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Review

The neuroligins and the synaptic pathway in Autism Spectrum Disorder

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Abstract

The genetics underlying autism spectrum disorder (ASD) is complex and heterogeneous, **and** *de novo* variants are found in genes converging in functional biological processes. Neuronal communication, including trans-synaptic signaling involving two families of cell-adhesion proteins, the presynaptic neurexins and the postsynaptic neuroligins, is one of the most recurrently affected pathways in ASD. Given the role of these proteins in determining synaptic function, abnormal synaptic plasticity and failure to establish proper synaptic contacts might represent mechanisms underlying risk of ASD. **More than 30 mutations have been found in the neuroligin genes. Most of the resulting residue substitutions map in the extracellular, cholinesterase-like domain of the protein, and impair protein folding and trafficking. Conversely, the stalk and intracellular domains are less affected.** Accordingly, several genetic animal models of ASD have been generated, showing behavioral and synaptic alterations. The aim of this review is to discuss the current knowledge on ASD-linked mutations in the neuroligin proteins and their effect on synaptic function, in various brain areas and circuits.

Keywords: animal model, genetics, behaviour, physiology, excitatory/inhibitory balance, synaptic plasticity, homeostatic mechanisms, misfolding, trafficking, unfolded protein response, endoplasmic reticulum

1. Genetics and Autism Spectrum Disorder risk genes

Autism Spectrum Disorder (ASD) is a neurodevelopmental syndrome characterized by impairments in social interaction and communication and by restricted and repetitive behaviors with an incidence higher than 1% of children worldwide (Lord et al., 2020). ASD has a strong genetic background and high heritability and it manifests in childhood. Large-scale exome and whole genome sequencing studies have identified hundreds of genes potentially implicated with the disorder (Vorstman et al., 2017). However, the genetic of ASD is very complex and heterogeneous not only for the number of involved genes but also for the nature of the genetic variants, **which range from rare, common, *de novo* and inherited** in both coding and non-coding regions of the genome (Abrahams and Geschwind, 2008). While common inherited variants seem to induce smaller effects with reduced penetrance (Kosmicki et al., 2017), rare *de novo* variations, mainly in the form of copy number variations (CNVs) and single nucleotide polymorphism (SNP), represent high-penetrance ASD-variations originating in the parent germ line depending on parental age (Sanders et al., 2012). Rare *de novo* variants may contribute to ASD in up to 10-30% of affected children by disrupting gene function (Iossifov et al., 2014; Ronemus et al., 2014). The challenge of the genetic studies over the last years is uncovering biological functional networks, cell types, or circuits involved in ASD. Although the susceptibility genes are numerous and most of them have yet to be identified, they seem to converge in two main biological pathways (Gilman et al., 2011): regulation of gene expression (Luo et al., 2012) and neuronal communication (De Rubeis et al., 2014; Grove et al., 2019; Pinto et al., 2014). Other pathways affected in ASD are related to WNT signaling, MAPK/JNK signaling, actin dynamics, and regulation of translational and degradation processes (Kumar et al., 2019). Inherited and *de novo* variations may impact distinct biological pathways interconnected at the level of gene expression. *De novo* alterations are enriched in transcriptional and chromatin regulation, while inherited variants are mainly related to ion transport, cell cycle and microtubule cytoskeleton. However, common pathways between *de novo* and inherited genes contribute to a common protein-protein interaction network (Ruzzo et al., 2019). A large-scale exome sequencing study has recently identified ASD susceptibility genes expressed in neurons and enriched in the human cortex between late mid-fetal development and infancy in maturing and mature neurons, in both excitatory and inhibitory neuronal systems (Satterstrom et al., 2020). ASD appears in the

first 3 years of life, a developmental period characterized by extensive neurite formation, synaptogenesis and refinement **(de la Torre-Ubieta et al., 2016), and** this is highly correlated to the role played by several risk genes in synaptic formation and function. For this reason, ASD is often referred to as a synaptic disease or is included among neurological disorders characterized by synaptic dysfunction, also called "synaptopathies" (Ebrahimi-Fakhari and Sahin, 2015; Zoghbi, 2003). Among the ASD-associated synaptic genes, a prominent role is played by the *NRXN, NLGN*, *SHANK*, *TSC1/2*, *FMR1* and *MECP2* genes, which converge on pathways intersecting at the synapse (Baig et al., 2017). These genes encode cell-adhesion molecules, scaffolding proteins and proteins involved in synaptic transcription, protein synthesis and degradation, affecting processes related to synapse formation and elimination, synaptic transmission and plasticity (Guang et al., 2018). Rare and recurrent variants in *NRXN* and *SHANK* genes are found in ASD patients, with *SHANK3* mutations representing one of the most prevalent monogenic causes of ASD (Arons et al., 2012; Bucan et al., 2009; Durand et al., 2007; Feng et al., 2006; Gouder et al., 2019). Both the neurexin (NRXN) and SHANK3 proteins interact with the neuroligin (NLGN) proteins, albeit with their extracellular and intracellular domains, respectively (Fig. 1), suggesting a role for the NLGN/NRXN signaling cascade in the neurobiological mechanisms underlying cognition and social behavior.

2. Neuroligins and ASD-associated mutations

NLGNs are cell-adhesion molecules anchored at the post-synaptic membrane (Fig. 1), where they act as synaptic organizers, involved in the identity specification and functional maturation of the synapse (Südhof and Malenka, 2008). **The human NLGNs derive from five genes, namely *NLGN1*, *2*, *3*, *4X*, and *4Y* or *5***, which are localized at chromosomes 3q26 (*NLGN1*), 17p13 (*NLGN2*), Xq13 (*NLGN3*), Xp22.3 (*NLGN4X*) and Yq11.2 **(*NLGN4Y also known as NLGN5*)**. The mature proteins are enriched at specific synapse types: NLGN1 is preferentially associated with glutamatergic excitatory synapses (Dong et al., 2007; Graf et al., 2004; Song et al., 1999); NLGN2 is found at GABAergic inhibitory synapses (Varoqueaux et al., 2004); NLGN3 is found at both glutamatergic and GABAergic synapses (Budreck and Scheiffele, 2007; Földy et al., 2013; Takács et al., 2013); **NLGN4X is found at excitatory synapses (Marro et al., 2019), but to our knowledge no information is available for NLGN4Y.** NLGNs are crucial for proper synaptic function as shown upon

NLGN1/2/3 triple knock-out (KO) in mice, resulting in death shortly after birth despite of a normal number of synapses and ultrastructural synaptic features (Varoqueaux et al., 2006).

The full-length NLGN protein consists, from N- to C-terminal, of an extracellular, globular cholinesterase-like domain, followed by a highly O-glycosylated stalk region, a single-pass transmembrane helix, and a short cytoplasmic tail containing one WW-, one gephyrin- and one PDZ-binding motifs thought to be necessary for the scaffolding of post-synaptic components (Bemben et al., 2015; Bolliger et al., 2001; Ichtchenko et al., 1995; Ichtchenko et al., 1996; Irie et al., 1997; Nguyen and Südhof, 1997). NLGNs can form non-covalent homo- and heterodimers of subunits associated through a four-helix bundle, similar to the cholinesterases (Araç et al., 2007; Comoletti et al., 2007, 2006, 2003; Fabrichny et al., 2007). In the synaptic cleft, the extracellular domains of a NLGN dimer bind to the extracellular domains of two NRXNs (Fig. 2) (Araç et al., 2007; Comoletti et al., 2007; Fabrichny et al., 2007; Leone et al., 2010; Miller et al., 2011). The NLGN-NRXN association is Ca^{2+}-dependent, and the affinity and specificity of individual NLGNs for distinct NRXNs is regulated by protein sequence and N-glycosylation, along with splicing variation in surface loops, with absence/presence of long splice inserts A1/A2 in NLGNs 1-4 and a shorter, N-glycosylated insert B in NLGN1 (Boucard et al., 2005; Comoletti et al., 2003; Koehnke et al., 2010; Talebizadeh et al., 2006). Moreover, it has been recently demonstrated that the MAM domain-containing GPI-anchored (MDGA) proteins are essential to regulate the NLGN-NRXN association. In fact, MDGA binding to NLGNs inhibits the NLGN-NRXN interaction, contributing to synaptic shaping (Elegheert et al., 2017; Gangwar et al., 2017; reviewed in: Connor et al., 2019; Thoumine and Marchot, 2017).

The NLGN-NRXN trans-synaptic bridge functionally couples the postsynaptic density with the presynaptic protein complex responsible for neurotransmitter release and stabilizes pre- and post-synaptic components (Cao and Tabuchi, 2017; Ichtchenko et al., 1995; Nguyen and Südhof, 1997). Moreover, depending on which NLGN is involved in the interaction, the synapse organization specifies for the formation of either glutamatergic or GABAergic synapses, contributing to the maintenance of the appropriate excitatory/inhibitory balance in specific neural connection (Craig and Kang, 2007; Levinson and El-Husseini, 2005).

Several point mutations in the NLGN genes have been found to be associated with ASD, with the majority resulting in substitutions mapping on the extracellular protein domain and only a few localizing in the stalk and intracellular domains. Interestingly, **among the substitutions in the extracellular domain,** only a few are proximal to the binding site for **NRXNβ1** (Table 1; Fig. 2), a feature suggesting alteration of the functional integrity of the protein.

2.1 Neuroligin1

To date, five rare ASD-associated mutations of *NLGN1* have been identified, corresponding to substitutions P89L, T90I, L269P, G297E and H795Y (Nakanishi et al., 2017) (Table 1, Fig. 2). Apart from H795Y, which localizes in the cytoplasmic domain of NLGN1, all these substitutions are found in the extracellular, cholinesterase-like domain of the protein where they potentially alter the structure. In particular, P89L and T90I affect the stability of a proline-rich loop at the protein surface (Araç et al., 2007; Chen et al., 2008). However, none of them are located within the NRXNβ1 binding site (Nakanishi et al., 2017). According to *in silico* prediction of deleterious effects, these NLGN1 variants can be divided into high-risk (P89L, L269P, G297E) and low-risk variants (T90I, H795Y). The high-risk variants, and in particular P89L and L269P, show altered protein trafficking with retention in the endoplasmic reticulum (ER), presumably due to protein misfolding. The low-risk variants do not show an altered localization in the cell, but H795Y shows reduced expression levels due to higher susceptibility to proteolytic cleavage and degradation. Knock-in (KI) mice for the P89L *NLGN1* variant display abnormal social behavior and impaired spatial memory (Nakanishi et al., 2017). Likewise, a *NLGN1* KO mouse exhibits deficits in spatial memory and increased repetitive behavior (Blundell et al., 2010; Jedlicka et al., 2015; Kim et al., 2008).

2.2 Neuroligin2

NLGN2 is strongly associated with the GABAergic system and it is crucial for maintaining the excitatory/inhibitory balance in the brain (Parente et al., 2017). *In vivo* studies have shown that changes in NLGN2 expression induce behavioral disturbances and defects (Blundell et al., 2009). Four rare missense mutations in the *NLGN2* gene have been found in schizophrenic patients, leading to substitutions R215H,

V510M, R621H and A637T (Sun et al., 2011). The first two are found in the extracellular cholinesterase-like domain of NLGN2 (Fig. 2), while R621H and A637T map in the extracellular stalk domain and the intracellular WW-binding domain, respectively. Although the R215H substitution is remote from both the NLGN dimerization interface and the NRXNβ1 binding site, it is probably the most affecting substitution since it causes retention of NLGN2 in the ER, thereby preventing exportation to the cell surface and extracellular binding to NRXNβ1, and creating a loss-of-function condition (Sun et al., 2011). HEK293T cells transfected with the R215H NLGN2 variant cannot aggregate with NRXNβ1-expressing cells, as cells expressing wild-type NLGN2 do, and in co-culture experiments this variant is unable to induce GABAergic synapse formation (Sun et al., 2011)). Homozygous KI mice expressing the R215H *NLGN2* variant show growth retardation, anxiety-like behavior, and impaired spatial learning and memory (Chen et al., 2017), while *NLGN2* KO mice present increased anxiety-like behavior and decreased pain sensitivity (Blundell et al., 2009; Jedlicka et al., 2011).

2.3 Neuroligin3

The involvement of the NLGNs in ASD has been first reported in a Swedish family presenting two affected siblings, of which one presented a *de novo* missense mutation in the coding region of the *NLGN3* gene inherited from the mother, consisting of a C to T nucleotide change and resulting in a R451C substitution in the cholinesterase-like domain of NLGN3 (Jamain et al., 2003). The Arg residue at position 451 in NLGN3 is highly conserved among cholinesterase-like proteins (De Jaco et al., 2012, 2006). The R451C substitution impairs NLGN3 folding and/or dimerization and trafficking to the cell surface, causing retention in the ER and degradation via the proteasome (Azoulay-Ginsburg et al., 2020; De Jaco et al., 2010, 2008). The substitution also affects the affinity of NLGN3 for NRXNβ1 (Chih et al., 2004; Comoletti et al., 2004). *NLGN3* KO mice are characterized by reduced ultrasound vocalization and deficits in social memory, which are symptoms reminiscent of ASD (Radyushkin et al., 2009). Although the mutation causes a 90% decrease of NLGN3 brain levels due to protein destabilization, the R451C *NLGN3* KI mice display autistic-like phenotypes in cognitive and social tasks (Tabuchi et al., 2007) (Norris et al., 2019) along with functional alterations in various brain regions not found in the *NLGN3* KO mice (**Etherton et al., 2011a**; Tabuchi et al.,

2007). However, R451C *NLGN3* KI and *NLGN3* KO mice also display common behavioral features, such as an increased repetitive motor routine and hyperactivity in the open-field test (Rothwell et al., 2014). In addition, the R451C *NLGN3* KI mice show alterations in the volume of several brain regions, with the hippocampus, striatum and thalamus being smaller than in WT mice (Ellegood et al., 2011). A *de novo* mutation was found, leading to the substitution G426S in the extracellular domain of the protein (Xu et al., 2014). This substitution was proposed to predispose to ASD through abnormal synaptic homeostasis and altered NRXN binding, although, its position is remote from the NRXNβ1 binding site (Fig. 2) (Xu et al., 2014). Another NLGN3 variant, V321A, was found in one man affected by intellectual disability (ID) and ASD (Yu et al., 2013). Recently, two *de novo* NLGN3 variants were found: R597W in two cousins with ID and ASD; and P514S in two brothers of another family (Quartier et al., 2019). These substitutions, which again map in the extracellular domain of NLGN3, cause protein misfolding (Table 1, Fig. 2)

2.4 Neuroligin4X

The largest number of ASD-associated mutations in the NLGNs are ascribed to the *NLGN4X* gene. A *de novo* frameshift mutation, 1186insT, was found in the same Swedish family where the R451C variation was found, in a sibling with Asperger syndrome (Jamain et al., 2003). This mutation generates a premature stop codon (D396X) leading to a non-functional protein containing only the first two thirds of the cholinesterase-like domain (Jamain et al., 2003). Several other mutations lead to the introduction of a premature stop codon: L211X, Q274X, Q329X, D429X and V454_A457X (Laumonnier et al., 2004; Martínez et al., 2017; Yu et al., 2013; C Yuen et al., 2017). To date, it has not been assessed if these presumed truncated proteins are effectively expressed. Moreover, several maternal inherited missense mutations have been identified in the *NLGN4X* coding region, leading to substitutions G84R, R87W, G99S, R101Q, V109L, Q162K, A283T, K378R, V403M, V522M, R704C and R766Q (Nguyen et al., 2020; Talebizadeh et al., 2006; Xu et al., 2014; Yan et al., 2005; Zhang et al., 2009). All these substitutions map in the extracellular domain of NLGN4X (fig.2), except R704C and R766Q that are localized in the intracellular domain (R704C just after the transmembrane span, R766Q in the gephyrin-binding domain).

The R87W variant, found in two ASD-affected brothers, was shown to suffer from severe misfolding of the extracellular domain and altered protein trafficking to the cell surface, when analyzed in transfected COS cells. However, this substitution does not impair NRXNβ2 binding, consistent with its position on the side of the subunit opposite to the NRXN binding site (Zhang et al., 2009).

In an autistic woman, it has been found a splicing variant of *NLGN4X* lacking exon 4 and resulting in the in-frame skip of 62 residues of the cholinesterase-like domain of the protein, but no further data on this variant have been published (Talebizadeh et al., 2006). **Mice with genetic knockout of *NLGN4*, the mouse homologue of human *NLGN4X*,** exhibit highly selective deficits in social interactions and communication, which are ASD-related behavioral features (Jamain et al., 2008).

It is noteworthy that several ASD-associated substitutions in NLGN4X are localized in the protein region between residues 75-125. Indeed, a single residue difference between NLGN4X and NLGN4Y within this region is responsible for the retention of NLGN4Y in the ER and its total absence at the cell surface when expressed in COS-7 cells (Nguyen et al., 2020).

3. Loss or gain of function?

Synaptic cell-adhesion molecules play a major role in the assembly and function of neuronal networks (Taoufik et al., 2018). In order to bind their partners, adhesion molecules from each side of the synapse must assume a precise orientation at the cell membrane, with their respective interaction domains exposed either in the synaptic cleft or inside the cell. Any perturbation of this orientation is much likely to alter their function (Baig et al., 2017).

NLGNs reach the post-synaptic membrane after passing through the secretory pathway (Ribeiro et al., 2018). They are translated by ER-associated ribosomes and translocated in the lumen of the organelle, where several resident chaperones cooperate for their folding. Along the secretory pathway they also undergo post-translational modifications such as disulfide bond formation, N- and O-glycosylation and/or phosphorylation, as required for their molecular and functional integrity (Jeong et al., 2017).

A fine quality-control system is active to avoid misfolding and prevent trafficking of non- or mis functional proteins beyond the ER (Hebert and Molinari, 2007), leading to the accumulation of

misfolded/unfolded proteins and causing a stress condition of the organelle. This ER overload potentially activates the Unfolded Protein Response (UPR), with the aim to restore the homeostasis in the organelle (Almanza et al., 2019; Chambers and Marciniak, 2014). Recently it has been shown that UPR mediators are implicated in the regulation of neuronal physiology, and that the IRE1-XBP1 axis regulates memory formation in the hippocampus by activating the transcription of BDNF (Martínez et al., 2016). Moreover, the regulation of protein synthesis mediated by the PERK-eIF2α branch was found to be implicated in synaptic plasticity (Di Prisco et al., 2014), suggesting that UPR activation could be involved in the regulation of neuronal functions. Furthermore, ER retention and UPR activation have been observed in HEK293 cells expressing D1129H CASPR2, a variant of the neuronal cell-adhesion protein, contactin-associated protein-like 2 (CASPR2), a member of the NRXN family, highly-linked to ASD (Falivelli et al., 2012). Here we summarize the effects of the mutations found in the NLGNs (Table 1), with respect to their effect on protein expression and trafficking, since absence of NLGNs at the cell surface leads to a loss-of-function phenotype, due to the lack of interaction with the synaptic partners.

The high-risk NLGN1 variants P89L, L269P and G288E cause retention in the ER and reduced amounts of both the membrane-localized and the secreted proteins (Nakanishi et al., 2017). Higher susceptibility of the misfolded variants to endogenous degradation, relative to the wild type, may also contribute to the lower extracellular protein levels. The resulting depletion of NLGN1 at the surface of transfected hippocampal neurons leads to a decreased number of spines. A different situation is observed for the low-risk H795Y variant, which shows unaltered trafficking along with enhanced cleavage and release, two features leading to both lower intracellular and higher extracellular protein levels (Nakanishi et al., 2017).

The R215H substitution in NLGN2 impairs protein trafficking, leading to its retention in the ER. As previously described for NLGN1 high-risk variants, drastic depletion of NLGN2 on the cell surface results in the inability of NLGN2 to bind NRXNβ1 and in defective synaptogenic functions (Sun et al., 2011). However, substitutions V510M, R621H, A637T, also found in schizophrenic patients, have no impact on NLGN2 levels, localization and ability to bind NRXNβ1 when expressed in HEK293T cells (Sun et al., 2011).

The most studied ASD-associated mutation in the NLGN family is the R451C substitution in NLGN3. This variant is the first for which an ER-retention phenotype associated with proteasomal degradation has been shown (De Jaco et al., 2010). However, a small amount of this variant can reach the cell surface, but it is unable to bind NRXNβ1 (Comoletti et al., 2004).

UPR activation has been described *in vitro*, in PC12 Tet-on cell lines with inducible expression of R451C NLGN3 (Ulbrich et al., 2016), and *in vivo*, in the R451C *NLGN3* KI mouse (Trobiani et al., 2018). Interestingly, UPR is selectively active in the cerebellum of the R451C *NLGN3* KI mouse where it modulates excitatory neurotransmission, resulting in a gain-of-function phenotype (Trobiani et al., 2018). This result is supported by studies showing that ER stress can alter spontaneous neurotransmission in hippocampal primary cultures (Nosyreva and Kavalali, 2010). Moreover, it is noteworthy that high levels of ER stress markers have been detected in the brain of ASD human patients and in a mouse model (Crider et al., 2017; Kawada and Mimori, 2018).

Recently, two other missense mutations have been found in the *NLGN3* gene, leading to substitutions P514S and R597W, which cause retention in the ER and a drastic decrease of protein levels (Quartier et al., 2019). These two *NLGN3* variants suffer from more severe misfolding of the extracellular domain than R451C. Since the higher degree of misfolding reflects stronger activation of the UPR, which in turn may result in a gain-of-function similar to that observed for the R451C *NLGN3* variant, it would be interesting to test if these substitutions cause electrophysiological alterations in animal models.

For a few only of the numerous mutations found in the *NLGN4X* gene, biochemical effects have been further studied. The truncated D396X NLGN4X variant shows unaltered protein expression levels compared to the wild type. However, this variant is highly retained in the ER and partially secreted, resulting in lower protein levels on the cell surface. Furthermore, it fails to promote presynaptic differentiation in transfected hippocampal neurons, confirming the loss-of-function phenotype induced by this mutation (Chih et al., 2004).

In 2009, Zhang and colleagues showed that the R87W substitution leads to retention of NLGN4X in the ER and to a drastic decrease in protein levels **(Zhang et al., 2009)**. Although the purified variant still binds NRXNβ2, its absence at the cell surface impairs synaptogenic properties in co-culture experiments,

indicating a loss-of-function phenotype. Whether R87W NLGN4X activates the UPR, possibly reflecting a gain-of-function phenotype, is unknown. A recent study has demonstrated that substitutions G99S, R101Q and V109L cause a drastic reduction of NLGN4X protein levels. Furthermore, G99S NLGN4X is unable to rescue mEPSC in hippocampal primary cultures in which the other NLGNs have been silenced by miRNA (Nguyen et al., 2020). Substitutions G84R, Q162K and A283T have also been described for NLGN4X (Xu et al., 2014). **Despite** *in silico* prediction of damaging effects, they do not alter NLGN4X levels, localization and ability to bind NRXNβ1 (Xu et al., 2017).

4. Synaptopathy in NLGN mouse models of ASD

Short or long-lasting forms of synaptic plasticity can be expressed by excitatory and inhibitory synapses in the central nervous system. Eventually, plasticity phenomena result in **strengthening or weakening of neurotransmitter release probability** at specific synapses. The simultaneous expression of different forms of plasticity at distinct synapses dynamically regulates the information flow in neural circuits. The expression of synaptic plasticity involves communication between presynaptic and postsynaptic neuronal compartments, accomplished through anterograde and retrograde messengers and cell adhesion molecules, and can implicate structural modifications of the synapse. Homeostatic mechanisms, necessary to maintain circuit function despite potentially destabilizing perturbations, coexist with these forms of plasticity (Fig. 3). Such mechanisms can act through the regulation of both the neuronal excitability and the **excitation/inhibition (E/I)** balance (Li et al., 2020; Monday et al., 2018; Turrigiano, 2017).

Many ASD-associated mutations affect processing of synaptic proteins or involve ion channels, receptors, synaptic scaffolding molecules, and adhesion molecules such as the NLGN proteins (Bourgeron, 2015; Chang et al., 2015; De Rubeis et al., 2014; Lord et al., 2020). In fact, the NLGNs are required not for initial formation of synaptic contacts but specifically for proper synapse maturation (Varoqueaux et al., 2006). Accordingly, manipulation of *NLGNs* genes induces altered expression of synaptic proteins in mouse models (for review see: Hu et al., 2015). For example, the synaptosomal levels of AMPA and NMDA receptors are reduced in the hippocampal CA1 area of *NLGN1* KO mice (Budreck et al., 2013). *NLGN2* loss

increases brain protein levels of Synaptobrevin2 in KO mice (Blundell et al., 2009), and alters levels of GABAAγ2 subunit, VGAT, and PV in R215H KI mutants (Chen et al., 2020). Additionally, knockdown (KD) of *NLGN2* decreases the expression level of the chloride transporter KCC2, impairing the functional switch of GABAA receptors from excitatory to inhibitory (Sun et al., 2013). R451C *NLGN3* KI mice show a significant increase in VGAT and the postsynaptic, intracellular protein gephyrin (Tabuchi et al., 2007).

4.1 Inhibitory synaptic transmission

Many studies investigated synaptic activity and plasticity in different brain areas of NLGN mouse models of ASD (Table 2). First evidence for dysfunction of inhibitory synaptic transmission came from *NLGN1-2-3* triple KO mice, which showed a reduced frequency of GABAergic and glycinergic spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs) in the brainstem (Varoqueaux et al., 2006). In conditional PV-Cre/*NLGN1-2-3* triple KO mice, with selective deletion of neuroligins from cerebellar inhibitory stellate interneurons and Purkinje cells, the latter showed nearly complete suppression of inhibitory synaptic transmission, despite a modest impairment in stellate cells (Zhang and Südhof, 2016). Specifically, both frequency and amplitude of sIPSCs were significantly reduced in Purkinje cells, whereas only a slight impairment in postsynaptic GABA/glycine receptors was observed in cerebellar interneurons. This different effect of the *NLGN1-2-3* triple KO on stellate and Purkinje cells points at a synapse- and neuron-specific role of NLGNs (Zhang and Südhof, 2016). L7-Cre/*NLGN1-2-3* Purkinje cell-selective triple KO mice provided further evidence of inhibitory transmission impairment, with increased size of inhibitory synapses, and a decrease in mIPSC frequency and amplitude (Zhang et al., 2015). Acute deletion of *NLGN1-2-3* at postnatal day 21 recapitulated the phenotype induced by germline *NLGN1-2-3* conditional KO (cKO) mice.

Deletion of *NLGN2* in Purkinje cells recapitulated the reduction of mIPSC frequency observed in triple *NLGN1-2-3* KO mice (Zhang et al., 2015). Indeed, as expected, given the localization of NLGN2 at inhibitory synapses, different brain areas of *NLGN2* KO mice showed impaired inhibitory transmission. In these mice, the amplitude of GABAergic evoked synaptic currents (eIPSCs), sIPSCs or mIPSCs was reduced in the Layer 2/3 of the somatosensory (barrel) cortex, dentate gyrus, and at specific neocortical synapses

(Chubykin et al., 2007; Gibson et al., 2009; Jedlicka et al., 2011). In the neocortex, deletion of *NLGN2* affected inhibitory synaptic transmission specifically at synapses connecting fast-spiking, but not somatostatin-positive, interneurons to excitatory neurons (Gibson et al., 2009). In the ventrolateral medulla, lack of NLGN2 lead to a reduced frequency of sIPSCs, a decreased frequency and amplitude of GABAergic and glycinergic mIPSCs, and a reduced eIPSCs amplitude (Poulopoulos et al., 2009). Both GABAergic and glycinergic agonist-evoked postsynaptic currents were also reduced, pointing to dysfunction of GABAA and glycine receptors. Similarly, reductions in the frequency of GABAergic sIPSC and mIPSC were observed in the CA1 region of the hippocampus, where altered kinetics of GABAergic and glycinergic mIPSCs onset were also reported. Accordingly, despite normal numbers of total synapses, postsynaptic specializations at perisomatic inhibitory synapses were reduced in the CA1 (Poulopoulos et al., 2009). A similar alteration of postsynaptic structures at perisomatic sites, despite normal numbers of total synapses, was observed in the amygdala, leading to impaired inhibitory synaptic transmission (Babaev et al., 2016). Further, an electron microscopy investigation in the CA1 and CA3 hippocampal regions showed a specific decrease in the density of inhibitory VGAT-positive puncta, despite the absence of changes in the density of total synapses (Blundell et al., 2009) . A reduced number of gephyrin and GABAA receptor clusters, representing loss of somatic GABA**A** receptors, together with the decreased amplitude of mIPSCs, indicated an impairment of perisomatic inhibition also in the dentate gyrus of *NLGN2* KO mice (Jedlicka et al., 2011). In the retina, GABAA receptor clustering, the expression level of the GABAAγ2 subunit and the number of GABAAγ2-immunoreactive puncta were reduced by NLGN2 loss (Hoon et al., 2009). Similar to germline KO models, in *NLGN2* mPFC-cKO mice conditional deletion of *NLGN2* in adult medial prefrontal cortex caused a reduction of both amplitude and frequency of GABAergic mIPSCs (Liang et al., 2015). The R215H substitution, decreasing NLGN2 protein level, caused an impairment of GABAergic transmission similar to KO animals, with reduced frequency and amplitude of mIPSCs (Chen et al., 2020). Conversely, NLGN2 overexpression increased mIPSCs frequency in the prefrontal cortex of transgenic mice (Hines et al., 2008).

A similar increase in inhibitory transmission was reported in different brain areas of NLGN3 mouse models. The frequency of mIPSCs was enhanced in the CA3 area of the hippocampus (Pizzarelli and Cherubini, 2013) and in the somatosensory cortex (Tabuchi et al., 2007) of R451C *NLGN3* KI mice, as well as

in the hippocampal CA1 area of *NLGN3* KO mice (**Etherton et al., 2011a**). In the CA1 region of the hippocampus, the R451C *NLGN3* substitution caused synapse-specific effects. IPSC amplitude and success rate were increased at CCK basket cells synapses, but reduced at PV basket cells synapses, an observation showing that this **substitution** can produce opposite changes at two different inhibitory synapses impinging on pyramidal neurons (Földy et al., 2013). Analysis of these synapses in *NLGN3* KO mice showed that these opposite synaptic alterations observed in R451C *NLGN3* KI mice were mediated by loss- and gain-of-function effects of the mutation, respectively. In fact, in the KO mice these authors found an increase in GABAergic transmission at CCK synapses, similar to what observed in KI animals, but lack of changes at PV synapses. In further support of the diverse context-specific functions of NLGN3, in the basolateral amygdala of R451C *NLGN3* KI mice, mIPSCs amplitude was reduced (Hosie et al., 2018), whereas mIPSCs frequency was reduced in the population of D1 receptor positive-spiny projection neurons (D1-SPN) of the nucleus accumbens of both the KO and KI models (Rothwell et al., 2014). Furthermore, a recent report found that NLGN3 loss decreased the frequency of sIPSCs in the CA2 area of the hippocampus and reduced the perisomatic inhibition mediated by CCK-containing GABAergic interneurons (Modi et al., 2019). The CCK but not the PV neuron-mediated component of the eIPSC was selectively reduced in the *NLGN3* KO animals. However, CCK positive puncta were not significantly altered in the CA2 pyramidal layer. Overall, in *NLGN3* KO mice no change in the density of inhibitory synapses was detected either in the somatosensory cortex or in hippocampal CA1, CA2 and CA3 areas. Conversely, VGAT-positive puncta were increased in the same regions of the R451C *NLGN3* KI model (Tabuchi et al., 2007).

The composition of perisomatic inhibitory synapses was altered also by genetic KO of *NLGN4*, the mouse homologue of human *NLGN4X*, in the hippocampal CA3 area, supporting a primary role of NLGN4 at inhibitory synapses in mice in this brain region (Hammer et al., 2015). Accordingly, sIPSCs frequency and amplitude were reduced, and a selective weakening of the inputs with the fastest kinetics, originating from perisomatic inhibitory synapses, was recorded. In thalamocortical slices, KO of *NLGN4* resulted in lower frequency, and increased rise and decay time of mIPSCs recorded from Layer 4 spiny stellate cells (Unichenko et al., 2018). Increased paired-pulse ratio and decreased numbers of readily releasable vesicles at GABAergic synapses suggested a reduced presynaptic GABA release probability, which the authors

attributed to possible trans-synaptic retrograde signaling alterations induced by NLGN4 loss. *NLGN4* KO affected inhibitory transmission in the retina as well, where the number of glycine receptors mediating fast glycinergic transmission was reduced and ganglion cells exhibited slower glycinergic mIPSCs (Hoon et al., 2011).

4.2 Excitatory synaptic transmission

The analysis of excitatory synaptic transmission in different brain areas of the NLGN rodent models of ASD provided different scenarios. In accordance with the localization of NLGN1 to excitatory synapses, acute RNAi knockdown of *NLGN1*, but not of *NLGN3*, abolished LTP and reduced both AMPA and NMDA receptor-mediated currents in the hippocampus (Shipman and Nicoll, 2012). Such loss of NLGN1 in adulthood led to a reduction in the number of synapses rather than a reduction in the number of AMPA or NMDA receptors per synapse, as confirmed by a decrease in spine density. *NLGN1* KO mice showed reduced magnitude of LTP in the dentate gyrus (Jedlicka et al., 2015) and loss of Spike-Timing-Dependent (STD)-LTP at thalamo-amygdala synapses (Jung et al., 2010). In accordance with LTP impairment, NMDA-mediated glutamatergic transmission was reduced in the amygdala, in cortical Layer 2/3, in the CA1 region of the hippocampus, and at cortico-striatal synapses of the dorsal striatum (Blundell et al., 2010; Chubykin et al., 2007; Jedlicka et al., 2015; Jung et al., 2010; Kim et al., 2008; Kwon et al., 2012). Notably, LTP was impaired in the CA1 hippocampus of both heterozygous and homozygous *NLGN1* KO mice, whereas LTD expression was altered selectively in *NLGN1*$^{+/-}$ mice (Dang et al., 2018). Specifically, NMDA receptor-dependent LTD was impaired, whereas mGlu receptor (mGluR)-dependent LTD was enhanced. Indeed, mGluR-LTD is associated with co-internalization of synaptic surface NLGN1 and PSD95, suggesting an inhibitory role for NLGN1 in the regulation of mGluR-LTD in the hippocampus. On the other hand, the selective impairment of activity-dependent synaptic plasticity was ascribed to a reduced phosphorylation of AMPA receptors. Accordingly, the amount of surface **GluA2 AMPA receptor** colocalized to PSD95 was significantly reduced, and the size of GluA2-positive puncta was significantly smaller in *NLGN1*$^{+/-}$ cultured hippocampal neurons (Dang et al., 2018). However, in the CA1 area of the hippocampus, the LTP of extracellular field excitatory postsynaptic potentials (fEPSP) was impaired also by overexpression of *NLGN1* (Dahlhaus et al., 2010),

suggesting a gene dosage requirement for a physiological expression of synaptic plasticity. This work also described increased amplitude of excitatory postsynaptic currents (EPSCs) and changes in hippocampal synapse morphology, pointing to an enhanced proportion of mature spines.

A decreased frequency of glutamatergic sEPSCs was observed in the brainstem of *NLGN1-2-3* triple KO mice (Varoqueaux et al., 2006). In PV-Cre/*NLGN1-2-3* cKO mice, conditional deletion of *NLGN1-2-3* in PV-expressing inhibitory cerebellar interneurons and Purkinje cells caused a selective decrease in NMDAR-mediated extrasynaptic EPSCs recorded from stellate cells (Zhang and Südhof, 2016). Lysates of total cerebellum from PV-Cre/*NLGN1-2-3* cKO mice also exhibited a reduction of total content of the NMDAR subunit NR1. This study showed similar alterations of excitatory synaptic transmission in PV-Cre/*NLGN1* cKO mice, suggesting that NLGN1 is responsible for recruiting extrasynaptic NMDARs at parallel fiber/stellate cell cerebellar synapses. Purkinje cell-specific L7-Cre/*NLGN1-2-3* cKO mice showed a decrease in the size and density of climbing-fiber synapses, accompanied by a decrease in the amplitude of climbing-fiber EPSCs, which was replicated in single *NLGN1* or *NLGN3* cKO mice (Zhang et al., 2015).

In *NLGN2* KO mice the amplitude of EPSCs at cortical synapses between excitatory and fast-spiking inhibitory interneurons was increased (Gibson et al., 2009). Conversely, in *NLGN3* KO mice a significant increase in sEPSCs frequency, but not amplitude, was observed in the CA2 area of the hippocampus (Modi et al., 2019). In the cerebellum, loss of NLGN3 caused a decrease in the amplitude of mEPSCs recorded from Purkinje cells, and impairment of mGluR-dependent LTD at parallel fiber synapses (Baudouin et al., 2012, but see: Zhang et al., 2015). Similarly, we found that the R451C substitution caused the loss of LTD at corticostriatal synapses of the dorsal striatum (Martella et al., 2018), where the paired-pulse ratio of glutamatergic synaptic currents, as well as the frequency and amplitude of sESPCs recorded from striatal spiny projection neurons revealed the absence of changes in the presynaptic release of glutamate. Of interest, two different substitutions in NLGN3 produced opposite effects on glutamatergic transmission in the CA1 region of the hippocampus: R451C increased (**Etherton et al., 2011a**), whereas R704C reduced, the fEPSP slope (**Etherton et al., 2011b**). Furthermore, while AMPA receptor-mediated excitatory transmission was increased in pyramidal neurons of R451C *NLGN3* KI mice (**Etherton et al., 2011a**), it was decreased in R704C *NLGN3* KI animals (**Etherton et al., 2011b**). Indeed, the R704C variation did not significantly alter

synapse formation, since the number of vGlut1-positive synapses was unchanged, but caused a selective impairment of excitatory synaptic transmission (**Etherton et al., 2011b**). Specifically, the frequency of mEPSCs was reduced, but the NMDA/AMPA ratio was increased and the NMDA-dependent LTP was unchanged, indicating a selective impairment of AMPA receptor-mediated synaptic transmission. Conversely, the R451C substitution exerted an opposite effect in CA1 pyramidal neurons, namely an increase in excitatory synaptic transmission. In fact, enhanced mEPSCs frequency, increased NMDA/AMPA ratio and augmented decay time of NMDA receptor-mediated EPSCs were observed in R451C *NLGN3* KI mutant mice (**Etherton et al., 2011a**). These findings indicate an enhanced NMDA-dependent excitatory transmission and are in line with the observation of an increased LTP of fEPSP, supported by up-regulation of NMDA receptors containing NR2B subunits (**Etherton et al., 2011a**).

Genetic KO of *NLGN4*, the mouse homologue of human *NLGN4X,* decreased the frequency of mEPSCs recorded from cortical Layer 4 spiny stellate cells and the size of the ready releasable pool, indicating that excitatory transmission in Layer 4 of the barrel cortex is reduced mostly presynaptically, possibly due to alterations of a trans-synaptic retrograde signaling (Unichenko et al., 2018). Recently, the involvement of NLGN4X in the regulation of excitatory synaptic transmission has been reported in human neurons (Marro et al., 2019). Neurons derived from human embryonic stem cells carrying the syndromic autism variant R704C NLGN4X formed more excitatory synapses, with increased functional synaptic transmission. This substitution targets a residue conserved in all NLGNs; when introduced in NLGN3, R704C induced a selective decrease in AMPA-mediated glutamatergic transmission in neurons derived from embryonic fibroblasts, in mouse hippocampus, and in the calyx of Held giant synapse of the auditory brainstem (Chanda et al., 2013; **Etherton et al., 2011b**; Zhang et al., 2017). Of note, at the calyx of Held synapse, KO of *NLGN3* did not alter synaptic transmission, whereas R451C *NLGN3* KI decreased excitatory synaptic transmission, indicating a spectrum of effects induced by different mutations in the *NLGN3* gene (Zhang et al., 2017).

Thus, distinct single point variations in synaptic cell adhesion molecules such as the NLGNs can cause very different context-dependent changes in synaptic transmission. Such diverse effects are consistent with the broad impact of *NLGNs* mutations on mouse model phenotypes and human behaviors.

In fact, NLGNs specify and validate synapses via an activity-dependent mechanism in a synapse-specific manner, likely contributing to the use-dependent wiring of neural circuits (Chubykin et al., 2007). On the other hand, synaptic activity can regulate the phosphorylation, expression, and cleavage of NLGNs (Hu et al., 2015), thus taking part in the homeostatic regulation of brain activity.

5. Excitatory/inhibitory balance

As previously mentioned, complex homeostatic mechanisms coexist with synaptic plasticity phenomena to preserve a correct brain function (Fig. 3). Among them, the maintenance of a proper E/I balance can stabilize neuronal circuits to prevent the overcoming of the computational capacity of the network and a dangerous shift towards hyperexcitability (Monday et al., 2018). In particular, a correct balance between excitation and inhibition is required for normal brain development, since a prevailing strength of GABA synapses might preclude synaptogenesis, whereas excessive glutamatergic transmission can cause excitotoxicity (Ben-Ari, 2002). In line with this view, an E/I imbalance has been implicated in ASD pathophysiology, based on data from human patients and animal models (Antoine et al., 2019; Bozzi et al., 2018; Gogolla et al., 2009; Horder et al., 2018; LeBlanc and Fagiolini, 2011; Nelson and Valakh, 2015; Sohal and Rubenstein, 2019). An E/I imbalance might result from alterations in synapse development, in synaptic transmission, plasticity or signaling pathways, and in intrinsic neuronal excitability. Rubenstein and Merzenich (Rubenstein and Merzenich, 2003) proposed a model postulating that some forms of autism are caused by an increased E/I ratio in specific functional networks of the brain, resulting in a reduction of signal-to-noise in key neural circuits. Indeed, a lower inhibition might provide an explanation for the hyper-excitability of cortical circuits (Robertson et al., 2016), and, in turn, for the propensity of autistic individuals to develop seizures at a rate up to 25 times higher than that of the general population (Bolton et al., 2011). Conversely, some other forms of ASD, such as those associated with *SHANK3* and *PSD95*, may be hypoglutamatergic disorders, characterized by a shift in the E/I balance towards inhibition (Carlsson, 1998; Nelson and Valakh, 2015). A precise balance of excitatory and inhibitory neurotransmission is required for experience-based sculpting of neuronal circuits during specific time windows called critical periods. In consideration of the reported E/I imbalance, which might cause an impairment of critical periods, autism

was proposed as a critical-period disorder (LeBlanc and Fagiolini, 2011). The perinatal period of spine pruning and shaping of neuronal circuits, during which GABA plays a key role, is followed by a phase of synapse stabilization in response to neuronal activity, probably requiring the NLGN–NRXN–SHANK pathway (Bourgeron, 2009). Synaptic adhesion molecules play a crucial role in development and can determine the identity and function of synapses, thereby having a direct influence on E/I balance. Indeed, several lines of evidence obtained from mouse models suggest that altering the expression level of distinct NLGNs may disrupt the E/I balance. *NLGN1* overexpression was shown to increase the E/I ratio in the CA1 region of the hippocampus, consistent with an increase in both the number of excitatory synapses and the level of their markers (Dahlhaus et al., 2010). Similarly, in hippocampal dentate gyrus *NLGN2* deletion increased the E/I balance, impairing inhibitory transmission (Jedlicka et al., 2011). Differently from *NLGN1*, overexpression of *NLGN2* decreased the VGluT/VGAT proportion in the prefrontal cortex, revealing a reduction of the E/I ratio (Hu et al., 2015). In medial prefrontal cortex, both *NLGN2* cKO and R215H *NLGN2* KI mice showed a reduction in synaptic inhibition and a consequent increase in the E/I ratio (Chen et al., 2020; Liang et al., 2015). Accordingly, mPFC gamma oscillations, whose generation requires a proper E/I balance, were reduced in R215H *NLGN2* KI mice (Chen et al., 2020). An increased E/I ratio was also described in the Layer 4 of the somatosensory cortex of R451C *NLGN3* KI mice (Cellot and Cherubini, 2014), as well as in the basolateral amygdala, where a decrease of the inhibitory transmission and a concomitant increase of the excitatory activity were recorded (Hosie et al., 2018). Similarly, *NLGN3* KO mice exhibited a cell type-specific imbalance between synaptic inhibition and excitation in the nucleus accumbens (Rothwell et al., 2014), while in the hippocampus a strong E/I imbalance in the CA2 region (Modi et al., 2019) and reduced gamma oscillations in the CA1 area (Polepalli et al., 2017) were observed. Interestingly, we observed a shift in the E/I balance toward inhibition, accompanied by higher levels of NLGN1 and NLGN2, in the prefrontal cortex of BTBR-Pah^{enu2} (ENU2) mice, an animal model of phenylketonuria (PKU), a metabolic disease with a well-documented association with ASD (De Jaco et al., 2017).

Based on the physiological evidence summarized above and in the previous section, both excitatory and inhibitory transmission are functionally altered in ASD, suggesting some considerations. First, mutations in a single gene may produce multiple synaptic phenotypes, in a brain area- and synapse- specific

manner, directly affecting local E/I ratios. Second, such primary changes may engage homeostatic mechanisms responsible for secondary compensatory changes, involving adjustments either of synaptic strength (homeostatic plasticity) or of E/I balance. As discussed in a recent review, despite the numerous and powerful homeostatic mechanisms acting in the brain, evidences **obtained** from both autistic patients and animal models show that these responses fail to restore normal circuit activity following pathological changes deriving from ASD-linked mutations (Nelson and Valakh, 2015). Such ineffectiveness could derive from the inability of homeostatic mechanisms to compensate **for** the magnitude of the initial insult or from an "overload" due to the accumulation of pathological changes. This hypothesis may explain why symptoms in ASD patients are mild in the early phases of development and worsen in later phases. Finally, homeostatic mechanisms may become maladaptive, **which are** initially adequate to counteract the original dysfunction but can eventually result in the opposite dysfunction.

6. Conclusions

Despite advances in our understanding of the genetic bases of ASD, due to the heterogeneity of these syndromes both for etiologies and clinical presentations in the population, symptomatic therapies remain limited and disease-modifying approaches are lacking (Baranova et al., 2020). Genome-wide association and bioinformatic analyses of functional networks encompassing rare ASD genes revealed the importance of cellular processes regulating synaptic function and plasticity changing our view of autism as a paradigmatic synaptopathy (Basilico et al., 2020). Animal models confirmed genes involved in synaptic homeostasis being relevant in ASD behavioral and functional alterations, suggesting they may represent a target for novel disease-modifying therapeutics (Delorme et al., 2013). The availability of a multitude of animal models for different forms of ASD has been instrumental for identifying promising targeted therapies based on shared biological processes and molecular targets (Basilico et al., 2020). In particular, trans-synaptic signaling involving the cell-adhesion proteins NRXNs/NLGNs and intracellular scaffold protein Shank, represent one of the most recurrently affected pathways in ASD due to the NLGNs playing a crucial role by specifying synapse identity in a given neuronal circuit, and by intervening in synaptic maturation and homeostasis (Zhang et al., 2015). In fact, a multitude of studies focusing on the functional

alterations due to mutations in the NLGNs genes report synaptic dysfunction specific for distinct brain regions and for the different NLGNs forms. However, molecular mechanisms underlying NRXN-NLGN-Shank trans-synaptic signaling are only starting to be uncovered. **Several substitutions of conserved residues** of the NLGNs proteins have been characterized and most of them map to the extracellular globular protein-domain where they interfere with proper folding, trafficking and binding to pre-synaptic partners. **They can not only promote loss of function phenotypes but also favor ER-retention** due to the mutant protein not passing ER quality control check points, and cause ER stress conditions in the organelle. Therefore, targeted therapies may employ specific compounds in order to rescue cell surface trafficking, ER homeostasis and binding to presynaptic partners.

Dissecting the molecular mechanisms that underlie these processes is expected to provide new strategies for selectively restoring a proper synaptic function in specific neuron populations thereby ameliorating the symptoms observed in patients.

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Figures

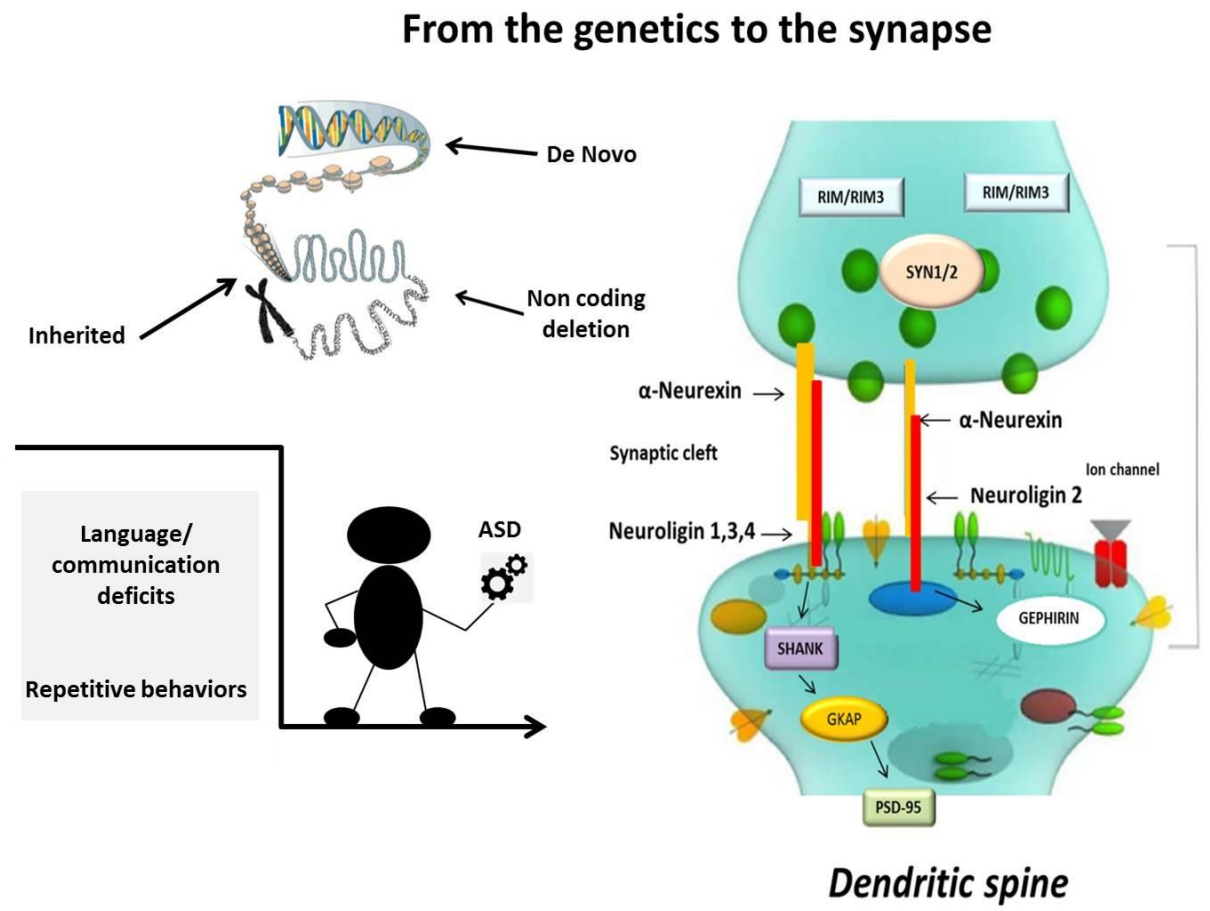

Figure 1. Synaptopathy in ASD. Several *de novo* and inherited mutations have been reported in genes encoding proteins involved in synaptic function belonging to the NLGN-NRXN signaling pathway, such as the NLGNs, NRXNs and SHANK proteins.

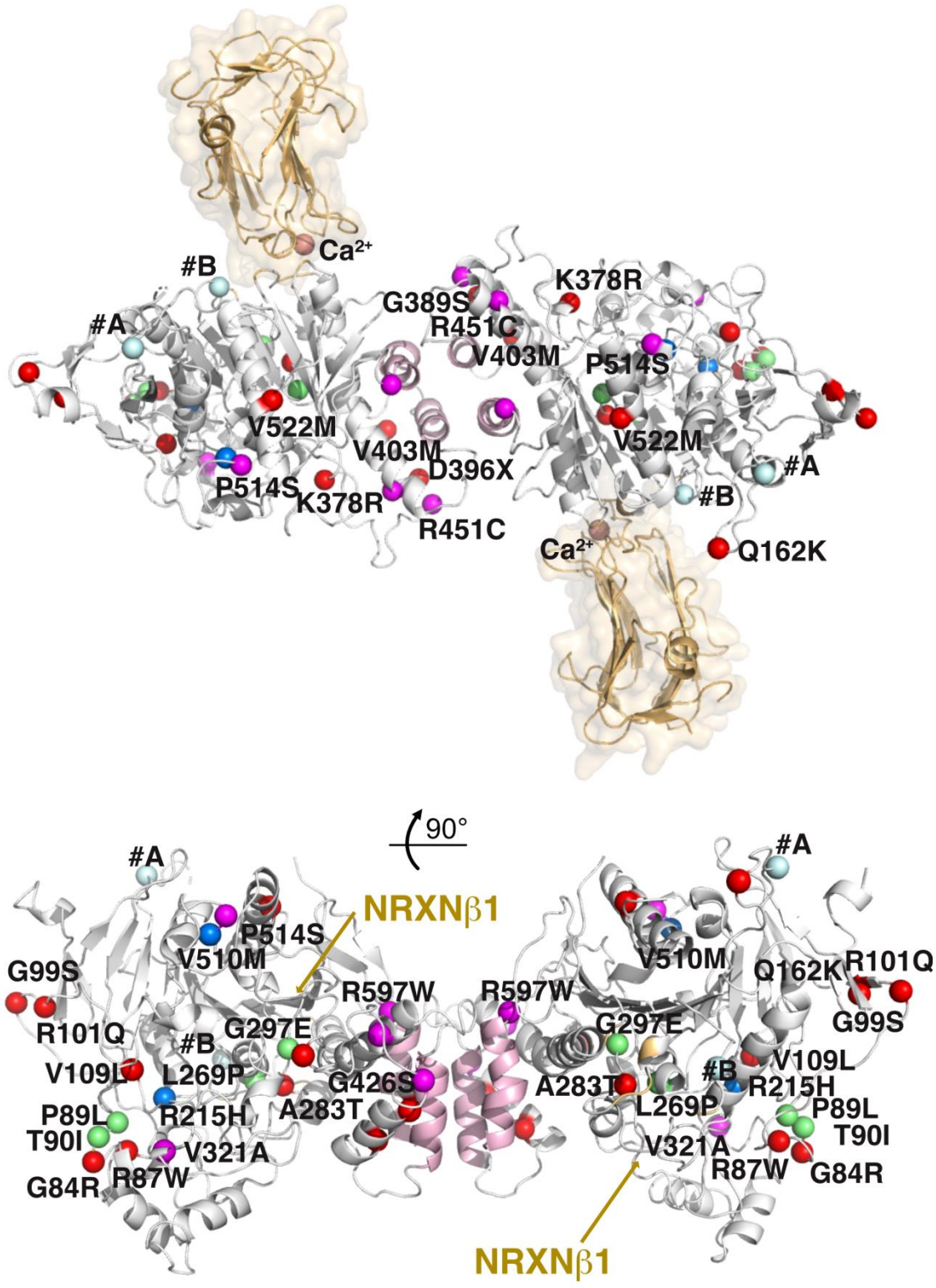

Figure 2. ASD-linked substitutions in the NLGNs 1-4X extracellular domains, mapped onto the crystal structure of NRXNβ1-bound NLGN4X. The NRXNβ1-bound NLGN4X dimer (RCSB-PDB accession code 2XB6) is displayed in two orientations through a 90° rotation along the dimer longer axis. Only for the top orientation are the two NRXNβ1 molecules displayed (brown ribbon, transparent molecular surface). For

the bottom orientation they would be before the left NLGN4X subunit and behind the right subunit, respectively. The four-helix bundle at the dimer interface is highlighted in light pink. The positions for residue substitutions in NLGN1, NLGN2, NLGN3 and NLGN4X are pinpointed by green, marine, magenta and red spheres, respectively, and labeled accordingly to their genuine positions in the respective NLGNs **(see references in Table I).** Substitutions leading to a premature STOP codon with no evidence for protein expression are not displayed. The positions for alternative splice inserts A in NLGNs 1-4 and B in NLGN1 are indicated by light blue spheres. Calcium ions trapped at the two NRXNβ1-NLGN4X binding interfaces are displayed as salmon-colored spheres.

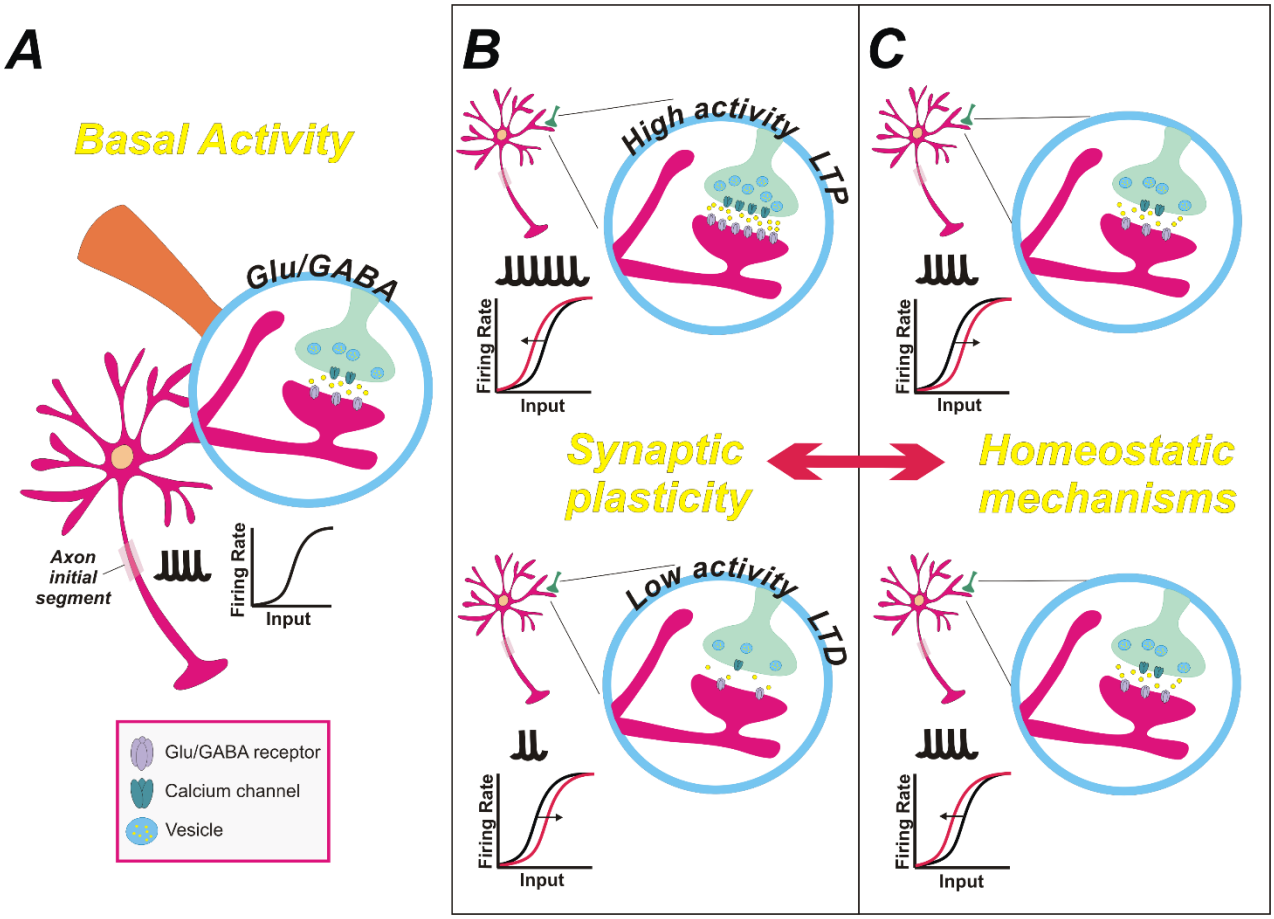

Figure 3. Synaptic plasticity and homeostatic mechanisms. (A) Basal neuronal activity is determined by steady-state presynaptic inhibitory and excitatory inputs, as well as by postsynaptic excitability. (B) Different forms of synaptic plasticity can increase (top, LTP) or decrease (bottom, LTD) synaptic strength, either presynaptically, by modifying vesicular release, or postsynaptically, by changing receptor availability, **ultimately altering neural output.** (C) When these persistent changes in neuronal activity represent potentially destabilizing perturbations in neural circuit activity, diverse homeostatic mechanisms can be activated in order to increase (top) or decrease (bottom) synaptic strength and/or neuronal excitability and restore basal levels of neuronal activity. Glu, glutamate; LTP, long-term potentiation; LTD, long-term depression.

Table 1. Residue substitution identified across human neuroligins NLGN1-4, and their equivalent positions in NLGN4X.

Protein and reference	Isoform	Residue substitution	Protein domain	Associated disorder	Homologous residue in NLGN4	Color on figure 2
NLGN1						
Nakanishi et al., 2017	1, 2	P89L (APPTG)	Extracellular	OCD	P82 (SPPTG)	Green
	1, 2	T90I (PPTGE)	Extracellular	ASD	T83 (PPTGE)	Green
	2	L269P (VNLLT)	Extracellular	ASD	L262 (VSLLT)	Green
	2	G297E (QSGTA)	Extracellular	ASD	G281 (QSGTA)	Green
	2	H795Y (PLHTF)	Intracellular	ASD	H793 (PLHTF)	*Not shown (domain not present in crystallized NLGN4 protein)*
NLGN2						
Sun et al., 2011	1	R215H (NYRLG)	Extracellular	SCZ	R204 (NYRLG)	Marine
	1	V510M (FGVPM)	Extracellular	SCZ	I499 (FGIPM)	Marine
	1	R621H (TTRLP)	Extracellular	SCZ	K612 (TTKVP)	*Not shown (present in crystallized NLGN4 protein but not resolved in the structure)*
	1	A637T (AGAPG)	Stalk	SCZ	I649 (AKIWP)	*Not shown (domain not present in crystallized NL4 protein)*
NLGN3						
Yu et al., 2013	3	V321A (ELVEQ)	Extracellular	ASD, ID	I327 (ELIQQ)	Pink
Xu et al., 2014	1	G426S (VSGTD)	Extracellular	ASD	P392 (VTPND)	Pink
Jamain et al., 2003	2	R451C (TRRKT)	Extracellular	ASD	R437 (TRRKT)	Pink
Quartier et al., 2019	2	P514S (GVPMV)	Extracellular	ASD, ID	P500 (GIPMI)	Pink
	2	R597W (HYRAT)	Extracellular	ASD, ID	R583 (HYRAT)	Pink
NLGN4X						
Xu et al., 2014	1	G84R	Extracellular	ASD	G84 (PTGER)	Red
Zhang et al., 2009	1, 2	R87W	Extracellular	ASD	R87 (ERRFQ)	Red
Yan et al., 2005	1, 2	G99S	Extracellular	ASD	G99 (WTGIR)	Red
Nguyen et al. 2020	1, 2	R101Q	Extracellular	ASD	R101 (GIRNT)	Red
	1, 2	V109L	Extracellular	ID	V109	Red

					(AAVCP)	
Xu et al., 2014	1	Q162K	Extracellular	ASD	Q162 (HDQNS)	Red
Yuen et al., 2017	1	L211X	Extracellular	ADHD	L211 (GFLST)	*Not shown (premature STOP codon)*
	1, 2	Q274X	Extracellular	ADHD	Q274 (LFQKA)	*Not shown (premature STOP codon)*
Xu et al., 2014	1	A283T	Extracellular	ASD	A283 (GTALS)	Red
Yu et al., 2013	1	Q329X	Extracellular	ASD	Q329 (IQQTI)	*Not shown (premature STOP codon)*
Talebizadeh et al., 2006	1	K378R	Extracellular	ASD	K378 (GLKFV)	Red
Jamain et al., 2003; Chih et al., 2004	1	D396X insertion	Extracellular	ASD	D396 (DFDFS)	Red *(truncated protein)*
Xu et al., 2014	1	V403M	Extracellular	ASD	V403 (NFVDN)	Red
Laumonnier et al., 2004	1	D429X deletion	Extracellular	ASD, ID	D429 (WADKE)	*Not shown (premature STOP codon)*
Martinez et al., 2016	1	V454_A457X deletion	Extracellular	ID	V454_A457X (AVATAD)	*Not shown (premature STOP codon)*
Wang et al., 2018	1	V522M	Extracellular	TD	V522 (SAVVM)	Red
Xu et al., 2014	1	R704C	Intracellular	ASD	R704 (KRRHE)	*Not shown (domain not present in crystallized NLGN4 protein)*
Yu et al., 2013	1	R766Q	Intracellular	ASD	R766 (LRRSP)	*Not shown (domain not present in crystallized NLGN4 protein)*

Insertions/deletions are reported with the wild-type residue number followed by a X (see also Figure 2). Isoform numbering (depending on presence/absence of splice inserts) and residue numbering (comprising the signal peptide) are those in UNIPROT. Abbreviations are: ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; OCD, obsessive compulsive disorder; ID, intellectual disability; SCZ, schizophrenia; TD, tardive dyskinesia.

Table 2. Main behavioral and physiological impairments reported in NLGNs rodent models.

Rodent Model	Physiology properties	References
NLGN1 KO$^{-/-}$ ↓ Social interaction ↓ Spatial working memory ↑ Grooming ↑ Repetitive behavior	***Hippocampus (CA1)***: ↓ LTP magnitude, NMDAR/AMPAR ratio, NMDAR EPSC; ↔ AMPAR EPSC; IPSC ***Hippocampus (DG)*: ↓** slope of fEPSP, LTP, fEPSP population spike; ***Somatosensory cortex*: ↔** mIPSC frequency and amplitude; ***Cortico-striatal synapses***: ↓ NMDAR/AMPAR ratio; ***Thalamo-amygdala synapses***: ↓ NMDAR/AMPAR ratio and NMDAR-dependent EPSC, STD-LTP; ***Cortico-amygdala synapses***: ↓ NMDAR/AMPAR ratio and NMDAR-dependent EPSC; ↔ cortical STD-LTP; ***Cortical layer 2/3 pyramidal neurons*: ↓** NMDAR uEPSC and NMDAR/AMPAR ratio;	Blundell *et al.*, 2010; Chubykin *et al.*, 2007; Jedlicka *et al.*, 2015; Jiang *et al.*, 2017; Jung *et al.*, 2010; Kim *et al.*, 2008; Kwon *et al.*, 2012; Tabuchi *et al.*, 2007
NLGN1 KO$^{+/-}$ ↑ Grooming ↓ Recognition memory	***Hippocampus (CA1)***: ↓ NMDAR-LTD and NMDAR-LTP; ↑ mGluR-LTD;	Dang *et al.*, 2018
NLGN1 cKO	***Cerebellum (Stellate cells):*** ↓NMDAR-mediated EPSC; ↔ AMPA-mediated EPSC; ***Cerebellum (Purkinje cells):*** ↓ climbing-fiber EPSCs amplitude;	Zhang and Südhof, 2016; Zhang *et al.*, 2015
NLGN1 TG ↓ Spatial working memory and spatial reference memory	***Hippocampus (CA1)***: ↓ STP and LTP; ↑ EPSC amplitude, E/I ratio; ↔ IPSC amplitude;	Dahlhaus *et al.*, 2010
NLGN1 KD ↓ Memory acquisition	***Hippocampus (DG)*: ↓** LTP, NMDAR-dependent EPSC, AMPAR-dependent EPSC; ***Hippocampus (CA1)*: ↓** spontaneous action potential frequency, mEPSC frequency; ↔ LTP, NMDAR/AMPAR ratio, mEPSC amplitude, sEPSC frequency and amplitude, spontaneous action potential amplitude; ***Amygdala*: ↓** NMDAR EPSC, NMDAR/AMPAR ratio, LTP; ↔ AMPAR-dependent EPSC, mEPSC frequency and amplitude; ***Cortical layer 2/3 pyramidal neurons*: ↓** mEPSC frequency, NMDAR/AMPAR ratio, NMDAR uEPSC; ↔ mEPSC amplitude and AMPAR uEPSC;	Fang *et al.*, 2016; Kim *et al.*, 2008; Kwon *et al.*, 2012; Schnell *et al.*, 2014; Shipman and Nicoll, 2012
NLGN2 KO ↑ Anxiety-like behavior ↓ Pain sensitivity ↓ Motor coordination Irregular breathing patterns	***Somatosensory cortex***: ↓ IPSC amplitude; ***Cortical FS interneuron-excitatory neuron synapses*: ↓** unitary IPSC amplitude and mIPSC amplitude; ↑ quantal content; ***Cortical excitatory neuron-FS interneuron synapses*: ↑** EPSC amplitude; ***Ventrolateral Medulla*: ↓** GABA/glycinergic eIPSC amplitude, GABA/glycinergic sIPSC and mIPSC frequency and amplitude, GABAergic and glycinergic mIPSC onset slope, postsynaptic response to GABAergic and glycinergic agonists; ↑ ePSC and GABA/glycinergic eIPSC failure rate ***Hippocampus (CA1)***: ↓ GABAergic sIPSC and mIPSC frequency, GABAergic mIPSC onset slope; ***Hippocampus (DG)*: ↓** threshold for epileptiform discharge induction, GABAergic mIPSC amplitude; ***Amigdala*: ↓** mIPSC frequency; ↔ mEPSC amplitude; ***Retina*: ↑**basal activity; ↓ amplitude of light response;	Babaev *et al.*, 2016; Blundell *et al.*, 2009; Chubykin *et al.*, 2007; Gibson *et al.*, 2009; Hoon *et al.*, 2009; Jedlicka *et al.*, 2011; Poulopoulos *et al.*, 2009
NLGN2 cKO ↓ Anxiety-like behavior ↓ Fear conditioning	***Medial prefrontal cortex*: ↓** mIPSC and sIPSC frequency and amplitude; ↔ mEPSC frequency and amplitude; ↑E/I ratio; ***Cerebellum (Purkinje cells)*: ↑** climbing-fiber EPSC**;** ***Basket/stellate cell synapse*: ↓** IPSC amplitude, mIPSC frequency	Liang *et al.*, 2015; Zhang *et al.*, 2015

R215H NLGN2 KI ↓ Fear conditioning ↑ Anxiety-like behavior ↑ Prepulse inhibition	***Medial prefrontal cortex*: ↓** mIPSC frequency and amplitude, Paired-pulse ratio; ↔ mEPSC frequency and amplitude; ↑ E/I ratio;	Chen *et al.*, 2017, 2020
NLGN2 TG ↑ Limb clasping ↑ Startle response ↑ Anxiety-like behavior ↓ Social interaction	***Prefrontal cortex*: ↑** mIPSC frequency; ↓ E/I ratio; ↔ mIPSC amplitude, mEPSC frequency and amplitude;	Hines *et al.*, 2008
NLGN3 KO ↑ Motor activity ↓ Social interaction ↓ Buried food finding ability ↓ Ultrasonic vocalization ↓ Fear conditioning memory ↑ Spatial working learning	***Hippocampus*** **CA1: ↑** mIPSC frequency; ↓ mEPSC frequency; ↔ NMDAR/AMPAR ratio, fEPSP slope and LTP; **PV basket cell-pyramidal neuron synapse: ↔** IPSC amplitude and success rate; **CCK basket cell-pyramidal neuron synapse**: ↑ IPSC amplitude and success rate; **CA2: ↑** pyramidal cell spontaneous firing frequency, sEPSC and sIPSC frequency; ↓ CCK-eIPSC**; ↔** mIPSC, sEPSC and sIPSC amplitude; PV-eIPSC; inhibitory short-term depression; ***Somatosensory cortex*: ↔** eEPSC and eIPSC amplitude; mIPSC frequency and amplitude ***Cerebellum*: ↓** mEPSC amplitude and mGluR-LTD; ***Nucleus Accumbens***: ↓ mIPSC frequency in D1-MSNs; ↑ E/I ratio; ↔ mIPSC amplitude	Baudouin *et al.*, 2012; M. Etherton *et al.*, 2011; Földy *et al.*, 2013; Modi *et al.*, 2019; Radyushkin *et al.*, 2009; Rothwell *et al.*, 2014; Speed *et al.*, 2015; Tabuchi *et al.*, 2007
NLGN3 cKO	***Cerebellum (Purkinje cells):* ↓** climbing-fiber EPSCs amplitude;	Zhang *et al.*, 2015
R451C NLGN3 KI ↓ Social interaction ↑ Spatial learning ↑ Repetitive and stereotyped behavior	***Somatosensory cortex layer 2/3***: ↑ mIPSC frequency, eIPSC amplitude, E/I balance; ↔ short term synaptic depression, NMDAR/AMPAR ratio, mEPSC frequency and amplitude, mIPSC amplitude, eEPSC amplitude; ***Hippocampus*** **CA1**: ↑ fEPSP linear slope, LTP amplitude, mEPSC frequency, NMDAR/AMPAR ratio, NMDAR-EPSC decay time, AMPAR-EPSC; ↔ mEPSC amplitude, mIPSC frequency and amplitude; **CA3: ↑** giant depolarizing potentials frequency, mIPSC frequency; ↔ mIPSC amplitude, AMPAR-mEPSC, NMDAR/AMPAR ratio; **PV basket cell-pyramidal neuron synapse: ↓** IPSC amplitude and success rate; **CCK basket cell-pyramidal neuron synapse: ↑** IPSC amplitude and success rate; ***Nucleus Accumbens***: ↓ mIPSC frequency in D1-MSNs; ↔ mIPSC amplitude; ***Striatum*: ↓** LTD; ***Basolateral amygdala*: ↑** mEPSC amplitude**; ↓** mIPSC amplitude;	Cellot and Cherubini, 2014; M. Etherton *et al.*, 2011; Földy *et al.*, 2013; Hosie *et al.*, 2018; Martella *et al.*, 2018; Pizzarelli and Cherubini, 2013; Rothwell *et al.*, 2014; Speed *et al.*, 2015; Tabuchi *et al.*, 2007
R704C NLGN3 KI	***Hippocampus CA1***: ↓ mEPSC frequency, fEPSP linear slope; ↑ NMDAR/AMPAR ratio; ↔ mEPSC amplitude, mIPSC frequency and amplitude; NMDA-LTP;	M. R. Etherton *et al.*, 2011
NLGN3 KD	***Hippocampus*** ***DG*: ↓**NMDAR-dependent EPSC, AMPAR-dependent EPSC; ↔ LTP ***CA1*: ↔** LTP, NMDAR-dependent EPSC, AMPAR-dependent EPSC;	Shipman and Nicoll, 2012
NLGN4 KO ↓ Social interaction ↓ Ultrasonic vocalization	***Somatosensory cortex*: ↓** mIPSC and mEPSC frequency; ↑ mIPSC rise time**,** mIPSC decay time**,** paired pulse ratio; ↔ mIPSC and mEPSC amplitude; ***Hippocampus CA3*: ↓** sIPSC frequency and amplitude; ↑ sIPSC rise time and decay tau; mIPSC rise time and decay tau; ↔ mIPSC, sEPSC and mEPSC frequency and amplitude;	Hammer *et al.*, 2015; Hoon *et al.*, 2011; Unichenko *et al.*, 2018

	***Retina*: ↑** decay time of glycinergic mIPSC**; ↓**latency in triggering action potential; **↔** glycinergic mIPSC amplitude and frequency, GABAergic mIPSC amplitude, frequency and decay time ;	
NLGN1/2/3 triple KO Irregular and flat breathing movements	***Brainstem*: ↓** GABAergic/glycinergic sPSC frequency and amplitude, GABAergic/glycinergic mPSC, sEPSC, and mEPSC frequency; **↑** evoked GABAergic/glycinergic transmission failure rate; **↔** GABAergic/glycinergic mPSC, sEPSC, and mEPSC amplitude;	Varoqueaux *et al.*, 2006
NLGN1/2/3 triple cKO	***Cerebellum*** **Stellate cells: ↓** mIPSC amplitude, NMDAR-mediated EPSC; **↔** mIPSC frequency, sEPSC amplitude and frequency, paired-pulse facilitation, AMPAR-EPSC rectification index; **Purkinje cells: ↓** sIPSC frequency and amplitude, mIPSC frequency, amplitude, and decay time, climbing-fiber EPSCs amplitude; **↔** DHPG-LTD, paired-pulse ratio; **Basket/stellate cell synapses: ↓** IPSC amplitude; mIPSC frequency, amplitude, and decay time	Zhang and Südhof, 2016; Zhang *et al.*, 2015

Reported changes: ↑ increase; ↓ decrease; ↔ no significant change. KO, knockout; KI, knockin; KD, knockdown; LTP, long-term potentiation; LTD, long-term depression; STD-LTP, spike timing-dependent LTP; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; sEPSC(IPSC), spontaneous EPSC(IPSC); mEPSC(IPSC), miniature EPSC(IPSC); fEPSP, extracellular field EPSP.