



FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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AUTISM AND SYNAPTIC GENES: CLINICAL CASES

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE PEDIATRIA

Trabalho realizado sob a orientação de:

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MARÇO 2018

Autism and synaptic genes: clinical cases

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Abstract

Introduction: Autism Spectrum Disorder (ASD) is a complex, early onset and life-long neurodevelopmental disorder. The prevalence of ASD has increased over the past decade. Moreover, the fundamental molecular pathways involved in this disorder are still largely uncharacterized. Although autism is a descriptive diagnosis, it has a strong genetic influence and heterogeneity and abundant evidence pointing to many hundreds of genetic variants involved, accounting for roughly 20% of the cases. Studying susceptibility genes is needed to define more valid genotype-phenotype relationships. Mutations in genes encoding neurexins (NRXN) and neuroligins (NLGN) have been associated with susceptibility for ASD and other neurodevelopmental disorders. Neurexins and neuroligins are cell adhesion molecules that connect presynaptic and postsynaptic neurons at synapses. They are emerging as central organizing molecules for excitatory and inhibitory synapses in the central nervous system. Unbalance synaptic transmission that results from dysfunctions in NRXN/NLGN mediated signalling, may lead to different neurodevelopmental disorders.

Objective: To characterize the full phenotypic spectrum associated to NRXN and NLGN mutations, reporting clinical features of individuals followed in our specialized Neurodevelopmental and Autism Unit of Child Developmental Center in Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra (HP-CHUC).

Methods: Using microarray-based comparative genomic hybridization (array-CGH), five probands were identified with mutations of the *NRXN* and *NLGN* genes, in patients diagnosed with ASD and attending the Neurodevelopmental and Autism Unit of Child Developmental Center in Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra (HP-CHUC). The ASD diagnosis was confirmed based on the Autism Diagnostic Interview – Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS), fulfilment of DSM-5 criteria and on clinical evaluation by a specialized neurodevelopmental multidisciplinary team. Furthermore, psychomotor development was evaluated using the Ruth Griffiths Developmental Scale and the Vineland Adaptive Behaviour Scale and the global severity of the autism was assessed using the Childhood Autism Rating Scale (CARS). Complete genetic and metabolic evaluations were performed to exclude other medical conditions.

Results: Patient 1 presented a CNV loss in the *NRXN* gene and was diagnosed with ASD and mild intellectual disability. Patient 2 presented a CNV gain in the *NRXN* gene and was diagnosed with ASD without intellectual disability. Patients 3, 4 and 5, presented a missense mutation in the *NLGN* gene and were diagnosed with severe ASD with severe intellectual disability, mild ASD without intellectual disability and severe Global Development Delay, respectively. Patient 3 has a younger brother with a diagnosed language disorder, who also carries the same variant and their mother, who is heterozygous for the same variant, had a documented learning disability. Patient 4's mother, heterozygous for the same variant, presented a learning disability. Patient 5 had also comorbid epilepsy, and his mother and grandmother, who are carriers of the same variant, showed no apparently signs of ASD or other cognitive disability.

Discussion and conclusions: Our findings revealed intellectual phenotypical heterogeneity and ASD severity level. Variable gene expression and incomplete penetrance is observed, which implies that other factors must cooperate to produce the ASD phenotype. Therefore, the presence of a mutation in these genes may contribute to ASD susceptibility but does not necessarily imply the manifestation of this disease. Although mutations in the *NRXN* and *NLGN* genes are implicated in rare cases of ASD, our findings emphasize their importance in the pathogenesis of neurodevelopmental disorders. Further studies are necessary on the functional properties of the proteins and may provide new insights on synaptic pathways and personalized therapeutic strategies for ASD.

Keywords

Autism; Autism spectrum disorder; Neuroligins; Neurexins; Synapse; Synaptic gene

Abbreviations:

- ABC: Adaptive Behaviour Composite;
- AChE: Acetylcholinesterase;
- ADI-R: Autism Diagnostic Interview - Revised;
- ADOS: Autism Diagnostic Observation Schedule;
- ASD: Autism Spectrum Disorder;
- CAMs: Cell Adhesion Molecules;
- CARS: Childhood Autism Rating Scale;
- CGH - Comparative Genome Hybridization;
- CNS: Central Nervous System;
- CNV: Copy Number Variant;
- DQ: Developmental Quotient;
- DSM 5 - Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition;
- EGF: Epidermal Growth Factor;
- GDQ: Global Developmental Quotient;
- IDD: Intellectual Developmental Disability;
- LDQ: Language Development Quotient;
- LNS: Laminin, Neurexin and Sex hormone;
- MRI: Magnetic Resonance Imaging;
- NLGN: Neuroligin;
- NRXN: Neurexin;
- PSDQ: Personal-Social Development Quotient;
- PDQ: Performance Development Quotient;
- SNV: Single Nucleotide Variation;
- SS: Splice Site.

Introduction

Autism spectrum disorder (ASD) is a complex chronic neurodevelopmental disorder that is characterized by impairments social interaction and communication, as well as by repetitive and limited patterns of behaviour and interests¹. ASD is a multifactorial brain dysfunction, with a high worldwide prevalence and a general ratio of four males (M) to one female (F)²⁻⁴.

These symptoms must be present from early childhood and limit or impair everyday functioning and are not better explained by intellectual developmental disorder (IDD) or global developmental delay (GDD). However, IDD and ASD frequently co-occur. ASD is a chronic neurodevelopmental disorder with a significant social impact¹.

Although ASD is a descriptive diagnosis, without implying an underlying pathology, it has a strong genetic influence and heterogeneity and abundant evidence pointing to hundreds of susceptibility genetic variants involved⁵. Heritability estimates for ASD have ranged from 37% to higher than 90%, based on twin concordance rates, making it the most heritable of all neurodevelopmental disorders⁶. Multiple converging research strategies to account for ASD genetic liability have identified a variety of genetic causes that account for roughly 20% of ASD cases⁷. ASD is polygenic and multifactorial in aetiology. The genetic complexity of ASD maybe mirrors their phenotypic complexity⁷.

Recent breakthroughs have advanced our understanding of ASD from the standpoint of human genetics and neuropathology, highlight the period of early brain development and neurological connectivity⁸. In fact, most of the features of ASD manifest in the first few years of life, at the time of brain development when sensory experience is modifying excitatory synapse maturation and elimination, and promoting the development of inhibitory synapses⁹.

Rare mutations in multiple members of the neurexin (NRXN) and neuroligin (NLGN) families have repeatedly been found to be associated with ASD (**Table 1**).

Proper brain function is based on a balance between excitation and inhibition, which are mainly mediated by two major neurotransmitters, glutamate and GABA, respectively¹⁰. Neurexins and neuroligins are emerging as central organizing molecules for excitatory glutamatergic and inhibitory GABAergic synapses¹¹. They function as synaptic cell-adhesion molecules (CAMs), namely, neurexins and neuroligins form Ca²⁺ dependent complexes at synapses in the central nervous system (CNS) (**Figure 1**).

Several lines of evidence suggest that neurexins and neuroligins play a role during the very initial steps of synaptogenesis¹².

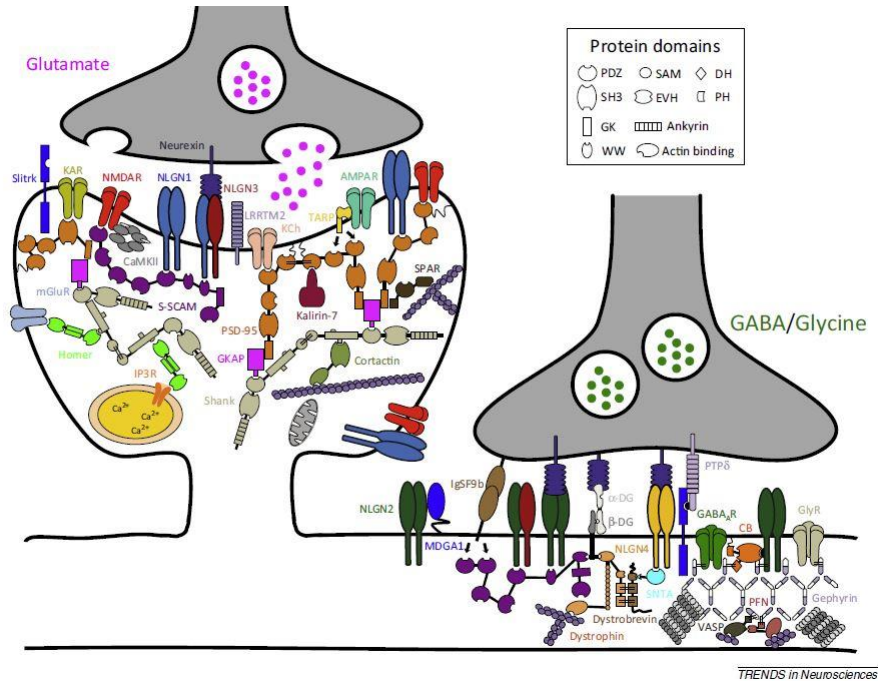


Fig. 1 – NLGN binding partners at excitatory and inhibitory synapses.

Adapted from Bembem MA *et al.* The cellular and molecular landscape of neuroligins. Trends in Neurosciences 2015.

NRXNs are type I membrane proteins and are predominantly located on the presynaptic compartment⁶. There are three neurexin genes in mammals, *NRXN* 1-3, each of which has both an upstream promoter that is used to generate the larger α -neurexin and a downstream promoter that is used to generate the smaller β -neurexin¹³. A γ -neurexin is transcribed from an internal promoter in the *NRXN1* gene and splices into the NRXN1 α and NRXN1 β ¹⁴. The three neurexin genes are transcribed in brain at similar levels, with α -neurexins being much more abundant than β -neurexins¹⁴. The two types of neurexin contain different amino-terminal extracellular sequences but identical carboxy-terminal transmembrane regions and cytoplasmic tails^{6,15}. Extracellularly, β -neurexins contain a single LNS domain (laminin, neurexin and sex hormone binding protein domain), whereas α -neurexins contain six LNS domains organized into modules with three epidermal growth factor (EGF)-like domains¹¹.

Furthermore, each α -neurexin has six canonical splice sites (referred to as SS1 to SS6), two of which are also present in the β -neurexin isoforms (SS4 and SS5)¹⁴. Alternative splicing at these sites allows the generation of thousands of alternative NRXN isoforms^{6,13}. Comparison of neurexin genes shows that *NRXN1* and *NRXN3* are more closely related to each other than to *NRXN2*, suggesting that evolutionarily *NRXN2* diverged from a common progenitor of *NRXN1* and *NRXN3*¹⁴.

NLGNs are endogenous neurexin ligands⁶. They are type I membrane proteins, like NRXNs, but have a simpler domain structure, are less diverse and are located exclusively at the post-synaptic compartment¹⁶. There are five neuroligin genes in humans: *NLGN1*, *NLGN2*, *NLGN3*, *NLGN4* and *NLGN4y/5*, whereas other mammals express only three or four¹². In humans, both *NLGN3* and *NLGN4* are localized to the X-chromosome and the *NLGN4* gene is complemented on the Y-chromosome by a similar *NLGN5*¹². The major extracellular domain of neuroligins is homologous to acetylcholinesterase (AChE) but lacks cholinesterase activity. Thus, instead of mediating enzyme/substrate interaction, this domain is thought to participate in receptor/ligand-like interaction¹³. The AChE-homologous region of neuroligins contain alternative splice site A (SSA), and there is an additional splice site B (SSB) within this region specifically in *NLGN1*¹¹. *NLGN1* is localized to excitatory synapses and *NLGN2* to inhibitory, dopaminergic, and cholinergic synapses possibly because dopaminergic and cholinergic synapses use GABA as a co-transmitter^{6,14,17}. *NLGN3* is found in both excitatory and inhibitory synapses, and *NLGN4* is found in glycinergic synapses^{14,17}. The high degree of homology between neuroligin isoforms suggests similar biological activities, yet their distinct localizations and different synapse specificities indicate divergent functions¹⁷. Recruitment of specific neuroligin homologues may direct the development of individual synapses towards either an excitatory or inhibitory fate¹⁶. Thus they are able to control the balance between excitatory and inhibitory synapse formation¹⁰.

Neuroligins bind to both α -neurexins and β -neurexins with nanomolar affinities. These binding affinities differ characteristically between various pairs of neuroligins and neurexins, and they are controlled by alternative splicing of both neurexins and neuroligins⁶.

Neuroligins and neurexins both have relatively short intracellular domains that terminate in PDZ-domain-binding sites, which are presumably important for linking them to other synaptic proteins. Intracellularly, the cytoplasmic sequence of neurexins contains a C-terminal binding site for class II PDZ domains that binds to the PDZ domain of CASK and related proteins, and a membrane-proximal binding site for protein 4.1. Through these interactions, neurexins may control the synaptic vesicle release machinery. Like neurexins, neuroligins bind to intracellular PDZ-domain proteins, but in contrast to neurexins, neuroligins bind to class I PDZ domains such as those contained in PSD95, which are centrally involved in recruiting glutamate receptors (NMDA- and AMPA-receptors) at postsynaptic sites. PSD95 binds to GKAP, which, in turn, binds to SHANK proteins and this complex may further recruit other post synaptic proteins to the excitatory synaptic junctions^{6,12}.

Neuroligins and α -neurexins are essential for synaptic function, but not for synapse formation. It was proposed that rather than mediating synaptogenesis, trans-synaptic interactions between neurexin and neuroigin may be more important for synapse maturation and specifically activity dependent modification of synaptic strength^{6,16}.

It has become widely accepted that impairments in neurexins and neuroligins caused by mutations may disturb the balance between excitatory and inhibitory activity that is thought to be critical for the underlying mechanisms in neuropsychiatric and neurodevelopmental diseases, such as schizophrenia and ASD, the mechanisms of which have proved difficult to ascertain^{6,12,14,15}.

The role of neurexins and neuroligins in synaptic function almost predestine them for a role in neurodevelopmental diseases⁶. The complexity of understanding neurodevelopmental diseases, is that they may arise from subtle changes in a subset of synapses in a neural circuit, as opposed to general impairment of all synapses in all circuits^{6,12}. As a result, the same molecular alteration can produce different circuit changes and neurological symptoms, which are then classified as distinct neurodevelopmental diseases. Conversely, markedly different molecular changes can produce similar syndromes, as exemplified by the different mutations that are associated with ASD⁶.

Several mutations and Copy Number Variants (CNVs) in *NRXN1-3* have been found to be associated with ASD with the prevalence highest for mutations in *NRXN1*⁶ (**Table**

1). Mutations in the *NRXN* and *NLGN* genes have been linked to the pathology of both ASD and schizophrenia. Similarly, *NLGN1-4* genes have been implicated in the pathogenesis of ASD with *NLGN3* and *4* being the most prevalent⁶ (**Table 1**).

Table 1 – Mutations in *NRXN* and *NLGN* genes linked to ASD.

NRXN

Missense mutation in exon 1 of *NRXN1β* gene¹⁸
 Deletion of coding exons from *NRXN1*¹⁹
 Ultra-rare mutation within *NRXN1α*²⁰
 De novo deletion of *NRXN1* gene²¹
 Disruption of *NRXN1* gene²²
 Intragenic rearrangements in *NRXN1*²³
 Deletions in *NRXN1*^{24,25}
NRXN1 and *NRXN2* disruptions²⁶
 Single nucleotide polymorphism within *NRXN 1*²⁷
 Micro-deletions within *NRXN3*²⁸
NRXN1β mutations close to initiation of translation associated with attention-deficit/hyperactivity disorder²⁹

NLGN

Point mutations within *NLGN3* and *NLGN4*³⁰
 Deletion in exon 5 of *NLGN4*³¹
 Missense mutations within *NLGN4* gene³²
NLGN4 isoform lacking exon 4; *NLGN3* lacking exon 7³³
 Deletion of exons 4, 5, and 6 in *NLGN4*³⁴
 Missense mutation in the promoter region, associated with an increased level of the *NLGN4* transcript³⁵
 Missense mutation in exon 5 of the *NLGN4* gene³⁶
 Synonymous substitutions in *NLGN3* and *NLGN4*³⁷
NLGN3 intronic mutations³⁸

Adapted from Bang *et al.* A matter of balance: Role of neurexin and neuroligin at the synapse. *Neurochem.* 2013

Our aim, is to characterize the full phenotypic spectrum associated with *NRXN* and *NLGN* mutations, reporting clinical features of individuals followed in our specialized neurodevelopmental and autism unit of Child Developmental Center in Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra (HP-CHUC).

Methods

In this retrospective study, five probands were identified with exonic mutations of the *NLGN* and *NRXN* gene, one female and four males currently with 2 to 21 years old, diagnosed with ASD and attending the Child Developmental Center in Hospital Pediátrico (a tertiary referral hospital) at the Centro Hospitalar e Universitário de Coimbra, which is a national reference for neurodevelopmental disorders, especially for ASD, which follow a paediatric population (0-18 years old).

A comprehensive clinical and biological database of more than 5000 patients with neurodevelopmental disorders (more than 2000 with ASD) is available. A large amount of this population is regularly followed two to three times per year, with an intake of new ASD cases around 150 per year from all over the country, half from the centre region of Portugal.

ASD diagnosis was confirmed based on gold standard instruments, such as Autism Diagnostic Interview-Revised (ADI-R)³⁹ and the Autism Diagnostic Observation Schedule (ADOS)⁴⁰ and on clinical evaluation by a specialized neurodevelopmental multidisciplinary team. The ADI-R is a structured interview conducted with the parents/caregivers of individuals who have been referred for the evaluation of possible autism spectrum disorder. Social interaction deficits were analysed using the score of social interaction area of the ADI-R (0-30 range) and the higher the score, the higher the social interaction impairment. The ADOS is a semi-structured standardized ASD diagnostic assessment, which consists in standard activities that allows the examiner to observe behaviours that have been recognized as critical to the diagnosis of ASD. All patients had positive results in the ADI-R and ADOS for ASD and met the criteria from the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. (DSM-5)¹.

Global clinical severity of autism was assessed using the Childhood Autism Rating Scale (CARS)⁴¹. According to the score, severity of autism can be classified as mild (< 30), moderate ($\geq 30 \leq 36$) or severe (≥ 37).

Global developmental quotient (GDQ) and subquotients in different dimensions (hearing and language, eye and hand coordination, personal-social and performance) were obtained using the Ruth Griffiths Developmental Scale (RGDS)⁴². Normal GDQ and subquotients is 100 ± 15 and the higher the score, the better the level of child's global development. The classification of the intellectual disability rank based on the

GDQ levels distinguishes four categories: mild intellectual developmental disability (50-69), moderate IDD (35-49), severe IDD (34-20) and profound IDD (below 20).

Global adaptive functioning was assessed by Vineland Adaptive Behaviour Scale⁴⁵ and three main domains (communication, socialization and daily living skills) were evaluated. The VABS has a total score, the Adaptive Behaviour Composite (ABC). Normal score is 100 ± 15 (standard deviation) and the higher the score, the better the individual functional - adaptive level.

Age for onset of independent walking was defined as the age (in months) at which the child takes unaided gait. Age for onset of first words was defined as the age (in months) at which the child first produced single words, other than “mama” and “dada,” in a consistent and meaningful way for the purposes of communication. Age for onset of first phrases was defined as the age (in months) at which the child first produced sentences composed of two or more words, one word being a verb, routinely used⁴⁶.

A blood sample was collected from each patient for the classical cytogenetic study (GTG banding) and for the last decade genetic study through Agilent CGH arrays (microarray-based comparative genomic hybridization or array-CGH, 4x180 configuration). Microarray-based comparative genomic hybridization allows the possibility to screen the whole genome at once and with high resolution. It is currently assumed that array-CGH should be the first genetic test offered to detect genomic imbalances in patients with ID, learning difficulties and ASD⁴⁷, after excluding other common diagnosis, such as fragile X.

From our database, three cases were selected with mutation of the gene *NLGN* and two cases with mutation of the gene *NRXN*, which is object of our study. We proceeded with a more detailed analysis of the clinical data regarding the current clinical history, the personal antecedents with attention to the milestones of neurodevelopment, the pre and perinatal history, the co-morbidities presented and family history.

The physical examination was performed with morphological and somatic evaluation (looking for possible dysmorphism and signs of neuro-cutaneous syndromes), anthropometric measurements, the search for visual or auditory sensory deficits, classical neurological examination and research of epilepsy. Complete genetic and

metabolic evaluations were also carried out to exclude accompanying medical conditions.

Finally, the parents of the children with the chromosomal or molecular alteration were studied at laboratory level to determine the inheritance pattern of the variants.

Ethics Statement

This study and all the procedures were conducted in accordance with the declaration of Helsinki.

Results

Five probands were identified with exonic mutations of the *NLGN* and *NRXN* genes, one female and four males, diagnosed with ASD. Their clinical and genetic results are summarised in **Table 2**.

Table 2 – Clinical and genetic characterization.

Patient	Age at diagnosis (years)	Diagnosis	Genetic alteration
#1	2y	ASD and mild IDD	CNV loss in the <i>NRXN1</i> gene
#2	4y	ASD no IDD	CNV gain in the <i>NRXN1</i> gene
#3	3y	ASD and severe IDD	Putative missense mutation in the <i>NLGN4</i> gene
#4	4y	ASD no IDD	Putative missense mutation in the <i>NLGN4</i> gene
#5	4y	ASD and severe IDD	Putative missense mutation in the <i>NLGN4</i> gene

Legend: ASD- Autism spectrum disorder; CNV – Copy Number Variant; IDD – intellectual developmental disorder;

Patient #1: a boy who was the first and only child of healthy, unrelated parents. He was born at 38 gestational weeks by caesarean section. It was a risk pregnancy, due to the advanced mother's age but early postnatal history was normal, without complications. His growth was normal, weight in 75th percentile, height and head circumference in the 50th percentile. Early motor skills were acquired at normal age range, with a walking age of 12 months old. The first parental concerns were evident in the second year of life exhibiting language development delay (absence of first words or phrases) and abnormal social interaction skills. In addition, autism features, such as restricted interests, repetitive behaviour and impaired social interaction were also present. No dysmorphic features, except dolichocephaly, were found. Hypopigmented or hyperpigmented macules were excluded as well as other skin abnormalities. Neurological examination was normal. Growth maintained in the normal range for sex and age. Normal hearing and vision skills were confirmed. He was diagnosed at age 2 years with ASD (with above cutoffs scores on the three domains of the ADI-R for autism) and mild IDD (GDQ of 64).

A CNV loss at chromosome 2p16.3 involving *NRXN1* gene was identified. It is a micro intragenic deletion, *de novo*, with 47Kbp extension.

Patient #2: a boy who was the second child of healthy, unrelated parents. He was born at 39 gestational weeks by caesarean section. His pre-natal growth was normal, suitable for age and gender. Early postnatal history was normal, without complications. Early motor skills were acquired at normal age range, with walking age of 14 months old. The first parental concerns were evident in the second year of life exhibiting language delay. He had first words by 19 month of age and phrases at 36. He was referred at the age of 3 years old due to a deviant neurodevelopment and obsessions. No dysmorphic features were found and excluded neurocutaneous stigma and had a normal neurological examination. General growth was in the normal range for sex and age. Normal hearing and vision skills were confirmed. He was diagnosed at age 4 years with ASD (a positive ADI-R and a CARS score 35), without IDD (GDQ of 72). A CNV gain at chromosome 2p16.3 involving *NRXN1* gene was identified.

Patient #3: a girl who was the seventh child of unrelated parents. She was born at 38 gestational weeks by vaginal delivery. Her pre-natal growth was normal, suitable for age and gender. Early postnatal history was normal, without complications. Early motor skills were acquired at normal age range but walking age was 18 months. Since the beginning, was noticed a delay in global neurodevelopmental acquisitions. The first parental concerns were evident in the third year of life exhibiting severe language development delay (absence of words) as well as behavioural problems characterized by severe and excessive tantrums and eating difficulties.

In addition, autism features, such as restricted interests, repetitive behaviour and impaired social interaction were also present. She was referred at the age of 3 years old by behaviour problems and language impairment. Diagnosed at age 3 years with severe autism [the CARS score was 52, and scores on the three domains of the ADI-R, social interaction, nonverbal communication and repetitive behaviours were 26 (normal range <10), 12 (normal range <7) and 4 (normal range <3), respectively] and severe IDD (GDQ of 31, with a cognitive profile characteristic of autism children: performance DQ of 39 and language DQ of 18). The patient belongs to a large sib ship, with the mother, who also carries the variant, and four sibs reporting learning disability without autism. Patient #3 has a younger brother with a diagnosed language disability and a global developmental quotient below the mean (GDQ of 89 in Ruth Griffiths Mental Developmental Scale), who also carries the G99S variation, a putative *missense* mutation was identified in the *NLGN4* gene. Their mother, who is heterozygous for the

variation, had a documented learning disability. No major or minor dysmorphic features were found neither neurocutaneous stigmata. General growth was in the normal range for sex and age. Normal hearing and vision skills were confirmed.

Patient #4: a boy who was the third child of healthy, unrelated parents. He was born at 38 gestational weeks by caesarean section. His pre-natal growth was normal, suitable for age and gender. Early postnatal history was normal, without complications. Early motor skills were acquired at normal age range, with walking age was 13 months old. The first parental concerns were evident in the second year of life exhibiting regression in language (cease to produce words that previously seemed a permanent part of his lexicon). development with first words by 18 months of age, but phrases only appeared by 48 months. He only was referred to our unit at the age of 4 years old motivated by behaviour problems and language delay. No major or minor dysmorphic features were found. Hypopigmented or hyperpigmented macules were excluded as well as other skin abnormalities. Global motor and sensorial neurological examination was normal. General growth was in the normal range for sex and age. Normal hearing and vision skills were confirmed. He was diagnosed at age 4 years with mild ASD (the CARS score was 27, and scores on the three domains of the ADI-R, social interaction, nonverbal communication and repetitive behaviours were 20 [normal range <10], 12 (normal range <7) and 5 (normal range <3), respectively] without IDD (GDQ of 70). A putative *missense* mutation was identified in the *NLGN4* gene with a K378R variation. His mother, who is heterozygous for the variation had learning disability. There was no family history of neurological or psychiatric disease.

Patient #5: a boy who was the third child of healthy, unrelated parents. He was born at 38 gestational weeks by vaginal delivery. At 31 gestational weeks, a right ventriculomegaly was identified by foetal MRI. His pre-natal growth was normal, suitable for age and gender. Early postnatal history was abnormal, with presence of axial hypotonia. He had a delay in motor skills, with a walking age of 24 months. The first parental concerns were evident in the first year of life. He was referred at the age of 2 years old GDD. He maintained severe language development delay (absence of first words or phrases). No major or minor dysmorphic features were found. Neurological examination was abnormal, with MRI findings such as mega cisterna magna, small lesions at the right thalamic nucleus and discreet dilatation of the ventricular system. General growth was in the normal range for sex and age. Normal hearing and vision

skills were confirmed. He was diagnosed at age 4 years with severe global development delay (GDQ of 34) with ASD features. Although this patient has a ADOS positive score, he was not formally diagnosed with ASD until his mental age, assessed by the Ruth Griffiths Developmental Scale and Vineland, was superior to 2 years old. He was also diagnosed with epilepsy. A *missense* mutation was identified in the *NLGN4* gene. Family studies revealed that this variation was also present in the mother and grandmother of the child, both healthy (apparently with no signs of intellectual disability) and absent in brothers and cousins. The variant was a c.1519C>G (p.Leu507Val) in *NLGN4* gene.

In **Table 3** are represented neurodevelopmental milestones of the index cases, with patients 1, 3 and 5 were and persist non-verbal.

Table 3 – Neurodevelopmental milestones

Patient	Walking age (months)	First words age (months)	First sentences age(months)
#1	12	Absent	
#2	14	19	36
#3	18	Absent	
#4	13	18	48
#5	24	Absent	

The patients's neurodevelopmental evaluation with Griffiths and Vineland adaptive behaviour results is summarized in **Table 4**.

Table 4 – Participants neurodevelopmental and adaptive behaviour levels

Patient	#1	#2	#3	#4	#5
Ruth Griffiths Developmental Scale:					
Age of DQ assessment (years and months)	1y 9m	4y 1m	4y 1m	9y 10m	3y 11m
GDQ	64	72	31	70	34
LDQ score	44	82	18	64	37
EHDQ score	52	94	32	85	29
PSDQ score	66	75	28	71	34
PDQ score	66	94	39	64	27
Vineland Adaptive Behaviour Scale:					
Age of assessment (years and months)	2y	15y	17y 11m	18y	3y 2m
Adaptive behaviour composite	62	67	20	45	50
Autonomy score	70	64	20	65	56
Communication score	62	90	20	25	53
Socialization score	62	64	20	56	53

Legend: DQ: Developmental Quotient; GDQ: Global Developmental Quotient; LDQ: Language Development Quotient; EHDQ -Eye and Hand Coordination Development Quotient; PSDQ: Personal-Social Development Quotient; PDQ: Performance Development Quotient;

Discussion

ASD are highly heritable, and genomic studies have revealed that a substantial proportion of ASD risk resides in high-impact rare variation, ranging from chromosome abnormalities and CNV to single-nucleotide variation (SNV). These studies have highlighted a striking degree of genetic heterogeneity, implicating both *de novo* germline mutation and rare inherited ASD variation distributed across numerous genes. A genetic overlap between ASD and other neuropsychiatric conditions has also been increasingly recognized⁴⁸.

As previously mentioned, the prevalence for mutations in the *NRXN1* and *NLGN4* genes is higher in comparison their other family members, in this context. This tendency is also observed in our sample with 2 probands presenting CNVs in the *NRXN1* gene and 3 probands presenting SNVs in the *NLGN4* gene.

According to Sudhof (2017)¹⁴, *NRXN1* CNVs are not fully penetrant and, although *NRXN1* CNVs are extremely rare in the general population, their incidence is increased in apparently normal relatives of affected individuals. The same author also refers that although mutations in *NLGN3* and *NLGN4* were the first to be linked to apparently idiopathic autism, they are less common than *NRXN1* CNVs, but seem to be more penetrant. Moreover, although neuroligin mutations are not associated with a specific syndrome but a range of clinical presentations, this range is narrower and involves ASD more often than the range of presentations associated with *NRXN1* variants.

A study conducted by the Autism Genome Project Consortium (2007)¹⁹, detected a hemizygous deletion of coding exons from *NRXN1* for a pair of affected siblings. According to the study, both girls presented with typical autism, including characteristic developmental delays (one appeared nonverbal, whereas her sister had mild language regression) and neither parent had clinically notable features. Also, recent studies, such as Schaaf C. *et al.* (2012)²⁵ concluded that exonic deletions of *NRXN1* are associated with GDD, IDD of various degrees and ASD. Our findings corroborate with these observations, with proband 1, presenting a CNV loss in the locus of *NRXN1* gene, with ASD and mild ID diagnosis.

Another study from Wisniewiecka-Kowalnik B. *et al.* (2010)²³ found tandem intragenic duplications of *NRXN1-b* sequences in two families that were associated to

autism phenotypes and cognitive delays. Our findings are also in according to this study, with proband 2 presenting a CNV gain in the *NRXN1* gene.

Previous studies, such as Jamain S. *et al.* (2003)³⁰ and Laumonier F. *et al.* (2004)³¹ suggested that mutations in the *NLGN4* gene may contribute to a wide spectrum of phenotypes, ranging from mild IDD without communication deficits to ASD with or without IDD. In our sample, we presented proband 3,4 and 5 with mutations in the *NLGN 4* gene, presenting ASD and severe IDD (3 and 5 participants) and ASD without IDD in proband 4.

Interestingly, in our study, a variable gene expression is observed in proband 3 who has a younger brother, carrier of the same variation, with language disability and a GDQ below the mean but in normal range without ASD, and their mother, heterozygous for the variation, with a documented learning disability, both without ASD. Also, proband 4's mother, heterozygous for the same variation, presented a LD. Incomplete penetration is observed in proband 5, whose mother and grandmother, carriers of the same variant, are both healthy (apparently with no signs of intellectual disability). The fact that these variations are not fully penetrant indicates that other factors must cooperate to produce the ASD phenotype. The presence of a mutation in these genes may contribute to ASD susceptibility but does not necessary imply the manifestation of disorder. It also suggests that disruption of core elements of synapse function may be associated with many other neuropsychiatric conditions.

According to the literature, onset of motor skills, which classically did not associate with cognitive development, has proved to be affected in patients with autism⁴⁶. In fact, the onset of independent walking was delayed in all our subjects, except for proband 2, when compared to those with the same age and a typical development, in whom onset of walking occurs at the age of 12 months, on average⁴⁶. At this set delayed motor skills may represent an unspecified mark of neurological dysfunction. According to current evidence, ASD associated with language impairment is linked to the worse outcome in adulthood. This underlines the importance of developing the functional language skills in these patients. Within the neurodevelopmental milestones, we can highlight that delayed onset of first phrases seems to be related in a positive manner to a greater severity of ASD⁴⁶. In our study there is objectified a significant delay on the onset of first phrases in all subjects, when compared to those with the same age and a typical development, in whom onset of first phrases occurs at the age of 24 months, on

average⁴⁹. Remarkably, three probands still remain non-verbal, with low functional level and more severe ASD.

The limitations of this study are the inherent to a retrospective study, namely: (1) The completeness and accuracy of the clinical phenotype identified in these patients is entirely dependent on the clinical information that was documented in the medical database of these subjects. (2) Important data may not be available. In this case, the parents were not formally assessed to ascertain their cognitive, physical, and behavioral phenotypes. But the strengths of this work are the large expertise of the professionals dealing with all spectrum of neurodevelopmental disorders specially with autism in a daily basis.

Although the current data indicate that neurexin and neuroligin mutations are implicated in rare cases of ASD, our findings emphasize their importance in the pathogenesis of neurodevelopmental disorders, such as ASD and IDD. This is in according to recent literature reviews that highlight a *spectrum* in phenotypes of neurodevelopmental disorders, including schizophrenia. Therefore, it is recommended that genomic analysis, with high-resolution microarrays, be used in clinical assessment of all patients presenting with ASD features or IDD or even LD without a known medical condition.

Our cases, coming from a large ASD sample, adds knowledge to a better phenotype-genotype description of putative role of NRXN/NLGN in ASD, as a synaptic disorder.

Further studies on the functional properties of NRXN and NLGNs are necessary to provide insights into the fundamental mechanisms of neuronal circuits, such as how synapse function is compromised in neuropsychiatric diseases and may provide new understanding in pathophysiology and, in future, a personalized and precise therapeutic strategy for ASD with known etiology.

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