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ORIGINAL ARTICLE

Homozygous frameshift variant in *NTNG2*, encoding a synaptic cell adhesion molecule, in individuals with developmental delay, hypotonia, and autistic features

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Abstract

Regulation of neuronal connectivity and synaptic communication are key to proper functioning of the brain. The Netrin-G subfamily and their cognate receptors are vertebrate-specific synaptic cell adhesion molecules with a role in synapse establishment and function, which seem to have co-evolved to contribute to higher brain functions. We identified a homozygous frameshift variant in *NTNG2* (NM_032536.3: c.376dup), encoding Netrin-G2, in eight individuals from four families with global developmental delay, hypotonia, secondary microcephaly, and autistic features. Comparison of haplotypes established this as a founder variant. Previous studies showed that *Ntng2*-knockout mice have impaired visual, auditory, and motor coordination abilities required for demanding tasks, as well as possible spatial learning and memory deficits. Knockout of *Ntng2* in a cellular model resulted in short neurites, and knockout of its trans-synaptic partner Ngl2/Lrrc4 in mice revealed autistic-like behavior and reduced NMDAR synaptic plasticity. The *Ngl2/Lrrc4*-knockout mouse phenotype was rescued by NMDAR activation, suggesting a mechanistic link to autism spectrum disorder. We thus propose *NTNG2* as a candidate disease gene and provide further support for the involvement of Netrin-G2 in neuropsychiatric phenotypes.

Keywords *NTNG2* · Netrin family · Synaptic adhesion molecules · Autism spectrum disorder · Exome sequencing

Introduction

Processing of information in the brain depends on precise neuronal connectivity and synaptic communication. Developing neurons must target neurites, establish synaptic contacts, designate presynaptic and postsynaptic roles, and prepare for signal transmission [1, 2]. Dysregulation of synaptic communication has been implicated in neuropsychiatric disorders such as autism spectrum disorder, schizophrenia, and intellectual disability [3–5].

Synaptic cell adhesion molecules, such as neuroligins, neurexins, and EphB, are critical regulators of synapse establishment and function [6]. Netrins mediate cell repulsion, attraction, or adhesion in the nervous system and other tissues [7]. Classic netrins are phylogenetically conserved, diffusible signaling molecules which interact with diverse single pass cell surface receptors. The Netrin-G subfamily is an independent subfamily of the UNC6/netrin family, exclusive to vertebrates. It consists of the neuronal ligands Netrin-G1 and Netrin-G2, which are not soluble but rather glycosylphosphatidylinositol (GPI)-anchored and enriched in

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presynaptic terminals. Netrin-G1 and Netrin-G2 bind their cognate NetrinG ligand (NGL) trans-synaptic partners to promote axon outgrowth, induce and maintain excitatory synapse formation, and contribute to elaboration of the complex and highly organized central nervous system (CNS) structure unique to vertebrates [8]. NGL1 and NGL2 exhibit distinct, complementary patterns of expression in the developing and maturing brain, suggesting that their differential expression and selective binding may contribute to the specificity of neuronal circuits [9, 10].

Abnormal expression of Netrin-Gs has been associated with behavioral phenotypes in mice, and with possible involvement with schizophrenia, bipolar disease, temporal lobe epilepsy, and Rett syndrome in humans [11]. In this study, we report a homozygous frameshift variant in *NTNG2* encoding the Netrin-G2 cell adhesion molecule, identified in 8 individuals from 4 apparently unrelated Arab Muslim families who presented with developmental delay, hypotonia, secondary microcephaly, and autistic behaviors. We present the clinical and molecular features of this newly described syndrome in light of current knowledge of Netrin-G contribution to higher brain functions.

Materials and methods

Exome analysis Following informed consent, exome analysis was pursued on DNA extracted from whole blood of the proband in each family (Fig. 1a). Exonic sequences from DNA were enriched with the SureSelect Human All Exon 50 Mb V4/V5 Kits (Agilent Technologies, Santa Clara, CA, USA). Sequences were generated on a HiSeq2500 (Illumina, San Diego, CA, USA) as 125-bp paired-end runs. Read alignment and variant calling were performed with DNAnexus (Palo Alto, CA, USA) using default parameters with the human genome assembly hg19 (GRCh37) as reference. Exome analysis of the probands yielded 42.5–60.1 million reads, with a mean coverage of 66–81 ×.

Sanger validation and segregation of the variant Amplicons containing the *NTNG2* variant were amplified by conventional PCR of genomic DNA from probands and all available parents and siblings, and analyzed by Sanger dideoxy nucleotide sequencing.

Results

Clinical reports

Eight affected individuals, including six males and two females from four consanguineous families (see pedigrees, Fig. 1a) were referred to genetics due to global developmental delay and hypotonia. Age at initial evaluation was 15 months at the earliest, and last follow-up was 9 years at the latest. A summary of the clinical information is presented in Table 1. The families were all of Arab Muslim origin residing in the Jerusalem and Hebron areas though no known relationship was documented between the families.

The affected children were all born after uneventful pregnancies at term with birth weights appropriate for gestational age. Perinatal course was normal. Initial concerns regarding psychomotor development were raised at 3–6 months of age when the children failed to achieve milestones. No regression was ever noted, but development stagnated at a level of approximately 9 months of age. The affected children were thus able to sit, albeit briefly for some, had no expressive verbal language, and did not use their hands purposefully beyond grasping and bringing objects to the mouth. Repetitive stereotypies of the hands and bruxism were noted in most affected individuals. Growth parameters were also delayed, and failure to thrive was diagnosed at a few years of age with head circumference more severely affected than weight or height. Seizures or involvement of other organs was not documented.

Initial examination was notable for appendicular and trunk hypotonia in all affected children with no pyramidal, cerebellar, or extrapyramidal signs present. Minor dysmorphic features consisting of beaked nose, pectus excavatum, and bilateral 5th finger clinodactyly were noted in the proband of Family 4 (IV-5 in Fig. 1a). Hearing and vision were not affected though cortical visual impairment could not be ruled out. Numerous ancillary investigations were variably carried out and yielded normal results for metabolic and electrophysiologic tests as well as for brain MRI (Table 1). The clinical features were thus similar in all affected individuals and consisted of nonsyndromic severe intellectual disability without regression and without seizures. Hypotonia and failure to thrive with relative microcephaly were also present in most.

Exome sequencing identifies a homozygous frameshift variant in *NTNG2*

To identify an underlying genetic diagnosis, exome sequencing was pursued on the proband in all four families. Following alignment to the reference genome [hg19] and variant calling, variants were filtered out if the total read depth was less than 8 ×, and if they were off-target (> 8 bp from splice junction), synonymous, or had minor allele frequency (MAF) > 0.001 in the GnomAD database. Variants that survived filtering may be found in Table S1. The affected individuals all shared a homozygous frameshift variant in *NTNG2* (NM_032536.3: c.376dup, p.(Ser126PhefsTer241)), found on a shared haplotype of ~ 4.35 Mb. This single nucleotide insertion is expected to result in a frameshift at Ser126 of 531 amino acids. The identified variant was absent from the GnomAD database (https://gnomad.broadinstitute.org/). Within our in-house

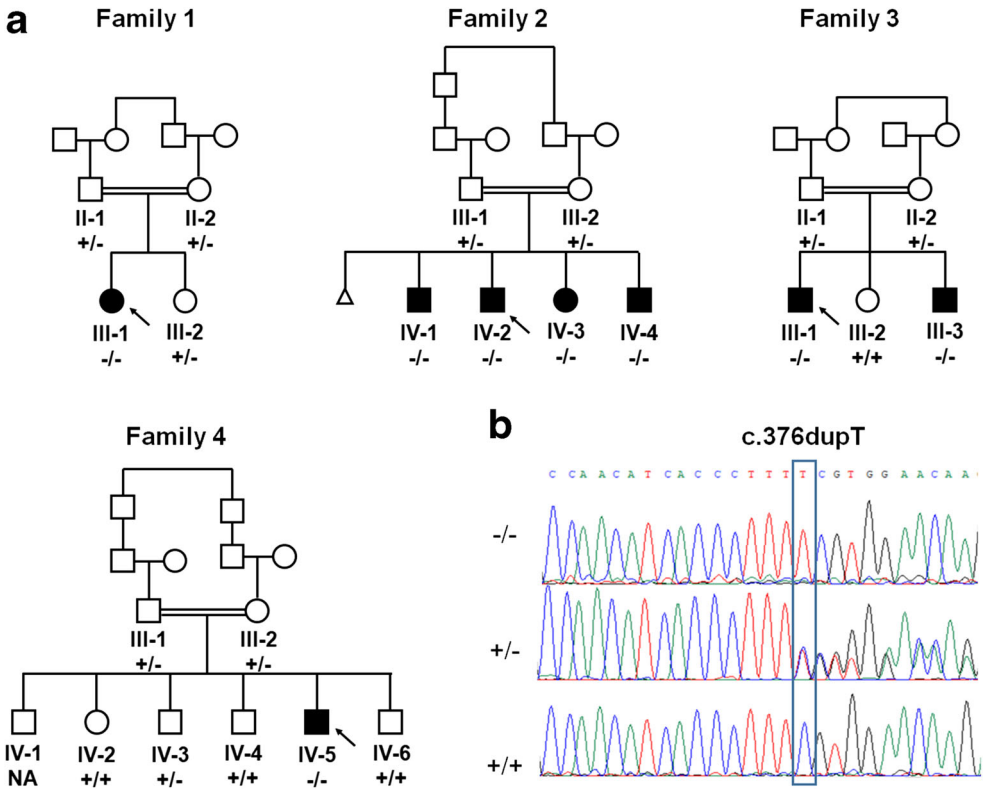

Fig. 1 Pedigrees and molecular analysis of affected families. **a** Pedigrees of the four consanguineous families, indicating segregation of the homozygous *NTNG2* frameshift variant. Plus (+) and minus (−) signs indicate wild-type and variant alleles, respectively. **b** Sanger traces of parents and affected child in Family 4, showing the c.376dup frameshift variant

exome database comprising ~3500 individuals with 50% Arab Muslims, two other unrelated probands were heterozygous for the *NTNG2* variant. Ascertainment of the connection between the families was done by a gene-first approach, whereby the in-house database was probed for homozygous variants in *NTNG2*. The c.376dup variant segregated with the disease status in all affected individuals and available family members (Fig. 1a, b).

Discussion

In this study, we report 8 individuals from 4 families of Arab Muslim consanguineous descent with a consistent phenotypic presentation of global developmental delay, hypotonia, and autistic features including reduced eye contact, absent verbal communication, and repetitive hand movements (Table 1). Genetic analysis revealed a homozygous frameshift variant in *NTNG2* (NM_032536.3: c.376dup), suggesting a founder variant in the Arab Muslim population.

NTNG2 encodes Netrin-G2 of the vertebrate-specific Netrin-G/NGL family whose members seem to have co-evolved to contribute to higher brain functions. In the hippocampus, parietal cortex, and piriform cortex, NGL-1 and NGL-2 are concentrated in the dendritic segments corresponding to the lamina-specific termination of netrin-G1-positive and netrin-G2-positive axons, respectively, and indicating specificity of neuronal circuits. Neither netrin-G1 nor netrin-G2 appear indispensable for axonal guidance; rather, netrin-G proteins seem to transneuronally determine local dendritic properties through their receptors on dendrites after completion of axonal projection [8]. Netrin-G2-knockout (KO) mice have impaired visual, auditory, and motor coordination abilities required for demanding tasks, as well as reduced anxiety levels and possible spatial learning and memory deficits. At a cellular level, Netrin-G2-KO mice have aberrations of NGL2 localization. In the absence of its presynaptic ligand, NGL2 undergoes lateral diffusion on dendrites and substantial amounts of NGL2 are internalized in postsynaptic sites. Thus, Netrin-G2 regulates lateral translocation of NGL2 along dendrites and vertical translocation of NGL2 at postsynaptic sites [12].

NGL2, the Netrin-G2 trans-synaptic partner also known as LRRC4, is a leucine-rich repeat-containing postsynaptic adhesion molecule that interacts intracellularly with the excitatory postsynaptic scaffolding protein PSD-95 [13]. Functionally, it serves as a polarity regulator with a role in axon differentiation and excitatory synapse development and transmission [14]. *Ngl2/Lrrc4*-KO mice display mild social interaction deficits and repetitive behaviors, with suppressed N-methyl-D-aspartate receptor (NMDAR)–dependent synaptic plasticity in the hippocampus. NGL2 has been proposed to promote synaptic stabilization of NMDARs and to regulate NMDAR-dependent synaptic plasticity. Accordingly, *Ngl2*-KO mice show rapid improvement in autistic-like behavior when provided with pharmacological NMDAR activation, providing

Table 1 Clinical features of the affected individuals

Individual	Family 1, III-1	Family 2, IV-1	Family 2, IV-2	Family 2, IV-3	Family 2, IV-4	Family 3, III-1	Family 3, III-3	Family 4, IV-5
Gender	F	M	M	F	M	M	M	M
Age at last follow-up	9 y	12 y	11 y	9 y	6.5 y	8 y	4 y	8.5 y
Weight (*Z*-score)	14 kg (−5.74)	20.5 kg (−4.68)	21.5 kg (−3.56)	11.7 kg (−7.91)	13 kg (−4.98)	16 kg (−4.24)	11.5 kg (−3.38)	15.4 kg (−5.28)
Height (*Z*-score)	108 cm (−4.40)	123 cm (−3.7)	123 cm (−3.04)	109 cm (−4.20)	104 cm (−2.8)	107 cm (−3.79)	88 cm (−3.42)	116 cm (−2.57)
Head circumference	48 cm (−3.2 SD)	52 cm (11th %ile)	51 cm (5th %ile)	47 cm (−4.1 SD)	50 cm (10th %ile)	48.5 cm (−2.7 SD)	46.5 cm (−2.9 SD)	49.5 cm (−3.9 SD)
Global DD	+	+	+	+	+	+	+	+
Peak verbal development (age-equivalent)	8 mo	8 mo	8	6 mo	7 mo	8 mo	4 mo	9 mo
Peak motor development (age-equivalent)	9 mo	8 mo	9 mo	8 mo	8 mo	9 mo	6 mo	10 mo
Hypotonia	+	+	+	+	+	+	+	+
Poor eye contact	+	+	+	+	+	+	+	+
Cognitive delay	+	+	+	+	+	+	+	+
Absent speech	+	+	+	+	+	+	+	+
Stereotypic hand movements	+	+	+	+	+	+	+	+
Seizures	–	–	–	–	–	–	–	–
Joint laxity	+	+	+	+	+	+	+	+
MRI	Normal	Normal	Normal	NA	NA	Normal	NA	Normal
EEG	Normal	Normal	Normal	Normal	Normal	Normal	NA	Normal
Previous evaluations	Negative *MECP2* and *SMN1* sequencing, negative metabolic screening (SAA, UOA, lactate), and negative muscle biopsy	None	Negative metabolic screening (SAA, UOA, ammonia, lactate)	None	None	Ophthalmic exam normal; negative metabolic screening (SAA, UOA, lactate, ammonia), normal CMA	None	Negative metabolic screening (SAA, UOA, ACP, lactate, VLCFA) and negative muscle biopsy, normal CMA

ACP acylcarnitine profile, *CMA* chromosomal microarray, *DD* developmental delay, *EEG* electroencephalogram, *mo* months, *MRI* magnetic resonance imaging, *NA* not available, *SAA* serum amino acids, *UOA* urine organic acids, *VLCFA* very long chain fatty acids, *y* years

further support to the hypothesis that NMDAR dysfunction contributes to autism spectrum disorders [3].

In humans, alterations in NetrinG/NGL family members have been associated with diverse neuropsychiatric phenotypes. For example, polymorphisms in NetrinG genes have been associated with development of schizophrenia [15, 16], altered expression levels of NetrinG2 have been linked to bipolar disorder [17], and NGL2 dysfunction has been implicated in autism spectrum disorder [18]. A de novo balanced translocation, 46,XX,t(1;7)(p13.3;q31.3), interrupting *NTNG1* was identified in a female with characteristic features of Rett syndrome yet no pathogenic variants in *MECP2* or *CDKL5*, suggesting that *NTNG1* may be a candidate gene for atypical Rett syndrome [19]. Moreover, CDKL5, associated with a neurodevelopmental disorder similar to Rett syndrome, has been shown to bind and phosphorylate NGL1 and to thus promote formation of dendritic spines in cultured neurons [20].

Ntng2 has been shown to be downregulated in cells with *KDM5C* variants, associated with lower levels of H3K4 methylation at its promoter. *KDM5C* encodes X-linked lysine (K)-specific demethylase 5C, and pathogenic variants in this gene are associated with neuropsychiatric symptoms such as intellectual disability, delayed language development, epilepsy,

and impulsivity. Interestingly, knockout of *Ntng2* in the control mouse neuroblastoma cell line Neuro2a resulted in short neurites, phenotypically similar to cells with *KDM5C* mutations. *Ntng2* overexpression in *KDM5C*-mutant cells rescued this morphological phenotype [21], providing further support to the connection between Netrin-G2 and neurodevelopmental phenotypes.

In conclusion, our data suggest *NTNG2* as a candidate disease gene associated with a clinical presentation characterized by global developmental delay, autistic mannerisms, and hypotonia. Based on available mouse models and cellular data, we propose that the mechanism may involve disruption of proper synaptic formation and transmission and altered NMDAR synaptic plasticity that may benefit from pharmacological modulation of NMDAR function.

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