Segregation of Nogo66 receptors into lipid rafts in rat brain and inhibition of Nogo66 signaling by cholesterol depletion

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Abstract NogoA, a myelin-associated component, inhibits neurite outgrowth. Nogo66, a portion of NogoA, binds to Nogo66 receptor (NgR) and induces the inhibitory signaling. LINGO-1 and p75 neurotrophin receptor (p75), the low-affinity nerve growth factor receptor, are also required for NogoA signaling. However, signaling mechanisms downstream to Nogo receptor remain poorly understood. Here, we observed that NgR and p75 were colocalized in low-density membrane raft fractions derived from forebrains and cerebella as well as from cerebellar granule cells. NgR interacted with p75 in lipid rafts. In addition, disruption of lipid rafts by β-methylcyclodextrin, a cholesterol-binding reagent, reduced the Nogo66 signaling. Our results suggest an important role of lipid rafts in facilitating the interaction between NgRs and provide insight into mechanisms underlying the inhibition of neurite outgrowth by NogoA.
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Keywords: Lipid raft; Nogo66; Nogo66 receptor; p75; Signal transduction; Cholesterol depletion

1. Introduction

Axon regeneration in the adult mammalian central nervous system (CNS) is limited after injury due partially to the presence of inhibitory myelin-associated components [1,2]. Numerous reports have implicated that an interaction of NogoA on the oligodendrocyte surface with Nogo66 receptor (NgR) on axons plays a key role in this process [3–6]. The extracellular 66-residue segment of NogoA appears to possess the ability to inhibit neurite growth in vitro and it is this portion of Nogo that binds NgR [3,7–10]. NgR is a 473-residue, glycosylphosphatidylinositol (GPI)-anchored membrane protein and acts as a convergent receptor of three known myelin-associated inhibitors: NogoA [3], myelin-associated glycoprotein (MAG) [11,12] and oligodendrocyte-myelinglycoprotein (OMgp) [13,14]. The signal transduction of NgR has been suggested to depend on the association with LINGO-1 and p75 neurotrophin receptor (p75), the low-affinity nerve growth factor (NGF) receptor, which may convey a signal into the cell through Rho family GTPases and consequently promote growth cone collapse and inhibit neurite extension [15–18].

Proteins and lipids in cell membrane form spatially differentiated microdomains. This lateral heterogeneity of the cell membrane presumably results from preferential packing of cholesterol and sphingolipids into platforms called "rafts," onto which specific proteins attach on both sides of the lipid bilayer. The unique lipid composition of rafts make them resistant to non-ionic detergent extraction using Triton X-100 and can be isolated from non-raft domains [19–23]. Disruption of the liquid ordered phase, by removal of cholesterol with β-methylcyclodextrin (β-MCD) which selectively and rapidly extract cholesterol from the plasma membrane [24–26], leads to increased solubility of raft-associated proteins in Triton X-100 [27]. Emerging evidence indicates that rafts serve as platforms to concentrate signaling components and other molecules [20,28]. They are implicated in various cellular functions, including neuronal differentiation and survival [29], neuritogenesis [30], neuronal cell adhesion, axon guidance [31,32] as well as synaptic transmission [33].

Previous reports have demonstrated that HSVWTNgR transfected in HEK293T cells and NgR, NgRH1 as well as NgRH2 transfected in CHO-K1 cells localize primarily to lipid rafts [34,35]. In addition, NgR and p75 localize primarily to lipid rafts in cerebellar granule cells [36,37]. However, it is unclear whether NgR is colocalized with p75 and RhoA in vivo and whether lipid rafts play a role in Nogo66 signaling in primary neurons. Here, we report that NgR, p75 and RhoA are associated with lipid rafts in vivo. Disruption of lipid rafts by depletion of cholesterol blocks the Nogo66 signal transduction.

2. Materials and methods

2.1. Reagents and antibodies

β-Methylcyclodextrin (M1356) was purchased from Sigma Chemical Co. (St. Louis, MO). Optiprep was obtained as a 60% (wt/vol) stock in water from AXIS-SHIELD PoC AS (Oslo, Norway). Rabbit polyclonal antibodies against human NgR (NGR11-A) were from Alpha Diagnostic, International (San Antonio, TX). Rabbit polyclonal

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Abbreviations: NgR, Nogo66 receptor; GPI, glycosylphosphatidylinositol; p75, p75 neurotrophin receptor; GST, glutathione *S*-transferase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TfR, transferrin receptor; β-MCD, β-methylcyclodextrin; CNS, central nervous system

antibodies against human p75 (G3231) were from Promega (Madison,WI). Mouse monoclonal antibodies against mouse flotillin-1 (610820) were from BD Transduction Laboratories. Mouse monoclonal antibodies against human PSD-95 (05-427) were from Upstate Biotechnology. Rabbit polyclonal antibodies against transferrin receptor (TfR, sc-9099) and mouse monoclonal antibodies against human RhoA (sc-418) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Lipid rafts preparation

All experimental procedures were under the approval of the Animal Experiment Committee of Chinese Academy of Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used. Lipid rafts were prepared as described previously [38,39]. For in vivo samples, ~3 g of forebrains and cerebella from postnatal days 8 (P8) or adult rats (Sprague–Dawley) were homogenized in 3 ml of buffer A containing 20 mM Tris/Cl (pH7.4), 50 mM NaCl, 250 mM sucrose, 1 mM DTT, 0.5 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml pepstain A, and 1 µg/ml leupeptin, respectively. After passing through a 22G needle three times, homogenates were spun at 960×g at 4 °C for 10 min. The supernatant was collected and mixed with OptiPrep (final concentration of 35%, 4.8 ml) and placed at the bottom of SW41 centrifugation tubes. The sample was overlaid with three layers of OptiPrep (30%, 20%, and 5% in buffer A, 2.5 ml each) and subjected to centrifugation at 200 000 ×g at 4 °C for 3 h. The fraction (900 µl aliquot each) in the 5–20% interface was collected and incubated with 600 µl of buffer A, containing 0.1% Triton X-100 (final concentration) at 4 °C for 20 min. The solubilized preparation was mixed with OptiPrep (final concentration of 35%, 3.6 ml) and placed at the bottom of SW41 tubes. The sample was overlaid sequentially with 7.5 ml of 30% OptiPrep in buffer A containing 0.1% Triton X-100 and another layer of buffer A containing 0.1% Triton X-100 (1.5 ml). The gradient was centrifuged at 200 000 ×g at 4 °C for 4 h and eight fractions (1.5 ml each) were collected from the top.

For in vitro samples, cerebellar granule cells from postnatal day 7 (P7) rats were cultured in chemically defined Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) on poly-D-lysine (Sigma) for 22–24 h. For some experiments, cells were treated with 10 mM β-MCD for 10 min before collection. Cell membrane was col-

lected and resuspended in 450 µl buffer B containing 50 mM Tris/Cl (pH7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 0.5 mM sodium orthovanadate, 1 µg/ml pepstatin A, 10 µg/ml aprotinin, and 1 µg/ml leupeptin. After homogenized by passing them through a 22G needle three times, the homogenates were mixed with OptiPrep (final concentration 35%, 1.08 ml), loaded at the bottom of an SW41 centrifuge tubes and overlaid with 10.5 ml of 30% OptiPrep in buffer B and 0.9 ml of buffer B. The final concentration of Triton X-100 of each layer was adjusted to 0.1%. The sample was centrifuged at 200 000×g at 4 °C for 4 h. Six fractions (2 ml each) were collected from the top. For some experiments, cell pellet was lysed in 800 µl buffer C containing 1% Triton X-100, 10% glycerol, 20 mM HEPES (pH 7.2), 100 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstain A. The lysates were centrifuged at 16 000×g at 4 °C for 15 min. The supernatant was designated as the Triton-soluble fraction (S). The pellet was washed with phosphate buffered saline (PBS), resuspended, and dissolved by sonication in 200 µl buffer C containing 0.5% sodium dodecyl sulfate (SDS). The supernatant was subsequently collected by centrifugation at 16 000×g at 4 °C for 10 min and designated as the Triton-insoluble fraction (IS).

2.3. Immunoprecipitation and immunoblotting

Triton-soluble and Triton-insoluble fractions (~300 µg of protein) were incubated directly with preimmuned IgG or indicated antibodies in RIPA lysis buffer (PBS buffer, pH 7.4, containing 1% NP-40, 0.1% SDS, 1% sodium deoxycholate, 10 mM EDTA, 10 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 mM sodium orthovanadate, and 1 mM PMSF) at 4 °C overnight on a rotating platform. They were then incubated with protein-A-agrose beads at 4 °C for 2 h on a rotating platform. Following centrifugation, beads were washed 4–5 times with RIPA lysis buffer. Bound proteins were eluted with 2× SDS-sample buffer at 95 °C. Protein samples from OptiPrep gradient fractions and immunoprecipitation were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto PVDF membranes, which were incubated in 5% non-fat dried milk dissolved in Tris/Cl buffered saline with 0.2% Tween (TBST) at room temperature for 1 h. Incubations with primary antibodies were carried out overnight at 4 °C. The membranes were then incubated with the respective peroxidase-conjugated secondary antibody at room temperature for 1 h. Immunoreactive bands
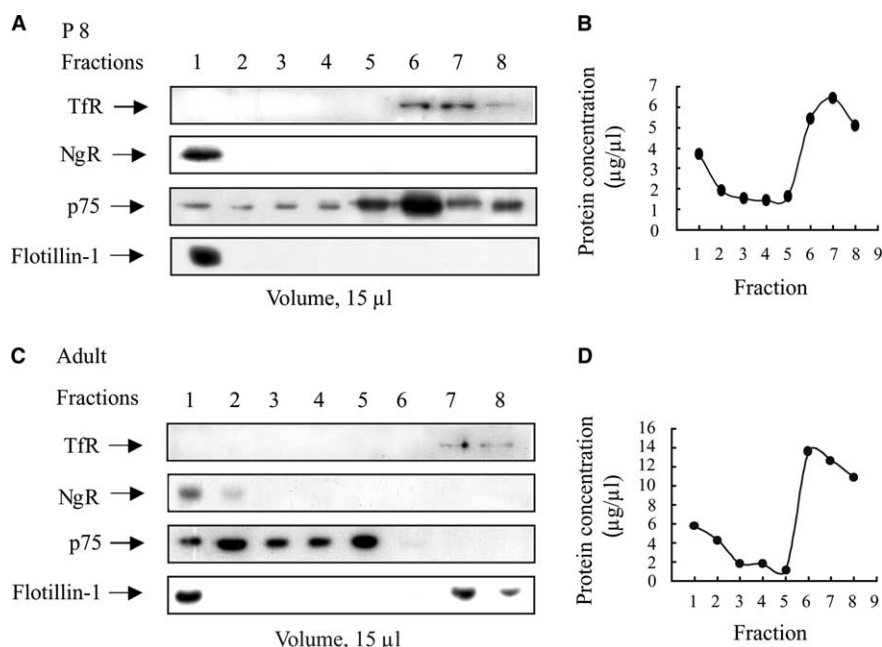

Fig. 1. NgR and p75 are associated with lipid rafts of the brain. (A, C) OptiPrep density gradient fractions were prepared from membranes of P8 and adult rat brains. Equal aliquots (15 µl) of the eight fractions were resolved on SDS–PAGE and immunoblotted with the indicated antibodies. Flotillin-1 (fractions 1–2) and TfR (fractions 6–8) were used as loading controls as raft and non-raft markers, respectively. Representative blots of three independent experiments with similar results are shown. (B, D) Protein concentrations in fractions in A and C.

were visualized using enhanced chemiluminescence substrate (Pierce, Rockford, IL). Intensities of the detected bands were quantified from scanned immunoblots using Metamorph software (Universal Imaging Corporation, West Chester, PA).

2.4. RhoA activity assays

Glutathione S-transferase (GST) and GST–Nogo66 (aa1026–1091) were prepared as described previously [7,40]. Measurement of RhoA activities was performed as described in the previous reports [41,42]. Cerebellar granule cells from P7 rat were grown (2×10^7 cells) on poly-D-lysine substrate for 24 h. Cells were treated with GST (6 μg/ml) or GST–Nogo66 (6 μg/ml) for 5 min. For some experiments, cells were treated with 5 mM β-MCD for 45 min before stimulation with GST or GST–Nogo66. 10% of the total volume of the cell lysate was used for assessment of total RhoA content. The remaining lysate was diluted with appropriate binding buffer (25 mM HEPES, pH7.5, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstain A) containing 20 μg of GST-Rho-binding domain of mouse rhotekin (GST-RBD) coupled to glutathione beads for GTP-bound RhoA and incubated at 4 °C for 2 h. Beads were then washed five times with binding buffer and bound proteins were eluted with 2× SDS-sample buffer and detected by immunoblotting using a mouse monoclonal anti-human RhoA antibody.

3. Results

3.1. NgR and p75 were present in the lipid raft fractions of the brain

Insolubility in cold non-ionic detergents and flotation on sucrose density gradients or Optiprep density gradients are the well-established criteria for identification of lipid raft-associated proteins [38,39,43]. Using these criteria, we tested whether NgR and p75 were localized to lipid rafts in vivo. Cell membrane mixtures from P8 and adult rat forebrains and cerebella were isolated, respectively, from which lipid raft fractions were prepared. Segregation of lipid raft from non-lipid raft regions was confirmed by separated distribution of lipid raft-specific and non-lipid raft-specific markers in the gradient. Flotillin-1, a lipid raft specific marker [44], mostly floated to low density fractions, whereas TfR, a non-lipid raft marker [45], stayed in high density fractions (Fig. 1A and C). In P8 brains, when equal volumes (15 μl) of different fractions were analyzed, NgR was detected exclusively in the raft associated fractions (Fig. 1A, panel 2), whereas p75 was detected in all the fractions, with a low level of p75 in the raft associated fractions and high amounts of p75 in the Triton X-100-soluble bottom fractions (Fig. 1A, panel 3). Interestingly, in adult rat brains, while NgR was still detected exclusively in the raft-associated fractions (Fig. 1C, panel 2), p75 was not detected in the soluble bottom fractions (Fig. 1C, panel 3). These results indicated that localization of p75 into lipid rafts is possibly correlated with developmental stages. Quantitative analysis showed that the majority of the proteins were present in the lipid raft and soluble fractions (Fig. 1B and D).

3.2. NgR interacted with p75 in detergent-insoluble fraction of neurons

P75 is the coreceptor of NgR and interacts with NgR on the cell surface of cerebellar granule cells and in cerebellum [15,16]. To investigate whether NgR interacts with p75 in detergent-insoluble fraction, we isolated Triton-soluble (S) and Triton-insoluble fractions (IS) from cultured cerebellar granule cells. Consistent with the above results, NgR proteins were detected exclusively in the Triton-insoluble fraction, whereas p75 was
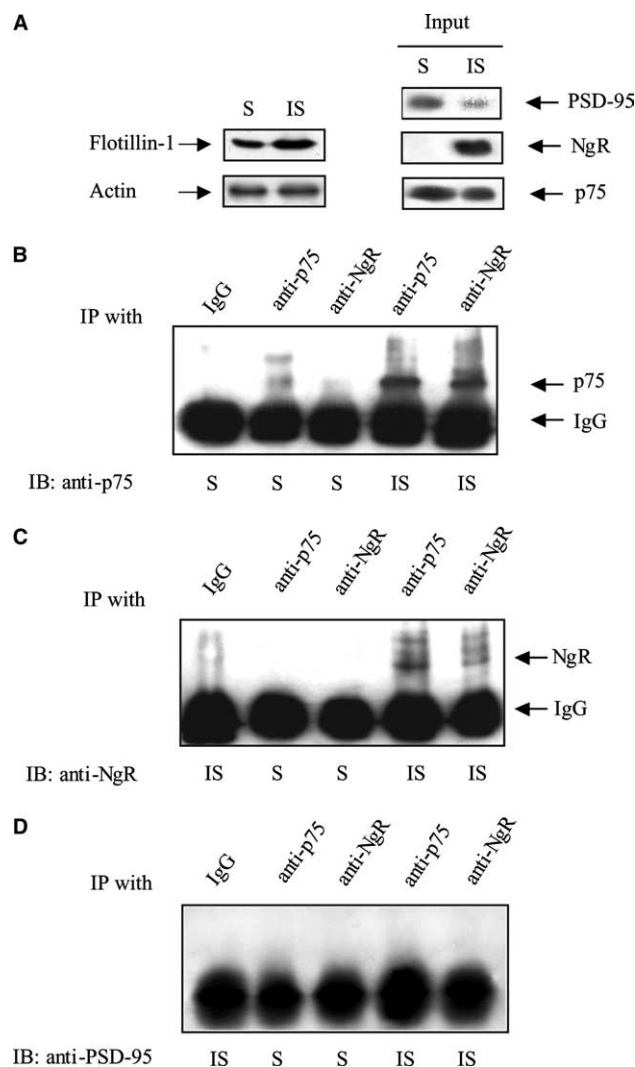

Fig. 2. Interaction of NgR with p75 in the detergent-insoluble fraction of cerebellar granule cells. Triton-soluble (S) and Triton-insoluble fractions (IS) were isolated from cultured cerebellar granule cells. (A) Equal amounts (20 μg) of the fractions were resolved on SDS–PAGE and immunoblotted with the indicated antibodies. (B, C, D) Immunoprecipitation of Triton-soluble and Triton-insoluble fractions (~300 μg of protein) were carried out and then immnoblotted with the indicated antibodies, respectively. Preimmuned IgG was used as a negative control. The inputs of proteins used in B–D are shown in A. Representative blots of two independent experiments with similar results are shown.

found in both the Triton-soluble and Triton-insoluble fractions. PSD-95, another raft-associated protein, was also found in both the Triton-soluble and Triton-insoluble fractions (Fig. 2A, right). Flotillin-1 was detected in the two fractions, with a majority of it in the Triton-insoluble fraction (Fig. 2A, left). Based on these results, the Triton-soluble and Triton-insoluble fractions were then processed for immunoprecipitation, respectively. The immunoprecipitation results showed an interaction between p75 and NgR in the Triton-insoluble fraction (Fig. 2B and C). However, PSD-95 did not interact with p75 and NgR in the Triton-insoluble fraction (Fig. 2D). These results indicate that lipid rafts might act as platforms for the Nogo66-mediated signaling.
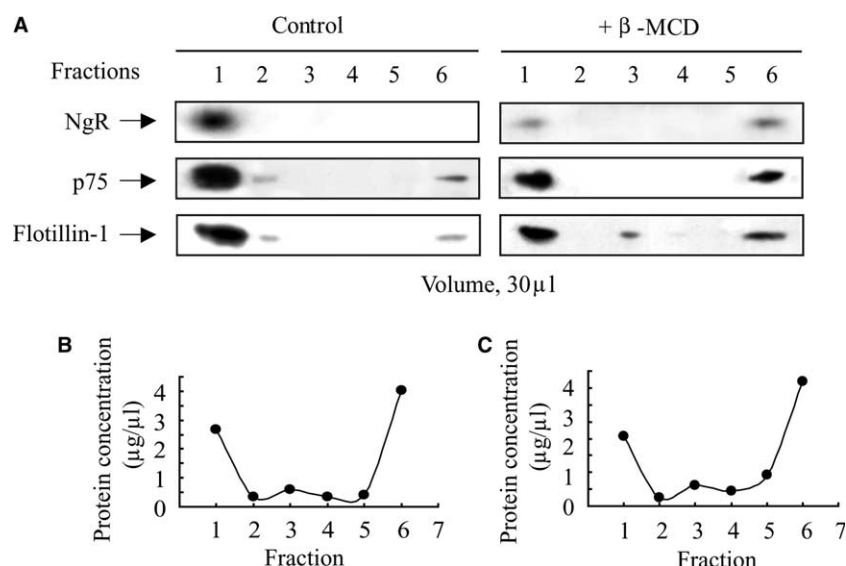
Fig. 3. Disruption of lipid rafts reduces NgR and p75 in lipid rafts. (A) OptiPrep density gradient fractions were prepared from cerebellar granule cells of P7 rats. Equal aliquots (30 μl) of the six fractions were resolved on SDS–PAGE and immunoblotted with the indicated antibodies. Control, cells without any treatment. +β-MCD, cells treated with 10 mM +β-MCD for 10 min. All of these Western blots used the samples from the same OptiPrep gradient centrifugation. Representative blots of two independent experiments with similar results are shown. (B, C) Protein concentrations in fractions in A.

3.3. β-Methylcyclodextrin reduced the localization of NgR and p75 in the lipid rafts

To confirm that the detergent insolubility of NgR and p75 is dependent on their localization to lipid rafts, we used β-MCD to disrupt rafts. Lipid raft fractions were prepared from cultured cerebellar granule cells. Consistent with the previous report, when equal volumes (30 μl) of different fractions were analyzed, NgR was detected exclusively in the lipid raft fractions, whereas p75 was detected both in the lipid raft and non-lipid raft fractions (Fig. 3A, left). In contrast, in neurons treated with 10 mM β-MCD for 10 min before lipid raft preparation, the level of NgR and p75 in lipid rafts were decreased while a larger portion of NgR and p75 was detected in the soluble fraction. Meanwhile, the level of flotillin-1 was also increased in the non-lipid raft fractions (Fig. 3A, right). Quantitative analysis showed that the majority of the proteins were present in the lipid raft and soluble fractions (Fig. 4B and C). These results suggested that the integrity of lipid rafts might be important for Nogo66 signaling.

3.4. Disruption of lipid rafts inhibited Nogo66 signaling

Previous studies have shown that Nogo66 induced inhibition of neurite outgrowth of cerebellar granule cells requires the involvement of the RhoA-associated kinase ROCK [40]. In endothelial, RhoA GTPase is localized to caveolae-enriched membrane domains [46]. To investigate whether RhoA is present in lipid rafts in CNS, we used mouse anti-human RhoA monoclonal antibodies to test the lipid raft and non-lipid raft fractions segregated from adult forebrains and cerebella. As expected, we observed that there was colocalization of RhoA with flotillin-1 in the lipid rafts (Fig. 4A). To further determine whether NgR in lipid rafts is important for Nogo66 signaling, we added soluble Nogo66 to stimulate cultured cerebellar granule cells and measured the amounts of cellular active GTP-bound RhoA. GST–Nogo66 induced a marked activation of RhoA after stimulation for 5 min. We then

treated cerebellar granule cells with 5 mM β-MCD for 45 min to disrupt lipid rafts before stimulation with GST–Nogo66 and examined the RhoA activation in response to GST–Nogo66. As shown in Fig. 4, GST–Nogo66 no longer induced obvious RhoA activation compared to control after pretreatment with β-MCD. Meanwhile, β-MCD itself did not affect the normal basal level of RhoA activation (Fig. 4B and C). Taken together, these results indicate that lipid rafts are essential for Nogo66 signaling.

4. Discussion

In this study, we have shown that NgR, p75 and RhoA were present in the lipid rafts of the brain. NgR was found to interact with p75 in detergent-insoluble fraction. Acute cholesterol depletion of cerebellar granule cells prior to detergent extraction reduced the level of NgR and p75 in lipid rafts. In addition, Nogo66 induced RhoA activity was abolished by β-MCD treatment.

Lipid rafts are characterized by their accumulation of cholesterol and sphingolipids. Typically, this fraction is enriched in GPI-linked glycoproteins [30]. NgR is predominantly expressed in neurons and their axons and is attached to the outer leaflet of the plasma membrane by a GPI moiety in the CNS [4,47]. Using well-established biochemical techniques to isolate lipid rafts, we have demonstrated that NgR, p75 and RhoA were localized to these membrane microdomains (Figs. 1–4). Interestingly, while NgR was detected exclusively in the lipid raft fractions at the two developmental stages examined, p75 showed a developmental change of localization into lipid rafts (Fig. 1). Similar developmental translocation of L1 and Ncad into lipid rafts has been reported previously [44]. The mechanism is possibly in part dependent on palmitoylation at their membrane-spanning domain [48]. A similar palmitoylation site is present in p75 at its membrane-spanning domain [49] and it
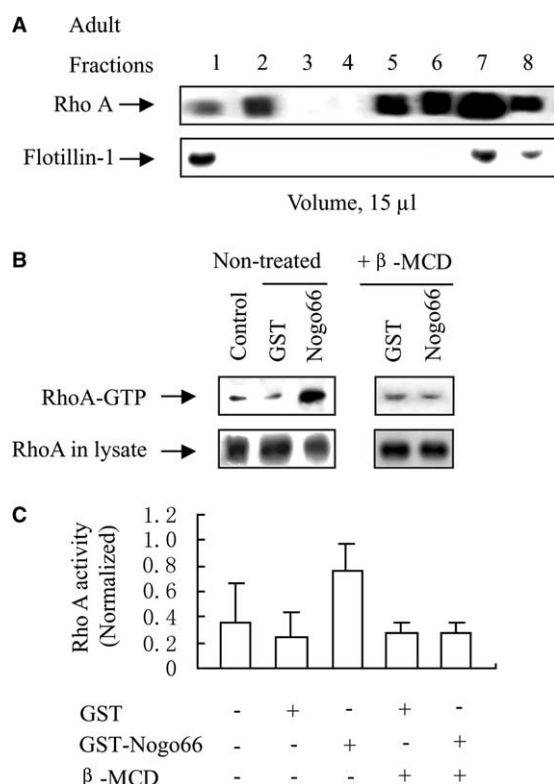
A Adult

Fractions   1 2 3 4 5 6 7 8

Rho A →

Flotillin-1 →

Volume, 15 µl

B   Non-treated   + β -MCD

Control GST Nogo66   GST Nogo66

RhoA-GTP →

RhoA in lysate →

C

Rho A activity (Normalized)

GST	-	+	-	+	-
GST-Nogo66	-	-	+	-	+
β -MCD	-	-	-	+	+

Fig. 4. β-MCD treatment of neurons blocks RhoA activation induced by GST–Nogo66. (A) OptiPrep density gradient fractions were prepared from membranes of adult rat brains. Equal aliquots (15 µl) of the eight fractions were resolved on SDS–PAGE and immunoblotted with indicated antibodies. (B) Soluble GST (6 µg/ml) and GST–Nogo66 (6 µg/ml) were used to stimulate cerebellar granule cells in culture for 5 min or treated with 5 mM β-MCD for 45 min before stimulation with GST and GST–Nogo66, respectively. The amounts of cellular active GTP-bound RhoA were measured. (C) RhoA activities are indicated by the amount of GST-RBD bound RhoA normalized to the amount of total RhoA content in the lysates. Results are means ± S.E.M. from three experiments.

is possible that p75 translocation is also in part dependent on the similar mechanism. A more recent report suggested that a lipid-dependent mechanism was involved in the recruitment of neuronal Src to lipid rafts in the brain [50]. Their findings may suggest an alternative mechanism of p75 translocation. Though the physiological importance remains unknown, we postulate that p75 translocation to lipid rafts might be correlated with more efficient transduction for signaling and thus provide a cue for studying the mechanisms of poorer axonal regeneration in older rats.

The importance of p75 in mediating NgR-dependent inhibitory signaling [15,16] is further confirmed by our co-immunoprecipitation results showing an interaction of p75 with NgR in detergent-insoluble fractions (Fig. 2). Considering the exclusive presence of NgR in the lipid rafts, we infer that localizing in lipid rafts may facilitate the interaction of NgR and p75 as well as with other molecules involved in the signal transduction.

Cholesterol is critical to the integrity of caveolae and other lipid raft structures. Cholesterol depletion will eventually lead to the disruption of lipid rafts and caveolae [51]. β-MCD is a cholesterol-binding reagent that can acutely deplete cellular cholesterol [21,52]. In our study, we found that β-MCD

treatment dramatically reduced the amounts of NgR and p75 in the lipid raft fractions and thus may affect the signal transduction of Nogo66.

Activation of the small GTPase RhoA has been shown to be a crucial step in the signal transduction of inhibitory cues in various neurons [53,54]. The most important effector of GTPases RhoA in the growth cone is probably the serine–threonine kinase Rho-kinase ROCK [55]. Inhibition of the RhoA–ROCK pathway greatly improved neurite outgrowth response of cerebellar granule cells [40]. Previous studies demonstrate that small GTPase Rho, Ras, c-Src and FAK are present in raft fractions [56,57]. In our study, we also detected RhoA in the detergent-insoluble fractions of the brain. In addition, we found that activation of RhoA in cerebellar granule cells by GST–Nogo66 was inhibited by β-MCD. Our results demonstrate that disruption of lipid rafts blocked the Nogo66 signaling and may consequently inhibit the neurite outhgrowth.

Based on these results and previous data, we suggest the following mode of action for Nogo66. Nogo66 binds NgR on the cell surface and simultaneously or subsequently interacts with p75 and other co-receptors in the lipid rafts. These interactions induce activation of RhoA and the upregulation of RhoA activity results in inhibition of neurite outgrowth. As LINGO-1 was recently reported to be an important component of the NgR1/p75 receptor complex, it remains to be investigated whether it is also located in lipid rafts. In addition, previous report has implied a mechanism of PKA regulated location of p75 into lipid rafts [37]. Considering the relatively low level of p75 and RhoA in the lipid rafts in younger rat brains (data not shown), it remains to be investigated whether p75 and RhoA would be recruited to lipid rafts by Nogo66 stimulation for more efficient signaling.

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