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Review

A review on the current neuroligin mouse models

XU Jun-Yu[1,*], XIA Qiang-Qiang[1], XIA Jun[2]

[1]Department of Neurobiology, Key Laboratory of Medical Neurobiology of Ministry of Health, Zhejiang Province Key Laboratory of Neurobiology, Zhejiang University School of Medicine, Hangzhou 310058, China; [2]Division of Life Science, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Abstract: Neuroligins (NLs) are postsynaptic membrane proteins expressed in the brain and mediate synaptogenesis. Neuroligin family proteins can specifically induce either excitatory or inhibitory synapses. Deletions or point mutations in neuroligin genes are found in patients with autism spectrum disorders (ASD) or mental retardations. The dysfunctions of these mutations have been tested in multiple neuroligin mouse models. In most of the models, including the human autism-linked NL3 and NL4 mutation mice, there are social interaction defects, memory impairment and repetitive behaviors. Researchers also found the excitatory/inhibitory synapse ratio altered in those mice, as well as receptor subunit composition. However, inconsistencies and debates also exist between different research approaches. In this review, we summarize the neuroligin mouse models currently available, examine the detailed alterations detected in those mice and compare the differences within different mouse models or different investigation methods, to obtain an overall picture of the current progress on neuroligin mouse models.

Key words: neuroligin; synaptogenesis; synapse function; autism; mice model

关于现有Neuroligin小鼠模型的总结

许均瑜[1,*]，夏强强[1]，夏 军[2]

[1]浙江大学医学院神经生物学系，卫生部医学神经生物学重点实验室，浙江大学神经生物学重点实验室，杭州 310058；[2]香港科技大学生命科学部，香港

摘 要：Neuroligin是位于神经元突触后膜并介导突触生成的一类细胞粘附分子。其家族蛋白可特异性介导兴奋性或抑制性突触形成。已有多项研究显示，neuroligin基因突变与自闭症等精神发育迟滞疾病相关。而因neuroligin蛋白功能异常引起的系列变化亦在小鼠模型中得到印证。neuroligin基因缺失，敲入或变异基因替代等小鼠模型，尤其是自闭症相关小鼠模型出现社交能力减弱，学习与记忆能力减退及强迫症相关表型，与人类自闭症症状相似。研究更进一步显示，该类小鼠中存在兴奋性突触及抑制性突触失调现象，并伴随有谷氨酸受体亚单位成分变化。然而，在小鼠模型研究中亦存在与体外研究结论不符情况，甚或出现相悖结论。在此综述中，我们总结了近几年所报道的小鼠模型，研究报道中的小鼠在分子，细胞及行为水平的各种异常现象，并归纳分析在各类小鼠中出现的相似及相异结果，希望能从中了解到neuroligin小鼠模型研究的进展及整体成果。

关键词：neuroligin；突触生成；突触功能；自闭症；小鼠模型
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*Corresponding author. Tel: +86-571-88981367; Fax: +86-571-88208248; E-mail: junyu@zju.edu.cn

Neuroligins (NLs) are cell adhesion proteins specifically localized on the postsynaptic membrane [1]. They normally compose of an extracellular acetylcholinesterase homology domain without esterase activity, a highly conserved single transmembrane domain and a cytoplasmic tail with PDZ domain binding motif [2]. There are 5 neuroligin genes in human (NLGN1, NLGN2, NLGN3, NLGN4 and NLGN4Y) and at least 4 in rodents (NLGN1–4) [2–4]. NLGN3 and NLGN4 genes are X-chromosome linked genes, while NLGN4Y is a NLGN4 equivalent located on the Y-chromosome [3, 4]. Subcellularly, neuroligin 1 (NL1) and Neuroligin 3 (NL3) proteins are expressed mainly on glutamatergic synapses, while neuroligin 2 (NL2) is exclusively localized on GABAergic synapses [5–7]. NL3 was later found to be localized on both glutamatergic and GABAergic synapses [8]. Recent data also show that neuroligin 4 (NL4) in a retina system is preferentially localized to glycinergic postsynapses [9].

Neuroligins bind to neurexins, which primarily localize on the presynaptic membrane in a splicing-specific manner [10–12]. Through a coculture system, neuroligins were shown to induce presynaptic structure formation through neurexins [13, 14], and vice versa, artificial neurexin-coated beads also cause aggregation of neuroligins and induce the formation of postsynaptic structure [15]. Different neuroligins show potencies in inducing distinct forms of synapses. NL1 preferentially induces excitatory synapse formation while NL2 induces inhibitory synapses [5–7, 14]. Since there are a large number of splicing isoforms in the neuroligin and neurexin families with different distribution patterns [11, 12, 16–18], it is highly possible that the molecular diversity of those two proteins contribute to the synapse diversity in the CNS.

Through *in vitro* assays, neuroligins are demonstrated to promote synapse formation not only by initiating synaptic contact, but also in establishing synapse specialization by recruiting synaptic elements. The C-terminus of neuroligin has been found to bind with the third PDZ domain of postsynaptic scaffold protein PSD-95 [19] which anchors a variety of signaling molecules and surface receptors including NMDA receptors and potassium channels [6, 20–22]. Moreover, interaction between neuroligins and PSD-95 regulate the balance of excitatory and inhibitory synapse formation [23, 24]. NL2 is able to cluster $GABA_A$ receptors through interacting with scaffold protein gephyrin and collybistin [25, 26].

In addition, neuroligins have been linked with many psychiatric disorders, especially the Autism Spectrum Disorders (ASD). The point mutation in the neuroligin 3 gene, where amino acid 451 of neuroligin 3 was changed from arginine to cysteine (NL3 R451C), was found in Swedish brothers with autism and Asperger syndrome, and the truncated mutation of neuroligin 4 (NL4 D396X) was found in another set of Swedish brothers [27]. Other forms of NL4 and NL4Y point mutations were also found to be associated with heritable autism and mental retardations; these include the NL4 R87W mutation [28] and NL4 G99S, K378R, V403M and R704C mutations [29] and NL4Y I679V mutation [30]. NL4 gene exon 4 deletion [31], exons 4, 5 and 6 deletion [32], NL4 gene 2 base pair deletion which leads to the D429X mutation, [33] associate with autism as well. A genome-wide copy number variation analysis also shows that NL1 could be linked to autism [34]. Even though there are other reports which claim that no significant or modest correlation between neuroligin and autism was found in their research [35–40], it is a possibility that neurexin-neuroligin regulates the neuronal connectivity and is one of the key factors implicated in cognitive diseases.

However, in disagreement with the findings in cultured neurons, *in vivo* studies revealed a greater potential of neuroligins in mediating synapse specification and modulation other than synapse formation; some of the neuroligin mouse models failed to show a strong correlation with the ASD phenotype. In this review, we will research the neuroligin mouse models through the documented literature, looking at the molecular, cellular and behavioral abnormalities in these mice, and from which, discuss the neuroligin functions in synapse development, neuronal functioning and psychiatric diseases.

1 Neuroligin knockout mice

The first neuroligin knockout (KO) mouse was generated by the Brose and Südhof groups, with the single NL1/2/3 KO, and the combination of two or three genes KO by interbreeding [41]. Despite the normal appearance of single or double KO mice, the NL1-3 triple KO mice showed reduced body weight, irregular and flat breathing movement and died within 24 h after birth. The abnormal breathing behavior was found to be due to reduced neurotransmission in the brain stem respiratory network pre-Bötzinger complex (PBC)[41]. In acute PBC slice, 80% reduction in GABAergic/

glycinergic mPSC and sPSC frequencies was detected as compared with single KO mice [41]. The spontaneous glutamatergic and GABAergic/glycinergic transmission frequencies were reduced by 4 and 10 folds respectively, with a remarkable increased evoked failure rate [41]. The single KO mice had similar electrophysiological activities as wildtype (WT) controls in PBS, while NL1,3 double KO mice showed similar defects in synaptic transmission as triple KO but to a lesser extent [41].

Biochemical studies of the newborn triple KO mice brain revealed a decreased expression level of synaptic proteins like synaptotagmin1, vGluT1, NR1 and others [41]. In the PBC region, there was a 20% increase in vGluT cluster density, a 20% reduction in VIAAT and 30% reduction in $GABA_AR\alpha1$ [41]. Even though around 15% reduction of GABAergic/glycinergic synapse density and 15% increase in glutamatergic synapse density were observed in PBC and inferior olive of brain stem, the overall synapse numbers remain unchanged [41], or at least had no significant effects compared with the *in vitro* siRNA knocking down experiments [5]. These *in vivo* studies imply that NL proteins play a subtle role in synapse formation but a major role in synapse functioning. It was also shown that NLGs stabilize and strengthen synapses in an activity-dependent manner [42].

Acute NL1 siRNA knock down has been performed in amygdala of Sprague-Dawley rats by lentiviral injection [43]. Around 50% of the total neurons in the lateral nucleus of the amygdala (LA) were infected, which led to a 40%–50% reduction of NL1 protein levels in that area [43]. The researchers failed to detect any spine density alteration in the LA region, but succeed in detecting a 55% reduction in NMDA receptor dependent EPSC, and abolished LTP in thalamo-amygdala synapses on principal neurons [43].

Further examination of the NL1 single KO mice was carried out afterwards, and defects in NMDA receptor mediated transmission was detected in some brain regions. Deficiency in NL1 caused a 50% reduction in NMDAR/AMPAR ratio on Schaffer collateral-CA1 synapses in the hippocampus [42]. Both thalamo-amygdala synapse and cortico-amygdala synapse showed 50% reduction in NMDAR/AMPAR ratio [44], and a 30% reduction of the ratio on cortico-striatal synapse [45]. The decreased NMDAR-dependent EPSC and abolished LTP were both observed on the thalamo-amygdala synapses [44]. The LTP in the CA1 region also showed a 20% reduction [45]. Interestingly, the NMDA receptor level was not changed, as well as the number of excitatory synapses [45]. Therefore, the changes observed on NMDAR/AMPAR ratio and LTP may due to the defects in synapse function, though the detailed mechanism is not fully understood. As supportive evidence, the expression levels of NL3, neurexin, synapsins, and other synaptic proteins were detected to change [45]. However, with further knock down of NL3 in NL1 KO mice, there was no further change on NMDAR/AMPAR ratio in CA1 hippocampus, suggesting a dominant role of NL1 in maintaining synaptic transmission [46]. Further behavioral tests showed that a deficit of NL1 would cause some autism-like symptoms in mice, such as slightly decreased social interaction in three-chambered social interaction test and impaired spatial working memory in Morris water maze task, and enhanced repetitive grooming activities [45]. During the fear conditioning tasks, the NL1 knockdown rats also have deficits in the storage of associative fear memory [43].

The deficient of NL2 mainly causes the major defects in inhibitory neurotransmission. A 50% reduction in IPSC amplitude in the acute cortial slices [42] was observed. In the thalamocortical region, the parvalbumin positive-fast spiking (FS) interneuron onto excitatory neuron synapse were affected, with 40%–50% reductions in IPSC amplitudes from both layer 2/3 and layer 4 neurons [47]. The researchers believed that NL2 plays an important role in synaptic scaling, as the unitary IPSC amplitude also decreased by 2 folds. It can be caused by the quantal release defects in NL2 KO mice, as the mIPSC amplitude and quantal amplitude showed a significant reduction [47], but not the synapse density. NL2 deficiency also caused about 2-fold increase in the EPSC amplitude onto FS neurons, either due to NL2's role on excitatory neurotransmission or a compensatory upregulation on the inhibited FS-excitatory neurotransmission [47].

Different from the *in vitro* studies that NL2 plays a role on GABAergic synapses [5, 7, 25, 26], *in vivo* data from NL2 KO mice showed an critical control of NL2 on both GABAergic and glycinergic neurotransmissions, but not on the synapse formation on the early stage of brain stem development [26]. The researchers found that NL2 KO mice exhibit irregular breathing patterns as the NL1-3 triple KO mice [41]; therefore they examined the neurotransmission in the brain stem slices containing the ventrolateral medulla [26]. Dramatic reductions on both GABAergic and glycinergic sIPSC and mIPSC amplitudes (15%–30% reduction) and frequencies

(50%–75% reduction) were detected, without changes on the excitatory synapses.

In hippocampal slices of NL2 KO mice, the functional reduction on GABAergic synapses were also detected [26]. Further examination showed intact excitatory and inhibitory synapses on similar density [26, 48] , but a 40% reduction of vGAT density was detected in CA1 region [48]. NL2 KO mice also showed a 40% reduction on the $GABA_A$ receptor γ2 subunit puncta density and 3-fold reduction in gephyrin puncta density in str. pyramidale of the CA1 region of the hippocampus, while large cytoplasmic aggregates of gephyrin aggregates were observed to have about 7-fold increase [26]. In the granule cell layer of dentate gyrus, there were around 40% reductions on gephyrin and $GABA_A$ receptor γ2 subunit clusters, as well as the coclusters of the two proteins [49]. *In vivo* recording showed the enhanced excitability on preforant path-granule cell synapses with 50% increased amplitude of population spikes and 45% lower threshold frequency for the induction of epileptiform discharges (i.e. seizure-like responses) in the KO mice [49]. 30% reduction in GABAergic mIPSC amplitude also was recorded from slice recording of KO mice [49]. Similar reductions in $GABA_A$ receptors was detected in retina of NL2 KO mice, leading to an increased action potential firing of retinal ganglion cells but decreased amplitudes of responses to light stimuli [50]. Electroretinogram recording also showed smaller ganglion cell action potential and increased neuronal excitability [50]. The data suggests that NL2 plays an important role in postsynaptic scaffolding and receptor recruitment, which enhances the excitability of neurons, other than the synapse formation.

The altered neuronal excitability in some brain regions may account for the abnormal behaviors of NL2 KO mice. From the open field and dark/light box tests, the NL2 KO mice always increased in anxiety-like behavior. The decrease in pain sensitivity was also obvious from both hotplate sensitivity and footshock sensitivity tests [48]. A slight decrease in motor coordination was also found from the accelerating rotarod test [48]. The NL2 KO mice showed normal social interaction.

For the NL3 and NL4 single KO mice, there are few examinations on the neuronal architectures, functions or molecular compositions. 25% decreased NL1 protein level in total brain homogenate was detected in NL3 KO mice [51]. In CA1 hippocampal region, but no other brain regions of NL3 KO mice, a slight increase in mIPSC frequency and slight reduction in mEPSC frequency were detected [52]. However, in CA1 region, the fEPSP, LTP or NMDAR/AMPAR ratio all showed a normal level [52]. A normal NMDAR/AMAPR ratio was also observed in the somatocortex region [52]. Magnetic resonance imaging revealed that both KO mice have a slight reduction in the total brain volume, 5% in NL3 KO mice and 1.5% in NL4 KO mice [53, 54]. About 4% reductions in cerebellum and brainstem volumes were also detected in NL4 KO mice [53]. In addition, both KO mice showed impairment in social interactions [53, 54]. NL3 KO mice also showed reduced social novelty preference from the three-chambered social test while NL4 KO mice did not [53, 54]. However, NL4 KO mice showed reduced social interaction during the open arena reciprocal social interaction test [53]. For NL4 KO mice, either the duration, direct contacting with other mice or the time attacking intruders was greatly reduced (45% and 68% reductions respectively compared to WT mice) [53]. Ultrasonic vocalization tests showed that for both KO male mice, there were about 3-fold increase in latency of start calling and 48% reduction in total number of calls when encountering a female mouse in estrous [53, 54] . Moreover, NL3 KO mice show higher motor activity in both open field tests and elevated plus maze tests [54]. These mice found buried food more slowly; had 47% decreased freezing responses in the contextual fear conditioning test and 25% decreased freezing responses in cued fear conditioning [54]. NL3 KO mice also show a slight increased learning ability during the reversal task of Morris water maze [54]. Though the underlying molecular or neuronal circuitry mechanisms leads to the behavioral defects are still under examination, it is clear that NL3 and NL4 are associated with the social ability, and especially for NL3, with certain learning abilities.

2 Neuroligin transgenic mice

In addition to NL null mice, HA-NL1 and HA-NL2 transgenic (TG) mice were also generated for functional studies [55] by the El-Husseini group. NL1 single or NL1-3 triple KO show no difference in synapse density [41, 45]. However, EM study of NL1 TG mice showed that with 12% elevated NL1 expression level, a 70%–79% increase in asymmetric synapse number was detected in CA1 hippocampus of different TG lines [56], together with the morphological increase in the average length and curvature of synaptic contacts, and average length of PSD [56]. Golgi impregnation also revealed similar in-

creases in spine density and mature spine populations [56]. Surprisingly, the AMPA receptor subunit content was shown to be altered by both quantitative western blot and immunofluorescent labeling [56]. An increase in GluA1 (22% in Western blot and 46% in IHC) and reduction in GluA2/3 (30% in Western blot and 58% in IHC) were constantly found from the CA1 hippocampus of NL1 TG mice [56]. Excitatory synaptic markers also show significant increases from both approaches, such as vGAT and vGluT [56]. Though there was a 26% increase in gephyrin expression levels, there was no change in the symmetric synapse number or morphology [56]. The imbalance between the excitatory and inhibitory synapses was measured by the E/I ratio and shown to have a 64%–73% increase [56]. In the CA1 region of transgenic mice, reductions in STP (5 folds) or LTP (3–4 folds) was detected from field recordings, and reductions in EPSC amplitude (2.5 folds) was detected from whole cell recordings [56]. The impaired synaptic plasticity may account for the learning deficits in the NL1 TG mice. From plus shaped water maze tests, the mice showed impaired spatial working memory acquisition for the hidden platform location in forward training and impaired new memory formation in reverse training. Similar results were obtained from the Morris water maze test [56]. After changing the platform position, the mice also showed impairment in spatial reference memory formation [56]. In addition, reduced threatening behavior in resident-intruder paradigm was observed in the NL1 TG mice [57]. Compared with the NL1 KO mice study, it is interesting to find out that both deficit and overexpression in NL1 protein levels could lead to impaired synaptic plasticity and the memory deficits [45, 56]. Perhaps one is caused by the reduced ion channel conductivity and the other is caused by the increased basal excitability.

Another interesting study is the HA-NL1 transgenic in a fragile X syndrome mouse model, FMR1 KO mice [57]. The crossed transgenic line is named TgN1F. Overexpression of NL1 in FMR1 KO mice would rescue the spine size reduction, PSD and vGluT puncta reduction in FMR1 KO mice [57]. The increased inhibitory synapse number in FMR1 KO mice was also reduced to a lower level (58% increase in FMR KO mice and a 18% increase in TgN1F mice) [57]. The social deficits in FMR1 KO mice was rescued by overexpression of NL1 from the three-chambered social interaction test, direct social interaction test, the resident-intruder task and the open field task [57]. This *in vivo* investigation suggests that NL1 may work downstream of FMR1 and be involved in the fragile X syndrome.

Altered E/I synapse ratio was also detected in the NL2 TG mice. An increase in total synapse number, symmetric and asymmetric synapse number was observed in the medial prefrontal cortex [55]. However, with a much greater increase in symmetric synapse number, the E/I ratio significantly decreased [55]. The expression level of NL3 was reduced in NL2 TG mice [55]. Although both vGAT and vGluT expression level and puncta intensity were increased in the TG mice, the ratio of vGluT/vGAT intensity was decreased, supporting the E/I ratio reduction [55]. Patch clamp study recorded an increased mIPSC frequency in the prefrontal cortex layer II/III pyramidal neurons of NL2 TG mice [55], but without any significant difference in RGCs from another NL2 TG mice line [58]. In contrary of the increase anxiety-like behavior in NL2 KO mice [48], the NL2 TG mice showed a decreased anxiety-like behavior from the open field paradigm, light/dark exploration test and elevated plus maze [55]. The NL2 KO mice showed no difference in social behaviors [48], while the NL2 transgenic mice showed reduced interaction with novel target mice in both reciprocal social interaction test and three-chambered social interaction test [55].

3 Neuroligin knockin mice

Among the neuroligin mutations found in autism patients, two of them have been investigated using knockin (KI) mouse models: the NL3 arginine 451 to cysteine mutation (R451C) and the NL4 arginine 704 to cysteine mutation (R704C) but in a NL3 context [51, 52, 59–63].

The first NL3 R451C KI mice study was reported 5 years ago [51]. The NL3 R451C mutation replacement of the WT NL3 gene caused a significant reduction in the endogenous NL1 level, as the R451C was reported to have ER retention and surface expression problems [64–66], the NL3 level was also reduced [51]. In the forebrain homogenate, increased expressions of vGAT and gephyrin were detected [64, 65]. 50%–80% increase in vGAT density was observed in somatosensory cortex as well as CA1 and CA3 regions of hippocampus [51]. The synapses in the somatosensory cortex region of the mice were carefully examined with electron microscopy, to have no alteration in synapse number in layer II/III neurons [51]. However, on enhanced inhibitory transmission, a 50% increase in both mIPSC frequency and amplitude from the electrophysiological recording of P13-16 mice were

observed [51]. Further behavioral study showed the mice had a reduced interaction time with another caged novel mouse in a caged social interaction test and three-chambered social interaction test, and enhanced spatial learning and memory in finding the hidden platform in Morris water maze test and reversal water maze test [51]. From the results, the autism-linked NL3 R451 KI mice do show autism-like symptoms. Defects in excitatory synaptic transmission in somatosensory cortex may be one of the causes.

However, the debate arises from individually generated NL3 R451 KI mice. In these mice, a series of behavioral tasks were conducted including the social interaction tests, anxiety-related tests, learning and memory tests and so on. The reciprocal social play or the three-chambered social test showed no difference in social behavior between KI and WT mice [59]. From the Morris water maze acquisition and reversal tests, the learning and memory abilities were intact [59]. In addition, contextual and cued fear conditioned learning and memory were also unaffected in the KI mice [59]. The authors believed that reduced surface NL3 level on synapses was not sufficient to cause social or cognitive defects. They challenged the results from the previous KI mice study based on the following reasons: different genetic backgrounds, improper experimental design, improper parameter measurements and improper statistical analyses.

The Sudhof group then answered the questions by re-examining the NL3 R451 KI through caged adult social interaction tests and three-chamber sociability and social novelty test. They extended the habituation time to 3 sessions of 10 min, the interaction time to 10 min per session, and found their KI mice spent more time sniffing at novel stranger mice, when analyzed by paired *t* tests [52]. For the caged adult social interaction time, the interaction duration was extended to 5 min, but they found their KI mice spent less time exploring or interacting with caged novel mice, when analyzed by unpaired *t* tests [52]. By examining both lines of KI mice, they found in the CA1 hippocampus region, both mice showed a large increase in fEPSC linear slope and NMDAR/AMPAR ratio [52]. Interestingly, NL3 R451C KI leads to different synaptic transmission changes in the hippocampus and somatosensory cortex: the mEPSC increase was only detected in hippocampus but not in somatosensory cortex, while the mIPSC increase only in somatosensory cortex [52]. Although the synapse number or morphology were unchanged in the CA1 stratum radiatum, the dendritic complexity increased by more dendritic braching and dendritic intersections [52]. Moreover, NL3 R451C KI made a significant switch in NMDA receptor subunit composition by boosting NR2B level to about 2 folds higher, as revealed from both electrophysiology and western blot approaches [52]. The AMPA receptor function in the hippocampus was also affected by 54% and 13% decreased AMPA receptor mediated mEPSC frequency and amplitude respectively [62]. The GluA1 subunit content was reduced in both signal intensity and puncta density [62]. However, the GluA1 cluster localized on synapse was not changed, indicating that NL3 R451C substitution affected the unitaty AMPA receptor content at single synapses but not the mature synapse number containing AMPA receptors [62]. A loss of PV-positive inhibitory neurons in somatosensory cortex was also detected, as found in many ASD mouse models [61]. This inhibitory neuron loss may cause large defects in neuronal γ-oscillation and alter the neuronal synchrony and activity in local circuitry. Indeed, *in vitro* overexpression studies show that the expression of NL3 R471C affects synchrony in dissociated rat hippocampal neurons [67]. The brain anatomical study reveals that, from the MRI study of fixed brain tissues, the NL3 R451C KI mice show significantly smaller brain volumes in various regions [60], which was commonly found in autism patients [68].

The NL3 KI mouse model carrying the NL4 autism-associated mutation R704C was generated (NL3 R704C KI) [29, 63]. Despite the lack of change in synaptic markers such as vGAT, vGluT and synaptophysin, the R704C substitution caused about 35% reduction in NL3 expression level, and there are 20%–25% increases in AMPA receptor subunits GluA1 and GluA3 expression level [63]. The influence in AMPA receptor mediated synaptic transmission was obvious from the culture hippocampal neurons of the KI mice, that the AMPA receptor-mediated EPSC amplitude was reduced by 40% [63]. It caused the major defects in excitatory synaptic transmissions measured by fEPSP and mEPSCs [63]. No behavioral examinations had yet been carried out in this NL3 R704C KI mouse model.

4 Summary

Neuroligins have been found to be associated with autism spectrum disorders and mental retardations. Table 1 summarizes the detected changes in the above

Table 1. Abnormalities in neuroligin mouse models.

Mice model	Behavior	IHC	ICC	WB	EM	EP	Others	Reference
NL3 R451C KI	Social interaction ↓ Spatial learning ↓	**NC** PV-cell number ↓, GAD65 puncta number ↑ **SSC and HP** vGAT density ↑ **HP**, SAP-102 & GluA1 density, GluA1 intensity ↓		**Whole brain**, vGAT & Gephyrin ↑, NL1 & NL3 ↓ **HP**, NL3 ↓, PSD-95, SAP-102, NR2A, NR2B ↑	**CA1, str. rad.**, spine area, bouton area, total number of vesicles per bouton ↓	**SSC**, mIPSC frequency, IPSC amplitude ↑ **CA1**, fEPSP linear slope, LTP amplitude, NMDAR/AMPAR ratio, mEPSC frequency, NMDAR-dependent EPSC decay time, ifenprodil sensitivity ↑, AMPAR-mediated mEPSC amplitude and frequency ↓	MRI, brain volume ↓ DTI, FA values ↓, RD values ↑ Sholl analyses on biocytin-filled **CA1** pyramidal neurons, dendritic branch points↑, dendritic intersections number in str. rad.↑ and in Lac. Mol.↓	[51, 52, 60–62]
NL3 R451C KI (individual line)	No significant changes in series of behavioral tests					**CA1**, fEPSP linear slope, NMDAR/AMPAR ratio ↑		[52, 59]
NL3 R704C KI				**Whole brain**, GluA1, GluA3 ↑, NL3 ↓		**Cultured HP neuron**, AMPAR-mediated EPSC amplitude ↓		[63]
NL1/2/3 triple KO	Irregular and flat breathing movements	**PBC and IOM**, vGluT density ↑, $GABA_AR\alpha1$ & VIAAT density ↓		**Whole brain**, complexin2, synaptobrevin2, synaptophysin1, synaptotagmin1, vGluT1, KCC2 & NR1 ↓		**PBC**, GABAergic/glycinergic mPSC and sPSC frequency, spontaneous glutamatergic transmission frequency, spontaneous GABAergic/gly-cinergic transmission frequency ↓, evoked GABAergic/glycinerg-ic transmission failure rate ↑	Body weight ↓ died within 24 h after birth	[41]
NL1 KO	Social interaction ↓ spatial working memory ↓ Grooming behavior ↑, rescued by NMDAR coagonist DCS			**Whole brain**, NL3, synapsin-1a/b ↑, α-neurexin, β-neurexin, Munc, CSP and liprin↓		**CA1**, LTP magnitude & NMDAR/AMPAR ratio ↓ **Str, corticostriatal synapses**, NMDAR/AMPAR ratio ↓ **Thalamo-amygdala synapses**, NMDAR/AMPAR ratio & NMDAR-dependent EPSC ↓, abolished STD-LTP **Cortico-amygdala synapses**, NMDAR/AMPAR ratio & NMDAR-dependent EPSC ↓		[42, 44, 45]
NL1 Knockdown rat in lateral amygdala	Contextual- and cued-fear conditioning memory ↓ Nest building skills ↓		**AMG**, 50% infection efficiency	**AMG**, NL1 ↓		**Thalamo-amygdala synapses**, NMDAR/AMPAR ratio & NMDAR-dependent EPSC amplitude ↓, LTP abolished		[43]

(Continued)

NL2 KO	Anxiety-like behavior ↑ Pain sensitivity ↓ Motor coordination ↓ irregular breathing patterns	**CA1 and CA3,** vGAT density ↓ **CA1**, gephyrin and $GABA_AR$ γ2 density in str. Pyramidale ↓, gephyrin cytoplasmic aggregates ↑ **Retina**, $GABA_AR$α1 intensity, $GABA_AR$α3 cluster number and intensity, $GABA_AR$γ2 cluster number and intensity ↓ **GCL-DG**, gephyrin density, $GABA_AR$γ2 density, percentage of gephyrin colocalized with $GABA_AR$γ2 ↓	Cultured **HP neuron**, cytoplasmic aggregates of gephyrin ↑, postsynaptic gephyrin cluster at perisomatic synapses ↓	**Whole brain**, synaptobrevin2 ↑	**SSC**, IPSC amplitude ↓ **Thalamocortical FS interneuron-excitatory neuron synapses**, unitary IPSC amplitude and mIPSC amplitude ↓, quantal content (CV) ↑ **Thalamocortical excitatory neuron-FS interneuron synapses**, EPSC amplitude ↑ **VLM**, mPSC frequency and amplitude, GABA/glyciner-gicsIPSC and eIPSC frequency and amplitude, GABAergic and glycinergic sIPSC and mIPSC frequency and amplitude, slope of GABAergic and glycinergic mIPSC onset, postsynaptic response in GABAergic and glycinergic amplitude upon extracellualr agonist ejection ↓; failure rate in ePSC and GABA/glycinergic eIPSC↑ **HP**, GABAergic sIPSC and mIPSC frequencies, slope of GABAergic mIPSC onset ↓ **Retina**, basal activity ↑, amplitude of light response ↓ **DG**, population spike amplitude ↑, threshold frequency for induction of epileptiform discharges, GABAergic mIPSC amplitude ↓ **Computational model of the dentate gyrus circuitry**, PPI ↓, perisomatically targeting interneurons ability in preventing action potential generation in GCs ↓	Electroretinogram recordings, STA peak amplitude and lantency, biphasic STA Peak-to-peak amplitudes and inflection point latencies ↓	[26, 42, 47–50]
NL3 KO	Motor activity ↑ Social interaction ↓ Buried food-finding ability ↓ Ultrasonic vocalization ↓ Contextual- and cued-fear conditioning Memory ↓ Spatial working learning ↑			**Whole brain**, NL1 ↓	**CA**1, fEPSP linear slope & mIPSC frequency ↑, decreased mEPSC frequency ↓	MRI, total brain volume ↓	[51, 52, 54]

(Continued)

NL4 KO	Social interaction ↓ Ultrasonic vocalization ↓						MRI, total brain volume, cerebellum volume, brainstem volume ↓	[53]
NL1 TG	Spatial working memory acquisition and new memory formation ↓ Spatial reference memory formation ↓	**CA1**, GluA1, vGluT1 and vGAT puncta area ↑, GluR2/3 puncta area ↓		NL1, PSD95, VGluT, Gephyrin, VGAT, GluA1 ↑; GluA2/3 ↓	**CA1**, asymmetric synaptic contact length and curvature, PSD length, asymmetric synapse number, E/I synapse ratio ↑	**CA1**, STP & LTP ↓, EPSC amplitude ↑	Golgi Cox impregnation**, CA1**, mushroom-shaped spine head size and density ↑, spine neck length ↓	[56]
TgN1F (NL1 TG in FMR1$^{-/-}$ mice)	Rescue the social interaction defects in FMR1$^{-/-}$ mice	**CA1**, rescue excitatory synapse loss in FMR1$^{-/-}$ mice; reduce the increased inhibitory synapse number in FMR1$^{-/-}$	Rescue the spine size reduction in FMR1$^{-/-}$ mice **HP neurons**					[57]
NL2 TG	Have limb clasping behavior basal activity ↑ Startle response to a 90 dB click ↑ Anxiety-like behavior ↑ Social interaction ↓	**FC**, vGAT and vGluT intensity ↑, ratio of vGluT/vGAT intensity ↓		**Forebrain**, vGAT, vGluT and syntaxin↑, NL3 ↓	**mPFC**, vesicle number in reserve pool, symmetric and asymmetric presynaptic compartment area, symmetric synaptic contact length, total and symmetric synapse densities ↑, asymmetric/symmetric synapse ratio ↓	**PFC**, mIPSC frequency ↑	Lifespan ↓, died by 24 postnatal weeks failed to produce viable offspring Body weight ↓ Limb clasping, straub tail, transient episodes of kyphosis bilateral bursting activity in neck EMG	[55]

IHC: immunohistochemistry; ICC: immunocytochemistry; WB: Western blot; EM: electron microscopy; EP: electrophysiology; KI: knockin; KO: knockout; TG: transgenic; ↑: increase; ↓: decrease; str. rad.: stratums radiatum; NC: neocortex; SSC: somatosensory cortex; HP: hippocampus; MRI: magnetic resonance imaging; DTI: diffusion tensor imaging; FA: fractional anisotropy; RD: radial diffusivity; Lac. Mol.: lacunosummoleculare; PBC: pre-Bötzinger complex; IOM: inferior olive; Str: dorsal striatum; AMG: lateral amygdala; GCL: granule cell layer; DG: dentate gyrus; FS: fast-spiking; VLM: ventrolateral medulla; FC: frontal cortex; mPFC: medial prefrontal cortex.

mentioned mouse models from biochemical, electrophysiological, or behavioral approaches (Table 1). Behavioral abnormalities related with ASD, such as repetitive behavior, social interaction and vocalization, and learning and memory abilities, have been found in several mouse models [45, 51–56]. From the investigation of detailed mechanisms, abnormal neurotransmission from excitatory or inhibitory synapses was found in almost all models (Table 1). These synaptic functional changes could be due to altered receptor targeting and ion channel kinetics, the altered neuronal excitability or reduced essential synaptic components. Less or no significant effects on synapse density or synapse morphologies were observed, except for the neuroligin knockin mice [55, 56]. Those findings were quite different from the *in vitro* studies. Even from the same mouse model, different mice lines could show controversial results [51, 52, 59].

The disagreements between the reports may due to the following reasons: (1) the neuron and synapse development is different in the cultured system and intact animals. The critical developmental windows of synaptogenesis and synapse maturation could be irrelevant to each other. Spine saturation is another issue that could be accounted for in different systems. In animals, there are more complicated circuitry formation and experience-dependent synaptic modification existing, which bring many additional factors into consideration when investigation purely the synaptogenic effects of neuroligins. (2) The experimental duration is an issue. *In vitro* approaches monitor the immediate consequences of overexpression expression or acute knock down of neuroligins; while in the mouse models, what can be observed are always due to chronic effects. Therefore, the pure consequences of neuroligins could be buried by the compensatory effects. (3) The time windows of examination in mice differ in different research groups. Due to the respiratory abnormalities, the NL1-3 KO and some of the NL2 KO mice were examined early after birth or during postnatal days 2–4 [26, 41]. Others carried out experiments in mice ranging from 2–12 week-old mice or older adult mice. The juvenile mice or adult mice could show different synaptogenic effects and behave within different extents, which could show difference significance if analyzed with the same standard. (4) The brain region matters when doing research. Such as in the NL3 R451C mouse model, there are selective effects in synaptic transmissions detected from hippocampus and somatosensory cortex [52]. Due to the splicing form and distribution variances of different neuroligins, the effects on synapse formation and function could be region specific. (5) Experimental measurements and quantifications could differ between different research groups.

Nevertheless, there is growing evidence supporting the importance of neuroligins in neuron development and synapse function, and their associations with ASD and mental retardations. In addition, mutations in neuroligin binding partners have been implicated in the same diseases as well [69–72]. Those proteins could together form a network initializing the synapse function and circuitry formation.

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