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Functional dependence of neuroligin on a new non-PDZ intracellular domain

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Abstract

Neuroligins, a family of postsynaptic adhesion molecules, are important in synaptogenesis through a well-characterized trans-synaptic interaction with neurexin. In addition, neuroligins are thought to drive postsynaptic assembly through binding of their intracellular domain to PSD-95. However, there is little direct evidence to support the functional necessity of the neuroligin intracellular domain in postsynaptic development. We found that presence of endogenous neuroligin obscured the study of exogenous mutated neuroligin. We therefore used chained microRNAs in rat organotypic hippocampal slices to generate a reduced background of endogenous neuroligin. On this reduced background, we found that neuroligin function was critically dependent on the cytoplasmic tail. However, this function required neither the PDZ ligand nor any other previously described cytoplasmic binding domain, but rather required a previously unknown conserved region. Mutation of a single critical residue in this region inhibited neuroligin-mediated excitatory synaptic potentiation. Finally, we found a functional distinction between neuroligins 1 and 3.

Synaptic junctions are points of both functional and anatomical connection between neurons. The functional connection, in the form of a chemical synapse, underlies the unique ability of a neuron to relay signals, allowing any given cell to integrate information received

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AUTHOR CONTRIBUTIONS

E.S. initiated the project and generated preliminary data. S.L.S. designed experiments, performed all electrophysiology and all imaging in slice, constructed all new constructs, produced virus, conducted data analysis and wrote the manuscript. T.H. and B.-S.C. performed all biochemical experiments. T.H. designed and carried out imaging in dissociated neurons. R.A.N. and K.W.R. supervised the project. R.A.N., K.W.R. and E.S. provided comments on the manuscript.

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in thousands of discrete units from distant or nearby cells. Prior to the assembly of the components necessary for functional chemical transmission, however, synaptic points of contact must be established anatomically. This initial step is thought to occur through a physical interaction between trans-acting synaptic adhesion molecules of various types[1–4]. Indeed, neuroligins, a critical family of adhesion molecules on the postsynaptic side, are able to induce the formation and maturation of synapses through an interaction with presynaptic neurexin[5–8]. This synapse-spanning complex is capable of inducing both pre- and postsynaptic specializations in artificial synapse assays using beads[9] as well as non-neuronal cells[10–12]. Furthermore, extensive evidence suggests that mutations in neuroligin genes cause cognitive impairments in humans, including autism[5,13–15], and behavioral and memory alterations in mice[16–18].

Neuroligins are a family of single-pass transmembrane proteins with an extracellular acetylcholinesterase-like domain that both interacts with neurexin and is responsible for dimerization between neuroligins[19,20]. In addition, a short cytoplasmic tail contains a number of motifs thought to be necessary for the scaffolding of postsynaptic components, including a PSD-95/Discs large/zona occludens-1 (PDZ) ligand, a WW-binding domain and a gephyrin-binding site[21–23]. Neuroligins 1–4 (NLGN1–4) are expressed in both rodents and humans and are thought to share many of the same functions. However, some noted specializations have been described. NLGN1 is primarily localized to excitatory synapses, whereas NLGN2 is localized to inhibitory synapses and NLGN3 is found at both[7,9,24–26]. The existence of such specialization is supported by single knockouts and overexpression of NLGN1 or NLGN2 individually[27], although, paradoxically, the individual knockdown of NLGN1 does not reduce excitatory currents[28], and overexpressed proteins of both subtypes are capable of localizing to and potentiating either type of synapse[25,29]. The presence of compensation with single knockdown, as well as the loss of synapse-type segregation with overexpression, may result from the ability of these proteins to heterodimerize with NLGN3, which is located at both excitatory and inhibitory synapses[26]. The fourth member of the family is less well characterized and, although it possesses a characteristic neuroligin structure in humans, is not well conserved in mice[30].

Although several interactions between the cytoplasmic tail of neuroligin and postsynaptic scaffolding molecules have been described biochemically, resolving the relative importance of these interactions for the function of endogenous neuroligin in neurons has proven to be difficult. Indeed, the overexpression of truncation mutants lacking the canonical binding domains often reveal little or no phenotypic difference from overexpression of wild-type protein[31,32]. These negative results could be explained by dimerization between overexpressed mutated protein and endogenous neuroligin, which may obscure the effects of the mutation. Unfortunately, one cannot study the function of neuroligins in the absence of dimerization, as dimerization itself is required for normal neuroligin function[33]. Further complication is introduced by the demonstration that neuroligins can heterodimerize[26], limiting the use of overexpression on the background of any single knockout. Finally, a neuroligin 1–3 triple knockout dies at birth, making extensive experimentation on a null background impossible thus far[34].

We tested the functional importance of the cytoplasmic domain of neuroligin on a background of reduced endogenous neuroligin expression, which we achieved with a triple knockdown strategy. We found that the canonical protein binding sites previously identified in the cytoplasmic tail of neuroligin have no obvious role in its ability to potentiate synaptic transmission. Instead, we identified a single critical residue in the cytoplasmic tail that is essential for the postsynaptic functional effects of neuroligins at excitatory synapses. Given the prior consensus in the field that the PDZ motif mediates neuroligins' primary

postsynaptic interactions, these findings suggest a redirection of study on a new molecular determinant of neuroligin function.

RESULTS

Neuroligin cytoplasmic tail is critical for functional replacement

To study the postsynaptic role of neuroligin, we used a dual whole-cell recording configuration in organotypic hippocampal slice cultures (Fig. 1) to simultaneously record evoked excitatory postsynaptic currents (EPSCs) in transfected and nearby control cells. We first characterized neuroligin 3 (NLGN3) because it is endogenously expressed at both excitatory and inhibitory synapses and it has been implicated in the pathogenesis of autism[15,26]. We introduced exogenous human NLGN3 because it is not susceptible to knockdown with RNAi targeting rat NLGN3 as a result of differences at the nucleotide level between the rat and human neuroligins, despite their highly conserved amino acid sequences[6]. On a wild-type background, overexpression of a full-length version of NLGN3 yielded an increase in AMPA receptor (AMPAR)-evoked currents (Fig. 1g), consistent with previous studies that reported increases in the number of synaptic contacts onto neuroligin-expressing cells[25,29,31]. However, a truncated version of NLGN3 (NLGN3Δ90) also yielded increases in AMPAR-evoked currents of comparable magnitude, despite the loss of most of the cytoplasmic tail (Fig. 1g).

One possible explanation for the lack of an effect following the severe truncation of the cytoplasmic tail would be if endogenous neuroligin were able to compensate for the deficits of the truncation mutant. Dimerization and heterodimerization, which can occur between neuroligins[19,20,26], might complicate the interpretation of results with exogenously expressed mutants. In COS cells, we confirmed that heterodimerization can occur between our NLGN1 and NLGN3 constructs and that full-length NLGN1 can dimerize with a truncated version of NLGN3 (NLGN3Δ90; Supplementary Fig. 1). Moreover, in dissociated neuronal cultures, we found that exogenous NLGN1 can heterodimerize with endogenous NLGN3, as seen by co-immunoprecipitation of endogenous NLGN3 with virally expressed, hemagglutinin (HA)-tagged exogenous NLGN1 (Supplementary Fig. 1). Such dimerization between truncated and full-length neuro-ligin might mask the detection of a differential effect on AMPAR-evoked currents between the full-length and truncated neuroligins.

To minimize any possible influence of endogenous neurolgins, we employed a triple knockdown strategy using three exogenous microRNAs, one each to knock down neuroligins 1, 2 and 3, chained together and expressed in a single transcript with GFP (Fig. 1b). These chained targeting sequences, previously characterized individually[6], yielded an efficient knockdown of all three neuroligins from one construct (Fig. 1c). Expression of the neuroligin microRNAs (NLmiRs) substantially reduced both AMPAR- (Fig. 1d) and NMDA receptor (NMDAR)-evoked currents (Fig. 1e), demonstrating the integral nature of this protein family at excitatory synapses. Although neuro-ligins are known to affect the maturation of presynaptic terminals through their interaction with neurexin[8], removal of neuroligins had no effect on paired-pulse ratio, a measure of presynaptic release probability (Fig. 1f). Critically, when the same full-length or truncated versions of NLGN3 are coexpressed with the NLmiRs, the truncated version is no longer capable of potentiating AMPAR-evoked currents (Fig. 1h), which both illustrates the confounding influence of endogenous protein and indicates that the cytoplasmic tail is necessary for full neuroligin function.

Neuroligin AMPAR effect independent of established motifs

Given the clear functional dependence of neuroligin on its cytoplasmic tail, we next characterized the relative importance of several previously described protein binding motifs found there. To do so, we first coexpressed the NLmiRs with wild-type NLGN3 or NLGN3 that lacked the PDZ motif (NLGN3Δ4). Expression of wild-type NLGN3 on the reduced neuroligin background yielded a marked increase in evoked AMPAR current amplitude. However, the elimination of the canonical PDZ-binding motif did not affect the enhancement of AMPAR currents compared to wild-type NLGN3 (Fig. 2a). This finding is in contrast with that of a previous study[10] that found reductions in AMPAR- and NMDAR-evoked currents with the expression of a cyan fluorescent protein–tagged NLGN1 construct lacking the PDZ-binding motif. However, subsequent studies have not reported such dominant-negative phenotypes with the expression of PDZ-truncated neuroligins on a wild-type background[6,35]. The difference in findings may result from the different constructs used and perhaps the inclusion of an extracellular cyan fluorescent protein tag.

To further characterize the role of previously described cytoplasmic-binding domains, we made subsequent truncations to eliminate the WW-binding domain (NLGN3Δ46) and the gephyrin-binding domain (NLGN3Δ77) (Fig. 2a). Similarly, we saw no effect on NLGN3 function, indicating that interactions between NLGN3 and PSD-95, S-SCAM and gephyrin mediated by these sites in the cytoplasmic tail are not required for enhancement of excitatory postsynaptic currents by neuroligin.

Novel intracellular region required for synaptic effect

As none of the previously identified binding sites affected the post-synaptic NLGN3 phenotype, we made further truncations to ascertain whether other sites on the cytoplasmic tail contribute to the post-synaptic function of this protein. Indeed, we found that removing more than 77 residues (NLGN3Δ90, NLGN3Δ99, NLGN3Δ109) from the C terminus eliminated the enhancement of AMPAR currents by NLGN3 as compared with control cells (Fig. 2a,b), signifying the presence of a previously undescribed critical region in the cytoplasmic tail that is essential for postsynaptic potentiation. To define this critical region more narrowly, we next selectively deleted a total of 13 amino acids between amino acids 77 and 90, counting from the C terminus (NLGN3Δ77–90). Similar to the truncation mutants, this deletion eliminated the enhancement of AMPAR currents on the reduced-neuroligin background (Fig. 2a,b) despite the inclusion of PDZ, WW-binding and gephrin-binding domains. We next took advantage of a recent finding that neuroligin 4 (NLGN4), when overexpressed, causes a dominant negative–like reduction in excitatory currents[36], in stark contrast with the enhancement of currents that results from the overexpression of the other neuroligins. There are three amino acid differences in NLGN4 in the critical region that are conserved between NLGN1 and NLGN3, one of which is a substitution from a negatively charged glutamic acid to a polar asparagine. To test the dependence of neuroligin function on this residue, we made a NLGN3 point mutant, switching the glutamic acid at position 740 for asparagine (NLGN3 E740N). Notably, this single point mutation eliminated the enhancement of AMPAR currents by NLGN3 when coexpressed with the NLmiRs (Fig. 2a,b).

As expected, overexpression of NLGN3 postsynaptically induced a presynaptic effect, as evidenced by a decrease in paired-pulse ratio, a measure of release probability (Fig. 2c). This increase in the probability of release was seen with all of the truncation mutants that did not abolish the AMPAR enhancement, but was not present in the deletion mutant or point mutant that lacked an enhancement of AMPAR currents (Fig. 2c). This indicates that functional enhancement of the postsynaptic side is necessary for neuroligin-induced maturation of presynaptic terminals. However, this increase in release probability on

overexpression of full-length NLGN3 and near truncation mutants is not sufficient to explain the enhancement of AMPAR currents, as wild-type NLGN3 did not induce a similar enhancement of NMDAR currents, which would be expected if the effect were primarily presynaptic (Fig. 2d). Moreover, the effect of the point-mutation is not a result of a deficit in surface trafficking, as surface levels of the point mutant are identical to those of the full-length protein (Supplementary Fig. 2). Notably, the effects of these various mutants could only be seen on the background of reduced endogenous neuroligin expression. Indeed, expression of the point mutant (NLGN3 E740N) on a wild-type background resulted in enhancements of AMPAR currents that were indistinguishable from expressing full-length NLGN3 on a wild-type background (Supplementary Fig. 2).

To test whether this site is necessary for the function of neuroligin in general or is specific to NLGN3, we made the same amino acid swap in the corresponding residue (E747) of an RNAi-proof version of NLGN1 (NLGN1* E747N) (Fig. 3a). As with NLGN3, this single point mutation in NLGN1* markedly reduced the enhancement of AMPAR currents induced by NLGN1* expression on the NLmiRs background (Fig. 3b). The effect of NLGN3 was primarily restricted to AMPARs and not NMDARs (Fig. 2d), consistent with a preferential increase in AMPARs at new synapses or an unsilencing of synapses that previously contained only NMDARs. However, NLGN1* enhanced both AMPAR- and NMDAR-mediated EPSCs (Fig. 3c). This difference between NLGN1 and NLGN3 has not previously been reported and was observed only on a background of reduced endogenous neuroligin and not on a wild-type background, presumably because of hetero-dimerization between exogenous NLGN3 and endogenous NLGN1 that occurs on a wild-type background (Supplementary Fig. 3). Introducing the point mutation E747N to NLGN1* also reduced the enhancement of NMDAR EPSCs (Fig. 3c), indicating that the effect of this mutation is upstream of a specific enhancement of either AMPAR or NMDAR currents. Similar to NLGN3, the effect of the NLGN1* E747N point mutation was only apparent on the background of reduced endogenous neuroligin (Supplementary Fig. 3). Finally, as this amino acid swap was generated on the basis of the sequence of NLGN4, we made the opposite mutation to NLGN4 (NLGN4 N726E) in the hope of transferring the NLGN3 phenotype to NLGN4. In fact, although the wild-type human NLGN4, when expressed singly, reduced both AMPAR and NMDAR currents, the point-mutant, which more closely matches the other neuroligins, enhanced AMPAR currents and did not affect NMDAR currents (Fig. 3d,e), indicating that this critical region is sufficient for the functional enhancement of AMPAR current by neuroligin. This effect could also be seen on the reduced-neuroligin background (Supplementary Fig. 3).

Effect of mutations is specific to excitatory synapses

We next tested whether this newly identified site is specific to excitatory synapses or extends to all synapse-promoting functions of neuroligin. The knockdown of neuroligins with the NLmiRs also reduced the amplitude of inhibitory postsynaptic currents (IPSCs) as compared with control cells (Fig. 4a,b), implicating this family of proteins in inhibitory as well as excitatory synapse function. Neuroligin 2 (NLGN2) is the member of the family most closely associated with inhibitory synapses[24]. Thus, along with the NLmiRs, we coexpressed an RNAi-proof version of neuroligin 2 (NLGN2*), which markedly enhanced evoked IPSCs (Fig. 4a,c). In this case, unlike the excitatory phenotype, a mutation of the analogous amino acid in NLGN2 (NLGN2* E740N) had no effect on the magnitude of enhancement of inhibitory currents (Fig. 4a,d). NLGN3 produced only a modest rescue of the knocked-down currents and the point mutant NLGN3 E740N also produced a modest rescue that was indistinguishable from that achieved by expressing NLGN3 (Fig. 4a).

These results indicate that this site is critical for excitatory synaptic function, but does not extend to inhibitory synapses. It is interesting that expression of NLGN3 on the knockdown

background produced only a modest rescue of inhibitory currents, whereas NLGN2* expression increased inhibitory currents substantially above baseline, given that both neuroligins have been shown to localize to inhibitory synapses[9,26]. To explore this effect further, we examined the effect of NLGN3 expression on a wild-type background and found that this expression resulted in large increases in evoked IPSCs (Supplementary Fig. 4). This raises the intriguing possibility that the presence of NLGN2 may be required for the function of NLGN3 at inhibitory synapses.

Neuroligin effect remains after synaptic blockade

It was previously reported that chronic blockade of NMDARs or CaM kinase II in dissociated neuronal cultures mitigates the effect of NLGN1 overexpression on evoked EPSCs and synapse density[27]. This finding of activity dependence has been used to support a model of neuroligin function in which the role of neuroligin is confined to the maturation rather than the genesis of synapses[5]. Thus far, our findings could result from changes in either synaptic maturation or synaptogenesis. The slice culture system and dual whole-cell configuration provide an excellent opportunity for studying pharmacologic manipulations, as both the control and transfected cells experience identical pharmacologic conditions.

To test whether our neuroligin effects are dependent on synaptic activity, we coexpressed either NLGN3 or NLGN1*, each with the NLmiRs as before, but maintained the slices in 6-nitro-2,3-dioxo-1,4-dihydro benzo[f]quinoxaline-7-sulfonamide (NBQX) and d(-)-2-amino-5-phosphonovaleric acid (AP5) to block all fast excitatory synaptic transmission for the duration of the transfection (Fig. 5a). We found that the effects of NLGN1* and NLGN3 persisted when excitatory synaptic activity was blocked. Indeed, in these experiments, as in the previous experiments, NLGN3 enhanced AMPAR-mediated, but not NMDAR-mediated, currents (Fig. 5b) and NLGN1* enhanced both AMPAR- and NMDAR-mediated currents (Fig. 5c). Similar results were obtained on coexpressing NLGN3 and the NLmiRs in the presence of only AP5 (Supplementary Fig. 5). These findings are consistent with a previous report[37], which found no change in the ability of neurexin to cluster AMPARs at sites of NLGN/neurexin contact in the presence of either AP5 or TTX.

Neuroligin induces the assembly of new functional synapses

Finally, we examined miniature EPSCs (mEPSCs) to further define the basis for the enhancement of excitatory transmission by neuroligins. mEPSCs were recorded in both the wild-type and point-mutant forms of NLGN3, each coexpressed with the NLmiRs. We found that the wild-type form greatly increased the frequency of mEPSCs with no effect on amplitude, whereas the point mutant displayed a modest increase in frequency, but had a significant reduction in amplitude ($P < 0.05$; Fig. 6a,b). Moreover, when we imaged both transfected and control neurons to examine spine density, an anatomical approximation of synapse number, we found that, although the NLmiRs reduced the density of spines, both the wild-type and point-mutant forms of NLGN3 increased the density of spines (although the point mutant did so to a lesser extent; Fig. 6c). In contrast, NLGN3Δ77, which displayed the same effect as the wild type with respect to AMPAR currents, also induced an increase in spine density of comparable magnitude to that of the wild type on the background of the NLmiRs (Supplementary Fig. 6).

Together, these results suggest that the enhancement of AMPAR currents by neuroligin results, at least in part, from the assembly of new functional synapses. Furthermore, given that mEPSC amplitude was reduced by the expression of NLGN3 E740N even though both spine density and mEPSC frequency were higher than in wild type, it appears that the point mutant displayed a specific postsynaptic deficit with respect to the recruitment of AMPARs to functional synapses. Notably, the effect of the point mutation is not a result of a

trafficking deficit, as GFP fusion constructs of both the full-length and the point-mutant protein clearly localized to spines (Fig. 6c). In further support of a specific postsynaptic deficit imparted by the point mutation, we found that both wild-type and point-mutant versions of NLGN1 and NLGN3 increased presynaptic VGLUT1 staining to similar magnitudes when expressed on the background of the NLmiRs in dissociated neurons (Fig. 6d). This suggests that the trans-synaptic effects of neuroligin, via the interaction with presynaptic neurexin, are not affected by the cytoplasmic point-mutation and that only the ability to functionally enhance the postsynaptic site is perturbed.

DISCUSSION

We used a combination of approaches to examine the molecular determinants of neuroligin function at the postsynaptic site. Our findings confirm the integral role of the neuroligin family of proteins in the function of both excitatory and inhibitory synapses, as evidenced by reductions in AMPAR- and NMDAR-mediated EPSCs, as well as IPSCs in neurons with experimentally reduced levels of neuroligins 1–3. Furthermore, we found, on this reduced background, that the canonical postsynaptic protein binding sites that are thought to mediate necessary interactions between neuroligin and other postsynaptic scaffolding molecules, such as S-SCAM and the MAGUK family (including PSD-95), are not required for the potentiation of excitatory synaptic currents seen when neuroligins are overexpressed. Instead, we identified a previously uncharacterized site in the cytoplasmic tail that is indispensible for the postsynaptic function of neuroligin at excitatory synapses. On the basis of these findings, future research should focus on this new molecular domain rather than on the PDZ-mediated interactions of neuroligin, given the lack of a functional dependence on the PDZ motif. Ideally, our extensive molecular dissection of the relative importance of regions in the cytoplasmic domain of neuroligin will allow subsequent biochemical studies to identify new protein interactions at the postsynaptic site, among them those that occur earliest during the formation and assembly of excitatory synapses.

Our experiments required a reduced background of endogenous neuroligin to prevent a confounding influence observed in the presence of endogenous protein, which could occur as the result of dimerization between mutant and endogenous protein. Indeed, we found that such heterodimerization does occur. We believe that this represents the most parsimonious explanation of the effects that we observe. However, as dimerization is required for the full function of neuroligin[33], we cannot definitively state that heterodimerization is the cause of this confounding influence, but only that the presence of endogenous neuroligin does not allow for accurate study of exogenous mutant neuroligin. We believe that this represents an important step forward in the study of this family, as well as other families, of partially redundant proteins. Indeed, a confounding effect in the presence of endogenous neuroligin may explain the differing results that have been reported when neuroligin is expressed in either neurons or non-neuronal cells that have no endogenous neuroligin[31].

Our findings also shed light on the synapse specificity of neuroligin. The critical site identified in our experiments is specifically necessary for the function of neuroligin at excitatory, but not inhibitory, synapses. The gephyrin binding site in the cytoplasmic tail of NLGN2 (ref. [23]) may account for its ability to enhance IPSCs, although we found that the gephyrin binding is not required for the action of neuroligin at excitatory synapses. The role of neuroligins in the balance of excitation and inhibition has been the topic of much research[38]. In fact, the mutations in NLGN3 that have been found in individuals with autism may exert their phenotypic effect through a shift in the ratio of excitation to inhibition[39,40]. However, the critical single residue identified in this study is present in both the neuroligins found endogenously at excitatory synapses, NLGN1 and NLGN3, and NLGN2, found exclusively at inhibitory synapses. This is reminiscent of the finding that the gephyrin-

binding domain is present in all four neuroligins even though NLGN2 appears to be specifically required for the assembly of inhibitory synapses[23]. Clearly, an unknown mechanism independent of specific, identified interaction sites in the cytoplasmic tail must segregate neuroligin subtypes to their respective synapses.

In addition to the subtype specializations at excitatory and inhibitory synapses, we report a difference in phenotype between NLGN1 and NLGN3. Specifically, the overexpression of NLGN1 enhanced both AMPAR- and NMDAR-mediated excitatory currents, whereas the effect of NLGN3 overexpression was relatively restricted to AMPARs. This difference may reflect a primary role for NLGN3 in the unsilencing of synapses. Indeed, its overexpression mimics the known effects of synapse unsilencing that occur during synapse development and long-term potentiation, in which the selective increase in evoked AMPAR EPSCs is accompanied primarily by an increase in mEPSC frequency[41,42]. However, given that NLGN3 rescued the NMDAR current from the knockdown level and increased spine density, it is unlikely that the sole effect of NLGN3 results from the unsilencing of synapses. Conversely, the more equivalent enhancements of both AMPAR and NMDAR currents seen with NLGN1 may reflect a role that is shifted toward the creation of new functional synapses. The functional segregation between NLGN1 and NLGN3 raises a number of interesting issues. For instance, matched increases in AMPARs and NMDARs, as are observed with the expression of NLGN1, are reminiscent of a developmental global increase in synapses, whereas specific modulation of AMPARs, as is the case with the expression of NLGN3, mirrors the changes that are most often seen during long-term potentiation[43].

In contrast with our results, several studies have presented evidence in support of a functional interaction between neuroligin and PSD-95 or S-SCAM, either in the form of enhanced neuroligin phenotypes by coexpression of PSD-95 (ref. [29]) or S-SCAM[22], or an alteration in the neuroligin overexpression phenotype by the reduction of PSD-95 (ref. [28]) or S-SCAM[44]. One possible scenario to explain the different conclusions is that PSD-95 and S-SCAM interact indirectly with the neuroligins during synapse formation. Indeed, it has been shown that, although the clustering of neuroligin induces a rapid accumulation of NMDARs, the same clustering is followed by a much slower recruitment of PSD-95 (ref. [45]). Others have also reported that neuroligin localization to synaptic sites lacks dependence on the PDZ or WW-binding domains when expressed on a wild-type background[32]. It is important to note that, although we found no requirement for the PDZ motif in the synaptogenic phenotype induced by neuroligin in this set of experiments, it is not our intention to categorically exclude its involvement in all aspects of synaptic function. It is possible that a PDZ-mediated interaction could be involved at other developmental stages, in other synaptic adhesion molecules, or in other aspects of synaptic function not tested in this set of experiments. Although we believe that this replacement strategy and culture system provide a reasonable surrogate for CNS physiology, they do represent a particular developmental stage and may introduce peculiarities that are specific to the experimental setup. Future studies should seek to determine the relevance of this newly defined critical region to adult CNS physiology.

In addition to its synaptogenic properties, postsynaptic neuroligin is thought to exert a maturational effect on apposing presynaptic terminals[34]. In fact, differing roles have been suggested for the extracellular domain, which has been implicated in the assembly of presynaptic terminals through an interaction with neurexin, and the intracellular domain, which is required for the maturation of those same terminals[8]. Our results suggest that there is an increase in release probability with the overexpression of neuroligin, which is consistent with a maturational effect on presynaptic terminals. Notably, the point mutants and deletion mutants of NLGN3 that did not induce enhancements of AMPAR currents also do not have this presynaptic effect. We believe this is consistent with the previous finding

that an intact cytoplasmic tail of neuroligin is required for the maturation of presynaptic terminals. However, a change in release probability is unlikely to underlie the major increase in AMPAR currents, as there is no equivalent change in NMDAR currents with the expression of those same functional proteins. Instead, these findings suggest that the majority of the increase in AMPAR currents results from a postsynaptic increase in AMPARs. Notably, the knockdown of neuroligins with the NLmiRs, which reduces both AMPAR- and NMDAR-evoked currents, does not affect the paired-pulse ratio. This finding, when viewed in combination with the reduction in spine density that accompanies expression of the NLmiRs, is consistent with a loss of synapses, rather than an alteration in maturational state at existing synapses.

Our findings contribute to the body of literature concerning the pathogenesis of autism, as mutations in NLGN3 and NLGN4 have been associated with the disease[15]. However, the loss-of-function mutations presented here are substantially different than those found in individuals with autism. The previously described autism-related neuroligin mutations have all been localized to the extracellular domain and the most well-characterized one (NLGN3 A451C) appears to result in the endoplasmic reticulum retention of the mutant protein[46]. Thus, individuals with these mutations may exhibit a total loss of synaptic NLGN3 or NLGN4. In contrast, the cytoplasmic mutation that we found imparts a specific deficit in postsynaptic function, whereas the protein retains synaptic targeting and trans-synaptic function. Thus, we cannot directly relate our findings to the pathogenesis of autism, but believe this represents a step forward in the understanding of neuroligin function at the synapse and may indirectly contribute to our understanding of autism.

The triple knockdown of NLGN1–3 markedly reduced, but did not eliminate, excitatory and inhibitory transmission. We do not know if the remaining current is a result of residual neuroligin expression or rather neuroligin-independent synaptic assembly. There is certainly evidence that the neuroligin/neurexin complex is not the only mechanism of synaptic assembly, as neurons cultured from neuroligin triple knockout animals have synapses present in a density comparable to wild type, as determined by electron microscopy[34]. Other families of postsynaptic adhesion molecules, most notably the LRRTM family, have also been shown to be capable of inducing the formation of synapses in neurons[4,47,48]. Regardless of the nature of the remaining current, the triple knockdown was clearly sufficient to allow us to measure the functional importance of the neuroligin cytoplasmic tail in mammalian neurons.

There is a notable difference between the effect of an acute knockdown of NLGN1–3 that we observed and the previous characterization of the triple NLGN1–3 knockout mouse. Although we found that the acute reduction of NLGN1–3 resulted in decreased AMPAR- and NMDAR-evoked EPSCs, as well as decreased IPSCs, the triple knockout did not appear to affect excitatory currents and did not change the total number of synapses in cultured neurons[34]. It is possible that there may be compensation by other synaptic adhesion molecules, such as the LRRTM family, when endogenous neuroligin is absent throughout the embryonic development of an animal. Indeed, the fact that knockout of NLGN1 alone results in decreased NMDAR currents[27] and that the triple knockout does not affect NMDAR currents suggests that such compensation is possible.

These results advance our understanding of the family of neuroligins. At an experimental level, our results emphasize the importance of performing experiments that target this family of proteins on a background of reduced endogenous expression. Using this approach, we found that the postsynaptic role of neuroligins does not depend on binding to S-SCAM and PSD-95, as was previously believed. Instead, we define a new region and critical residue in the intracellular domain. This shift in focus, away from PDZ-mediated neuroligin

interactions and toward a new molecular site of importance in the cytoplasmic domain, should redirect the course of future research into this family of proteins. It will be interesting to explore the nature of this new domain, as it may regulate the function of neuroligins with respect to known components of the postsynaptic site or may mediate new, unexpected interactions between neuroligin and other proteins that have an unknown role in the assembly or maintenance of a postsynaptic site. A number of interesting candidates for a possible interaction at this site may be found in the results of a yeast two-hybrid screen using the cytoplasmic tail of NLGN2 lacking the terminal PDZ motif[23]. Moreover, given the relevance of neuroligins to human disease, with specific mutations of NLGN3 and NLGN4 associated with autism[15], understanding the molecular determinants of neuroligin function has implications for clinical, as well as basic science, research.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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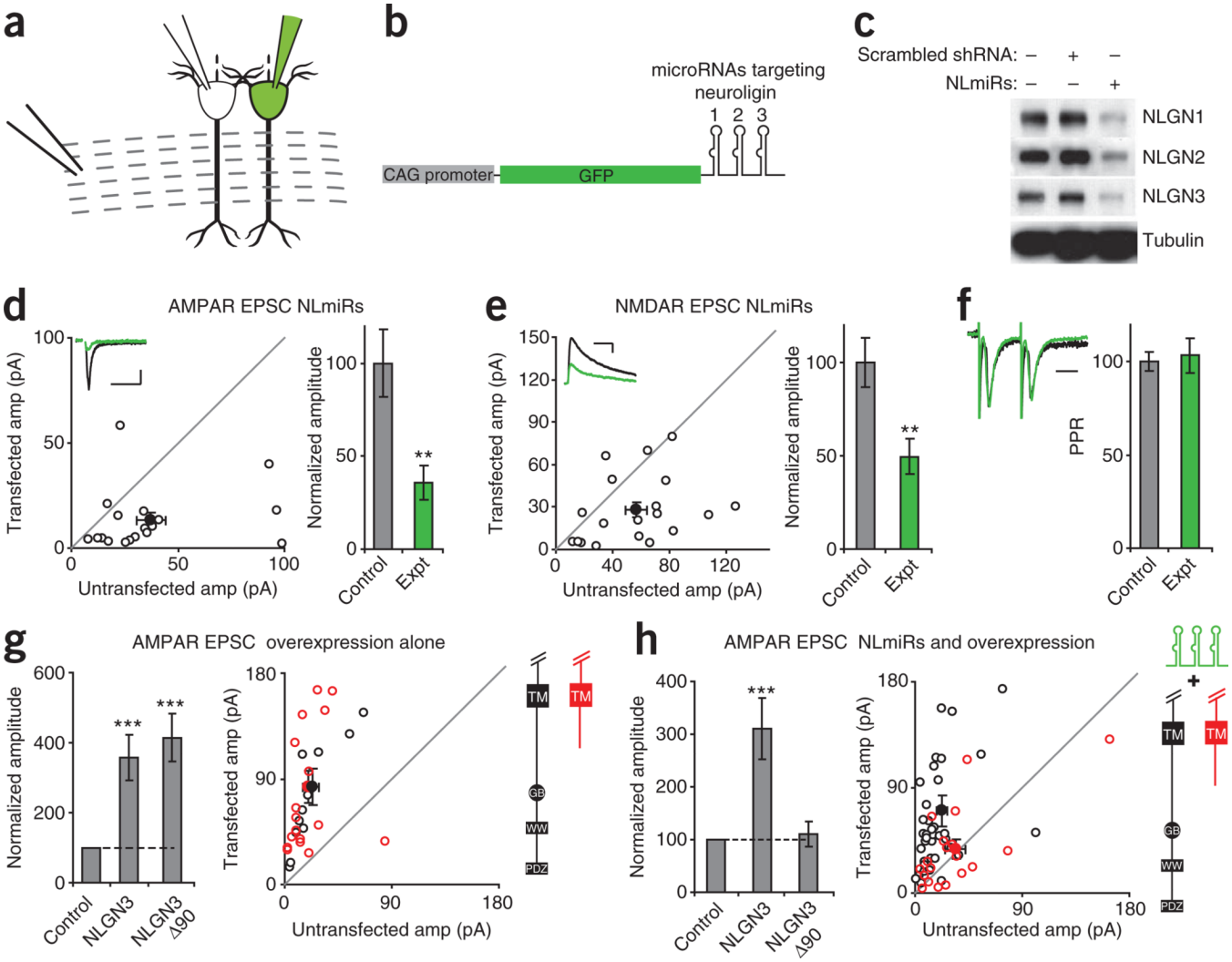

Figure 1.

Knockdown of neuroligin family necessary for functional study of cytoplasmic tail. (**a**) Dual whole-cell recording configuration. Leftmost pipette is the stimulating electrode. Dashed lines indicate Schaffer collaterals. (**b**) *CAG* promoter driving GFP expression and chained microRNAs targeting neuroligins 1–3. (**c**) Western blot analysis of knockdown. (**d**) AMPAR-mediated EPSC scatter plots showing reductions in amplitude (amp) in NLmiR-transfected neurons (expt) compared with control, untransfected neurons ($P < 0.005$, $n = 18$). Open circles are individual pairs, filled circle is mean ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 10 pA and 50 ms. Bar graph plots transfected amplitude normalized to control ± s.e.m. (**e**) NMDAR-mediated EPSC scatter plots and normalized amplitude showing reductions in amplitude in NLmiR-expressing cells ($P < 0.005$, $n = 19$). Scale bars represent 20 pA and 100 ms. (**f**) Paired-pulse ratio (PPR), second EPSC over first EPSC, ± s.e.m. for consecutive stimuli separated by 40 ms ($P > 0.05$, $n = 10$). Example traces normalized at first EPSC. Scale bar represents 20 ms. (**g**) AMPAR-mediated EPSCs showing no difference in phenotype between full-length NLGN3 ($P < 0.0005$, $n = 12$) and truncated NLGN3 ($P < 0.0005$, $n = 18$) when overexpressed alone. Bar graph and scatter plot are as described for **d**, full-length in black, truncation mutant in red. Schematic to the right illustrates truncation with previously described domains (PDZ; WW, WW-binding; GB, gephyrin-binding; TM, transmembrane). (**h**) AMPAR-mediated

EPSCs showing a clear phenotypic difference between full-length NLGN3 ($P < 0.0005$, $n = 40$) and truncated NLGN3 ($P > 0.05$, $n = 19$) when coexpressed with NLmiRs. Bar graph and scatter plot are as described for **d**, schematic is as described for **g**.

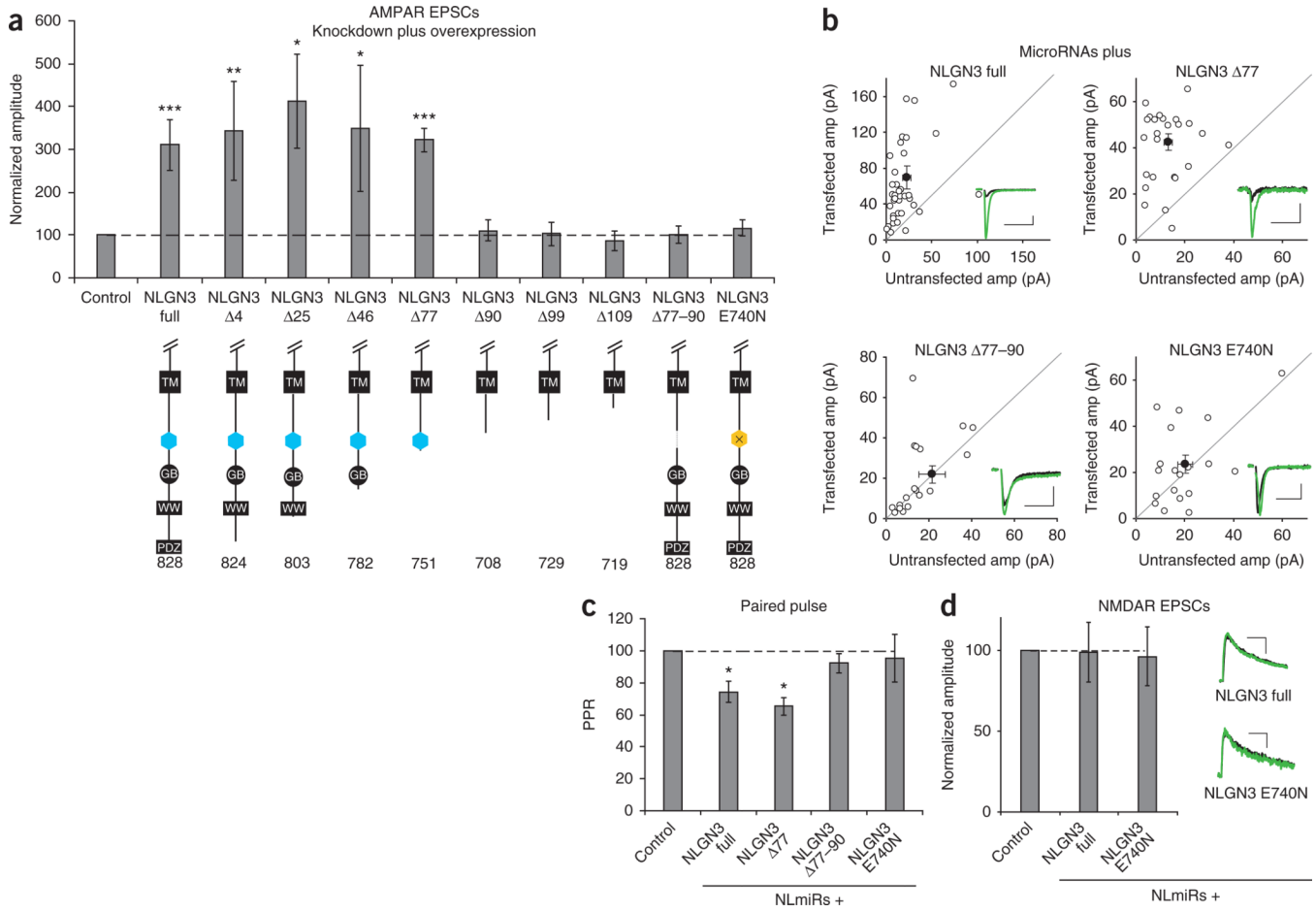

Figure 2.

Replacement of endogenous NLGNs with wild-type and mutated NLGN3 reveals that AMPAR enhancement is dependent on a single residue in the cytoplasmic tail. (**a**) Evoked AMPAR EPSC amplitudes (amp) as percent of control ± s.e.m. for each NLGN3 construct coexpressed with the NLmiRs (NLGN3 full, $P < 0.0001$, $n = 40$; NLGN3 Δ4, $P < 0.005$, $n = 14$; NLGN3 Δ25, $P < 0.05$, $n = 9$; NLGN3 Δ46, $P < 0.05$, $n = 11$; NLGN3 Δ77, $P < 0.0001$, $n = 26$; NLGN3 Δ90, $P > 0.05$, $n = 19$; NLGN3 Δ99, $P > 0.05$, $n = 7$; NLGN3 Δ109, $P > 0.05$, $n = 10$; NLGN3 Δ77–90, $P > 0.05$, $n = 19$; NLGN3 E740N, $P > 0.05$, $n = 19$). Delta number indicates number of truncated amino acids from C terminus. Schematic below graph represents cytoplasmic tail of NLGN3. Bar graphs for NLGN3 full and NLGN3 Δ90 are presented as in Figure 1g. (**b**) AMPAR EPSC scatter plots for notable replacements. Open circles are individual pairs, filled are mean ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 20 pA and 50 ms. The scatter plot for NLmiRs plus NLGN3 full, originally shown with NLGN3 Δ90 in Figure 1h, is repeated here for clarity. (**c**) Paired-pulse ratios, second EPSC over first EPSC, for notable replacements, normalized to control ± s.e.m. (NLGN3 full, $P < 0.05$, $n = 26$; NLGN3 Δ77, $P < 0.05$, $n = 16$; NLGN3 Δ77–90, $P > 0.05$, $n = 14$; NLGN3 E740N, $P > 0.05$, $n = 15$.) (**d**) Normalized NMDAR EPSC amplitudes for full-length NLGN3 ($P > 0.05$, $n = 19$) and NLGN3 E740N ($P > 0.05$, $n = 19$) expression on background of triple NLGN knockdown ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 20 pA and 100 ms. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

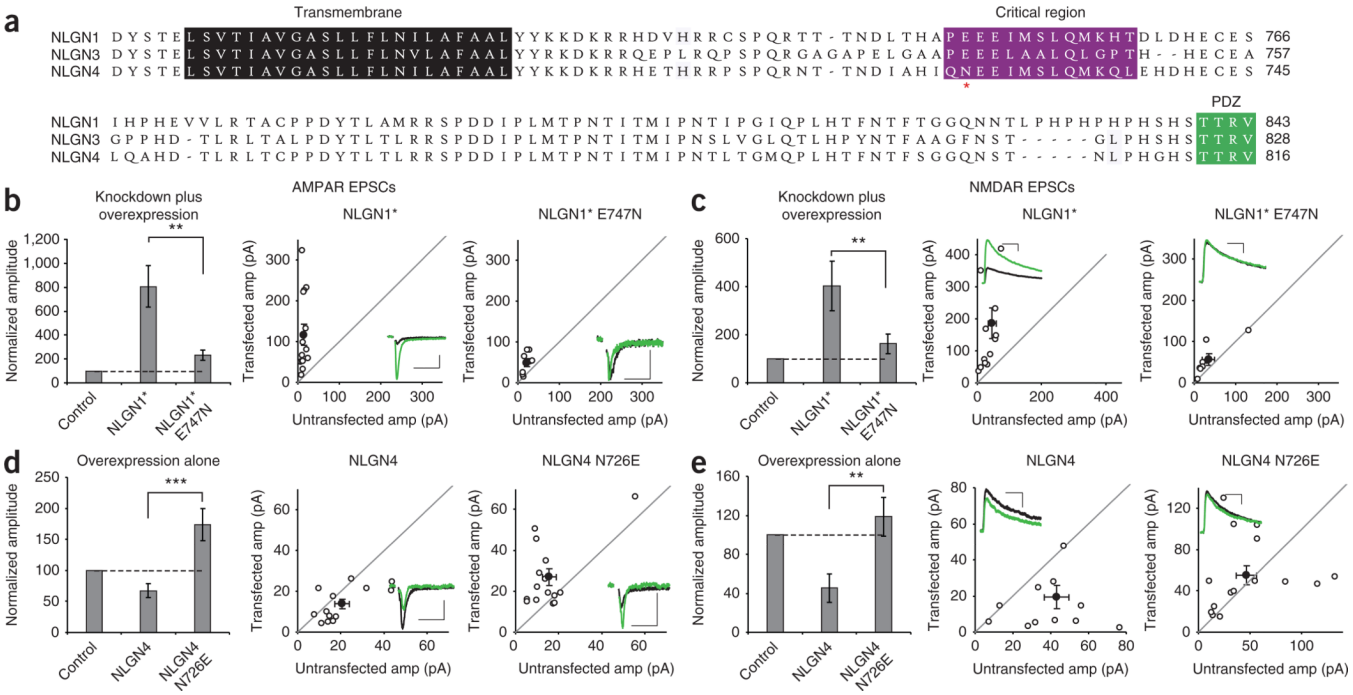

Figure 3.

Critical region identified in NLGN3 is also crucial for the function of NLGN1 and NLGN4. (**a**) Alignment of the transmembrane domain and c tails of NLGNs 1 (mouse), 3 (human) and 4 (human). Critical region identified in NLGN3 is highlighted with a star indicating the position of the single AA swap. The PDZ domain is highlighted in green. (**b**) AMPAR EPSC scatter plots and normalized amplitudes (amp) for NLGN1* (RNAi-proof) and NLGN1* E747N on the background of the triple knockdown demonstrating a significant difference ($P < 0.005$; $n = 14$, NLGN1*; $n = 8$, NLGN1* E747N). Scale bars represent 20 pA and 50 ms. (**c**) NMDAR EPSC scatter plots and normalized amplitudes for NLGN1* (RNAi-proof) and NLGN1* E747N on the background of the triple NLGN knockdown also demonstrating a significant reduction in current amplitude ($P < 0.005$; $n = 13$, NLGN1*; $n = 8$ NLGN1* E747N). Scale bars represent 40 pA and 100 ms. (**d**) AMPAR EPSC scatter plots and normalized amplitudes for NLGN4 and NLGN4 N726E on a wild-type background demonstrating the gain-of-function by this mutation ($P < 0.0001$; $n = 13$, NLGN4; $n = 15$, NLGN4 N726E). Open circles are individual pairs, filled circles are mean ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 20 pA and 50 ms. Bar graphs plot transfected amplitude normalized to control ± s.e.m. (**e**) NMDAR EPSC scatter plots and normalized amplitudes for NLGN4 and NLGN4 N726E on a wild-type background ($P < 0.005$; $n = 12$, NLGN4; $n = 15$, NLGN4 N726E). Scale bars represent 20 pA and 100 ms. Open circles are individual pairs, filled circles are mean ± s.e.m. Black sample traces are control, green are transfected. Bar graphs plot transfected amplitude normalized to control ± s.e.m. $^{**}P < 0.005$, $^{***}P < 0.0005$.

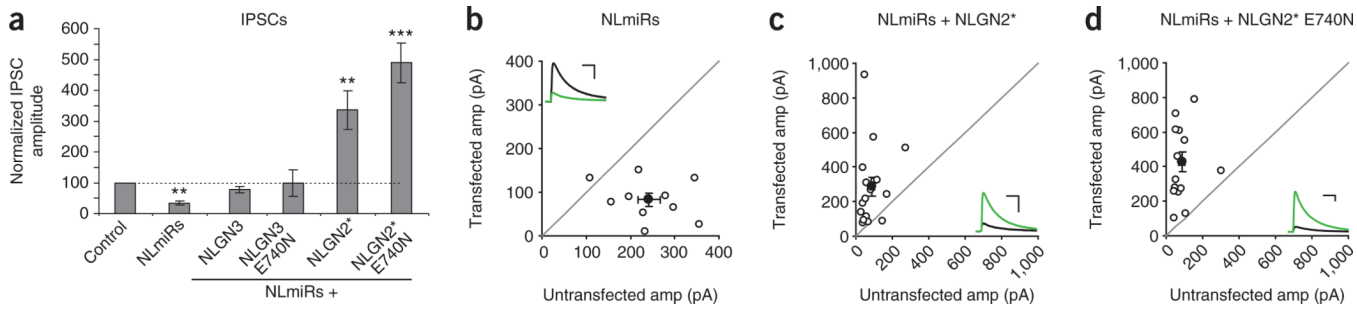

Figure 4.

Mutation of the critical residue does not affect inhibitory synapses. (**a**) IPSC amplitudes ((amp); recorded at 0 mV) normalized to control ± s.e.m. for neurons expressing NLmiRs ($P < 0.005$, $n = 10$) or the combination of NLmiRs with NLGN3 ($P > 0.05$, $n = 10$), NLGN3 E740N ($P > 0.05$, $n = 11$), NLGN2* (RNAi-proof, $P < 0.0001$, $n = 17$) and NLGN2* E740N ($P < 0.0001$, $n = 15$). No significant difference exists between NLGN2* and NLGN2* E740N ($P > 0.05$). (**b**) Scatter plot of NLmiR-expressing cells versus untransfected control cells demonstrating the reduction in IPSCs induced by the NLmiRs. (**c**) Scatter plot of cells expressing both NLmiRs and NLGN2* versus control demonstrating the enhancement of inhibitory currents induced by the expression of NLGN2*. (**d**) Scatter plot of cells expressing NLmiRs and NLGN2* with the glutamic acid at residue 740 mutated to asparagine versus control, which are not different than the NLGN2* without the point mutation. Open circles are individual pairs, filled circles are mean ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 75 pA and 30 ms. $^{**}P < 0.005$, $^{***}P < 0.0005$.

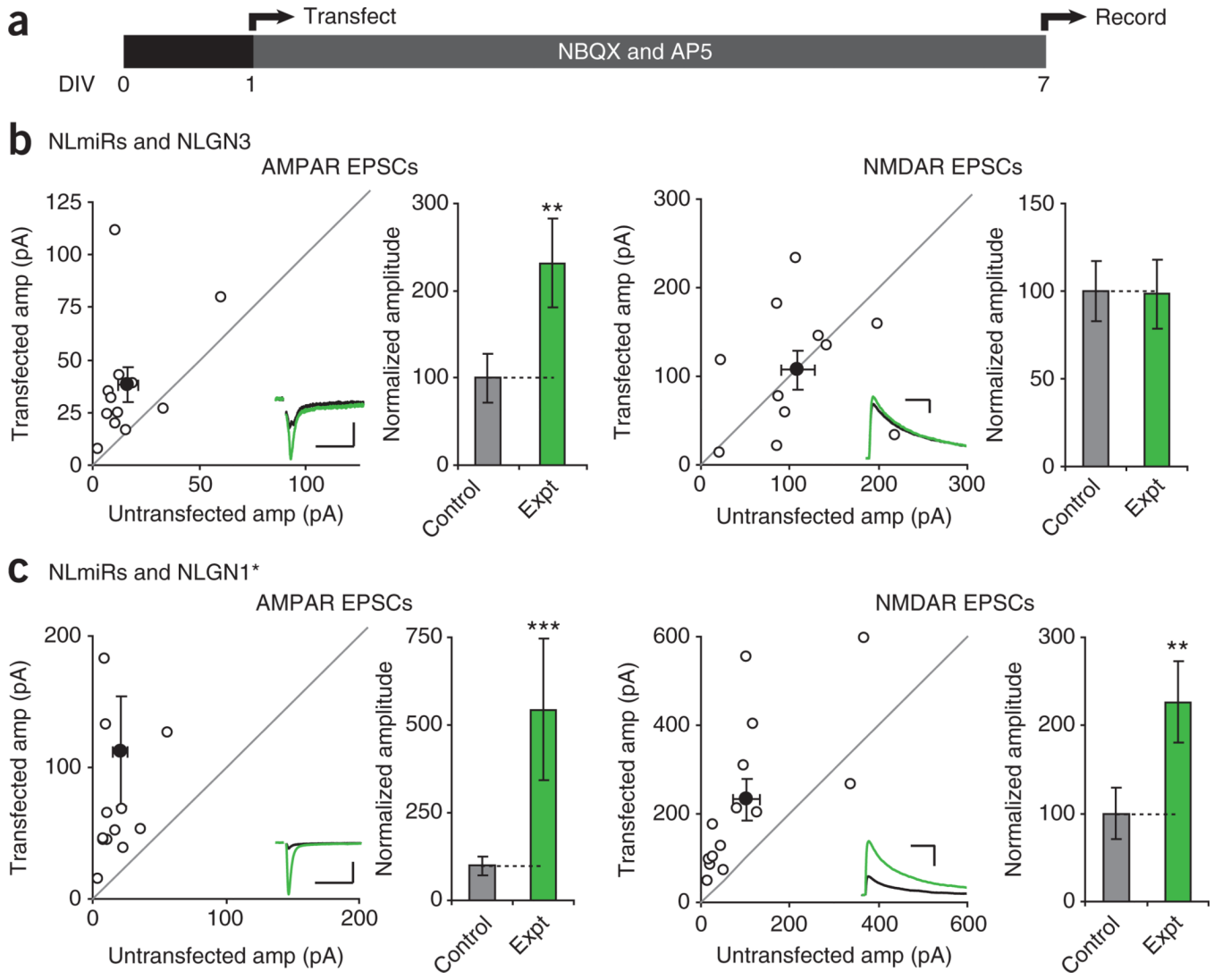

Figure 5.

The effects of neuroligin on excitatory synapses are independent of excitatory synaptic activity. (**a**) Schematic depicting the time course of these experiments. Days *in vitro* (DIV) are noted below the bar. (**b**) AMPAR- and NMDAR-mediated currents for cells expressing NLmiRs plus NLGN3 full-length in the presence of NBQX and AP5. Scatter plots show transfected cells as compared to simultaneously recorded control cells. Open circles are individual pairs, filled circles are mean ± s.e.m. Black sample traces are control, green are transfected (expt). Scale bars represent 15 pA and 50 ms (AMPAR) and 50 pA and 100 ms (NMDAR). Bar graphs plot experimental amplitudes (amp) normalized to control ± s.e.m. As was the case in the absence of drugs, NLGN3 induced an increase only in AMPAR-mediated currents ($P < 0.005$, $n = 12$) and not NMDAR-mediated currents ($P > 0.05$, $n = 11$). (**c**) AMPAR- and NMDAR-mediated currents for cells expressing NLmiRs plus NLGN1* (RNAi-proof) in the presence of NBQX and AP5. Scatter plots show transfected cells as compared to simultaneously recorded control cells. Open circles are individual pairs, filled circles are mean ± s.e.m. Black sample traces are control, green are transfected. Scale bars represent 60 pA and 50 ms (AMPAR) and 200 pA and 100 ms (NMDAR). Bar graphs plot experimental amplitudes normalized to control ± s.e.m. As in the absence of drug,

NLGN1* expression results in increased AMPAR-mediated ($P < 0.0005$, $n = 13$) and NMDAR-mediated ($P < 0.005$, $n = 14$) currents. **$P < 0.005$, ***$P < 0.0005$.

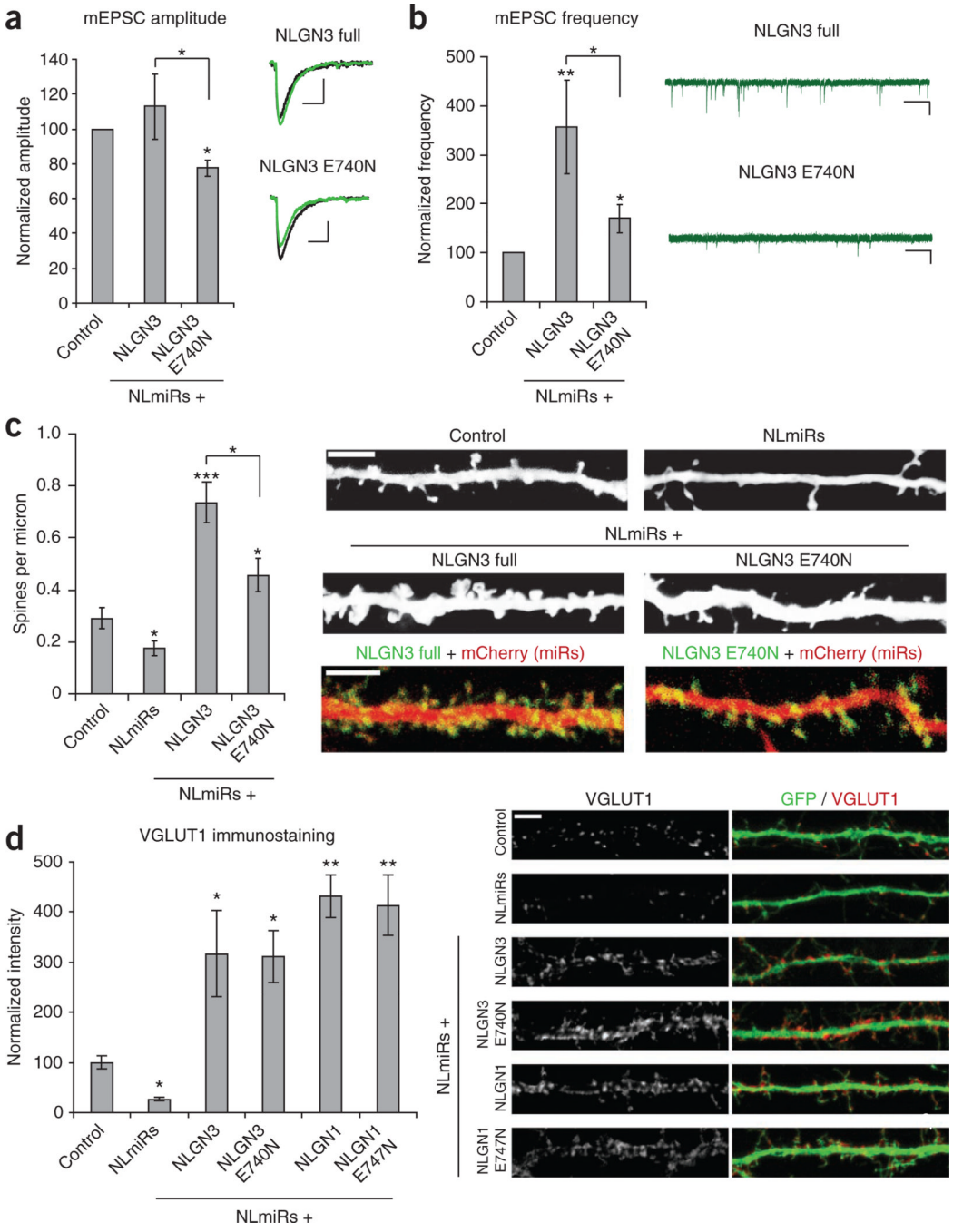

Figure 6.

Mechanism of synaptic enhancement by neuroligins and specific deficits of the mutant. (**a**) Spontaneous mEPSC amplitude in neurons expressing NLGN3 and NLGN3 E740N on triple NLGN knockdown background. Amplitudes are expressed as percentage of control ± s.e.m.; on knockdown background, amplitudes reduced by NLGN3 E740N compared with control ($P < 0.05$, $n = 13$) and full-length NLGN3 ($P < 0.05$, $n = 10$). Black sample traces are control; green are experiments. Scale bars represent 4 pA and 10 ms. (**b**) Spontaneous mEPSC frequencies with neuroligin replacements on triple knockdown background expressed as percentage of control ± s.e.m., showing increases by both NLGN3 ($P < 0.005$, $n = 10$) and NLGN3 E740N ($P < 0.05$, $n = 13$). Frequency increase was significantly larger

with full-length than with NLGN3 E740N ($P < 0.05$). Scale bars represent 20 pA and 0.5 s. (**c**) Spine density expressed as spines per micron ± s.e.m. with expression of NLmiRs ($P < 0.05$, $n = 12$) or expression of NLmiRs in combination with NLGN3 ($P < 0.0001$, $n = 11$) or NLGN3 E740N ($P < 0.05$, $n = 9$). A significant difference existed between NLmiRs plus NLGN3 and NLmiRs plus NLGN3 E740N ($P < 0.05$). The top four right-hand panels show representative images from each condition. Scale bar represents 5 μm. The bottom two panels show localization of full-length and mutated neuroligin to spines. (**d**) Normalized VGLUT1 immunostaining intensity ± s.e.m. onto neurons transfected with either GFP ($n = 16$), NLmiRs or neuroligin constructs with NLmiRs. VGLUT1 intensity decreased with NLmiRs expression ($P < 0.001$, $n = 20$); VGLUT1 intensity increased with all NLGN constructs on NLmiRs background (NLGN3, $P < 0.01$, $n = 15$; NLGN3 E740N, $P < 0.01$, $n = 16$; NLGN1*, $P < 0.001$, $n = 18$; NLGN1* E747N, $P < 0.001$, $n = 14$). Representative images show neurons immunolabeled with VGLUT1 (left, red in merge) and GFP (green in merge). Scale bar represents 2 μm. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.