studied in detail. It has been shown that its neutralization in vivo in intact adult rats produces a transitory growth response of Purkinje axons and the corticospinal tract (25–27).

In the last few years, the application of systems biology approaches based on large-scale analysis of proteins has been successfully applied to complex biological networks, e.g. for the analysis of neurodegenerative disorders and infections (28, 29). In this study, we used systems biology profiling based on two proteomic approaches (two-dimensional gel electrophoresis and isotope-coded affinity tagging (ICAT)) to address the role of Nogo-A in the unlesioned adult CNS. We compared the adult CNS of C57BL/6 naïve, unlesioned, Nogo-A KO and wild-type (WT) mice, and using a combination of approaches, we report that the depletion of Nogo-A or functional blockade of neuronal Nogo-A in the adult as well as postnatal intact nervous system causes the reorganization of the cytoskeletal growth cone machinery at both the molecular and morphological levels and that the LIMK1/cofilin phosphorylation state is critical for this process.

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¶ The abbreviations used are: CNS, central nervous system; KO, knock-out; DRG, dorsal root ganglia; ICAT, isotope-coded affinity tagging; WT, wild-type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; HPLC, high performance liquid chromatography; P0, postnatal day 0; NGF, nerve growth factor.

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EXPERIMENTAL PROCEDURES

Animals—Male C57BL/6 Nogo-A KO mice with a strain purity of >99.98% and back-crossed for >10 generations (12) and male C57BL/6 WT mice were used. All animal experiments were performed with the approval of and in strict accordance with the guidelines of the Zurich Cantonal Veterinary Office. All efforts were made to minimize animal suffering and to reduce the number of animals required.

Antibodies—Rabbit anti-cofilin (Western blot, 1:1000; Chemicon), rabbit anti-phospho-cofilin Ser3 (Western blot, 1:1000; Chemicon), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (Western blot, 1:15000; Abcam), rabbit anti-LIMK1 (Western blot, 1:1000; immunocytochemistry, 1:100; Abcam), mouse anti-Nogo-A 11C7 (Western blot, 1:15000; neutralization assay, 3 μg/ml; Novartis), rabbit anti-phospho-LIMK1 Thr508 (Western blot, 1:1000; immunocytochemistry, 1:150; Abcam), rabbit anti-β-tubulin (immunocytochemistry, 1:150; Abcam), mouse anti-β-tubulin III (immunocytochemistry, 1:2000; Promega), horseradish peroxidase-coupled goat anti-mouse IgG (Western blot, 1:15000; Pierce), Cy3-coupled goat anti-rabbit IgG (H+L; immunocytochemistry, 1:3000; Jackson ImmunoResearch Laboratories), horseradish peroxidase-coupled donkey anti-rabbit IgG (Western blot, 1:10000; Pierce), rabbit IgG (Western blot, 1:1000; Pierce), Alexa 488-conjugated phalloidin (immunocytochemistry, 1:40; Molecular Probes), glutathione S-transferase-tagged rhothekin Rho-bind- ing domain protein (1:100; Cytoskeleton Inc.) antibodies and rabbit anti-glutathione S-transferase polyclonal antibody (1:1000; Abcam) were from the indicated manufacturers.

Two-dimensional Gel Electrophoresis—Lumbar spinal cords of three adult Nogo-A KO and WT mice were dissected and transferred to CHAPS lysis buffer (50 mM NaH2PO4 (pH 8.0), 150 mM NaCl, 0.5% CHAPS, protease inhibitor mixture (Roche Applied Science)) on ice. Tissues were disrupted using a rotor-stator homogenizer. After 30 min on ice, samples were centrifuged (15 min, 2000 × g, 4 °C), and the total protein concentration of the supernatant was determined using a NanoDrop Technologies spectrophotometer system. 600 μg of both samples were reduced with tributylphosphine and labeled with ICAT reagents (Applied Biosystems) for 2 h at 37 °C. After quenching with dithiothreitol, samples were combined and digested with trypsin overnight at 37 °C. The peptide mixture was fractionated by HPLC in a KCl gradient. Biotinylated peptides were purified on Applied Biosystems ICAT avidin cartridges according to the manufacturer’s protocol. Fractions were dried by speed-vacuuming, biotin-cleaved with Applied Biosystems cleaving reagents, and cleaned with Sep-Pak cartridges (Vac C18, 1 cc; Waters Corp.). ICAT peptides were analyzed by nano-liquid chromatography/mass spectrometry using an LTQ FT mass spectrometer (Thermo Electron Corp.). Peptides were separated on a nano-HPLC system (Agilent Technologies) online prior to mass spectrometry analysis on a homemade C18 reversed-phase column (Magic, 5 μm, 100A C18 AQ; Michrom) using an acetonitrile/water system at a flow rate of 200 nl/min. Tandem mass spectra were acquired in a data-dependent manner. Typically, four tandem mass spectrometries were performed after each high accuracy spectral acquisition range survey. Data analysis was performed through the Sisyphus platform (developed by the group of B. W.); ProteinProphet probability score and ASAPRatio were applied.

Dissociated DRG—DRG from three postnatal day 0 (P0) and three adult KO and WT mice per experiment were dissected and trypsinized at 37 °C. After trituration, cells were filtered, plated at low density (1.5 × 10^4, glass coverslips coated with 20 μg/ml poly-L-lysine and 5 μg/ml laminin), and cultured (L-15 medium with L-glutamine, N1 additives, 50 μg/ml nerve growth factor (NGF), and 0.25% NaHCO3 at 5% CO2 and 37 °C) for 6 h (P0) or 14 h or 1–3 days in vitro (adult). Each experiment was repeated three times with four experimental replicates each unless indicated otherwise.

Parameters of neuronal morphology (branching points, number of neurites, number of filopodia, lamellipodial area, lamellipodial perimeter, tubulin-containing core area) were measured using Image software (National Institutes of Health) in 20 neurons per experiment in three independent experimen-
ments for a total of 60 neurons. The percentage of enlarged growth cones and growth cone morphology were assessed for all growth cones of the total number of neurons in three independent experiments. The total length of the neurite tree was measured using Metamorph software (Molecular Devices). The parameters of neuronal morphology for P0 dissociated DRG were measured using ImageJ software in at least 10 neurons per coverslip in four coverslips (experimental replicates) in two separate experiments for a total of at least 80 neurons. P0 neurons were manually traced using ImageJ software with the NeuronJ plug-in (30). Data analysis was performed using Prism 4.0 (GraphPad Software).

**Nogo-A Neutralization**—Highly purified mouse anti-Nogo-A monoclonal antibody 11C7 or a control highly purified mouse IgG antibody (3 μg/ml) was applied to Nogo-A KO and WT adult low density dissociated DRG neurons at plating time. Cells were cultured for 14 h. Each experiment was repeated three times, with four experimental replicates each. The percentage of enlarged growth cones was calculated for the total number of neurons in three independent experiments. Data analysis was performed using Prism 4.0.

**COS-7 Cultures**—Native COS-7 cells were cultured overnight in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 1% gentamicin and then treated for 1 h with increasing concentrations (5, 10, 20, and 40 μg/ml) of the S3 peptide or reverse-sequence control RV peptide.

**PC12 Cultures**—PC12 cells were cultured for 3 days in Dulbecco’s modified Eagle’s medium, 6% horse serum, 6% newborn calf serum, and 1% penicillin/streptomycin. Following trypsinization, cells were counted and plated on poly-L-lysine-coated coverslips (20 μg/ml) at a density of 50,000 cells/100 μl in differentiating medium (Dulbecco’s modified Eagle’s medium, 1% bovine serum albumin, and 100 ng/ml NGF). Cells were cultured for

![FIGURE 1. Protein expression changes in Nogo-A KO versus WT adult mouse spinal cord as shown by two-dimensional gels.](image1.png)

![FIGURE 2. Quantitative proteomic analysis of Nogo-A KO versus WT adult mouse spinal cord by ICAT analysis.](image2.png)
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TABLE 1
Two-dimensional gel electrophoresis spots differentially expressed in the spinal cords of Nogo-A KO versus WT mice in the cytoskeleton and signaling categories

| Spot No. | Protein* | Mean change (p ≤ 0.05) | IPG strip |
|----------|----------|------------------------|-----------|
|          | Cytoskeleton |                        |           |
| 1837     | Actin (β, γ, α) | 0.76                   | 4–7       |
| 1926     | α-Centrin (actin-related protein 1) | 1.22** | 4–7 |
| 2032     | α-Internexin (66-kDa filament) | 0.05** | 4–7 |
| 2155     | α-Internexin (66-kDa filament) | 20.00* | 4–7 |
| 2147     | α-Internexin (66-kDa filament) | 20.00* | 4–7 |
| 4102     | Cofilin, non-muscle isoform (cofilin-1) | 0.79 | 3–10 |
| 4180     | Cofilin, non-muscle isoform (cofilin-1) | 1.70 | 3–10 |
| 4173     | GFAP | 1.50 | 3–10 |
| 4505     | GFAP | 20.00* | 3–10 |
| 4526     | GFAP | 20.00* | 3–10 |
| 3951     | GFAP | 1.92 | 3–10 |
| 4502     | GFAP | 20.00* | 3–10 |
| 4058     | GFAP | 1.50 | 3–10 |
| 1864     | GFAP | 2.94 | 4–7 |
| 2027     | GFAP | 0.60 | 4–7 |
| 2038     | GFAP | 0.05** | 3–10 |
| 2018     | GFAP | 0.60 | 4–7 |
| 4007     | Neurofilament-68 | 0.63 | 3–10 |
| 4339     | Neurofilament-68 | 0.05** | 3–10 |
| 1679     | Neurofilament-68 | 0.62 | 4–7 |
| 1674     | Neurofilament-68 | 0.62 | 4–7 |
| 1753     | Neurofilament-68 | 1.50 | 4–7 |
| 4119     | Myosin regulatory light chain 2, skeletal muscle isoform | 0.46 | 3–10 |
| 4333     | Myosin regulatory light chain 2, skeletal muscle isoform | 0.05** | 3–10 |
| 1678     | Peripherin | 1.25 | 4–7 |
| 1776     | Tropomyosin α-chain (tropomyosin 1) | 0.61 | 4–7 |
| 4261     | Tropomyosin β-chain (tropomyosin 2) | 0.27 | 3–10 |
| 1914     | Tubulin α-chain | 0.78 | 4–7 |
| 1870     | Tubulin α-chain | 1.50 | 4–7 |
| 1908     | Tubulin β-chain | 0.75 | 4–7 |
| 2012     | Tubulin β-chain | 0.69 | 4–7 |
|          | Signaling |                        |           |
| 2621     | 14-3-3 protein β/α (PKC inhibitor protein) | 1.34 | 4–7 |
| 1774     | 14-3-3 protein (14-3-3σ) | 0.46 | 4–7 |
| 4347     | Acidic FGF (FGF-1) | 0.05** | 3–10 |
| 3931     | Astrocytic phosphoprotein PEA-15 | 1.50 | 3–10 |
| 1881     | Dihydropyrimidinase-related protein 2 (CRMP2) | 0.90 | 4–7 |
| 1681     | Guanine nucleotide-binding protein (transducin α-chain) | 2.00** | 4–7 |
| 2164     | Guanine nucleotide-binding protein (transducin β-chain 2) | 20.00* | 4–7 |
| 4020     | Rho-GDP dissociation inhibitor 1α | 1.55 | 3–10 |
| 4521     | Rho-GDP dissociation inhibitor 1α | 20.00* | 3–10 |
| 1748     | Serine/threonine protein phosphatase 2A | 1.80 | 4–7 |

* Spots correspond to statistically significant (p ≤ 0.05, one-way analysis of variance; normalization per gel as well as to the median of the three biological replicate gels and a call (present/absent) filter (two out of three biological replicates, WT and/or KO) were applied) regulated cytoskeletal and signaling proteins.

** Spots present only in KO or WT protein lysates were assigned the arbitrary values of 20 (20-fold increase; present only in the KO protein lysates) and 0.05 (20-fold decrease; present only in the WT protein lysates).

48 h and then treated for 1 h with increasing concentrations (5, 10, 20, and 40 μg/ml) of the S3 peptide or reverse-sequence control RV peptide.

Retinal Ganglion Neuron Cultures—Immortalized retinal ganglion neurons (RGC-5; provided by Dr. Krishnamoorthy (Department of Cell Biology and Genetics, University of Texas Health Science Center, Fort Worth, TX) were grown for 3 days in Dulbecco’s modified Eagle’s medium + GlutaMAX (Invitrogen), 10% fetal bovine serum, and 1% penicillin/streptomycin. Following trypsinization, cells were counted and plated on poly-L-lysine-coated coverslips (20 μg/ml) at a density of 10,000 cells/100 μl. Cells were grown overnight and then treated for 1 h with 20 μg/ml S3 peptide or reverse-sequence control RV peptide.

Time-lapse Video Microscopy—Dissociated DRG from adult Nogo-A KO and WT mice were cultured for 14 h in 2-well Lab-Tek chambered coverglass (Nalge Nunc International). Samples were then observed under a Leica wide-field IRBE microscope by phase contrast at 37 °C and 5% CO2 with a ×100 oil immersion objective. Images were captured with a Hamamatsu camera using Openlab 3.1.7 software (Improvision). At least five KO as well as WT neurons were randomly chosen, and the largest growth cone of each of them was imaged. Six independent experiments were performed. For each growth cone, 60 images at intervals of 15 s were acquired for a total real time of 15 min of recordings. Movies were further processed using Openlab and QuickTime (Apple) (0.15 s/frame). Statistical data analysis was performed using Prism 4.0. For S3 and RV peptide treatments, after 13 h of culturing, KO and WT adult dissociated DRG were treated with 20 μg/ml peptide for 1 h and then imaged as described above with a ×40 oil immersion objective.

Immunoblotting—The spinal cords from eight adult Nogo-A KO as well as WT mice were processed for protein lyses as described for two-dimensional gel electrophoresis. Samples (30 μg/lane) were resolved on 7–14% NuPAGE (Invitrogen) and transferred onto polyvinylidene difluoride membranes, followed by blocking and overnight incubation...
with primary antibodies at 4 °C. After washing, membranes were incubated with secondary antibodies. Proteins were detected using a chemiluminescent substrate (SuperSignal West Pico, Pierce). Images were captured with the Stella system (Agilent Technologies). Densitometry was performed with AIDA software (Raytest GmbH). Data analysis was performed using Prism 4.0.

**Immunocytochemistry**—Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized (0.1% Triton X-100 and 1X phosphate-buffered saline). After blocking, cells were incubated for double staining with primary antibodies, followed by incubation with the appropriate secondary antibody for 30 min and with 4',6-diamidino-2-phenylindole. Activated Rho was detected by probing cells with the Rho-binding domain from the Rho-GTP-interacting protein rhotekin tagged with glutathione S-transferase. Antibodies against glutathione S-transferase were subsequently used. Preparations were mounted in Mowiol/1,4-diazabicyclo[2.2.2]octane before examination under a conventional fluorescence microscope (Axio Scope 2 MOT Plus, Zeiss). Imaging was performed using an AxioVision system.

**RESULTS**

**Profiling of Nogo-A KO Versus WT Unlesioned Adult Mouse Spinal Cord by Two-dimensional Gel Electrophoresis**

and ICAT—We analyzed the spinal cords of C57BL/6 adult Nogo-A KO versus WT mice by two-dimensional gel electrophoresis (pH 4–7 and 3–10 isoelectric focusing) strips both used to increase the number of identified regulated spots (Fig. 1 and supplemental Fig. 1) and ICAT (Fig. 2), followed by HPLC peptide separation and subsequent run and analysis through a liquid chromatography/tandem mass spectrometer. With two-dimensional gel electrophoresis, 93 regulated spots were identified, corresponding to 62 different known proteins/protein subunits. 16 of the identified proteins were represented in more than one regulated spot, pointing to a different post-translational modification state in Nogo-A KO versus WT tissue (Table 1). Up- and down-regulation were equally represented (51.6% up-regulated and 48.4% down-regulated spots). Assignment to functional categories on the basis of GeneOntology and literature mining showed that the highest number of regulated proteins or spots fell into the cytoskeleton category (17% and 33%, respectively). The other major functional categories significantly represented (% proteins and % spots) were neuroprotection (17% and 13%), metabolism (12% and 10%), and transport (7% and 6%) (Fig. 1). Only one regulated myelin protein was observed (myelin basic protein, 0.52-fold). The complete list of protein changes is shown in supplemental Table 1. The quantitative ICAT analysis allowed the identification of a high number of regulated proteins, in particular of hydrophobic membrane proteins, due to the avoidance of the loading/separation in a gel matrix. 407 proteins were identified with a ProteinProphet probability score of ≥0.9 (71.7%, ≥2 unique peptides) (Fig. 2A and supplemental Table 2) with a normal distribution of ~1.19 ± 0.02 (mean ratio) (Fig. 2B). All ratios have been normalized to this value, assuming a correct mean ratio of 1.000, to correct for possible errors in protein quantification. 219 proteins showed a -fold change ratio of between 0.5 and 2 in Nogo-A KO versus WT spinal cord. Proteins outside of this interval were normally distributed in terms of up-regulation (16.5%) and down-regulation (15.5%). 104 proteins showed statistically significant regulation according to adjusted ASAPRatio and S.E. (supplemental Table 2) and were categorized into functional categories using Panther Version 6.1 software and literature mining (Fig. 2C). The results reflected those observed with the two-dimensional gel electrophoresis approach: signaling (14%), metabolism (14%), transport (11%), cytoskeleton (9%), and neuroprotection (5%) were the major represented categories (Fig. 2C).
**TABLE 2**

Differentially expressed cytoskeleton and signaling proteins in the spinal cords of Nogo-A KO versus WT mice by ICAT

| Description                                                                 | Adjusted ratio |
|----------------------------------------------------------------------------|----------------|
| **Cytoskeleton**                                                           |                |
| 1 Transgelin-3                                                             | 2.04 0.22      |
| 1 Tetraspanin-2                                                             | 0.34 0.11      |
| 1 Splice isoform 2 of AP-2 complex subunit β1, splice isoform 1 of AP-2 complex subunit β1 | 0.19 0.04      |
| 1 Predicted: similar to coflin-1 (collin, non-muscle isoform)               | 0.66 0.27      |
| 0.98 Predicted: microtubule-associated protein (MAP2)                       | 2.05 0.62      |
| 1 Microtubule-associated protein RP/EB family member 3                       | 1.81 0.59      |
| 1 Lap3 protein                                                               | 0.29 0.06      |
| 1 In vitro-fertilized egg cDNA, RIKEN full-length enriched library, clone 7420442C15, product γ-actin, cytoplasmic, full insert sequence; bone marrow macrophage cDNA, RIKEN full-length enriched library, clone I830072C08, product β-actin, cytoplasmic, full insert sequence; actin, cytoplasmic 1; actin, cytoplasmic 2 | 1.23 0.22      |
| 1 Impact protein                                                            | 0.72 0.21      |
| 1 F-actin-capping protein α3-subunit                                        | 0.69 0.15      |
| 1 Cytoplasmic dynein heavy chain, dynein heavy chain, cytosolic             | 1.72 0.30      |
| 1 Complexin-1                                                               | 1.57 0.43      |
| 1 AP-2 complex subunit α2                                                   | 0.35 0.06      |
| 1 117-kDa protein, ankyrin 2, brain                                         |                |
| **Signaling**                                                               |                |
| 1 Splice isoform 2 of 14-3-30, splice isoform 1 of 14-3-30                   | 1.70 0.55      |
| 1 Rho-related GTP-binding protein RhoB precursor                            | 0.34 0.12      |
| 1 Ras-related protein Rab-3D                                                | 0.22 0.06      |
| 0.99 Predicted: similar to 14-3-3η,14-3-3η                                  | 1.48 0.30      |
| 0.98 Predicted: hypothetical protein LOC75471; GTP-binding nuclear protein Ran, testis-specific isoform; GTP-binding nuclear protein Ran | 0.50 0.13      |
| 1 Mammary gland RCB-0527 Jyg-MC(B) cDNA, RIKEN full-length enriched library, clone G930005L08, product Ras-related C3 botulinum substrate 1, full insert sequence | 0.68 0.23      |
| 1 Guanine nucleotide-binding protein Gα-α-subunit 2; guanine nucleotide-binding protein Gα-α-subunit 1 | 0.69 0.19      |
| 1 Guanine nucleotide-binding protein Gα-β-subunit 2                         | 0.71 0.09      |
| 1 Dihydropyrimidinase-related protein, 5.62 kDa (CRMP5)                     | 0.72 0.24      |
| 1 14-3-3γ                                                                  | 1.28 0.25      |

Molecular Reorganization of the Neurite Outgrowth Machinery in the Intact Adult Nervous System of Nogo-A KO Mice—
The category “cytoskeleton” was the one mainly represented in the changes found in our proteomic approach. With two-dimen-
sional gel electrophoresis, we observed a higher number of regulated spots (33%) than of proteins (17%) (Fig. 1), indicating an important role of post-translational modifications in the regulation of the cytoskeleton in response to Nogo-A ablation. In addition to actin and tubulin isoforms, many of the differentially expressed proteins play an important role in actin and tubulin cytoskeleton remodeling (Fig. 3 and Table 1). Particularly interesting is cofilin, previously suggested to be downstream of Nogo-A/Nogo-66 receptor signaling (31); it showed two regulated spots, 0.79 (−1.27)- and 1.70-fold, respectively (Table 1), pointing to a diverse post-translational modification state of this protein. Cofilin phosphorylation state, regulated by LIMK1, is known to affect actin cytoskeleton dynamics, growth cone motility, and neurite outgrowth (Fig. 3). The changes in actin-binding proteins (myosin, tropomyosin) as well as actin itself (Table 1) suggested that the actin cytoskeleton was directly affected. In addition, neuronal intermediate filaments, e.g. peripherin, neurofilament-68, and α-internexin, which are highly expressed during development and in early neurite formation, were also regulated, suggesting the whole neuronal cytoskeleton to be affected by the lack of Nogo-A (Table 1). This conclusion was supported by the high number of spots (12%) and proteins (15%) falling into the category of signaling molecules (Fig. 1), the majority of which belonged to pathways responsible for cytoskeleton rearrangements and neurite outgrowth control. Examples are the 14-3-3 family of proteins (known to interact with cofilin as well as with its major regulator, LIMK1) and Rho-GDP dissociation inhibitor 1 (a key regul-
ulatory molecule of Rho-GTPase signaling pathways (Table 1), which can, upstream of LIMK1, affect its phosphorylation and therefore activity state).

We observed also the down-regulation of CRMP2, a protein playing a role in growth cone collapse (Table 1). The compar-
ison and integration of the ICAT data with the two-dimensional gel electrophoresis data permitted us to strengthen our obser-
avation of an important response at the level of the neuronal cytoskeleton and signaling machinery (Figs. 2 and 3 and Table 2). Cytoskeletal proteins known to affect actin polymerization (F-actin-capping protein, 0.69 ± 0.15), neurite outgrowth (microtubule-associated protein RP/EB3, 1.81 ± 0.59; MAP2, 2.05 ± 0.62), or microtubule-dependent transport (dynein, 15.54 ± 5.64) were shown to be regulated in a direction pointing to growth enhancement: down-regulation of actin-severing proteins and up-regulation of motor as well as microtubular proteins. The total level of cofilin presented a slight down-regulation (0.66 ± 0.27) (Table 2). This technique allowed only the quantification of the total level of relative cofilin expression but did not permit the discrimination between phosphorylated and unphosphorylated forms. Among the other regulated signaling proteins emerged again the 14-3-3 proteins (14-3-3Δ, 1.7 ± 0.55; 14-3-3η, 1.48 ± 0.30; 14-3-3γ, 1.28 ± 0.25) as well as a member of the CRMP protein family (dihydropyrimidinase-related protein 5, 0.72 ± 0.25). The Rho-GTPase signaling pathway was also affected (Rho-related GTP-binding protein
growth cones (KO, 1.46; WT, 1.0). The results show increased levels of phosphorylated LIMK1 in the growth cones of Nogo-A KO and WT adult dissociated DRG neurons (Fig. 5A). Densitometry confirmed increased levels of phosphorylated, and therefore inactive, LIMK1 in Nogo-A KO mice. It also showed increased levels of phosphorylated, and therefore active, LIMK1. No change was detected in total protein levels. These data support a role of the LIMK1 activation state in the regulation of cofilin phosphorylation and inactivation (Fig. 4, A and B) in the intact spinal cords of adult Nogo-A KO mice.

To clarify if these changes could be ongoing in Nogo-A KO adult neurons, as a recent study from Endo et al. (34) has shown that LIMK1 regulates actin filament assembly at the tips of the growth cones of chick DRG neurons and as DRG neurons are one of the few populations of adult neurons with axons in the CNS that can be maintained in culture, we analyzed by immunocytochemistry the levels of phosphorylated and total LIMK1 in the growth cones of Nogo-A KO and WT adult dissociated DRG neurons after 14 h in culture (Fig. 5A). The results show that the levels of phosphorylated LIMK1 were increased in the growth cones (KO, 1.46 ± 0.16; WT, 1.0 ± 0.08) of Nogo-A KO compared with WT neurons (Fig. 5A). However, no change could be detected for the total level of expression of LIMK1 (KO, 1.158 ± 0.06; WT, 1.0 ± 0.06) (Fig. 5A).

LIMK1 phosphorylation is regulated through activation of ROCK by Rho-GTP and of PAK1 by Rac and Cdc42. Interestingly, our proteomic data already pointed to a potential regulation of Rho-GTPases in the intact nervous system of Nogo-A KO mice. Up to now, Nogo-A has been known to affect only RhoA activation. Therefore, we tested the level of Rho-GTP in vitro in Nogo-A KO and WT adult dissociated DRG and observed up-regulation of activated Rho in the growth cones of Nogo-A KO neurons (KO, 1.56 ± 0.1; WT, 1.0 ± 0.04), pointing to its potential involvement in the increase of LIMK1 phosphorylation (Fig. 5B).

Neurons from Adult Nogo-A KO Mice Show Altered Growth Cone Morphology and Increased Growth Cone Motility—The LIMK1/cofilin pathway, shown recently to directly affect neurite outgrowth and actin filopodial formation at the tip of the growth cone in chicken DRG (34), as well as the observed modulation of other signaling molecules participating in this pathway and/or known to affect cytoskeletal dynamics (14-3-3 and CRMP proteins and Rho-GTPases) and the changed expression levels of several additional cytoskeletal and cytoskeleton-binding proteins such as dynnein and myosin II, which have been suggested to increase neurite and growth cone motility when up-regulated (35–37), could influence the morphology and dynamic aspects of the neuronal cytoskeleton of Nogo-A KO adult neurons (Fig. 3). Nogo-A KO adult dissociated DRG neurons plated onto a polylysine/laminin substrate and kept 14 h in culture did not show significant differences in the number of emitted neurites or in branching points compared with WT neurons (supplemental Fig. 2). However, they showed a clear shift toward larger growth cones (≥100 μm²) and a corresponding reduction in smaller growth cones (≤50 μm²) compared with WT DRG neurons (Fig. 6A). Taking the overall morphology and distribution of F-actin and microtubules into account, we categorized the growth cones into dystrophic (38), normal, and enlarged (Fig. 6A). The percentage of neurons presenting enlarged growth cones was significantly higher in Nogo-A KO dissociated DRG cultures (KO, 35 ± 6.6%; WT, 13.30 ± 6.72%) (Fig. 6A). A more accurate analysis of the
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A

Phalloidin-Alexa488  pLIMK1-Cy3  Merged

WT

KO

Phalloidin-Alexa488  Total LIMK1-Cy3  Merged

WT

KO

Phalloidin-Alexa488  IgG-Cy3  Merged

WT

KO

B

Rho-GTP-Cy3

WT

KO

Normalized pLIMK1 staining (Mean Gray Value - Background)

Normalized LIMK1 staining (Mean Gray Value - Background)

Normalized Rho-GTP staining (Mean Gray Value - Background)
growth cones has shown that the increase in growth cone area is specifically due to an increase in the area covered by actin-containing lamellipodia (KO, 2.28 ± 0.16; WT, 1.0 ± 0.19), whereas no difference could be observed in the tubulin-containing core area (KO, 1.2 ± 0.14; WT, 1.0 ± 0.17). These data are reflected in the measured decrease in the percentage of the tubulin-containing core area over the total area of Nogo-A KO versus WT growth cones (KO, 0.61 ± 0.03; WT, 1.0 ± 0.05). To test whether more F-actin polymerization could result in an increase in both lamellipodia and filopodia, we counted the number of filopodia per growth cone. No significant change was present in the total filopodial number (KO, 0.99 ± 0.07; WT, 1.0 ± 0.08). However, due to the larger lamellipodial area, a decrease in the number of filopodia in relation to the perimeter of the growth cone was observed (KO, 0.47 ± 0.03; WT, 1.0 ± 0.16) (Fig. 6B).

To check the specificity of the observed morphological changes, we performed an additional set of experiments in which we specifically and acutely blocked Nogo-A, present on the cell membranes of adult DRG (24), in culture by adding anti-Nogo-A antibodies (11C7) (Fig. 7, A and B) to dissociated DRG from WT mice. An IgG antibody was applied as a control, and Nogo-A KO adult dissociated DRG were treated as well with both antibody 11C7 or IgG as an additional control (Fig. 7B). The specific neutralization of Nogo-A reproduced the neuronal phenotype observed in Nogo-A KO neurons, showing an increase in the percentage of neurons with enlarged growth cones (Fig. 7B), suggesting that the observed phenotype is specifically due to the lack of...
neuronal Nogo-A and not to additional compensating molecules, e.g. Nogo-B.

**Neuronal Nogo-A Modulation of Growth Cone Morphology**

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**Neuronal Nogo-A Influences Growth Cone Morphology Also in Newborn DRG Neurons**—To further understand whether the lack of Nogo-A signaling affects the growth cone machinery only of Nogo-A KO adult neurons or also during development, when Nogo-A expression is strictly neuronal, we dissociated DRG neurons from Nogo-A KO and WT newborn (P0) mice (Fig. 7C). As in adult neurons, no significant difference was detected in the number of branch points (KO, 8.26 ± 0.64; WT, 7.2 ± 0.45) or in the number of neurites per cell body (KO, 3.82 ± 0.18; WT, 3.53 ± 0.20) (data not shown). However, we were able to observe a substantial enlargement in the growth cone area of Nogo-A KO neurons (KO, 168.2 ± 16.71; WT, 102.30 ± 10.94) (Fig. 7C) as well as a major increase in the number of actin filopodia emitted from both the growth cone (KO, 21.41 ± 1.07; WT, 11.93 ± 0.51) and the cell body (KO, 22.43 ± 1.17; WT, 16.65 ± 8.61) (Fig. 7C). Furthermore, these filopodia were significantly longer (KO, 5.31 ± 0.22; WT, 3.79 ± 0.17) (Fig. 7C). These data further support a role of neuronal Nogo-A in actin cytoskeleton remodeling.

**Increased Growth Cone Motility in Nogo-A KO Adult Dissociated DRG Neurons**—To observe whether the changes at the morphological level shown in growth cones of Nogo-A KO neurons could also cause a functional modification in growth cone motility, we used time-lapse microscopy (supplemental Videos 1 and 2). As expected from the previous results, we found a clear increase in the mean area of growth cones of Nogo-A KO DRG neurons (initial area, 96.6 ± 9.9 μm²; final area, 105.5 ± 10.6 μm² over a 15-min observation period) compared with WT DRG neurons (initial area, 54.6 ± 5.4 μm²; final area, 61.1 ± 5.3 μm²) (Fig. 8A). Furthermore, in 15 min, Nogo-A KO growth cones covered double the distance covered by WT growth cones (KO, 8.9 ± 0.9 μm; WT, 4.3 ± 0.7 μm), showing a strong increase in growth speed (Fig. 8B). Nogo-A KO growth cones showed higher minimum speed (KO, 0.16 μm/min; WT, 0.00 μm/min) as well as higher maximum speed (KO, 1.38 μm/min; WT, 0.94 μm/min). This increase was not due to a continuous enlargement over time in the area of Nogo-A KO growth cones or a restriction of WT ones, as the initial and final areas for each genotype were not statistically different (Fig. 8A). To test whether the observed speed difference was a secondary effect caused by different turning behavior of KO compared with WT growth cones, the rotation angle was measured, but no significant difference was observed (supplemental Fig. 3). If
Nogo-A KO growth cones advance more rapidly, longer neurites could be expected. We measured the total length of the neuritic tree of WT and Nogo-A KO adult dissociated DRG neurons after 1–3 days in vitro. The increase in growth cone area and speed did translate into a significant increase in the overall size of the neuronal tree (Fig. 8C).

Inhibition of LIMK1 Activity Decreases Growth Cone Motility in Nogo-A KO Adult Dissociated DRG Neurons—Endo et al. (34) recently found that a cell-permeable peptide containing the N-terminal sequence of cofilin and therefore its Ser3 phosphorylation site (the S3 peptide) inhibits LIMK1 activity and reduces phosphorylated cofilin levels in the growth cones of chick DRG neurons. We confirmed that the S3 peptide, but not the reverse-sequence control RV peptide, can decrease cofilin phosphorylation and F-actin in vitro in native COS-7 cells (supplemental Fig. 4), NGF-primed PC12 cells, and retinal ganglion neurons (Fig. 9). To test whether the activity of LIMK1 could be directly responsible for the increased growth cone motility observed in Nogo-A KO adult DRG neurons, we inhibited LIMK1 in Nogo-A KO as well as WT dissociated DRG neurons. Time-lapse video analysis of S3 peptide-treated KO cultures showed clearly reduced growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 min⁻¹, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 min⁻¹ (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A). The inhibition of LIMK1 in WT dissociated DRG neuronal cultures with the S3 peptide did not produce a significant decrease in growth cone motility compared with the control RV peptide-treated Nogo-A KO cells: KO S3 peptide-treated growth cones grew 6.93 ± 0.93 μm in 15 min, and KO RV peptide-treated growth cones grew 10.45 ± 0.88 μm in 15 min (Fig. 10A).
DISCUSSION

Molecular Reorganization of the Neurite Outgrowth Machinery in the Intact Adult Nervous System of Nogo-A KO Mice—Nogo-A KO mice have been extensively studied as a regeneration model following a CNS lesion (12–15), but the possible functions of Nogo-A in the intact CNS are still rather unclear. Therefore, in this study, we addressed this question using a double proteomic approach to investigate the molecular changes in the adult intact nervous system of Nogo-A KO mice. In line with previous reports of enhanced sprouting and fiber growth following antibody-mediated Nogo neuronalization in the unlesioned rat adult CNS (25, 26), in our study, a reorganization and enhancement of the Nogo-A KO neuronal growth machinery at both the molecular and morphological levels could be observed.

We found that a large proportion of differentially expressed proteins in the Nogo-A KO intact adult nervous system are components of the cytoskeleton, cytoskeleton-binding proteins, signaling molecules involved in cytoskeleton remodeling and growth, early neurite growth markers, and axonal transport constituents. Numerous proteins that are known to play an important role in actin and tubulin cytoskeleton remodeling (e.g. F-actin-binding protein, dynein, myosin, and tropomyosin) were found to be regulated in a direction pointing to growth enhancement: down-regulation of actin-severing proteins and up-regulation of motor as well as structural proteins. The regulation of many neuron-specific cytoskeletal proteins, in particular neurofilament-68, peripherin, and α-internexin, which are highly expressed during development and in early neurite formation, as well as of other neu-
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Increased Growth Cone Motility in Nogo-A KO Neurons through the LIMK1/Cofilin Pathway—The analysis of the observed regulated signaling pathways in our proteomic approach had highlighted the Rho-GTPase/LIMK1/cofilin signaling pathway as a potential, even if not unique, candidate in the regulation of neuronal cytoskeletal proteins in the absence of Nogo-A signaling. Our experiments further supported this: the Western blot analysis showed increased levels of phosphorylated LIMK1 as well as cofilin in the intact spinal cords of adult Nogo-A KO mice. Moreover, LIMK1 phosphorylation was up-regulated in growth cones of DRG neurons dissected from adult Nogo-A KO mice. LIMK1 phosphorylation depends on the activity of ROCK, regulated by GTP-bound Rho, and of PAK1, regulated by Rac and Cdc42. Interestingly, Nogo-A is already known to modulate RhoA activity. Our data confirm a role for Rho activation in Nogo-A-mediated neuronal cytoskeleton rearrangements, as an increase in activated GTP-bound Rho could be observed in growth cones of Nogo-A KO neurons. Cofilin is inactivated by phosphorylation at Ser3 by...
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active LIMK and is known as an essential regulator of actin dynamics through its filament-severing activity (34, 39). Its high expression at the growth cone has pointed to its possible involvement in the regulation of growth cone motility: cofilin increased the length of neurite outgrowth when expressed in rat cortical neurons in primary cultures (43), suggesting that it plays a critical role in neurite extension and growth cone motility. Endo et al. (34) recently found that a cell-permeable cofilin fragment (S3 peptide) containing the Ser\textsuperscript{3} phosphorylation site binds to and inhibits the endogenous LIMK1 activity. The resulting reduced endogenous phosphorylated cofilin levels in the growth cones suppressed growth cone motility and extension in chick DRG neurons, suggesting that LIMK1 stimulates neurite extension through its cofilin-phosphorylating activity. In addition, it has been shown that LIMK1 activation state can influence dendritogenesis (44). Because of all these numerous observations, the observed modulation of the LIMK1/cofilin pathway in the spinal cords of unlesioned adult Nogo-A KO mice and at the growth cones of Nogo-A KO adult neurons could influence growth cone morphology and motility and neurite outgrowth. In fact, we observed that growth cones of Nogo-A KO adult DRG neurons are larger and more motile compared with WT ones. Moreover, the increase in the growth cone area is due to an enlargement of lamellipodia but not of the microtubule-containing core of the growth cone, pointing to a key role of the actin cytoskeleton and to its modulation. The increase in growth cone motility translated in long-term cultures into a noticeably increased neuronal total outgrowth, as described previously (12). The specificity of the observed phenotype was confirmed by its reproducibility by antibody-mediated acute neuronal Nogo-A neutralization in WT adult DRG neurons and by its reproducibility in Nogo-A KO postnatal dissociated DRG neurons. These results point to neuronal Nogo-A as major contributor to the observed phenotype and highlight the specificity of the observed effects, which could be otherwise attributed to the regulation of compensating molecules like Nogo-B, which has been described to be highly up-regulated in Nogo-A KO mice (12).

More evidence of a direct link between the observed phenotype of increased growth cone motility and the observed regulation of the LIMK1/cofilin pathway was obtained by the fact that the presence of the S3 peptide, which activated cofilin by decreasing its phosphorylation, caused the reversion of the observed phenotype \textit{in vitro} in Nogo-A KO growth cones: they moved at a lower speed, which was comparable with that of WT growth cones.

Summary—This study provides mechanistic insights into the function of neuronal Nogo-A in the unlesioned CNS and the related molecular signaling pathways. Our results suggest that Nogo-A might act as neuronal local growth suppressor in the intact nervous system both in adulthood and during development: its ablation causes modulation of Rho-GTP and of the LIMK1/cofilin pathway through phosphorylation regulation, which leads to actin cytoskeleton remodeling, increased growth cone motility, and neurite outgrowth in an intact system. In conclusion, we suggest that neuronal Nogo-A could play a role in the fine regulation of neuronal growth control through growth cone motility modulation in the intact adult as well as developing nervous system.

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