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High frequency of neurexin 1β signal peptide structural variants in patients with autism

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Abstract

Neuroligins are postsynaptic membrane cell-adhesion molecules which bind to β-neurexins, a family of proteins that act as neuronal cell surface receptors. To explore the possibility that structural variants in the β-neurexin genes predispose to autism, the coding regions and associated splice junctions of three β-neurexin genes were scanned with detection of virtually all mutations-SSCP (DOVAM-S) in 72 Caucasian patients with autism. In addition, segments of the neurexin 1β gene were sequenced in 131 additional Caucasian and 61 Afro-American patients with autism from South Carolina and the Midwest. Two putative missense structural variants were identified in the neurexin 1β gene in four Caucasian patients with autism and not in 535 healthy Caucasian controls (4/203 vs. 0/535, $P = 0.0056$). Initial family data suggest that incomplete penetrance may occur. In addition, no structural variant was found in the neurexin 2β gene and the neurexin 3β gene. In the context of all available data, we conclude that mutations of the neurexin 1β gene may contribute to autism susceptibility.

Keywords: Autism; β-Neurexin genes; NLGN4 gene; Mutation detection

Autism is a complex developmental disorder that is characterized by deficits in social interaction and communication along with restricted and stereotyped patterns of behavior.

An excess of affected males is observed, consistent with a role for X-chromosomal genes in the etiology of autism. MECP2 missense and 3′ UTR variants have been associated with autism [20]. In addition, mutations in the neuroligin 3 (NLGN3, MIM #300336) and neuroligin four genes (NLGN4, MIM #300427) were identified in patients with autism or mental retardation [9,11,27] (Schwartz, unpublished data).

Neuroligins are postsynaptic membrane cell-adhesion molecules which bind to β-neurexins, a family of proteins that act as neuronal cell surface presynaptic receptors [16]. The three neurexin genes are designated NRXN1 (MIM #600565), NRXN2 (MIM #600566), and NRXN3 (MIM #600567). The neurexin genes have two independent promoters which generate two classes of mRNAs: The longer mRNAs encode α-neurexins and the shorter mRNAs encode β-neurexins [8]. The domain organization of α-neurexin is as follows: An N-terminal signal peptide, three "neurexin repeats," a carbohydrate attachment region, a transmembrane domain, and a short cytoplasmic domain. The neurexin repeats are composed of two lamanin/neurexin/sex (LNS) hormone-binding globulin domains (A and B) flanking an EGF like domain. The beta neurexins contain a different signal peptide, a short region unique to beta neurexins, the LNS [B] domain from the last neurexin repeat followed by the carbohydrate attachment region, the transmembrane and the cytoplasmic domains.

Neurexins display an evolutionarily conserved pattern of extensive alternative splicing. Multiple neurexin proteins can be generated from each of the genes. There are five sites of alternative splicing in the α-neurexin transcripts, two of which are shared with the β-neurexin transcripts. As a result, the total number of neurexins in brain probably exceeds 2000 [24].

The human neurexin genes (NRXN1, 2, 3) span 1.1 Mb, 106 kb, and 1.6 Mb, respectively [10,23]. The NRXN1 and NRXN3 genes have very large introns. The CpG rich promoter

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Table 1
Gender and ethnicity of autism patients and controls

	Caucasian		Afro-American		Total
	Male	Female	Male	Female	
Patients					
Scanned[a]					
South Carolina	48				48
Midwest	21	3			24
Total	69	3			72
Exon 1 sequenced[b]					
South Carolina	45	22	45	7	119
Midwest	52	12	8	1	73
Total	97	34	53	8	192
Controls					
Exon 1 sequenced[b]					
South Carolina		335		194	
Midwest		200			
Total		535		194	729

[a] Patients were scanned for mutations in the neurexin 1β, 2β, and 3β genes.
[b] Exon 1 of the neurexin 1β gene was sequenced.

for β-neurexins is located downstream of exon 17 of the neurexin α genes [17]. The NRXN1β, NRXN2β and NRXN3β transcripts contain 6, 6, and 7 exons, respectively, including 442, 666, and 432 amino acids, respectively.

The interaction of neuroligin and β-neurexin in cultured neurons induces morphological events resembling synapse formation [6], suggesting that neurexins function in the specification and/or initiation of synapse formation. Dean et al. [3] found that β-neurexin clustering was sufficient to trigger the recruitment of synaptic vesicles through interactions that require the cytoplasmic domain of neurexin.

To explore the possibility that structural variants in the β-neurexin genes could predispose to autism, the three β-neurexin genes were scanned in patients with autism.

The study was approved by the Institutional Review Board. The South Carolina autism project (SCAP) enrolled ethnically diverse families from South Carolina. The SCAP population has been described in detail [19] (Table 1). All patients have had a clinical genetic examination, and all meet the DSM IV-R criteria for autism. All patients were initially classified as having autism based on the childhood autism rating scale [18]. In addition, the autism diagnostic interview-revised (ADI-R) [14], was administered for 175 patients, 167 of whom (139 males, 28 females) met the criteria for autism by this instrument. The Vineland adaptive behavior scale (VABS) [22] was administered to every patient as part of the SCAP. Cognitive testing was performed through the individual child's school system or the South Carolina Department of Disabilities and special needs, using standardized tests chosen according to the individual child's level of verbal ability [19,13].

The autism patients from Midwest were previously described [2] (Table 1). The inclusion criteria were [2]: (1) diagnosis of autistic disorder, by use of the ADI-R [14] and age-appropriate versions of the autism diagnostic observation schedule [15] or the pre-linguistic autism diagnostic observation schedule [4]; (2) mental age >18 mo, as assessed by the VABS, Sparrow et al. [22]; (3) nonverbal IQ >35; (4) confirmation of the diagnosis of autistic disorder, by a child psychologist; and (5) exclusion of known etiologies of autistic disorder, by physical examination, including neurological examination and Wood's lamp examination to exclude tuberous sclerosis [21].

The three β-neurexin genes were scanned with detection of virtually all mutations-SSCP (DOVAM-S), a robotically enhanced, high throughput, and multiplex form of SSCP: Half a megabase of genomic sequence is analyzed on one gel under five generic conditions [12]. Three hundred and ninety-one unique mutations (525 total mutations) in six genes were detected in blinded analyses [5]. To detect NRXN mutations, genomic DNA and standard PCR conditions [1] were used to generate separate PCR segments labeled with [α^{33}P] dATP (Amersham, Boston, Massachusetts) which included all coding exons and splice junctions of the NRXN genes. Sequences of the primers are available upon request. The segments were amplified and pooled by the ABI PRISM 877 integrated thermal cycler (Applied Biosystem, Inc., Foster City, CA), denatured, and electrophoresed under five non-denaturing conditions in which gel matrix, buffer, temperature, and additive were varied as previously described [1].

A total of 1.9 Megabases of genomic sequence of three β-neurexin genes were initially scanned in 72 patients with autism (48 South Carolina Caucasians and 24 Midwest US Caucasians, 69 males and three females) and 48 Caucasions without autism, followed by sequencing of exon 1 of neurexin 1β in 131 additional Caucasian and 61 Afro-American patients with autism from South Carolina and the Midwest. Two missense variants (p.S14L and p.T40S) were identified in the neurexin 1β gene in four South Carolina Caucasian patients (Table 2). An in-frame deletion of five GGC triplets and two different in-frame insertions (a single GGC triplet and a two GGC triplet repeats) were identified in the neurexin 1β gene in a total of nine patients (Table 2). No structural variants were found in the neurexin 2β gene and the neurexin 3β gene.

Sequencing of exon 1 of neurexin 1β in 535 healthy Caucasian controls from the Midwest, South Carolina and 194 healthy Afro-American controls from South Carolina did not reveal these two missense variants or any other missense changes (4/264 vs. 0/729, $P = 0.0049$; Caucasian 4/203 vs. 0/535, $P = 0.0056$ Fisher exact test).

The in-frame insertion of a single GGC triplet repeat in neurexin 1β was identified in three controls (2/264 vs. 3/729, $P = 0.40$). The in-frame deletion of a five GGC triplet repeats was found in Afro-American patients and controls only (6/61 vs. 11/194, $P = 0.19$, Fisher exact test), indicating this variant is a common polymorphism in Afro-Americans. No in-frame insertion of the two GGC triplet repeats in neurexin 1β was identified in 535 healthy Caucasian controls and 194 healthy Afro-American controls (1/264 vs. 0/729, $P = 0.27$).

Patient #8139 met ADI-R criteria for autism. He is heterozygous for the p.S14L missense mutation as is his sister. He has an IQ of 50, suffers from seizures, and has some mild facial dysmorphism and hypopigmented areas on his body. The sister has an IQ of 123, a central auditory processing problem, and decreased social interaction, but she does meet criteria for Asperger syndrome. The father of patient 8139 is heterozygous for the p.S14L

Table 2
Structural variants identified in the neurexin 1β gene in patients with autism

No.	Patient ID	Gender	Ethnicity	Exon	NT change	AA change	Family members
1	8139	Male	Caucasian	1	c.41 C>T	p.S14L	Mother wt, father ±, sister ±
	15107	Male	Caucasian	1	c.41 C>T	p.S14L	Mother wt, father ±, brother 1 ±, brother 2 ±
	17160	Female	Caucasian	1	c.41 C>T	p.S14L	n/a
2	13296	Male	Caucasian	1	c.118 A>T	p.T40S	Father wt, mother ±, sister ±
3	1653	Male	Caucasian	1	c.78 ins GGC	p.26 ins G	
	13302	Female	Afro-American	1	c.78 ins GGC	p.26 ins G	
4	2154	Female	Caucasian	1	c.78 ins GGCGGC	p.26 ins GG	
5	Common[a]			1	c.50 G>T	p.G17V	
6	Common[b]			1	c.64–78 del 5 GGC	p.22–26 del 5G	

[a] More than 24 patients.
[b] Six African-American patients.

missense mutation, is good at math. The mother who does not have the p.S14L change, has above average cognitive function and good social skills. However, she reported she stammered as a child. There is a paternal history of learning disabilities.

Patient #15107 has autism by ADI-R criteria. He has a seizure disorder, mild dysmorphic facial features and hypopigmented lesions of the skin at various locations. His IQ was 68. The p.S14L missense mutation is also present in one brother who has learning disorder and hyperactivity. Another brother, who does not carry this variant, is in law school. He displays some hyperactivity. The father, who carries p.S14L, is hyperactive. The mother of patient 15107 does not carry the p.S14L variant, is normal but there is a history of mental retardation on her side.

Patient #13296 who is heterozygous for the p.T40S missense variant, has autism as determined by AD1-R criteria. His IQ is 58 and he has a flat midface. He has a sister who also carries the p.T40S missense mutation. The sister is not reported to have any behavior or learning problems. The mother, who is heterozygous for the p.T40S variation, had learning problems and experienced periods of depression. Additionally, she has a family history of learning problems as does the father of patient 13296, who does not carry the p.T40S variant.

Two missense variants were identified in exon 1, in which the N-terminus of all three β-neurexins contains an unusual cleaved signal sequence [25]. The signal sequences of the β-neurexins are not homologous to each other, but they share several characteristics. They are unusually long (46 amino acids for neurexins 1β and 2β, 35 for neurexin 3β), quite hydrophobic, contain high concentrations of short chain amino acids and do not have the characteristics of a cleaved signal sequence [7,26]. The unusual structures of the signal sequences of the β-neurexins raise the possibility that there may be more mechanistic heterogeneity in signal sequence function than currently appreciated. Thus, mutations in this region may affect the signaling function.

The following caveats should be mentioned: (i) Family data was limited to mostly written records from telephone conversations or brief interviews; (ii) Incomplete penetrance in neuropsychiatric disease is generally expected from lack of concordance in monozygotic twins, but it is also possible that the lack of penetrance may arise from partial equilibrium with a linked causative mutation; (iii) In 6 out of 1000 case control analyses, a *P*-value of equal or greater than this magnitude would be expected by chance; (iv) The patients with autism were non-Hispanic Caucasians of European origin, but detailed ethnicity data are not available, so population stratification cannot be excluded. However, 335 of the controls were ascertained in the same South Carolina population; sequencing of exon 1 of the neurexin-1β gene did not reveal either p.S14L, or the p.T40S, or any novel missense structural variant. As the three neurexin beta genes are similar in function and interact with neuroligins, the absence of variants in the related neurexin 2β and 3β may reflect the limitations of sample size (80% power to detect an attributable risk of 2.2%).

In conclusion, neurexin-1β missense variants are associated with autism in a case-control study ($P < 0.0056$). This finding and the binding of neurexin to NLGN give biological plausibility for a causative link. However, additional studies in other autism populations, as well as analyses of appropriate *in vitro* and animal model systems, are clearly necessary.

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References

[1] C.H. Buzin, C.Y. Wen, V.Q. Nguyen, G. Nozari, A. Mengos, X. Li, J.S. Chen, Q. Liu, R.A. Gatti, F.K. Fujimura, S.S. Sommer, Scanning by DOVAM-S detects all unique sequence changes in blinded analyses: Evidence that the scanning conditions are generic, BioTechniques 28 (2000) 746–753.
[2] E.H.J. Cook, R.Y. Courchesne, N.J. Cox, C. Lord, D. Gonen, S.J. Guter, A. Lincoln, K. Nix, R. Haas, B.L. Leventhal, E. Courchesne, Linkage-disequilibrium mapping of autistic disorder, with 15q11-13 markers, Am. J. Hum. Genet. 62 (1998) 1077–1083.
[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.C. DiLavore, C. Lord, M. Rutter, The pre-linguistic autism diagnostic observation schedule, J Autism Dev. Disord. 25 (1995) 355–379.
[5] J. Feng, J. Yan, W. Li, J. Chen, S.S. Sommer, Candidate gene analyses by scanning or brute force fluorescent sequencing; A comparison of

DOVAM-S with gel-based and capillary-based sequencing, Genet. Test. (in press).
[6] C.C. Garner, J. Nash, R.L. Huganir, PDZ domains in synapse assembly and signaling, Trends Cell Biol. 10 (2000) 274–280.
[7] M. Hortsch, D.I. Meyer, Transfer of secretory proteins through the membrane of the endoplasmic reticulum, Int. Rev. Cytol. 102 (1986) 215–242.
[8] 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.
[9] S. Jamain, H. Quach, C. Betancur, M. Rastam, C. Colineaux, I.C. Gillberg, H. Soderstrom, B. Giros, M. Leboyer, C. Gillberg, T. Bourgeron, Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism, Nat. Genet. 34 (2003) 27–29.
[10] J.J. Kleiderlein, P.E. Nisson, J. Jessee, W.B. Li, K.G. Becker, M.L. Derby, C.A. Ross, R.L. Margolis, CCG repeats in cDNAs from human brain, Hum. Genet. 103 (1998) 666–673.
[11] F. Laumonnier, F. Bonnet-Brilhault, M. Gomot, R. Blanc, A. David, M.P. Moizard, M. Raynaud, N. Ronce, E. Lemonnier, P. Calvas, B. Laudier, J. Chelly, J.P. Fryns, H.H. Ropers, B.C. Hamel, C. Andres, C. Barthelemy, C. Moraine, S. Briault, 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.
[12] Q. Liu, J. Feng, C. Buzin, C. Wen, G. Nozari, A. Mengos, V. Nguyen, J. Liu, F. Crawford, F.K. Fujimura, S.S. Sommer, Detection of virtually all mutations-SSCP (DOVAM-S): A rapid method for mutation scanning with virtually 100% sensitivity, BioTechniques 26 (1999) 932–942.
[13] F. Lobo-Menendez, K. Sossey-Alaoui, J.M. Bell, S.A. Copeland-Yates, S.M. Plank, S.O. Sanford, C. Skinner, R.J. Simensen, R.J. Schroer, R.C. Michaelis, Absence of MeCP2 mutations in patients from the South Carolina autism project, Am. J. Med. Genet. B Neuropsychiatr. Genet. 117 (2003) 97–101.
[14] C. Lord, M. Rutter, A. LeCouteur, Autism diagnostic interview-revised: A revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders, J. Autism Dev. Dis. 24 (1994) 659–685.
[15] C. Lord, E. Schopler, The role of age at assessment, developmental level, and test in the stability of intelligence scores in young autistic children, J. Autism Dev. Disord. 19 (1989) 483–499.
[16] M. Missler, T.C. Sudhof, Neurexins: Three genes and 1001 products, Trends Genet. 14 (1998) 20–26.
[17] L. Rowen, J. Young, B. Birditt, A. Kaur, A. Madan, D.L. Philipps, S. Qin, P. Minx, R.K. Wilson, L. Hood, B.R. Graveley, Analysis of the human neurexin genes: Alternative splicing and the generation of protein diversity, Genomics 79 (2002) 587–597.
[18] E. Schopler, R.J. Reichler, R.F. DeVellis, K. Daly, Toward objective classification of childhood autism: Childhood autism rating scale (CARS), J. Autism Dev. Disord. 10 (1980) 91–103.
[19] R.J. Schroer, M.C. Phelan, R.C. Michaelis, E.C. Crawford, S.A. Skinner, M. Cuccaro, R.J. Simensen, J. Bishop, C. Skinner, D. Fender, R.E. Stevenson, Autism and maternally derived aberrations of chromosome 15q, Am. J. Med. Genet. 76 (1998) 327–336.
[20] A. Shibayama, E.H. Cook Jr., J. Feng, C. Glanzmann, J. Yan, N. Craddock, I.R. Jones, D. Goldman, L.L. Heston, S.S. Sommer, MECP2 structural and 3′-UTR variants in schizophrenia, autism and other psychiatric diseases: A possible association with autism, Am. J Med. Genet. B Neuropsychiatr. Genet. 128 (2004) 50–53.
[21] S.L. Smalley, P.E. Tanguay, M. Smith, G. Gutierrez, Autism and tuberous sclerosis, J. Autism Dev. Disord. 22 (1992) 339–355.
[22] S.S. Sparrow, D.A. Balla, D.V. Cicchetti, Vineland Adaptive Behavior Scales, American Guidance Service, Circle Pines, MN, 1984.
[23] K. Tabuchi, T.C. Sudhof, Structure and evolution of neurexin genes: Insight into the mechanism of alternative splicing, Genomics 79 (2002) 849–859.
[24] B. Ullrich, Y.A. Ushkaryov, T.C. Sudhof, Cartography of neurexins: More than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons, Neuron 14 (1995) 497–507.
[25] Y.A. Ushkaryov, Y. Hata, K. Ichtchenko, C. Moomaw, S. Afendis, C.A. Slaughter, T.C. Sudhof, Conserved domain structure of beta-neurexins. Unusual cleaved signal sequences in receptor-like neuronal cell-surface proteins, J Biol. Chem. 269 (1994) 11987–11992.
[26] G. Von Heijne, A new method for predicting signal sequence cleavage sites, Nucleic Acids Res. 14 (1986) 4683–4690.
[27] J. Yan, G. Oliveira, A.M. Coutinho, C. Yang, J. Feng, C. Katz, J. Sram, A. Bockholt, I.R. Jones, N. Craddock, E.H.J. Cook, A.M. Vicente, S.S. Sommer, Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients, Mol. Psychiatr. 10 (2005) 329–335.