Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 41–46
BBRC
www.elsevier.com/locate/ybbrc

Neuroligins 3 and 4X interact with syntrophin-γ2, and the interactions are affected by autism-related mutations

Hidekuni Yamakawa [a], Satoko Oyama [a], Hiroaki Mitsuhashi [a], Noboru Sasagawa [a], Shigeo Uchino [b], Shinichi Kohsaka [b], Shoichi Ishiura [a,*]

[a] *Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, 3-8-1 Komaba, Meguro-ku, 153-8902, Japan*
[b] *Department of Neurochemistry, National Institute of Neuroscience, Tokyo, 4-1-1 Ogawahigashi, Kodaira, 187-8502, Japan*

Received 4 January 2007
Available online 31 January 2007

Abstract

Recently, neuroligins (NLs)3 and 4X have received much attention as autism-related genes. Here, we identified syntrophin-γ2 (SNTG2) as a *de novo* binding partner of NL3. SNTG2 also bound to NL4X and NL4Y. Interestingly, the binding was influenced by autism-related mutations, implying that the impaired interaction between NLs and SNTG2 contributes to the etiology of autism.

Keywords: Autism; Neuroligin; Scaffolding protein; Syntrophin

Neuroligin (NL) is a neural cell adhesion molecule, which was identified as a ligand for β-neurexin [1]. Rodents possess three NLs, all of which interact with β-neurexin, while humans have five NLs. The trans-synaptic interaction between NL and β-neurexin strongly induces both pre- and postsynaptic maturation [2–4]. Interestingly, mutations of human *NL3* and *NL4X* are implicated in autism and mental retardation [5–9]. Autism is characterized by impaired reciprocal social interaction and communication and restricted, stereotyped patterns of interests and activities. Here, we investigated the *de novo* binding partners of NL3 using yeast two-hybrid screening, and found that syntrophin-γ2 (SNTG2) interacted with NL3, NL4X, and NL4Y, which are autism-related NL isoforms. Interestingly, the interactions between the NLs and SNTG2 are very noteworthy in terms of the etiology of autism because they are influenced by autism-related mutations.

Materials and methods

Plasmid construction. Tag sequences and cDNA fragments were cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) or in pECFP vector (Clontech, Palo Alto, CA) using conventional molecular biological techniques. The nucleotide sequences of the DNA inserts were confirmed using sequence analysis. The following NLin (intracellular region of human neuroligin) constructs were made: NL3in (731–848 aa), NL4Xin (698–816 aa), and NL4Yin (698–816 aa). For ΔPBM mutants, the C-terminus of NLs (six amino acids: HSTTRV) was deleted. For ΔTMD32 mutants, TMD32, the N-terminus 32 amino acids of NL3in, was deleted (see Fig. 1). The YFP-NLs contained each NL signal sequence followed by the enhanced yellow fluorescent protein (EYFP) and the mature N-terminus of each of the NLs inserted into pcDNA3.1 expression vector. The Myc × 6 tagged NLs also contained each NL signal sequence followed by Myc × 6 tags and the mature N-terminus of each of the NLs inserted into pcDNA3.1 expression vector. The full-length human syntrophin-γ2 (NCBI Accession No. NP_061841, hSNTG2) was inserted into the revised pACT2 vector to express the GAL4AD-fusion proteins in the yeast two-hybrid system. The FLAG-tagged SNTG2 contained three FLAG tags followed by the N-terminus of SNTG2. The Myc-tagged SNTG2 contained SNTG2 followed by six Myc tags.

Antibodies. We purchased the following rabbit polyclonal antibodies: anti-GFP (Molecular Probes, Eugene, OR) and anti-FLAG (Sigma, St. Louis, MO). In addition, we obtained the following mouse monoclonal antibodies: anti-synaptophysin (Chemicon), anti-MAP2, anti-FLAG M2 (Sigma), anti-Myc, and anti-V5 (Invitrogen).

Abbreviations: NL, neuroligin; PDZ, PSD/Dlg/ZO-1 homology; PSD, postsynaptic density; DIV, days *in vitro*; CNS, central nervous system.

* Corresponding author. Fax: +81 3 5454 6739.
E-mail address: cishiura@mail.ecc.u-tokyo.ac.jp (S. Ishiura).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.01.127

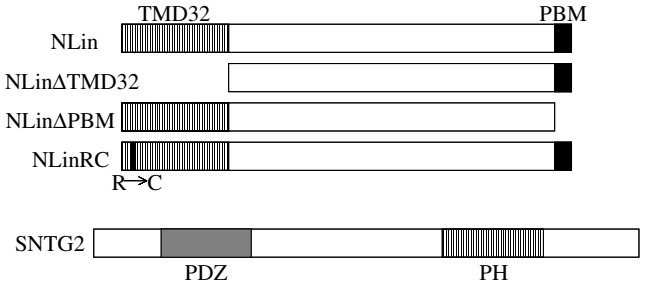

Fig. 1. Schematic depiction of the constructs of NLs and SNTG2. Vertical-striped, black, gray, and horizontal-striped rectangles show the TMD32 domain, PDZ binding motif (PBM), PDZ domain, and PH domain, respectively.

Western blotting. Samples were run on 7.5% SDS–polyacrylamide gels and transferred to PVDF membranes (Immobilon-P; Millipore). The membranes were blocked with 5% skim milk in TPBS (PBS with 0.05% Tween®20) for 1 h at room temperature and then incubated for 1–2 h with primary antibodies in TPBS. After washing, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies. The immunoreactive bands were visualized using ECL.

Yeast two-hybrid screening. Yeast two-hybrid assays were performed using the MATCHMAKER GAL4 Two-Hybrid System (Clontech). The cDNA fragments encoding the intracellular region of human NL3 (711–828 aa, NCBI Accession No. NP_061850, hNL3in) were amplified by PCR and cloned into the *Eco*RI and *Bam*HI restriction sites of the pAS2-1C vector to serve as bait in the yeast two-hybrid screening. The pAS2-1C vector is the pAS2-1 vector (Clontech) revised as a single-copy plasmid in yeasts. The nucleotide sequences of the DNA inserts were confirmed by sequence analysis to verify that the inserts did not contain mutations. The human fetal brain MATCHMAKER cDNA library in the pACT2 vector (Clontech) served as prey in the yeast two-hybrid screening. The inserts were expressed as fusion proteins with the DNA-binding and DNA-activating domains of GAL4. Details of the other constructs used in the yeast two-hybrid assays are presented in Fig. 1.

Yeast was transformed using the lithium acetate method with 30% polyethylene glycol 4000. In screening the NL3in-binding partners, 5 μg cDNA library per plate were introduced into transformants of NL3in in pAS2-1C. Transformants were selected on −LWHA and −LWH + 3-AT plates, and then the surviving transformants were selected using the β-galactosidase assay. We screened more than 1×10^6 cells. The intensity of the interactions between NLs and SNTG2 was examined using the 3-AT assay.

Immunoprecipitation. COS-7 cells were transfected with Myc-tagged constructs of NL and FLAG-tagged constructs of SNTG2 using FuGENE 6 (Roche, Basel, Switzerland). Cells from two 10-cm plates were homogenized in 500 μl of Lysis Buffer (50 mM Tris/HCl [pH 8.0] containing 200 mM NaCl, 5 mM DTT, 1 mM EDTA, 1% (w/v) Triton X-100, and protease inhibitor cocktail). Then, the lysates were centrifuged at 100,000*g* for 15 min at 4 °C. The supernatant was precleared with protein G Sepharose 4 fast flow beads (Amersham Biosciences, Piscataway, NJ) for 1 h and then incubated with 1 μl of anti-FLAG M2 antibody fixed on 15 μl beads. After the beads were washed five times with homogenization buffer, the precipitates were analyzed using SDS–PAGE and immunoblotted with either anti-Myc or anti-FLAG antibody.

Immunocytochemistry and image analysis. Cortical neurons and COS-7 cells were fixed with PBS containing 2% or 4% (w/v) paraformaldehyde for 15 min, and permeabilized with 0.1% (w/v) Triton X-100 in PBS for 15 min. After the buffer was exchanged for 3% (w/v) BSA in PBS, the cells were incubated with the first antibody in 3% BSA in PBS for 1 h, washed with PBS, and then incubated with the second antibody in 3% BSA in PBS for 1 h. After washing with PBS, the samples were embedded in Mowiol (Calbiochem, La Jolla, CA). When only the cell surface was stained, the cells were incubated with the first antibody in PBS for 20 min, washed with PBS, and incubated with the second antibody for 20 min before washing and fixing. Z-stacks of four to 10 images were acquired on a Zeiss LSM510 meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Brightest point projections of the Z-stacks were used for image analysis.

Neuronal cultures and transfection. Neurons were dissociated from the cerebral cortices of E18 rats and cultured at a density of 5×10^4 cells/cm^2 in Neurobasal medium with 2% B27 supplement (Invitrogen), 200 mM L-glutamine, and 10 mM L-glutamate at 37 °C under a controlled atmosphere containing 10% CO_2. After 7 or 11 days *in vitro*, the neurons were transfected with 1 μg DNA per well in 12-well plates using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's directions.

Results

Library screening identified syntrophin-γ2 as an NL3in-binding protein

While some reports have implied that GABA synapses are somehow involved in the pathology of autism [10,11], no scaffolding protein which exclusively localizes in the inhibitory synapse has been identified as a binding partner of NL3, which is thought to be strongly tied to autism [5,6]. NLs have been reported to be associated with both excitatory and inhibitory synapses. Therefore, we hypothesized that some unknown scaffolding proteins were associated with NL in inhibitory synapses, and that the interactions were involved in the pathology of autism. To identify these unknown scaffolding proteins, we performed yeast two-hybrid screening using the intracellular region of NL3 (NL3in) as bait to screen a human fetal brain cDNA library. Syntrophin-γ2 (SNTG2) was identified as a putative candidate. We confirmed the authenticity of the clone by transforming it back into yeast with NL3in.

Moreover, to determine the SNTG2-binding regions in NLs, we performed the HIS3 assay using various constructs (Fig. 1) that express mutant NL-fusion proteins in yeasts (Table 1). We examined the binding of NL3, 4X, and 4Y to SNTG2 in yeasts. All of the autism-related

Table 1
Results of the HIS3 assay of the NL mutants

Construct	HIS3 assay
Vector (DBD)	+
NL3in	++++++
NL3inΔPBM	+
NL3inR737C	+++
NL3inR737CΔPBM	+
NL3inΔTMD32	+++
NL4Xin	++++++
NL4XinΔPBM	+
NL4XinR704C	+++
NL4XinR704CΔPBM	+
NL4Yin	++++
NL4YinR704C	+++

We examined the strength of the interactions between NL or NL mutants and SNTG2 using the HIS3 assay. Since the HIS3 gene product, which is required for cell growth on plates lacking histidine, is competitively inhibited by 3-AT, the concentration of 3-AT at which yeast transformants can grow represents the activity of HIS3. Therefore, it reflects the strength of the interaction. Six, five, four, three, two, and one plus (+) indicate 3-AT concentrations of 20, 15, 10, 5, 1, and 0 mM, respectively.

NLs bound to SNTG2. The deletion of the PDZ binding motif (PBM) from NL3, 4X, and 4Y disrupted this binding to SNTG2. Notably, the introduction of an R→C point mutation, an autism-related mutation [9], weakened the binding. Similarly, the deletion of TMD32, the region including the autism-related mutation, also weakened the binding of NL3 to SNTG2. These results suggest that the binding of NLs to SNTG2 depends mainly on PBM and is stabilized by TMD32.

NLs bound to SNTG2 in COS-7 cells

Next, we confirmed that NLs would bind to SNTG2 in COS-7 cells using immunoprecipitation. Myc-NL3 and Myc-NL4X were co-immunoprecipitated with SNTG2 when FLAG-SNTG2 was immunoprecipitated using anti-FLAG antibody, whereas Myc-NL3 and Myc-NL4X were not immunoprecipitated by normal IgG, suggesting that NL3 and NL4X specifically interacted with SNTG2 (Fig. 2). In addition, Myc-NL4Y bound to FLAG-SNTG2 in COS-7 cells (data not shown).

We also examined the binding of NL mutants to SNTG2 in COS-7 cells. As with the HIS3 assay, the deletion of PBM in NL drastically weakened the binding to SNTG2 (Fig. 3A), indicating that the interaction between SNTG2 and NL in mammalian cells is mostly dependent on PBM. In contrast, the autism-related R704C mutation in NL4X did not seem to alter the binding (Fig. 3B). This result seems to be inconsistent with HIS3 assay. However, we suppose that we couldn't detect the effect of the autism-related R704C on the interaction between NL4X and SNTG2 because immunoprecipitation is not sensitive enough to detect minute differences of affinity. We also examined the regions of SNTG2 involved in its binding to NLs in COS-7 cells. The deletion of either the N- or C-terminal half of SNTG2 dramatically weakened the binding to NL3 and NL4X (Fig. 3C), suggesting that both the N-terminal part, including the PDZ domain, and the C-terminal half of SNTG2 are involved in the binding to NL.

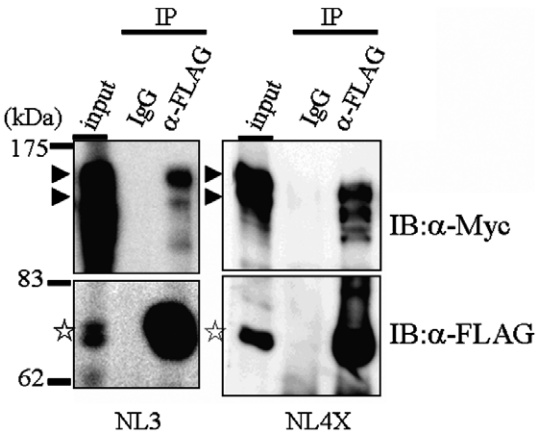

Fig. 2. The binding between SNTG2 and NLs in COS-7 cells. Myc-NLs were co-immunoprecipitated with FLAG-SNTG2 using anti-FLAG M2 antibody in Lysis buffer. Immunoprecipitation using normal mouse IgG was the negative control. Arrowheads and stars indicate Myc-NLs and FLAG-SNTG2, respectively. Both arrowheads may denote the posttranslational modification of NLs.

NLs partially colocalize with SNTG2 in both COS-7 cells and rat cortical neurons

To determine whether NL and SNTG2 colocalize because of their interaction, we immunostained COS-7 cells expressing YFP-NLs and SNTG2-myc. When YFP-NL3 or YFP-NL4X was expressed after transfecting COS-7 cells, it formed a small cluster on the cell surface (Fig. 4A). When SNTG2-myc was also expressed, it partially colocalized with YFP-NL (Fig. 4B); however, SNTG2-myc did not alter the localization of YFP-NL on the cell surface substantially, and vice versa.

Next, we examined the localization of exogenous NL3 and SNTG2 in rat cortical neurons at DIV9. When YFP-NL3 was expressed by transfection, YFP-NL3 formed small clusters on the surface of soma and dendrites, just like endogenous NL3 (Fig. 4C). Clusters of YFP-NL3 often colocalized with SNTG2-myc when neurons were doubly transfected, supporting the idea that NL3 is associated with SNTG2 in neurons and that SNTG2 functions as a scaffolding protein.

Discussion

This study revealed functional differences among NLs, which were related to binding to syntrophin-γ2 (SNTG2). Here, SNTG2 was identified as a *de novo* binding partner of autism-related NLs using yeast two-hybrid screening. SNTG2 is a member of the syntrophin family, whose members have a PDZ domain near the N-terminus and a PH domain near the C-terminus. Syntrophins play a key role as scaffolding proteins in both neuromuscular junctions and the CNS, where they interact with dystrophin and dystrobrevin [12]. Interestingly, the CNS syntrophins have been reported to localize exclusively in the inhibitory postsynaptic regions by binding to dystrophin and dystrobrevin [13,14]. Therefore, SNTG2 may be an unknown NL-binding scaffolding protein in inhibitory synapses.

The interaction between SNTG2 and NLs was mainly dependent on the PBM-PDZ interaction because it was disrupted by the deletion of PBM in NLs (Table 1 and Fig. 3A). Meanwhile, the binding of NLs to SNTG2 was influenced by factors other than the PBM-PDZ interaction, involving TMD32 in NLs and the region including PH in SNTG2 (Table 1 and Fig. 3B and C). Since deletion of the PBM disrupted the binding, we postulated that these non-PBM-PDZ factors somehow stabilize the PBM-PDZ core interaction. In particular, the effect of TMD32 on binding is interesting because it was previously reported to be necessary for the proper localization of NL1 [15]. In addition, TMD32 is variable

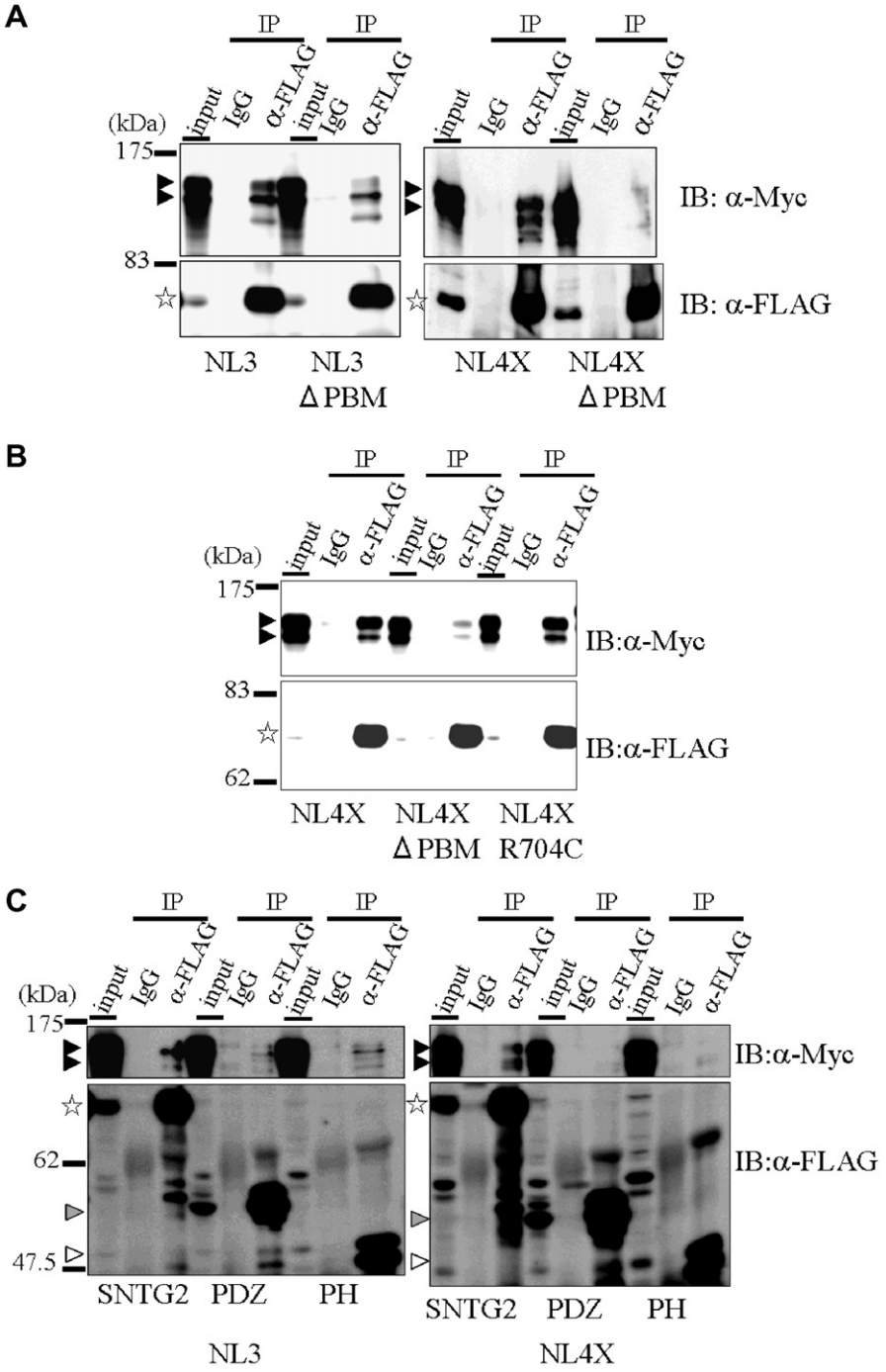

Fig. 3. (A) Effects of the deletion of PBM in NLs on the binding between SNTG2 and NLs in COS-7 cells. Various Myc-tagged NLs were co-immunoprecipitated with FLAG-SNTG2 in Lysis buffer. The deletion of PBM in NLs weakened the binding of NLs to SNTG2. Arrowheads and stars indicate Myc-NLs and FLAG-SNTG2, respectively. (B) The effect of R704C in NL4X, the autism-related mutation, on the binding to SNTG2 in COS-7 cells. Various Myc-tagged NL4Xs were co-immunoprecipitated with FLAG-SNTG2 in Lysis buffer. Under these conditions, the effect of the R704C mutation on the binding to SNTG2 was not detected. (C) The role of PDZ or the PH domain of SNTG2 in binding to NL. Whenever the regions containing the PDZ or PH domain were deleted, binding to NL was weakened. Therefore, both these regions might be involved in the interaction between NL and SNTG2. Black, gray, and white arrowheads and the star indicate Myc-NLs, the FLAG-tagged region containing the PDZ domain, the FLAG-tagged region containing the PH domain of SNT2G2, and the FLAG-tagged full-length SNTG, respectively.

in comparison with other parts of NL. These findings led us to hypothesize that due to the diversity of TMD32, NLs are functionally different from each other and the sequence of TMD32 for each NL determines whether the NL localizes in the inhibitory synapse. In this experiment, we could not examine the interaction between SNTG2 and NL2, which has been reported to localize in the inhibitory synapse exclusively. However, NL2 is

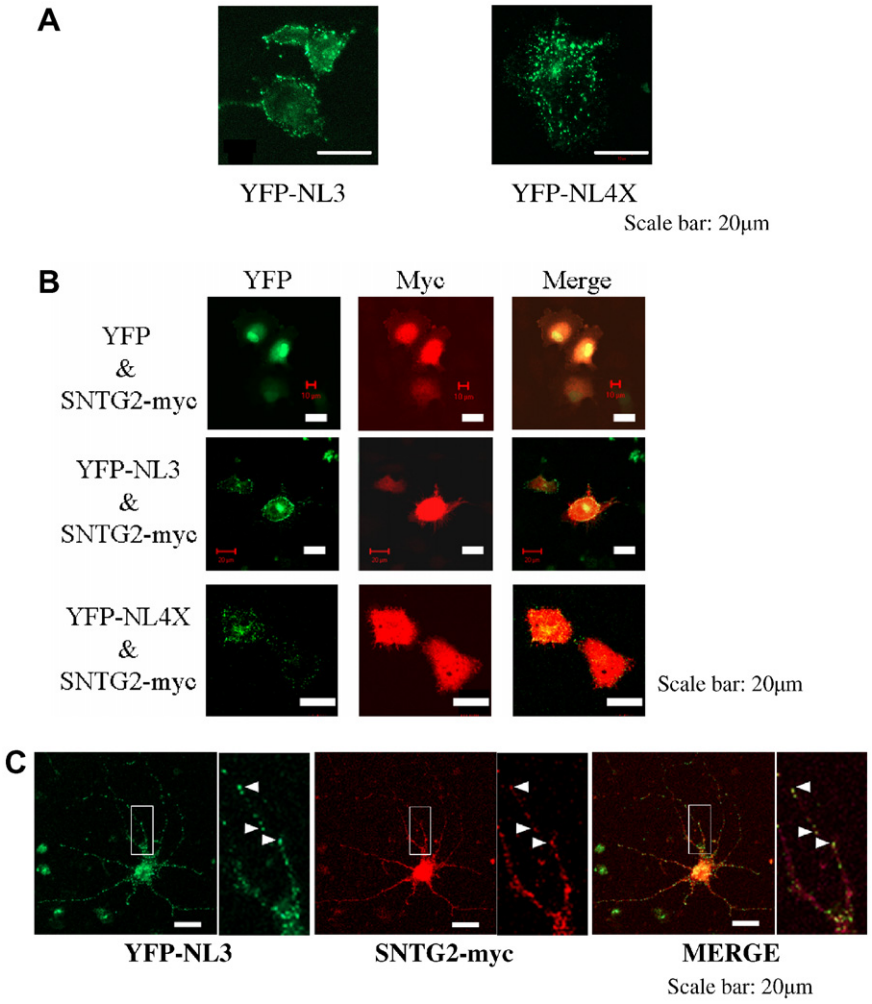

Fig. 4. (A) Localization of NL3 and NL4X on the surface of COS-7 cells. Only YFP-NL3 and YFP-NL4X on the cell-surface were immunostained using anti-GFP antibody and the secondary antibody conjugated with Oregon Green 488 without membrane permeabilization. They were clustered on the cell surface. (B) Localization of NLs and SNTG2 in COS-7 cells. The upper panels are negative controls. YFP or YFP-NL of cell-surface, SNTG2-Myc and merged images are shown on the left, center, and right, respectively. Co-transfected YFP-NL and SNTG2-Myc partially colocalized. Scale bar, 20 μm. (C) Localization of NL3, NL4X, and SNTG2 in rat cortical neurons. Neurons were transfected with YFP-NL3 and SNTG2-Myc at DIV7 using Lipofectamine 2000. After a 48-h incubation, the neurons were fixed, immunostained, and observed under confocal microscopy. The boxed area is enlarged in the bottom row. The clusters of YFP-NL3 of cell-surface sometimes colocalized with the cluster of SNTG2-Myc (arrowhead). Scale bar, 20 μm.

expected to bind to SNTG2 much stronger than any other NL, considering that both NL2 and SNTG2 dominantly localize in the inhibitory synapse [13,14,16]. The interaction will be determined in future.

Recently, some reports have suggested that the balance between excitatory and inhibitory synapses is disrupted in patients with autism [17,18]. A postmortem analysis of the brains of subjects with autism indicated that glutamic acid decarboxylase (GAD) was reduced in the autistic parietal and cerebellar cortices [11], suggesting that the impairment of GABAergic synaptic transmission causes autism. Meanwhile, the binding of NLs to SNTG2, a putative inhibitory-synaptic scaffolding protein, were affected by autism-related mutations in *NLs* in our experiments. Totally, the interactions between NLs and SNTG2 at the inhibitory synapse may be deeply involved in the etiology of autism. In future, screening autistic individuals for *SNTG2* mutations may elucidate the association between autism and the binding of SNTG2 to NLs.

References

[1] K. Ichtchenko, Y. Hata, T. Nguyen, B. Ullrich, M. Missler, C. Moomaw, T.C. Sudhof, Neuroligin 1: a splice site-specific ligand for beta-neurexins, Cell 81 (1995) 435–443.
[2] E.R. Graf, X. Zhang, S.X. Jin, M.W. Linhoff, A.M. Craig, Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins, Cell 119 (2004) 1013–1026.
[3] C. Dean, F.G. Scholl, J. Choih, S. DeMaria, J. Berger, E. Isacoff, P. Scheiffele, Neurexin mediates the assembly of presynaptic terminals, Nat. Neurosci. 6 (2003) 708–716.

[4] P. Scheiffele, J. Fan, J. Choih, R. Fetter, T. Serafini, Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons, Cell 101 (2000) 657–669.
[5] S. Jamain, H. Quach, C. Betancur, M. Rastam, C. Colineaux, I.C. Gillberg, H. Soderstrom, B. Giros, M. Leboyer, C. Gillberg, et al., Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism, Nat. Genet. 34 (2003) 27–29.
[6] B. Chih, S.K. Afridi, L. Clark, P. Scheiffele, Disorder-associated mutations lead to functional inactivation of neuroligins, Hum. Mol. Genet. 13 (2004) 1471–1477.
[7] D. Comoletti, A. De Jaco, L.L. Jennings, R.E. Flynn, G. Gaietta, I. Tsigelny, M.H. Ellisman, P. Taylor, The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing, J. Neurosci. 24 (2004) 4889–4893.
[8] F. Laumonnier, F. Bonnet-Brilhault, M. Gomot, R. Blanc, A. David, M.P. Moizard, M. Raynaud, N. Ronce, E. Lemonnier, P. Calvas, et al., X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family, Am. J. Hum. Genet. 74 (2004) 552–557.
[9] J. Yan, G. Oliveira, A. Coutinho, C. Yang, J. Feng, C. Katz, J. Sram, A. Bockholt, I.R. Jones, N. Craddock, et al., Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients, Mol. Psychiatry 10 (2005) 329–332.
[10] M.M. Menold, Y. Shao, C.M. Wolpert, S.L. Donnelly, K.L. Raiford, E.R. Martin, S.A. Ravan, R.K. Abramson, H.H. Wright, G.R. Delong, et al., Association analysis of chromosome 15 gabaa receptor subunit genes in autistic disorder, J. Neurogenet. 15 (2001) 245–259.
[11] S.H. Fatemi, A.R. Halt, J.M. Stary, R. Kanodia, S.C. Schulz, G.R. Realmuto, Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices, Biol. Psychiatry 52 (2002) 805–810.
[12] M.E. Adams, N. Kramarcy, T. Fukuda, A.G. Engel, R. Sealock, S.C. Froehner, Structural abnormalities at neuromuscular synapses lacking multiple syntrophin isoforms, J. Neurosci. 24 (2004) 10302–10309.
[13] G. Piluso, M. Mirabella, E. Ricci, A. Belsito, C. Abbondanza, S. Servidei, A.A. Puca, P. Tonali, G.A. Puca, V. Nigro, Gamma1- and gamma2-syntrophins, two novel dystrophin-binding proteins localized in neuronal cells, J. Biol. Chem. 275 (2000) 15851–15860.
[14] I. Brunig, A. Suter, I. Knuesel, B. Luscher, J.M. Fritschy, GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABA(A) receptors and gephyrin, J. Neurosci. 22 (2002) 4805–4813.
[15] T. Dresbach, A. Neeb, G. Meyer, E.D. Gundelfinger, N. Brose, Synaptic targeting of neuroligin is independent of neurexin and SAP90/PSD95 binding, Mol. Cell Neurosci. 27 (2004) 227–235.
[16] F. Varoqueaux, S. Jamain, N. Brose, Neuroligin 2 is exclusively localized to inhibitory synapses, Eur. J. Cell Biol. 83 (2004) 449–456.
[17] H. Cline, Synaptogenesis: a balancing act between excitation and inhibition, Curr. Biol. 15 (2005) R203–R205.
[18] N.K. Hussain, M. Sheng, Neuroscience. Making synapses: a balancing act, Science 307 (2005) 1207–1208.