SUPPLEMENTARY MATERIAL

Materials and Methods

Families

Families (n=226) with at least one child with ASD were recruited by the PARIS (Paris Autism Research International Sibpair) study at specialized clinical centers in seven countries (France, Sweden, Norway, Italy, Belgium, Austria, and the United States); 163 families had one child with ASD and 63 families had at least two children with ASD. Diagnosis was based on clinical evaluation by experienced clinicians, DSM-IV criteria[1], and the Autism Diagnostic Interview-Revised (ADI-R)[2]. In Sweden, the Diagnostic Interview for Social and Communication Disorders (DISCO-10)[3] was used instead of the ADI-R in some cases. Like the ADI-R, the DISCO-10 utilizes algorithms based on ICD-10 and DSM-IV research criteria to diagnose autism. However, it can also be used to diagnose other pervasive developmental disorders, such as pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger syndrome. The diagnosis of Asperger syndrome was confirmed using the Asperger Syndrome Diagnostic Interview (ASDI)[4]. The sample consisted of 177 males and 50 females; there were 194 patients with autistic disorder and 29 with Asperger syndrome; 4 individuals narrowly missed the criteria for autistic disorder and were considered to have atypical autism (PDD-NOS). Laboratory tests to rule out medical causes of autism included standard karyotyping, fragile-X testing, and metabolic screening; brain imaging and EEG were performed when possible. Patients diagnosed with medical disorders, such as fragile X syndrome or chromosomal anomalies, were excluded from the study. The local research ethics boards reviewed and approved the study. Informed consent was obtained from all families. There were 206 Caucasian, 7 Black, 1 Asian and 13 families of mixed ethnicity.

The control sample (n=270) comprised 120 Caucasians from France and 150 Caucasians living in Sweden. An additional control group of 63 individuals from China was screened for rare variants because one proband carrying the R300C mutation had parents originating from this country. For exons with no mutation in ASD (2, 3, 9, 11, 13-17), 190 controls (95 from France and 95 from Sweden) were sequenced. Using this minimum sample size (n = 380 chromosomes), we can detect a polymorphism with a frequency of 1% with 95% power[5]. The controls from Sweden were part of two population-based cohorts living in Göteborg, originally recruited for a study of obesity and body fat distribution[6,7], who volunteered to provide a blood sample for genetic studies. Women (n=30) were born in 1956 and men (n=65) were born in 1944; subjects with one or two parents probably or certainly being non-Caucasian were excluded.

The controls from France (40 females and 55 males) were healthy volunteers, between 19 and 65 years old, interviewed with the Diagnostic Interview for Genetic Studies (DIGS) and the Family Interview for Genetic Studies (FIGS) to confirm the absence of both personal and family history of psychiatric disorders in first- and second-degree relatives.

Genomic structure and RT-PCR analyses of *SHANK3*

The genomic structure of *SHANK3* was deduced using the published data from Wilson *et al.* [8], the Expressed Sequence Tags (ESTs), and the rat cDNA (AJ133120) published in databases. *SHANK3* transcripts were detected in human brain regions from three independent controls (two females and one male) and in human tissues using the Clontech cDNA panel (Clontech Laboratories Inc). Total

RNA was isolated from brain tissues by the acid-guanidium thiocyanate phenol chlorophorm method and reverse transcribed by oligodT priming using SuperScript™ II Reverse Transcriptase (Invitrogen). Primer sequences for RT-PCR are indicated in Supplementary Table 2. Before sequencing the alternatively spliced exons, the breakpoint in family ASD 1 and the frame-shift mutation in family ASD 2, the PCR products were cloned with TOPO–TA cloning kit (Invitrogen). Prediction of the Quadruplex forming G-Rich Sequences (QGRS) at chromosome 22q13.3 was performed using the GQRS mapper software (http://bioinformatics.ramapo.edu/QGRS/index.php)[9].

Mutation analysis

DNA was extracted from blood leukocytes or B lymphoblastoid cell lines with the phenol chlorophorm method[10]. In the mother of family ASD 2, DNA from buccal cells was extracted using the BuccalAmp DNA extraction kit (Tebu-bio). For mutation analysis, the 24 coding exons of *SHANK3* were amplified from genomic DNA with specific primers (Supplementary Table 2). Amplification was performed on 20 ng of DNA template with HotStar Taq polymerase (Qiagen) for all exons except for exons 1, 11, 21, 22, 22b, and 22c, for which amplification was performed with Taq polymerase from Eurobio and 10% GC melt (Clontech GC rich kit). Two PCR protocols were used: (i) Standard protocol: 95° for 15 min, followed by 35 cycles at 95°C for 30 sec, 55 to 65°C for 20 sec, 72°C for 30 sec to 1 min, with a final cycle at 72°C for 10 min; and (ii) Touchdown protocol: 95°C for 15 min followed by 20 cycles at 95°C for 30 sec, 70°-60° or 65-55°C for 30 sec, and 72°C for 30 sec, followed by 20 cycles at 95°C for 30 sec, 60° or 55°C for 10 sec, and 72°C for 30 sec, with a final cycle at 72°C for 10 min. Sequence analysis was performed by direct sequencing of the PCR products, using a 373A automated DNA sequencer (Applied Biosystems).

Detection of 22q13/*SHANK3* deletions and duplications

For quantitative analysis, the forward primer was labeled with fluorescent 6-carboxyfluorescein (6-FAM). PCR amplification (25 cycles) was as described above for standard protocol. Fluorescence-labeled PCR products were run on a 373A automated DNA sequencer (Applied Biosystems) with GENEFLO 625 DNA Ladder, ROX l (EurX). Following data collection, samples were analyzed with Genescan 3.7 software program. Two independent *SHANK3* PCRs (exon 9 and exon 17) were compared to two autosomal control genes *NLGN1* and *ANKRD15*, located on chromosome 3q26.31 and 9p24.3, respectively. The peak ratio of each PCR was used to calculate the dosage quotient (DQ) value. DQ= (PCR *SHANK3*/PCR in control region) in tested individual / (PCR *SHANK3*/PCR in control region) in control individual. Thus, DQ gives a theoretical value of 0.5 for a deletion and 1.5 for a duplication. A total of 155 individuals (58 with autism, 38 with Asperger, and 59 controls) were screened for deletion/duplication of *SHANK3*. All primers are indicated in supplementary table 2.

***In vitro* mutagenesis and transfection studies in hippocampal neurons**

Full-length rat *Shank3* cDNA (sequence AJ133120) was cloned into a pEGFP-C2 vector (Clontech). Mutagenesis was made using the QuickChange II XL site directed mutagenesis kit (Invitrogen) on 100 ng of wild type plasmid. Each clone was purified with Endofree Plasmid Maxi kit (Qiagen) and entirely sequenced to rule out additional mutations in the *Shank3* cDNA. Moreover, the GFP constructs were transfected into Cos cells and analyzed by Western Blot using a GFP antibody. The preparation of rat hippocampal cultures and the Shank3 localization experiments were performed

essentially as described previously[11,12]. Neurons were transfected after 14 days in culture. For co-localization of transfected Shank3 full length construct or the mutated constructs with the pre- and postsynaptic marker proteins SAP-90/PSD95, synaptophysin or Bassoon[13], cells were fixed on day 17 in 4% paraformaldehyde for 20 min at room temperature (20°C). Secondary antibodies conjugated to Cyanine 3 (Cy3) fluorophore were used (anti mouse Cy3 , anti-rabbit Cy3, Chemicon, Temecula, CA, USA), and the cells were visualized by fluorescence microscopy. All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the National Institutes of Health and The Max Planck Society.

Quantitative and qualitative measurement of GFP expression

Quantitative and qualitative measurement of GFP expression was performed essentially as described by Craven and Bredt[14]. In brief, transfected neurons were chosen at random from three independent transfections of each construct (4-5 cells per construct). The perimeter of the dendrites was traced (excluding spine heads/clusters), and the average pixel intensity was calculated. Similarly, the spine heads/clusters were traced, and the average pixel intensity was obtained. The ratio of the average pixel intensity in dendrites *vs*. spine heads/clusters was defined as the synaptic clustering ratio (SCR): a ratio of zero indicates complete synaptic clustering whereas a ratio of ≥ 0.7 indicates diffuse dendritic fluorescence with no clusters. Consecutively, we determined the synaptic localization of the clustering constructs by immunostaining with an antibody directed against the presynaptic marker protein Bassoon[13]. Between 250 and 300 synapses per construct were analyzed and the percentage of co-localization between Bassoon and the different GFP-constructs was determined (P/B in percentage).

References

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.*, (American Psychiatric Press, Washington D.C., 1994).
2. Lord, C., Rutter, M. & Le Couteur, A. *J Autism Dev Disord* **24**, 659-685 (1994).
3. Wing, L., Leekam, S.R., Libby, S.J., Gould, J. & Larcombe, M. *J. Child Psychol. Psychiatry* **43**, 307-25 (2002).
4. Gillberg, C., Rastam, M. & Wentz, E. *Autism* **5**, 57-66 (2001).
5. Collins, J.S. & Schwartz, C.E. *Am J Hum Genet* **71**, 1251-2 (2002).
6. Rosmond, R., Lapidus, L., Marin, P. & Bjorntorp, P. *Obes Res* **4**, 245-52 (1996).
7. Rosmond, R. & Bjorntorp, P. *Obes Res* **6**, 338-45 (1998).
8. Wilson, H.L. et al. *J Med Genet* **40**, 575-84 (2003).
9. Kikin, O., D'Antonio, L. & Bagga, P.S. *Nucleic Acids Res* **34**, W676-82 (2006).
10. Sambrook, J. & Russell, D.W. *Molecular Cloning, a Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).
11. Boeckers, T.M. et al. *J Neurochem* **92**, 519-24 (2005).
12. Dresbach, T. et al. *Mol Cell Neurosci* **23**, 279-91 (2003).
13. tom Dieck, S. et al. *J Cell Biol* **142**, 499-509 (1998).
14. Craven, S.E. & Bredt, D.S. *J Biol Chem* **275**, 20045-51 (2000).