Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism

Many studies have supported a genetic etiology for autism. Here we report mutations in two X-linked genes encoding neuroligins NLGN3 and NLGN4 in siblings with autism-spectrum disorders. These mutations affect cell-adhesion molecules localized at the synapse and suggest that a defect of synaptogenesis may predispose to autism.

Autism is characterized by impaired reciprocal social interaction and communication and restricted and stereotyped patterns of interests and activities[1]. Asperger syndrome is characterized by higher cognitive abilities and more normal language function[2]. The recurrence risk of autism in siblings is approximately 45 times greater than in the general population, and twin studies have documented a higher concordance rate in monozygotic (60–91%) than in dizygotic twins (0–6%; ref. 3). The male-to-female ratio is 4:1 in autism and 8:1 in Asperger syndrome. Male predisposition to autistic disorder has not been explained, although abnormalities of the sex chromosomes are frequently associated with autistic spectrum disorders[4]. At least two loci on the X chromosome have been suggested to be associated with a predisposition to autism. At Xp22.3 *de novo* chromosomal deletions have been observed in three autistic females[5], and a second locus at Xq13–21 has shown greater allele sharing around markers *DXS7132* (52 cM) and *DXS6789* (62 cM) in analyses of pairs of affected siblings in two independent genome scans[6,7].

Within the deleted interval on Xp22.3, we identified the transcript *KIAA1260*, which corresponds to *NLGN4*, a member of the neuroligin family[8]. Notably, *NLGN3* (ref. 9), a homolog of *NLGN4*, is located at Xq13 (55–56 cM) within the second locus on chromosome X associated with autism. These genes encode cell-adhesion molecules present at the postsynaptic side of the synapse[10,11] and may be essential factors for the formation of functional synapses[12]. Five genes encoding neuroligins have been identified in the human genome, localized at 3q26 (*NLGN1*), 17p13 (*NLGN2*), Xq13 (*NLGN3*), Xp22.3 (*NLGN4*) and Yq11.2 (*NLGN4Y*). Neuroligin phylogeny suggests that *NLGN3* is the common ancestor with *NLGN4* and *NLGN4Y* (data not shown). Sequence comparison showed that all amino acids known to be essential

for neuroligins are conserved in NLGN4 and NLGN4Y, including the cysteines, the transmembrane domain and the postsynaptic density 95–discs large–zona occludens-1 (PDZ) binding domain.

We determined the expression profiles of *NLGN* genes by specific RT–PCR in individual male and female adult brain tissues (Fig. 1). We sequenced all RT–PCR products and found that they correspond to alternative splicing isoforms. We detected transcripts from *NLGN1*, *NLGN2* and *NLGN3* in all brain regions. We detected *NLGN4* and *NLGN4Y* at similar levels in male brains, with no considerable differences in regional distribution. There is an alternative promoter 693 bp downstream of *NLGN4* exon 1a, in which exon 1b is substituted in place of exon 1a. Exon 1b is specific to *NLGN4* and contains an alternative donor-splice site.

We screened for mutations in *NLGN3*, *NLGN4* and *NLGN4Y* in 36 pairs of affected siblings and 122 trios with autism or Asperger syndrome (140 males and 18 females). In one Swedish family with two affected brothers, one with typical autism and the other with Asperger syndrome, we identified a frameshift mutation (1186insT) in *NLGN4* (Fig. 2*a*). This mutation creates a stop codon at position 396, leading to premature termination of the protein before the transmembrane domain. The mutation was present in the mother but was absent in the maternal grandmother and in two maternal aunts. The unaffected maternal grandfather was deceased and could not be studied. A false paternity of the maternal grandfather was excluded by studying eight microsatellites markers from the X chromosome (data not shown). These results indicate that 1186insT is a *de novo* mutation in the mother. The mutation was not found in an unaffected brother or in 350 unrelated controls (250 females and 100 males).

In a second Swedish family with two affected brothers, one with typical autism and the other with Asperger syndrome, we identified a C→T transition in *NLGN3*. This mutation was inherited from the mother and changes a highly conserved arginine residue to cysteine (R451C) within the esterase domain (Fig. 2*b*). Arg451 is located in a predicted EF-hand domain conserved in all known neuroligins (including *Drosophila melanogaster* NL and
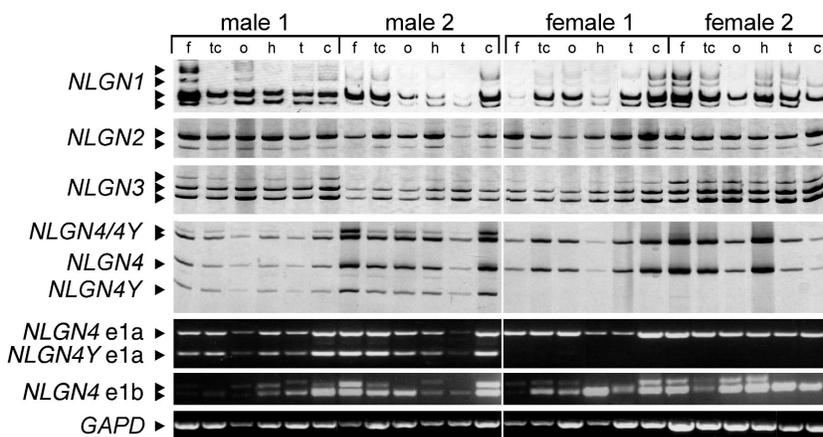
Fig. 1 Expression of *NLGN* genes in the human brain. Specific RT–PCRs were carried out on total RNA from different brain regions using primers in exon 2 and 5 to analyze the different alternatively spliced transcripts. To distinguish between *NLGN4* and *NLGN4Y* mRNA, RT–PCRs were digested by Nco I. Using specific forward primers in exon 1a (e1a) or exon 1b (e1b) and reverse primers in exon 2, the two alternative *NLGN4* promoters were identified. The size of the PCR products corresponding to *NLGN4* and *NLGN4Y* differ by 193 bp. The ages of the two males and the two females studied were 74, 42, 55 and 36 years with a post-mortem delay of 10, 21, 24 and 2 h, respectively. f, frontal cortex; tc, temporal cortex; o, occipital cortex; h, hippocampus; t, thalamus; c, cerebellum. Normal control human brains were obtained at autopsy under guidelines approved by the ethics committee. DNA amplification and RT–PCRs were carried out as described[15]. Alignment and sequences of all NLGN proteins and isoforms are available from the authors.
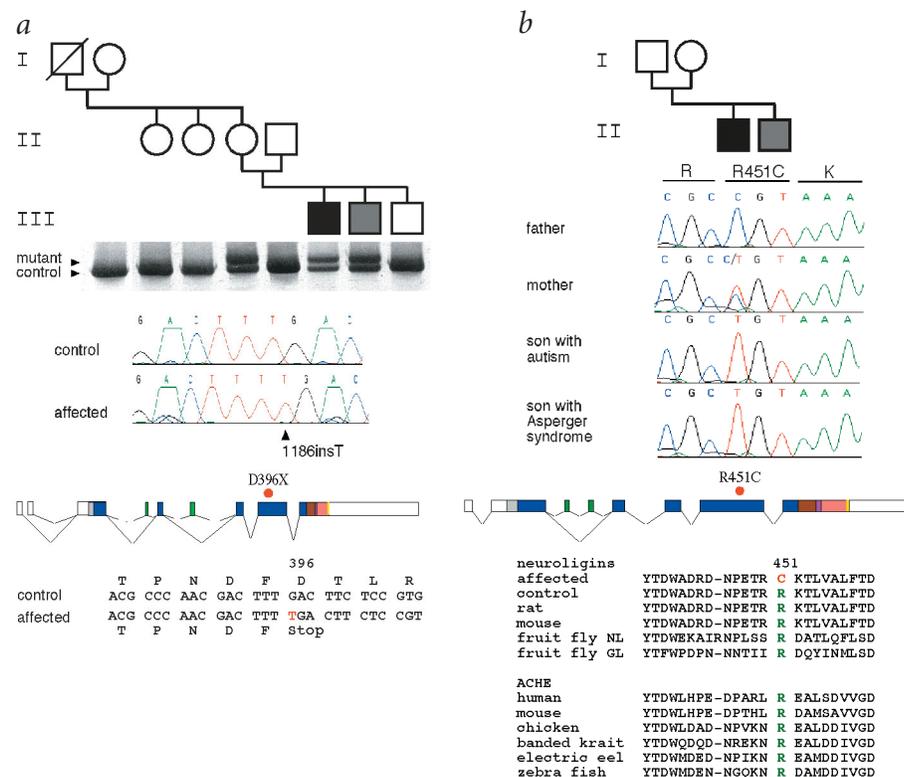
Fig. 2 Screening for mutations in *NLGN3* and *NLGN4* in autistic individuals. *a*, Identification of the frameshift *NLGN4* mutation in two individuals with autism and Asperger syndrome. Pedigree structure and the abnormal single-strand conformation polymorphism are shown. Using *NLGN4*-specific primers, sequence of the PCR product identified a 1-bp insertion (1186insT) in the mother and her two affected sons, which was absent in the father and the unaffected son. This insertion occurs in exon 5 of *NLGN4* and causes a frameshift that leads to the premature termination of NLGN4, which thus lacks 421 amino acids (51% of the protein), including the transmembrane domain. *b*, Identification of the mutation in *NLGN3*. The family includes a son with autism and his younger brother with Asperger syndrome. The R451C mutation is localized in the esterase domain of the protein and modifies a highly conserved arginine residue present in neuroligins and acetylcholine esterase (ACHE). Affected individuals are indicated by filled symbols (black for autism and gray for Asperger syndrome). Squares represent males, circles represent females and a line through a symbol indicates that the person is deceased. We carried out single-strand conformation polymorphism analysis with a GenePhor apparatus (Pharmacia-Biotech) and direct sequencing with BigDye terminator cycle Sequencing Kit (Applied Biosystems) on an ABI 3100 Automated Sequencer.

gliotactin) and in all sequenced esterases from mammals, fish and birds[13]. EF-hand domains are known to confer structural integrity and Ca^{2+}-dependent functional properties. R451C may therefore modify the binding of neuroligins to their presynaptic partners, neurexins, as binding is only observed in the presence of Ca^{2+} (ref. 14). This mutation was absent in 200 controls (100 females and 100 males). See Supplementary Note 1 online for detailed clinical information for these families.

Three independent lines of evidence strongly suggest that mutations in *NLGN3* and *NLGN4* are involved in autistic spectrum disorders. First, deletions at Xp22.3 that include *NLGN4* have been reported in several autistic individuals[5]. Second, the point mutations in *NLGN3* and *NLGN4* cause severe alterations of the predicted protein structure. Third, a mutation in *NLGN4* appeared *de novo* in one affected individual's mother. Among the various proteins involved in the establishment of the neural networks, cell-adhesion molecules are essential factors for the identification of the appropriate partner cell and the formation of a functional synapse. Therefore, we hypothesize that a defect in NLGN3 or NLGN4 may abolish formation, stabilization or recognition of specific synapses essential for the communication processes that are deficient in individuals with autistic spectrum disorder.

URL. All primers used in this study are available on request or at http://www.im3.inserm.fr/autism/Recherche.htm.

GenBank accession numbers. The cDNA sequences of *NLGN2*, *NLGN4* and *NLGN4Y* are deposited in GenBank under accession numbers AF376802, AF376803 and AF376804, respectively.

Note: Supplementary information is available on the Nature Genetics website.

Competing interests statement
The authors declare that they have no competing financial interests.

Stéphane Jamain[1], Hélène Quach[1], Catalina Betancur[2], Maria Råstam[3], Catherine Colineaux[2,4], I. Carina Gillberg[3], Henrik Soderstrom[3], Bruno Giros[2], Marion Leboyer[2,5], Christopher Gillberg[3,6], Thomas Bourgeron[1] & Paris Autism Research International Sibpair Study[7]

[1]*Laboratoire de Génétique Humaine et Fonctions Cognitives, Université Paris 7, Institut National de la Santé et la Recherche Médicale E0021, Institut Pasteur, 75015 Paris, France.* [2]*Institut National de la Santé et la Recherche Médicale U513, Faculté de Médecine, Université Paris XII, 94000 Créteil, France.* [3]*Department of Child and Adolescent Psychiatry, Göteborg University, 41119 Göteborg, Sweden.* [4]*Department of Child and Adolescent Psychiatry, Hôpital Robert Debré, 75019 Paris, France.* [5] *Department of Psychiatry, Hôpital Albert Chenevier et Henri Mondor, 94000 Créteil, France.* [6]*Saint George's Hospital Medical School, SW17 ORE London, UK.* [7]*Christopher Gillberg, Maria Råstam, Carina Gillberg, Agneta Nydén & Henrik Söderström, Department of Child and Adolescent Psychiatry, Göteborg University, Göteborg, Sweden; Marion Leboyer, Department of Psychiatry, Hôpital Albert Chenevier et Henri Mondor, Créteil, France; Catalina Betancur, Anne Philippe & Bruno Giros, Institut National de la Santé et la Recherche Médicale U513, Faculté de Médecine, Créteil, France; Catherine Colineaux, Deborah Cohen, Nadia Chabane & Marie-Christine Mouren-Siméoni, Service de Psychopathologie de l'Enfant et l'Adolescent, Hôpital Robert Debré, Paris, France; Alexis Brice, Institut National de la Santé et la Recherche Médicale U289, Hôpital de la Salpêtrière, Paris, France; Eili Sponheim & Ingrid Spurkland, Centre for Child and Adolescent Psychiatry, University of Oslo, Oslo, Norway; Ola H. Skjeldal, Department of*

Pediatrics, Rikshospitalet, University of Oslo, Oslo, Norway; Mary Coleman, Department of Pediatrics, Georgetown University School of Medicine, Washington D.C., USA; Philip L. Pearl, Children's National Medical Center, George Washington University School of Medicine, Washington, D.C., USA; Ira L. Cohen & John Tsiouris, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, USA; Michele Zappella, Grazia Menchetti & Alfonso Pompella, Divisione di Neuropsichiatria Infantile, Azienda Ospedaliera Senese, Siena, Italy; Harald Aschauer, Department of General Psychiatry, University Hospital, Vienna, Austria; Lionel Van

Maldergem, Centre de Génétique Humaine, Institut de Pathologie et de Génétique, Gerpinnes, Loverval, Belgium. Correspondence should be addressed to T.B. (e-mail: thomasb@pasteur.fr).

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders* 4th edn. (American Psychiatric Press, Washington D.C., 1994).
2. Gillberg, C. *Br. J. Psychiatry* **172**, 200–209 (1998).
3. Folstein, S.E. & Rosen-Sheidley, B. *Nat. Rev. Genet.* **2**, 943–955 (2001).
4. Gillberg, C. *J. Autism Dev. Disord* **28**, 415–425 (1998).
5. Thomas, N.S. *et al. Hum. Genet.* **104**, 43–48 (1999).
6. Shao, Y. *et al. Am. J. Med. Genet.* **114**, 99–105 (2002).
7. Auranen, M. *et al. Am. J. Hum. Genet.* **71**, 777–390 (2002).
8. Bolliger, M.F., Frei, K., Winterhalter, K.H. & Gloor, S.M. *Biochem. J.* **356**, 581–588 (2001).
9. Philibert, R.A., Winfield, S.L., Sandhu, H.K., Martin, B.M. & Ginns, E.I. *Gene* **246**, 303–310 (2000).
10. Ichtchenko, K., Nguyen, T. & Sudhof, T.C. *J. Biol. Chem.* **271**, 2676–2682 (1996).
11. Song, J.Y., Ichtchenko, K., Sudhof, T.C. & Brose, N. *Proc. Natl. Acad. Sci. USA* **96**, 1100–1105 (1999).
12. Scheiffele, P., Fan, J.H., Choih, J., Fetter, R. & Serafini, T. *Cell* **101**, 657–669 (2000).
13. Tsigelny, I., Shindyalov, I.N., Bourne, P.E., Sudhof, T.C. & Taylor, P. *Protein Sci.* **9**, 180–185 (2000).
14. Nguyen, T. & Sudhof, T.C. *J. Biol. Chem.* **272**, 26032–26039 (1997).
15. Jamain, S. *et al. Mol. Psychiatry* **7**, 302–310 (2002).

Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders

Dietary fat is an important source of nutrition. Here we identify eight mutations in *SARA2* that are associated with three severe disorders of fat malabsorption. The Sar1 family of proteins initiates the intracellular transport of proteins in COPII (coat protein)-coated vesicles. Our data suggest that chylomicrons, which vastly exceed the size of typical COPII vesicles, are selectively recruited by the COPII machinery for transport through the secretory pathways of the cell.

The small intestine is the site of absorption of dietary fat and fat-soluble vitamins. The fats enter the luminal surface of the absorptive cell as lipolytic products and emerge from the basolateral surface as chylomicrons. Each particle carries a large neutral lipid core surrounded by a molecule of apolipoprotein (Apo) B48 and a monolayer of phospholipids and cholesterol. Dietary lipid is an important source of energy, but in excess, it may be stored in fat depots. Chylomicrons also supply the liver with lipids. These are re-packaged as lipoproteins containing ApoB100, elevated levels of which constitute a risk factor for cardiovascular disease.

Table 1 • Mutations in CMRD, Anderson disease and CMRD-MSS.

Family, individual	Diagnosis	Origin	Status	Mutation	Amino-acid change	Predicted effect on protein
1, II-1	AD[4]	Algerian	Hom	109G→A	G37R	No affinity for GDP/GTP
1, II-2	AD[4]	Algerian	Hom	109G→A	G37R	No affinity for GDP/GTP
2, II-1	CMRD[1]	White Canadian	Hom	409G→A	D137N	Reduced affinity for GDP/GTP
2, II-2	CMRD[1]	White Canadian	Hom	409G→A	D137N	Reduced affinity for GDP/GTP
3, II-1	CMRD[1]	White Canadian	Het	409G→A	D137N	Reduced affinity for GDP/GTP
			Het	75–76delTG	L28fsX34	Translation arrested after 34 residues
4, II-1	CMRD[1]	White Canadian	Hom	537T→A	S179R	No affinity for GDP/GTP
5, II-1	CMRD[2]	Turkish	Hom	555–558dupTTAC	G187fsX199[a]	Substitution of amino acids forming helix 6 and reduced affinity for ER membrane[11]
5, II-2	CMRD[2]	Turkish	Hom	555–558dupTTAC	G187fsX199	Substitution of amino acids forming helix 6 and reduced affinity for ER membrane[11]
6, II-1	CMRD-MSS[5]	Italian	Hom	349–1G→C	Null allele[b]	No translated product
					D116VSX119	Translation arrested after 118 residues
					S117–K160del	Loss of secondary structure
6, II-2	CMRD-MSS[5]	Italian	Hom	349–1G→C	Null allele	No translated product
					D116VSX119	Translation arrested after 118 residues
					S117_K160del	Loss of secondary structure
7, II-1	CMRD-AD[c]	Pakistan	Hom	536G→T; 542T→C	S179I+L181P[d]	No affinity for GDP/GTP
8, II-1	AD[3]	Algerian	Hom	109G→A	G37R	No affinity for GDP/GTP

Nucleotides are numbered from A of the initiation codon (ATG). AD, Anderson disease. [a]The mutated allele replaces amino acids 187–198 of Sar1b with the amino-acid sequence LRRRLPLDGTVH. [b]The outcome of this mutation could include exon skipping, activation of a nearby cryptic splice site or production of an unspliced mRNA. All would substantially disrupt the protein. [c]See Supplementary Note 1 online. [d]Each parent was heterozygous on one allele with respect to both sequence variants. hom, homozygous; het, heterozygous; fs, frameshift; del, deletion; dup, duplication; X, stop.