Proteolytic control of neurite outgrowth inhibitor NOGO-A by the cAMP/PKA pathway

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Protein kinase A (PKA) controls major aspects of neurite outgrowth and morphogenesis and plays an essential role in synaptic plasticity and memory. However, the molecular mechanism(s) of PKA action on neurite sprouting and activity are still unknown. Here, we report that in response to neurotrophin or cAMP stimulation the RING ligase praja2 ubiquitinates and degrades NOGO-A, a major inhibitor of neurite outgrowth in mammalian brain. Genetic silencing of praja2 severely inhibited neurite extension of differentiating neuroblastoma cells and mesencephalic neurons and axon outgrowth and sprouting of striatal terminals in developing rat brain. This phenotype was rescued when both praja2 and NOGO-A were depleted, suggesting that NOGO-A is, indeed, a biologically relevant target of praja2 in neuronal cells. Our findings unveil a novel mechanism that functionally couples cAMP signaling with the proteolytic turnover of NOGO-A, positively impacting on neurite outgrowth in mammalian brain.

cAMP | PKA | proteasome | ubiquitin | NOGO-A

Neurite outgrowth plays an essential role in embryonic development, neuronal differentiation, and central nervous system (CNS) plasticity. Outgrowth can be also altered in several neurological disorders, as well as by neuronal injury and degeneration (1, 2). Extracellular signals, such as neurotrophins (NTFs) and neurotransmitters, regulate neurite outgrowth, dendritic arborization, and synaptic activity, establishing a dynamic neuronal network in developing and adult CNS. NTFs and neurotransmitters act at the cell membrane by generating intracellular second messengers that, in turn, reversibly modulate the activity of signaling proteins and effector enzymes (3, 4).

cAMP is an ancient second messenger that controls a variety of biological cues. In neurons, essential functions such as neurite outgrowth and morphogenesis, synaptic transmission, and plasticity require tightly regulated responses to cAMP/protein kinase A (PKA) stimulation (5). PKA holoenzyme localizes in subcellular microdomains through interactions with A-Kinase-Anchor-Proteins (AKAPs). AKAP forms a local transduction unit, which includes signaling/metabolic enzymes, receptors, ion channels, adaptor molecules, and mRNAs (6, 7). Space-restricted activation of PKA provides a control mechanism to direct, integrate, and locally attenuate the cAMP cascade (8). praja2 belongs to a growing family of mammalian RING ligases abundantly expressed in the brain that finely tune the stability of intracellular substrates and play an essential role in critical aspects of cell signaling. In response to cAMP stimulation, praja2 couples ubiquitination and proteolysis of the inhibitory PKA regulatory (R) subunits by the proteasome to a sustained cAMP/PKA signaling, significantly impacting on synaptic plasticity and long-term memory (9). In addition to enhancing cAMP signaling, the role of praja2 in neuronal differentiation and dendritic network in the CNS and the molecular targets involved are unknown.

NOGO-A is a member of the reticulon (RTN) family of integral membrane proteins with a conserved C terminus reticulon homology domain (RHD) and abundantly expressed in oligodendrocytes and in distinct neuronal subpopulations (10, 11). NOGO-A was originally identified as a potent inhibitor of neurite outgrowth (1, 10). In the adult CNS and in injured neurons, NOGO-A restricts the capacity of an axon to grow and regenerate. Genetic ablation of NOGO-A promotes neuritogenesis and fasciculation of oligodendrocytes and culture of dorsal root ganglion neurons, functionally improving neuronal plasticity and recovery of post-ischemic adult rat brain (12).

Although the role of NOGO-A in neurite outgrowth is well established, regulation of NOGO-A levels in differentiating neurons and the mechanism(s) involved have been, to date, unknown. Here, we report a novel mechanism of neuritogenesis based on proteolytic turnover of NOGO-A (13). In response to cAMP or neurothrophin stimulation, RING ligase praja2 ubiquitinates and degrades NOGO-A. Proteolysis of NOGO-A by praja2 is functionally linked to neurite outgrowth in both differentiating neurons and developing rat brain.

Results

praja2 Interacts with NOGO-A. We isolated novel praja2 interactors/substrates by screening a human brain cDNA library. Two positive clones encoding for the conserved C terminus of RTNs were further characterized. Among different RTNs with still unclear functions, RTN4 variants, also known as NOGO-A, NOGO-B, and NOGO-C, play a role in inhibiting axonal growth, neuronal regeneration, and synaptic activity.

First, we demonstrated that praja2 interacts with NOGO-A in cell extracts. Recipient cells were transiently transfected

Significance

Damage of the central nervous system (CNS) and neurodegenerative disorders represent the principal cause of morbidity and mortality among adults. An injured CNS is unable to regenerate dendritic or neurite connections because they spontaneously occur in the peripheral nervous system. Inhibitory influences of the glial cells and of myelin-associated inhibitors oppose the spontaneous regeneration of CNS neurons. Here, we identify a positive mechanism of neuronal differentiation, which acts in response to activation of intracellular pathway(s) to remove an inhibitory constraint of neurite outgrowth. Unveiling the molecular events regulating the regenerative capacity of neurons will impact on therapeutic initiatives to stem the course of brain damage or neurodegeneration.

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The authors declare no conflict of interest.

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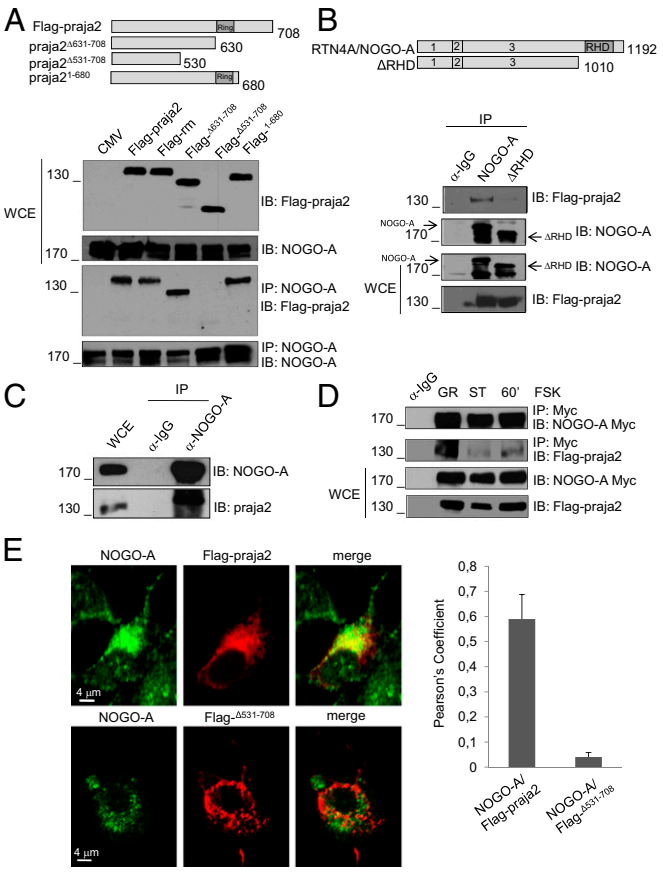

Fig. 1. praja2 interacts with NOGO-A. (*A*) Schematic representation of human praja2 constructs (*Upper*). Flag-praja2, the RING mutant (praja2rm), or the deletion mutants (Δ631–708, Δ531–708, and 1–680) were transiently transfected in HEK293 cells. Cells were treated with MG132 (10 μM) for 6 h before harvesting. Lysates were subjected to coimmunoprecipitation with anti–NOGO-A and anti-Flag antibodies (*Lower*). WCE, whole-cell extracts. (*B*) Schematic representation of NOGO-A constructs. Coimmunoprecipitation of flag-praja2 and NOGO-A or NOGO-ΔRHD (*Lower*). (*C*) Isolation of endogenous NOGO-A and praja2 complex from lysates (2 mg) of SH-SY5Y cells. (*D*) Transfected cells were left untreated (GR, growing) or serum-starved (ST) overnight and then stimulated with FSK. Lysates were subjected to coimmunoprecipitation assay with the indicated antibodies. All of the coimmunoprecipitation experiments were repeated at least three times. (*E*) Immunofluorescence analysis of SH-SY5Y cells transiently transfected with Flag-praja2 and Flag-Δ531–708 vectors. The bar graph represents the Pearson's coefficient of four indipendent experiments. At least 25 cells for each experimental group were analyzed. The data are expressed as mean ± SEM.

with Flag-tagged praja2 and Myc-NOGO-A (the longest NOGO variant). Coimmunoprecipitation assays demonstrated that flag-praja2 and the coexpressed NOGO-Myc formed a stable complex (Fig. 1*A*). praja2 ligase activity was not required for NOGO-A binding, as a praja2 inactive mutant carrying an alanine substitution of two critical residues within the RING domain (cys634 and cys671) (Flag-praja2rm) was able to bind NOGO-A as well as the wild-type protein (Fig. 1*A*). Deletion mutagenesis and binding analysis identified residues 531–708 of praja2 as the segment required for binding to NOGO-A (Fig. 1*A*). The RHD of NOGO-A mediates interaction with praja2 because a NOGO-A mutant lacking the C-terminal segment did not bind praja2 (Fig. 1*B*). A complex containing endogenous praja2 and NOGO-A was isolated from cell lysates (Fig. 1*C*). praja2 is phosphorylated by PKA (9). Moreover, Fig. 1*D* shows that serum deprivation significantly reduced the amount of praja2/NOGO-A complex recovered in the immunoprecipitates. The binding was only partially restored by forskolin treatment after 60 min of stimulation.

Because the RHD motif is highly conserved among RTNs, we tested if other family members form a complex with praja2. Fig. S1 shows that RTN2C and RTN3C bind praja2, suggesting that binding to praja2 is a general feature of the RTN protein family.

In situ immunostaining analysis of human neuroblastoma cells demonstrated that flag-praja2, but not its deletion mutant flag-Δ531–708, partially colocalized with NOGO-A. Overlapping signals were detectable in the perinuclear region, cytoplasm, and cell membrane, supporting the existence of a complex in intact cells (Fig. 1*E*).

Proteolysis of NOGO-A by praja2. By acting as E3-ubiquitin ligase, praja2 should destabilize RTNs. Indeed, wild-type praja2 severely reduced the levels of coexpressed NOGO-A (Fig. 2 *A* and *B*). In contrast, in cells expressing the praja2 ring mutant (Flag-praja2rm), NOGO-A accumulated to the same extent as in control cells that were not transfected with praja2 (Fig. 2 *A* and *B*). A similar effect was observed also on the other RTN variants (Fig. S2). Proteasome inhibition by MG132 treatment prevented

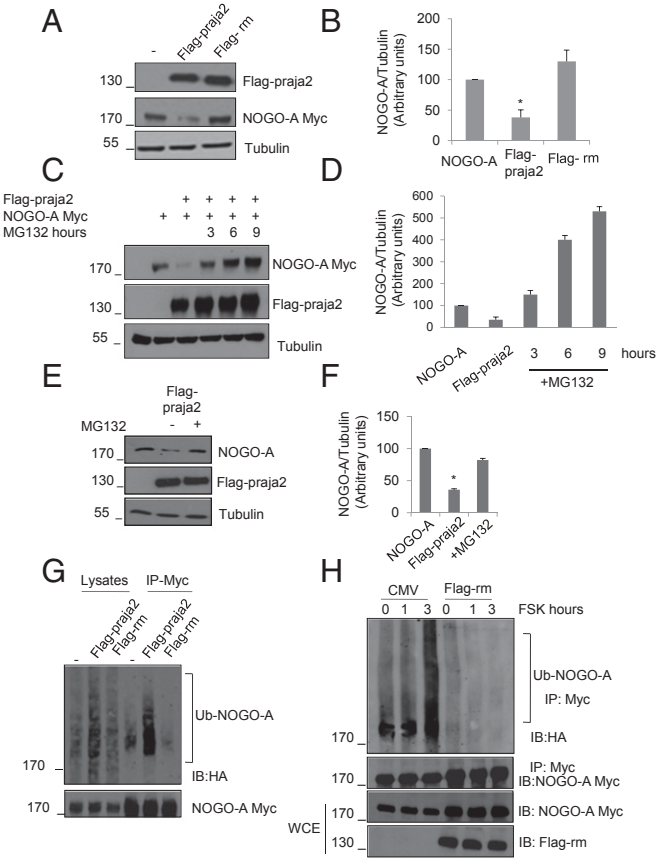

Fig. 2. praja2 ubiquitinates and degrades NOGO-A. (*A*) Immunoblot of lysates from cells transiently transfected with vectors encoding Flag-praja2 (either wild-type or RING mutant) and Myc-tagged NOGO-A. (*B*) Quantitative analysis (mean ± SEM) of three independent experiments shown in *A*. **P* < 0.01 versus control (NOGO-A). (*C*) Immunoblot analysis of lysates from cells transfected with NOGO-A alone or with Flag-praja2. MG132 (10 μM) was added 6 h before harvesting. (*D*) Quantitative analysis of (mean ± SEM) three independent experiments shown in *C*. (*E* and *F*) HEK293 cells were transfected with flag-praja2 and treated for 3 h with MG132 (20 μM) before harvesting. Lysates were immunoblotted with the indicated antibodies. (*F*) Quantitative analysis of three independent experiments shown in *E*. **P* < 0.01 versus NOGO-A and flag-praja2+MG132. (*G*) Lysates from transfected cells were immunoprecipitated with anti-Myc antibody. The precipitates were immunoblotted with anti-HA and anti-Myc. (*H*) Same as in *G*, with the exception that transfected cells were serum-deprived overnight and then stimulated with FSK.

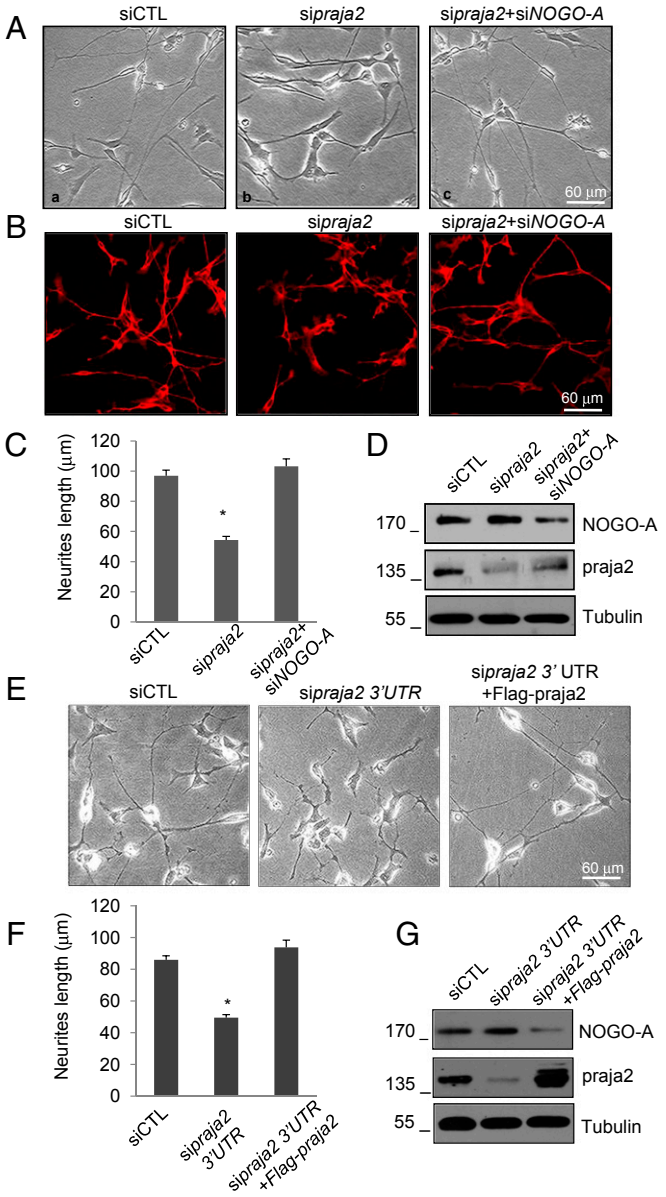

Fig. 3. praja2 controls axonal outgrowth. (*A*) Differentiating SH-SY5Y cells were transiently transfected with the indicated siRNAs. Images were collected and analyzed by phase-contrast microscope. (*B*) Same as in *A*, with the exception that cells were immunostained with anti–α-tubulin antibody and analyzed by confocal microscopy. (*C*) Cumulative data are expressed as a mean value ± SEM of four independent experiments. $*P < 0.05$ versus siCTL. Number of cells analyzed: control siRNA (50), *praja2 siRNA* (50), *praja2 siRNA +NOGO-A siRNA* (59). (*D*) Immunoblot analysis of SH-SY5Y–transfected cells. (*E*) Differentiating SH-SY5Y cells were transiently transfected with the indicated siRNAs and with Flag-praja2 vector. (*F*) Cumulative data of three independent experiments shown in *E*. $*P < 0.01$ versus siCTL and *sipraja2 3′ UTR*+Flag-praja2. (*G*) Immunoblot for NOGO-A, praja2, and tubulin using lysates from SH-SY5Y–transfected cells.

praja2-mediated NOGO-A degradation and induced a time-dependent rise of exogenous (Fig. 2 *C* and *D*) and endogenous (Fig. 2 *E* and *F* and Fig. S3) NOGO-A levels. Next, we investigated if praja2 promotes ubiquitination of NOGO-A. As expected, overexpression of praja2, but not praja2rm, leads to accumulation of poly-ubiquitinated NOGO-A (Fig. 2*G*). praja2 activity is induced by PKA (9). Therefore, we tested if PKA activation regulates ubiquitination of NOGO-A. Cells were serum-starved overnight and subsequently treated with forskolin (FSK), an adenylate cyclase activator. As shown in Fig. 2*H*, raising intracellular cAMP levels positively impacted on NOGO-A ubiquitination, even at 3 h from forskolin treatment. Expression of Flag-praja2rm downregulated both basal and FSK-stimulated NOGO-A ubiquitination, confirming the role of praja2 in the control of NOGO-A stability.

praja2 Is Required for NOGO-A Degradation and Neurite Outgrowth in Differentiating Neurons. In light of the inhibitory role of NOGO-A in neurite outgrowth of developing or injured neurons, we asked whether, by affecting NOGO-A stability, praja2 regulates neurite elongation in differentiating neuronal cells. To this end, we used SH-SY5Y neuroblastoma cells as a model system. SH-SY5Y cells differentiate in vitro in the presence of retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (14). RA-treated cells exhibit a neuron-like morphology and express neuron-specific proteins. Addition of BDNF into the medium of RA-treated cells induces additional major morphological changes and promotes neurite formation. We reproduced this neural-like phenotype by sequentially treating SH-SY5Y cells with RA and BDNF (Fig. 3 *A*, a). Genetic knockdown of praja2 using a mixture of four siRNAs targeting praja2 reversed the neural-like phenotype of treated cells (Fig. 3 *A*, b and *C*). Similar effects were obtained using two independent siRNAs targeting distinct coding regions (Fig. S4) or the 3′ UTR of praja2 mRNA. Moreover, the neurite extension in siRNA-transfected cells could be rescued by re-expression of exogenous praja2 (Fig. 3 *E* and *F*). The relative abundance of NOGO-A and praja2 in transfected cells is shown (Fig. 3*G*).

The effects of praja2 downregulation on neurite extension might result from either impairment of cAMP signaling or the accumulation of NOGO-A. To discriminate between these possibilities, we performed a double knockdown for praja2 and NOGO-A. Downregulation of NOGO-A restored, at least in part, neurite extension in praja2-silenced neuroblastoma cells (Fig. 3 *A*, c and *C*). The data were confirmed by immunostaining differentiating SH-SY5Y cells with anti–α-tubulin antibody (Fig. 3*B*). Fig. 3*D* shows the levels of praja2 and

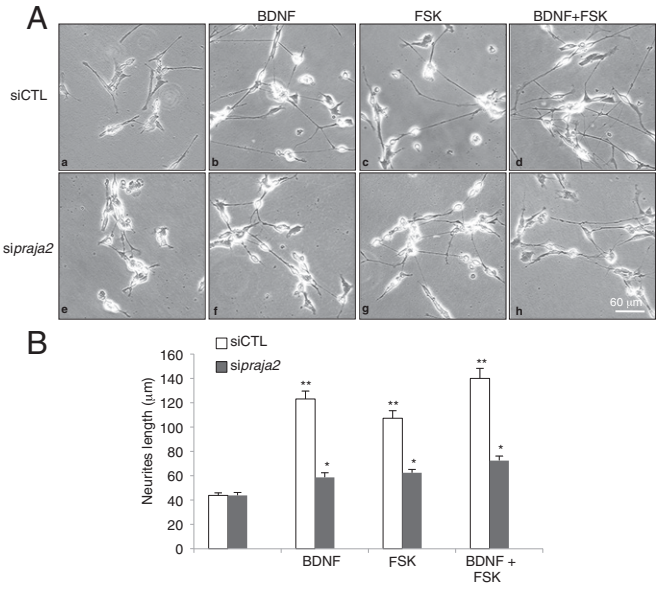

Fig. 4. praja2 is required for cAMP-induced neurite outgrowth. (*A*) Differentiating neuroblastoma cells transfected with control siRNA or *praja2* siRNA were left untreated (a, e) or treated with BDNF (50 ng/mL) (b, f), FSK (40 μM) (c, g), or with both agonists (FSK and BDNF) (d, h) for 3 d. Images were collected by phase-contrast microscope. (*B*) A mean of three independent experiments that gave similar results is shown. $*P < 0.01$ versus BDNF, FSK, and BDNF/FSK-stimulated, siCTL; $**P < 0.05$ versus unstimulated cells.

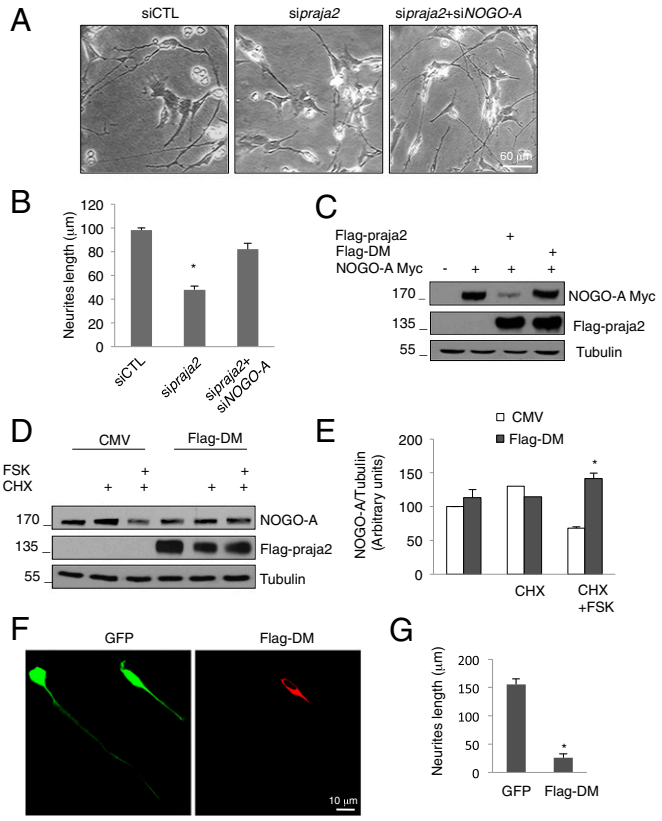

Fig. 5. praja2 mediates cAMP effects on neurite outgrowth. (*A*) Differentiating SH-SY5Y cells were transfected with the indicated siRNAs and treated with FSK (10 μM), and neurite lengths were scored 3 d later. (*B*) Cumulative data of three independent experiments as a mean value ± SEM are shown. **P* < 0.05 versus siCTL. (*C*) Expression levels of Flag-praja2, Flag-DM, and NOGO-A. (*D*) Immunoblot analysis of lysates from SH-SY5Y cells transiently transfected with CMV and praja2-DM treated with cycloheximide (100 μg/mL) and forskolin (40 μM). (*E*) Cumulative data of five independent exeperiments are expressed as a mean value ± SEM. **P* < 0.05 versus controls [CMV ± cycloheximide (CHX)] and flag-DM (CHX ± FSK). (*F*) Differentiating SH-SY5Y cells cotransfected with praja2-DM and GFP vector were immunostained with anti-Flag antibody and analyzed by confocal microscopy. (*G*) Cumulative data of three experiments as a mean value ± SEM are shown. **P* < 0.01 versus GFP.

NOGO-A in siRNA-transfected cells. Although praja2 regulates RTN stability, no major morphological changes of the endoplasmic reticulum were observed following praja2 knockdown (Fig. S5). Moreover, in situ immunostaining analysis showed overlapping signals between NOGO-A and calreticulin, whereas only a partial colocalization could be assigned to praja2 and calreticulin (Fig. S6).

praja2 Mediates cAMP Effects on NOGO-A Stability and Neurite Outgrowth. Activation of the cAMP/PKA pathway positively regulates neurite outgrowth in a wide variety of species, profoundly impacting brain development and synaptic plasticity (15–17). Accordingly, treating neuroblastoma cells with forskolin or BDNF promoted neurite outgrowth and extension (Fig. 4 *A*, b and c, and *B*). These effects were similar when cells were cotreated with both agonists (FSK and BDNF) (Fig. 4 *A*, d and *B*). Silencing of praja2 prevented neurite extension induced by FSK and BDNF (Fig. 4 *A* and *B*, f and g), this phenotype was rescued when both praja2 and NOGO-A were depleted (Fig. 5 *A* and *B*), suggesting that praja2 is, indeed, a relevant downstream effector of cAMP signaling on neuronal differentiation. The neurite extention induced by FSK was PKA-dependent, as treatment with the PKA inhibitor H89 reduced neurite elongation in both control and praja2-depleted cells (Fig. S7). PKA phosphorylation of praja2 at Ser342/Thr389 is required for ligase activity (9). Therefore, we monitored NOGO-A levels in cells expressing a praja2 mutant carrying substitution of Ser342/Thr389 to Ala (double mutant, praja2DM). As suspected, praja2DM failed to degrade coexpressed NOGO-A, compared with wild-type praja2 (Fig. 5*C*). When expressed in differentiating neuroblastoma cells, praja2DM prevented FSK-induced proteolysis of NOGO-A (Fig. 5 *D* and *E*) and severely reduced neurite extension (Fig. 5 *F* and *G*).

Next, we investigated the role of praja2 in mesencephalic neurons both in culture and in developing rat brain. As expected, praja2 silencing significantly reduced neurite extension of cultured mesencephalic neurons. The total length of neurites in *praja2* siRNA-treated neurons was significantly lower compared with controls, as observed at three different time periods after transfection (Fig. 6 *A* and *B*). Notably, after 6 d of silencing, the length of tyrosine hydroxylase (TH)-positive axons, clearly recognizable from the typical axonal varicosities, was reduced by 55.5% in *praja2* siRNA-treated neurons (neurite lengths were

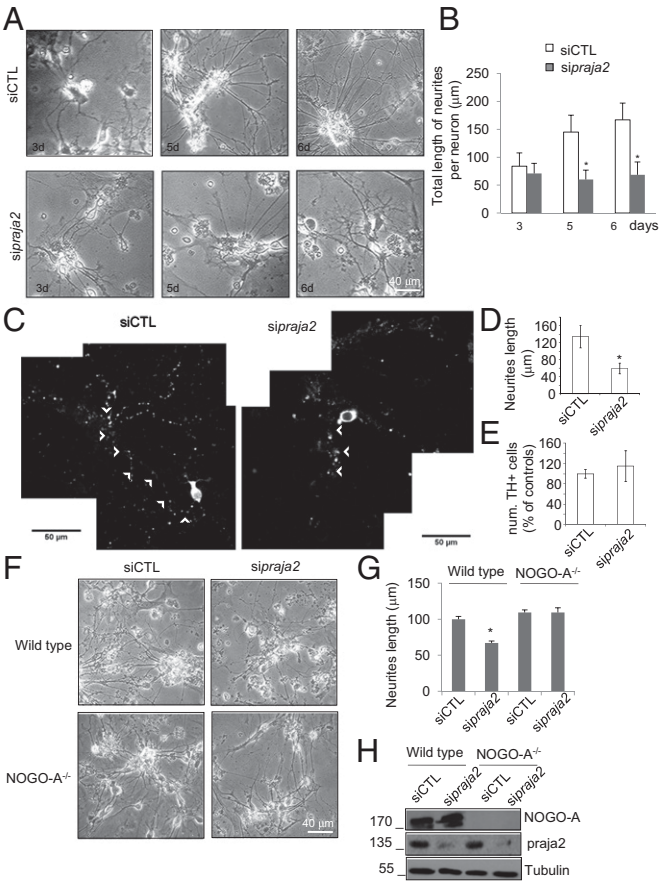

Fig. 6. praja2 controls neurite outgrowth in mesencephalic neurons. (*A*) Midbrain neurons were transiently transfected with *praja2* siRNA or control siRNA, and neurite extension was calculated at the indicated days from transfection. (*B*) Neurite lengths are expressed as mean ± SEM; **P* < 0.05 versus siCTL of three independent experiments. (*C*) Neurite branching of TH-positive neurons in vitro (midbrain primary cultures) under basal conditions and after downregulation of praja2 with siRNA. (*D* and *E*) Quantification of principal neurite lengths and number of TH-positive neurons in culture. Neurite lengths of TH+ mesencephalic neurons from control (34) and siRNA praja2-transfected (24) cells. (*F*) Neurite extension ofcontrol and NOGO-A KO neurons (NOGO-A$^{-/-}$) transiently transfected with *praja2* siRNA or control siRNA. (*G*) Quantification of neurite lengths from *F* of three independent experiments. **P* < 0.05 versus siCTL and sipraja2 in NOGO-A$^{-/-}$. (*H*) NOGO-A and praja2 levels in mesencephalic neurons from *F* and *G*.

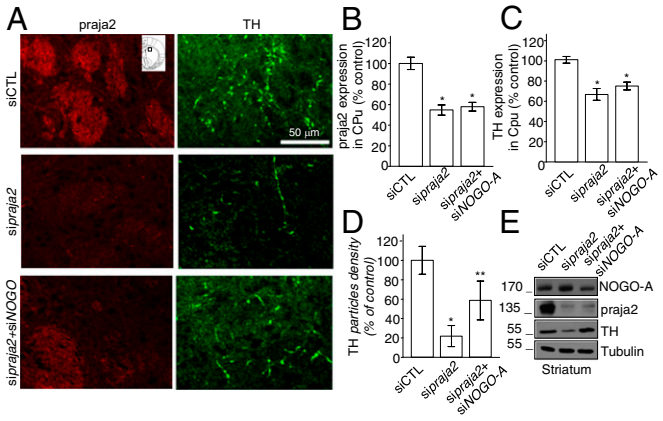

Fig. 7. praja2 controls neurite outgrowth in developing mammalian brain. (*A*) Immunofluorescence images of TH and praja2 in the CPu from rat brain perfused in the lateral ventricle with either control siRNA, *praja2* siRNA, *NOGO-A* siRNA, and *praja2 siRNA+NOGO-A* siRNA. (*Inset*) The position of the region analyzed. Quantification of total fluorescence intensity of praja2 (*B*) and TH (*C*) in CPu is reported. (*D*) The density of TH-positive varicosities in the dorsal striatum was assessed on average of six sections. To automatically delineate the fibers, the images were first thresholded and subsequently quantified with the Analyze particles tool of Image J. One-tailed statistical test was used. In particular, $*P < 0.05$ versus siCTL and $**P < 0.05$ versus *sipraja2*. (*E*) Immunoblot analysis of whole lysates from striatum of neonatal mice perfused with the indicated siRNAs.

123.5 ± 26.5 μm in control siRNA condition and 59.8 ± 12.8 μm in *praja2* siRNA neurons) (Fig. 6 *C* and *D*). Reduction of axonal length with *praja2* siRNA was not accompanied by a significant loss of TH-positive cell bodies (Fig. 6*E*). Furthermore, genetic knockdown of praja2 in mesencephalic neurons isolated from NOGO-A KO mice had no effects on neurite elongation, compared with wild-type neurons. These results indicate that praja2 controls neurite elongation by targeting NOGO-A (Fig. 6 *F*–*H*).

praja2 Regulates NOGO-A Levels and Axon Outgrowth in Developing Rat Brain. High levels of praja2 are found in the nigro-striatal pathway, a system involved in several neurological disorders, such as Parkinson's disease and attention deficit hyperactivity disorder. Therefore, we tested the influence of praja2 on axonal sprouting in the caudate-putamen (CPu) of postnatal rat brain. Interestingly, genetic knockdown of praja2 in this area decreased the overall intensity of TH signal, a marker of mesencephalic neurons, and significantly reduced the number of TH-positive dopaminergic varicosities in the dorsal striatum (Fig. 7 *A*–*D*). The data suggest that the effects of siRNA are the consequence of downregulation of the protein at presynaptic levels. It is also possible that the injected siRNA may work on postsynaptic neurons that in turn regulate dopaminergic endings via NOGO-A. To understand whether the effects of praja2 were mediated by NOGO-A, a third group of animals received both siRNAs for praja2 and NOGO-A. Results show that concomitant downregulation of NOGO-A partly reversed the effects of praja2 silencing on the number of axonal varicosities (Fig. 7 *A*–*D*).

Immunoblot analysis of lysates from the CPu area confirmed that silencing of praja2 reduced TH expression (Fig. 7*E*). To address whether the reduction of TH-positive terminals in the CPu was due to neuronal loss, we measured the number of dopamine (TH-positive) neurons in the substantia nigra pars compacta (SNpc), the region of origin of CPu dopamine fibers. Fluorescence intensity, neuronal density, and neuronal diameter were not significantly changed in the SNpc of siRNA-treated animals (Fig. S8).

Discussion

Here, we demonstrate that cAMP promotes degradation of NOGO-A through the ubiquitin proteasome system. We identify praja2 as the E3 ubiquitin ligase that mediates cAMP destabilization of NOGO-A. Degradation of NOGO-A by praja2 is a mechanism that underlies cAMP-induced neurite outgrowth of differentiating neuronal cells. Genetic downregulation of praja2 prevents NOGO-A degradation and inhibits axon outgrowth and branching in cultured neurons and in the nigro-striatal dopaminergic pathway of rat brain.

BDNF is essential for neuronal development, survival, and differentiation. Acting through a dedicated receptor at the cell membrane, BDNF stimulates a number of second messenger pathways that, in turn, activate distinct classes of adaptors, scaffolds, and signaling enzymes (18). cAMP and its effector enzyme PKA have been implicated in mediating some of the effects of BDNF stimulation on neuronal differentiation and activity. BDNF stimulation of PLCγ induces transient release of calcium from intracellular stores, activating calcium-sensitive adenylate cyclases and consequent elevation of cAMP levels (19, 20). Activation of PKA by cAMP increases the number and extension of neurites and varicosities, accelerating neuritogenesis and neurite outgrowth rate in a variety of mammalian neurons (21, 22). Conversely, genetic or pharmacological inhibition of PKA severely downregulated neurite outgrowth and extension, supporting the role of the cAMP/PKA pathway in critical aspects of neuronal differentiation (16). PKA phosphorylation of CREB and CREB-directed gene transcription represents the principal mechanism of cAMP action in promoting and sustaining neuronal differentiation (23). Additionally, a variety of intracellular substrates and downstream effectors of cAMP signaling have been identified and mechanistically linked to neuronal differentiation (24, 25).

Our results disclose a previously unrecognized mechanism of cAMP action on neuronal differentiation based on the regulated proteolysis of NOGO-A, a major inhibitor of neurite outgrowth in mammalian brain (26). cAMP induces proteolysis of NOGO-A through the ubiquitin proteasome system (UPS). Decrease of NOGO-A levels removes the inhibitory constraint on neurite outgrowth, preventing growth cone collapse and sustaining neurite extension and morphogenesis (Fig. 8). We identified praja2 as the ubiquitin ligase responsible for ubiquitination and proteolysis of NOGO-A. praja2 is phosphorylated by the associated PKA, which stimulates its ubiquitin ligase activity (9). Genetic knockdown

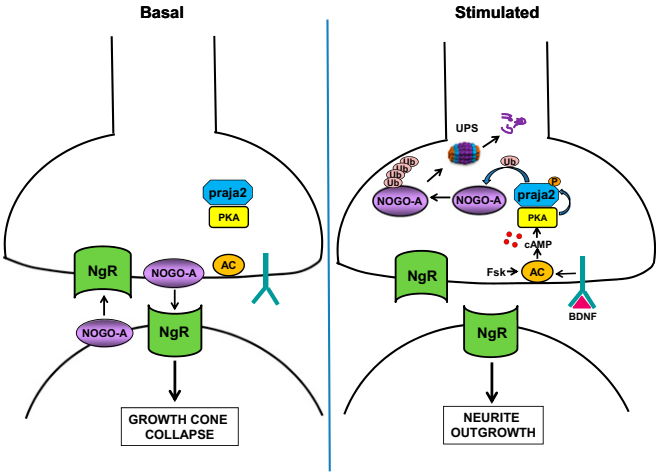

Fig. 8. Model of praja2–NOGO-A pathway. Under basal conditions, NOGO-A inhibits neurite outgrowth. Activation of adenylate cyclase (AC) by BDNF or by Forskolin (Fsk) increases cAMP levels and dissociates PKA holoenzyme. Active PKA catalytic subunit phosphorylates praja2, which in turn ubiquitinates and degrades NOGO-A through the UPS. The drop in NOGO-A levels promotes neurite outgrowth.

of praja2 or expression of a phosphorylation-defective praja2 mutant prevented cAMP-induced NOGO-A degradation and severely inhibited neurite extension in differentiating neurons. Notably, downregulation of NOGO-A levels in praja2-silenced cells restored neurite outgrowth to control values. Moreover, praja2 silencing was ineffective in downregulating neurite extension in NOGO-A KO neurons. Altogether, these findings are consistent with the notion that inhibition of neurite outgrowth by praja2 silencing depends on increased NOGO-A levels, rather than on global downregulation of cAMP signaling.

In conclusion, negative regulation of the NOGO-A pathway by cAMP and praja2 constitutes a novel UPS-driven signaling circuit that promotes and sustains biological processes underlying neuronal differentiation and synaptic activity. Unveiling the mechanism(s) regulating praja2 expression and activity in neurons and identifying relevant praja2 substrates operating in vivo will contribute to our understanding of the role of cAMP and UPS in the regulation of brain development and plasticity and may well impact on therapeutic initiatives for neurodegenerative disorders.

Materials and Methods

Cell Lines. HEK293 and a neuroblastoma cell line (SHSY-5Y) were maintained in DMEM supplemented with 10% heat-inactivated FBS at 37 °C, 5% (vol/vol) CO_2, and 95% humidity. To induce differentiation, neuroblastoma cells were treated for 5 d with 10 μM of all-trans RA (Sigma) and then with BDNF (14, 27). Midbrain primary cultures were obtained from brains of 16-d-old Wistar rat embryos (28) (29). Primary dopaminergic neuron cultures were isolated from NOGO-A KO mouse brain (30).

siRNA Administration into the Rat Brain. Postnatal Wistar rats were studied in a period of partial immaturity of the mesostriatal dopamine system (31). Experiments were performed according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the University of Naples Federico II. Three-day-old Wistar rats were intracerebroventricularly injected with targeting naked siRNAs (2 μL, 0.5 μmol/L in cerbrospinal fluid with 0.05% trypan blue) or control naked siRNAs, according to previously published protocol for neonatal mice (32). siRNA injection was repeated at postnatal day 6 (P6), and animals were killed at P9. $n = 7$ for each experimental group.

Plasmids and Transfection. Flag-praja2rm was generated by site-directed mutagenesis, whereas praja2-deletion mutant was generated by PCR. NOGO-A ΔRHD was generated by PCR from GenScript. All of the RTN vectors, including NOGO-A, were previously described (11, 33).

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