INT. J. BIOL. BIOTECH., 22 (2): 235-242, 2025.

CLINICAL AND MOLECULAR GENETICS OF NEUROLIGINS IN AUTISM SPECTRUM DISORDER

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ABSTRACT

Autism spectrum disorder (ASD) is a group of early-onset complex neurodevelopmental diseases, including classical autism, pervasive developmental disorders, and Asperger syndrome, characterized by defects in social interaction and communication or repetitive behavior. While high heritability estimates have been reported in ASD, their basis remains unexplained owing to the high heterogeneity of candidate susceptibility genes. The number of ASD susceptibility genes has been substantially expanding owing to genetic biotechnological advances and the growing number of searchable genome databases. ASDs are multifactorial, heterogeneous disorders resulting from multiple genetic variants. Over 30 mutations have been identified in the neuroligin gene family and have been implicated in the pathogenesis of ASD. However, reviews on neuroligin genes are rare, particularly in non-western countries. In this review, we have discussed the epidemiology, genetic etiology, and neuroligin gene family in ASD, including its variable expressivity and interactions with other conditions. Further, we discussed the mechanisms by which ASD-related mutations in neuroligin proteins influence synaptic activity in various brain areas and circuits.

Keywords: Autism Spectrum Disorder; neuroligin gene (*NLGN*); clinical features; molecular genetics

INTRODUCTION

Autism spectrum disorder (ASD, MIM 209850) is an early-onset neurodevelopmental disorder characterized mainly by difficulties in social interaction, communication and repetitive/ restricted behaviors, with an incidence of more than 1% in children worldwide (Lord *et al.,* 2020). Patients with ASD are more likely to have psychological problems, such as depression, anxiety, obsessive-compulsive disorder, and eating disorders (American Psychiatric Association, 2013). ASD is considered as immensely heritable; however, it pertains complex genetic variabilities, and manifests various copy numbers (CNs) (copy number-variants (CNVs); single nucleotide-variants (SNVs) (Sebat *et al.,* 2007; Liu *et al.,* 2014; Weitlauf *et al.,* 2014; Geschwind and Flint, 2015; Sanders *et al.,* 2015). Genetic studies at larger scale have identified numerous candidate genes involved as complex risk factors/variables in ASD (Iossifov *et al.,* 2014; Sanders *et al.,* 2015).

Neuroligins (NLGNs)-the postsynaptic proteins involved in cell adhesion in human have five types (NLGNs-1-3, and NLGNs-4X & 4Y) (Südhof, 2008; Krueger *et al.,* 2012). It was revealed that NLGN1 and NLGN2 has less relevance than the NLGN3 and NLGN4 that initially associate with nonsyndromic type of ASD (Jamain *et al.,* 2003; Laumonnier *et al.,* 2004; Yan *et al.,* 2005). The patients having ASD as well as other neuro-psychiatric complications were identified repeatedly for variations in NRXN and SHANK (Durand *et al.,* 2007; Szatmari *et al.,* 2007; Berkel *et al.,* 2010; Sato *et al.,* 2012). The ASD shows CNVs as well as SNVs for binding partners-NLGN1, the EPAC2 & MDGA2 (Bacchelli *et al.,* 2003; Bucan *et al.,* 2009). Although the existing literature does not fully present the information about the causative factors for synaptic connectivity for ASD, it was suggested that NRXN-NLGN and downstream setup process the cognitive/social expression of behavior.

Patients with ASD currently have a better prognosis than they did 50 years ago. An increasing number of individuals with the disorder can communicate and learn, and some children can become symptom-free by adulthood and have a social life. However, most patients cannot live independently. Novel risk trends have been discovered owing to advances in genetics and neuroscience; however, currently, these are of not much practical benefit. Further studies are needed to find effective therapeutic and medical therapies (Lord *et al*. 2018). There are two types of ASD: syndromic and non-syndromic (Jamain *et al*. 2003). Syndromic ASD refers to the co-existence of another neurological complication, e.g., patients having fragile X-syndrome (FXS), Rett-syndrome or tuberous-sclerosis (TSC), exhibit ASD-like expressions. Non-syndromic ASD is not related to any other neurological disorders (Jamain *et al*. 2003).

EARLY HISTORY OF AUTISM

Autism was first described in the early 1940s by psychiatrists Leo Kanner (1943) in the United States and Hans

Asperger in Austria (1944). However, it remained largely unknown outside psychiatry until the 1980s (Werling and Geschwind, 2013). Mothers were blamed for their children's illnesses, arguing that their children's lack of social development was owing to their emotional demeanor, and were therefore labeled "refrigerator mothers" (Miles, 2011). ASD is a category of etiologically and clinically heterogeneous disorders (American Psychiatric Association, 2013). Early autism twin studies have revealed that autism is highly heritable. In instances where one twin is diagnosed with autism, the other identical twin exhibits an 80% likelihood of developing the disorder. In fraternal twins, the corresponding rate is approximately 40%. In addition to genetic factors, environmental factors can contribute to autism or intensify its features.

EPIDEMIOLOGY

Children with ASD have a decreased ability to communicate with others from as early as 1 to 2 years of age and can be diagnosed before age 2. However, the disorder is most often diagnosed between the ages of 2 and 4, when children begin to acquire more advanced communication and social skills, such as learning to play with others. Boys are four or five times more likely than girls to develop ASD (Werling and Geschwind, 2013). The prevalence of autism is 4–10 per 10,000, whereas that of ASD is 10–60 per 10,000 (Bailey *et al*. 1995). Twin and family studies have revealed a strong genetic component; however, the genetic mechanisms underlying the predisposition to the disease remain largely unknown (Chakrabarti and Fombonne, 2001).

CLINICAL FEATURES OF ASD

ASD features appear before the age of three. Infants with ASD rarely want to be kissed or cuddled, and they rarely reach out to be picked up. Further, they avoid making eye contact. Despite early warning signs, children with ASD often get medical help only in their second year of age, when there are noticeable language delays (Hosseini and Molla, 2021). There are two types of characteristics associated with ASD. 1) Problems with communication and social interaction, including difficulties in regular communication, decreased exchange of desires or feelings, difficulties in reacting to social cues such as eye contact and facial expressions, and deficiencies in establishing and deficits in developing and understanding relationships. 2) Restricted and repetitive patterns of actions, interests, or activities, including a desire for a routine and unusual activities (Khan *et al*. 2012). Although the exact causes of ASD are unknown, research suggests that genes combined with environmental factors affect the development of ASD. The causes and mechanisms of ASDs are being actively investigated (Chaste and Leboyer, 2012).

CLINICAL DIAGNOSTIC CRITERIA FOR ASD

There are standard procedures for collecting knowledge about a child's strengths and weaknesses in various fields. A psychologist's assessment generally entails evaluating available data, making observations, interviewing both the parents and the infant, and administering tests. Sometimes, children are referred to another multidisciplinary practitioner, such as a psychiatrist or voice, occupational, or physical therapist. After gathering the required data, the findings are documented in a detailed report that includes a diagnosis and recommendations. The diagnostic assessment involves formal testing methods to ascertain or rule out differential diagnoses (Klin *et al*. 2005). Typically, a follow-up meeting with the parents is arranged to review the results and recommendations and to answer their questions.

The Statistical Manual of Mental Disorders (DSM-V) was updated and published in 2013 (American Psychiatric Association, 2013). In this revision, the way autism is classified and diagnosed is modified, and various disorders are grouped under ASD. The DSM-V criteria for ASD (Frazier *et al*. 2012) are as follows: 1) social contact and communication deficits, 2) limited interests, repetitive actions, and activities, and 3) impaired daily functioning. The screening tool for autism in toddlers and young children comprises a 20-min monitoring period (Weitlauf *et al.*, 2014), whereas the autism diagnostic observation schedule includes 45-min monitoring by a professional and is available in different formats for various ages (12 months to adulthood) and language levels (Weitlauf *et al.* 2014).

DIAGNOSTIC SYMPTOMS OF ASD

Various scales (Weitlauf *et al*. 2014), including the childhood autism rating scale, social responsiveness scale, social communication questionnaire, and intelligence quotient (IQ), can be used to assess ASD symptoms in children. Often, ASD is accompanied by intellectual disabilities. Clinicians and families should monitor the child's cognitive and language skills during their development (Wingate *et al*. 2014). As the first point of contact for parents, clinicians play an essential role in the early detection of ASD. Therefore, doctors must be able to identify the different signs and symptoms of the disorder, including qualitative impairments in social, language, and communication abilities and repetitive interests and actions (Johnson and Myers, 2007), as these should alert

physicians of the possibility of autism or its associated conditions. Symptom severity substantially varies among children with ASD. Even though the impairments typically manifest before three years of age, they may be subtle and not detectable before school age (Mattila *et al*. 2007). Approximately 11%–65% of school-age children with ASD have intellectual disabilities (IQ < 70) (Baron-Cohen *et al.* 2001).

GENETIC AND NON-GENETIC FACTORS

Various gene variants are thought to influence the risk of developing ASD; however, not everyone carrying the variant will be affected. Most gene variants have a minor impact, and environmental risk factors must be considered when assessing an individual's risk of developing this complex disorder. Non-genetic factors may account for up to 40% of the risk of ASD (Modabbernia *et al*. 2017). Mutations in more than one gene can be involved in an individual with ASD, and different patients can have mutations in different genes. There may be complex interactions between mutations in different genes or between the environment and mutated genes. Numerous candidate genes have been identified based on the discovery of genetic markers associated with autism in family studies. Unlike 22q13 deletion syndrome or FXS, autism is not caused by a single chromosome defect or single gene mutation (Müller, 2007).

CANDIDATE ASD GENES

Twin and family studies have indicated that genes are involved in the risk of autism; however, the exact genetic etiology of the disorder remains unclear. There is evidence for some candidates, but their involvement in the disease etiology remains to be demonstrated. Linkage, correlation, cytogenetic, and CNV studies and next-generation sequencing are used to identify candidate genes. When a candidate gene is discovered, it is typically tested using samples from various patients. Replication is critical for elevating a candidate gene to a risk gene, particularly for studies that depend on case–control comparisons (Muhle *et al*. 2004; Morrow *et al*. 2008). Several candidate genes, including *SHANK3*, *CNTNAP2*, *NRXN1*, *PTEN*, *FMR1*, and *TSC1* pertain mutations linked to ASD and intellectual disability (ID) (Geschwind and State, 2015).

NEUROLIGINS (NLGNS) AND ASD

The larger extracellular-region (N-terminal) has esterase homology-domain and the cytoplasmic tail (C terminal) in NLGM members (Ichtchenko *et al*. 1995). Transsynaptic structure with presynaptic neurexin (NRXN) proteins through extracellular domain is formed by NLGN proteins, and on the other hand, PSD95, EPAC, SHANK, and MDGA (postsynaptic molecules) interact with the cytoplasmic domain (Südhof, 2008; Woolfrey *et al*. 2009; Krueger *et al*. 2012; Connor *et al*. 2016). Although the underlying cause of ASD remains largely unclear, recent advances in genome sequencing have facilitated the identification of several disease-related genes. Mutations in synaptic proteins, such as cell-adhesion molecules, are closely linked to ASD (Laumonnier *et al*. 2004). Neuroligins are cell-adhesion molecules found on the postsynaptic side of synapses. Neuroligins interact with β-neurexins to form functional synapses. There are five neuroligin gene family members: *NLGN1* located on chromosome 3q26, *NLGN2* on 17p13, *NLGN3* on Xq13, *NLGN4* on Xp22, and *NLGN4Y* or *NLGN5* on Yq11 (Jeong *et al*. 2017).

NEUROLIGIN FAMILY MEMBERS

Neuroligins are cell-adhesion proteins on the postsynaptic membrane that help neurons shape and maintain synapses. They act as ligands for neurexins, which are presynaptic cell-adhesion proteins. Neuroligin and β-neurexin synaptic transmission is mediated by neuroligins and neurexins, resulting in interactions between two neurons, forming a synapse (Geschwind and State, 2015) (Fig. 1).

Neuroligins influence neural network properties by defining synaptic roles and mediating signaling by recruiting and stabilizing main synaptic components. Some postsynaptic proteins interact with neuroligins to help locate channels and neurotransmitter receptors (Laumonnier *et al*. 2004). Alterations in genes encoding neuroligins have been suggested to contribute to autism (Yu *et al.* 2013). Because of their essential role at synapses, NLGNs are of particular interest among ASD-associated genes discovered via human genetic screens.

Neuroligin-1

The substitutions P89L, T90I, G297E, L269P, and H795Y in NLGN1 (MIM 600568) have been associated with ASD (Nakanishi *et al.* 2017). Except for H795Y, which is located in the NLGN1 cytoplasmic domain, all of these mutations are located in the extracellular cholinesterase-like domain, altering its structure (Suzuki *et al*. 2012). P89L and T90I, in particular, influence the stability of a proline-rich loop on the protein surface. However, none of them are located in the NRXN1 binding site. The NLGN1 variants can be classified as high (P89L, L269P, and G297E) and low (P89L, L269P, and G297E) risk. Based on *in silico* prediction of deleterious effects (T90I, H795Y), protein

trafficking is altered in the high-risk variants. Particularly, P89L and L269P are retained in the endoplasmic reticulum (ER), supposedly owing to protein misfolding. Although the low-risk variants do not alter cell localization, H795Y results in lower expression levels owing to a higher susceptibility to proteolytic cleavage and degradation (Chih *et al.* 2004, Suzuki *et al*. 2012)

P89L NLGN1 knockin (KI) mice have poor spatial memory and abnormal social activity behavior (Nakanishi *et al*. 2017). NLGN1 knockout (KO) mice have problems with repetitive actions and spatial memory (Jedlicka *et al.* 2015). The mechanisms at the cellular level including decreased levels of protein and disordered localization in the respective cells, are associated with the NLGN1 variants having pathogenic property causing neural impairment (Nakanishi *et al*. 2017).

Neuroligin-2

The excitatory/inhibitory balance in the brain is maintained by NLGN2 (MIM 606479), which is strongly linked to the GABAergic system. Changes in NLGN2 expression cause behavioral abnormalities (Pelkey *et al*. 2017). In patients with schizophrenia, four rare missense mutations in *NLGN2*, resulting in the substitutions R215H, 7V510M, R621H, and A637T, have been discovered (Craddock *et al*. 2007). R215H and V510M are located in the extracellular cholinesterase-like domain, whereas R621H and A637T are located in the extracellular stalk domain and intracellular WW-binding domain, respectively (Sun *et al*. 2011). The R215H substitution likely is the most detrimental because it results in ER retention of NLGN2, preventing export to the cell surface and extracellular binding to NRXN1. HEK293T cells transfected with the R215H NLGN2 variant did not aggregate with NRXN1-expressing cells as cells transfected with wild-type NLGN2 did, and this variant did not induce GABAergic synapse formation in co-culture experiments (Sun *et al.* 2011). Homozygous R215H NLGN2 KI mice exhibited developmental retardation, anxiety-like activity, and impaired spatial learning and memory, whereas NLGN2 KO mice demonstrated increased anxiety and reduced pain sensitivity (Jedlicka *et al.* 2015).

Neuroligin-3

The first case of NLGN3 (MIM 300336) involvement in ASD was discovered in a Swedish family of two affected siblings. One of them had a *de novo* C-to-T missense mutation in *NLGN3*, encoding arginine instead of cysteine in the extracellular domain of NLGN3 at amino acid 451 (Quartier *et al*. 2019). The R451C substitution prevents NLGN3 from folding or dimerizing and trafficking to the cell surface, resulting in ER retention, and it affects the affinity of NLGN3 for NRXN1 (Liu *et al.* 2017).

NLGN3 KO mice are characterized by reduced ultrasound vocalization and social memory deficits, similar to ASD. The R451C mutation decreases protein instability by 90%, and R451C NLGN3 KI mice exhibited autistic-like phenotypes in social and cognitive functions, as well as functional anomalies (Norris *et al.* 2019). R451C NLGN3 KI and NLGN3 KO mice shared some behavioral characteristics, such as increased repetitive activity, whereas they exhibited different phenotypes in terms of social interaction and spatial memory in an open-field test (Quartier *et al*. 2019). The NLGN3 mutants produce repetitive behavior that is processed by D1-dopamine receptors (DRs)-expressing, but not the D2 type of DRs for medium forms of spiny neurons (Rothwell *et al*. 2014).

Further research into spatial memory, social memory, social interaction, and phenotypic effects is required for uncovering the mode of occurrence of deficit in ASD behavior. Different synaptic phenotypes caused by NLGN3 R451C, which is a single point mutation, indicated that wild-type NLGN3 works in a context-dependent manner, depending on the relative expression of NLGN1 (Rothwell *et al.* 2014). It could be applied to clarify in the animals (Letellier *et al*. 2020). Further, investigating the mechanisms by which NLGN3 functions in growth is crucial. Synaptic transmission is inhibited with the late deletion of NLGN3 in development when it is deleted early. Further, if NLGN3 is deleted in the early stages of growth, cerebellin-1 will offset the lack of NLGN3 (Zhang *et al.* 2017). Another NLGN3 mutation, V321A, was discovered in a man diagnosed with ASD and ID (Yu *et al.* 2013). Recently, two new *de novo* NLGN3 variants were discovered: P514S in two brothers and R597W in two cousins with ASD and ID (Letellier *et al*. 2020). These mutations occur in the extracellular domain of NLGN3 and result in protein misfolding.

Neuroligin-4X

Variants in *NLGN4X* (MIM 300427) have been identified in patients with ASD and Asperger's syndromes. A mutation has been discovered as causative of ASD. This mutation (D396X) causes a premature stop codon, resulting in a non-functional protein that only contains the first two to thirds of the cholinesterase-like domain. (Jamain *et al*. 2003). The mutations L211X, Q274X, Q329X, D429X, and V454 introduce a premature stop codon (Yu *et al.* 2013). Several missense mutations in the *NLGN4X* coding region, resulting in substitutions G99S, R101Q, G84R, R87W, V109L, Q162K, A283T, V522M, R704C, K378R, V403M, and R766Q, are inherited maternally. Except for

R704C and R766Q, which are located in the intracellular domain of NLGN4X (R766Q in the gephyrin-binding domain and R704C right after the transmembrane span), all of these substitutions map to the extracellular domain of the protein (Nguyen *et al*. 2020).

A deletion in *Xp22.3* was discovered in early genetic studies in patients with ASD. Notably, *NLGN4X* is located within this region. Genetic pedigree studies have uncovered the causal connection in human; therefore, the *NLGN4*-like gene identification in mice gained attention because it enabled studies of the function of *NLGN4* in ASD in rodents (Nguyen *et al*. 2020).

Despite substantial progress in our understanding of the synaptic functions of NLGNs 1 to 3, those of NLGN4 isoforms remain elusive because of their rapid divergence between humans and rodents (Maxeiner *et al*. 2019). Sex associated role of *NLGN4* in human, with *NLGN4X* & *NLGN4Y* interacting to make a pair of X-Y gene, whereas *NLGN4* in mice is called as *NLGN4*-like. Furthermore, mouse *NLGN4*-like evolves rapidly, resulting in protein sequence changes (Maxeiner *et al*. 2019). Notably, *NLGN4* from some rodents keep the human-NLGN4X similarity, but that from other rodents, including mice, does not. Collectively, a decade of research has revealed a disparity in the roles of *NLGN4* genes between humans and rodents, challenging the previously held assumption of their functional similarities.

Neuroligin-4Y

With only 19 amino acid variations between them, NLGN4X and NLGN4Y (MIM 400028) are highly conserved; accordingly, the two proteins are thought to have the same function (Südhof, 2018). However, until recently, this theory had not been tested experimentally. As it is known that the sex-associated genes are *NLGN4X/Y*, sex bias in *NLGN4X* expression is important to consider. Some of the Y-linking genes serve as homologous to those that are X-linkage genes. The X-Y gene pairs play a crucial role in the processes of transcription as well as translation, and stability of proteins (Hughes and Page, 2015). *NLGN4X* and *NLGN4Y* expression differ between males and females; *NLGN4Y* is expressed only in males, whereas *NLGN4X* is expressed at similar levels in females and males. However, it was revealed that higher is the expression of *NLGN4X* in cortex of females than in males (Trabzuni *et al.* 2013). While the expression of gene was extensively studied, protein functions of NLGN4X and NLGN4Y is lagging.

Notably, several ASD-linked variants have been discovered in NLGN4X, while mutation of one missense discovered in the NLGN4Y. Furthermore, more number of males are affected than females for NLGN4X by ASD-associated mutations without known explanation that has alarmed the researchers for concentrating on the NLGN4Y (Trabzuni *et al*. 2013). A recent comparison of NLGN4X and NLGN4Y revealed that NLGN4Y could not be trafficked to the surface to form synapses (Nguyen *et al*. 2020). The major difference between these is a 93rd position amino acid, where NLGN4X has amino acid proline, & NLGN4Y has another amino acid serine. An S93P variant of NLGN4Y was capable of efficiently trafficking to the surface and inducing synapses (Nguyen *et al*. 2020).

CONCLUSIONS

The evidence presented in this review suggests that synaptic dysfunction plays a role in the development of ASD. ASD phenotypes can be viewed as the culmination of various genetic and environmental factors. Insights into the mechanisms by which functions are disrupted in mouse model studies indicate the expressive forms of ASD A number of studies have ignored sex chromosomes. Several X-chromosome genes have been noted to be linked in ASD based on analyses of proband pedigrees. However, the importance of the Y chromosome is often overlooked. Though many aspects of characterization are conducted for the NLGNs that are sex linked, it still requires further research into synaptic control and the development of novel treatments.

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(*Accepted for publication April 2025*)