SCAMP5, NBEA and AMISYN: three candidate genes for autism involved in secretion of large dense-core vesicles

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Received November 10, 2009; Revised and Accepted January 11, 2010

Autism is a neurodevelopmental disorder characterized by impaired social reciprocity, impaired communication and stereotypical behaviors. Despite strong evidence for a genetic basis, few susceptibility genes have been identified. Here, we describe the positional cloning of SCAMP5, CLIC4 and PPCDC as candidate genes for autism, starting from a person with idiopathic, sporadic autism carrying a de novo chromosomal translocation. One of these genes, SCAMP5 is silenced on the derivative chromosome, and encodes a brain-enriched protein involved in membrane trafficking, similar to the previously identified candidate genes NBEA and AMISYN. Gene silencing of Nbea, Amisyn and Scamp5 in mouse β-TC3 cells resulted in a 2-fold increase in stimulated secretion of large dense-core vesicles (LDCVs), while overexpression suppressed secretion. Moreover, ultrastructural analysis of blood platelets from the patients with haploinsufficiency of one of the three candidate genes, showed morphological abnormalities of dense-core granules, which closely resemble LDCVs. Taken together, this study shows that in three independent patients with autism three different negative regulators of LDCV secretion are affected, respectively, suggesting that in at least a subgroup of patients the regulation of neuronal vesicle trafficking may be involved in the pathogenesis of autism.

INTRODUCTION

Autism and autism spectrum disorder is a frequent and severe developmental disorder of the central nervous system, with an estimated prevalence of about 6/1000 (1). Given the lack of reliable diagnostic neurobiological or genetic markers, the diagnosis of autism is still achieved on purely clinical grounds. There is compelling evidence of a genetic cause for autism in the majority of cases (2). In the last few years, the general idea has grown that genes involved in the pathogenesis of autism play a role in the overall processes of neuronal signal transmission, including molecules such as synaptic scaffolding proteins, cell adhesion molecules, proteins involved in

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second-messenger systems, secreted proteins, receptors and transporters (3–7). Moreover, a number of cell adhesion proteins coordinating synaptic connectivity between dendrites and axons were shown to be involved in autism, e.g. the post-synaptic neurexin (NRXN1) (8–12), a presynaptic neurexin (NRXN3 and 4) (13,14), binding partner of the neureligins SH3 and multiple ankyrin repeat domains 3 (SHANK3) (15,16) and neurexin superfamily member contactin-associated protein-like 2 (CNTNAP2) (17–19). Recently, Ca$^{2+}$-dependent activator protein for secretion 2 (CADPS2), involved in the regulation of large dense-core vesicle (LDCV) release and synaptic vesicle fusion (20), has been added to this list of genes implicated in autism (21,22).

The identification and characterization of additional genes involved in autism is expected to increase our understanding of the pathogenesis of this disorder. Chromosomal anomalies are found in ~5% of individuals with autism and their study has contributed much towards the identification of autism candidate genes. The characterization of balanced translocations is particularly useful compared with chromosomal deletions or duplications, since the identification of the breakpoint regions allows the selection of a small number of candidate genes at or near the breakpoints (23,24).

Here we report on the positional cloning of novel candidate genes starting from a patient with autism carrying a de novo balanced chromosomal translocation. Together with two previously reported candidate genes for autism, NBEA (25) and AMISYN (26), we present SCAMP5 as a third novel candidate gene predicted to play a role in neuronal vesicle trafficking. Functional studies show that the three proteins function as negative regulators of secretion of LDCVs.

**RESULTS**

**Case report**

The subject is a 40-year-old male, living in a group home for adults with autistic disorder. He is the second child of healthy, unrelated parents. Family history is negative with regard to autism or mental retardation. Pregnancy and delivery were uneventful, and he was born at term with birth weight 4.5 kg. Early psychomotor developmental milestones were mildly delayed. Language and social development were markedly delayed. At 5 years, he was referred for child psychiatric assessment. A diagnosis of ‘early infantile autism’ was made based on a lack of responsiveness to other people, a very disturbed language development and stereotyped movements. Testing with the Merrill-Palmer scale showed an IQ of 63 with an uneven profile. Visuomotor skills were good, language skills were impaired. Psychiatric examination at the age of 15 years confirmed a diagnosis of autistic disorder according to the DSM-III-R criteria (27). Ritualistic behavior and stereotyped body movements were very pronounced. Other psychiatric problems at this age included mood changes and anxiety, episodic aggression and automutilation. Clinical examination at the age of 18 years revealed no dysmorphic features. Height was 188 cm, head circumference 54 cm. He carried a de novo apparently balanced translocation with karyotype 46,XY t (1;15)(p36.11;q24.2).

**Molecular analysis**

Genome-wide array Comparative Genome Hybridisation (aCGH, BAC/PAC array with resolution of 1 Mb) revealed that no additional deletions or duplications larger than 1 Mb were present in the genome of the reported subject. By means of fluorescent in situ Hybridization (FISH), the breakpoint on chromosome 1 was found to be flanked by BAC RP11-373M8 (NCBI AL445648) and cosmid c418b12 at the telomeric side, and BAC RP3-39819 (NCBI AL02396) and cosmid 208b4 at the centromeric side (Supplementary Material, Fig. S1A). BAC RP11-1089J (NCBI AL662924) spanned the breakpoint (Supplementary Material, Fig. S1B). The breakpoint must therefore be located in the chloride intra-cellular channel 4 gene (CLIC4), most likely between exon 1 and exon 4 (Supplementary Material, Fig. S1A). Since there are no alternative CLIC4 transcripts known lacking the 5’ end, the translocation is predicted to disrupt CLIC4 expression from this allele. This was confirmed on northern blot, showing a decreased expression of the CLIC4 gene in an Epstein–Barr virus (EBV) transformed leukocytes from the patient (Supplementary Material, Fig. S1C) using a multiple tissue northern blot, expression was detected in all tissues investigated, including in brain (Supplementary Material, Fig. S1D).

On chromosome 15, BAC RP11-151H2 (NCBI AC015720) and cosmids 32h10 and 76d1 spanned the breakpoint (Fig. 1A and B). Southern blot analysis with a probe from these cosmids showed rearranged fragments in the DNA of the patient using two different restriction enzymes (Fig. 1C), localizing the breakpoint to a 3.18 kb XbaI restriction fragment. This indicated that the breakpoint is located between the putative exons 1 and 3 of the recently characterized PPCDC gene (Fig. 1A). A CpG island (CpG34 - covering exon 1 of PPCDC) and a putative promoter were identified by the Promoter Inspector program (Genomatix). The gene encodes a transcript of 2.3 kb, with an ATG start in exon 2. However, there is evidence for the presence of an alternative splice variant lacking the first 2 exons and containing a truncated last exon (PPCDC*, Fig. 1A) (28), and we cannot exclude that the transcription of this shorter transcript is unaffected by the translocation. The PPCDC gene is ubiquitously expressed and codes for an enzyme involved in the biosynthesis of coenzyme A. Since this cofactor is essential in the synthesis and oxidation of fatty acids, and the oxidation of pyruvate in the citric acid cycle, we do not further consider PPCDC a candidate gene for autism.

The SCAMP5 gene is located 10 kb centromeric to the breakpoint on chromosome 15q (Fig. 1A). This gene is almost specifically expressed in brain (Fig. 1D). Expression studies were carried out by means of quantitative real-time PCR (qRT-PCR) on cDNA from EBV transformed leukocytes of the patient versus five normal controls. SCAMP5 expression was robustly detectable (Ct ≤ 30) but slightly variable between five control individuals (1.0 ± 0.23, n = 5). Relative quantification of SCAMP5 expression in two different RNA extracts revealed a reduction in expression to <50% (0.39 ± 0.08, n = 2) in the patient, consistent with haploinsufficiency (Fig. 1E). By single nucleotide polymorphism (SNP) analysis, we investigated the expression of this gene in the patient. At the genomic level, the patient was found to be
Figure 1. The PPCDC gene is disrupted and SCAMP5 expression is affected by the translocation on 15q24.2. (A) Physical map of 15q24.2 (UCSC Genome Browser, July 2003 version—centromere on the left). The position of the BAC ends (AC125435 and AC113208) and cosmids used for FISH analysis are shown. Arrows indicate the position of the translocation breakpoint with regard to the genomic clone (right arrow, distal; left arrow, proximal). Genes in the genomic locus are depicted (SCAMP5, PPCDC and alternative transcript PPCDC'), and exons of all these genes are numbered, indicating direction of transcription. (B) FISH analysis on metaphase spread of the patient shows that BAC AC015720 (left) and cosmid 32h10 span the breakpoint on chromosome 15. Der, derivative chromosome. (C) Southern blot analysis with a probe from C32h10 reveals rearranged fragments (indicated by arrow) in the patient (P) compared with a control (C). (D) Multiple tissue northern blot (Heart, Brain, Placenta, Lung, Liver, Skeletal muscle, Kidney, Pancreas) for human SCAMP5. (E) Quantitative RT–PCR expression study of SCAMP5 in leukocyte cDNA shows haplo-insufficiency for the patient with the translocation. Relative quantification results and respective standard deviations are shown for two different patient cDNA samples, normalized to the mean expression value in 5 controls. (F) Expression analysis of SCAMP5 in the patient. Sequence analysis on gDNA of the patient showed heterozygosity for rs8033925 (A/G), an SNP at the 3'-UTR of SCAMP5. cDNA analysis revealed expression of the A allele was significantly reduced, compared with equal expression from both alleles observed in four controls (one shown). Sequencing analysis of rs8033925 in the dissected amplified DNA from der (15) of the patient showed the A allele, consistent with affected expression from the aberrant chromosome 15.
heterozygous for rs8033925, an SNP (A/G) in the 3'UTR of SCAMP5 (Fig. 1F). Subsequent analysis of the corresponding mRNA fragment was performed in duplo on two different RNA extracts from the patient. This study revealed altered expression of SCAMP5, since the expression from the A allele was consistently found to be strongly reduced (Fig. 1F), unlike four heterozygous controls (one shown in Fig. 1F). Microdissection of the derivative chromosome 15 containing the SCAMP5 gene, and subsequent amplification and sequencing showed the presence of the A allele on this aberrant chromosome (Fig. 1F), consistent with affected expression of the SCAMP5 gene from the translocated chromosome.

Novel candidate genes involved in regulated secretion of LDCVs

To investigated the role of SCAMP5, CLIC4 and the previously identified candidate genes NBEA and AMISYN in the secretion of LDCVs, RNA interference (RNAi)-mediated gene silencing and overexpression experiments were performed. In this assay, we used recombinant Agouti-related protein (AGRP) as a marker for LDCV cargo. AGRP is a soluble protein that is efficiently sorted to the regulated secretory pathway (29).

By means of RT–PCR and western blot analysis, we studied the endogenous expression of the four candidate genes in both neuronal and endocrine mouse cell lines (Fig. 2A and B). Nbea, Scamp5 and Clc4 were found to be expressed in all cell lines investigated, whereas Amisyn was only expressed in the insulinoma cell line β-TC3. RT–PCR confirmed that Synaptotagmin IX (SytIX), used as a control, is also expressed in this cell line (data not shown). Constructs expressing silencing short hairpin RNAs (shRNA) were designed and cloned for the candidate genes Nbea, Scamp5, Amisyn and Clc4 as well as for the controls SytIX and MALT1. Knockdown efficiency of these shRNA constructs was tested in β-TC3 cells by transfection with the silencing construct and an epitope tagged recombinant construct representing the gene of interest (Fig. 2C).

β-TC3 cells have previously been shown to display low tonic release of recombinant AGRP while high secretion was observed after stimulation with secretagogues, such as forskolin and IBMX (29). In a pilot experiment it was found by means of immunofluorescence analysis that endogenous Nbea protein was almost undetectable 72 h after transfection with the appropriate silencing construct (data not shown). This timepoint was chosen for further experiments.

Results of the regulated secretion assay are shown in Figure 2D. Knockdown of the SNARE regulator SytIX resulted in a 2-fold reduction of stimulated secretion of LDCVs, consistent with reported data (30). As expected, we observed no effect with the shRNA construct for human MALT1 (31), as it does not have a target in mouse. Knockdown of Nbea, Scamp5 and Amisyn resulted in a significant 1.8–2.2-fold increase of regulated secretion. Consistent with these findings, overexpression of Scamp5 and Amisyn resulted in a significant 4-fold and 2-fold decrease of regulated secretion, respectively.

Figure 2. NBEA, SCAMP5 and AMISYN are involved in the regulated secretion of LDCVs. (A) Analysis of the endogenous expression of the candidate genes in endocrine and neuronal mouse cell lines (α-TC1-6, β-TC3, AFT-20 and Neuro2A) by RT–PCR (A) and western blot analysis (B). Since no antibody directed against Scamp5 was available, endogenous Scamp5 could not be detected. (C) Western blot analysis showing the silencing capacity of the RNAi constructs (R) by means of transient co-transfection of β-TC3 cells with a corresponding expression construct (E). (M) indicates the mock-transfected lanes. For each gene, the results of the mU6pro RNAi construct with the best silencing capacity is shown. (D) Effect of silencing and overexpression of the genes of interest on regulated secretion in β-TC3 cells. Stimulated secretion (addition of IBMX and forskolin) of Flag-AGRP was assayed 3 days after transient co-transfection with a shRNA (Nbea, Scamp5, Amisyn, Clc4, SytIX and MALT1) or full-length expression construct (Scamp5, Amisyn and Clc4). Number of experiments are six for each construct. Mean data are presented, standard deviations are depicted and asterisks indicate P-values <0.01.
In β-Tc3 cells, Nbea could not be overexpressed detectably, probably related to its large size (~9 kb cDNA). For Clic4, no effect on the regulated secretion of LDCVs was observed, neither by overexpression nor by knockdown.

Ultrastructural analysis of dense-core granules in blood platelets of the patients

In order to provide evidence for a defect in regulated secretion in the patients, ultrastructural analysis of blood platelets of the reported patients was performed. Platelets were chosen as a model because they are accessible and they contain dense granules. Many components involved in the secretion of dense granules are conserved with LDCVs in neuronal and endocrine cells (32). RT–PCR confirmed that all of the candidate genes are expressed in platelets (data not shown). The morphology of the platelets of controls (Fig. 3A) and all three patients with chromosomal rearrangements (Fig. 3B–D) was examined by electron microscopy. In the patients with chromosomal rearrangements affecting genes NBEA (B inset), SCAMP5 (C inset) and AMISYN (D inset) the dense core of the granules are smaller, more irregular, frequently peripherally localized and poorly delineated.

In order to establish a genotype–phenotype correlation between candidate genes described above and autism, mutation analyses were performed. Resequencing of all 7 coding exons of SCAMP5, including the intron–exon boundaries, in a cohort of 192 persons with autism revealed no mutations. Given its large size, mutation analysis of the NBEA gene was limited to the detection of small deletions or duplications. In a cohort of 248 persons with autism, no copy-number alterations were detected in eight regions spanning the NBEA gene.

DISCUSSION

This study describes the identification of novel autism candidate genes in a person with autism, carrying a de novo translocation involving chromosomes 1 and 15: 46,XY t(1;15)(p36.11;q24.2). In the subject, two genes were found to be directly disrupted, CLIC4 (Chloride Intracellular Channel 4 gene) at chromosome 1, and PPCDC (PhosphoPantothenoylCysteine DeCarboxylase...
gene) at chromosome 15. Since recently several autism genes have been proposed with a role in synaptic processes (3,4,33), CLIC4, coding for a protein predicted to play a role in neuronal vesicle trafficking (34), was considered a good functional candidate gene for autism. In contrast, the PPCDC gene codes for an enzyme involved in the biosynthesis of coenzyme A (35,36). Since this metabolic process is unlikely to be involved in autism, this gene was not included in any further studies. However, SCAMP5 (Secretory Carrier Membrane Protein 5), located 10 kb proximal to this breakpoint on chromosome 15q, was considered an excellent additional functional candidate gene. SCAMP5 is most abundantly expressed in brain late during development, coinciding with the elaboration of mature synapses, and has a probable function in membrane trafficking (37,38). Chromosomal breakpoints may have effects on the expression of genes located at a distance (26,39–41), and in the patient with a translocation breakpoint 10 kb downstream of SCAMP5, the gene’s expression was found reduced to ~40%. Analysis of rs8033925, an SNP in the 3’-UTR of SCAMP5, supports this haploinsufficiency for SCAMP5 in the reported patient. Moreover, our SNP data on microdissected derivative chromosome 15 is consistent with reduced expression of the translocated allele.

Besides SCAMP5 and CLIC4, two other autism candidate genes we recently identified, NBEA (25) and AMISYN (26), also play a possible role in neuronal vesicle trafficking and synaptogenesis (42–46). Therefore, the genes considered as candidate genes in all further studies are SCAMP5, CLIC4, AMISYN and NBEA. All these genes are expressed in neuronal and/or endocrine cells, specialized cell types in which regulated secretory pathways are present. Neuronal vesicle transport includes LDCVs and small synaptic vesicles (SSVs), whereas (neuro)endocrine cells contain only LDCVs (47). Since all candidate genes are endogenously expressed in the endocrine β-TC3 cell line, we were able to specifically investigate their role in regulated secretion of LDCVs. However, it should be stated that our data do not exclude an additional role in secretion of SSVs. Our results indicate that SCAMP5, NBEA and AMISYN function as negative regulators of secretion, whereas for CLIC4, no effect could be demonstrated.

SCAMPs (secretory carrier membrane proteins) are proposed to play a role as positive regulators of exocytosis (38), most likely by setting up SNARE interactions between secretory vesicle and plasma membranes or facilitate fusion pore formation (48). However, we show here that Scamp5 functions as a negative regulator of exocytosis. Most likely this is due to the absence of an N-terminal NPF repeat, found only in SCAMP1-3, combined with the presence of the so-called E-peptide, located between transmembrane regions 2 and 3 and conserved in all SCAMPs. This 11-residue peptide inhibits the fusion of secretory vesicles with plasma membrane in mast cells (49,50) and PC12 cells (48), whereas fusion of the N-terminal NPF repeat of Scamp2 with full-length Scamp5 resulted in a 2.5-fold increase in secretion in our assay (Fig. S2). In addition, it has been recently reported that expression of SCAMP5 is markedly increased in the striatum of Huntington disease patients, an increase positively regulates the aggregation of mutant huntingtin protein via the endocytotic pathway (51). Taken together, this suggests that SCAMP5, and possibly also SCAMP4, might play a role as negative regulator of exocytosis, possibly by influencing the formation of fusogenic SNARE complexes.

AMISYN has previously been described as a member of the inhibitory class of SNAREs or i-SNAREs (52,53). The presence of a VAMP2-like C-terminal coiled-coil domain, interacting with t-SNARE complex syntaxin-1/SNAP25, together with the unique N-terminus lacking the hydrophobic stretch that may serve as a transmembrane anchor to the vesicle, AMISYN cannot function as a classical v-SNARE (45). The formation of so-called nonfusogenic complexes prevents proper v/t-SNARE assembly and subsequent fusion of vesicle with the plasma membrane (46). Not only do our data support this role for AMISYN as negative regulator of secretion, we also confirmed the dominant inhibitory effect on exocytosis observed by overexpressing the C-terminal coiled-coil domain (Supplementary material, Fig. S2) as reported earlier in rat PC12 cells (45).

Disruption of the interaction of protein kinase A (PKA) with AKAPs in Xenopus laevis melanotrophic cells has been shown to result in induced secretion (54), consistent with negative regulation of secretion. Besides compartmentalization of PKA, the interaction with AKAPs, has been suggested to participate in regulating PKA activity (54). Tight regulation of kinase activity is essential for many processes, e.g. the phosphorylation status of SNAREs, both t-SNAREs and regulating i-SNAREs, alters the ability to assemble and form fusogenic SNARE complexes (46,55–57).

These observations support our finding that all three proteins identified function as negative regulators of LDCV secretion, possibly via the regulation of SNARE complex formation. Therefore, haploinsufficiency of SCAMP5, NBEA and/or AMISYN in the patients most likely results in increased secretion of LDCVs, and possibly of SSVs. In line with this hypothesis, elevated expression of t-SNARE syntaxin 1A in lymphocytes was observed to be associated with high functioning autism (58), likely resulting in elevated formation of fusogenic vesicles and subsequent secretion. Interestingly, a knock-in mouse model of the gain-of-function mutation R451C in NLGN3, previously reported to be involved in autism (8), showed an increase in inhibitory synaptic transmission with no apparent effect on excitatory synapses (59). Although all these reports support the idea that elevated neuronal vesicle trafficking may contribute to the pathogenesis of (a subgroup of) persons with autism, the latter is not in line with the theoretical model postulating that some forms of autism are caused by an increase in ‘noise information’ resulting from an increased ratio of excitation/inhibition in neural circuits that mediate language and social behaviors (60). However, several functional studies on the physiological consequences of certain mutations in candidate genes have reported ‘contradictory and incompatible results’, suggesting that affecting processes such as exocytosis or synaptogenesis in either direction might lead to autism. For instance, the NLGN4 gene on chromosome X, is associated with autism and mental retardation (8). Further functional analysis of the reported D396X frame shift mutation showed intracellular retention of the NLGN4 mutant protein and loss of synaptic function (61). In addition, an R87W missense mutation was shown to impair neuroligin-4 folding and endoplasmic
reticulum export (11), and a novel NLGN4 isoform lacking exon 4 resulting in monoallelic expression was identified in an autistic female (62). Although all these reports support the idea that a point mutation in NLGN4 can cause autism by a loss-of-function mechanism, very recently, a de novo mutation in the regulatory sequence of NLGN4 associated with autism was shown to result in increased expression of the gene (12), suggesting that also a gain in functional NLGN4 protein can cause autism.

Regulated secretion is not restricted to neurons and (neuro)-endocrine cells, but also occurs in a conserved manner in specialized cell types like blood platelets (32). It has been stated that platelet research can be used to unravel mechanisms involved in a neurological disorder, such as depression and autism (63). Moreover, platelet hyperserotonemia (described in one-third of patients with autism), the only biochemical anomaly repeatedly observed in autism (64,65), supports the idea of blood platelets representing an endophenotypic model for autism. Since platelets contain DCGs that resemble LDCVs both biochemically and morphologically, and, most importantly, platelets are easily accessible from patients, our in vitro results prompted us to perform ultrastructural analysis in vivo on blood platelets of all three patients and a number of controls. We detected similar structural abnormalities of the DCGs in the platelets of all three patients. Moreover, analysis of platelet ultrastructure in an autistic patient with a deletion of the 22q13 region, including the well-established autism candidate gene SHANK3, revealed the same structural aberration in the DCGs (Supplementary material, Fig. S3).

Although we do not exclude an additional role for these proteins in secretion of SSVs or endocytic processes, we showed a clear functional and morphological involvement of all three candidate gene products in the regulated secretion of LDCVs. In neurons, these LDCVs contain neurotrophins, like brain-derived neurotrophic factor (BDNF) and nerve growth factor. These factors regulate neuronal structure and synaptic plasticity, and their uncleaved precursors mediate apoptosis of neurons (66). In line with the hypothesized increased regulated secretion of LDCVs in our patients, two recent studies have reported increased concentrations of BDNF in a group of persons with autism (67,68). It has been suggested that these impaired levels reflect an abnormal status of prenatal or early postnatal neuronal development, which may lead to autism (67,69). However, since the autism susceptibility gene CADPS2 is a positive regulator of neurotrophin secretion (21,22), it is also possible that both increased and decreased LDCV release may contribute to the development of autism. Since LDCV transport is implicated in a wide range of neuronal processes, such as proliferation of neuronal precursors, apoptosis, outgrowth of neurites, synapse formation and synaptic transmission, it is not yet clear how altered regulated secretion affects the biology of neuronal cells.

Recently, high-resolution techniques, such as aCGH, have detected de novo submicroscopic chromosomal aberrations in a significant number of individuals with idiopathic autism (70–72). For both SCAMP5 and NBEA several independent patients carrying microdeletions harboring the gene are reported. In the five cases with the novel 15q24 microdeletion syndrome that includes SCAMP5, autism has not been recorded (73,74), whereas the three cases with a microdeletion including NBEA have autism (75–77). In addition to that, NBEA is located in a linkage region for autism (78,79). In order to determine to what extent these candidate genes contribute to the cause of autism in the whole population, mutation and association studies need to be performed. Denaturing high performance liquid chromatography (DHPLC) analysis and resequencing of the coding exons and all intron–exon boundaries of SCAMP5 did not reveal any functional mutations in a cohort of 192 persons with autism. Similarly, the previously reported mutation analysis of AMISYN revealed no functional mutations in a cohort of 227 patients (26). Since NBEA appears to be located at the common fragile site FRA13A (80,81), the region might be predisposed to breakage and rearrangements. Nevertheless, Multiplex Amplicon Quantification (MAQ) analysis of the gene in a cohort of 248 persons with autism did not reveal any copy-number variations (CNVs) in this genomic region. These findings are in agreement with previous reports showing a very low frequency of mutations and CNVs in established candidate genes for autism (for instance NLGN3 and NLGN4) (82–85), underscoring the high genetic heterogeneity of this condition (86).

MATERIALS AND METHODS

Electronic-database and primer information is provided online as Supplementary material.

Positional cloning of the translocation breakpoints (including strategy, genomic resources, FISH, aCGH, Southern and northern blotting) was performed as described before (25,87). Primers used for designing the Southern and northern probes are listed as Supplementary material.

qRT-PCR and calculation of relative expression

Primers were designed using the web-based Roche Probefinder software and ordered from Eurogentec (Seraing, Belgium). Sequences of these primers are available as Supplementary material. Primer pairs were validated with a standard dilution series. The 3 primer pairs for housekeeping genes ACTB, GUSB, CLK2 were validated as stable housekeeping gene primer pairs in EBV cell-lines with the online GEnorm software (88). qRT-PCR was carried out on the Roche Lightcycler 480 instrument using Roche Sybrgreen Mastermix in 15 μL total volumes. cDNA samples of the patient (two different RNA extracts) and five independent controls were diluted 1:15, all reactions were done at least in duplicate and a non-template control was included in each series. If necessary, series were replicated to obtain a ΔCt <0.3 for all duplicates. All non-amplification and non-template controls were negative with Ct >35. Relative gene-expression values were calculated with qBase software v.1.3.5 by use of a ΔCt relative quantification model with PCR efficiency correction (89). The results were normalized to the mean expression levels of SCAMP5 in the five control persons.
Study of gene expression using SNPs

Exonic SNPs were used to study the effect of a breakpoint on the expression of the SCAMP5 gene nearby the breakpoint at chromosome 15q24. First, by sequencing the genomic DNA (gDNA) corresponding to coding exons and 5' and 3' untranslated regions (UTR) of these genes, we showed that the subject was heterozygous for reported SNP rs8033925. Next, the presence or absence of this heterozygous SNP was analyzed in the patients mRNA (performed in duplo on each of the two different RNA extracts) by sequencing the corresponding cDNA fragments, and biallelic expression was tested in three independent experiments.

Isolation of gDNA and mRNA from the patient's EBV transformed leukocytes, reverse transcription of mRNA to cDNA and gDNA/cDNA SNP analysis by subsequent PCR amplification and sequencing was performed as described (41). Specific primers used for amplification and sequencing of the regions of interest are listed as Supplementary material.

Microdissection of derivative chromosome 15

Microdissection was performed as described (90), with minor modifications. To identify the der (15) a paint probe for 1pter was designed by random prime labeling of 10 subsequent DOP amplified BAC clones from the 1-Mb clone set at 1pter. This resulted in the hybridization signals on 1pter on the normal chromosome 1 and on 1pter translocated to the distal end of der (15). Reverse painting using the DOP amplified, dissected DNA labeled with Cy3-dCTPs by random prime labeling showed hybridization signals to the proximal part of chromosome 15q but not to the distal end of chromosome 15q and to the distal end of chromosome 1p. This confirmed that the aberrant chromosome 15 was dissected and not the normal chromosome 15. PCR amplification and subsequent sequencing for the SNP rs8033925 using the dissected amplified DNA as template, including gDNA as a positive control and a blank as negative control, was performed using primers listed as Supplementary material (Study of gene expression using SNPs).

Mutation analysis

Mutation analysis was done by means of DHPLC (Supplementary material) and subsequent sequencing analysis on all coding exons and exon–intron boundaries of the SCAMP5 gene. The NBEA gene was screened for CNVs using MAQ (Supplementary material).

Analysis of endogenous gene expression in cell lines

Endogenous expression of the candidate genes was tested by RT–PCR on mRNA from mouse endocrine and neuronal cell lines (α-TC1-6, β-TC3, AtT-20 and Neuro2A).

Gene silencing and overexpression constructs

For RNA interference, 19-mer target regions were designed inside the coding sequences of the different mouse genes of interest making use of Ambions' siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). Several target sequences were selected at different positions along the length of the gene. 60-mer oligonucleotides encoding the corresponding silencing short hairpin RNA (shRNA) were designed as described (29) and cloned in the mU6pro vector (kindly provided by Dr Turner, MI, USA). The efficiency was confirmed in HEK293T and β-TC3 cells by co-transfection of the silencing shRNA construct with expression vectors encoding the target mRNA in a 4/1 ratio. In general, the expression construct encoded the complete coding sequence. However, for Nbea the construct only encoded a C-terminal part (AA1863–2566) including its target sequence.

In order to obtain full-length expression constructs for Clic4, Scamp5 and SytIX, PCR was performed on cDNA obtained by RT–PCR of total RNA from mouse embryonic brain. PCR fragments were purified (QIAquick gel extraction kit, Qiagen), followed by a digestion and another purification step (QIAquick PCR purification kit, Qiagen) before the inserts were cloned in the expression vectors. Inserts were cloned into the pcDNA3.1/HisA vector (Invitrogen), except for Scamp5 that was cloned into the pcDNA3 vector (Invitrogen) with addition of an N-terminal HA-tag. Although a full-length mouse Nbea expression construct was made (overexpression was observed in HEK293T cells), we were unable to obtain detectable overexpression in the β-TC3 cell line.

Detailed information concerning the primers used for the construction of all these plasmids is provided as Supplementary material.

Cell lines

Cells from the human embryonic kidney cell line HEK293T, the mouse pancreatic cell lines β-TC3 and α-TC1-6, the mouse pituitary tumor cell line AtT-20 and the mouse neuronal cell line Neuro2A were grown in Dulbecco’s modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C, 5% CO2. Transfection of cells was performed using Fugene 6 Transfection Reagent (Roche) for HEK293T cells, and Lipofectamine 2000 (Invitrogen) with Plus reagent (Invitrogen) for β-TC3 cells.

Western blot analysis

Cells were lysed in Sample Buffer (60 mM Tris–HCl pH 6.8, 12% glycerol, 4% SDS) and proteins in media were precipitated in 4 volumes of methanol (–20°C) in the presence of albumin and dissolved in Sample Buffer containing 4% 2-mercaptoethanol. Proteins were separated by SDS–PAGE on 10% Tris–Glycine gels, with the exception of endogenous Nbea (5% SDS–PAGE) and Agouti-related protein (AGRP) samples of the ‘regulated secretion’ assay (NuPage 10% Bis–Tris Gel (Invitrogen). Gels were electroblotted and processed as described (29). The polyclonal rabbit antibody used to detect endogenous neurobeachin was raised against the synthetic peptide TKVSDDILGNSDPRGCS-Cys-KLH-MBS (Eurogentec). Primary antibodies used for detection of recombinant tagged constructs are anti-Xpress (Invitrogen), anti-HA (Roche) and anti-Flag M2 (Sigma-Aldrich). Anti-Clic4
and anti-Amisyn antibodies were kindly provided by Dr Yuspa (Bethesda, MD, USA) and Dr Scheller (Stanford, CA, USA), respectively. Secondary antibodies used were horseradish peroxidase-linked (DAKO). Chemiluminescent signal (Renaissance, Perkin Elmer Life Sciences) was either detected by X-ray film (Fujiﬁlm) or directly quantiﬁed using Kodak Digital Science (Kodak Imager with 1D Image Analysis Software, version 3.0).

Regulated secretion assay

The assay was performed essentially as described previously (29,91). Brieﬂy, β-TG3 cells were transiently transfected (Lipofectamine 2000) with an shRNA mU6pro construct or overexpression construct, together with the recombinant AGRP construct (C-terminally Flag-tagged). For Scamp3 knockdown experiments, the AGRP construct was co-transfected with 20 μM of synthetic siRNA (Dharmacon) instead of an shRNA construct. As a control, β-TG3 cells were co-transfected with the Flag-tagged AGRP construct and the empty mU6pro vector or the ‘non-targeting’ siRNA respectively. After 2 days the cells were washed and incubated for 18 h in serum-free medium to reduce the tonic release of stored material induced by serum factors. Subsequently, the cells were incubated for 3 h in fresh serum-free medium (tonic release fraction), followed by 3 h incubation in serum-free medium containing secretagogues [10 μM forskolin (Sigma) and 0.1 mM IBMX (1-Methyl-3-isobutylxanthine, Sigma)] (induced secretion fraction). Forskolin is an adenylyl cyclase activator and IBMX is a phosphodiesterase inhibitor, both stimulating secretion of LDCVs through activation of the PKA pathway.

Both medium fractions and lysates were collected and the amount of AGRP was quantiﬁed. Regulated secretion was deﬁned as the amount of AGRP secreted after stimulation with forskolin/IBMX minus tonic release and normalized for the total amount of AGRP in medium samples plus lysates. Regulated secretion in the absence of any silencing or overexpression constructs was arbitrarily set at 1, and the other samples were adjusted using the same correction factor.

Transmission electron microscopy

Platelet-rich fractions from the patients and controls were prepared by centrifugation (15 min at 150g) of whole blood anticoagulated with 3.8% (w/v) trisodium citrate (9:1) as described (92). Both pellets of β-TG3 cells and platelet-rich plasma were immediately ﬁxed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C overnight. After 1 h post-ﬁxation in 1% osmium tetroxide and 0.3% potassium ferricyanide in 0.1 M phosphate buffer at 4°C, the samples were dehydrated in graded series of alcohol and embedded in epoxy resin. Ultra-thin sections of 50–60 nm were cut, stained with uranyl acetate and lead citrate and examined at 50 kV using a Zeiss EM 900 electron microscope (Oberkochen, Germany). Images were recorded digitally using a Jenoptik Progress C14 camera system operated using Image-Pro express software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank the patients and their families for their cooperation. Reinhilde Thoelen and Rob Deckers are gratefully acknowledged for their excellent technical assistance and Bernard Thienpont for performing the aCGH experiments.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from Cure Autism Now, the Interuniversity Attraction Poles Program-Belgian Science Policy (IUAP 5/25), ‘Geconcerteerde Onderzoeksaaktes’ GOA/2006/12 and GOA/2008/16, ‘Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO)’ G0264.04 and G.0124.02 and ‘interdisciplinaire onderzoekprogramma (IDO)’. K.V. is supported by a grant from the Instituut voor de aanmoediging van Innovatie door wetenschap en technologie in Vlaanderen (IWT). J.S. is supported by a grant of the Clinical Research Fund U.Z. Leuven. K.D. is a Senior Clinical Investigator of FWO, K.F. is postdoctoral fellow of FWO, A.C. is PhD fellow of FWO.

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