The *Caenorhabditis elegans* voltage-gated calcium channel subunits UNC-2 and UNC-36 and the calcium-dependent kinase UNC-43/CaMKII regulate neuromuscular junction morphology

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**Abstract**

**Background:** The conserved *Caenorhabditis elegans* proteins NID-1/nidogen and PTP-3A/LAR-RPTP function to efficiently localize the presynaptic scaffold protein SYD-2/α-liprin at active zones. Loss of function in these molecules results in defects in the size, morphology and spacing of neuromuscular junctions.

**Results:** Here we show that the Cav2-like voltage-gated calcium channel (VGCC) proteins, UNC-2 and UNC-36, and the calmodulin kinase II (CaMKII), UNC-43, function to regulate the size and morphology of presynaptic domains in *C. elegans*. Loss of function in unc-2, unc-36 or unc-43 resulted in slightly larger GABAergic neuromuscular junctions (NMJs), but could suppress the synaptic morphology defects found in nid-1/nidogen or ptp-3/LAR mutants. A gain-of-function mutation in unc-43 caused defects similar to those found in nid-1 mutants. Mutations in egl-19, Cav1-like, or cca-1, Cav3-like, α1 subunits, or the second α2/δ subunit, tag-180, did not suppress nid-1, suggesting a specific interaction between unc-2 and the synaptic extracellular matrix (ECM) component nidogen. Using a synaptic vesicle marker in time-lapse microscopy studies, we observed GABAergic motor neurons adding NMJ-like structures during late larval development. The synaptic bouton addition appeared to form in at least two ways: (1) *de novo* formation, where a cluster of vesicles appeared to coalesce, or (2) when a single punctum became enlarged and then divided to form two discrete fluorescent puncta. In comparison to wild type animals, we found unc-2 mutants exhibited reduced NMJ dynamics, with fewer observed divisions during a similar stage of development.

**Conclusions:** We identified UNC-2/UNC-36 VGCCs and UNC-43/CaMKII as regulators of *C. elegans* synaptogenesis. UNC-2 has a modest role in synapse formation, but a broader role in regulating dynamic changes in the size and morphology of synapses that occur during organismal development. During the late 4th larval stage (L4), wild type animals exhibit synaptic morphologies that are similar to those found in animals lacking NID-1/PTP-3 adhesion, as well as those with constitutive activation of UNC-43. Genetic evidence indicates that the VGCCs and the NID-1/PTP-3 adhesion complex provide opposing functions in synaptic development, suggesting that modulation of synaptic adhesion may underlie synapse development in *C. elegans*.

**Keywords:** Calcium channels, Synaptogenesis, Extracellular matrix, Nidogen, LAR-RPTP, α-liprin, Cell adhesion, Calmodulin kinase II
Background

Changes in the cell membrane potential can open voltage-gated calcium channels (VGCC) to permit calcium entry. VGCCs are formed by α1, α2/δ, and β subunits and can include a γ subunit [1]. The α1 subunit forms the channel pore, while the auxiliary β and α2/δ subunits affect channel trafficking and physiology [2,3]. The α2/δ subunits are synthesized as a single polypeptide that undergoes proteolytic cleavage, but remains covalently associated [4-6]. VGCCs have been classified by their pharmacological and electrophysiological properties [1]. Ca_{2+}-type channels are localized to the presynaptic active zone, where they function in vesicle exocytosis. Ca_{1} channels localize more broadly and have been associated with events including gene regulation, local translation and dendritic growth [7].

In addition to synaptic vesicle exocytosis, there have been reports describing a role for VGCCs in regulating presynaptic development. Loss of calcium signaling through the α1 subunit cacophony resulted in reduced neuromuscular junction (NMJ) growth during Drosophila larval development [8]. At the vertebrate NMJ, a role for synaptic calcium channels has been discovered in mediating synaptic stability. The Ca_{2+}1 and Ca_{2+}2 calcium channels were found to bind directly to the extracellular matrix proteins laminin-10 and −11 and this interaction is critical for synaptic maintenance [9]. In the central nervous system, the α2/81 protein is a receptor for the extracellular matrix (ECM) protein thrombospondin, and together they act to promote synaptogenesis [10]. These results demonstrate how an interaction between VGCCs and the synaptic ECM can regulate synaptic development and morphology.

Previously, we have shown the synaptic ECM components nidogen (nid-1) and collagen XVIII (cle-1) exhibit distinct roles in the formation of Caenorhabditis elegans NMJs [11]. Mutations in nid-1 cause a disruption in the size, shape and function of these synapses by disrupting the accumulation of the LAR receptor tyrosine phosphatase, PTP-3A, and the intracellular-adaptor protein α-liprin/SYD-2 at active zones [12].

During a screen for nid-1 genetic modifiers we recovered an allele of unc-2, which encodes the single C. elegans Ca_{2+}-like α1 subunit [13]. Loss-of-function (LOF) mutations in unc-2 result in presynaptic contacts being slightly enlarged, but also in suppression of the defects caused by the nid-1 mutation. Using time-lapse analysis we show that GABAergic NMJs exhibit dynamic shape changes during the late 4th larval stage (L4), and that new NMJs can be formed by the elongation and division of established presynaptic domains. These dynamic changes were dependent on functional UNC-2. We also find that unc-43, the C. elegans Calmodulin kinase II (CaMKII) homolog, regulates GABAergic synapse formation. LOF in unc-43 suppresses nid-1, while a gain-of-function (GOF) mutation in unc-43 causes nid-1-like defects in NMJ morphology. Our results find a novel interaction between the ECM and VGCCs, where, during synaptic development, they appear to function antagonistically.

Results and discussion

unc-2 regulates the size, shape and morphology of presynaptic domains

We have found that the ECM protein nidogen, nid-1, affects the morphology and function of C. elegans NMJs [11,12]. We visualized GABAergic NMJs using a SNB-1::GFP (synaptobrevin) chimeric marker, juIs1 [14], as an indicator of presynaptic size and placement. In young adult (yAd) wild type (wt) animals GFP-labeled synaptic vesicles cluster in discrete, regularly sized puncta that have a smooth morphology (Figure 1A), with an average area of 0.81 ± 0.01 μm² (Mean ± S.E.M.). Strong LOF nid-1(cg119) animals have a synapse defective (Syd) phenotype such that fluorescent puncta often exhibit a rough or disorganized appearance and have an apparent area of 1.45 ± 0.08 μm² (P <0.05) (Figure 1B).

To better understand how nidogen affects NMJs, we conducted a screen for genetic modifiers of the nid-1 phenotype (see Methods). We recovered ju493, which appeared to largely suppress the synaptic vesicle accumulation defects present in nid-1(cg119) animals (Figure 1C). By themselves, the ju493 animals were uncoordinated (Unc), and also displayed synaptic morphology defects (Figure 1D). We mapped the allele to the left arm of linkage group X, and found ju493 failed to complement unc-2(e55), a strong LOF allele [13]. By PCR amplification and sequence analysis, we found that ju493 animals had a large deletion within the unc-2 coding region, suggesting that it is likely a strong LOF allele (Figure 1K). unc-2 is one of three C. elegans genes encoding VGCC α1 subunits. We tested LOF alleles in the other two VGCC, egl-19 and cca-1, and did not observe any suppression of nid-1 defects [See Additional file 1: Figure S1], indicating the observed effect is specific to unc-2.

We further characterized the effect of unc-2 LOF using the e55 allele, which causes a premature stop codon in exon 9 (Figure 1K). Presynaptic domains of unc-2(e55) mutants had smooth and oval-shaped SNB-1::GFP puncta, but the area of the puncta was significantly increased over wt by approximately 20% (1.04 ± 0.07 μm², P <0.05) (Figure 1E) in yAd animals. We also found that the total number of presynaptic clusters was slightly reduced in unc-2(e55) mutants (wt 25.6 ± 0.2 puncta/100 μm versus unc-2 22.8 ± 0.7 puncta/100 μm (P <0.05)). Like ju493, the e55 mutation suppressed vesicle-accumulation defects found in nid-1(cg119) (Figure 1F). Puncta formed in nid-1;unc-2 double mutants had an
**Figure 1 unc-2 mutations suppress SNB-1::GFP defects in nid-1.** (A) In wild type animals synaptic vesicles accumulated in evenly spaced puncta (arrow) with smooth morphology (arrowhead). Cell bodies (asterisks) are also visible. Scale bar equals 10 μm. (B) Animals lacking nidogen accumulated synaptic vesicles in elongated (arrow) puncta. Diffuse GFP was often present in regions between puncta (arrowhead). (C) The ju493 mutation suppressed the large aggregations of SNB-1::GFP seen in nid-1 mutants. (D, E) Mutations in unc-2 (ju493, e55) resulted in slightly larger puncta, but the morphology and distribution were more similar to wild type than nid-1, even when nid-1 was absent (F). (G) unc-13(s69) animals had a slight decrease in the number of puncta, but the morphology was not grossly affected. (H) In the nid-1(cg119);unc-13(s69) double mutants puncta were elongated similar to nid-1 single mutants. (I, J) We quantified puncta area (K) and circularity (L) for each analyzed genotype and plotted them as mean (circle) with the 5 to 95% confidence interval (whiskers). (K) A cartoon of the genomic region of unc-2 with the position of alleles used indicated. Exon/introns are to scale, except for dashed lines, which indicate larger introns. The e55 allele is a G > A nucleotide change in exon 9 that results in Q571 to stop (Wormbase: CE42155). The ju493 deletion removes a large part of the coding segment, corresponding to the exons encoding AA144-1888. All animals in these micrographs are young adults (1 to 2 days post 4th larval stage (L4)).
average area of 1.15 ± 0.10 μm² (P < 0.05 versus nid-1 or wt; P > 0.05 versus unc-2), and appeared morphologically similar to wt.

To more quantitatively assess the NMJ morphology, we calculated the circularity of the SNB-1::GFP fluorescent puncta (Figure 1J) (see Methods). Wild type SNB-1::GFP puncta are ovoid, and have an average circularity of 0.70 ± 0.01. By comparison, in unc-2(e55) the puncta varied slightly, but significantly, from wt (0.66 ± 0.03, P < 0.05), while in nid-1 mutants, the puncta were more elongated and less circular (0.60 ± 0.01, P < 0.05). The nid-1;unc-2 double mutants were like wt (0.74 ± 0.02, P < 0.05 versus nid-1 or unc-2; P > 0.05 versus wt), indicating that the elongated morphology in nid-1 was ameliorated by removing unc-2 function.

UNC-2 is the single Ca,2-like VGCC α1 subunit present in C. elegans, and is localized to the presynaptic active zone [15]. At synapses UNC-2 regulates calcium ion entry that facilitates synaptic vesicle exocytosis [16]. It is the reduced neural transmission from motor neurons to muscles that likely results in the reduced locomotor activity and the Unc phenotype. To address that it is the reduced neural transmission from motor neurons are added after the first larval stage, axons of these neurons, to accommodate the increase in body size, must grow and add synapses accordingly. Since all previous observations were made in yAd animals, we examined synaptic puncta of wt animals in the early, mid and late L4 stages, using the maturation of the vulva as a guide for the developmental stage [22]. In the early L4 stage, SNB-1::GFP puncta in GABAergic motor neurons are added after the first larval stage, axons of these neurons, to accommodate the increase in body size, must grow and add synapses accordingly. Since all previous observations were made in yAd animals, we examined synaptic puncta of wt animals in the early, mid and late L4 stages, using the maturation of the vulva as a guide for the developmental stage [22]. In the early L4 stage, SNB-1::GFP puncta in GABAergic motor neurons appeared of normal size (0.79 ± 0.05 μm²) (Figure 2A). The average area of the puncta noticeably increased starting during the mid-L4 stage (0.87 ± 0.08 μm²) and through the late-L4 stage (1.27 ± 0.08 μm²), and the puncta shapes were frequently found to be elongated, often with a disorganized perimeter (Figure 2B, C). Elongated, disorganized puncta were infrequently observed in yAd stage animals (0.81 ± 0.01 μm²) (Figure 2D), indicating that in wt, presynaptic domains have developmentally dynamic morphologies.

unc-2 mutants displayed fewer presynaptic domains than wt animals (Figure 1). We wondered if this was an effect of the failure to establish synaptic domains during development or the deterioration of existing connections. We found that the puncta in early (0.90 ± 0.05 μm²) and mid L4 (0.79 ± 0.04 μm²) were evenly sized and regularly shaped (Figure 2E, F). However, in late L4 unc-2(e55) animals, enlarged puncta were observed, although to a lesser degree than wt (1.07 ± 0.06 μm²), but did not appear disorganized. Similarly sized puncta were observed in young unc-2 adults (1.04 ± 0.07 μm²) (Figure 2H).

To assess the frequency of enlarged puncta relative to the total number of presynaptic domains observed, we binned individual puncta measured into categories, based on measured area (Figure 3). During the late L4 stage, approximately 35% of the puncta in wt animals appeared enlarged, with an area greater than 1.4 μm² (approximately nid-1-like), compared to yAd, where less than 15% of puncta fit into that category. The increased number of enlarged puncta came at the expense of normal-sized puncta, suggesting a natural size change occurs during this period. By comparison, there was no change in the proportion of the population (26%) of
puncta that measured larger than 1.4 μm² in unc-2(e55) when we compared late L4s to yAds (Figure 3).

To investigate more precisely the dynamic changes in synapse morphology during the late-L4 stage, we performed time-lapse confocal microscopy on live, non-anesthetized animals (see methods). In wt animals, we saw the following dynamic behaviors: SNB-1::GFP puncta forming de novo, puncta that disappeared, puncta that changed shape by increasing and decreasing in size, and at a low frequency, an existing punctum divided such that two puncta were generated (Figure 4A-C). Overall, per segment of the nerve cord examined (approximately 150 to 200 μm), a net increase of 1 puncta/hour in wt animals was observed.

By contrast in unc-2(e55) animals we found little evidence of dynamic shape changes. Puncta still formed de novo, puncta did enlarge, but rarely did those enlarged puncta shrink or divide (Figure 4D-F) as was seen in wt. Overall, per segment of the nerve cord examined unc-2 mutants displayed a net loss of approximately 3 NMJ puncta/hour under our time-lapse protocol. It is not clear whether these were budding events that initiated and failed, or were delayed. The decreased number of puncta seen by GFP imaging may be due to a failure in unc-2 mutants to add and/or maintain synapses during development. Alternatively, our observations are also consistent with a significant delay in synaptic development, although not a total failure of synaptic addition.

Due to the presence of de novo appearance of SNB-1::GFP puncta in unc-2(e55) mutants during the time-lapse imaging, we infer the observed synaptic dynamics likely relies on UNC-2 function after, not during, initial NMJ formation. Further, preliminary analyses indicate that synapses formed in the L3 stage of unc-2 animals can also be enlarged, indicate the function of UNC-2 is not confined to the L4 developmental stage.
unc-36 genetically interacts with unc-2 to regulate neuromuscular junction morphology

Mutations in unc-36, one of two VGCC α2/δ subunit genes in the *C. elegans* genome, often phenocopy unc-2 [15,23,24], so we tested if unc-36 was required for normal synaptic morphology. Similar to unc-2, unc-36(e251) caused an increase in SNB-1::GFP area in yAd animals (1.10 ± 0.07 μm²) (*P* < 0.05) (Figure 5A). A slight, but not significant, decrease in puncta number (23.1 ± 1.5) (*P* > 0.05) was also observed. unc-36(e251);nid-1(cg119) double mutants appeared like unc-36 synapses alone, with an apparent area of 1.11 ± 0.07 μm² (*P* < 0.05 compared to nid-1(cg119) and *P* > 0.05 versus unc-36(e251)) (Figure 5B). Double mutants of unc-2 and unc-36 had puncta that resembled each of the single mutants (1.08 ± 0.05 μm², *P* > 0.05 versus unc-2 or unc-36 alone) (Figure 5C). unc-2(e55);unc-36(e251);nid-1(cg119) triple mutants displayed no significant differences from either of the double mutant combinations (1.15 ± 0.06 μm², *P* > 0.05 versus unc-2;nid-1 or unc-36;nid-1) (Figure 5D). unc-36 was also able to suppress ptp-3A defects (1.50 ± 0.09 μm² (*P* < 0.05) versus unc-36(e251);ptp-3A(tm352) = 0.80 ± 0.10 μm² (*P* < 0.05) (Figure 5E,F). From these data,
we concluded unc-2 and unc-36 likely function in a linear genetic pathway to interact with ptp-3A and nid-1 in synapse development.

We also tested the second C. elegans α2/δ subunit-encoding gene, T24F1.6/tag-180 [25]. A LOF allele, ok779, exhibited a minor effect on SNB-1::GFP area (0.73 ± 0.03 μm², P < 0.05) [see Additional file 1: Figure S1], and number (28.8 puncta/100 μm², P > 0.05) in yAd animals. tag-180(ok779);nid-1(cg119) double mutants were more like nid-1(cg119) than the ok779 (1.65 ± 0.12 μm², P < 0.05 versus nid-1 alone), indicating the suppression of nid-1 defects is specific to unc-36. Double mutants of ok779 with unc-2 (1.02 μm², P > 0.05 versus unc-2 alone) or unc-36 (1.14 μm², P > 0.05 versus unc-36 alone) similarly showed no significant change from the unc-2 or unc-36 single mutants.

Next we addressed whether the role of unc-36 is cell autonomous. We were able to rescue the Unc (not shown) and Syd (Figure 5G) defects in yAd unc-36(e251) animals by reintroducing a wild type copy of UNC-36 under the control of the endogenous promoter (N = 4 lines) (puncta area - 0.84 ± 0.03, P < 0.05 versus unc-36(e251)). The unc-36 promoter is broadly active in the animal, including the muscles and nervous system [15,25,26]. To determine whether unc-36 could be functioning cell autonomously, we specifically expressed UNC-36 solely in the D-type motor neurons in unc-36(e251);juIs1 animals. As expected, the locomotor defects were not rescued, but the size of the presynaptic puncta in these animals was significantly reduced relative to unc-36(e251) yAd animals lacking the transgene (0.69 ± 0.05, P < 0.05 versus unc-36(e251)) (Figure 5H) (N = 1
line), indicating that *unc-36* is capable of cell autonomously affecting the GABAergic NMJs.

α2/δ subunits have been implicated in synapse formation and development [10,27,28]. In the vertebrate central nervous system (CNS), thrombospondin (TSP) molecules act as ligands for α2/δ1. Overexpression of α2/δ1 results in increased synaptogenesis, while interfering with the TSP-α2/δ1 interaction inhibited synaptogenesis. The C-terminal region of TSP, that contains EGF repeats, binds directly to the von Willebrand factor-A domain (VWF-A) present in the α2 portion of the α2/δ1 protein [10]. Since NID-1 contains EGF repeats and localizes near NMJs [11,29], and UNC-36 has a VWF-A domain, it is possible that NID-1 might physically interact with UNC-36. However, at GABAergic NMJs, the loss of *nid-1* causes morphological defects that are suppressed by removing *unc-36*. Also, LOF in *unc-36* has a very modest reduction in synapses formed. Thus, it seems unlikely that NID-1 acts as a ligand for UNC-36 to promote synapse addition.

There is evidence from *Drosophila* that α2/δ subunits can have effects on synaptic development independent from the α1 subunits [28]. However, our data suggest *unc-36* and *unc-2* are acting in the same genetic pathway. Work from the Bargmann lab has demonstrated that UNC-36, along with the calcium channel chaperone CALF-1, is required for the localization of UNC-2 to the synaptic plasma membrane [15]. Thus, a simple explanation for the synaptic patterning defects would be a failure to direct UNC-2 to the synaptic plasma membrane in *unc-36(e251)* mutants. In this model, we would assume that UNC-2 is the key player required to drive changes in synaptic development, although we cannot rule out UNC-36 may have additional functions at NMJs.

**unc-36** function is required during the 4th larval stage period for synaptic development

Next, to address the temporal requirement of VGCC, we used the α2/δ-antagonist gabapentin [30] to acutely inhibit VGCC function specifically during the L4 stage of development. In cultured vertebrate neurons gabapentin inhibits the trafficking of α2/δ subunits from the endoplasmic reticulum to the plasma membrane [31]. Wild type early L4 animals were exposed to gabapentin (100 μM) for 24 hours. When imaged as yAds, these animals had a significant increase in SNB-1::GFP area (1.00 ± 0.06 μm², *P* < 0.05 versus vehicle alone - 0.83 ± 0.06) (Figure 5I). This suggests that gabapentin is phenocopying *unc-36* LOF, although since presynaptic domains in double mutants of *unc-36* with *tag-180* resembled those found in *unc-36* alone, we cannot rule out that gabapentin was broadly affecting α2/δ function. Gabapentin treatment of *nid-1*(*cg119*) mutant L4 animals, resulted in a significant decrease in the SNB-1::GFP area (0.96 ± 0.05 μm², *P* < 0.05 versus *nid-1* alone), indicating that acute gabapentin treatment of *nid-1*(*cg119*) animals during the L4 stage was able to, at least partially, suppress presynaptic defects (Figure 5J). We conclude from our analysis that the synaptic patterning that occurs in late L4 animals requires intact α2/δ function during that period, rather than being fixed in an earlier developmental event. *nid-1* mutants have defective presynaptic morphologies prior to L4, and these appear to be ameliorated by LOF in *unc-2*. Thus, we do not rule out a function for VGCCs prior to L4, and this result would suggest that synaptic morphologies are broadly dynamic during L4 development.

**CaMKII regulates synaptic morphology**

To identify molecules that might utilize changes in local calcium concentrations to shape presynaptic domains, we undertook a candidate-molecule approach. The first gene we examined was *unc-43/CaMKII*. CaMKII has been extensively linked to both pre- and postsynaptic development in multiple organisms (for review see [32,33]). *unc-43* has been shown to work downstream of *unc-2*, and *unc-43* localization is dependent on UNC-2. CaMKII proteins have been shown to affect synapse development in multiple systems, including *C. elegans* where *unc-43* functions in the synaptic development of the glutamatergic neurons [34-36]. Because the complete loss of *unc-43* is embryonic lethal, we used a hypomorphic LOF allele, *e408*. *e408* yAd animals have slightly enlarged SNB-1::GFP (Figure 6A) (1.03 ± 0.08 μm², *P* < 0.05 versus wt). Thus, reduced *unc-43* function has a similar manifestation at synapses as the loss of *unc-2* and *unc-36*.

Like LOF in *unc-2* or *unc-36*, the *unc-43(e408)* LOF suppressed defects present in *nid-1* (Figure 6B) (1.06 ± 0.07 μm², *P* < 0.05 versus *nid-1*) and *ptp-3A* yAds (*ptp-3A*(*ok244*)) (1.03 ± 0.12 μm², *P* < 0.05 versus *ptp-3A*(*ok244*)) (1.42 ± 0.09 μm²). In contrast, *unc-43(m498)* GOF [37], bearing a constitutively activating mutation, E108K, in the active site core [35], SNB-1::GFP puncta were elongated and disorganized in yAds (1.26 ± 0.11 μm², *P* < 0.05 versus wt) (Figure 6C), and similar to *nid-1* (*P* > 0.05 versus *nid-1*).

We next examined whether *unc-43* was functioning cell autonomously by expressing an mCherry-tagged *unc-43* cDNA, under the control of the *unc-25* promoter, in the *unc-43(e408); juIs1* animals. Replacing UNC-43D (Wormbase: CE28054) specifically in the GABAergic neurons rescued the defects in *e408*, (0.83 ± 0.05 μm², (N = 6 lines) *P* > 0.05 versus wt and *P* < 0.05 versus *e408*). The RFP::UNC-43 chimera was localized throughout the cytoplasm, including being present at synapses, as evidenced by co-localization with the SNB-1::GFP (Figure 6D-G). The localization of UNC-43
suggests it could be directly functioning at NMJs to locally affect morphology.

**Conclusions**

**VGCCs contribute to synaptic development in *Caenorhabditis elegans***

Here we show that mutations in the single Cav2, **unc-2**, result in changes to the number, size and shape of presynaptic domains that form during development. The overall reduction in the number of synapses formed in **unc-2** mutants is modest, suggesting that the primary role of VGCCs is not to promote *de novo* synapse addition. Rather, **unc-2** function is most evident in the maintenance of presynaptic morphology, as **unc-2** mutants display areas that are enlarged relative to wild type.

The function of **unc-2** in regulating presynaptic morphology is also observed when the synaptically associated adhesion molecules NID-1 or PTP-3A are absent. Removing UNC-2 from *nid-1* or *ptp-3* loss-of-function backgrounds ameliorates the mislocalization of presynaptic proteins and synaptic overgrowth in those mutants. This indicates that the morphological changes associated with the loss of NID-1-mediated adhesion require functional UNC-2, but independent of synaptic vesicle exocytosis.

**Regulation of adhesion and growth at synapses**

Synapses are dynamic structures that can be added or removed, or change shape to accommodate functional changes in neural networks and/or organismal growth. In Drosophila, transient changes in adhesion have been shown directly to permit switching between synaptic stability and synaptic growth [38-43]. Molecularly, both Fas2 and *discs large* (DLG) appear to be important for synaptic stability. Down regulation of Fas2-mediated adhesion by activation of CaMKII has been shown to specifically induce synaptic growth [40,42,43].

**DLAR**, the Drosophila PTP-3 homolog, has also been shown to complex with different ligands to switch between synaptic stability and synaptic growth [39]. In vertebrates, cell adhesion molecules like laminins and nidogens are required for NMJ maintenance. In cultured vertebrate neurons, regulation of liprin-α, via CaMKII, has been shown to regulate LAR to affect dendritic spine stability at excitatory synapses [40]. Overall, these findings suggest that changes in synaptic morphology that
Cooperative versus antagonistic interactions between the extracellular matrix and voltage-gated calcium channels in synaptic maintenance

Previous work has shown that the ECM and the VGCCs work cooperatively to stimulate synapse development [9,10,19]. For example, the synaptically associated laminin β2 subunit can directly bind to an extracellular loop in the VGCC α1 polypeptide. In cultured neurons, this interaction induces the clustering of synaptic vesicles at the binding site. However, neither the genetic ablation of either Ca,2.1 or laminin β2, nor disrupting the binding of these proteins in vivo results in a total failure in synapse formation [9]. Rather, a defect in synaptic growth and/or maintenance was observed in those animals. Similarly, NMJs in mice lacking nidogen-2 do form normally, but fail to develop, beginning to fragment around three weeks after birth, suggesting a role in synaptic growth and/or maintenance was observed in those animals. Thus, in contrast to the apparent co-operative interaction observed in vertebrates, our study indicates that the loss of unc-2 also limits synapses from elongating during specific periods of development or when nid-1 is absent. A simple way of thinking about this is that in the absence of UNC-2, NMJs are too stable, unable to respond to signals that instruct them to enlarge or shrink. This would be a novel finding for this class of VGCCs: that they function as key regulators of dynamic changes in synaptic morphology. Going forward our goal will be to identify how UNC-2 can regulate these seemingly distinct functions at NMJs.

Methods

Caenorhabditis elegans strains

All C. elegans strains were maintained at 20 to 22.5°C as described [45]. The following alleles were used in this report: N2 (var. Bristol), nid-1(cg119), unc-2(ju493), unc-2(e55), unc-36(e251), tag-190(ok779), egl-19(n582), eca-1(ad1650), ptp-3A(tm352), ptp-3A(tm352); ptp-3A(ok244), ptp-3(mu256), unc-43(n498), unc-43(e408), nid-1(cg119) rpm-1(ju44), unc-13(s69), tra-2(q276)/mnC1. The following integrated strain was used: juls1 [Punc-25::SNB-1::GFP]. Transgenic animals were generated by germ line transformation as described [46]. To conduct the ju493/ e55 non-complementation, tra-2(q276);juls1/+;ju493/+ XX males were crossed to unc-2(e55) hermaphrodites. Cross progeny were identified by presence of GFP (juls1), non-complementation was determined by presence of UncGfp animals.

Genetic modifier screen

nid-1(cg119) rpm-1(ju44);juls1 animals were mutagenized using 50mM ethane methyl sulfonate (EMS). F2 animals were scored for a hypercontracted uncoordinated phenotype that is observed in syd-2 rpm-1 double mutants. Individual HypUnc animals were allowed to self-fertilize to insure transmission of the phenotype. The animals were outcrossed to either nid-1(cg119) or rpm-1(ju44) single mutant backgrounds to determine the effect of new mutations.

Image analysis

Synapse morphology of D type neurons was visualized by juls1 [Punc-25 SNB-1::GFP]. All images were collected on either a Zeiss Pascal confocal microscope or an Olympus FV1000 confocal microscope equipped with Fluoview software (Olympus America Inc., Center Valley, PA USA). Images were acquired using multi-track parameters when necessary (unc-36 and unc-43 rescue), with either a 63X or 60X Plan-apochromat objective, respectively. Animals were anesthetized using 0.5% phenoxy-propanol (TCI America, Portland, OR U.S.A.) in M9 and mounted on 2% agarose pads. Measurements of SNB-1::GFP were as described with minor modifications [11]. All images were collected using the exact same microscope settings. Briefly, confocal images were projected into a single plane using the maximum projection and exported as a tiff file with a scale bar. Using Imagemj the files were converted to a binary image using ImageJ the files were converted to a binary image using
the threshold command, so that the binary image resembled the RGB image. A region of interest was drawn around the relevant region of the nerve cords. The following measurement options were selected: Area, Center of Mass, Circularity, Perimeter, Fit Ellipse, and Limit to Threshold. Scaling was set by measuring the scale bar. The “Analyze Particle” command was used with a minimum of four pixels and no maximum size. The following options were selected: Outline Particles, Ignore Particles Touching Edge, Include Interior Holes and Reset Counter. The resulting measurements were exported to Microsoft Excel and GraphPad Prism for statistical analysis. Comparisons of single mutants to the wild type were tested by Students two-tailed t-test, while double and triple mutant combinations were compared within the group using a Kruskal-Wallis test with a Dunn’s Multiple Comparison post hoc test. Circularity is a measure of how close to a perfect circle an object is, where 0 is a line and 1 is a perfect circle. The test relates the area of the observed object to the area of a circle with the same radius:

\[
\text{Circularity} = \frac{4\pi \times \text{area}}{\text{perimeter}^2}
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**UNC-36 rescue**

To rescue the unc-36(e251) defect we generated a PCR product including the putative promoter and endogenous 3′UTR using the following primers (unc-36promF1: 5′-ccagcagttgaagaagtgcac-3′ and unc-36 3′UTR R1: 5′-caaggcagtggaaagtgcac-3′). The PCR product was TOPO cloned into pCRXLI (Life Technologies, Grand Island, NY USA) to generate pBA234. pBA234 was injected at 10 ng/μl into unc-36(e251) juIs1 animals along with pPD118.33 (Pmyo-2::gfp) as a co-injection marker, plus pBA186 (Punc-25::mCherry) to mark the GABAergic motor neurons containing the transgene.

**Cell-specific rescue**

A genomic fragment covering the unc-36 coding region was amplified using the following primers (unc-36promF1: 5′-ccagcagttgaagaagtgcac-3′ and unc-36 3′UTR R1: 5′-caaggcagtggaaagtgcac-3′). The PCR product was T/A cloned into the pCR8/GW/TOPO vector (Life Technologies), and then was recombined using L/R clonase (Life Technologies) into pBA153, creating pEVL404 (Punc-25::unc-36). pEVL404 was injected into unc-36(e251) juIs1 animals at 10 ng/μl along with pPD118.33 (Pmyo-2::gfp) as a co-injection marker. For unc-43, we isolated a full-length cDNA for UNC-43D isoform by RT-PCR from wild type RNA isolated by Trizol using the following primers (unc-43cDNA F1: 5′-atgatgaacgcaagcaca-3′ and unc-43cDNA R1: 5′-ctagaattctgaactttgattttg-3′). Using the InFusion (Clontech Laboratories, Inc., Mountain View, CA USA) enzyme this product was recombined into pEVL387 (Punc-25::mCherry::unc-43 3′ UTR) to generate pEVL400 (Punc-25::mCherry::unc-43E::unc-43 3′ UTR). The pEVL400 plasmid was injected into unc-43(e408) juIs1 at 5 ng/μl. Any additional information about sequences or cloning procedures is available upon request.

**Time-lapse analysis**

L4 animals were immobilized on 10% agarose pads in the presence of 5% (w/v) polystyrene beads (Bangs Laboratory, Fishers, IN USA). Animals were imaged at 5-minute intervals for 1 hour. Animals that died during the acquisition process (as determined by a rapid and dramatic increase in intestinal autofluorescence) were excluded from the analysis. Images were then exported to ImageJ. Z-stacks were produced for each time point, and then times 0 and 60 minutes were thresholded, converted to masks and overlaid as false colored, green and red respectively. This allowed simple determination of spots that were added or removed, grew or shrank or divided during the analysis period. The total number of puncta added/lost during the hour session was determined by comparing initial time and final time points for the appearance or disappearance of puncta.

**Pharmacology**

Gabapentin (Sigma Aldrich, St. Louis, MO USA) was resuspended in DMSO at 100 mM, and then diluted in 1:10 in PBS and added to standard NGM plates seeded with OP50 E. coli to achieve the final desired concentration. Using plates with an increasing dose of gabapentin, we found that animals reared throughout development on NGM plates containing 100 μM gabapentin phenotypically resembled unc-36, appearing thin, with poor movement (data not shown), thus we used this concentration for our experiments. Plates were permitted to dry overnight and then 20 L4 animals of each genotype were placed on the media. Animals were imaged 24 hours later (as young adults).

**Additional file**

**Additional file 1: Figure 1.** Specificity of interaction between unc-2/unc-36 and nid-1. (A) The egl-19 LOF allele, n582, animals had normal appearing SNB-1::GFP puncta. (B) In egl-19(n582); nid-1(e119) the puncta appear enlarged and disorganized. (C) A deletion in the Cav-3-like channel, cca-1, had no obvious effect on SNB-1::GFP morphology. (D) Removing cca-1 from nid-1 mutants did not suppress the morphological changes, as puncta were observed to be elongated and disorganized. (E) Loss of the second α2/δ subunit, tag-180, had only a modest effect on SNB-1::GFP puncta, but did not suppress the defects found in nid-1 mutants. (F) (G) A plot of the puncta area measured by genotypic as mean (circle) with the 5 to 95% confidence interval (whiskers). All animals in these micrographs are young adults (1 to 2 days post larval stage 4 (L4)).
Abbreviations
CaMKII: calcium-modulin kinase II; CNS: central nervous system; ECM: extracellular matrix; GABA: y-amino butyric acid; GOF: gain of function; LOF: loss of function; L4: 4th larval stage; NMJ: neuromuscular junction; S.E.M.: standard error of the mean; Syd: synaptic defective; Unc: uncoordinated movement; VGCC: voltage-gated calcium channel; wt: wild type.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RCC and BDA carried out the molecular, genetic and cell biological studies and collected and analyzed the data. YJ and BDA designed and conducted the genetic screen to isolate jur493. BDA drafted the manuscript. All authors have read and approved the final manuscript.

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