Mutations affecting synaptic levels of neurexin-1β in autism and mental retardation

Rafael J. Camacho-García, Mª. Inmaculada Planelles, Mar Margalef, Maria L. Pecero, Amalia Martinez-Mira, Rafael Martínez-Leal, Francisco Aguilera, Elisabet Vilella

Abstract

The identification of mutations in genes encoding proteins of the synaptic neurexin-neuroligin pathway in different neurodevelopmental disorders, including autism and mental retardation, has suggested the presence of a shared underlying mechanism. A few mutations have been described so far and for most of them the biological consequences are unknown. To further explore the role of the NRXN1β gene in neurodevelopmental disorders, we have sequenced the coding exons of the gene in 86 cases with autism and mental retardation and 200 controls and performed expression analysis of DNA variants identified in patients. We report the identification of four novel independent mutations that affect nearby positions in two regions of the gene/protein: i) sequences important for protein translation initiation, c.−3G>T within the Kozak sequence, and c.3G>T (p.Met1), at the initiation codon; and ii) the juxtamembrane region of the extracellular domain, p.Arg375Gln and p.Gly378Ser. These mutations cosegregate with different psychiatric disorders other than autism and mental retardation, such as psychosis and attention-deficit/hyperactivity disorder. We provide experimental evidence for the use of an alternative translation initiation codon for c.−3G>T and p.Met1 mutations and reduced synaptic levels of neurexin-1β protein resulting from p.Met1 and p.Arg375Gln. The data reported here support a role for synaptic defects of neurexin-1β in neurodevelopmental disorders.

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Introduction

Neurodevelopmental disorders such as autism spectrum disorders (ASD) and mental retardation (MR) show a complex genetic basis with extensive genetic heterogeneity. ASD comprise a spectrum of clinical phenotypes characterized by qualitative impairment in communication and social interaction, and restricted and stereotyped patterns of behavior, interests, and activities (American Psychiatric Association, 1994). As a group, ASD are highly prevalent disorders, ranging from 27/10,000 to 116/10,000 (Baird et al., 2006; Fombonne, 2003). The clinical heterogeneity of ASD is further complicated by the fact that 55% and 73% of cases of ASD and classical autism, respectively, have MR (IQ<70) (Baird et al., 2006).

Neurexins belong to a family of synaptic adhesion molecules involved in ASD. In humans, neurexins are encoded by three genes (NRXN1, NRXN2 and NRXN3), each with two alternative promoters that give rise to the long, alpha, and the short, beta, isoforms (Missler and Sudhof, 1998). The N-terminal domain of beta-neurexins includes a specific translation initiation site, not shared with alpha-neurexins, and an unusual signal peptide (Ushkaryov et al., 1994). At the synapse, presynaptic neurexins bind to postsynaptic neuroligins and mediate glutamate and gabaergic differentiation through the interaction of splicing isoforms (Boucard et al., 2005; Chih et al., 2006).

In the last years, mutations in neurexin and neuroligin genes have been associated with ASD. The identification of a missense and a frameshift mutation in the NLG3 and NLG4 genes, respectively, in two families with typical autism and Asperger syndrome (Jamain et al., 2003) led to the hypothesis that altered synapse function could underlie aspects of ASD. The subsequent identification of mutations in NRXN and SHANK genes (Berkel et al., 2010; Durand et al., 2007; Feng et al., 2006; Gauthier et al., 2011; Moessner et al., 2007; Pinto et al., 2010; Szatmari et al., 2007) has strengthened the role of the NRXN–NLGN–SHANK pathway in the pathophysiology of ASD (Bourgeron, 2009). These genes show both intra- and interfamily phenotypic variability and have been involved in a number of neuropsychiatric disorders. Mutations in NRXN1 have been identified in heterozygosis in ASD, schizophrenia, MR and Tourette syndrome (Ching et al., 2010; Gauthier et al., 2011; Kirov et al., 2008; Sundaram et al., 2010; Szatmari et al., 2007; Zahir et al.,
2008; Zweier et al., 2009). A common trend among the variants identified in these genes (point mutations, copy number variants, and chromosomal abnormalities) is that although most mutations are rare, they may collectively explain a considerable proportion of cases and could reveal mechanisms of disease (McClellan and King, 2010; State, 2010).

Based on the hypothesized role of neurexin-1 in neuropsychiatric diseases and the scarcity of functional studies with the identified variants, we have performed a genetic study of NRXN1β in patients with a combined diagnosis of autism and MR. We have identified four novel mutations that cluster in two different regions of the gene/protein, the translation initiation and the juxtamembrane region of the extracellular domain. Expression studies show the use of an alternative translation initiation codon by the mutations affecting the initiation site and reduced synaptic levels derived from mutations p.Met1 and p.Arg375Gln.

Material and methods

Subjects

A sample of 86 patients were selected by two independent clinical evaluators from an initial group of 115 Caucasian patients with a combined diagnosis of autism and MR. The sample consisted of adult patients (mean ± SD age = 41.45 ± 7.80 years) with profound (83.5%) or severe mental retardation (16.5%), 67.1% of whom were male. A diagnosis of epilepsy was present in 65.9% of the participants. A detailed description of patient selection is presented in the Supplementary information.

Inclusion of patients in the study was performed after informed and written consent was obtained from their relatives or other legal figures. The study was approved by the local ethical committees (Committee of Hospital Sant Joan de Reus and Committee of the University of Seville).

Mutational analysis

The full coding sequence of human NRXN1β gene, including two alternatively spliced exons, and flanking intronic sequences were analyzed by PCR-direct sequencing in 86 patients and 200 ethnically matched controls. PCR conditions and sequence analysis are given in the Supplementary information. DNA variants were confirmed by a second, independent PCR amplification and bi-directional sequencing. The frequency of rare mutations in cases and controls was compared using the Fisher exact test. The common variant rs13413205 and the indel alleles within the glycine repeat region (Table 1) were excluded from this comparison. ClustalX v.2.0.12 software was used for sequence alignment.

Cell culture, transfection and Western blot analysis

The cDNA sequences of wild-type and mutant neurexin-1β with or without a HA-tag were cloned into the pCAGGS expression vector (see Supplementary information for cloning details). Human embryonic kidney (HEK293) cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4; 100 mM NaCl, 5 mM MgCl2; 1% Triton X-100; 0.1% SDS) containing protease inhibitors (Roche, Basel, Switzerland). Western blot analysis was performed using the following antibodies: a chicken anti-pan-neurexin antibody recognizing the common cytoplasmic tail of neurexin proteins (Dean et al., 2003) and anti α-actin antibody (Sigma, St. Louis, MO, USA). Immunoreactivity was detected with cross-absorbed secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Chemiluminescence was detected using ECL (GE Healthcare, Munich, Germany) in a Chemidoc XRS apparatus (BioRad, Hercules, CA, USA). Protein images were quantified with the ImageJ software within a linear range of detection. Data were evaluated with one-way ANOVA and Holm–Sidak method for multiple comparisons.

Hippocampal tissue was dissected from embryonic day 18 rat brains. Dissociated cells were plated on poly-D-lysine coated glass coverslips at a density of 25,000/cm² and maintained in neurobasal medium (Invitrogen) supplemented with 2 mM Glutamax, B27 and penicillin/streptomycin (Invitrogen). Hippocampal neurons were transfected at 7–9 days in vitro with Lipofectamine 2000 (Invitrogen) and analyzed 3 days after transfection.

Animal procedures were performed in accordance with institutional and national guidelines for the care and use of laboratory animals following approval by the Ethics Committee of the University of Seville.

Fluorescence microscopy, image acquisition and quantification

Cell cultures were fixed with 4% paraformaldehyde in phosphate buffer containing 4% sucrose and permeabilized with PBS containing 0.05% Triton X-100. The primary antibodies used were: mouse anti-synaptobrevin 2 (Synaptic Systems, Goettingen, Germany) and rat anti-HA (Roche). Immunoreactivity was detected with cross-absorbed secondary antibodies raised in donkey and conjugated to Cy3 or Cy5 (Jackson ImmunoResearch).

Table 1

<table>
<thead>
<tr>
<th>Subject/group</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Aminoacid change</th>
<th>Frequency in patients</th>
<th>Frequency in controls</th>
<th>Other populations</th>
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<td>-</td>
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<td>p.Gly17Val (rs13413205)</td>
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<tr>
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<td>p.Gly26_Ala27insGly</td>
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<td>1/200</td>
<td>2/264 ASD patients&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>p.Gly26del</td>
<td>0/86</td>
<td>1/200</td>
<td>-</td>
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</table>

<sup>a</sup> Feng et al. (2006).

<sup>b</sup> Gauthier et al. (2011). In this study, variant p.Gly26_Ala27insGly was also identified in 2/143 and 1/94 patients with schizophrenia and non-syndromic mental retardation, respectively.

<sup>c</sup> These two variants are located within a glycine repeat region where different indel alleles have been reported in patients and controls (Feng et al., 2006; Gauthier et al. 2011).
Confocal images were acquired on an Olympus Fluoview FV1000 microscope with a 60x Plan-Apochromat oil immersion objective. Maximal projections of Z-stacked images were analyzed with ImageJ software. For experiments in which GFP was co-transfected with HA-tagged neurexin-1β in hippocampal neurons, the GFP expressing regions of axonal segments were selected by thresholding. The threshold was set such that background noise in axons expressing only GFP was excluded and most of the specific HA-NRXN1β signal was retained. For quantification of neurexin intensity at synaptic terminals, the GFP segments were imported into the synaptobrevin channel. Thresholds for intensity and area were set such that the synaptobrevin puncta within the GFP transfected axons were selected as region of interests (ROIs). The mean intensity of HA-tagged neurexin-1β proteins within the ROIs was then calculated. Experiments were repeated in three independent neuronal cultures and the data obtained from one representative experiment are shown in the Results section. Statistical significance was determined by Kruskal-Wallis with dependent post-hoc Dunn’s multiple comparison test.

Results

Genetic analysis

The full coding sequence of NRXN1β, including two exons subject to alternative splicing, and flanking intronic regions were sequenced in all 86 selected patients and 200 control subjects. Four novel point mutations were identified in heterozygosis in four unrelated patients (Table 1). These mutations cluster in two regions of the gene: exon 18, which encodes the signal peptide and is specific to NRXN1β, and exon 24, common to alpha and beta neurexin isoforms (Table 1). A G-to-T transition at cDNA position −3, c.−3G>T, located within the Kozak sequence, and a G-to-T transition at position +3, c.3G>T (p.Met1), affecting the initiation codon, were identified in exon 18, involving sequences important for translation initiation (Fig. 1). Furthermore, two point mutations were identified in exon 24, an A-to-G transition at cDNA position 1124, leading to the missense mutation p.Arg375Gln, and an A-to-G transition at position 1132, resulting in mutation p.Gly1453Ser (Fig. 1). The mutations in exon 24 correspond to p.Arg1450Gln and p.Gly1453Ser in neurexin-1α isoform (accession #: NM_001135659.1). These four variants were absent from the 200 control subjects studied here, as well as over 700 controls reported elsewhere (Feng et al., 2006; Gauthier et al., 2011; Kim et al., 2008; Yan et al., 2008).

Proband #1 is heterozygous for the c.−3G>T mutation, within the Kozak sequence (Fig. 1). Together with a combined diagnosis of autism and profound mental retardation, she received a diagnosis of psychosis and suffers from seizures (Table S1). She has a brother with paranoid personality disorder who is also a carrier of the mutation (Fig. 1). Her brother showed incoherent discourse, immature personality and borderline intellectual functioning, with an idle and disorganized attitude. He had never worked. Her mother and one of her cousins presented borderline intellectual functioning and two paternal uncles received a diagnosis of schizophrenia (Table S2).

Proband #2 is heterozygous for mutation p.Met1, at the translation initiation codon (Fig. 1). He was diagnosed with autism and profound mental retardation and has had no epileptic seizures (Table S1). Two of his sisters, a nephew, and a niece were found to be heterozygous carriers of the mutation (Fig. 1). His two carrier sisters were diagnosed with major depressive disorder and mood disorder, respectively, and his nephew has attention-deficit/hyperactivity disorder, antisocial personality and substance abuse. His niece, who is also a carrier of the mutation, has a normal learning history and no reported mental problems. On his paternal side, the proband’s father and grandmother had Alzheimer’s disease and dementia not otherwise specified, respectively (Table S2).

Proband #3 is heterozygous for the missense mutation p.Arg375Gln (Fig. 1) and met the diagnosis of autism and profound mental retardation (Table S1). Segregation analysis in the participating family members showed that the mutation was present in her mother, without any reported mental problem, and was not present in her brother, with severe intellectual disability and autism diagnosis (Fig. 1). A maternal cousin, not available for the study, has mental retardation of unknown etiology and her father was diagnosed with Parkinson’s disease (Table S2).

Proband #4 is heterozygous for the missense mutation p.Gly378Ser (Fig. 1) and received a diagnosis of autism and profound mental retardation and compulsive personality disorder (Table S1). Analysis of the mutation in the participating family members showed that the mother of the patient, diagnosed with a non-psychotic non-specified mental disorder (Table S2), is a heterozygous carrier, while the mutation was not present in a healthy maternal cousin (Fig. 1).

Together with the aforementioned mutations, we identified variant p.Ser14Leu in one control individual (Table 1), which had been previously found in 4 ASD patients (Feng et al., 2006; Gauthier et al., 2011). In all, we observed an enrichment of rare variants in patients when compared to controls (4/86 vs 1/200, p = 0.03, Fisher exact test).

Sequence conservation

Translation initiation in eukaryotes is mediated by the Kozak sequence, a short recognition sequence that facilitates the assembly of the ribosome to the mRNA and controls the initiation of translation (Kozak, 1986). The Kozak consensus sequence in mammalian species is GCCRCCaugG, where R = purine. Along with the start codon, the most highly conserved position in vertebrates is −3R and a G nucleotide at position +4 (Kozak, 1987). Human NRXN1β contains a Kozak sequence GCCGCaugT with a purine nucleotide at position −3 (−3G) and lacks a G nucleotide at position +4. Interestingly, the identified ASD-associated mutations c.−3G>T and p.Met1 affect nucleotide positions important for translation initiation (Figs. 2A–B). Mutation c.−3G>T affects the most conserved position of the Kozak sequence. Accordingly, alignment of orthologous NRXN1β nucleotide sequences shows complete conservation of this position in mammals (Fig. 2B). Mutation p.Met1 affects the third position of the initiation codon (Fig. 2B). Alignment of all described vertebrate NRXN1β genes shows the start codon at this position (Fig. 2B). On the other hand, missense mutations p.Arg375Gln and p.Gly378Ser affect residues highly conserved in neurexin-1 proteins, located within an O-glycosylation region in proximity to the transmembrane region (Figs. 2A, C).

Expression of ASD-associated mutations in HEK293 cells

To study the effect of the identified mutations, we cloned the human NRXN1β cDNA and generated the four ASD-associated mutations. As a first approach, we performed Western blot analysis in HEK293 cells, a cell line that does not express neurexins, using a previously reported pan-neurexin antibody (Dean et al., 2003).

Given that two of the identified mutations affect conserved sequences involved in translation initiation of neurexin-1β, we proceeded to evaluate whether translation of the mutant proteins was affected. In lysates of cells transfected with wild-type NRXN1β, the pan-neurexin antibody recognized a major band corresponding to the mature glycosylated-protein (80 kDa) and a minor band corresponding to the non-glycosylated core protein (50 kDa) (Fig. 3A). These bands were also observed in lysates derived from cells expressing c.−3G>T and p.Met1 constructs, indicating that translation was not abolished by these mutations under overexpression conditions (Fig. 3A).

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Whereas protein expression could be expected in the case of the c.−3G>T mutation, it was somehow unexpected for p.Met1, which abolishes the predicted start codon. Based on these results, we assessed the existence of an alternative initiation site. Neurexin-1β contains a downstream in-frame Met residue at position +5, which is evolutionarily conserved (Fig. 2B). Therefore, we considered the possibility that this Met+5 residue was being used as an alternative translation initiation site from which the mutant proteins could be translated. To test this hypothesis, we generated NRXN1β cDNAs bearing a mutated Met at position +5 (Met5Ile), within the context of the wild-type allele (WT_Met5Ile) and the two mutant alleles (−3G>T_Met5Ile and Met1_Met5Ile) (Fig. 3A). Protein expression obtained from each neurexin-1β construct was analyzed by Western blot experiments in HEK293 cells (Fig. 3A). Mutation of the Met+5 residue had a minor effect on wild-type neurexin-1β expression (WT_Met5Ile vs WT: 79.53±10.42%) (Fig. 3B), indicating that Met+1 is used as the major translational start site in a control situation. On the contrary, expression was inhibited when the Met at position +5 was mutated in the context of mutation p.Met1 (Met1_Met5Ile vs Met1: 3.27±0.88%) (Fig. 3B), suggesting that p.Met1 shifts the translation initiation site to Met+5. A partial inhibition of neurexin-1β expression was observed when the Met at position +5 was...
mutated in the context of the c.-3G>T allele, showing a contribution of both start sites (p.Met1, p.Arg375Gln and p.Gly378Ser in HEK293 cells showed a mobility shift for p.Arg375Gln in Western blot experiments that affected the 50- and 80-kDa bands (Fig. 3C). The reason for the slower migration of the p.Arg375Gln protein might reflect the loss of a positive charge caused by the mutation.

Decreased synaptic expression of ASD-associated mutations in cultured hippocampal neurons

Given the role of neurexins as synaptic proteins, it is thought that synaptic levels and/or function of neurexins might be affected by disease-associated mutations (Sudhof, 2008). Moreover, the identification by other groups of deletions in NRXN1 in patients with ASD (Szatmari et al., 2007) and other psychiatric conditions (Guilmatre et al., 2009; Rujescu et al., 2009) points at a possible neurexin-1 dosage effect in neurodevelopmental disorders. To evaluate whether the identified ASD-mutations in neurexin-1 β affect synaptic expression levels, we generated HA-tagged versions of the corresponding human neurexin proteins by inserting a HA-tag epitope after the signal sequence. Cultured hippocampal neurons transfected with GFP and HA-tagged neurexins were analyzed by immunofluorescence using an HA antibody and the synaptic vesicle marker synaptobrevin to identify synaptic puncta. HA-NRXN1 β showed a punctate distribution that partially overlapped with synaptobrevin, as similarly described for endogenous neurexins (Dean et al., 2003) (Fig. 4A). However, we observed a reduction of HA-neurexin levels in neurons overexpressing HA-NRXN1 βMet1 and HA-NRXN1 βArg375Gln (Fig. 4). Quantification analysis revealed a decrease of ~30% for HA-NRXN1 βMet1 and ~60% for HA-NRXN1 βArg375Gln, as compared with axons overexpressing HA-NRXN1 βMet1 (Fig. 4B). On the other hand, the slight reduction observed for HA-NRXN1 β−3G>T and HA-NRXN1 βGly378Ser did not reach statistical significance (Fig. 4B). In summary, expression analysis showed a decrease in synaptic levels for neurexin point mutations p.Met1 and p.Arg375Gln.

Discussion

With the aim of studying the role of neurexin genes in neurodevelopmental psychiatric disorders, we have performed a genetic analysis of NRXN1 β in patients with autism and MR. We
The frequency of mutations in NRXN1β in patients with autism and MR reported here (4.65%) is in line with previous analyses that identified mutations in NRXN1α and NRXN1β in 2–5% of ASD patients (Feng et al., 2006; Gauthier et al., 2011; Kim et al., 2008; Yan et al., 2008). In addition, mutational search in SHANK2 and SHANK3 revealed mutations in 1–2% of patients, including deletions, nonsense and missense mutations (Berkel et al., 2010; Durand et al., 2007; Moessner et al., 2007). Finally, mutations in NLGN3 and NLGN4 account for around 1% of the studied ASD cases (Persico and Bourgeron, 2006). In all, the results reported here in a relatively small population reinforce the role of rare mutations in genes of the NRXN–NLGN–SHANK pathway in ASD.

Analysis of the mutations showed partial cosegregation within each family, with asymptomatic mutation carriers as well as carriers affected with non-ASD psychiatric disorders. This is not surprising considering the expected genetic architecture of complex disorders, where a combination of a number of rare and/or common DNA variants could be contributing to the clinical phenotype. This partial cosegregation has also been shown by other groups. Bucan et al. (2009) reported the identification of copy number variants in the NRXN1 gene in familial autism that were either shared by affected and unaffected relatives, or were transmitted to only some of the affected children. This also seems to be the case for SHANK2 and SHANK2 mutations, where non-ASD parents transmitted the mutated allele to their affected offspring (Berkel et al., 2010; Gauthier et al., 2011). As also suggested by other authors (State, 2010), these findings reflect the fact that rare mutations can act as risk alleles, and not only as fully-penetrant disease alleles. Novel approaches such as exome sequencing may help draw a more accurate picture of the genetic architecture of ASD (O’Roak et al., 2011).

A shared genetic basis has been suggested for different neurodevelopmental disorders, such as autism, MR and schizophrenia (Mitchell, 2011). The mutations in NRXN1β reported here cosegregate with autism and MR, together with a number of different psychiatric conditions such as psychosis, compulsive personality disorder and attention-deficit/hyperactivity disorder, among others. Similarly, mutations in genes of the NRXN–NLGN–SHANK pathway have been reported in phenotypes ranging from autism with or without MR, and MR alone (Jamain et al., 2003; Laumonnier et al., 2004). The existence of a shared genetic basis is not restricted to ASD and MR, but includes other disorders such as schizophrenia and Tourette syndrome (Guilmatre et al., 2009; Rujescu et al., 2009; Sundaram et al., 2010). Together, these findings would point at shared biological pathways among different mental disorders, blurring classifications solely based on clinical data (Abrahams and Geschwind, 2008; State, 2010).

At the molecular level, the four independent mutations reported in this study cluster in two conserved regions of the gene, where they alter nearby positions (Fig. 2A). Mutations c.–3G>T and p.Met1 are unique to the NRXN1β isoform and affect key positions involved in the initiation of protein translation. On the other hand, missense mutations p.Arg375Gln and p.Gly378Ser result in non-conservative changes and introduce a polar aminoacid in an O-glycosylated region adjacent to the transmembrane domain. Interestingly, previous studies have reported mutations that affect the signal peptide and the juxtamembrane region of neurexin-1β in patients with ASD and schizophrenia (Feng et al., 2006; Gauthier et al., 2011) (Fig. 2A). This clustering might suggest a functional relevance of the affected regions. In fact, we provide experimental evidence for reduced synaptic levels of neurexin-1β derived from mutations affecting both regions (p.Met1 and p.Arg375Gln). On the other hand, we detected variant p.Ser141Leu, within the signal peptide, in one control subject. Although this variant had been previously found only report the identification of four novel mutations in a sample of 86 patients. These mutations cosegregate with a number of different psychiatric disorders within each family, consistent with a role of NRXN1 in a broad spectrum of mental diseases. Expression analysis in cultured hippocampal neurons shows reduced synaptic levels of neurexin-1β as a consequence of mutations p.Met1 and p.Arg375Gln.

Fig. 3. Expression of ASD-mutations affecting NRXN1β. (A) Schematic diagram showing the sequence of constructs harboring a mutation in the Met residue at position +5 (Met5Ile). Expression of neurexin-1β proteins by Western blot analysis in non-transfected HEK293 cells (control) or HEK293 cells transfected with the indicated constructs. Expression of neurexin-1β was not inhibited when Met+5 residue was mutated in the context of the wild-type protein (WT_Met5Ile), whereas it was inhibited in the context of the p.Met1 mutation (Met5Ile). The -80 and -50 kDa bands corresponding to the full-length and the non-glycosylated core protein, respectively, are marked by arrowheads. Expression of α-actin is shown as a loading control. (B) Expression of constructs containing a Met5Ile mutation was normalized to their corresponding control without the mutation and shown as percentage. Error bars indicate SEM. Data obtained from 4 independent experiments. **p<0.01; ***p<0.001. (C) Western blot of HEK293 cells transfected with NRXN1β, NRXN1β mutant or NRXN1β Gly378Ser. A mobility shift corresponding to mutation p.Arg375Gln can be observed.
in ASD patients (Feng et al., 2006; Gauthier et al., 2011), Gauthier et al. (2011) recently reported that p.Ser14Leu does not alter cell surface trafficking. These results further stress the need of functional approaches for rare variants.

To explore the possibility of a common underlying mechanism, we studied the c.−3G>T and p.Met1 variants. Using site-directed mutagenesis and expression analysis, we show the use of an alternative initiation codon by the c.−3G>T and p.Met1 alleles. The efficiency of translation initiation depends on the nucleotides flanking the AUG start codon, the so-called Kozak sequence (Kozak, 1986). In vertebrates the consensus Kozak sequence (GCCRCCaugG) contains a purine at position −3 in 90–95% of cases (Kozak, 1987; Pesole et al., 2000; Suzuki et al., 2000). Interestingly, several studies have reported disease-associated mutations within the Kozak sequence that affect protein expression (Kozak, 2002). In a spontaneous mouse model of human anophthalmia, mutational inactivation of the major start codon of the Rcx/rax gene, located in an optimal context, leaves only an upstream in-frame AUG codon surrounded by a weak sequence context. This results in reduced levels of the Rcx protein (Tucker et al., 2001). Mutation c.−3G>T identified here affects the conserved G at position −3 in NRXN1β, while p.Met1 changes the third nucleotide of the start codon. Our results revealed that these ASD-associated mutations shifted the initiation site from Met+1 to a downstream, in-frame AUG at position +5, located in a weaker sequence context. Furthermore, we observed that the effect of each mutation depends on the affected position. While the c.−3G>T mutated allele resulted in a mixed phenotype, with translation starting at Met+1 and Met+5, the p.Met1 allele was mostly initiated...
at Met+5. These findings indicate that in normal situations translation of neurexin-1β is mainly initiated at Met+1, and that the ASD mutations shift the initiation start codon to Met+5. As a consequence, the use of Met+5 generates a 4-aminoacid truncation within the signal sequence. Beta-neurexins have a long, unusual signal peptide of 40–50 aminocids, which is cleaved during protein maturation (Ushkaryov et al., 1994). The truncation of the signal peptide reported here does not seem to grossly alter the molecular weight and subcellular distribution of mutant neurexin-1β (Fig. 3A and data not shown). However, we observed a reduction of the synaptic levels of neurexin-1β when p.Met1 mutation was overexpressed in cultured hippocampal neurons. Although an effect on the stability or transport of the truncated protein cannot be ruled out, these results suggest that the use of a start codon located in a weaker sequence context (Met+5) leads to reduced synaptic levels in neurons. The observation that the c.→3G>T mutation did not significantly reduce synaptic levels suggests that the partial inhibition of translation from Met+1 caused by this mutation could have a more subtle effect, not detectable in overexpression experiments.

On the other hand, mutation p.Arg375Gln also leads to reduced synaptic levels of neurexin-1β. Given the location of the mutated residue, it is expected that this mutation affects protein levels through a mechanism not involving translation initiation. Recently, we and others have identified a proteolytical processing of neurexins by metalloproteases and γ-secretase, which takes place at the juxtamembrane and intramembrane regions, respectively (Bot et al., 2011; Saura et al., 2011). Importantly, this proteolytical processing could regulate neurexin levels at the synapse (Saura et al., 2011). Thus, based on their location on the juxtamembrane region of neurexin-1β, mutations p.Arg375Gln and p.Gly378Ser could affect residues important for this proteolytical processing. Identification of the cleavage sites will help to explore the role of these mutations in the processing of neurexin-1β.

In summary, the results reported here support a role for defects in NRXN1β as a shared mechanism associated with different mental disorders, including ASD and MR (Mitchell, 2011). Despite the fact that the underlying mechanism is not completely known for most of the mutations, the identification of heterozygous deletions in neurexin genes (Sztamari et al., 2007) suggests a dosage effect as a susceptibility factor (Toro et al., 2010). Importantly, a possible dosage effect has also been proposed for truncating mutations in NRXN1 and NRXN2 in schizophrenia and ASD (Gauthier et al., 2011) and reduced synaptic expression has been shown for ASD-associated mutations in NLGN3 and NLGN4 (Chih et al., 2004; Zhang et al., 2009). Accordingly, our data showing the effect of point mutations would be in agreement with reduced protein levels as a risk factor for ASD and MR. Further genetic analysis of NRXN genes in larger populations combined with functional analysis of ASD-associated mutations will help clarify the disease mechanisms.

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Appendix A. Supplementary data

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References


