Disorder-associated mutations lead to functional inactivation of neuroligins

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Autism is a neuro-developmental syndrome that affects 0.1–0.5% of the population. It has been proposed that alterations in neuronal circuitry and/or neuronal signaling are responsible for the behavioral and cognitive aberrations in autism patients. However, the cellular basis of such alterations is unknown. Recently, point mutations in a family of neuronal cell adhesion molecules called neuroligins have been linked to autism-spectrum disorders and mental retardation. We investigated the consequences of these disease-associated mutations on neuroligin function. We demonstrate that the point mutation at arginine 451 and a nonsense mutation at aspartate 396 of neuroligin-3 and -4 (NL3 and NL4), respectively, result in intracellular retention of the mutant proteins. Over-expression of wild-type NL3 and NL4 proteins in hippocampal neurons stimulates the formation of presynaptic terminals, whereas the disease-associated mutations result in a loss of this synaptic function. Our findings suggest that the previously identified mutations in neuroligin genes are likely to be relevant for the neuro-developmental defects in autism-spectrum disorders and mental retardation since they impair the function of a synaptic cell adhesion molecule.

INTRODUCTION

Autism-spectrum disorders are a group of heterogeneous neuro-developmental syndromes that are characterized by deficits in social behavior and communication (1–3). Autism is accompanied frequently by mental retardation but there are individuals with autism-spectrum disorders (e.g. Asperger syndrome) who have normal cognitive abilities. Currently, there is only very limited information available on the etiology and neurobiological basis of these disorders. While the role of environmental factors has been discussed, there is strong evidence for a genetic basis. The recurrence risk in siblings is 4.5%, which represents a 9–45-fold higher relative risk compared with the general population incidence of 0.1–0.5%. Moreover, the concordance rate for monozygotic twins is >50%, strongly supporting a high degree of heritability (4–6).

Imaging studies, histological analysis and the observation that seizure disorders are associated frequently with autism suggest that perturbations in neuronal circuitry underlie the behavioral and cognitive defects in autistic patients (reviewed in 7). Autistic disorders are manifested typically in the second to third year of postnatal life, which corresponds to the developmental time period when neuronal circuits are elaborated and remodeled (8). Moreover, postmortem analysis has revealed aberrant neuronal morphologies, with a reduction in dendritic branching and stunted neuronal arbors (9–11).

Several recent studies have implicated a family of neuronal cell adhesion molecules called neuroligins (NL) in autistic disorders. In humans, this protein family is encoded by five genes, NLGN1, -2, -3, -4 and -Y. Notably, the NLGN1, NLGN3 and NLGN4 genes map to three loci, which are associated with a predisposition to autism [3q26, Xp22.3 and Xq13, respectively (12–15)]. Direct sequencing of the NLGN4 coding sequence in 158 families with autistic individuals led to the identification of a nonsense mutation at aspartate 396 (D396X) in two affected brothers of one family (16). The mutation was absent in a third, unaffected brother in the same family and occurred de novo in the mother. Sequencing of NLGN3 in the same 158 families also revealed a point mutation arginine 451 to cysteine (R451C) in another family which was present in two affected brothers and their unaffected mother but absent from the unaffected father (16).

The link between neuroligins and autism potentially derives from their role in synaptogenesis (7). Neuroligin-3 and -4 proteins (NL3 and NL4) are ~70% identical in amino acid sequence to NL1, which was first identified as a transmembrane ligand of the neuronal cell surface receptor beta-neurexin (17).
In mice, NL1 is concentrated in the postsynaptic density of excitatory synapses (18) and functional in vitro studies revealed that adhesive interactions between NL1 and beta-neurexin can trigger the assembly of functional presynaptic terminals in axons (19,20). These findings suggested that NL–beta-neurexin complexes might contribute to the formation or remodeling of synapses during nervous system development.

Given the high sequence similarity between neuroligin family members, and that mouse NL1 and NL2 have similar activities in vitro (19,21,22), NL3 and NL4 might also function as neuronal adhesion molecules. Consistent with this, a recent study has shown that NL4 interacts with the postsynaptic scaffolding molecule PSD-95 (23).

Here, we address several questions concerning the possible contribution of NL3 and NL4 to nervous system disorders. We show that the autism-associated mutations NL3 R451C and NL4 D396X lead to intracellular retention of neuroligin proteins. We then demonstrate that wild-type NL3 and NL4 have synapse-promoting activity in cultured neurons and that this activity is lost or greatly reduced in the disease-associated mutants. Our findings provide insight into the cellular origins of autism-spectrum disorders and support the hypothesis that autism is caused by defects in neuronal circuitry.

RESULTS

Expression of autism-spectrum disorder-associated NL3 and NL4 mutants

Given the association of the R451C mutation in NLGN3 and the D396X nonsense mutation in NLGN4 with autism-spectrum disorders (16), we investigated the cellular consequences of these mutations. We generated expression constructs for human wild-type NL3 and NL4 proteins and the disease-associated mutant NL3 R451C and NL4 D396X (Fig. 1). Since currently no specific antibodies are available for human NL3 and NL4 proteins we inserted a HA-epitope tag at the mature N-terminus of the proteins for detection. Western blotting of total cell lysates of transfected COS cells revealed immunoreactive bands of ~130 kDa for the wild-type proteins, corresponding to the expected molecular weight of carbohydrate-modified full-length proteins (Fig. 2A). The NL3 R451C mutant yielded a protein of slightly different electrophoretic mobility, possibly due to changes in glycosylation, whereas the nonsense mutation in NL4 D396X resulted in a truncated protein of ~60 kDa. The higher molecular weight species >250 kDa was reproducibly observed and probably represents misfolded SDS-resistant aggregates of the protein.

Decreased surface transport of NL3 R451C

NL1 and NL2 have been shown previously to function as cell surface receptors (19,24). To investigate whether the mutant NL3 and NL4 proteins are transported to the cell surface with similar efficiency as the wild-type proteins, we transfected the COS cells with the wild-type and mutant NL3 and NL4 constructs and performed surface biotinylations with a membrane-impermeable amino-group reactive biotin derivate. Wild-type NL3 and NL4 were abundant in the cell surface fractions (Fig. 2B). Levels of NL3 R451C and NL4 D396X in the total cell lysates were similar to the wild-type proteins (Fig. 2A). However, we recovered only a small amount of NL3 R451C and no NL4 D396X in the surface fraction. The ratio of surface to lysate NL3 was 3.3 ± 0.9 for the wild-type protein but only 0.7 ± 0.6 for NL3 R451C \((n = 3)\), indicating that surface levels of the mutant protein are reduced 4–5-fold in transfected cells. Since NL4 D396X lacks the transmembrane domain we tested whether the truncated protein might be released from cells. Indeed a significant amount of NL4 D396X could be immunoprecipitated from the culture medium (Fig. 2C). These results demonstrate that the single amino acid change in the disease-associated NL3 R451C mutant results in substantially reduced cell surface levels of the protein, whereas NL4 D396X is absent from the cell surface.

NL3 R451C and NL4 D396X are retained in the endoplasmic reticulum

To further investigate the intracellular transport of NL3 R451C and NL4 D396X, we analyzed the subcellular localization of the wild-type and mutant NL3 and NL4 proteins in the transfected COS cells by immunocytochemistry. HA-tagged wild-type NL3 and NL4 were localized mostly to the cell surface as judged by the staining pattern, which was similar in permeabilized and non-permeabilized cells (Fig. 3 and data not shown). Consistent with the surface biotinylation experiments, mutant NL3 and NL4 did not show significant levels of cell surface staining, but were concentrated in an intracellular compartment (Fig. 3). This intracellular pool co-localized extensively with the endogenous endoplasmic reticulum (ER) protein calnexin. These findings suggest that both mutant proteins are retained in ER and are not appropriately delivered to the plasma membrane, likely due to failure to pass the cellular ER quality control (27).
was recovered specifically from the NL4 D396X expressing cells. Where detected from mock-transfected cells, whereas secreted truncated NL4 bodies. Only bands corresponding to heavy and light chains of the antibody anti-HA antibodies and detected by western blotting with mouse anti-HA anti-

NL4 D396X was immunoprecipitated from the culture medium with rat weight standards are indicated in kDa. (A) Shows aliquots of the corresponding total cell lysate fractions. (B) Secreted NL4 D396X was immunoprecipitated from the culture medium with rat anti-HA antibodies and detected by western blotting with mouse anti-HA antibod-

ies. Only bands corresponding to heavy and light chains of the antibody were detected from mock-transfected cells, whereas secreted truncated NL4 was recovered specifically from the NL4 D396X expressing cells.

Functional inactivation of NL3 R451C and NL4 D396X in hippocampal neurons

In our previous studies, we have shown that mouse NL1 and NL2 can promote the formation of functional presynaptic elements in vitro (19,20). We therefore tested whether wild-type NL3 and NL4 might have similar activity and whether the disease-associated mutations might result in a loss of this function. We transfected the epitope-tagged forms of mouse NL1 and wild-type and mutant human NL3 and NL4 into cultured rat hippocampal neurons [at 10 days in vitro (DIV)] and determined the number of presynaptic vesicle clusters formed on the transfected cells 2 days after transfection. As shown previ-

ously (20), expression of NL1 led to a robust increase in the number and size of synapsin-positive puncta on NL1 expressing cells [21.9 ± 0.5 puncta/10 μm dendrite length compared with 5.6 ± 0.3 puncta/10 μm in EGFP expressing cells, Fig. 4 (Supplementary Material, Fig. S1)]. Wild-type NL3 and NL4 showed similar though somewhat lower synaptogenic activities (19.0 ± 0.6 puncta/10 μm for NL3 and 18.0 ± 0.6 puncta/10 μm for NL4, Fig. 4). Importantly, the mutant NL proteins failed to promote synaptogenic differentiation. As in the COS cells, NL3 R451C and NL4 D396X were accumulated mostly in the cell body and not detected readily at the cell surface (Supplementary Material, Fig. S2). Moreover, there was no signifi-

cant increase in the number of synapsin-positive clusters formed on the transfected cells when compared with EGFP expressing control cells (Fig. 4B). However, we noted in some experiments that a fraction of the mutant protein reached the cell surface in a subset of cells with unusually high levels of NL3 R451C expression (5–20% of all cells expressing NL3 R451C, depending on the experiment). In this minority of cells (‘NL3 451C high’ in Fig. 4), the number of synapsin-positive puncta was increased when com-
pared with control cells but did not reach the level observed with wild-type NL3 [12.9 ± 0.8 puncta/10 μm dendrite length in NL3 R451C expressing cells compared with 5.6 ± 0.3 puncta/10 μm for EGFP and 19.0 ± 0.6 puncta/10 μm for wild-type NL3]. These findings suggest that the primary defect caused by the disease-associated R451C mutation is intracellular retention of NL3, whereas the mutant protein retains some synaptogenic activity.

DISCUSSION

Our studies support the hypothesis that mutations in human neuroligin genes contribute to neuro-developmental disorders (7,16). We demonstrate that the wild-type NL3 and NL4 pro-

teins can promote the formation of presynaptic elements in cultured hippocampal neurons and that two autism-associated mutations lead to a loss of this activity.

It is intriguing that the NLGN3 and NLGN4 genes are encoded on the X-chromosome considering that the incidence of autism is 4-fold higher in males than in females (1,2). However, since neuroligin mutations appear to occur only at low frequencies in autistic individuals it is currently unclear whether they are indeed a common cause of autism-spectrum disorders. While this work was in progress, a nonsense mutation in NLGN4 similar to D396X was found to be associated with mental retardation or autism in several members of a large French family (28). Therefore, the neuro-

ligin mutations might be associated primarily with mental retardation and have pleiotropic effects that contribute to the development or diagnosis of autism. Further analysis of all five neuroligin genes in larger groups of patients with autism and/or mental retardation should resolve this question in the future.

Several of our observations suggest that the mutation D396X in NL4 leads to a complete functional inactivation of the protein. First, the protein does not localize to the cell surface. Second, we do not detect any synapse-promoting activity of this mutant protein, even in cells with the highest expression levels that can be obtained in our experimental system. Third, the truncated protein lacks extracellular sequences that are predicted to form an oligomerization inter-

face (26,29,30), which we have shown previously to be essential for the synapse-promoting function of NL1 (20). Finally, we have not observed a significant dominant-interfering activity of this mutant, and no phenotype has been observed in patients heterozygous for the mutation (16). The additional nonsense mutation in NLGN4 which was described recently (26) results in a termination site 33 amino acids downstream of D396X. Therefore, it should generate a similar truncated NL4 protein, which is likely to lack synapse-promoting func-

tion as we have shown here for NL4 D396X. It still remains to be seen whether these truncated proteins are in fact expressed in vivo since their mRNA contains a premature termination codon, which would be expected to target the mRNA for nonsense-mediated decay (31).
In the case of the NL3 R451C mutation, the loss of synaptogenic activity is to a large extent due to inefficient surface transport and retention of the mutant protein in the ER. The extracellular domain of neuroligin proteins contains four disulfide bridges (25). The introduction of a cysteine at position 451 in NL3 R451C may result in aberrant disulfide bridge formation due to inappropriate pairing with the additional cysteine and therefore in misfolding of the protein. Alternatively, the free, unpaired cysteine might lead to ER retention due to persistent interaction of the mutant protein with the ER resident protein calnexin. The scale bar is 10 μm.

**Figure 3.** Retention of neuroligin mutants in the ER. Cells expressing HA-tagged wild-type neuroligin (NL3wt or NL4wt) or mutant neuroligin (NL3 R451C or NL4 D396X) were immunostained with antibodies against the HA-epitope tag (left column, red) and against the ER marker calnexin (middle column, green). While wild-type neuroligins are transported efficiently to the cell surface, the mutant proteins accumulate in the ER as confirmed by the overlap with the ER resident protein calnexin. The scale bar is 10 μm.
protein with protein-disulfide isomerase. This latter mechanism has been reported to mediate the ER retention of other proteins, including immunoglobulin M or pro-collagen peptides (32,33). In either scenario, NL3 R451C would be retained in ER due to the failure to pass the ER quality control.

We observed that the mutant NL3 R451C was transported to the cell surface in a subset of transfected hippocampal neurons. (A) Rat hippocampal neurons were transfected with NL expression constructs at 10 DIV and analyzed 48 h later by immunohistochemistry. The distribution of EGFP which was co-transfected with all NL expression constructs to visualize the morphology of the entire cell (green in merge) is shown in the left. The column labeled HA shows the distribution of the HA-tagged NL proteins (red in merge). The distribution of synaptic vesicles detected with rabbit anti-synapsin antibodies is shown in the column labeled synapsin (blue in merge). Note that the disease-associated mutations NL3 R451C and NL4 D396X led to inefficient transport of the mutant proteins to the dendritic processes. In a subset of NL3 R451C expressing cells with exceptionally high expression level (less than 20% of all the transfected cells, variable between different experiments), a fraction of the mutant protein reached the cell surface and did lead to an increase in the number of synapsin-positive puncta on the cell (‘NL3 R451C high’). The scale bar is 5 μm.

(B) The number of synapsin puncta formed on the transfected hippocampal neurons was quantitated by thresholding of confocal images. The diagram shows the average density of synapsin puncta from six cells or more cells for each construct and standard errors. Quantitation of data from several independent experiments yielded similar results.

Figure 4. Function of NL mutants in dissociated hippocampal neurons. (A) Rat hippocampal neurons were transfected with NL expression constructs at 10 DIV and analyzed 48 h later by immunohistochemistry. The distribution of EGFP which was co-transfected with all NL expression constructs to visualize the morphology of the entire cell (green in merge) is shown in the left. The column labeled HA shows the distribution of the HA-tagged NL proteins (red in merge). The distribution of synaptic vesicles detected with rabbit anti-synapsin antibodies is shown in the column labeled synapsin (blue in merge). Note that the disease-associated mutations NL3 R451C and NL4 D396X led to inefficient transport of the mutant proteins to the dendritic processes. In a subset of NL3 R451C expressing cells with exceptionally high expression level (less than 20% of all the transfected cells, variable between different experiments), a fraction of the mutant protein reached the cell surface and did lead to an increase in the number of synapsin-positive puncta on the cell (‘NL3 R451C high’). The scale bar is 5 μm.

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We observed that the mutant NL3 R451C was transported to the cell surface in a subset of transfected hippocampal
neurons with very high expression level. These cells did show an increase in the number of synaptic vesicle clusters formed on the cell, indicating that the primary defect in NL3 R451C is protein export and that the mutant protein retains some synapticogenic activity. This is reminiscent of mutants in CFTR, a chloride transporter mutated in patients with cystic fibrosis, which is retained in ER due to misfolding. Conditions that promote folding and surface transport of the mutant protein restore chloride transport indicating that the channel is functional despite the mutation (34). In several recent studies, protein-selective agonists and synthetic compounds have been demonstrated to restore normal function of disease-associated mutant proteins by specifically improving protein folding in vitro and in vivo (35–38). In the future, it might therefore be promising to adopt a similar strategy for NL3 R451C to test whether rescuing surface transport indeed rescues the synaptic function of this disease-associated mutant protein.

**MATERIALS AND METHODS**

**DNA constructs**

Human NL3 and NL4 cDNAs were obtained from EST clones (Invitrogen clone ID 5258980 and 4702963, for human NL3 and NL4, respectively). For expression and detection a HA-epitope tag was introduced at the mature N-terminus after the signal sequence and cDNAs were inserted into the expression vector pcDNA3.1 under control of the CMV promoter for expression in the COS cells and into pCAG containing the beta-actin promoter for neuronal expression. The NL mutations were introduced by PCR or Quick-change mutagenesis (Stratagene). All DNA constructs were verified subsequently by DNA sequencing.

**Cell culture and transfections**

The COS cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. Rat hippocampal neurons were isolated from rats at embryonic day 18 and maintained in serum-free culture medium. At 10 DIV, neurons were transfected with Lipofectamine 2000 reagent (Invitrogen) as described (39). Cells were analyzed 48–72 h after transfection.

**Biochemistry**

The COS cells were analyzed 24–36 h after transfection. For western blot analysis, cells grown on 35 mm dishes were harvested in 200 μl phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 0.1% SDS and 2 mM dithiothreitol (DTT) at 4°C and lysates were cleared by 5 min centrifugation at 12,000g. Proteins in the supernatants were analyzed by PAGE and western blotting with mouse or rat antibodies directed against the HA-epitope tag (Roche), followed by chemoluminscent detection (ECL, Amersham).

For surface biotinylations, the transfected COS cells were washed twice with ice-cold PBS, pH 8.0 and incubated with 2 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS, pH 8.0 for 30 min on ice. Subsequently, cells were washed twice for 5 min with PBS containing 0.2 mg/ml bovine serum albumin and lysed in PBS containing 1% Triton X-100, 0.2% SDS, 5 mM EDTA, 2 mM DTT and protease inhibitors (‘complete’, Roche) at 4°C. Lysates were cleared by 5 min centrifugation at 12,000g, and after removal of an aliquot, the supernatant was incubated with streptavidin–agarose (Pierce) for 6 h at 4°C. Beads were washed four times with lysis buffer and bound proteins were eluted by boiling in 2% SDS and analyzed by western blotting. Ratios of surface to lysate pools of neuroligin proteins were estimated by densitometric scanning of blots derived from three independent experiments.

**Immunocytochemistry**

For immunocytochemistry, cells were fixed with 4% paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.2 containing 4% sucrose. Cells were permeabilized with 0.1% Triton and unspecific binding sites were blocked with 10% goat serum in PBS. The following primary antibodies were used: rabbit anti-calnexin (Stressgen), rat and mouse anti-HA-tag (Roche) and rabbit anti-synapsin (Chemicon). Secondary antibodies were from Jackson ImmunoReasearch. Z-stacks of 5–10 images were acquired on a Zeiss LSM510meta laser scanning confocal microscope. For quantitation of synaptic vesicle clustering in hippocampal neurons, confocal images were acquired with identical laser power and photomultiplier settings. Brightest point projections of the Z-stacks were used for image analysis.

**Image analysis and quantitation**

Image analysis was carried out with Metamorph software (Universal Imaging Corporation) as described (39). Briefly, the synaptic marker channel was thresholded to select synaptic puncta. Thresholding settings were identical for all images analyzed and were chosen such that all recognizable synaptic puncta in control cells were included in the analysis. Dendrites of the transfected cells were visualized by EGFP fluorescence and identified by their morphology. The number of synapsin-positive puncta per dendrite length was determined for dendrites of at least 10 cells for each condition. Quantitation of cells from three independent experiments gave similar results.

Confocal images were assembled into figures for publication using Adobe Photoshop (Adobe Systems).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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