Cooperation of Syd-1 with Neurexin synchronizes pre- with postsynaptic assembly

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Synapse formation and maturation requires bidirectional communication across the synaptic cleft. The trans-synaptic Neurexin-Neuroigin complex can bridge this cleft, and severe synapse assembly deficits are found in *Drosophila melanogaster* *neuroigin* (*Nlg1*, *dnlg1*) and *neurexin* (*Nrx-1*, *dnrx*) mutants. We show that the presynaptic active zone protein Syd-1 interacts with *Nrx-1* to control synapse formation at the *Drosophila* neuromuscular junction. Mutants in *Syd-1* (*RhoGAP100F*, *dsyd-1*), *Nrx-1* and *Nlg1* shared active zone cytomatrix defects, which were nonadditive. Syd-1 and *Nrx-1* formed a complex *in vivo*, and Syd-1 was important for synaptic clustering and immobilization of *Nrx-1*. Consequently, postsynaptic clustering of *Nlg1* was affected in *Syd-1* mutants, and *in vivo* glutamate receptor incorporation was changed in *Syd-1*, *Nrx-1* and *Nlg1* mutants. Stabilization of nascent Syd-1–Liprin- α (DLiprin- α) clusters, important to initialize active zone formation, was *Nlg1* dependent. Thus, cooperation between Syd-1 and *Nrx-1*–*Nlg1* seems to orchestrate early assembly processes between pre- and postsynaptic membranes, promoting avidity of newly forming synaptic scaffolds.

Understanding the process of synapse assembly in molecular and cell-biological detail is a prerequisite for understanding neural circuit development and activity-mediated remodeling, and thus is important for unraveling learning and memory processes (structural plasticity)^{1–3}. Functional chemical synapses are characterized by two apposed compartments that must be coestablished with high spatiotemporal precision: the presynaptic active zone, where regulated and rapid fusion of neurotransmitter-filled synaptic vesicles takes place, and the postsynaptic density (PSD), which embeds neurotransmitter receptors.

Glutamatergic neuromuscular junction (NMJ) terminals of *Drosophila* larvae grow to meet the requirements of the growing muscle fibers, a process in which new synapses are continuously added⁴ (where a synapse is defined as a single active zone opposed by a single PSD¹). *In vivo* imaging has shown that presynaptic Syd-1 and Liprin- α clusters initiate active zone formation⁵. On the postsynaptic side, initial PSD growth depends on incorporation of a glutamate receptor (GluR) containing the GluRIIA subunit. Later PSD maturation is marked by the incorporation of GluRIIB-containing receptor complexes⁶. Synapse assembly is concluded at presynaptic active zones by the incorporation of the active zone scaffold component Bruchpilot (BRP)⁵.

Coordinating synapse assembly requires signaling across the synaptic cleft, which separates pre- from postsynaptic membranes. Trans-synaptic cell adhesion molecules are obvious candidates for coupling active zone and PSD assembly. *In vitro*, the Neurexin-Neuroigin (Nrx-Nlg)

complex can mediate the trans-synaptic signaling required for synapse assembly^{7,8}. How this signaling axis integrates with the additional assembly machinery, however, has remained largely unclear.

Here, we provide evidence of a dual role for Syd-1 in early assembly of NMJ synapses. It retains Liprin- α clusters at active zones and is important for clustering of presynaptic *Nrx-1*, likely through a direct and PDZ-domain-dependent interaction. Consequently, Syd-1 is also needed for clustering of postsynaptic *Nlg1*, which organizes postsynaptic assembly. Coincident action of Syd-1 with *Nrx-1*–*Nlg1* appears to allow active zone scaffolds to pass through an initial, still fragile assembly phase. We suggest that binding between Syd-1 and *Nrx-1*–*Nlg1* is a means to coordinate pre- with postsynaptic assembly. Our study shows an example of how coincident action of a presynaptic active zone scaffold protein and a trans-synaptic cell adhesion protein module can spatiotemporally orchestrate synapse assembly.

RESULTS

Initially described in cell culture systems (for a review, see ref. 9), interaction between mammalian presynaptic *Nrx* proteins and postsynaptic *Nlg* molecules was proposed to be important for proper synapse assembly. However, genetic ablation of three *Nlg* (*Nlgn*) genes in mice¹⁰ does not result in a substantial structural phenotype, potentially reflecting a strong capacity for compensatory processes *in vivo*.

Nonetheless, severe deficits in the *in vivo* assembly of *Drosophila* NMJ synapses^{11–13} were reported for mutants of *Nrx-1* and *Nlg1* (for domain organization, see Fig. 1a). These findings provided an opportunity for

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Figure 1 Double mutants analysis of *Nrx-1*, *Nlg1* and *Syd-1*. **(a)** Domain structures. *Nrx-1* includes extracellular laminin G (LAM G) and epidermal growth factor (EGF)-3 domains. The short intracellular domain has a PDZ-binding motif. The extracellular part of *Nlg1* comprises an acetylcholinesterase-like (AChE) domain, while the cytoplasmic part has a PDZ-binding motif. The D356R point mutation (*) in the AChE-like domain interferes with *Nrx-1* binding. The cytoplasmic part is deleted in *Nlg1ΔCyto*

comprises a PDZ domain, a C2 domain and a putative RhoGAP domain. PDZ* indicates the *Syd-1* R165A L170A point mutations (*Syd-1*^{PDZ*}). **(b)** CD8-GFP-Sh expression in NMJs 1 and 9. Scale bar, 20 μ m. **(c)** Quantification of bouton numbers on NMJs 1 and 9 relative to muscle area. Control: 5.185 ± 0.171 boutons per $10^4 \mu\text{m}^2$, $n = 20$; UAS-*Nlg1ΔCyto*: 0.850 ± 0.205 , $n = 20$; UAS-*Nlg1ΔCyto*^{D356R}: 3.84 ± 0.221 , $n = 20$; *Nrx-1*: 3.225 ± 0.178 , $n = 20$; *Nrx-1*, UAS-*Nlg1ΔCyto*: 3.205 ± 0.209 , $n = 20$; *Liprin-α*: 3.935 ± 0.164 , $n = 20$; *Liprin-α*, UAS-*Nlg1ΔCyto*: 1.374 ± 0.269 , $n = 20$; *Syd-1*: 3.953 ± 0.199 , $n = 20$; *Syd-1*, UAS-*Nlg1ΔCyto*: 3.537 ± 0.21 , $n = 20$; *Nlg1*: 3.105 ± 0.170 , $n = 19$; *Syd-1*, *Nrx-1*: 3.505 ± 0.230 , $n = 20$; *Syd-1*, *Nlg1*: 3.200 ± 0.246 , $n = 18$. All single and double mutant data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison test (test statistic $K = 48.77$).

Each single mutant was compared to UAS-*Nlg1ΔCyto* expressed in the mutant background using the Mann-Whitney *U*-test: *Nrx-1* versus *Nrx-1*, UAS-*Nlg1ΔCyto* $P = 0.59$, $U = 179.5$; *Liprin-α* versus *Liprin-α*, UAS-*Nlg1ΔCyto* $P < 0.001$, $U = 15.0$; *Syd-1* versus *Syd-1*, UAS-*Nlg1ΔCyto* $P = 0.22$, $U = 138.0$. Control, UAS-*Nlg1ΔCyto* and UAS-*Nlg1ΔCyto*^{D356R} were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison test ($K = 44.73$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant ($P > 0.05$); all values are mean $\pm$ s.e.m.

an efficient study of how this trans-synaptic signaling complex integrates into synapse assembly in a genetically tractable *in vivo* model.

We started by searching for additional factors involved in the *Nlg1*-instructed role of *Nrx-1*. Previously, we found that muscle (*Mef2-Gal4*-driven) expression of GFP-tagged *Nlg1* lacking its C terminus (UAS-*Nlg1ΔCyto*^{GFP} (ref. 12), **Fig. 1a**) results in marked under-growth of NMJs (**Fig. 1b,c**). Therefore, postsynaptic *Nlg1ΔCyto* has a dominant negative effect on the addition of presynaptic boutons (and thus active zones) during terminal expansion. Although the mechanism underlying this phenotype is still elusive, we noted that introducing a point mutation interfering with Neurexin binding¹⁴ (UAS-*Nlg1ΔCyto*^{D356R}, **Fig. 1a**) largely suppressed the dominant negative effect of *Nlg1ΔCyto* (**Fig. 1c**). Likewise, expression of *Nlg1ΔCyto* in the *Nrx-1* mutant background did not lead to the severe under-growth *Nlg1ΔCyto* caused in the control background. Instead, NMJ structures and bouton numbers were indistinguishable from those of *Nrx-1* mutants (**Fig. 1b,c**). Thus, as the *Nlg1ΔCyto* phenotype depends on *Nrx-1*, testing for suppression of *Nlg1ΔCyto* might be a way to identify additional presynaptic factors implicated in *Nrx-1* signaling.

In both *Drosophila* and *Caenorhabditis elegans*, presynaptic *Syd-1* (for domain organization, see **Fig. 1a**) and *Liprin-α* (*Syd-2*) promote presynaptic assembly^{5,15–18}. When we expressed *Nlg1ΔCyto* in a *Liprin-α* mutant background, NMJ terminals still showed severe under-growth compared to those in the *Liprin-α* mutant background alone (**Fig. 1b,c**). In contrast, the *Syd-1* mutant background fully suppressed the *Nlg1ΔCyto*-induced NMJ under-growth (**Fig. 1b,c**), implying that *Syd-1* and *Nlg1* may operate in the same pathway.

Similar synaptic phenotypes of *Syd-1*, *Nrx-1* and *Nlg1*

We previously showed that *Nrx-1* and *Nlg1* behave nonadditively: the reduced NMJ bouton numbers in *Nlg1* mutants are not further diminished in a *Nlg1*, *Nrx-1* double mutant¹². To test whether *Syd-1* genetically interacts with *Nrx-1* or *Nlg1*, we constructed the respective double mutants. NMJ size is reduced in *Nlg1*, *Nrx-1* and *Syd-1* single mutants, as well as in *Liprin-α* single mutants^{5,11,12,18}. As expected, all these single mutants showed significantly reduced bouton numbers as compared to controls (**Fig. 1b,c**). Both *Syd-1*, *Nrx-1* and *Syd-1*, *Nlg1* double mutants reached third instar larval stage, allowing bouton number analysis at mature NMJs (whereas *Syd-1*, *Liprin-α* double mutants die as embryos⁵). Combination of *Nrx-1* or *Nlg1* with the *Syd-1* mutant did not reduce bouton numbers further than those of the individual single mutants (**Fig. 1b,c**).

Apart from reduced NMJ size, all three mutants (*Nlg1*, *Nrx-1* and *Syd-1*) show defects in the organization of the remaining active zones^{5,11,12}. We compared their active zone organization by using stimulated emission depletion (STED¹⁹) microscopy at ~80 nm resolution (instead of the ~250 nm of a standard confocal microscope) and electron microscopy. In electron micrographs of controls, active zone dense bodies (T-bars) were properly shaped (**Fig. 2a**). In contrast, an aberrant, star-shaped T-bar morphology was regularly observed at the remaining active zones of *Syd-1* (compare ref. 5), *Nrx-1* and *Nlg1* mutants, but never at control active zones (**Fig. 2a–f**).

The protein BRP is a molecular building block of the electron-dense T-bar^{5,20}. At control NMJs, diffraction-limited BRP spots mark individual active zones²¹ that appear as ring-shaped structures when imaged at higher resolutions with STED^{20,22} (**Fig. 2g,h**). We stained

Figure 2 Comparison of active zone ultrastructure for *Nrx-1*, *Nlg1* and *Syd-1* mutants. (a-f) Electron microscopy of presynaptic electron-dense projections (T-bars). (a) A normal presynaptic electron-dense projection (T-bar) of a control bouton. (c) Serial reconstruction of the T-bar shown in a. T-bars of *Syd-1* (b), *Nrx-1* (d), and *Nlg1* (e) mutant active zones often show star-like shapes. Arrowheads indicate T-bars. (f) Serial reconstruction of the *Nlg1* mutant T-bar shown in e. Scale bar, 100 nm. (g,h) Examples of confocal (g) and STED (h) images of BRP spots at a control NMJ (muscle 4). (i-n) BRP rings are irregular and interconnected (arrowheads) in *Nrx-1* (i), *Nlg1* (j) and *Syd-1* (k) single mutants and *Syd-1,Nlg1* (l), *Nrx-1,Nlg1* (m) and *Syd-1,Nrx-1* (n) double mutants. STED images were deconvolved with a Lorentz point spread function of 80 nm using a Wiener filter (0.05 regulation parameter). Scale bar, 500 nm. (o) Quantification of BRP ring diameter. Control: $0.233 \pm 0.012 \mu\text{m}$, $n = 34$; *Nlg1*: 0.305 ± 0.015 , $n = 43$; *Syd-1*: 0.301 ± 0.011 , $n = 47$; *Nrx-1*: 0.322 ± 0.013 , $n = 54$; *Nrx-1,Nlg1*: 0.315 ± 0.013 , $n = 52$; *Syd-1,Nlg1*: 0.321 ± 0.013 , $n = 46$; *Syd-1,Nrx-1*: 0.328 ± 0.017 , $n = 43$. Kruskal-Wallis test with Dunn's multiple comparison test ($K = 29.2$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant; all values are mean $\pm$ s.e.m.

Nrx-1 (Fig. 2i) and *Nlg1* (Fig. 2j) mutants for BRP and imaged active zones using STED. BRP rings were frequently interconnected and over-grown (Fig. 2i,j). We had previously observed this phenotype in STED images of *Syd-1* mutants⁵ (Fig. 2k). In fact, BRP ring diameters were increased in all *Syd-1* (compare ref. 5), *Nrx-1* and *Nlg1* single mutants, which were statistically indistinguishable from each other and from the double mutants (Fig. 2i-o).

Syd-1 is needed to cluster *Nrx-1* and *Nlg1* at NMJ synapses

Endogenous *Nrx-1* forms distinct clusters at the presynaptic terminal (Fig. 3a)^{11,12}. We performed immunostaining to investigate whether *Syd-1* could influence the distribution of *Nrx-1*. As with *Nlg1* mutants (Fig. 3a,b), *Nrx-1* intensity was significantly reduced in *Syd-1* mutants (Fig. 3a,b).

Nlg1 normally clusters opposite presynaptic *Nrx-1*. These clusters are no longer detectable in *Nrx-1* mutants (compare ref. 12). Consistent with *Syd-1* organizing *Nrx-1* clustering, *Nlg1* clusters also dropped below the detection limit in *Syd-1* mutants (Fig. 3c,d).

Both *Syd-1* and *Nrx-1* mutant terminals show reduced glutamate release^{5,11}. Thus, lack of *Nlg1* clustering could be a mere consequence of reduced synaptic activity. To test this, we quantified *Nlg1* intensity (as a measure of *Nlg1* clustering) in mutants for presynaptic *brp* (*brp*^{1.3})²⁰. The *brp* mutant larvae show a stronger decrease in presynaptic neurotransmitter release than any of the mutants discussed above^{5,11,20,22,23}. *Nlg1* clusters, however, were not reduced in *brp* mutants (Fig. 3c,d), indicating that the observed reduction of *Nlg1* in *Syd-1* and *Nrx-1* mutants was neither due to reduced transmission nor a reflection of a general active zone assembly deficit (which is very pronounced in *brp* mutants²²). Instead, *Syd-1* seems to be specifically involved in clustering postsynaptic *Nlg1* adjacent to synapses.

The finding that the cytoplasmic scaffold protein *Syd-1* is needed for both *Nrx-1* and *Nlg1* clustering is consistent with *Nrx-1* directly

organizing the synaptic clustering of *Nlg1*. We also found that muscle-specific *Nlg1* expression markedly increased presynaptic *Nrx-1* clustering in the control background (Supplementary Fig. 1a) and that there was a trend for motor neuron-driven UAS-*Nrx-1* to increase postsynaptic *Nlg1* (Fig. 3e,f). We reasoned that if *Nrx-1* availability was rate limiting for *Nlg1* clustering in *Syd-1* mutants, overexpression of *Nrx-1* in *Syd-1* mutants might allow restoration of postsynaptic *Nlg1* clustering. Indeed, *Nlg1* clustering was partially restored when we overexpressed *Nrx-1* in the *Syd-1* mutant background (Fig. 3e,f). Of note, immunostainings for endogenous *Syd-1* showed that the protein was still detectable at both *Nrx-1* and *Nlg1* mutant NMJs (Supplementary Fig. 2). Taken together, these results show that *Syd-1* is needed to effectively cluster *Nrx-1*, which likely affects the clustering of postsynaptic *Nlg1*.

Syd-1 and *Nrx-1* form an *in vivo* complex

These findings motivated us to analyze whether *Syd-1* and *Nrx-1* might be part of a common complex. We performed immunoprecipitations from an active zone protein-enriched preparation, derived from *Drosophila* head extracts, using antibodies recognizing *Nrx-1* (ref. 24). There was robust coimmunoprecipitation of *Syd-1* with *Nrx-1*, whereas specificity controls were negative (Fig. 4a).

We next sought the molecular link joining *Syd-1* and *Nrx-1*. *Syd-1* comprises a PDZ domain, and *Nrx-1* has a PDZ-binding motif (Fig. 1a). A large-scale *C. elegans* yeast two-hybrid screen recently identified a direct interaction of the *Syd-1* PDZ domain with *C. elegans* *Nrx* *in vitro*²⁵. We found the same interaction using the corresponding stretches of the fly proteins (Supplementary Fig. 3a). We introduced two point mutations (*Syd-1* R165A L170A (*Syd-1*^{PDZ*}); compare ref. 26) into the *Syd-1* cDNA to destroy the PDZ domain's ligand-binding capability. When tested in a yeast two-hybrid assay, binding of the *Nrx-1* C terminal to the mutated *Syd-1* PDZ domain was greatly reduced as compared to its binding to the unmutated *Syd-1* PDZ domain (Supplementary Fig. 3a).

Expressing *Nrx-1*^{GFP} (Fig. 4b) and *Syd-1* tagged with the fluorescent protein mStrawberry (*mStrawSyd-1*) (Fig. 4c) in larval salivary gland cells, we tested this interaction *in vivo*. As expected, when expressed by itself, the transmembrane protein *Nrx-1* labeled the plasma membrane (Fig. 4b). In contrast, *Syd-1* or *Syd-1*^{PDZ*} expressed on its own had an intracellular distribution (Fig. 4c,d). When expressed with *Nrx-1*, however, *Syd-1* was clearly recruited to the plasma membrane (Fig. 4e), whereas *Syd-1*^{PDZ*} was not (Fig. 4f). In the reverse experiment, *Nrx-1* lacking its PDZ-binding motif (UAS-*Nrx-1*^{PDZ*,no-tag}; ref. 24) failed to

Figure 3 Reduced endogenous Nr x -1 and Nlg1 clusters at *Syd*-1 mutant NMJs. (a) Muscle 4 boutons of control, *Nlg1*, *Syd*-1 and *Nrx*-1 larvae immunostained for Nr x -1 and BRP. (b) Quantification of Nr x -1 signal intensities from NMJs on muscle 4, normalized to control. Residual background (mean intensity of *Nrx*-1 mutants) was subtracted for all genotypes and data sets were then normalized to control. Control: 100 ± 12.40 , $n = 21$; *Syd*-1: 29.44 ± 6.58 , $n = 19$; *Nlg1*: 34.46 ± 5.06 , $n = 18$; *Nrx*-1: 0.00 ± 1.43 , $n = 18$; control versus *Syd*-1 $P < 0.001$; control versus *Nlg1* $P < 0.01$; control versus *Nrx*-1 $P < 0.001$; *Syd*-1 versus *Nlg1* $P > 0.05$; Kruskal-Wallis test with Dunn's multiple comparison test ($K = 52.6$). (c) Muscle 4 boutons of control, *Nlg1*, *Syd*-1, and *brp* larvae immunostained for Nlg1 and BRP. (d) Quantification of Nlg1 signal intensity from NMJs on muscle 4. Residual background (mean intensity of *Nlg1* mutants) was subtracted for all genotypes (hence the negative values for *Syd*-1 mutants) and data sets were then normalized to control. Control: 100 ± 14.61 , $n = 14$; *Syd*-1: -20.8 ± 7.34 , $n = 9$; *Nlg1*: 0.0 ± 19.36 , $n = 8$; *brp*: 90.1 ± 38.95 , $n = 7$; control versus *Syd*-1 $P < 0.001$; control versus *Nlg1* $P < 0.01$; control versus *brp* $P > 0.05$; Kruskal-Wallis test with Dunn's multiple comparison test ($K = 22.4$). (e) Muscle 4 boutons of control larva and of control and *Syd*-1 larvae with UAS-Nr x -1^{GFP} driven in motor neurons. (f) Quantification of Nlg1 signal intensities normalized to control. Control: 100 ± 10.40 , $n = 12$; Nr x -1^{GFP}: 195.0 ± 45.59 , $n = 13$; *Syd*-1: 8.505 ± 1.390 , $n = 13$; Nr x -1^{GFP}, *Syd*-1: 36.43 ± 4.202 , $n = 12$; control versus Nr x -1^{GFP} $P > 0.05$; Mann-Whitney U -test ($U = 55.0$); *Syd*-1 versus Nr x -1^{GFP}, *Syd*-1 $P < 0.001$; Mann-Whitney U -test ($U = 0.0$). NS, not significant; ** $P < 0.01$, *** $P < 0.001$; all values are mean $\pm$ s.e.m. Scale bars, 2 μ m.

recruit mStraw^{Syd}-1, whereas the control, UAS-Nr x -1^{no-tag} (ref. 24), effectively recruited mStraw^{Syd}-1 to the plasma membrane (Supplementary Fig. 3b). Thus, Nr x -1 and *Syd*-1 can be found in a common complex *in vivo*, with their binding apparently mediated through a direct PDZ-domain interaction.

Syd-1 recruits Nr x -1 to active zones

We next performed *in vivo* imaging experiments⁶ (Fig. 5a) at NMJs of developing intact larvae. As expected⁵, motor neuron-driven (*ok6-Gal4*) UAS-mStraw^{Syd}-1 invariably marked active zones. UAS-Nr x -1^{GFP}, when expressed alone, however, distributed more diffusely over the presynaptic terminal membrane (Fig. 5b) and was only slightly enriched close to the active zones marked by BRP (data not shown). When we expressed both proteins under these conditions, Nr x -1^{GFP} became densely enriched in the *Syd*-1-positive active zones (compare Fig. 5b and Fig. 5c). Thus, overexpressed *Syd*-1 can direct exogenous Nr x -1 to active zones. Next we expressed UAS-*Syd*-1^{PDZ*}. UAS-*Syd*-1^{PDZ*} still localized to active zones to a fair extent (Fig. 5d), but coexpressed Nr x -1 appeared diffuse (Fig. 5d), with a distribution indistinguishable from that in the absence of *Syd*-1 expression (Fig. 5b). Thus,

Syd-1^{PDZ*} failed to recruit Nr x -1 to active zones. Liprin- α overexpression was not sufficient to trigger active-zone enrichment of Nr x -1^{GFP} (Supplementary Fig. 4a).

We also noted that endogenous postsynaptic Nlg1 clustering was reduced at terminals that presynaptically overexpressed UAS-*Syd*-1^{PDZ*} (Supplementary Fig. 1b). *Syd*-1^{PDZ*} likely competes with endogenous *Syd*-1 at active zones, impairing Nr x -1-*Syd*-1 interactions and, thereby, Nlg1 clustering.

We next used STED to investigate Nr x -1 localization relative to the active zone core marker BRP. At ~80 nm resolution, *Syd*-1 puncta surrounded the BRP core⁵. A good fraction of Nr x -1^{GFP} puncta also surrounded the BRP core when expressed with *Syd*-1. This active zone-associated distribution (Fig. 5e) was no longer apparent when we expressed Nr x -1 with *Syd*-1^{PDZ*} (Fig. 5f); in this case, Nr x -1 puncta not associated with the active zone dominated instead.

Figure 4 Biochemical and cell biological analysis of *Syd*-1-Nr x -1 complex. (a) Immunoblot of Nr x -1 immunoprecipitate (IP) from a *Drosophila* active zone protein-enriched preparation. *Syd*-1 coprecipitates with Nr x -1 but is not detected in the random mouse immunoglobulin G (IgG) control. For full blots, see Supplementary Figure 10. (b) UAS-Nr x -1^{GFP} localizes to the cell membrane of salivary gland cells, whereas (c,d) either UAS-mStraw^{Syd}-1 or UAS-mStraw^{Syd}-1^{PDZ*} expressed alone distributes diffusely throughout the cytoplasm. (e) Coexpression of UAS-mStraw^{Syd}-1 and UAS-Nr x -1^{GFP} directs UAS-mStraw^{Syd}-1 to the plasma membrane. (f) In contrast, localization of UAS-mStraw^{Syd}-1^{PDZ*} expressed with UAS-Nr x -1^{GFP} remains diffuse. Scale bar, 25 μ m.

Syd-1 retains Liprin- α and Nr1 at synapses

It is conceivable that the synapse assembly process may demand continuous trafficking and remodeling. Notably, in the hierarchy of active zone assembly, genetic analysis in *C. elegans* has placed Syd-1 upstream of Syd-2 (Liprin- α), a protein linked to long-range transport of vesicular axonal cargo^{27,28}.

We previously found that Liprin- α localization was severely disturbed in *Syd-1* mutants and proposed that Syd-1 might anchor clusters of Liprin- α ⁵. To address this, we imaged Liprin- α clusters of control and *Syd-1* NMJs *in vivo* in 30-min increments (Supplementary Fig. 5a) and scored mobile spots (comparing images taken at 0 and

60 min; see Online Methods). Numbers of mobile Liprin- α spots were elevated in the *Syd-1* mutant background (Supplementary Fig. 5b). Notably, this interaction was not reciprocal, as Syd-1 mobility appeared unchanged in *Liprin- α* mutants (data not shown).

Does Syd-1 retain Nr1 in a similar manner? Owing to the diffuse nature of overexpressed Nr1^{GFP}, tracking of individual clusters was problematic here. Instead, we performed fluorescence recovery after photobleaching (FRAP) experiments at Nr1^{GFP}-expressing synaptic terminals and measured recovery of the diffuse GFP signal (Fig. 5g,h). We compared NMJs expressing Nr1^{GFP} in *Syd-1* mutants; in a rescue experiment, with Syd-1 reexpressed in the *Syd-1*

mutant background; and with Syd-1^{PDZ*} re-expressed in the *Syd-1* mutant background (Fig. 5g,h). The *Syd-1* mutants showed higher Nr1^{GFP} mobility than rescue controls. Notably, UAS-Syd-1^{PDZ*} expression in the *Syd-1* mutant background also

Figure 6 *In vivo* imaging of Liprin- α -Syd-1 cluster dynamics at *Nlg1* mutant NMJs. (a) *In vivo*-imaged NMJ boutons from a UAS-GFP^{Syd-1} and UAS-Liprin- α ^{mStraw}-expressing larva (muscle 26/27). Junctions were reimaged after 30 and 60 min. (b) A new Liprin- α (red, arrowheads) and Syd-1 (green, arrowheads)-positive site emerging at 60 min (as expected⁵). (c) A nascent site disassembling again; arrowheads and colors in c,e,f as in b. (d) *In vivo*-imaged NMJ boutons from a UAS-GFP^{Syd-1} and UAS-Liprin- α ^{mStraw}-expressing *Nlg1* mutant. (e,f) New Liprin- α - and Syd-1-positive sites assembling (e), disassembling (f) and moving. Scale bar in a,d, 5 μ m. Scale bar in b,c,e,f, 1 μ m.

Figure 7 Analysis of *in vivo* glutamate receptor incorporation in *Nlg1*, *Nrx-1* and *Syd-1* mutants. (a) Time-lapse (24 h) of growing NMJs at muscles 26/27 for the determination of GluR content of newly forming PSDs. At control NMJs, most of the young PSDs (arrowheads) are rich in GluRIIA, with a few exceptions (asterisks). Young PSDs in *Nrx-1*, *Syd-1* and *Nlg1* mutants (genotypes indicated in black) show an inverse trend in GluRIIA-GluRIIB composition (arrowheads). (b) The GluRIIB/IIA composition⁶ in newly formed PSDs is significantly shifted toward GluRIIB, in comparison to control PSDs, for all *Nrx-1*, *Syd-1* and *Nlg1* mutants. Control: -0.283 ± 0.038 , $n = 23$; *Nrx-1*: -0.019 ± 0.038 , $n = 20$; *Syd-1*: 0.064 ± 0.035 , $n = 16$; *Nlg1*: 0.114 ± 0.026 , $n = 30$; control versus *Nrx-1* $P < 0.01$; control versus *Syd-1* $P < 0.001$; control versus *Nlg1* $P < 0.001$; Kruskal-Wallis test with Dunn's multiple comparison test ($K = 39.1$); $**P < 0.01$, $***P < 0.001$, all values are mean $\pm$ s.e.m. (c) Subsynaptic localization of GluRIIA- and GluRIIB-type glutamate receptor subunits at individual PSDs on muscle 4 of larvae (fixed preparation). GluRIIB localizes to the edge of a GluRIIA-rich core in most control PSDs. For *Nrx-1*, *Syd-1* and *Nlg1* mutant NMJs, examples are shown in which this distinct localization is reversed. Scale bars, 5 μ m.

resulted in a very high recovery of bleached *Nrx-1*^{GFP} signal. In contrast, *Nrx-1*^{GFP} recovery appeared unchanged in both *Liprin-α* mutants and a *Liprin-α*-overexpressing background (Supplementary Fig. 4b). These findings suggest that *Syd-1*, but not *Liprin-α*, is able to retain *Nrx-1*.

During these live imaging experiments, we observed that *Nrx-1*^{GFP} levels were reduced in *Syd-1* mutants, as compared to those in the control background. In the reverse situation, we observed a trend toward increased *Nrx-1* when overexpressing *Syd-1* in the *Syd-1* mutant background (Supplementary Fig. 6). Thus, it appears likely that complex formation with *Syd-1* not only retains but also stabilizes *Nrx-1*.

Collectively, these data suggest that, as expected, *Syd-1* promotes the anchoring of *Liprin-α* clusters. *Syd-1* also actively recruits and keeps *Nrx-1* levels high in active zone-proximal compartments.

Evidence for a spatially retrograde function of *Nlg1*

In vivo NMJ synapse assembly is a protracted process of roughly 10 hours at 25 °C and finally reaches a mature steady state. *Liprin-α* and *Syd-1* mark sites of synapse formation first. Postsynaptic GluRIIA follows next and finally BRP is incorporated⁵. To readdress this early assembly process, we imaged *Syd-1* and *Liprin-α* at 30-min intervals in living *Drosophila* larvae (Fig. 6a). As expected⁵, *Liprin-α* and *Syd-1* were tightly clustered (Fig. 6b). We noticed, however, that such *Liprin-α*-*Syd-1* clusters often dissolved again (Fig. 6c). In contrast, such disassembly events were hardly ever observed for GluRIIA (ref. 6; data not shown). Apparently not all early *Liprin-α*-*Syd-1* clusters entered the irreversible assembly phase marked by GluRIIA accumulation.

To address the influence of postsynaptic *Nlg1* on presynaptic assembly, we imaged *Liprin-α*-*Syd-1* clusters in *Nlg1* mutants (Fig. 6d). Notably, the distribution of *Syd-1* (Supplementary Fig. 7) and *Liprin-α* (data not shown) seemed to rapidly change over time at individual *Nlg1* mutant active zones. Likewise, *Liprin-α*-*Syd-1* clusters appeared highly mobile (Fig. 6e,f). We scored all mobile clusters,

evaluating the image for each fluorophore (representing *Syd-1* and *Liprin-α*) separately as well as the two together. The overall mobility of each of these—*Syd-1* spots, *Liprin-α* spots and *Syd-1*-*Liprin-α* clusters—was significantly elevated in *Nlg1* mutants over 60 min (Mann-Whitney *U*-tests: for *Syd-1* spots, control: 0.079 ± 0.006 mobile spots per μ m, mean $\pm$ s.e.m., $n = 8$; *Nlg1*: 0.176 ± 0.013 , $n = 8$; control versus *Nlg1* $P < 0.001$, $U = 0.0$; for *Liprin-α* spots, control: 0.080 ± 0.007 , $n = 8$; *Nlg1*: 0.189 ± 0.021 , $n = 8$; control versus *Nlg1* $P < 0.001$, $U = 1.0$; for *Liprin-α*-*Syd-1* clusters, control: 0.052 ± 0.009 , $n = 8$; *Nlg1*: 0.155 ± 0.017 , $n = 8$, control versus *Nlg1* $P < 0.001$, $U = 1.0$) Consistent with *Nrx-1* serving as a hub for *Nlg1* and *Syd-1*, we also found a tendency toward increased mobility of *Liprin-α* clusters in *Nrx-1* mutants (Supplementary Fig. 5).

Together, these data suggest that *Nlg1* is important for *Syd-1*-*Liprin-α* cluster stabilization in a spatially retrograde fashion. Lack of postsynaptic *Nlg1* seems to destabilize the corresponding presynaptic complex. Most likely this is due to deficits in the trans-synaptic promotion of *Nrx-1* clustering in *Nlg1* mutants (Fig. 3c,d), which ultimately leads to aberrant presynaptic active zones (Fig. 2).

GluR incorporation defects in *Nrx-1*, *Nlg1* and *Syd-1* mutants

Drosophila NMJ PSDs incorporate glutamate receptors containing either the GluRIIA or the GluRIIB subunit. Normally, most immature, young PSDs form by preferentially incorporating the GluRIIA complex, later followed by GluRIIB during subsequent PSD maturation⁶. Would presynaptic *Nrx-1* and *Syd-1* also influence postsynaptic assembly?

We addressed postsynaptic assembly at *Syd-1*, *Nrx-1* and *Nlg1* single mutant NMJs (Fig. 7a) by coexpressing fluorescently tagged

GluRIIA and GluRIIB from genomic constructs^{6,29}. Identified NMJs were reimaged after 24 h and PSDs were tracked over time in three dimensions. This method allowed us to identify newly formed PSDs. As expected, new PSDs in controls were predominantly rich in GluRIIA (**Fig. 7a,b**). By contrast, in *Syd-1*, *Nrx-1* and *Nlg1* single mutants, new PSDs were rich in GluRIIB (**Fig. 7a,b**). The early incorporation of GluRIIA, but not GluRIIB, is a rate-limiting driving force for PSD assembly and thus for synapse formation at growing NMJs^{6,29,30}. This deficit in early GluRIIA incorporation might contribute to fewer synapses forming in these mutants (**Fig. 1c,d**).

GluRIIA incorporation appears to be nearly irreversible⁶. As a result, PSDs often show a GluRIIA-rich core surrounded by a GluRIIB-rich edge (**Fig. 7c**). Notably, this concentric arrangement was regularly inverted in all three—*Nlg1*, *Nrx-1* and *Syd-1*—mutants (**Fig. 7c**), reflecting GluRIIB-rich PSDs that eventually incorporate GluRIIA (**Supplementary Fig. 8a**). As this suggests that a lack of *Nlg1* is ultimately responsible for this inversion, we tested whether receptor organization in *Nrx-1* mutants would profit from *Nlg1* overexpression. Indeed, the receptor distribution largely normalized after *Nlg1* overexpression (data not shown), indicating that lack of *Nlg1* was in fact responsible for the PSD assembly deficits of *Nrx-1* mutants (and by extension also for those in *Syd-1* mutants). Of note, a fraction of PSDs ultimately over-grew in all three mutants (**Supplementary Fig. 8b,c**).

In summary, *Nlg1*, *Nrx-1* and *Syd-1* mutants share a specific deficit in the early, PSD growth-promoting GluRIIA incorporation, and are likely directly responsible for this specific assembly deficit in the subcellular PSD distribution of GluR subunits. In our model, *Syd-1* supports the aggregation of *Nrx-1*, which in turn clusters postsynaptic *Nlg1*, coupling pre- to postsynaptic assembly (**Supplementary Fig. 9**).

DISCUSSION

The *Nrx* and *Nlg* families include autism susceptibility genes^{31,32}, and their proteins are needed for proper synapse formation during circuit development. It has so far, however, remained largely unclear how they molecularly integrate into the synapse formation process, particularly in regard to the assembly of the presynaptic active zone scaffold. Thus, identifying proteins coupling *Nrx*-*Nlg* to the assembly process itself and defining where in the sequence of events *Nrx*-*Nlg* acts is critical for a deeper understanding of synapse formation and remodeling.

Independent work in model organisms has identified and characterized proteins guiding active zone assembly, with *Syd-1* proteins functioning upstream of *Syd-2* (Liprin- α)^{5,15,16}. *In vivo* imaging demonstrated that both *Syd-1* and Liprin- α accumulate very early during synapse assembly (**Supplementary Fig. 9**)—earlier than postsynaptic GluRs, and much earlier than presynaptic BRP⁵. *In vivo* FRAP analysis now suggests that *Syd-1* increases the dwell time of *Nrx-1* near active zones and can actively recruit *Nrx-1* in a PDZ-dependent manner (**Supplementary Fig. 9**). Likewise, Liprin- α cluster mobility was elevated in the *Syd-1* mutant background, implying a retention function of *Syd-1* for both *Nrx-1* and Liprin- α at assembling active zones. This study also suggests that the assembly of initially forming *Syd-1* and Liprin- α scaffolds is reversible (**Supplementary Fig. 9**). The success rate of establishing stable *Syd-1* and Liprin- α scaffolds dropped in the absence of *Nlg1* (**Fig. 6d–f**). As postsynaptic overexpression of *Nlg1* increased the expression of presynaptic *Nrx-1* (**Supplementary Fig. 1a**), interaction of these initial active zone scaffolds is likely to be directly dependent on local *Nrx-1* interacting with *Syd-1*. It is tempting to speculate that the *Nrx-1*-*Syd-1* interaction provides binding sites at newly forming active zones to drive the accumulation of Liprin- α scaffolds^{33,34} past a critical point, to enter

an essentially irreversible maturation process (characterized by the onset of GluRIIA incorporation). Such a cooperative scheme might be optimized for the integration of regulatory elements and protect the system from untimely and aberrant assembly. In fact, the active zone component BRP has been shown to be under constitutive phosphorylation to avoid premature assembly^{35,36}.

We found here that mutants for *Nlg1* and *Nrx-1* showed aberrant active zone organization reflected in over-grown (star-shaped) T-bars (**Fig. 2**). We previously observed these phenotypes in *Syd-1* mutants⁵. All three mutants (*Syd-1*, *Nrx-1* and *Nlg1*) assemble fewer active zones per NMJ^{5,11,12}. Consequently, levels of unused active zone scaffold components, such as BRP, might locally accumulate along their NMJ terminals. This increase in building blocks in turn might result in over-growth of the remaining active zone scaffolds. Additionally, *Syd-1*, *Nrx-1* and *Nlg1* might define an assembly sequence, which in turn could be a precondition to properly terminating assembly. One might speculate that an improperly assembled scaffold could retain free valences and that the scaffold could outgrow improperly. As active zone localization of *Syd-1* clustering did not strongly depend on either *Nrx-1* or *Nlg1*, we suspect that a complex of *Syd-1* with *Nrx-1* might be important for the regulation of BRP incorporation. Potentially, binding to *Nrx-1* (in a trans-synaptic complex with *Nlg1*) might unmask additional domains of *Syd-1* for assembly and thereby allow the effective stabilization of Liprin- α scaffolds. Notably, mammalian *Nlg1* has also been implicated in induction and maturation of the presynaptic terminal^{37,38}.

Nrx-1 (ref. 11) and *Syd-1* (ref. 5) are both expressed throughout the CNS, whereas *Nlg1* is not¹². It is likely that other *Drosophila* *Nlgs* (see ref. 12) substitute for *Nlg1* at central synapses. Of note, we found star-shaped T-bars at adult CNS synapses of *Syd-1* mutants as well (data not shown), suggesting that similar mechanisms as here described for NMJ synapses apply to CNS synapses.

Although *Syd-1* remains cytoplasmic and depends on the presence of *Nrx-1* to localize to the plasma membrane in non-neural cells (salivary gland epithelial cells; **Fig. 4b–f**), *Syd-1* can also localize to active zones in the absence of *Nrx-1*. Consistently, *Syd-1* mutated in its PDZ-domain (*Gal4-UAS* expressed) still localized to active zones, at least to a fair extent. Thus, nascent active zones seemingly contain additional proteins providing binding sites for *Syd-1* (that also may be needed to stabilize a complex of *Syd-1* and *Nrx-1*). Binding sites are still present after deletion of either Liprin- α or BRP—despite a direct interaction of *Syd-1* with BRP⁵. Additional proteins representing potential upstream functions, such as the adaptor protein Neurabin that was shown to recruit *C. elegans* *Syd-1* and *Syd-2* to F-actin foci³⁹, are prime candidates for the localization of *Syd-1*.

Unlike those of endogenous *Syd-1*, levels of *Gal4-UAS*-expressed *Syd-1* depended on the presence of *Nrx-1* (**Supplementary Fig. 6**). Thus, uncomplexed, excessive *Syd-1* might be subjected to degradation. Of note, Liprin- α is a downstream effector and possible substrate of the E3 ubiquitin ligase APC/C (ref. 40).

We found that early and rapid GluRIIA-mediated growth of nascent PSDs (younger than 24 h) was selectively impaired in *Syd-1*, *Nrx-1* and *Nlg1* mutants, where young PSDs are characterized by a high GluRIIB content. Mutants for *GluRIIA*, but not for *GluRIIB*, fail to grow sufficient synapses per terminal when challenged by high-temperature rearing⁴¹. In addition, terminals of *Syd-1*, *Nrx-1* and *Nlg1* all suffer from under-growth of synaptic terminals^{5,11,12}. Thus, this under-growth might partially be a consequence of reduced initial GluRIIA incorporation. However, this leaves the question of how *Nlg1* dictates GluRIIA incorporation. *Nlg1* clusters, functionally associated with proteins regulating initial synapse assembly, might selectively promote

GluRIIA incorporation directly (**Supplementary Fig. 9**). Notably, Nrx-Nlg complexes have been associated with GluR subunit-specific recruitment into PSDs in mammals. In that system, overexpression of Nlg1 selectively decreases the surface mobility of GluA2-containing AMPA-type glutamate receptors, in a manner mediated by a PSD95-Nlg1 interaction, while having no effect on GluA1 homomers^{42,43}. Indeed, Nlg1 is able to recruit the PSD95 ortholog Discs large (Dlg) to the *Drosophila* NMJ¹². Nlg1 clustering instructed by Syd-1 and Nrx-1 might create a seed for GluRIIA clustering mediated by Dlg and other scaffold proteins. It should be noted, however, that GluRIIA receptors still incorporate at Nlg1-mutant PSDs, although at a later time point of assembly, with PSDs also overshooting in size (**Supplementary Fig. 8c**). Thus, Nlg1 seems particularly important for providing binding sites for GluRIIA complexes during early assembly, and choosing the right temporal sequence also seems important for the proper termination of the assembly process.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.O., O.K. and S.J.S. designed research. D.O., O.K., V.K.G., D.B., H.D., W.F., C.W., S.M. and E.R. performed experiments. D.O., O.K., V.K.G., D.B., H.D., W.F., C.W., S.M., H.A. and S.J.S. analyzed data. M.H. and S.E. shared protocols, reagents and advice. All authors commented on the paper. D.O., O.K. and S.J.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Genetics and molecular cloning. Fly strains were reared under standard laboratory conditions. For all experiments, both male and female larva were used for analysis. All mutations were kept in the *w¹¹¹⁸* genetic background. Double mutant combinations were verified via genomic PCR^{5,11,12} or, where possible, by complementation analysis. Mutants for *Syd-1* (*Syd-1^{ex1.2}/Syd-1^{ex3.4}*)⁵, *Nrx-1* (*Nrx-1²⁴¹/Nrx-1Df*)¹¹, *Nlg1* (*Nlg1^{ex1.9}/Nlg1^{ex2.3}* or *Nlg1¹⁹⁶⁰/Nlg1^{H324}*)¹², *Liprin-α* (*Liprin-α^{EPexR60}/Liprin-α^{F3ex15}*)¹⁸ and *brp* (*brp^{1.3}/Df(2)BSC29*)²⁰ were described previously. Flies carrying UAS-GFP-tagged *Nrx-1* (ref. 12), UAS-untagged-*Nrx-1* (ref. 24), UAS-untagged-*Nrx-1^{PDZ*}* (ref. 24), UAS-GFP or -mStraw-tagged *Syd-1* (ref. 5), UAS-GFP-tagged *Liprin-α* (ref. 20), UAS-GFP-tagged *Nlg1* (ref. 12) and UAS-GFP-tagged *Nlg1ΔCyto* (ref. 12) were described previously. mStrawberry-tagged UAS-*Liprin-α* was obtained using the Gateway vector pTWmStrawberry and *pENTR4/Liprin-α* (ref. 20). The D358R point mutation was introduced to GFP-tagged-*Nlg1ΔCyto* as previously described¹². UAS-*Syd-1^{PDZ*}* was constructed based on the UAS-*Syd-1cDNA* (also referred to as *Syd-1 Rescue*)⁵. Two fragments were obtained via PCR using (1) 5'-GAGCGCGCCGCGATGACG-3' and 5'-AGCCGCGCTGTGGCCGCGCCTTGACTA-3', and (2) 5'-TAGTCAAGGCGCCGGCCAGACGCGGGCTTGT-3' and 5'-CCGTTGACATTCTTCTCGAGGGACCCA-3', and joined using elongation PCR to create the PDZ* construct. UAS-*Syd-1cDNA* and -PDZ* were digested using NotI and XhoI and ligated. *pTmStrawberryW/Syd-1^{PDZ*}* was obtained using the Gateway system (Invitrogen). Point mutations were confirmed via double strand sequencing. For the yeast two-hybrid bait vector, a region encoding the cytoplasmic C terminus of *Nrx-1* was obtained via PCR on UAS-*Nrx-1cDNA* (ref. 12) using 5'-GATGCCATGGAGTCAATGGCGATCGTGGCT-3' and 5'-GTCTATGAA TTCTTTACACATACCACTCCTTGACGTCCT-3'. The product was digested using NcoI and EcoRI and ligated into *pGADT7-IIB* (modified *pGADT7*, containing only a single NcoI site). For the yeast two-hybrid prey vectors, a region encoding the *Syd-1* PDZ domain was obtained via PCR on UAS-*Syd-1cDNA* and UAS-*Syd-1^{PDZ*}cDNA*, respectively, using 5'-GTCTATGAATTCTGGTAGAAA TAGTCAAG-3' and 5'-GTCTATGGATCCCTACGTTGGCGGTCCAGGAG-3'. The products were digested using EcoRI and BamHI and ligated into *pGBKT7*.

Immunostainings and imaging. The relative number of synaptic boutons was quantified on dorsal muscles 1 and 9 in abdominal segment A3 of intact CD8-GFP-Sh third-instar larvae expressing UAS-*Nlg1ΔCyto^{GFP}* (*Mef2-Gal4*) in various genetic backgrounds. The relative number of synaptic boutons was calculated by adjusting the absolute bouton number to the approximate muscle surface areas.

Larval filets were dissected and stained as described previously⁵. The following primary antibodies were used: RbαDSyd-1 (ref. 5; 1:500), RbαGluRIID (ref. 5; 1:500), MαNc82 (refs. 20,22; 1:100) (provided by E. Buchner, Universität Würzburg), RbαNlg1 (ref. 12; 1:500) and GpαNrx-1 (ref. 11; 1:500) (provided by M. Bhat, School of Medicine, University of North Carolina). Secondary antibodies were diluted 1:500. For STED we used GαM (A11001) or GαChicken (A11039) Alexa488 (Molecular Probes, Germany).

Nlg1 and *Nrx-1* clusters were quantified from confocal stacks of NMJs converted to maximal projections. The signal of a Cy5αHRP antibody (23-175-021, Jackson ImmunoResearch, 1:250) was used as template for a mask, restricting the quantified area to the shape of the NMJ. After subtraction of background signals (where indicated), the absolute intensity per unit NMJ area was acquired using ImageJ and converted to the relative intensity in Microsoft Excel.

In vivo imaging: All UAS constructs (except for *Nlg1* constructs (*Mef2-Gal4*, see above)) were driven in motor neurons using *ok6-Gal4* (*P[GawB]OK6*; ref. 44). NMJs on muscles 26 and 27 were recorded. Salivary glands were also imaged from living larvae. All live imaging was performed as previously described^{5,6,20}. Briefly, larvae of different genotypes were mounted in the imaging chamber and single confocal sections were acquired. For FRAP experiments (all performed in the synaptic area of the NMJ), intense laser light was applied to a region of interest, bleaching green or/and red fluorescent fusion tags. The after-photobleaching image was taken approximately 1–2 min after the *t* = 0 min image. After an incubation time of 30 min, the junctions were reimaged and compared with the images taken before bleaching. For 30-min time-course experiments, larvae were awakened by applying fresh air but remained in the imaging chamber between the acquisition points. For 24-h imaging intervals, the larvae were reared in an incubator at 25 °C between acquisitions.

FRAP analysis was done following a previously described protocol¹²⁰.

Quantification of mobile *Liprin-α* and *Syd-1* spots was performed on a time series of three confocal stacks of individual junctions that were sequentially acquired at 30-min intervals (*t* = 0 min, *t* = 30 min, *t* = 60 min). These images were deconvolved using Autoquant X 2.1.1 from MediaCybernetics. Confocal stacks were merged into a single plane by using the maximum projection function of ImageJ. Subsequently, a Gaussian blur filter was applied (blur radius, 0.5 pixels) and the highest signal intensity was scaled to 255. Only spots with a mean intensity 2.5 times higher than the background intensity were counted. The spots were classified as mobile spots or immobile spots. Spots were considered to be 'immobile' spots if they remained at their initial position (relative to the surrounding spots) during all three time points, whereas 'mobile' spots were defined as spots that appeared and/or disappeared during the time interval of 60 min. All spots were manually counted, and the number of spots was normalized to the length of the junction (estimated using the line function in ImageJ).

For quantitative analysis of GluR fields the following genotypes were produced: [Df(2L)^{h4},GluRIIB-GFP/A22,GluRIIA-mRFP] for controls, [Df(2L)^{h4},GluRIIB-GFP/A22,GluRIIA-mRFP; *Syd-1^{ex1.2}/Syd-1^{ex3.4}*] for *Syd-1*, [Df(2L)^{h4},GluRIIB-GFP/A22,GluRIIA-mRFP; *Nrx-1²⁴¹/Nrx-1²⁴¹*] for *Nrx-1*, & [Df(2L)^{h4},GluRIIB-GFP/A22,GluRIIA-mRFP; *Nlg1^{ex2.3}/Nlg1^{ex1.9}*] for *Nlg1* mutants.

The *GluRIIA*,*GluRIIB* (Df(2L)^{h4}/A22) double mutation was rescued by the expression of fluorescently tagged genomic constructs of *GluRIIA* and *GluRIIB* under the control of their endogenous promoters⁶.

Confocal image stacks were analyzed using ImageJ and Imaris 6.15. 3D-surface masks were generated after background subtraction, scaling to the highest pixel intensity and application of Gaussian blur filters. These stacks were further analyzed in Imaris and single receptor fields (PSDs) were detected using the seed point detection algorithm combined with manual segmentation. Pixel intensities of unprocessed *GluRIIA* and *GluRIIB* channels in single PSDs were transferred to Microsoft Excel for further processing, as previously described^{6,12}.

Receptor topology at individual PSDs and *GluRIIA* intensities in control, *Nrx-1*, *Syd-1* and *Nlg1* larvae were evaluated in images acquired from muscle 4 of fixed preparations and analyzed as described previously¹².

All confocal images were acquired on a Leica TCS-SP5 microscope with a 63× magnification, NA 1.4 oil objective using Application Suite Advanced Fluorescence (LAS AF; Leica) software. Confocal stacks were processed with ImageJ software (<http://rsbweb.nih.gov/ij/>), Imaris 6.15 (Bitplane) and Autoquant X 2.1.1 (MediaCybernetics).

STED and electron microscopy. STED images were acquired on a Leica TCS STED CW. Images were deconvolved using the built-in deconvolution algorithms of the Leica LAS-AF software. The PSF was generated by using a 2D Lorentz function with the full-width half-maximum set to 80 nm (as calculated on the image using the Wiener filter algorithm; regulation parameter: 0.05). For preparation of the figures, a Gaussian blur filter of 0.5 was applied to the images.

Measurement of BRP ring diameters: deconvolved STED images of BRP stained NMJs (muscle 4) of 3rd instar larvae were processed in ImageJ. The diameters of planar oriented BRP rings were measured using the line tool of ImageJ. The distance from intensity maximum to intensity maximum was acquired in the plot window of individual hand-drawn lines and transferred to Microsoft Excel. Per genotype, 5–7 images were analyzed, resulting in 36–56 measurements per group.

Electron microscopy: Conventional transmission electron microscopy and 3D-serial reconstruction were conducted as described⁵.

Coimmunoprecipitation and yeast two-hybrid assay. For protein extracts used in coimmunoprecipitation experiments, we carried out a *Drosophila* head fractionation based on protocols from mammalian subcellular preparations⁴⁵. In brief, in the absence of detergents, *Drosophila* wild-type heads were sheared mechanically and differential centrifugation was applied to separate particles according to their size and density. Recovered membranes were then solubilized in aqueous immunoprecipitation buffer containing 20 mM HEPES pH 7.4, 200 mM KCl, 2 mM MgCl₂, 1% Triton X-100. Precleared protein extract was incubated for 10 h at 4 °C with either MαNrx-1 (mAb4, provided by W. Xie, Southeast University, Nanjing, China²⁴) or murine IgGs (Sigma-Aldrich) coupled to Protein A Sepharose-beads (Bio-Rad). After washing four times with immunoprecipitation buffer, proteins were eluted with 50 μl 2× SDS-PAGE sample

buffer. For immunoblot analysis, 4% of input and 10 μ l of the eluate were loaded. Membranes were probed with M α Nrx-1 (ref. 24) and Rb α Syd-1 (ref. 5).

For the yeast two-hybrid assay, *Saccharomyces cerevisiae* reporter strain Y187 (Clontech) was transformed with the Nrx-1 bait vector and prey vectors containing the unmutated PDZ domain or the mutated domain (PDZ*) of Syd-1. Clontech's control vectors (pGBKT7-Lam, pGBKT7-53 and pGADT7-T) were used as the negative and positive controls. Liquid cultures were assayed for β -galactosidase using ONPG (*o*-nitrophenyl- β -D-galactopyranoside) to compare for relative strength of the protein-protein interaction. The assay was performed as described in the Yeast Protocols Handbook (Clontech) with the exceptions that cells were permeabilized with SDS/chloroform⁴⁶ and that OD₄₀₅ was measured. Clones showing β -galactosidase activity twice that of the negative control values were analyzed.

Statistics. Data were analyzed with Prism (GraphPad Software). To compare two groups, nonparametric Mann-Whitney *U*-tests were used for all data sets. For comparison of more than two groups, nonparametric Kruskal-Wallis tests were used, followed by a Dunn's multiple comparison test. *P* values, *n* values, and *U* or *K* statistic are given in the figure legends or main text.

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