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Neuronal Nogo-A regulates glutamate receptor subunit expression in hippocampal neurons

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Abstract

Nogo-A and its cognate receptor NogoR1 (NgR1) are both expressed in neurons. In order to explore the function of these proteins in neurons of the central nervous system, we carried out a series of studies using postnatal hippocampal neurons in culture. Interfering with the binding of Nogo-A to NgR1 either by adding truncated soluble fragment of NgR1 (NgSR) or by reducing NgR1 protein with a specific siRNA, resulted in a marked reduction in Nogo-A expression. Inhibition of Rho-ROCK or MEK-MAPK signaling resulted in a similar reduction in neuronal Nogo-A mRNA and protein. Reducing Nogo-A protein levels by siRNA resulted in an increase in the post-synaptic scaffolding protein PSD95, as well as increases in GluA1/GluA2 AMPA receptor and GluN1/GluN2A/GluN2B NMDA glutamate receptor subunits. siRNA treatment to reduce Nogo-A resulted in phosphorylation of mTOR; addition of rapamycin to block mTOR signaling prevented the up-regulation in glutamate receptor subunits. siRNA reduction of NgR1 resulted in increased expression of the same glutamate receptor subunits. Taken together the results suggest that transcription and translation of Nogo-A in hippocampal neurons is regulated by a signaling through NgR1, and that interactions between neuronal Nogo-A and NgR1 regulate glutamatergic transmission by altering NMDA and AMPA receptor levels through an rapamycin sensitive mTOR dependent translation mechanism.

Keywords

Nogo-A; Nogo receptor; synaptic plasticity

Introduction

Nogo-A was originally identified as a growth inhibitor. Myelin derived Nogo-A binds preferentially to neuronal NgR1, triggering growth cone collapse and inhibition of neurite growth (Chen et al. 2000; Fournier et al. 2001). In the setting of CNS injury, Nogo-A expressed in oligodendrocytes plays important roles in blocking regeneration of central axons (Liu et al. 2006; Cheatwood et al. 2008a; Gonzenbach and Schwab 2008; Nash et al. 2009). Subsequent studies revealed that Nogo-A is expressed also by neurons (Huber et al. 2002), and neuronal Nogo-A expression is increased after injury and in some disease states; NogoA in hippocampus is increased after deafferentation or kainate-induced seizures, in human brains with Alzheimer disease or temporal lobe epilepsy, in cortical neurons after focal ischemia and in DRG after axonal injury (Meier et al. 2003; Bandtlow et al. 2004; Gil

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et al. 2006; Cheatwood et al. 2008b; Peng et al. 2010). We have previously reported that exposure to myelin inhibitors *in vitro* results in an increase in Nogo-A expression in DRG neurons; a effect mediated by NgR1 (Peng et al. 2010). On the other hand, reducing neuronal Nogo-A enhances growth cone motility and neurite growth, while reducing axon branching (Craveiro et al. 2008; Montani et al. 2009; Peng et al. 2010; Petrinovic et al. 2010).

NgR1 is expressed at both pre-synaptic and post-synaptic sites (Wang et al. 2002; Barrette et al. 2007), and expression levels correlate with synaptic activity (Josephson et al. 2003). After spinal cord injury, enhanced synaptic plasticity in sensory cortex correlates with downregulation of NgR1 (Endo et al. 2007). In visual cortex, NgR1 signaling modulates experience-dependent plasticity and the period of ocular dominance plasticity is prolonged in NgR1 null mice (McGee et al. 2005). NgR1 modulates activity dependent synaptic strength and spine morphology and Nogo66 peptide applied to CA1 dendritic field suppresses LTP in hippocampal slices (Raiker et al. 2010). LTD is reduced and the LTP response enhanced in Schaffer collateral-CA1 of NgR1 null mice, while mice over-expressing NgR1 have impairment of long term memory (Lee et al. 2008; Karlen et al. 2009). Conversely, overexpression of Nogo-A (with Nogo-B) in cerebellar Purkinje cells results in synaptic destabilization of GABAergic terminals (Aloy et al. 2006).

These observations suggest that neuronal Nogo-A may play a role in regulating glutamatergic synapses. To investigate the role of neuronal Nogo-A interactions with NgR1 in the development of synapses we studied post-natal hippocampal neurons in culture. We found that Nogo-A transcription in neurons is under control of NgR1 signaling through Rho-ROCK and MAPK pathways, and reducing neuronal Nogo-A with siRNA promoted increases in NMDA and AMPA receptor subunit expression and dendritic PSD95 through an mTOR mediated and rapamycin sensitive pathway.

Materials and Methods

Tissue culture and in vitro experiments

The study was reviewed and approved by our institutional animal studies committee. Hippocampal neurons were isolated from P2 rats and cortical neurons were obtained from E17 rat pups of both sexes obtained from litters produced by timed pregnant Sprague Dawley female rats (Charles River). The cells were cultured in defined Neurobasal medium (Gibco) containing B27, Glutamax I, Albumax I, and penicillin/streptomycin. A solution of mitotic inhibitors fluoro-2-deoxyuridine (2.5 μg/ml) and uridine (2.7 μg/ml) (Sigma) was added to the cultures twice weekly. Hippocampal neurons were maintained in vitro for 15-19 days (DIV15-19) for these studies. ROCK inhibitor Y-27632 (Calbiochem) or the highly selective and potent MEK1/2 inhibitor UO126 (Promega) were added for 24h. U0126 was chosen because of its pharmacokinetic properties and minimal, if any, effect on other kinase pathways (Favata et al. 1998). Similarly Y-27632 has been shown to be a specific inhibitor of ROCKI/II with K_i more than 100 fold lower than those for PKA, PKC,MLCK, PAK and does not affect ERK or JNK activity at the concentrations employed in these studies (Uehata et al. 1997; Davies et al. 2000; Ishizaki et al. 2000; Narumiya et al. 2000). Cortical neurons DIV7 were infected for 2h with HSV-based vectors QHNgSR expressing the soluble fragment of NgR1 (aa 1-310; NgSR), or QHGFP expressing GFP at a multiplicity of infection of 1. Media from transfected cortical neurons, containing NgSR released from QHNgSR or from QHGFP control vector was applied to the hippocampal neurons for 24h (Peng et al. 2010).

siRNA Preparation and Transfection

ON-TARGET plus SMARTpool siRNA directed against Nogo-A and NgR1 (Dharmacon, Chicago, IL). The siRNA sequences used for Nogo-A were as follows: sequence 1, 5′-CCAAAUCACUUACGAAAGA-3′; sequence 2, 5 ′- UCUAGAAGUAUCCGACAAA-3′; sequence 3, 5 ′-GAAUGAAGCCACAGGUACA-3′; sequence 4, 5′-GAAUAAAGGACUCGGGGAA-′; The siRNA sequences used for NgR1 were as follows: sequence 1, 5′- GCCCACGGCACAUCAAUGA-3′; sequence 2, 5 ′-AGAAAGAACCGCACCCGUA-3′; sequence 3, 5 ′-CUGCAGAAGUUCCGAGGUU-3′; sequence 4, 5′-GGAAGUGGGAGCAGUGGAA-′; O N-TARGET plus siCONTROL nontargeting pool siRNA(Dharmacon) was used as control. For siRNA transfection, 2.5 μl of siRNA in 47.5 μl of antibiotic-free cultured medium and 2 μl of DharmaFECT siRNA transfection reagent 3 (Dharmacon) in 48 μl of cultured medium were incubated for 20 min at RT. Hippocampal neurons (DIV15) were treated with siRNA transfection solution for 72 h.

RT-PCR

RNA was isolated from hippocampal neurons using TRIzol (Invitrogen). cDNA was amplified using the following primer sets: β-actin forward, 5′-CAG TTC GCC ATG GAT GAC GAT ATC-3′, and β-actin reverse, 5′-CAC GCT CGG TCA GGA TCT TCA TG-3′; Nogo-A forward, 5′-GAG ACC CTT TTT GCT CTT CCT G-3′, and Nogo-A reverse, 5′-AAT GAT GGG CAA AGC TGT GCT G-3′. All reactions involved initial denaturation at 94 °C for 5 min followed by 24 cycles (94 °C for 30 s, 68° C for 2 min, and 1 cycle 68 °C for 8 min using a GeneAmp PCR 2700 (Applied Biosystems). No changes in β-actin mRNA levels were observed across different experimental groups and β-actin was used as internal control.

Western Blot

Hippocampal neurons were collected in the lysis buffer (50 mM Tris, 10 mM NaCl, 1% Nonidet P-40, 0.02% NaN_3, protease inhibitor, and phosphatase inhibitor mixtures) and Western blot performed as described (Peng et al. 2010). Primary antibodies included the following: anti-NogoR that recognizes both full-length and truncated soluble NgR1 (R&D Systems), anti-Nogo-A (Santa Cruz Biotechnology) anti-PSD95 (UC Davis/NIH NeuroMab), anti-NR1 (GluN1, Millipore), anti-NR2A (GluN2A, Millipore), anti-NR2B (GluN2B, UC Davis/NIH NeuroMab), anti-GluR1 (GluA1, Santa Cruz Biotechnology), anti-GluR2 (GluA2, UC Davis/NIH NeuroMab), anti-synapsin I (Millipore), anti-synaptophysin (1:1000, Millipore), anti-mTOR (Cell Signaling), anti-phospho-mTOR (Cell Signaling) followed by peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology) and amplified using SuperSignal chemiluminscence reagent (Pierce). The membranes were then stripped and reprobed with β-actin antibody (Santa Cruz Biotechnology). Quantitation of chemiluminescence emitted from each protein band was done using ChemiDoc (BioRad). No changes in β-actin levels in 20 μg of protein load were observed in cell homogenates across different treatment groups and β-actin was used as internal control.

Immunocytochemistry

Hippocampal neurons were fixed, blocked, and probed overnight with the following antibodies anti-Nogo-A (Santa Cruz Biotechnology), anti-NogoR (R&D), anti-NeuN (Millipore), anti-MAP2 (Millipore), anti-MAP2 (Sigma-Aldrich), anti-SMI31 (Covance), anti-synaptophysin (Millipore), anti-PSD95 (UC Davis/NIH NeuroMab), anti-GluR2 (GluA2, Santa Cruz), anti-NR2A (GluN2A, Millipore), vGlut1 (Santa Cruz Biotechnology), GFAP (Sigma) or MBP (Millipore). The secondary antibodies were tagged-Alexa Fluor 594 or 488 (Invitrogen). Dendrites from randomly selected hippocampal neurons treated with

Nogo-A siRNA or control siRNA were selected for quantitation of boutons. Three or four dendrites per each neuron were selected and measured in length starting at 10 μm distance from the neuronal cell body to the endpoint. Total dendrite length analyzed was 600 μm per treatment condition. The number of GluN2A and PSD95 immunofluorescent punctae along the dendrites stained with MAP2 antibody were counted, and the value represented as number of boutons per 10 μm of dendrite length per treatment condition.

Data Analysis

The statistical significance of the difference between treated and control groups was determined by independent sample *t* test except for data presented in Figure 4A a which was analyzed by one-way *ANOVA* using SPSS 12.0 for Windows (SPSS Inc.). Data are expressed as means ± S.E., with $p < 0.05$ considered significant. All the experiments were performed independently at least three times.

Results

Transcriptional control of Nogo-A by NgR1 in hippocampal neurons

To evaluate the regulation and function of neuronal Nogo-A in the absence of myelin inhibition we used primary postnatal hippocampal neurons and the cellular composition of the cultures was assess by immunostaining. Approximately 80% of the neurons as determined by the neuronal marker NeuN express the vesicular glutamate transporter 1 consistent with a glutamatergic phenotype (Fig. 1A). Astrocyte visualized by GFAP staining accounted for less than 8% of the cells (Fig. 1B). No oligodendrocytes were detected using antibodies to MBP or MAG. Hippocampal neurons (DIV15-17) have committed dendritic-axonal differentiation and elaborate extensive processes that form synaptic contacts as indicated by punctuate distribution of synaptophysin on dendrites (Fig. 1C). Nogo-A and NgR1 were both detected in neuronal cell bodies and dendrites indicated by co-localization with MAP-2 immunostaining, extending the length of the dendrites (Fig. 2A). Nogo-A and NgR1 were also found in axons as determined by co-localization with SMI31, a marker of phosphorylated neurofilaments and tau (Fig. 2B). Staining for NogoA along axons appeared more intense that for NgR1 but accurate quantitation cannot be made because of differences in antibody source and binding affinities, and because variation in staining intensity between different fibers, seen also with SMI31 antibody, may represent the number of axons forming the bundle. There was no detectable Nogo-A in astrocytes and there were no oligodendroglial cells in these hippocampal cultures (data not shown).

We exposed hippocampal neurons to Nogo soluble receptor (NgSR) that recognizes the Nogo66 domain of Nogo-A, produced by release from HSV vector (QHNgSR) transfection of cortical neurons (Peng et al. 2010). NgSR treatment resulted in a significant downregulation of Nogo-A mRNA and protein (Fig. 3A,B). In order to determine if the effect of NgSR was due to capping of Nogo-A and internalization or by signal derived from receptor occupancy we used an RNAi approach. Treatment of hippocampal neurons with an NgR1-specific siRNA resulted in a 70% reduction in NgR1 protein as compared to neurons transfected with control siRNA (Fig. 4B). Reduction of NgR1 was accompanied by a marked decrease in Nogo-A mRNA and protein (Fig. 4A,B). Taken together, these experiments indicate that expression of Nogo-A in hippocampal neurons is regulated, in part, by signals that derived from interactions of neuronal-expressed Nogo-A with NgR1.

Rho-ROCK and MAPK pathways converge in the regulation of neuronal Nogo-A expression

NgR1 signals through Rho-GTPase and Rho activated kinase (ROCK) to regulate actin polymerization, microtubule stabilization, and changes in transcriptional activity (Arimura et

al. 2000; Fournier et al. 2001; Peng et al. 2010). To assess whether a similar signaling cascade might be involved in NgR1 regulation of Nogo-A expression in hippocampal neurons, we added the ROCK inhibitor Y-27632 for 24h. ROCK inhibition resulted in a marked decrease in Nogo-A levels (Fig. 5A). ROCK inhibition also significantly lowered the basal activity of ERK1/2 in the treated neurons (Fig. 5B), suggesting that the MAPK pathway lies downstream of ROCK. Expression of Nogo-A mRNA and protein was also decreased by treatment of the hippocampal neurons with the MEK inhibitor UO126 for 24h (Fig. 6A,B) that inhibited phosphorylation of ERK (Fig. 6C) indicating that Rho-ROCK and MAPK signaling pathways converge to regulate neuronal Nogo-A expression.

Regulation of NMDA and AMPA receptors by Nogo-A signaling

In order to evaluate the possibility that Nogo-A-NgR1 interactions known to alter synaptic plasticity may regulate glutamate receptor expression levels in differentiated postnatal hippocampal neurons, we designed an siRNA specific for Nogo-A and evaluated synaptic markers and glutamate receptor subunit proteins. Addition of Nogo-A siRNA to hippocampal neurons decreased Nogo-A protein without any change in the level of NgR1 (Fig. 7A), and resulted in increases in NMDA receptor GluN1, GluN2A and GluN2B subunit levels (Fig. 7B). The postsynaptic scaffolding protein PSD95, was increased in the Nogo-A siRNA-treated neurons (Fig. 7B). The ratio of GluN2A/GluN2B was not altered by knockdown of Nogo-A expression. Decreasing levels of Nogo-A by siRNA treatment of hippocampal neurons also resulted in an increase in AMPA receptor subunits GluA1 and GluA2 (Fig. 7B). By immunocytochemistry, representative subunits of AMPA and NMDA receptors as well as PSD95 showed punctuate staining along MAP2-positive dendrites (Fig. 7C-D) consistent with localization at synapses. Although we did not directly assess the relative levels of each subunit in different cellular compartments after siRNA treatment, the effect appeared to be most likely post-synaptic as knockdown of Nogo-A in hippocampal neurons by siRNA treatment did not result in any change in the amount of the SNARE proteins synapsin I and synaptophysin or the level of the vesicular glutamate transporter isoforms vGlut1 and vGlut2 (data not shown). This was supported by quantitative analysis of the number of GluN2A and PSD95 synaptic boutons on MAP2 stained dendrites that were significantly increased in Nogo-A siRNA treated neurons as compared to control siRNA (GluN2A per 10 μm = 3.12±0.13 in NogoAsiRNA and 2.63±0.15 in csiRNA *$P < 0.05$; PSD95 per 10 μm = 3.25±0.14 in NogoAsiRNA and 2.46±0.05 in csiRNA *$P < 0.05$).

Rapamycin sensitive mTOR pathway in the regulation of NMDA/AMPA by Nogo-A

mTOR and its downstream effectors eIF-4E-BP and eIF-4E, are localized in dendritic spines of hippocampal neurons, and play important roles in the development of late LTP (Vickers et al. 2005; Bekinschtein et al. 2007; Bramham and Wells 2007). mTOR activity measured by the amount of phospho-mTOR compared to total mTOR, was increased in hippocampal neuron cultures in which Nogo-A levels have been reduced by siRNA (Fig. 7E). Treatment of the neurons for 24h with rapamycin blocked mTOR phosphorylation (Fig. 8A) and prevented the increase in NMDA and AMPA subunits and PSD95 protein levels resulting from knocking down Nogo-A by siRNA (Fig. 8B). Rapamycin treatment did not significantly change the levels of NMDA or AMPA receptor subunits in neurons treated with control siRNA (Fig. 8C). The results suggest that neuronal Nogo-A, regulation of the activity state of the rapamycin sensitive mTOR signaling pathway is important for the translation and/or maintenance of glutamate receptor protein subunits in hippocampal neurons.

Is regulation of NMDA and AMPA receptors by Nogo-A dependent on NgR1?

NgR1 has been implicated in synaptic plasticity using transgenic models of NgR1 deletion and over-expression. In order to address if the regulation of NMDA and AMPA receptor

subunit expression by Nogo-A was mediated through NgR1 signaling we used siRNA to decrease NgR1 levels in hippocampal neurons. We found an increase in NMDA and AMPA receptor subunits and PSD95 protein in neurons treated with NgR1 siRNA that was similar to the changes caused by Nogo-A knock down, but not in neurons treated with a scrambled sequence control siRNA (Fig. 9). As the down regulation of NgR1 was accompanied by simultaneous decrease in Nogo-A levels (Fig. 4) the effects of NgR1 could not be fully dissociated from Nogo-A, but taken together these results suggest that decreased Nogo-A-NgR1 interaction as a result of reductions in either Nogo-A or NgR1 would favor enhanced glutamatergic transmission.

Discussion

The studies reported demonstrate four principal findings: 1) Nogo-A expression in neurons is under transcriptional control of NgR1; 2) Nogo-A expression is regulated through Rho-ROCK and MEK-MAPK pathways; 3) neuronal Nogo-A regulates NMDA and AMPA glutamate receptor subunit expression; 4) Nogo-A regulation of NMDA and AMPA receptor subunits expression is effected through the rapamycin-sensitive mTOR pathway.

The assembly and maintenance of neuronal networks involves neurite growth, synapse formation and plasticity. Recent studies suggest that Nogo-A interaction with NgR1 plays an important role in many of these cellular processes. In postnatal hippocampal neurons *in vitro*, at a time when these cells have developed a large and distinct axonal and dendritic arborization with extensive axo-dendritic synaptic contacts we found both Nogo-A and NgR1 in axons and dendrites, and that disengagement of NgR1 from Nogo-A, by either NgSR interference or by siRNA knockdown of NgR1, resulted in a reduction of Nogo-A mRNA and protein.

These results suggest the existence of a feedback loop in which interaction of NgR1 with one of its ligands regulates the level of Nogo-A. Ligand binding to NgR1 activates Rho-GTPases and downstream Rho-dependent kinase to mediate cellular responses responsible for growth cone collapse and axon growth inhibition. In hippocampal neurons inhibition of ROCK activity diminished Nogo-A expression and reduced the basal state of ERK phosphorylation and inhibition of the MEK-MAPK signaling also resulted in a decrease in Nogo-A expression. These results suggest that, in neurons, ROCK and ERK pathways converge to regulate Nogo-A expression. Rho-ROCK signaling regulates transcription through the binding of serum response factor (SRF) on serum response element (SRE). The SRF-SRE cassette utilizes as binding partners the transcription factors ternary complex factors (TCF), downstream of ERK, and the actin sensors MKL, downstream from RhoA to regulate early and late gene transcription necessary for essential cellular responses including neurite growth, axonal regeneration and synapse formation (Knoll and Nordheim 2009). The regulation of Nogo-A by Rho-ROCK and MEK-MAPK pathways is consistent with the convergence of these signaling pathways on SRE. The observed regulation of Nogo-A by pathways downstream of NgR1 does not exclude the possibility of similar or other effects that may be triggered by other myelin inhibitors (OMgp, MAG) through binding to NgR1.

In the hippocampal neuron culture system, downregulation of Nogo-A resulted in increased expression of NMDA (GluN1, GluN2A and GluN2B) and AMPA (GluA1 and GluA2) receptor subunits and the postsynaptic anchoring protein PSD95. The effects of Nogo-A downregulation appear to be postsynaptic, as there were no changes in presynaptic proteins (synapsin, synaptophysin vGlut1 or vGlut2), and there were no significant changes in neurite growth (Peng et al. 2010). These effects were mediated by mTOR signaling, as blocking the mTOR pathway with rapamycin prevented the effects of knocking down Nogo-A on the levels of NMDA and AMPA receptors. mTOR is found in dendritic spines where it

regulates the initiation of translation from local polyribosomal mRNAs to produce changes in synaptic function (Bourne et al. 2007) and where mTOR mediates translation of NMDA and AMPA receptors (Slipczuk et al. 2009). In dendritic spines, changes in synapse structure and function that occur dependent or independent of electrical activity, may persist for hours or days (Saneyoshi et al. 2010) These results suggest that enhanced mTOR activity by reduced Nogo-A signaling results in an increase in rapamycin sensitive translation of NMDA and AMPA receptor subunits.

Dendritic spines are highly motile structures (Fischer et al. 2000; Yuste and Bonhoeffer 2001) and changes in spine morphology and development of LTP/LTD reflects multiple processes including protein synthesis from local mRNAs in dendrites, trafficking of synaptic proteins and incorporation of new neurotransmitter receptor subunits (Malenka and Bear 2004; Lin et al. 2005). As dendritic spines undergo actin dependent shape changes regulated by glutamate neurotransmission, a two-step process has been proposed in which synaptic spines are formed in response to NMDAR activation and spines are subsequently stabilized by AMPAR mediated transmission (Fischer et al. 2000); the enhanced translation of NMDA and AMPA demonstrated by suppression Nogo-A-NgR1 signaling could contribute to changes in spine morphology. Considered in light of studies demonstrating that Nogo-A is involved in altering conformation of membrane structures (Voeltz et al. 2006), the observations that NgR1 knock-out mice display abnormal spines in apical dendrites of hippocampal CA1 neurons (Lee et al. 2008) and that neutralization of Nogo-A function alters dendritic and spine morphology in hippocampal pyramidal neurons (Zagrebelsky et al.), our results suggest that neuronal Nogo-A-NgR1 signaling may be involved in the refinement of glutamatergic connections in hippocampal neurons. These data are complementary to published observations that describe modulatory effects of Nogo-A and NgR1 on synaptic plasticity: the observation of NgR1 dependent Nogo66 suppression of NMDA mediated LTP (Raiker et al. 2010) and with the recent report that interfering with NgR1 or Nogo-A by function blocking antibodies increases LTP without affecting basal synaptic transmission, LTD or other measures of presynaptic function (Delekate et al. 2011).

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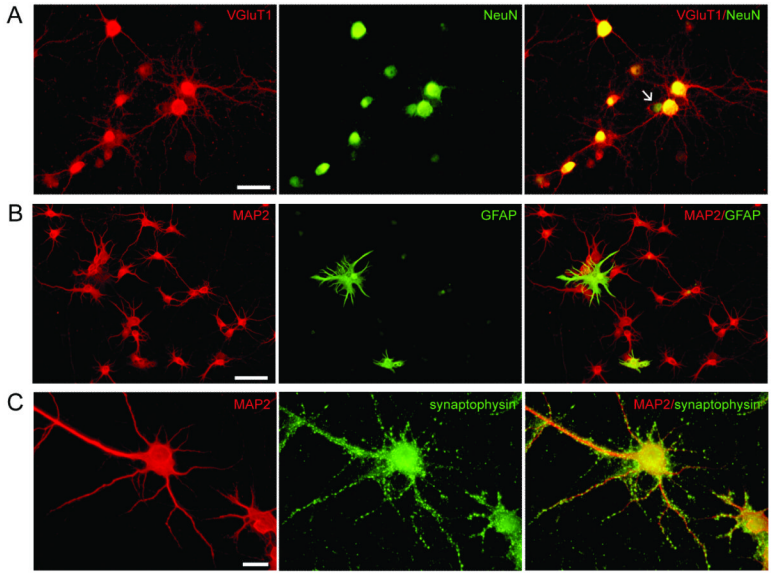

Figure 1. Cellular composition of hippocampal cultures

P2 hippocampal neurons cultured in the presence of mitotic inhibitors as described in Methods. A. Vesicular glutamate transporter 1 (red) protein expression was seen in 80% of neurons stained with NeuN antibody (green). Arrow shows neuron without vGlut1 staining in the field. Scale bar = 50 μm. B. Less than 10% of all cells were astrocytes characterized by GFAP staining (green) and MAP2 (red). Scale bar = 100 μm. C. Hippocampal neurons (DIV15) have a mature phenotype with axon dendritic differentiation and widespread synaptic contacts. Immunostaining with MAP2 (red) and synaptophysin (green) as indicated. Scale bar = 20 μm.

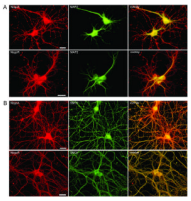

Figure 2. Localization of Nogo-A and NgR1 in hippocampal neurons

Hippocampal neurons (DIV15) show expression of Nogo-A and NgR1 in dendrites and axons. A. Nogo-A and NgR1 immunostaining in cell body and dendrites but also extending beyond the limits of MAP2 staining. Nogo-A (red) with MAP2 (green) and NgR (red) with MAP2 (green). Scale bar=20 μm. B. Nogo-A and NgR (red) are seen in most axons stained with SMI31 (red). Scale bar = 20 μm.

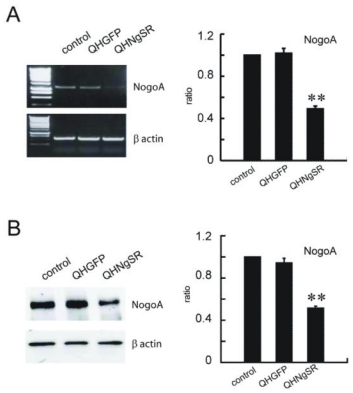

Figure 3. Soluble NgR1 reduces Nogo-A expression

Hippocampal neurons treated with or without NgSR released from QHNgSR for 24h. NgSR decreases Nogo-A mRNA (A) and protein (B) as compared to control (QHGFP). Each sample was calculated as a ratio to β-actin and presented as percentage of control. ** $p < 0.01$.

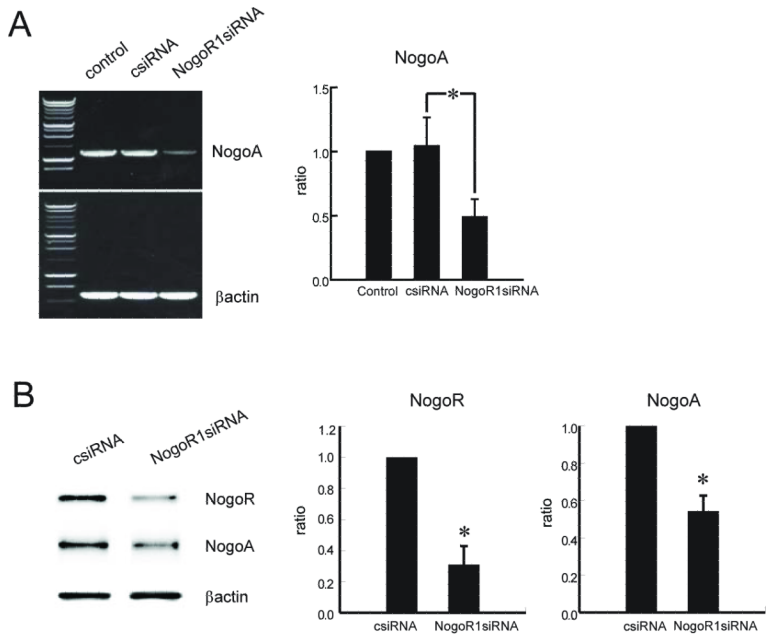

Figure 4. NgR1 knock-down causes a reduction of Nogo-A

Hippocampal neurons treated with control scrambled siRNA (csiRNA) or NgR1 siRNA for 72h. NgR siRNA decreases NgR1 protein (B). Nogo-A mRNA (A) and protein (B) were decreased in NgR1 siRNA as compared to csiRNA. Each sample was calculated as a ratio to β-actin and presented as percentage of control. * $p < 0.05$.

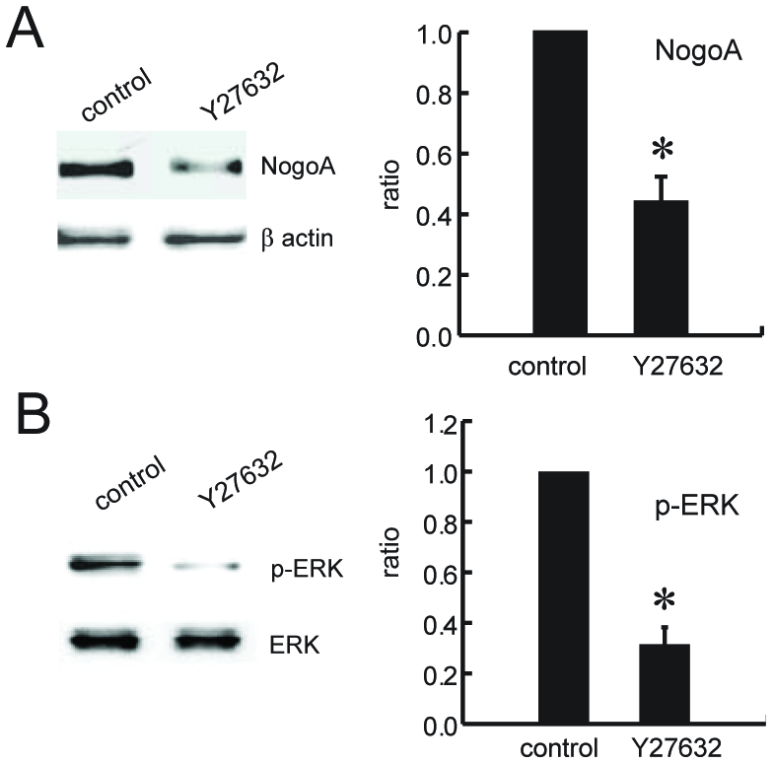

Figure 5. Inhibition of Rho dependent kinase decreases Nogo-A levels and inhibits MAPK

Addition of 10 μM Y27632 for 24h decreases Nogo-A (A) and pERK level (B). Each sample was calculated as a ratio to β-actin or total ERK and presented as percentage of control. * $p < 0.05$.

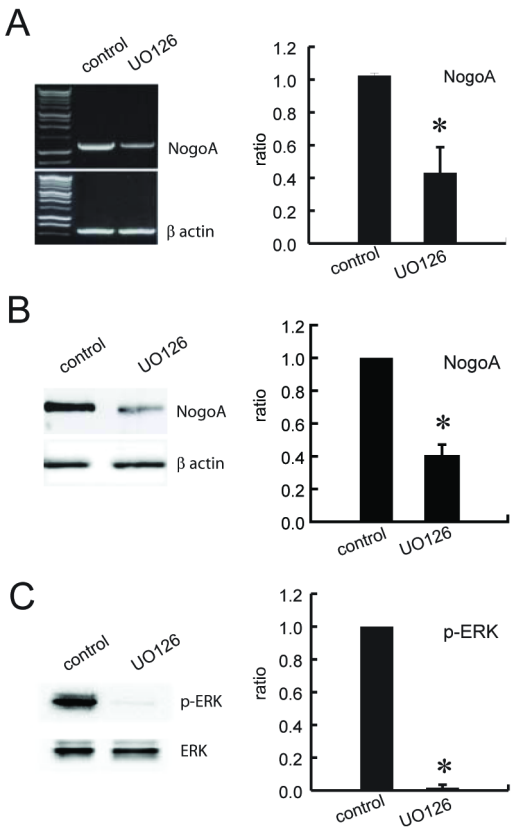

Figure 6. Inhibition of MEK-MAPK reduces Nogo-A expression

Addition of 10 μM UO126 for 24h reduces Nogo-A mRNA (A) and protein (B). U0126 prevented the phosphorylation of ERK (C). Each sample was calculated as a ratio to β-actin or total ERK and presented as percentage of control. * $p < 0.05$.

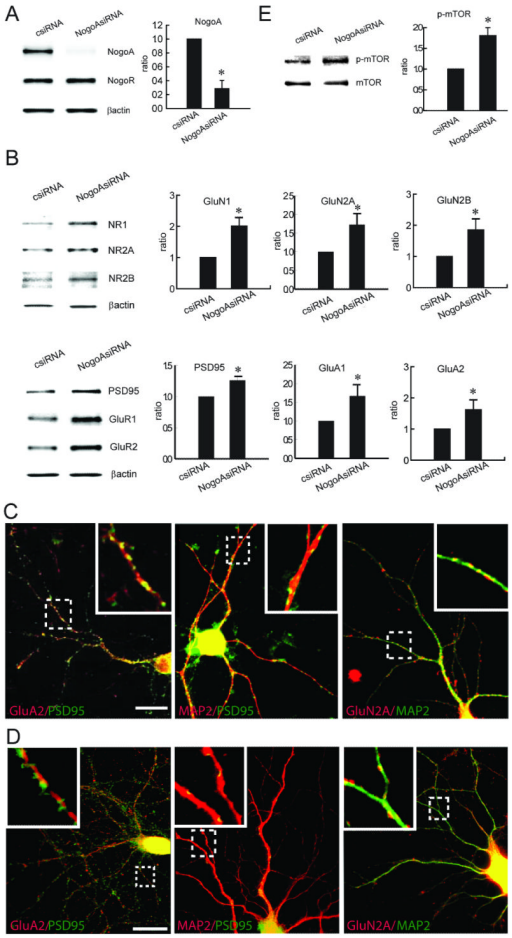

Figure 7. Down-regulation of Nogo-A increases NMDA and AMPA receptor subunits and PSD95. Phosphorylation of mTOR

Hippocampal neurons (DIV15) treated with csiRNA or Nogo-A siRNA. A. Nogo-A siRNA significantly decreased Nogo-A without altering NgR1 levels. B. Nogo-A siRNA caused increase in GluN1, GluN2A and GluN2B, PSD95, GluA1 and GluA2. C. Neurons treated with Nogo-A siRNA show dendritic synaptic immunolocalization of GluA2 (red) with PSD95 (green), of PSD95 (green) with MAP2 (red) and GluN2A (red) with MAP2 (green). D. Similar neurons treated with csiRNA and stained as in C. Scale bar = 30 μm. E. Nogo-A siRNA caused increase in p-mTOR as compared to csiRNA. Each sample was calculated as a ratio to β-actin or total mTOR and presented as percentage of control. * $p < 0.05$

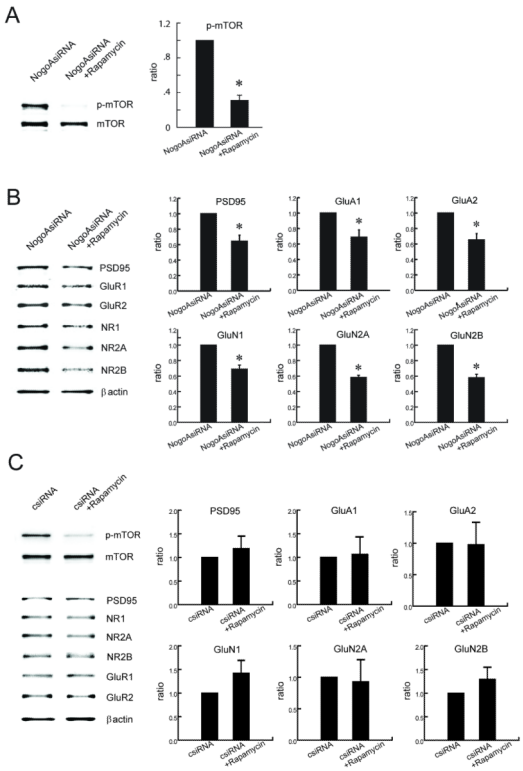

Figure 8. Reversal of NMDA, AMPA subunits and PSD95 expression by rapamycin in Nogo-A knock-down

Addition of 100 nM rapamycin to hippocampal neurons transfected with Nogo-A siRNA prevented the phosphorylation of mTOR (A) and resulted in decreased GluN1, GluN2A, GluN2B, PSD95, GluA1, and GluA2 (B). Addition of 100 nM rapamycin transfected with csiRNA lowered p-mTOR below basal level but did not significantly change NMDA and AMPA subunits or PSD95 levels (C). Each sample was calculated as a ratio to β-actin or total mTOR and presented as percentage of control. * $p < 0.05$.

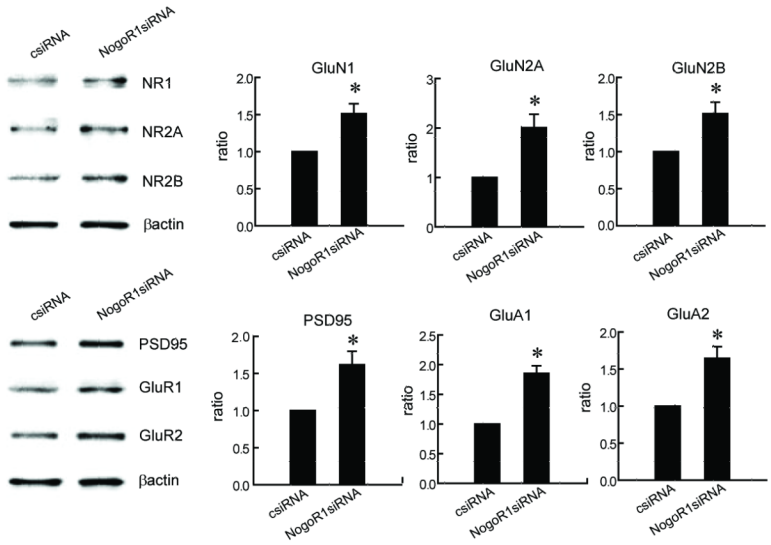

Figure 9. Down-regulation of NgR1 by siRNA increases NMDA and AMPA receptor subunits and PSD95

P2 hippocampal neurons cultured for 2 weeks and treated with csiRNA or NgR1 siRNA for 72h. NgR1 siRNA treatment results in increased GluN1, GluN2A, GluN2B, PSD95, GluA1 and GluA2. Each sample was calculated as a ratio to β-actin and presented as percentage of control. * $p < 0.05$.