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SCIENTIFIC CORRESPONDENCE

Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients

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SIR—Jamain *et al*[1] reported a frameshift and a mis sense mutation in the X-linked neuroligin 4 (NLGN4, MIM# 300427) and neuroligin 3 (NLGN3, MIM# 300336) genes, respectively, in Swedish families with autism. A frameshift mutation in NLGN4 appeared *de novo* in the mother, cosegregated with an affected brother with Asperger syndrome and was absent in a normal brother. This frameshift mutation was not present in 600 unrelated control X-chromosomes. A missense mutation in NLGN3, R451C, was found in the mother and two sibs, one with autism and another with Asperger syndrome, but no other relatives were studied. It was not found in 300 unrelated control X-chromosomes.

Laumonnier *et al*[2] reported a large French family in which 10 males had nonspecific X-linked mental retardation, two had autism and one had pervasive developmental disorder. All affected patients were found to have the same frameshift mutation (1253delAG) in the NLGN4 gene. One obligate female carrier had mild mental retardation.

The NLGN3 and NLGN4 genes map to Xq13 and Xp22.3, respectively. The NLGN3 gene spans 32 kb, and the NLGN4 gene spans 338 kb. NLGN3 has eight exons, encoding two alternatively spliced isoforms of 828 and 848 amino acids.[3] The NLGN4 gene contains six exons and codes for a protein of 816 amino acids. All neuroligins contain an N-terminal hydrophobic sequence with the characteristics of a cleaved signal peptide followed by a large esterase homology domain, a highly conserved single transmembrane region, and a short cytoplasmic domain.[4]

To better understand the relationship between the NLGN4 gene and autism, the coding regions and associated splice junctions of the NLGN4 gene were scanned for mutations with DOVAM-S (Detection of Virtually All Mutations-SSCP) and direct sequencing in the following subjects: 148 unrelated patients with autism (76 Midwest US Caucasians and 72 Portuguese Caucasians; 122 males and 26 females), 48 patients without autism, including 24 Midwest US Caucasian patients with attention deficit hyperactivity disorder (ADHD)[5] and 24 UK Caucasian patients with DSM-IV Bipolar I Disorder (BPD),[6] as well as 48 Portuguese healthy control subjects.

The Portuguese autistic patients were diagnosed using DSM-IV criteria, the Autism Diagnostic Interview-Revised (ADI-R) and the Childhood Autism Rating Scale (CARS). Idiopathic subjects were included in the study after clinical assessment and screening for known medical and genetic conditions associated with autism (fragile X, chromosomal disorders, neurocutaneous syndromes, metabolic disorders, infectious diseases). Neuropsychological evaluation was performed using the Ruth Griffiths Mental Developmental Scales or the Wechsler Intelligence Scale for Children (WISC), depending on the patient's age. The Midwest autistic patients were diagnosed as described previously.[7]

Putative missense mutations were identified once each in the NLGN4 gene in four separate autistic patients (Table 1). G99S and K378R were found in unrelated Portuguese patients. V403M and R704C were found in unrelated Midwest patients. G99, K378 and V403 are located in the esterase domain and R704 is located in the cytoplasmic domain.[4] Three of the structural changes, K378R, V403M and R704C, occur in asymptomatic mothers, while G99S occurs in a mother with learning disability.

Comprehensive mutation scanning of 48 Portuguese healthy controls and sequencing of the appropriate exons in 288 healthy controls including 96 Portuguese and 192 Midwest US Caucasians (144 males and 192 females; 528 X-chromosomes total) did not reveal these four missense variants or any other structural changes (4/148 *vs* 0/336, $P=0.009$ or 4/174 *vs* 0/528, $P=0.004$, when the Fisher exact test is performed with patients or X-chromosome alleles, respectively).

In addition, no structural variants were found in a pilot experiment performed on patients with ADHD and BPD (24 of each).

Patient #1 has a younger brother with a diagnosed language disability and a global developmental quotient below the mean (Ruth Griffiths Mental Developmental Scales score of 89), who also carries the G99S variation. Their mother, who is heterozygous for the variation, had a documented learning disability. As also found by Laumonnier *et al*, sequence variation in NLGN4 may be segregating with autism and cognitive disability in this family.

Patient #3 had an affected brother with V403M. He had three other unaffected sibs, including a sister without the variant and two brothers with V403M who had normal social function (making friends easily), normal school performance and no attentional problems, consistent with an absence of cosegregation between this variant and any phenotype. The three unaffected children had Social Communication Questionnaire (SCQ) (Lifetime)[8] scores of 0. Proband #3 was specifically of Irish descent. In all, 50 normal female controls of Irish descent were sequenced, none of them had V403M.

Table 1 Structural variants identified in the NLGN4 gene

ID	*NT change*	*A.A change*	*Conservation*[a]	*Clinical information*	*Family members*[b]
#1	759 G>A	G99S	NLGN2/3/4/4Y	Portuguese Caucasian, female Diagnosed at age 3 years with severe autism (the CARS score was 52, and scores on the three domains of the ADI-R, social interaction, nonverbal communication and repetitive behaviors were 26, 12 and 4, respectively) and mental retardation (GDQ of 31, with a cognitive profile characteristic of autistic children: performance DQ of 39 and language DQ of 18). The patient belongs to a large sibship, with the mother, who also carries the variant, and four sibs reporting learning disability without autism Patient #1 has a younger brother with a diagnosed language disability and a global developmental quotient below the mean (Ruth Griffiths Mental Developmental Scales score of 89), who also carries the G99S variation. Their mother, who is heterozygous for the variation, had a documented learning disability. As also found by Laumonnier *et al*, sequence variation in NLGN4 may be segregating with autism and cognitive disability in this family	Mother: +/− Father: + Mother has learning disability. A brother with language disability: −
#2	1597 A>G	K378R	NLGN1/2/3/4/4Y	Portuguese Caucasian, male Diagnosed at age 4 years with mild autism (scores on the ADI-R domains of social interaction, verbal communication and repetitive behaviors were 20, 12 and 5, respectively), with normal IQ. The parents reported regression from age 2 years in language development and social interaction skills. There was no family history of neurological or psychiatric disease	Mother: +/− Father: +
#3	1671 G>A	V403M	NLGN1/2/3/4/4Y *Drosophila* NL	Midwest Caucasian, male Diagnosed at age 4 years with PDD NOS[d] (scores on ADI-R domains of social interaction, verbal communication, and repetitive and restricted behaviors were 10, 9 and 7, respectively) with ratio nonverbal IQ of 84 and ratio verbal IQ of 79; ADOS was consistent with PDD NOS; head circumference 53.3 cm at 39 months Patient #3 had an affected brother with V403M. He had three other unaffected sibs, including a sister without the variant and two brothers with V403M who had normal social function (making friends easily), normal school performance, and no attentional problems, consistent with an absence of cosegregation between this variant and any phenotype	Mother: +/− Father: + Affected brother: − (Autistic disorder) Unaffected brothers (2): −
#4[c]	2574 C>T	R704C	NLGN1/3/4/4Y	Midwest Caucasian, male Diagnosed at age 6 years with autistic disorder (scores on ADI-R domains of social interaction, verbal communication, and repetitive and restricted behaviors were 27, 22 and 10, respectively) with nonverbal IQ of 85 ADOS was consistent with autistic disorder; head circumference 52.9 cm at 6 years One sister has PDD NOS, and the other, her dizygotic twin, is unaffected. A paternal first cousin has autism	Mother: +/− Father: + Sister with PDD NOS: + Unaffected sister: +/−

[a]Each of the amino-acid change is at residue strictly conserved in either four or five of the five members of the neuroligin gene family.
[b]+ wild type allele; − variant allele; +/− heterozygotes.
[c]R704C was discovered in one of 52 Midwest autism patients for which whole genome amplification was performed with multiple displacement amplification (MDA). The 52 MDA amplified samples passed stringent quality control.[14] The presence of R704C also was confirmed from remaining original genomic DNA.
[d]PDD NOS—Pervasive developmental disorder not otherwise specified.

The lack of cosegregation of V403M suggests caution in interpreting the results and highlights the need for future work. V403M may not be of functional significance. However, incomplete penetrance for V403M seems the likely conclusion, because case–control association studies routinely detect incomplete penetrance, for example, a hypothetical penetrance of 25%, which is compatible with segregation in this family, implies a 200-fold relative risk of autism imparted by V403M. Indeed, this case–control design cannot *a priori* distinguish full from partial penetrance. In addition, the epidemiological and biological context of our work is consistent with the interpretation that missense variants with incomplete penetrance predispose to autism as follows: (i) incomplete penetrance is the rule in psychiatric diseases (eg BPD, schizophrenia, autism) as the concordance (or concordance for disease spectrum phenotypes) found in monozygotic twins can vary from 43 to 92% and typically is 15% or lower in dizygotic twins who share similar environment and half of the genes;[9–11] (ii) the strict conservation of V403 in the five members of the neuroligin family (representing 2 + billion years of evolutionary divergence[12,13]) suggests that amino-acid substitution at V403 is deleterious; (iii) the affected sibs have V403M, consistent with a single genetic mechanism as generally expected in a single family with this uncommon disease (0.2% incidence) and (iv) the current case–control studies complement the data of Jamain *et al*[1] and Laumonnier *et al*[2] that strongly suggest protein-truncating mutations predispose to autism. Efforts are underway to ascertain extended families of the four probands and to characterize them clinically and genetically. Demonstration of cosegregation in all affected individuals will be helpful to confirm the putative association of each variant with autism. If the association is confirmed, study of unaffected individuals will allow an estimate of penetrance.

Patient #4 has two sisters who are dizygotic twins. One sister has PDD NOS (pervasive developmental disorder not otherwise specified), close but not quite mild mental retardation and lacks R704C. The other sister is heterozygous for R704C and is not affected. The lack of R704C inherited from the mother in the sister with PDD NOS seemingly falsifies the putative association, unless autism, autism spectrum disorder or mental retardation occurs in the paternal lineage. Indeed, exploration of the paternal lineage reveals that one of two paternal first cousins has autism. Thus, two genetic mechanisms may operate in this family. More work is needed to interpret the segregation pattern in this family.

No structural variants were found in the NLGN3 gene when the 96 unrelated patients with autism (24 Midwest and 72 Portuguese), 24 ADHD and 24 BPD patients were analyzed.

In conclusion, scanning and sequencing of 2.5 Mb of the NLGN3 and NLGN4 genes reveals an association of NLGN4 structural variants at highly conserved amino acids with an estimated attributable risk for autism of about 3% in these cohorts. This study suggests that missense changes in neuroligin 4 may contribute to autism susceptibility as well as the protein-truncating mutations reported by Jamain *et al*[1] and Laumonnier *et al*.[2]

Autism and language disability were present in hemizygous male relatives and a learning disability was present in one of four heterozygous mothers. The presence of V403M in two normal brothers raises the distinct possibility of incomplete penetrance. Male relatives with a missense change have language disability, autism or normal function, while female relatives who are heterozygous for a missense variant are normal, or have a learning disability. Incomplete penetrance has important clinical implications, for example, the presence of an NLGN4 mutation in a fetus does not necessarily imply autism or mental retardation.

GenBank Accession numbers:

The following protein sequences were used: AAM46112 for NLGN4, AAM46113 for NLGN4Y, AAF71230 for NLGN3, AAH32555 for NLGN1, AAM46111 for NLGN2 and AAF52450 for *Drosophila neuroligin*.

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Psychopathological symptoms during interferon-α and ribavirin treatment: effects on virologic response

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SIR—Interferon-α (IFN-α), the most effective treatment for chronic hepatitis by virus C (HCV), induces depression, anxiety, and fatigue.[1–3] We report here that depression and anxiety have different time courses compared to fatigue, and that patients experiencing more severe fatigue are less likely to respond to IFN-α by clearing the virus.

We studied all patients who started pegylated IFN-α (pegIFN-α-2b, 1.5 μg/kg, weekly, s.c.) and ribavirin (400 mg, b.d.) between February and July 2001: $n = 29$, 23 males and six females; 23 Caucasians; mean (SD) age 43 ± 7 years; 16 virus genotype 1, 13 virus genotype non-1. All subjects were HCV RNA positive, with histological evidence, by liver biopsy, of moderate or severe HCV-related injury. Treatment lasted 24 weeks for genotype non-1, and 48 weeks for genotype 1. In all, 18 patients (64%; one subject was lost to follow-up) were identified as sustained virologic responders, as indicated by serum HCV RNA undetectable (<100 copies/ml, Roche Amplicor System) at 24 weeks after the end of treatment. Approval was received from Kings College Hospital Research Ethics committee.

To monitor psychiatric adverse effects and quality of life, four self-report questionnaires were administered at baseline, weekly for the first 4 weeks of treatment, and four-weekly thereafter until the end of therapy: the Beck Depression Inventory (BDI),[4] the Stait and Trait Anxiety Inventory (STAI),[5] Chalder's Fatigue Questionnaire (CFQ);[6] and three items from the SF-36 (physical role limitation, emotional role limitation, and physical function).[7] At baseline, the scores were: BDI, 12 ± 9; STAI (added scores), 87 ± 21; CFQ, 16 ± 5; and SF-36 (added scores), 242 ± 77. Nine patients (31%) described a previous psychiatric history and were all included in the study; four patients were taking an antidepressant at baseline and continued with this throughout the treatment. The results of the questionnaires during the treatment were modeled as a polynomial function of time, generating curves fitted through all available points, describing both courses over time and changes from baseline using summary measures.

During the treatment, depression and anxiety increased linearly with time (depression: $t(26) = 4.3$, $P < 0.001$; anxiety: $t(26) = 3.1$, $P = 0.004$). Therefore, we used the slopes of the regression lines as summary measures. At 24 weeks, the estimated increases were 4.5 on the BDI (95% CI: 2.4–6.6) and 8.6 on the STAI (3.0–14.3). In contrast, fatigue increased, and quality of life decreased, predominantly during the first 8 weeks of treatment, while the curves remained relatively flat at later time points (fatigue: $t(25) = 3.3$, $P = 0.003$; quality of life: $t(25) = -7.4$, $P < 0.001$). Therefore, we used the differences between 8 weeks and baseline as summary measures. At 8 weeks, the estimated increase in the CFQ was 3.5 (1.3–5.7), and the estimated decrease in the SF-36 was 117.8 (85.1–150.5). Of note is that Capuron *et al*[8] examined patients treated with IFN-α for malignant melanoma, and also found that fatigue (with anorexia and pain) appears earlier than depression and anxiety. However, we are the first to describe that the reduction in quality of life follows the course of the fatigue, rather than that of depression or anxiety. Interestingly, better quality of life at baseline predicted a larger reduction in quality of life during the treatment ($P = 0.046$).

Baseline mental state did not predict virologic response (all P-values > 0.5), but changes in mental state did. Specifically, the 10 patients who did not respond had had greater deterioration in all four psychological variables during the treatment. This reached statistical significance for fatigue: the CFQ increase at 8 weeks was 7.4 in nonresponders and only 2.3 in responders (CI for the difference: 1.1–9.1; $P = 0.016$). This result is particularly striking as the fatigue was measured months before the time in which the viral response was measured. Moreover, this was not due to a difference in virus genotypes in responders *vs* nonresponders, as changes in mental state were not associated with virus genotype (all P-values > 0.5). Although these are preliminary findings in a small sample, our findings are consistent