Title page

Rare variants analysis of neurexin-1β in autism reveals a novel start codon mutation affecting protein levels at synapses.

Running Head: A novel mutation in the start codon of neurexin-1β in autism

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Abstract

Neurexins are synaptic plasma membrane proteins encoded by three genes (*NRXN1*, *-2*, *-3*) with alternative promoters. Mutations in neurexin genes have been identified in different neurodevelopmental disorders, including autism. Recently, two point mutations altering the translation initiation site of *NRXN1β* (c.-3G>T and c.3G>T) have been described in patients with autism and mental retardation. In this study, we analyzed the *NRXN1β* gene in a sample of 153 patients with autism. We report the identification of a novel mutation, c.3G>A (p.Met1), affecting the translation initiation site. Expression analysis showed that the c.3G>A mutation switches the translation start site of *NRXN1β* to an in-frame downstream methionine and decreases synaptic levels of the mutant protein in cultured neurons. These data reinforce a role for synaptic defects of *NRXN1β* in neurodevelopmental disorders.

Keywords:

autism, neurexin, *NRXN*, neuroligin, *NLGN*, mutation, synapse, translation

Text

Introduction

Autism is a complex neurodevelopmental disorder that appears in the first years of life and persists throughout adulthood. Autism is characterized by qualitative impairment in communication and social interactions, and restricted and stereotyped patterns of behavior, interests and activities (American Psychiatric Association, 1994). The disorder belongs to a larger group of neuropsychiatric conditions known as autism spectrum disorders (ASDs), which also include Asperger syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS). The prevalence of ASD is 6 in 1.000 individuals with a male to female ratio of 4:1 (Elsabbagh *et al.*, 2012).

In the last years, mutations in neurexin and neuroligin (Sudhof, 2008) genes have been found associated with ASD and other psychiatric disorders (Jamain *et al.*, 2003; Autism Genome Project *et al.*, 2007; Kirov *et al.*, 2008). Neurexins and neuroligins bind transynaptically to regulate important signaling events between presynaptic and postsynaptic terminals, pointing at synaptic defects as a potential pathological mechanism associated with mutations within this system (Sudhof, 2008). Neurexins are encoded by three genes in humans, each with two alternative promoters, generating the long, alpha, and the short, beta isoforms (Missler and Sudhof, 1998). Beta-neurexins are translated from a translation initiation site encoded by a specific first exon not shared with alpha isoforms. Recently, we reported two mutations within the *NRXN1β* specific exon in patients with autism and mental retardation that affect the translation initiation

site. These point mutations involve conserved residues within the Kozak sequence (c.-3G>T) and the start codon (c.3G>T) (Camacho-Garcia *et al.*, 2012).

In this report we evaluated the role of rare mutations in the etiology of autism by screening the *NRXN1β* gene in 153 ASD subjects. We identified in one patient a mutation not previously described (c.3G>A; p.Met1) that affects the translation initiation site. Expression studies showed that the c.3G>A mutation changes the initiation site and leads to reduced levels of neurexin-1β protein at the synapse. These results represent an additional evidence for a role of mutations that alter synaptic function of neurexin-1β in the risk to autism.

Methods

Patient samples

The autism cohort under study included 153 patients (male:female ratio: 4:1, mean age: 17.7 years) that met DSM-IV-TR criteria for autism, Asperger disorder and pervasive developmental disorder not otherwise specified (PDD-NOS) based on ADI-R (Autism Diagnostic Interview-Revised) and ADOS-G (Autism Diagnostic Observation Schedule-Generic) (Lord *et al.*, 1994; Lord *et al.*, 2000). Cytogenetic abnormalities and positive Fragile X test were considered as exclusion criteria. The control sample consisted of 400 healthy donors, sex-matched with the case sample (mean age: 47.7 years). Patients and controls were Caucasians and Spanish. The study was approved by the relevant ethical committee and written informed consent was obtained from all

parents. Genomic DNA was extracted from blood lymphocytes using the salting-out method.

Mutational analysis

Direct sequencing of the coding regions, splice junctions and of 560 bp of the 5' UTR and 131 bp of the 3' UTR regions of *NRXN1β* was performed as previously reported (Camacho-Garcia *et al.*, 2012). The identified variant was confirmed by an independent PCR amplification and re-sequencing with 5' and 3' primers.

Expression and Western blot analysis

The pCAGGS vectors expressing human NRXN1β, NRXN1β p.Met1(ATT), NRXN1β$^{Met1(ATT)_Met5Ile}$ and HA-NRXN1β have been reported elsewhere (Camacho-Garcia *et al*, 2012). A pCAGGS vector expressing NRXN1β p.Met1(ATA) was generated by amplifying the mutation-containing region from genomic DNA of the patient which contains the 5' UTR and the N-terminal region and replacing the corresponding sequence in the NRXN1β vector. Similarly, pCAG-NRXN1β$^{Met1(ATA)_Met5Ile}$ was generated by inserting a DNA fragment containing the p.Met1(ATA) mutation into NRXN1β$^{Met1(ATT)_Met5Ile}$. For expression in cultured neurons, HA-tagged wild-type and p.Met1(ATA) NRXN1β were cloned into a lentiviral vector under the control of the human synapsin promoter (Gascon *et al.*, 2008).

HEK293T cells were transfected with NRXN1β vectors using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, cells

were analyzed by Western blot with a pan-neurexin antibody (Dean *et al.*, 2003) and an α-actin antibody (Sigma, St. Louis, MO, USA) as previously described (Camacho-Garcia *et al.*, 2012). Data from three independent experiments were evaluated with one-way ANOVA and Dunn's method for multiple comparisons.

Primary hippocampal cultures were prepared from embryonic day 18 rat brains and plated at a final density of 25,000 cells/cm^2. For transfection experiments, cultures at 7–9 days *in vitro* were co-transfected with GFP and HA-NRXN1β or HA-NRXN1β p.Met1(ATA) 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.

Immunofluorescence analysis

Hippocampal cultures fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 were analyzed by immunofluorescence with synaptobrevin-2 (Synaptic Systems, Goettingen, Germany) and HA (Roche) antibodies, followed by incubation with secondary antibodies conjugated to Cy3 or Cy5 (Jackson ImmunoResearch).

Confocal images were captured in a Zeiss LSM 7 Duo microscope using a 60x Plan-Apochromat oil immersion objective. The GFP expressing regions of axonal segments were selected by thresholding, such that the specific HA-neurexin-1β signal was retained. Maximal projections of Z-stacked images were

analyzed with ImageJ software. Quantification of HA-neurexin-1β intensity and area at synaptic terminals was performed by selecting synaptobrevin-2 positive clusters within the GFP transfected axons as region of interest (ROI). Data were obtained from three independent neuronal cultures. Statistical significance was determined by Mann-Whitney test.

Results and Discussion

The coding sequence, splice junctions and the 5' UTR and 3' UTR regions of the *NRXN1β* gene were screened for rare variants in a sample of 153 patients with ASD. The only mutation found was a c.3G>A (p.Met1) identified in one male patient (Fig.1A). This variant was absent from a control sample of 400 individuals and from the 1000 Genomes and Exome Variant Server datasets. The patient was diagnosed with autistic disorder at the age of 3 years, with language delay and normal-high intellectual capacity (IQ =125). Segregation analysis showed that the c.3G>A (p.Met1) mutation is maternally inherited and is present in three of the proband's sisters. The mother has a major depression and socialization problems and one of the carriers was diagnosed with anorexia nervosa, while the other two carriers did not present any psychiatric trait (Fig.1B). Interestingly, mutations in *NRXN* genes have been described in patients with different psychiatric disorders, although they are also present in asymptomatic relatives (Bucan *et al.*, 2009; Gauthier *et al.*, 2011). This is in accordance with the multi-hit model for the molecular basis of this type of disorders, where the phenotype would be the result of the combined effect of several genetic variants.

The mutation described here, p.Met1(ATA), affects the initiation codon of the neurexin-1β, ATG>ATA. The mutated residue is evolutionary conserved and affects only neurexin-1β, sparing the alpha-isoform. As the p.Met1(ATA) mutation alters the Met start site, we performed Western blot analysis of transfected cell lysates to evaluate the translation of the mutant protein. We observed expression of neurexin-1β p.Met1(ATA), indicating that translation was not abolished. Interestingly, we had previously identified two different mutations in ASD patients that switch translation initiation of neurexin-1β to a downstream Met codon at position +5 (Met5). One of the reported mutations also affects the p.Met1 codon, p.Met1(ATT) (Camacho-Garcia *et al.*, 2012). To analyze whether this is also the case for the novel mutation identified here, we generated NRXN1β constructs where Met5 was mutated (p.Met5Ile) and protein expression was analyzed by Western blot. The results showed that neurexin-1β expression was inhibited when Met5 was mutated in the context of the p.Met1(ATA) mutant allele (Fig.1C-D). Similar results were obtained with p.Met1(ATT) mutation, as previously reported (Fig.1C-D) (Camacho-Garcia *et al.*, 2012). On the contrary, expression was preserved when the Met5 residue was mutated in the wild-type allele (Fig. 1C-D). These results suggest that mutation p.Met1(ATA) switches the translation initiation site to Met5, which is not normally used in control individuals.

Neurexins have a role at synapses regulating presynaptic function. To evaluate whether the p.Met1(ATA) mutation affects the synaptic levels of the protein, we

generated HA-tagged forms of neurexin-1β by inserting a HA-epitope after the signal peptide and transfected cultured hippocampal neurons. Thus, immunofluorescence analysis using an HA antibody allowed us to distinguish transfected neurexin over endogenous proteins. HA-neurexin-1β showed a distribution that partially colocalized with the presynaptic marker synaptobrevin, as previously shown (Fig. 2). However, expression of HA-neurexin-1β p.Met1(ATA) resulted in a reduction of protein levels in transfected axons. Quantification analysis revealed a decrease of ~30-40% in mean intensity and area of HA-neurexin-1β p.Met1(ATA) when compared with HA-neurexin-1β (Fig. 2). These data indicate that the autism-associated mutation c.3G>A (p.Met1) results in a reduction of the protein levels at the synapse. These results confirm previous data showing that protein translation form the alternative start site at Met5 leads to reduced synaptic levels of neurexin-1β (Camacho-Garcia *et al.*, 2012).

In conclusion, the novel mutation identified here, c.3G>A (p.Met1), provides an independent confirmation that variants that affect the translation initiation site of neurexin-1β uncover an alternative start codon at Met5. This novel start site is located in a weak consensus initiation sequence that is not the major start site in individuals not bearing the identified change. Translation from this alternative initiation site leads to a reduction in the synaptic levels of neurexin-1β in cultured neurons, reinforcing a dosage effect of neurexins in autism (Toro *et al.*, 2010).

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Captions

Figure 1: Identification of mutation c.3G>A (p.Met1) in *NRXN1β* in autism. (A) Sequence chromatogram showing the identified variant. (B) Pedigree of the proband's family showing the segregation of the mutation. Symbols are as follows: solid black: proband with autism; solid grey: major depression and socialization problems; striped grey: anorexia nervosa. The genotype for the c.3G>A mutation is indicated under each symbol (heterozygotes are shown in red). (C) Top panel. Sequence of *NRXN1β* showing the mutations affecting the start codon. The alternative start codon at position Met5 is also indicated. Lower panels. Expression of neurexin-1β proteins by Western blot analysis in non-transfected (control) or transfected HEK293 cells with the indicated constructs. Expression of neurexin-1β was not inhibited when Met5 residue was mutated in the context of the wild-type protein (WT_Met5Ile), whereas it was inhibited when Met5 is mutated in the context of p.Met1 mutations (Met1(ATA) and Met1(ATT)_Met5Ile). Arrowheads indicate the ~80 and ~50 kDa bands for the mature glycosylated and non-glycosylated proteins, respectively. Expression of α-actin is shown as a loading control. (D) The Met5Ile/Met5 ratio is calculated to quantify the protein expression that remains when the Met5 is mutated in the different alleles. Mutation of Met5 in p.Met1 mutants results in a significant reduction in protein levels. WT: 64.343±30.148%; Met1(ATA): 10.292±4.028%; Met1(ATT): 7.693±4.022%. Error bars indicate SEM. Data obtained from three independent experiments. ***p<0.001.

Figure 2: Mutation c.-3G>A (p.Met1) in *NRNX1β* results in reduced synaptic levels. (A) Confocal images of hippocampal neurons co-transfected with GFP and empty vector (control), HA-NRXN1β or HA-NRXN1β p.Met1(ATA) vectors, as indicated. Expression of GFP (green in the colocalization), HA-neurexin-1β (red in the colocalization) and synaptobrevin-2 (blue in the colocalization) was analyzed by immunofluorescence. Two sets of images per each HA-NRXN1β construct are shown. Arrowheads indicate localization of HA-neurexin-1β proteins and synaptobrevin. Mean intensity (B) and area (C) of HA-neurexin-1β at synaptic terminals of transfected neurons. HA-neurexin-1β synapses: intensity 102.106±2.92; area 67.209%±1.987. HA-neurexin-1β p.Met1(ATA) synapses: intensity 69.204±3.38; area 39.169%±1.968 (n=17 images, 287 ROIs and n=22 images, 398 ROIs for HA-neurexin-1β and HA-neurexin-1β p.Met1(ATA) synapses, respectively). Data were obtained from three independent experiments and expressed as means ± SEM. ***p<0.001. Scale bar: 5 μm.