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Brief Research Communication

No Evidence for Involvement of Genetic Variants in the X-Linked Neuroligin Genes *NLGN3* and *NLGN4X* in Probands With Autism Spectrum Disorder on High Functioning Level

Anne-Kathrin Wermter,[1]* Inge Kamp-Becker,[2] Konstantin Strauch,[3] Gerd Schulte-Körne,[2] and Helmut Remschmidt[2]

[1]*Clinical Research Group, Department of Child and Adolescent Psychiatry and Psychotherapy, Philipps-University of Marburg, Marburg, Germany*
[2]*Department of Child and Adolescent Psychiatry and Psychotherapy, Philipps-University of Marburg, Marburg, Germany*
[3]*Institute of Medical Biometry and Epidemiology, Philipps University of Marburg, Marburg, Germany*

Several lines of evidence indicate a role of mutations in the two X-linked genes neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4X*) in the etiology of autistic spectrum disorders. To analyze whether genetic variants in the *NLGN3* and *NLGN4X* genes occurs in patients with autistic disorders on high functioning level, we performed a mutation screen of both genes using SSCP in 107 probands with Asperger syndrome, high-functioning autism and atypical autism. We identified four polymorphisms (rs2290488, rs7049300, rs3747333, rs3747334) and one novel synonymous variant (A558) in the *NLGN4X*. The polymorphisms rs7049300, rs3747333, and rs3747334 did not cause any amino acid substitutions in the total of the eight detected carriers. A family-based association study for rs2290488 in 101 trios did not reveal association of this polymorphism with autistic disorders on high functioning level. We conclude that there is no evidence for an involvement of *NLGN3* and *NLGN4X* genetic variants with autism spectrum disorder on high functioning level in our study group.

KEY WORDS: neuroligin 3; neuroligin 4; autism spectrum disorder; Asperger syndrome; X-chromosome

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Autism spectrum disorders (ASD) are a family of neurodevelopmental disorders characterized by early-onset delays and deviance in the development of social, communicative skills and restricted, stereotyped pattern of interests and activities [Volkmar et al., 2004]. Despite the fact that there are many similarities within the spectrum of autistic disorders the condition is characterized by great variability of clinical presentations. They vary in terms of profile of symptomatology, degree of affectedness, IQ, verbal skills and associated physical disease. The awareness of the heterogeneity gave rise to the conception of "ASD," which includes basically autism, Asperger syndrome (AS) and atypical autism (AA) (pervasive developmental disorder-not otherwise specified, PDD-NOS) [Volkmar et al., 2004].

Twin and family studies have presented a strong genetic predisposition of ASD [Bacchelli and Maestrini, 2006]. Sex differences in the epidemiology of ASD [Fombonne, 2005] may be explained by genetic variations on the X-chromosome. This has prompted some authors to the formulation of an "extreme male brain" [Baron-Cohen et al., 2005] with impaired empathizing and superior systemizing for individuals with ASD.

It is conceivable that X-chromosomal loci may be responsible for these sex differences in social cognition. Linkage results in genomewide scans as well as cytogenetic abnormalities in individuals with ASD provide further evidence for an involvement of the X-chromosomal loci Xq12-q21 and Xp22 in the etiology of ASD [Vorstman et al., 2006].

Two credible candidate genes for ASD, the *neuroligin 3* (*NLGN3*) and *neuroligin 4* (*NLGN4X*) genes, are located in these X-chromosomal regions. Neuroligins belong to a family of postsynaptic cell adhesion molecules and may be involved in the synaptogenesis by interacting with β-neurexins. Neuroligins play a functional role in modulating the development of excitatory and inhibitory synapses and of their balance [Dean and Dresbach, 2006].

Recently, one non-synonymous genetic variant (R451C) in *NLGN3* and one frameshift mutation leading to a premature truncation of the protein (D396X) in *NLGN4X* were detected in two Swedish families in each case one brother with autism and one brother with AS [Jamain et al., 2003]. Both mutations led to an intracellular retention of neuroligin proteins and to their impaired function in the synaptogenesis compared with the wild-type neuroligin proteins [Chih et al., 2004]. In

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Gerd Schulte-Körne's present address is Department of Child and Adolescent Psychiatry, Psychosomatics, and Psychotherapy, University of Munich, Munich, Germany.

*Correspondence to: Anne-Kathrin Wermter, Department of Child and Adolescent Psychiatry and Psychotherapy, Philipps-University of Marburg, Schützenstr. 49, D-35033 Marburg, Germany. E-mail: Anne-Kathrin.Wermter@med.uni-marburg.de

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replication studies further non-synonymous genetic variants in the *NLGN3* and *NLGN4X* were detected in probands with autism, mental retardation (MR) or pervasive developmental disorders (PDD-NOS) [Laumonnier et al., 2004; Yan et al., 2005; Blasi et al., 2006]. Additionally, novel splice variants for *NLGN3* and *NLGN4* with possible implications in autism were identified [Talebizadeh et al., 2006]. In contrast, several studies failed to identify any non-synonymous variants in *NLGN3* and *NLGN4X* in samples of individuals with ASD [Talebizadeh et al., 2004; Vincent et al., 2004; Gauthier et al., 2005; Ylisaukko-oja et al., 2005].

Since the findings are controversial and replication in independent samples is necessary to confirm the role of the mutations for autistic individuals we aim to replicate the reported non-synonymous variants and to find possible novel genetic variants, respectively, in the *NLGN3* and *NLGN4X* genes in a sample of 107 individuals with autism on high functioning level.

We investigated a sample of 107 individuals (102 male, 5 female) with ASD [55 AS, 44 high-functioning autism (HFA), 8 AA]. All children and their parents or caregivers gave their written informed consent after having been informed about the details and the purpose of this study. The study was approved by the ethics committee of the University Hospital Marburg.

The autistic children were diagnosed by experienced clinicians according to the standard criteria of ICD-10 [WHO, 1993] and underwent an extensive psychiatric examination at the Department of Child and Adolescent Psychiatry, University Hospital Marburg. The expression of autistic symptoms was further assessed by the autism diagnostic observation scale (ADOS-G) [Lord et al., 2000] and a autism specific parent interview (ADI-R) [Le Couteur et al., 1989]. The IQ was assessed by the Wechsler Scales (WISC-III/WAIS-R) (age: mean = 12.5 ± 4.7; full scale IQ: mean = 99.84 ± 19, verbal IQ: mean = 107.0 ± 21, performance IQ: mean = 91.5 ± 19).

For mutation screening by single stranded conformation polymorphism analysis (SSCP) the sequence of all coding and 5′ UTR exons including splice junctions of the *NLGN3* and the *NLGN4X* were amplified from genomic DNA using PCR. SSCP was performed as described previously [Hinney et al., 1999] (see Supplementary Tables 1 and 2). For genotyping of four SNPs in the *NLGN4X* in the autistic sample polymerase chain reaction based restriction fragment length polymorphisms (PCR-RFLP) were performed (see Supplementary Table 3). Primers were designed in a careful manner to selectively amplify fragments only of *NLGN4X* and to avoid contamination with amplicons of the highly homologous *NLGN4Y*.

A family-based association test for rs2290488 in 101 trios (of the six remaining probands the DNA of both parents was not available) was performed using the program TDTPHASE of the UNPHASED package version 2.404 [Dudbridge, 2003]. The transmission/disequilibrium test, taking X-chromosomal inheritance into account, showed no association at this marker ($P = 0.68$).

It is hypothesized that a disproportionate high level of excitation or disproportionately weak inhibition in neural circuits leading to a more poorly functionally differentiated cortex and therefore to abnormalities in perception, memory, cognition, and motor control may explain some forms of autism [Rubenstein and Merzenich, 2003]. In both X-linked genes *NLGN3* and *NLGN4*, which may be involved in the synaptogenesis and the modulating of the ratio of excitatory and inhibitory synapses several mutations were detected in patients with ASD [Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005; Blasi et al., 2006; Talebizadeh et al., 2006].

In attempt to identify genetic variants in the *NLGN3* and *NLGN4X* genes we performed a mutation screen of both genes in 107 probands with autistic disorders on high functioning level. We identified one novel synonymous variant (A558) in the *NLGN4X* in one female patient with AS. In addition, we detected four previously known SNPs in the *NLGN4X*. In a family-based association study for rs2290488 in the 5′ UTR we found no evidence for transmission disequilibrium ($P > 0.05$). The remaining three SNPs were detected in a total of eight male patients in the hemizygous state. One boy with AS carried a mutation at the SNP rs7049300 (T311). Seven boys (five AS, two HFA) were carriers of mutations at all three SNPs: the synonymous SNP rs7049300, the non-synonymous SNP rs3747333 (L593) and the synonymous SNP rs3747334 (L593). However, the co-occurrence of rs3747333C > T leading to a substitution of leucine to phenylalanine and rs3747334C > G entails a double substitution of CTC to TTG in codon 593 and thus protect the amino acid leucine. In summary, we could neither replicate any described non-synonymous variants in both X-linked neuroligin genes nor detect any novel non-synonymous mutations in our sample. These results confirm other studies which failed to identify non-synonymous variants in the coding region of *NLGN3* and *NLGN4X* [Talebizadeh et al., 2004; Vincent et al., 2004; Gauthier et al., 2005; Ylisaukko-oja et al., 2005]. The contradictory results of different studies pertaining the detection of mutations in both X-linked neuroligin genes may be caused by the broad variety of included samples: probands with autism, AS, MR, and pervasive developmental disorder-not otherwise specified (PDD-NOS). The heterogeneity of the investigated samples in different studies and the heterogeneity of neurodevelopmental disorders combined in the Autism spectrum disorder limit the comparability of the samples and hampers the elucidation of genetic factors associated with autism and the neurobiological mechanism that underlie its behavioral symptoms [Tager-Flusberg and Joseph, 2003]. To enhance the possibility of finding relevant genetic causes, the phenotype variability in study samples has to be reduced. In the present study we avoid heterogeneity by examining a relatively homogeneously sample of autistic disorders on high functioning level. We suspected an increased likelihood to detect both mutations initially described in probands with autism and AS [Jamain et al., 2003] in our sample consisting mainly of individuals with AS and autism. The lack of confirmation of both mutations in our sample highlights the fact that both mutations may be not enriched in probands with AS and may disrupt function of neuroligins in basic neurodevelopmental mechanisms. Furthermore, the lack of confirmation of non-synonymous sequence variants affirm that mutations in X-linked neuroligin genes seem to explain the etiology of only a small proportion of cases with ASD.

In conclusion, we did not find any evidence for an involvement of both X-linked genes *NLGN3* and *NLGN4X* in the etiology of ASD. Thus our study provides a further indication that variants in the coding sequence of neuroligin genes do not play a causal role in the etiology of ASD or only account for a small proportion of autism individuals. Further studies are warranted to elucidate the function of neuroligins and thus to get insights in the relationship of neuroligins with particular symptoms of autistic or other neurodevelopmental disorders.

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