Homozygous Missense Variants in NTNG2, Encoding a Presynaptic Netrin-G2 Adhesion Protein, Lead to a Distinct Neurodevelopmental Disorder

Caroline M. Dias,1,2,3 Jaya Punetha,3,1,4,5 Céline Zheng,4,5 Neda Mazaheri,6,7 Abolfazl Rad,7,17 Stephanie Effthymiou,8 Andrea Petersen,9 Mohammadreza Dehghani,10 Davut Pehlivan,5,11 Jennifer N. Partlow,1,12 Jennifer E. Posey,3 Vincenzo Salpietro,8 Alper Gezdirci,13 Reza Azizi Malamir,14 Nihal M. Al Menabawy,15 Laila A. Selim,15 Mohammad Yahya Vahidi Mehrjardi,10 Selina Banu,16 Daniel L. Polla,17,18 Edward Yang,19 Jamileh Rezazadeh Varaghchi,20 Tadahiro Mitani,3 Ellen van Beusekom,17 Maryam Najafi,21 Alireza Sedaghat,22 Jennifer Keller-Ramey,23 Leslie Durham,9 Zeynep Coban-Akdemir, Ender Karaca,1,24 Valeria Orlova,4 Lieke L.M. Schaeken,17 Amir Sherafat,25 Shalini N. Jhangiani,26 Valentina Stanley,27 Gholamreza Shariati,6,28 Hamid Galedhari,3 Joseph G. Gleeson,27 Christopher A. Walsh,1,12 James R. Lupski,3,26,29,30 Elena Seiradake,4 Henry Houlden,8 Hans van Bokhoven,7 and Reza Maroofian8,*

NTNG2 encodes netrin-G2, a membrane-anchored protein implicated in the molecular organization of neuronal circuitry and synaptic organization and diversification in vertebrates. In this study, through a combination of exome sequencing and autozygosity mapping, we have identified 16 individuals (from seven unrelated families) with ultra-rare homozygous missense variants in NTNG2; these individuals present with shared features of a neurodevelopmental disorder consisting of global developmental delay, severe to profound intellectual disability, muscle weakness and abnormal tone, autistic features, behavioral abnormalities, and variable dysmorphisms. The variants disrupt highly conserved residues across the protein. Functional experiments, including in silico analysis of the protein structure, in vitro assessment of cell surface expression, and in vitro knockdown, revealed potential mechanisms of pathogenicity of the variants, including loss of protein function and decreased neurite outgrowth. Our data indicate that appropriate expression of NTNG2 plays an important role in neyotypical development.

Based on studies in invertebrates and chicken and mouse, netrins are considered to be the paradigmatic axon guidance molecules, yet the essential role of this family of proteins in humans remains unclear. Members of the classical netrin family are secreted proteins that include UNC6 (uncoordinated-6) in C. elegans and netrins NTN1–4 in vertebrates.1,2 Netrin-G proteins (NTNG1 and NTNG2) are distinct from classical netrins in that they are vertebrate-specific, membrane-bound proteins tethered to the plasma membrane by glycosyl phosphatidylinositol (GPI) anchors.3 NTNG1 (MIM: 608818) and NTNG2 are predominantly expressed in a non-overlapping and complementary pattern in specific neuronal subsets of the developing and mature central nervous system.1–6 The proteins interact with the extracellular region of their specific netrin-G ligand receptors NGL-1/LRRC4C (MIM: 608817) and NGL-2/LRRC4, respectively. Selectivity in binding between netrin-G molecules and their cognate receptors is

1Division of Genetics and Genomics, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA; 2Division of Developmental Medicine, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA; 3Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA; 4Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK; 5Department of Genetics, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, 6153783151, Iran; 6Nages Medical Genetics and Prenatal Diagnosis Laboratory, Kianpars, Ahvaz, 6155689467, Iran; 7Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar, 009851, Iran; 8Department of Neuromuscular Disorders, Queen Square Institute of Neurology, University College London, WC1N 3BG, London, UK; 9Randall Children’s Hospital at Legacy Emanuel, Portland, OR 97227, USA; 10Medical Genetics Research Centre, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; 11Section of Pediatric Neurology and Developmental Neuroscience, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA; 12Howard Hughes Medical Institute, Boston Children’s Hospital, Boston, MA 02115, USA; 13Department of Medical Genetics, Kanuni Sultan Suleyman Training and Research Hospital, Istanbul, 34330, Turkey; 14Department of Paediatric Neurology, Gozaltem Medical, Educational, and Research Center, Alvaz Jundishapur University of Medical Sciences, Alvaz 6163764648, Iran; 15Pediatric Neurology and Metabolic Division, Cairo University Children Hospital, Egypt; 16Department of Pediatric Neurology, ICH and SSH Hospital Mirpur, Dhaka, 1216, Bangladesh; 17Department of Human Genetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6500 HB, Nijmegen, the Netherlands; 18CAPES Foundation, Ministry of Education of Brazil, 549 Brasilia, Brazil; 19Department of Radiology, Boston Children’s Hospital, Boston, MA 02115, USA; 20Hasti Genetic Counseling Center of Welfare Organization of Southern Khorasan, Birjand, Iran; 21Genome Research Division, Human Genetics Department, Radioboud University Medical Center, 6500 HB, Nijmegen, the Netherlands; 22Health Research Institute, Diabetes Research Center, Alvaz Jundishapur University of Medical Sciences, Alvaz, Iran; 23GenExD, Gaienhurn, MD 20877, USA; 24Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA; 25Department of Neurology, Faculty of Medicine, Bam University of Medical Sciences, Bam, Iran; 26Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030, USA; 27Laboratory for Pediatric Brain Disease, Howard Hughes Medical Institute, Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA; 28Department of Medical Genetics, Faculty of Medicine, Alvaz Jundishapur University of Medical Sciences, Alvaz 6135715794, Iran; 29Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA; 30Texas Children’s Hospital, Houston, TX 77030, USA 31Present address: Department of Genetics and Genomic Sciences, Ichsun School of Medicine at Mount Sinai, New York City, NY 10029, USA

*Correspondence: r.maroofian@ucl.ac.uk

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mediated by the interactions of three loops of the laminin domain, and the extracellular leucine rich repeats (LRR) domain of NGLs results in a molecular hand-clasp interaction of high affinity.  

NTNG2 encodes netrin-G2, a vertebrate-specific protein that is part of a distinct functional sub-class of the highly conserved netrin family. The netrin family provides axonal guidance cues during central nervous system development. NTNG2 is located on 9q34.13, and the canonical transcript consists of eight exons including seven coding exons; it encodes a 530-amino-acid protein. NTNG2 demonstrates evidence of missense constraint in the ExAC database, with a Z score of 4.34, and review of population-based (gnomAD) and ethnically diverse in-house databases reveals an absence of homozygous damaging and/or deleterious variants. Despite this constraint, its potential role in human genetic disease is not clear. Here we show that bi-allelic missense variants in NTNG2 cause a distinctive neurological and behavioral disorder that highlights the importance of this family of genes in human nervous system development.

We have identified 16 individuals (from seven unrelated families) who have ultra-rare bi-allelic variants in NTNG2 and who present with shared clinical features of a neurodevelopmental disorder. Consent for clinical data and biological material collection, use, and storage was obtained from all participating families after written informed consent was provided, and studies for each family were approved by their respective institutional review boards (see Supplemental Data for further details). Following genomic DNA extraction from blood, exome sequencing, and homozygosity mapping, we identified 16 individuals in seven unrelated families from different parts of Iran (Families 1, 2, 3), Mexico (Family 4), Turkey (Family 5), Egypt (Family 6), and Bangladesh (Family 7) who have a similar clinical phenotype and have homozygous missense variants in NTNG2 (Figure S1, Table S1). Researchers and physicians for all families were connected using the GeneMatcher/Matchmaker exchange. All families except for Families 4 and 7 had a known history of consanguinity, and all of the variants were segregated in the affected families in accordance with Mendelian expectations for a recessive disease trait (Figure 1). Autozygosity mapping for Families 4 and 7 revealed distant relatedness, and parents of the proband in Family 7 come from the same village (Figure S2, Table S2). There was no evidence of neuropsychiatric disorders in the heterozygous family members presented here.

Clinical features of affected individuals are presented in Table 1. Affected individuals presented with global developmental delay with severe to profound intellectual disability; the majority were non-verbal and non-ambulatory. Most individuals also had features of autism and all were noted to have mood and/or behavioral challenges, many of which were similar to those seen in Rett syndrome, such as hand stereotypy, episodes of laughing and/or screaming, and bruxism, and in Angelman syndrome (Videos S1–S4). Gastrointestinal symptoms, including constipation and bloating, were also common. Growth parameters were below average, and four individuals had documented failure to thrive. Secondary microcephaly was also observed. Dysmorphic features were variable and included low-set ears, hypotelorism, and frontal

![Figure 1. Pedigrees of All Families with Affected Individuals and Variants and Segregation Findings](image-url)

+ indicates wild-type allele, - indicates variant allele, P indicates proband.
bossing (Figure 2A). Neurologically, hypotonia in infancy and muscle weakness and/or atrophy were common findings. Five individuals had early-onset seizures, and four were noted to have ocular findings of esotropia, nystagmus, or strabismus. Brain imaging, conducted in both infancy and childhood, demonstrated findings ranging from normal to mild brain atrophy with white matter abnormalities (Figure 2B). In summary, affected individuals display a neurodevelopmental disorder of severe-to-profound intellectual disability with marked motor involvement and mood and behavioral challenges including autistic features, as well as poor growth and facial dysmorphisms. Detailed phenotypic descriptions are provided in Table 1, Table S1, Figure 2, and the Supplemental Note: Case Reports in Supplemental Data.

The ultra-rare variants we identified from family based genomic studies in the above individuals were notable for several reasons (Table S2). All variants were absent from both local ethnically diverse in-house databases, as well as large population databases. Because most of the NTNG2 missense variants observed are rare to their specific “clan,” they may reflect variants that arose recently and according to the clan genomics hypothesis are therefore expected to have a larger influence on disease.12 All variants were predicted by a majority of prediction tools (FATHMM, MutationAssessor, MutationTaster, PolyPhen-2, SIFT, PROVEAN, and CADD) to be likely damaging to protein function, and genomic evolutionary rate profiling (GERP) indicated that these sites may be under evolutionary constraint (Table S2). In fact, all variants impact residues conserved in NTNG1, a result that gives further evidence for the argument that they are pathogenic. Annotation of the variant locations on the protein domains of NTNG2 revealed that they are not confined to one domain, but they fall within the laminin and EGF domains and are predicted to disrupt structural motifs within NTNG2 (Figure 3A).

No other alternative candidate variants common to the families were identified (Table S3). The available NGL2/netrin-G2 crystal structure contains a model of the netrin-G2 N terminus up to the first EGF domain. We used MODELER13 to create a homology for the EGF domains 2–4, which were not included in that crystal structure. Using these models, we found that the variants are located in the laminin, EGF2, or EGF4 domain (Figure 3B–3C, Figure S3). In addition to possible effects on specific protein-to-protein interaction sites, this suggests a more global mechanism of functional disruption. Strikingly, we found that four of the seven, i.e., 57% of the variants, involve the loss or addition of cysteine residues (GenBank: NM_032536.3: c.242G>A [p.Cys81Tyr], c.1065C>G
Figure 2. Clinical Features of Affected Individuals
(A) Representative photographs demonstrating clinical features of affected individuals; these features include facial features, muscular atrophy, and hand stereotypy. Top row from left to right: Family 2 IV:2, IV:3, IV:1; Family 1 IV:3, IV:2. Bottom row: Family 3 IV:1, IV:2; Family 6 IV:2, IV:1; Family 7 II:1.
(B) Representative MRIs of affected individuals, demonstrating decreased brain volume. From top to bottom: Family 6 IV:2; Family 5 IV:4; Family 5 IV:5.
(C) Bar graph summarizing proportions of various clinical findings affecting individuals.
[p.Cys355Trp], c.1076C>G [p.Ser359Cys], c.1367G>A [p.Cys456Tyr]). Given that the cysteine content of NTNG2 is only 7.9% in humans, the enrichment for cysteine variants in this cohort suggests a mechanism of pathogenicity. Due to the oxidizing environment in the endoplasmic reticulum (ER) and extracellular space, cysteine residues found in extracellular proteins typically appear in pairs and form disulfide bridges. Such bridges can stabilize a protein by reducing the entropy of the unfolded state and/or they can facilitate the path to the native state if they link parts of a protein that must come into contact early during a folding reaction.14 Unpaired exposed cysteines are detected by the ER quality control machinery and targeted for refolding or degradation.14 We hypothesize that the NTNG2 variants involving cysteine could have a negative effect on protein stability and cell surface expression.

Three out of the seven variants do not involve cysteines: c.599C>T (p.Ser200Leu), c.319T>G (p.Trp107Gly), and c.446T>C, (p.Met149Thr). For both p.Trp107Gly and p.Met149Thr, a large hydrophobic residue (Trp or Met) is changed to either one lacking a side chain (Gly) or one bearing a small polar side chain (Thr). Both of these residues form part of the hydrophobic core that stabilizes the folding of the netrin-G2 laminin domain. Disruption likely causes protein misfolding and lack of expression at the cell surface. Thus the consequence of both types of variants, cysteine-dependent or hydrophobic core disruptive, is potentially a similar reduction in protein stability and expression at the cell surface. The p.Ser200Leu variant does not fit into either of the above categories, with Ser200 located at the periphery of the laminin domain. It is also located ~0.9 nm away from the surface of NGL2.
With typical hydrogen bonds about 0.25 nm in length, Ser200 is not interacting directly with NGL2, although allosteric effects could still influence the netrin-G2 binding loops. We tested all variants for function by overexpressing wild-type (WT) and variant netrin-G2 constructs in HeLa cells and assessing their presence at the cell surface through the use of indirect immunofluorescence and immunoblotting validation. All variants displayed substantially decreased cell surface expression as compared to WT (Figures 3D–3E). Notably, some of these variants had more cell surface expression compared to others, suggesting that some netrin-G2 may still be localized in these individuals. The variants may nevertheless show deficient ligand-receptor binding or signaling since we did not observe a clear association with cell surface expression levels and clinical phenotype severity.

Given the decreased cell surface expression pattern observed in all seven variants, we sought to determine the more global effects of NTNG2 loss of function. Using siRNA to target endogenous Ntng2 expression in mouse N2A cells, we first confirmed that transfection with our Ntng2-specific siRNA led to decreased expression with quantitative polymerase chain reaction (Figure 4A). We next assessed neurite outgrowth and found a significant reduction for all parameters assessed, which included neurite number, neurite length, and convex hull area—a measurement used for measuring dendritic field (Figures 4B–4F). These findings demonstrate a potential mechanism by which the NTNG2 variants may contribute to pathological neurodevelopment.

Netrin signaling has been implicated in neurologic and psychiatric disorders. For example, conditional Ntn1 knockout in distinct neuronal subtypes is associated with...
alterations in fear and anxiety-like behaviors in rodent models, and abnormal expression of NTNG2 has been found in the human brain in refractory epilepsy. Studies of Ntng2 and Ngl2 knockout mice have shown that both types of mutant mice have an identical phenotype of lack of behavioral startle in response to acoustic stimulus, with no structural abnormalities noted in the inner ear. Single-nucleotide polymorphisms (SNPs) and differential expression patterns of NTNG1 and NTNG2 have been associated with schizophrenia and bipolar disorder in humans. A have been associated with schizophrenia and bipolar disorder in humans. A de novo genomic rearrangement involving NTNG1 was proposed to potentially cause features of Rett syndrome in an isolated individual. Additionally, de novo missense variants in NTNG1 were reported in two individuals with autism spectrum disorder. An in vitro study of variants in the histone demethylase KDM3C (Lysine demethylase 5C [MIM: 314690]), which is known to cause intellectual disability, showed that NTNG2 seems to be important in mediating effects on neurite growth and length; these results are consistent with our findings here. In fact, several clinical features, in addition to intellectual disability, are shared between these two disorders, including variable neurologic, behavioral, and dysmorphic features. Extensive behavioral battery on Ntng2 knockout mice demonstrated marked deficits in learning, memory, and visual and motor functioning. Although NTNG2 does not appear to be necessary for axon guidance, it has been shown to be important in the laminar distribution of its receptors and synaptic plasticity. A homozygous founder frameshift variant in NTNG2 has recently been identified in eight individuals from four families with a similar clinical phenotype, and this further strengthens the evidence supporting the pathogenicity of the variants presented here.

Other genes involved in netrin signaling have also been implicated in neurodevelopmental disorders in isolated case reports, some of which have involved examples of de novo variation. Specifically, variations in LRRC4C and LRRC4 have both been associated with intellectual disability and autism. Furthermore, functional work in mice has shown that LRRC4 expression regulates N-methyl-D-aspartate receptor (NMDAR)-dependent synaptic plasticity and prevents autistic-like behaviors. LRRC4C and LRRC4 have both been shown to be important in hippocampal synapse formation and function. The marked findings of severe intellectual disability and autistic features in our cohort are particularly intriguing given the unique role of NTNG2 in vertebrates. As we previously mentioned, netrin-g family members express in distinct, non-overlapping, and complementary neuronal circuits, suggesting a role in establishing appropriate neuronal patterning. This neuronal compartmentalization parallels distinct behavioral compartmentalization, as in mouse knockout models, Ntng2 knockouts demonstrated sensorimotor, spatial memory, working memory, procedural learning, and attentional deficits, while Ntng1 knockouts demonstrated distinct learning and fear conditioning deficits. Our findings here, in conjunction with the known role of NTNG2 in the control of synaptic plasticity and postsynaptic membrane organization, illustrate the clinical relevance of these neuronal functions to higher cognitive processes. In fact, given the profound finding of intellectual disability in the individuals presented here, it is intriguing that NTNG2 expression is enriched in the human claus-A

Our work provides the groundwork for establishing a genotype-to-phenotype relationship with NTNG2 variants, and establishes an initial description of the clinical spectrum. NTNG2 should be considered in the clinical evaluation of children with severe intellectual disability and neuropsychiatric symptoms. In addition to identification by exome sequencing, it will be important to add NTNG2 to clinical gene-panel tests for intellectual disability given the marked yet variable clinical phenotype. In summary, our results implicate rare bi-allelic missense NTNG2 variants in the pathobiology of a neurodevelopmental disorder consisting of severe intellectual disability, autistic features, and motor impairment. Our findings provide strong clinical and functional evidence for the importance of the appropriate expression of NTNG2 in neurodevelopment.

Accession Numbers
The accession numbers for the variants reported in this paper are ClinVar SCV000994967.1, SCV000994968.1, SCV000994969.1, SCV000994970.1, SCV000994971.1, SCV000994972.1, and SCV000994973.1. LOVD variant identification numbers are S97120–S97126.

Supplemental Data
Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.09.025.

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**Declaration of Interests**

BayCollege of Medicine (BCM) and Miraca Holdings have formed a joint venture with shared ownership and governance of Baylor Genetics (BG), which performs clinical microarray analysis and clinical exome sequencing. J.R.L. serves on the Scientific Advisory Board of BG. J.R.L. has stock options in Lasergen, and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial infections. The other authors declare no competing interests.

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**Web Resources**

- CADD, https://cadd.gs.washington.edu/
- ExAC, http://exac.broadinstitute.org/
- FATHMM web server, http://fathmm.biocompute.org.uk/
- GeneMatcher, https://genematcher.org/
- GERP, http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html
- gnomAD, https://gnomad.broadinstitute.org/
- MutationAssessor web server, http://mutationassessor.org/r3/
- MutationTaster, http://www.mutationtaster.org/
- Online Mendelian Inheritance in Man, https://www.omim.org
- PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
- PROVEAN, http://provean.jcvi.org/index.php
- SIFT, https://sift.bii.a-star.edu.sg/

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