The cell polarity scaffold Lethal Giant Larvae regulates synapse morphology and function

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Summary
Lethal Giant Larvae (LGL) is a cytosolic cell polarity scaffold whose loss dominantly enhances neuromuscular junction (NMJ) synaptic overgrowth caused by loss of the Fragile X Mental Retardation Protein (FMRP). However, direct roles for LGL in NMJ morphological and functional development have not before been tested. Here, we use confocal imaging and two-electrode voltage-clamp electrophysiology at the Drosophila larval NMJ to define the synaptic requirements of LGL. We find that LGL is expressed both presynaptically and postsynaptically, where the scaffold localizes at the membrane on both sides of the synaptic interface. We show that LGL has a cell autonomous presynaptic role facilitating NMJ terminal branching and synaptic bouton formation. Moreover, loss of both presynaptic and postsynaptic LGL strongly decreases evoked neurotransmission strength, whereas the frequency and amplitude of spontaneous synaptic vesicle fusion events is increased. Cell-targeted RNAi and rescue reveals separable pre- and postsynaptic LGL roles mediating neurotransmission. We show that presynaptic LGL facilitates the assembly of active zone vesicle fusion sites, and that neuronally targeted rescue of LGL is sufficient to ameliorate increased synaptic vesicle cycling imaged with FM1-43 dye labeling. Postsynaptically, we show that loss of LGL results in a net increase in total glutamate receptor (GluR) expression, associated with the selective elevation of GluRIIB subunit-containing receptors. Taken together, these data indicate that the presynaptic LGL scaffold facilitates the assembly of active zone fusion sites to regulate synaptic vesicle cycling, and that the postsynaptic LGL scaffold modulates glutamate receptor composition and function.

Key words: Synaptogenesis, Bouton, Neurotransmission, Active zone, Synaptic vesicle cycle, Glutamate receptor, Neuromuscular junction, Drosophila

Introduction
Lethal Giant Larvae (LGL) was initially characterized as a Drosophila tumor suppressor (Gateff and Schneiderman, 1969), but has since been implicated in diverse neuronal functions, including the regulation of asymmetrical cell divisions and targeted vesicle trafficking (Ohshiro et al., 2000; Bilder et al., 2000; Wang et al., 2011a). At the Drosophila neuromuscular junction (NMJ), genetic interaction between lgl and dfmr1 dominantly enhances the synaptic overgrowth caused by loss of the Fragile X Mental Retardation Protein (FMRP) (Zarnescu et al., 2005), a well-known translational regulator of synapse development, function and plasticity (Tessier and Broadie, 2012). LGL and FMRP also co-localize in mouse hippocampal cultures, and can be reciprocally co-immunoprecipitated from both mammalian and Drosophila cells (Zarnescu et al., 2005). Mammalian LGL1 selectively associates with the cortical membrane of developing axonal projections in hippocampal cultures, but is absent in developing dendrites (Wang et al., 2011a), likely reflecting conserved LGL roles in the differentiation of neuronal polarity (Rolls et al., 2003; Atwood and Prehoda, 2009; Haenfler et al., 2012). LGL1 overexpression increases axonal length, whereas RNAi knockdown causes axonal undergrowth (Wang et al., 2011a). Genetic and biochemical analyses indicate mammalian LGL1 directly interacts with Rab10 GTPase to deliver membrane vesicles to developing axonal projections (Wang et al., 2011a).

LGL is a membrane-associated scaffold that interacts with Discs Large (DLG) and Scribbled scaffolds, and apical Par6/aPKC complexes, to establish asymmetric polar determinant localization required for Drosophila neuroblast differentiation (Rolls et al., 2003; Atwood and Prehoda, 2009; Haenfler et al., 2012). Phosphorylation by apical aPKC disrupts the LGL binding interaction and dissociates it from the apical cortex, establishing LGL basal polarity (Betschinger et al., 2003; Betschinger et al., 2005). In mammals, the LGL1 scaffold physically interacts with myosin-IIA and the actin cytoskeleton (Betschinger et al., 2005). During active cell migration, the F-actin-associated LGL1 is cortically localized to focal adhesions where it inhibits assembly of myosin-IIA filaments (Dahan et al., 2012). Thus, LGL is a conserved membrane- and cytoskeleton-associated scaffold functioning in diverse cellular contexts to asymmetrically localize proteins and establish polarized structures. By extension, we hypothesized similar LGL mechanisms at neuronal synapses could drive the polarized vesicle delivery and molecular assemblies underlying direction al structural growth and the localized exocytosis mediating neurotransmission.

In this study, we use confocal imaging and two-electrode voltage-clamp electrophysiological recording to test LGL roles in the structural and functional differentiation of the Drosophila glutamatergic NMJ synapse. We show LGL has a polarized distribution in motor neurons and muscles, where it is membrane-associated in pre- and postsynaptic compartments, respectively.
We show LGL positively regulates both synaptic morphological growth and overall neurotransmission strength, while limiting the frequency and amplitude of vesicle fusion events at individual synapses. We show that targeted RNAi and Gal4/UAS-mediated rescue of LGL function reveals separable pre- and postsynaptic requirements. Presynaptically, LGL promotes NMJ morphological growth, active zone differentiation and synaptic vesicle cycling efficacy. Postsynaptically, LGL mediates glutamate receptor (GluR) abundance and subunit composition. Together, these results establish that the membrane-associated LGL scaffold functions on both sides of the synaptic interface to modulate NMJ morphogenesis and neurotransmission strength.

Results

LGL is localized at both pre- and postsynaptic membranes of the NMJ

Null lgl larvae (supplementary material Fig. S1) exhibit behaviors characteristic of mutants with impaired motor function including reduced escape movement behaviors elicited by noxious heat stimuli (supplementary material Fig. S2). Null lgl mutants also grow much more slowly in the presence of their heterozygous siblings than when reared as fully mutant populations, suggesting impaired competitive fitness. These traits are characteristic of the large number of previously analyzed mutants with defective neuromuscular junction (NMJ) function, suggesting that LGL is likely present at the NMJ, mediating mechanisms important for functional movement output. To test this hypothesis, we first used a characterized anti-LGL antibody (Ohshiro et al., 2000; Zarnescu et al., 2005) to probe LGL expression in the neuromusculature. We confirmed antibody specificity and the loss of LGL in our array of lgl mutant alleles by Western blot, showing the single ~110 kDa LGL band completely eliminated in null mutants (supplementary material Fig. S1). To examine LGL expression in the neuromusculature, wandering 3rd instars were triple-labeled for LGL, the neuronal marker horseradish peroxidase (HRP) and either (1) the presynaptic active zone protein Bruchpilot (BRP) or (2) the primarily postsynaptic subsynaptic reticulum scaffold Discs Large (DLG). These data are summarized in Fig. 1.

The LGL scaffold is abundantly expressed at NMJ synapses, with little or no detectable expression outside of the synaptic domain (Fig. 1A,B). Specifically, NMJ synapses exhibit robust LGL expression that co-localizes closely with the presynaptic HRP membrane marker throughout the synaptic arbor (Fig. 1A). High magnification images of synaptic boutons show LGL within the presynaptic domain demarcated by the HRP membrane marker (white outline), in close association with BRP-marked active zones (red). LGL is detected near the presynaptic membrane and is not observed in the synaptic bouton interiors (Fig. 1A). These results suggest that LGL is membrane-associated in presynaptic boutons. Co-labeling with the postsynaptic marker DLG indicates that LGL is also present in the postsynaptic domain (Fig. 1B). LGL is clearly expressed in the muscle outside of the HRP-labeled boutons (white outline), where the protein is strongly enriched only in the NMJ persynaptic region within the postsynaptic domains immediately apposing the synaptic boutons (Fig. 1B). LGL appears closely membrane-associated with an expression pattern largely overlapping the well-characterized postsynaptic membrane scaffold DLG (red). We conclude that LGL is enriched in the pre- and postsynaptic compartments of the NMJ, where it is membrane-associated in close proximity to BRP-labeled presynaptic active zones and DLG-labeled postsynaptic domains.

LGL promotes the growth and structural differentiation of NMJ synapses

Recent studies demonstrate a role for mammalian LGL1 in promoting axonal morphological development (Wang et al., 2011a), and aberrant synaptic morphology arises from the genetic interaction between lgl and dfmr1 at the Drosophila NMJ (Zarnescu et al., 2005). We therefore first tested whether loss of LGL affects synaptic architecture, and observed a striking reduction in NMJ growth and complexity in null lgl alleles (Fig. 2A). To confirm that the structural defects were due to the loss of LGL, we used the Gal4/UAS system (Brand and Perrimon, 1993) to reintroduce wild-type LGL into the mutant (lgl<sup>1<sub>/214a</sub></sup>) background with the ubiquitous uh1-Gal4 driver (Rohrbough and Broadie, 2010). This transgenic expression in otherwise null animals completely rescued NMJ architecture. Synaptic branches were defined as BRP/HRP positive projections consisting of ≥2 boutons. At the muscle 6/7 NMJ, the number of synaptic branches was reduced by ~50% in lgl mutants and was fully rescued by the uh1-Gal4 mediated rescue of LGL in the lgl<sup>1<sub>/214a</sub></sup> mutant background; normalized χ<sup>2</sup> test control 1.00±0.08 (n=12) compared with 0.52±0.05

A

B

Fig. 1. LGL expression at the neuromuscular synapse. LGL antibody labeling in the wandering third instar synaptic expression at neuromuscular junctions. (A) Muscle 6/7 NMJ triple-labeled for LGL (green), neuronal membrane marker HRP (blue) and presynaptic active zone marker BRP (red). Low (left column) and high (right column) magnification images show LGL within the cortex of the HRP-positive presynaptic membrane (white outline; bottom right). LGL localizes near, but does not co-localize with, individual BRP active zone puncta. (B) Muscle 6/7 NMJ triple-labeled for LGL (green), HRP (blue) and the postsynaptic marker DLG (red). Low (left) and high (right) magnification images show LGL enriched in the postsynaptic domain, co-localizing with DLG, and also visible within the opposing HRP-positive presynaptic membrane (white outline; right).
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Fig. 2. LGL positively controls NMJ axonal growth and structural development. Null–lgl mutant NMJs exhibit a loss of axonal branches and synaptic boutons, which is rescued by transgenic expression of wild-type lgl. (A) Representative images of muscle 6/7 NMJs in genetic background control (w1118), two lgl mutant combinations, transgenic control (uh1-Gal4/+), and uh1-Gal4 mediated UAS-lgl expression (UAS-lgl-/+; lgl1/u334, uh1-Gal4/+). Low (top) and high (bottom) magnification images show loss of NMJ structure in mutants, rescued by reintroduction of wild-type lgl. Synaptic boutons are defined as BRP/HRP-positive varicosities >1 μM in minimum diameter. Synaptic branches are defined as any axonal process with ≥2 boutons. (B, C) Quantification of synaptic branch number (B) and bouton number (C) for lgl mutant conditions versus control and transgenic rescue conditions. Sample sizes of n=12, 12, 14 and 14 NMJs, respectively, for branch number and n=14, 14, 16, 16 and 14 NMJs, respectively, for bouton number. *P<0.05, **P<0.01, ***P<0.001.

(lgl1/4, n=12), and 0.64±0.07 (lgl1/u334, n=12), restored back to 0.99±0.05 (uh1-Gal4 Rescue, n=14) compared with 1.00±0.06 (uh1-Gal4/+; n=14) (ANOVA P<0.0001; Tukey–Kramer P<0.001 (w1118 versus lgl1/4), P<0.01 (w1118 versus lgl1/u334), P<0.001 (lgl1/4 versus uh1-Gal4) and P<0.01 (lgl1/u334 versus uh1-Gal4 Rescue); Fig. 2B; see supplementary material Table S1 for all pairwise comparisons). Boutons were defined as any BRP/HRP positive puncta >1 μM in minimum diameter. Consistent with the observed branching phenotypes, the number of type I boutons was concomitantly reduced in lgl mutants and rescued by ubiquitous reintroduction of LGL driven by uh1-Gal4; normalized w1118 control 1.00±0.09 (n=14) compared with 0.68±0.08 (lgl1/4, n=14) and 0.69±0.06 (lgl1/u334, n=16), restored back to 1.05±0.06 (uh1-Gal4 Rescue, n=14) compared with 1.00±0.04 (uh1-Gal4/+ control, n=16) (ANOVA P<0.0001; Tukey–Kramer P<0.05 (w1118 versus lgl1/4), P<0.05 (w1118 versus lgl1/u334), P<0.01 (lgl1/4 versus uh1-Gal4 Rescue) and P<0.01 (lgl1/u334 versus uh1-Gal4 Rescue) (Fig. 2C). Thus, we demonstrate a requirement for LGL in promoting the morphological differentiation of the NMJ synapse.

We next used the Gal4/UAS system for targeted expression to determine the cell-specific requirement for LGL in synaptic morphogenesis (Fig. 3). Using the muscle-specific 24b-Gal4 driver (Brand and Perrimon, 1993) to express UAS-lgl (Roegiers et al., 2009) in the postsynaptic muscle cells of otherwise lgl null mutants, we observed improved larval motility and growth rates similar to heterozygous siblings, even when reared in competition (data not shown). At the NMJ, however, postsynaptic LGL expression provided no improvement of synaptic morphology (Fig. 3A). Axonal branch number and synaptic bouton numbers were nearly identical to the lgl null condition despite the dramatic behavioral improvement. The normalized mean branch number for the 24b-Gal4/+ control was 1.00±0.06 compared with 0.66±0.04 for the 24b-Gal4 postsynaptic UAS-lgl expression condition (Fig. 3B). Likewise, normalized bouton number for the

Fig. 3. Presynaptic LGL is specifically required to regulate NMJ synaptic architecture. Targeted neuronal expression of wild-type lgl is necessary and sufficient to rescue lgl NMJ mutant architecture.

(A) Representative images of muscle 6/7 NMJs with presynaptic elav–Gal4 (elav–Gal4/UAS-lgl; lgl1/u334) and postsynaptic 24b-Gal4 driven expression of lgl (UAS-lgl-/+; lgl1/u334, 24b-Gal4/+), (B) compared with controls elav–Gal4/+ and 24b-Gal4/+ respectively. Low (top) and high (bottom) magnification images show loss of NMJ structure in mutants, rescued by reintroduction of neuronal UAS-lgl only. (B) Quantification of synaptic branch and bouton numbers with muscle UAS-lgl expression versus control (n=14 NMJs each; branch number, Mann–Whitney test, ***P<0.0001; bouton number, **P<0.01). (C) Quantification of branch and bouton numbers by neuronal UAS-lgl expression versus control (n=12 NMJs each; branch number, Mann–Whitney test, P=0.18; bouton number, P=0.97).
two independent homozygous null alleles (Gal4/+ controls was 1.00, was comparable to controls; normalized branch number for elav

recording, we assayed an allelic panel of whether the loss of LGL impacts NMJ neurotransmission function. Muscle postsynaptic domain (Fig. 1B), yet plays no discernible synaptogenesis. Moreover, LGL is abundantly expressed in the genetic background control. Compared with the control value of 1.00 ± 0.07 (n = 10), EJC amplitudes were 0.62 ± 0.07 (lglu334/w1118, n = 10), 0.62 ± 0.03 (lglu334/w1118, n = 7), and 0.64 ± 0.08 (lglu334/w1118, n = 9). This uniform functional defect was rescued to 1.02 ± 0.12 (uh1-Gal4 Rescue; n = 8) compared with the normalized transgenic control of 1.00 ± 0.04 (uh1-Gal4/+ n = 9) [ANOVA P < 0.0001; Tukey–Kramer P < 0.01 (w1118 versus lglu334/w1118), P < 0.05 (w1118 versus lglu334/w1118), P < 0.05 (uh1-Gal4 drive versus lglu334/w1118), P < 0.05 (uh1-Gal4 drive versus uh1-Gal4 Rescue), and P < 0.05 (lglu334/w1118 versus uh1-Gal4 Rescue) and P < 0.05 (lglu334/w1118 versus uh1-Gal4 Rescue); Fig. 4B]. Supplementary material Table S1 shows all pairwise comparisons. Taken together, these data show that specific loss of LGL results in a ~40% reduction in NMJ neurotransmission.

We next tested whether this functional defect was pre- or postsynaptic, using both UAS-lgl RNAi in the wild-type background and UAS-lgl expression in the lgl null mutant background. We confirmed RNAi efficacy by Western blot, showing ubiquitous uh1-Gal4 driven knockdown completely eliminated detectable LGL protein (supplementary material Fig. S3). Using the RNAi strategy, we knocked-down LGL in presynaptic neurons with the elav-Gal4 driver and postsynaptically with the muscle-specific 24b-Gal4 driver (Fig. 4A). In both cases, there was a clear decrease in synaptic transmission strength relative to controls. Moreover, neither neuron nor muscle UAS-lgl expression alone was able to restore normal transmission in the lgl null mutant. Compared with the normalized control 1.00 ± 0.06 (elav-Gal4/+; n = 6), the mean EJC amplitude for neuron-specific knockdown was 0.74 ± 0.04 (elav RNAi; n = 6) and neuron-targeted UAS-lgl expression 0.63 ± 0.08 (elav Rescue; n = 8), both highly significant reductions [ANOVA P < 0.005; Newman–Keuls P < 0.05]

Loss of LGL strongly compromises neurotransmission strength at the NMJ (Fig. 4A). In all lgl mutant allelic combinations, there is a clear reduction in EJC transmission amplitude compared with genetic control (w1118/+/), with no significant difference between any of the mutant conditions. Importantly, ubiquitous uh1-Gal4 driven expression of UAS-lgl in the lglu334/w1118 mutant background completely restored the evoked EJC response to control levels (Fig. 4A).

For a quantified comparison of these results between genotypes, EJC amplitude values were normalized to the w1118 genetic background control. Compared with the control value of 1.00 ± 0.07 (w1118/+/), EJC amplitudes were 1.00 ± 0.04 (lglu334/w1118, n = 10), 1.00 ± 0.07 (lglu334/w1118, n = 7), and 1.00 ± 0.08 (lglu334/w1118, n = 9) [Fig. 4B].

Fig. 4. LGL is required both pre- and postsynaptically for NMJ synaptic function. Two-electrode voltage-clamp (TEVC) muscle recordings of motor nerve-evoked excitatory junctional currents (EJCs) at the wandering third instar NMJ. (A) Representative TEVC records for the 12 genotypes shown. Each record shows five superimposed traces from an individual of each genotype. Null lgl mutants exhibit strong reduction in evoked synaptic response with both pre- and postsynaptic lgl requirements clear from target RNAi knockdown and rescue experiments. (B) Quantification of mean EJC amplitude in mutants compared with w1118/+/ genetic control and UAS-lgl/RNAi+; lglu334/w1118, uh1-Gal4/+ backgrounds (n = 10, 10, 7, 10, 9 and 8 NMJs, respectively). (C) Quantification of elav-Gal4 driven UAS-lgl expression and knockdown in motor neurons compared with the elav-Gal4/+ transgenic control (n = 6, 6 and 8 NMJs, respectively). (D) Quantification of UAS-lgl/+; lglu334/w1118, 24b-Gal4/+ and 24b-Gal4/lgl RNAi knockdown in muscle compared with 24b-Gal4/+ transgenic control (n = 8, 11 and 11 NMJs, respectively). *P < 0.05, **P < 0.01.
LGL regulates spontaneous vesicle fusion frequency and amplitude

To better separate pre- and postsynaptic requirements for LGL, we next recorded miniature EJC (mEJC) events caused by the spontaneous fusion of individual synaptic vesicles with the presynaptic membrane (Long et al., 2008; Rohrbough and Brodie, 2010). The frequency of these fusion events represents presynaptic vesicle release probability, whereas mEJC amplitude represents the functional availability of the postsynaptic glutamate receptors. These data are summarized in Fig. 5. Using the same lgl alleles as above, we surprisingly found that both mEJC frequency and amplitude were significantly increased compared with controls (Fig. 5A). Compared with the normalized genetic control 1.00 ± 0.07 (w1118; n = 13), mean mEJC frequency was 1.97 ± 0.19 (lglu334; n = 9), 1.86 ± 0.13 (lglu334; n = 13) and 1.71 ± 0.18 (lgl1; n = 13). This was a highly significant increase in mEJC frequency over control in all three lgl allelic combinations, with no difference between any of the mutants (Fig. 5B). Moreover, the elevated fusion frequency was completely rescued by uh1-Gal4 ubiquitous expression of LGL: 1.00 ± 0.13 (uh1-Gal4/+; n = 8) compared with 1.09 ± 0.13 (uh1-Gal4 Rescue; n = 9; Fig. 5B). Statistical comparisons between all six genotypes, as well as pair-wise analysis between all mutant and control genotypes, show these effects to be highly significant [ANOVA P < 0.0001; Tukey–Kramer < 0.001 (w1118 versus lglu334), P < 0.001 (w1118 versus lglu334; n = 9), P < 0.01 (w1118 versus lgl1; n = 8) (lglu334 versus uh1-Gal4 Rescue), P < 0.01 (lglu334 versus uh1-Gal4 Rescue) and P < 0.05 (lgl1 versus uh1-Gal4 Rescue) (Fig. 5B)]. Likewise, compared with the normalized control 1.00 ± 0.02 (w1118; n = 19), mean mEJC amplitudes were also significantly increased in all three lgl mutant combinations; 1.22 ± 0.02 (lglu334; n = 17), 1.19 ± 0.04 (lglu334; n = 20) and 1.19 ± 0.03 (lgl1; n = 18; Fig. 5C). This defect was also fully rescued by ubiquitous uh1-Gal4 expression of wild-type LGL in the null mutant background; 1.00 ± 0.02 (uh1-Gal4/+; n = 8) compared with 1.01 ± 0.03 (uh1-Gal4 Rescue; n = 9; Fig. 5C). Although the magnitude of the elevated mEJC amplitude was small, these changes were highly significant (Kruskal–Wallis P < 0.0001; Dunn’s post-test P < 0.001 (w1118 versus lglu334), P < 0.01 (w1118 versus lglu334; n = 9), P < 0.01 (w1118 versus lgl1), P < 0.01 (lglu334 versus uh1-Gal4 Rescue), P < 0.05 (lglu334 versus uh1-Gal4 Rescue) and P < 0.01 (lgl1 versus uh1-Gal4 Rescue) (Fig. 5C). Taken together, these data show that specific loss of LGL results in an elevation of both presynaptic release probability and postsynaptic responsiveness.

As with the evoked neurotransmission experiments, we next used tissue-specific UAS-lgl expression to test pre- versus postsynaptic cellular requirements. We found that elav-Gal4 driven neuronal UAS-lgl expression effectively restored mEJC synaptic vesicle fusion probability, but not mEJC amplitude (Fig. 5D,E). The normalized mean mEJC frequency was 1.00 ± 0.011 (elav-Gal4/+; n = 9) compared with 1.13 ± 0.16 (elav-Gal4/++; n = 8).
[elav-Gal4 rescue condition; n=11; P=0.62 (NS)], whereas normalized mEJC amplitude was 1.00±0.01 (elav-Gal4/+; n=9) compared with 1.15±0.07 (elav-Gal4 rescue condition; n=11; **P=0.01). In intriguing contrast, UAS-LGL expression in the postsynaptic muscle restored both mEJC frequency and amplitude compared with the control (Fig. 5D,E). The normalized mean mEJC frequency was 1.00±0.12 (24b-Gal4/+ control; n=9) compared with 0.94±0.011 (24b-Gal4 rescue condition; n=11; P=0.86 (NS)), and mean mEJC amplitude was 1.00±0.06 (24b-Gal4/+ control; n=9) compared with 0.94±0.01 (24b rescue condition; n=11; P=0.91 (NS)). Taken together, these measurements strongly support the separability of pre- and postsynaptic requirements for LGL in regulating neurotransmission function at the NMJ.

Presynaptic loss of LGL alters active zone vesicle fusion site morphology

To begin testing possible mechanisms underlying the synaptic requirement for LGL, we first examined the abundance and localization of the active zone protein Bruchpilot (BRP; Fig. 6A), a critical component of synaptic vesicle release sites (Kittel et al., 2006; Wagh et al., 2006; Fouquet et al., 2009). For quantification of paired control and mutant samples, we measured synaptic BRP immunofluorescence intensity, number of BRP active zone puncta normalized to area and BRP puncta diameter to assess active zone size. The total BRP abundance at the NMJ terminal was unchanged in all lgl mutants compared with control (Fig. 6A). To examine changes in distribution, BRP puncta number/area was averaged from separable puncta in type IB boutons (±3 μM in diameter) at the muscle 6/7 NMJ, dividing by the mean bouton area. This active zone density was increased by 16–20% in lgl mutants over control (Fig. 6A). Compared with the normalized control 1.00±0.03 (w1118; n=12), the lgl mutants were 1.20±0.04 (lgll/u334; n=12) and 1.16±0.02 (lgl/4; n=12; Fig. 6B). This change in active zones was fully rescued by uh1-Gal4 expression of LGL in the null mutant; 1.00±0.04 (uh1-Gal4/+; n=12) compared with 1.01±0.02 (uh1-Gal4 Rescue; n=12). Statistical comparisons between all genotypes and between all paired genotypes show significant differences [ANOVA P<0.0001; Tukey–Kramer P<0.01 (w1118 versus lgll/u334), P<0.05 (w1118 versus lgl/4), P<0.01 (lgll/u334 versus uh1-Gal4 Rescue) and P<0.05 (lgl/4 versus uh1-Gal4 Rescue) (Fig. 6B)]. Next, data were processed by the addition of a Guassian filter to a background threshold and the maximum diameter of individual BRP puncta was measured using ImageJ. The average BRP puncta diameter was found to be decreased by ~15–20% in lgl mutants normalized to control; 1.00±0.02 (w1118; n=12) compared with 0.80±0.01 (lgl/4; n=12) and 0.85±0.02 (lgll/u334; n=12; Fig. 6C). This defect in active zone size was also rescued by uh1-Gal4 LGL expression; 1.00±0.02 (uh1-Gal4/+; n=12) compared with 1.01±0.02 (uh1-Gal4 Rescue; n=12). Statistical comparisons show the effect on active zone size to be highly significant [ANOVA P<0.0001; Tukey–Kramer P<0.001 (w1118 versus lgl/4), P<0.001 (w1118 versus lgl/4), P<0.01 (lgl/4 versus uh1-Gal4 Rescue) and P<0.001 (lgll/u334 versus uh1-Gal4 Rescue) (Fig. 6C)]. Taken together, these data show that specific loss of LGL changes active zone density.
zone organization by increasing the average density but decreasing the average size of synaptic vesicle release sites.

Since total BRP levels were unchanged in lgl-null mutants, the above results indicate a LGL role in the localization/aggregation of the synaptic vesicle release machinery. We next wished to test whether this LGL role is cell-autonomous within the motor neurons, or results as a consequence or trans-synaptic signaling or compensation. A cell-autonomous requirement in the presynaptic neuron was found because targeted neuronal expression of wild-type LGL rescued active zone defects in lgl mutants, whereas postsynaptic muscle LGL expression had no impact on the mutant phenotypes (Fig. 6D). In these rescue experiments, the normalized mean BRP puncta number/area was 1.00±0.07 in control (elav-Gal4/+; n=10) compared with 1.03±0.03 with targeted neuronal expression [elav-Gal4 rescue condition; n=10; P=0.62 (NS); Mann–Whitney U-test; Fig. 6E], whereas the active zone size was 1.00±0.06 in control (24b-Gal4/+; n=12) compared with 1.17±0.04 with targeted muscle expression (24b-Gal4 Rescue; n=12; P=0.03; Mann–Whitey U-test; Fig. 6F). Likewise, normalized BRP puncta size was 0.95±0.01 [elav-Gal4 rescue condition; n=10; P=0.33 (NS); Mann–Whitney U-test; Fig. 6E] and 0.85±0.01 (24b-Gal4 rescue condition; n=12; P<0.01; Mann–Whitney U-test; Fig. 6F). Thus, there is a clear presynaptic role for the LGL scaffold in mediating the clustering/aggregation of active zone synaptic vesicle release sites, correlating with the presynaptic functional requirement for LGL in the regulation of neurotransmission strength.

**LGL modulates synaptic vesicle cycling efficacy**

The changes in presynaptic active zones predict possible alterations in the synaptic vesicle cycling underlying neurotransmission defects in lgl mutants. This is also a particularly important mechanistic question because removal of presynaptic LGL reduces evoked neurotransmission strength for the entire NMJ (Fig. 4), yet increases in the frequency of vesicle fusion at individual active zones (Fig. 5). To test synaptic vesicle cycling in lgl mutants, we used the lipophilic dye FM1-43 to visualize endo/exocytosis (Fig. 7; Huang et al., 2006; Long et al., 2008; Vijayakrishnan et al., 2009). The muscle 6/7 NMJ was exposed to 10 μM FM1-43 in saline containing 90 mM KCl, to depolarize the synaptic terminal and label endocytosed synaptic vesicles (Fig. 7A, load). Quantified comparisons showed that the fluorescent intensity of loaded terminals was unchanged in lgl mutants normalized to the w^{1118} control; 1.04±0.05 (lgl^{1/4}, n=10) and 0.98±0.04 (lgl^{lgl/u334}, n=14; ANOVA P=0.76 (NS); Fig. 7B). The efficacy of synaptic vesicle exocytosis was next determined by a second depolarization in the absence of FM1-43 (Fig. 7A, unload). In this case, the unloaded fluorescent intensity was significantly reduced by ~20–24% in lgl mutants normalized to control; 0.76±0.05 (lgl^{1/4}, n=10; P=0.01) and 0.80±0.04 (lgl^{lgl/u334}, n=14; Fig. 7C). Statistical comparisons confirmed a significant change between all genotypes and in pair-wise combinations [ANOVA P=0.02; Newman–Keuls P<0.05 (w^{1118} versus lgl^{1/4}), P<0.05 (w^{1118} versus lgl^{lgl/u334}) (Fig. 7C)]. The ratio of unloaded/loaded intensity normalized to control was 0.73±0.05 (lgl^{1/4}, n=10) and 0.84±0.04 (lgl^{lgl/u334}, n=14; Fig. 7D). This confirmed a significantly increased synaptic vesicle cycling rate in the absence of LGL [ANOVA P=0.008; Newman–Keuls P<0.01 (w^{1118} versus lgl^{1/4}), P<0.05 (w^{1118} versus lgl^{lgl/u334}) (Fig. 7D)]. We further found that targeted neuronal expression of wild-type UAS-lgl was sufficient to rescue the observed mutant defect in vesicle cycling. Compared with the normalized control (elav-Gal4/+; n=10), the unloaded/loaded intensity ratio was 0.94±0.05 in the elav rescue condition (n=10; P=0.68), showing rescue of the defect (Wilcoxon matched-pairs test; Fig. 7E). Together these data demonstrate that synaptic vesicle cycling is significantly elevated in the absence of presynaptic LGL.

![Figure 7](image-url)
Postsynaptic LGL regulates glutamate receptor abundance and subunit composition

In addition to presynaptic requirements, the LGL scaffold is abundantly present at the postsynaptic membrane (Fig. 1), where it modulates evoked neurotransmission strength (Fig. 4) and postsynaptic responsiveness to spontaneous vesicle fusion events (Fig. 5). These results strongly predict alterations in the postsynaptic glutamate receptor (GluR) domains in lgl mutants. To test this hypothesis, we used immunocytochemistry to analyze both overall GluR abundance and subunit composition. NMJ ionotropic GluRs are thought to be tetrameric, consisting of 3 essential subunits (GluRIIC, D and E) and a fourth variable subunit, GluRIIA or GluRIIB (DiAntonio et al., 1999; Marrus et al., 2004; Featherstone et al., 2005). Subunits A and B compete for inclusion within the tetramer complex and confer distinct physiological characteristics to the channel (DiAntonio et al., 1999). It has been established that the two GluR classes are regulated independently. For example, FMRP in conjunction with Drosophila metabotropic glutamate receptor A (DmGluRA) differentially regulates the expression of GluRIIA versus GluRIIB (Pan and Broadie, 2007), and the membrane scaffold DLG, a target of FMRP translational repression (Darnell et al., 2011), selectively regulates only GluRIIB and not GluRIIA (Chen and Featherstone, 2005). To test total GluRs and each GluR class, we used specific antibody probes for the common ionotropic GluRs that are thought to be tetrameric, consisting of 3 essential GluR subunits (GluRIIA, D and E) and a fourth variable subunit, GluRIIB (Pan and Broadie, 2007), and the membrane scaffold DLG, a target of FMRP translational repression (Darnell et al., 2011), selectively regulates only GluRIIB and not GluRIIA (Chen and Featherstone, 2005). To test total GluRs and each GluR class, we used specific antibody probes for the common ionotropic GluRs that are thought to be tetrameric, consisting of 3 essential subunits (GluRIIC, D and E) and a fourth variable subunit, GluRIIA or GluRIIB (DiAntonio et al., 1999; Marrus et al., 2004; Rohrbough and Brodie, 2010) to measure total GluR abundance in the NMJ postsynaptic domains (Fig. 8A). Consistent with the observed LGL role in regulating transmission strength and the increase in lgl mutant mEJC amplitudes, we observed an >40% increase in GluRIIC immunofluorescence intensity in lgl mutants normalized to w1118 genetic background control; 1.43 ± 0.05 (lgl1/4; n = 12) and 1.42 ± 0.05 (lgl1/334; n = 12) (Fig. 8B). This total GluR increase was restored to control levels by uh1-Gal4-driven UAS-lgl expression in the otherwise null mutant background; 1.07 ± 0.03 (uh1-Gal4 Rescue; n = 13) (Fig. 8B). Statistical comparisons between all genotypes and in post-test pairwise combinations showed significant changes (Kruskal–Wallis P < 0.0001; Dunn’s post-test P < 0.001 (w1118 versus lgl1/4), P < 0.001 (w1118 versus lgl1/334), P < 0.05 (lgl1/4 versus uh1-Gal4 Rescue) and P < 0.05 (lgl1/334 versus uh1-Gal4 Rescue) (Fig. 8B). Taken together, these data show that specific loss of LGL elevates total glutamate receptor abundance at the NMJ.

Testing cellular requirements for LGL in GluR regulation, we found a cell-autonomous postsynaptic role (Fig. 8A). Targeted postsynaptic GluRIIC expression was necessary and sufficient to rescue GluRIIC expression to control levels, whereas presynaptic LGL had no effect on the lgl mutant phenotype. Normalized GluRIIC levels remained elevated at 1.34 ± 0.09 with neuronal elav-Gal4 driven UAS-lgl expression (n = 12; P = 0.03; Mann–Whitney U-test; Fig. 8C), but was restored to 0.99 ± 0.06 with postsynaptic muscle 24b-Gal4 driven UAS-lgl rescue [n = 14; P = 0.97 (NS); Mann–Whitney U-test; Fig. 8D]. We next tested GluR subunit composition using GluRIIA and GluRIIB antibodies (Fig. 8E). Intriguingly, GluRIIA abundance at lgl mutant synapses was not significantly different from controls, whereas GluRIIB level was increased by approximately the same 60% with neuronal elav-Gal4 driven UAS-lgl expression (n = 12; P = 0.03; Mann–Whitney U-test) (Fig. 8E). The normalized GluRIIA intensity was 0.90 ± 0.05 (lgl1/4; n = 12) and 0.95 ± 0.06 (lgl1/334; n = 12; ANOVA P = 0.62, which is not significant). GluRIIB intensity was 1.36 ± 0.09 (lgl1/4; n = 12) and 1.31 ± 0.09 (lgl1/334; n = 12; ANOVA P = 0.62 (NS); Fig. 8F), whereas GluRIIB intensity was 1.30 ± 0.05 (lgl1/4; n = 12) and 1.40 ± 0.05 (lgl1/334; n = 12; ANOVA P = 0.01 (**)). (G) Quantification of GluRIIC intensity in the elav-Gal4/+ control versus UAS-lgl expression condition (n = 12 NMJs each; P = 0.03, Mann–Whitney test). (H) Quantification of GluRIIB intensity in 24b-Gal4/+ control versus UAS-lgl expression condition (n = 12 NMJs each; P = 0.05, Mann–Whitney test). **P < 0.01, ***P < 0.001.
n=12; ANOVA P=0.005; Newman–Keuls P<0.01 (w^{lgl1/4} versus w^{lgl1/u334}) and P<0.01 (w^{lgl1/4} versus w^{lgl1/4n}) (Fig. 8G). Taken together, these data indicate that the postsynaptic LGL scaffold is specifically required to regulate the localization of GluRIIB-containing receptors, and that the observed increase in total GluR abundance in lgl mutants is due to an increase in GluRIIB class receptors.

**Discussion**

Lethal Giant Larvae (LGL) is a well-characterized scaffolding protein with numerous WD40 protein–protein and cytoskeletal interaction domains, which functions in polarized protein recruitment to localized cellular compartments. Defined first as a tumor suppressor (Gateff and Schneiderman, 1969), LGL has been studied primarily in asymmetrical cell fate determinant localization during neural stem cell division (Bilder et al., 2000; Ohshiro et al., 2000; Betschinger et al., 2005; Atwood and Prehoda, 2009; Ogawa et al., 2009; Haenfler et al., 2012). In addition, loss of LGL has also long been known to dominantly enhance the loss of Fragile X Mental Retardation Protein (FMRP) in control of NMJ architecture (Zarnescu et al., 2005). Recently, mammalian LGL1 was shown to directly interact with Rab10 GTPase in polarized delivery of membrane-precursor vesicles to distal axonal projections (Wang et al., 2011a). Based on these observations, we asked whether LGL might control synaptic morphogenesis and neurotransmission, using the well-characterized *Drosophila* NMJ synapse model. Here, we report clearly separable pre- and postsynaptic roles for the LGL scaffold in axonal growth, branching and bouton formation; organization and function of presynaptic active zones; and the composition and function of postsynaptic glutamate receptors. Together, these LGL activities strongly modulate neurotransmission strength.

In *Drosophila* larvae, LGL displays strong expression in NMJ synapses, where it closely overlaps with the well-characterized DLG synaptic scaffold. Interestingly, both LGL and DLG scaffolds localize basal determinants in embryonic neuroblasts (Bilder et al., 2000; Ohshiro et al., 2000; Atwood and Prehoda, 2009; Ogawa et al., 2009; Haenfler et al., 2012) and, consistently, both scaffolds are similarly enriched in the muscle postsynaptic domain and neuronal presynaptic boutons, tightly associated with the membrane cortex on both sides of the synaptic interface. Global LGL loss reduces NMJ architectural complexity, with fewer axonal branches and synaptic boutons, which is rescued by neuron specific LGL expression, paralleling the presynaptic cell-autonomous FMRP requirement in synaptic morphogenesis (Gatto and Broadie, 2008). Global LGL loss also causes a ~40% decrease in neurotransmission strength, but this requirement has both pre- and postsynaptic elements. LGL negatively regulates the frequency and amplitude of spontaneous synaptic vesicle fusion events, and likewise controls these properties in separable pre- and postsynaptic roles. Presynaptic loss of LGL cell-autonomously alters the density and size of BRP-containing active zones, although total BRP levels are unchanged, indicating a localization function. Similarly, postsynaptic loss of LGL causes a cell-autonomous increase in GluR localization, correlated with the selective upregulation of GluRIIB-containing receptors. Together, these data demonstrate distinct scaffolding requirements for LGL on either side of the NMJ synapse. Since mammalian FMRP binds LGL1 mRNA (Darnell et al., 2011), these results suggest a possible regulatory pathway controlling synaptic modulation.

Classical interpretations of miniature event records suggest frequency reflects presynaptic fusion probability, amplitude reflects the number/function of postsynaptic receptors, and that these properties additively predict evoked neurotransmission strength (Fatt and Katz, 1952; Cull-Candy and Miledi, 1982). Miniature event recordings of lgl-null synapses indicate increased presynaptic vesicle fusion probability and postsynaptic glutamate receptor number but, conversely, evoked records show a strong reduction in neurotransmission. Our FM1-43 data show elevated vesicle cycling at the individual bouton level, which is consistent with the increased mEJC frequency. Although the mEJC and FM1-43 data seemingly contradict our EJC records, the simplest interpretation is that increased neurotransmission at the individual bouton level is simply insufficient to compensate for the severe loss of bouton numbers in lgl mutants. Alternatively, many studies indicate that the relationship between spontaneous and evoked vesicle release may not be straightforward (Sara et al., 2005; Mathew et al., 2008; Fredj and Burrone, 2009; Chung et al., 2010), and there are numerous precedents for a similar disconnect. For example, loss of the Synaptotagmin calcium sensor causes increased spontaneous vesicle fusion, but a dramatic decrease in evoked response (Broadie et al., 1994; Xu et al., 2009). Similarly, loss of Fuseless (Fusl), a protein required for presynaptic calcium channel localization, simultaneously causes increased mEJC frequency and decreased evoked transmission (Long et al., 2008). Likewise, LGL may function to differentially regulate spontaneous and evoked transmission events. As LGL binds both membrane-associated proteins and actin cytoskeleton (Betschinger et al., 2005), and acts to asymmetrically localize proteins (Atwood and Prehoda, 2009; Ogawa et al., 2009; Fletcher et al., 2012; Haenfler et al., 2012), we propose LGL similarly regulates neurotransmission by controlling growth and localizing/clustering membrane-associated vesicle release machinery (e.g. BRP). Intriguingly, *Drosophila* Rab3 GTPase also regulates presynaptic BRP-containing active zone morphology (Graf et al., 2009). Although Rab3 loss results in the opposite defects to loss of LGL, this relationship may have some bearing on the recently defined interaction between mammalian LGL1 and Rab10 GTPase (Wang et al., 2011a).

In the postsynaptic domain, both mEJC recordings and GluR immunolabeling indicate that total glutamate receptor abundance is increased in lgl mutants, driven by selective recruitment of GluRIIB-containing receptors. This GluR shift may have important functional consequences, as single channel recordings show that GluRIIB-containing receptors repolarize nearly 10 times faster than GluRIIA-containing receptors (DiAntonio et al., 1999; Marrus et al., 2004), and evoked neurotransmission is strongly attenuated at NMJs containing only GluRIIB rather than GluRIIA channels (Schmid et al., 2008). In addition, these two GluR classes differentially regulate trans-synaptic signaling. For example, GluRIIA overexpression causes increased synaptic boutons, postsynaptic densities and presynaptic T-bar active zones, whereas the inverse changes occur with GluRIIB overexpression (Sigrist et al., 2002). Several studies indicate that GluRIIA selectively acts in a PKA/cAMP-dependent retrograde trans-synaptic signaling pathway that modulates presynaptic release properties (Petersen et al., 1997; Davis et al., 1998b; Sigrist et al., 2002; Yoshihara et al., 2005). During NMJ synaptogenesis, postsynaptic sites appear initially GluRIIA-enriched, but acquire more GluRIIB during maturation, and
alterations of this ratio are activity-dependent (Schmid et al., 2008). We show here that LGL is specifically required to inhibit postsynaptic localization of GluRIB-containing receptors, and thereby likely modulates synaptic functional properties, as well as the potential for trans-synaptic signaling. Intriguingly, the LGL-associated DLG scaffold similarly regulates only GluRIB-containing receptors (Chen and Featherstone, 2005). Moreover, genetically-interacting FMRP also differentially regulates GluRIB at the Drosophila NMJ (Pan and Brodie, 2007), and mammalian FMRP binds the mRNA of both LGL1 and the DLG homologue, PSD95 (Darnell et al., 2011). The co-localization of LGL and DLG in the postsynaptic domain, their known direct interaction, their common regulation by FMRP, and the common role regulating GluRIB-containing receptors (Ohshiro et al., 2000; Chen and Featherstone, 2005), all suggest a mechanism for selective control of this specific GluR class.

Although almost all of our data indicate strict cell-autonomous pre- and postsynaptic LGL requirements, we also show that LGL facilitates a retrograde trans-synaptic signal. Specifically, postsynaptic expression of LGL restores synaptic vesicle fusion probability and response amplitude to control levels, while presynaptic LGL expression restores only mEJC frequency. Moreover, while targeted pre- and postsynaptic RNAi against LGL shows clearly separable roles in neurotransmission, the loss of evoked synaptic current amplitude cannot be rescued by reintroduction of LGL into either pre- or postsynaptic cell alone, but requires LGL on both sides of the synapse. A possible mechanistic link is the known interaction between LGL and the trans-synaptic signal Wingless (Wg), the founding Drosophila Wnt protein (Mann, 1923; Waddington, 1940; Sharma, 1973). Indeed, Disheveled (Dsh) and Frizzled-8 (Fz8) Wnt receptors are required to localize vertebrate LGL to the cortex of Xenopus embryos (Dollar et al., 2005), in a manner similar to the Par6/aPKC-mediated basolateral localization of LGL required for the asymmetrical division of Drosophila neuroblasts (Rolls et al., 2003; Betschinger et al., 2003; Atwood and Prehoda, 2009; Haenfler et al., 2012). Similar signaling mechanisms may also control synaptic development. Considerable evidence shows proteins required for neuroblast differentiation continue to function during synaptogenesis, including aPKC/Par6/PP2A, LGL/ DLG/Scribble and Wg/Dsh/Fz (Zhang et al., 2007a; Zhang et al., 2007b; Ramachandran et al., 2009; Viquez et al., 2009; Mosca and Schwarz, 2010; Wang et al., 2011a; Wang et al., 2011b). Given the breadth of studies linking these proteins to NMJ synaptogenesis, and established links to FMRP regulation, it appears likely that these ‘polarity proteins’ may also mediate polarized synaptic assembly downstream of FMRP translational control.

This study is the first in vivo characterization of synaptic requirements for the membrane-associated LGL scaffold, demonstrating roles in modulating both synaptic architecture and neurotransmission strength. LGL requirements are largely separable into distinct pre- and postsynaptic cell-autonomous roles, both of which strongly impact the efficacy of neurotransmission. These synaptic modulatory functions are strongly reinforced by the long-known interactions between 1) LGL and FMRP (Zarnescu et al., 2005), an RNA-binding translational regulator of activity-dependent changes in synaptic structure/function (Tessier and Brodie, 2009); 2) LGL and DLG, likewise a tumor suppressor and cell-polarity scaffold (Ohshiro et al., 2000) with critical roles controlling synaptic architecture/ function (Mathew et al., 2002; Zhang et al., 2007a; Wang et al., 2011b); and 3) LGL and the Wnt/Wg signaling pathway, also a key regulator of cell polarity and synaptic structure/function (Dollar et al., 2005; Miech et al., 2008; Korkut et al., 2009). Although many of these interactions remain to be explored in the synaptic context, this study provides enticing evidence that LGL may link synaptic translational regulation and the directional localization of encoded proteins to signaling complexes on both sides of the synaptic cleft.

Materials and Methods

Genetic stocks

All lgl mutant alleles were maintained as GFP-balancer stocks, including w; lglU642/CyOKgFP; w; lglU642/CyOKGFP and w; lglU642/CyOKGFP. The lglU642 allele is a strong hypomorph containing a 10 kb deletion of the telomeric and regulatory regions upstream of the lgl locus, which maps to the proximal region of chromosome 2L (Mechler et al., 1985). The lglU642 and lglU642 lesions are characterized as full loss-of-function null alleles containing 22 kb and 18 kb deletions of the lgl locus, respectively (Mechler et al., 1985; Zarnescu et al., 2005; Betschinger et al., 2006). Western blot analysis confirmed that both lglU642 and lglU642 show no detectable LGL expression, whereas trace amounts of LGL were present in the lglU642 line, consistent with a strong hypomorph (supplementary material Fig. S1).

Phenotypically, lglU642 larvae were developmentally retarded and consistently smaller than either lglU642 or lglU642 and were therefore not used in the homogeneous condition. All experiments using transheterozygous allelic combinations were generated from virgin female w; lglU642/CyOKgFP, and all genetic background control experiments were performed using a w line derived from the lglU642 null background. RNAi experiments were performed using a w; lglU642-driven UAS-lgl RNAi line (w; P{GD4047}v51247; Dickson et al., 2007) crossed to either the muscle-specific Gal4 driver 24b (w; 24b-Gal4/24b-Gal4; Brand and Perrimon, 1993; Nitabach et al., 2006; Rohrbough and Brodie, 2010) or the neuron-specific Gal4-driver elav (w; elav-Gal4); Luo et al., 1994; Rohrbough and Brodie, 2010). Rescue experiments were done on lines generated from a cross between UAS-lgl; lglU642/CyOKgFP males (Roegers et al., 2009), and virgin females from (1) lglU642; croKrGFP; uh1-Gal4 for ubiquitous expression (w; +; uh1-Gal4; Wodarz et al., 1995), (2) lglU642/CyOKgFP; 24b-Gal4 for muscle expression or (3) elav-Gal4; lglU642/CyOKgFP for neuronal expression. To control for background effects of Gal4 drivers, a single copy of each Gal4 driver alone was crossed into the lgl control background (uh1-Gal4/+; 24b-Gal4/+ and elav-Gal4/+). All animals were reared at 25°C in a 12/12 light/dark cycle on standard food.

Behavioral assay

To assess motor activity in wandering third instar larvae, we used the well-characterized escape movement behavior elicited in response to noxious thermal stimulation (Tracey et al., 2003). The stereotyped rolling response requiring highly coordinated motor function was measured as described previously (Tracey et al., 2003). In brief, a small probe heated to a constant 45°C via a variac powersource and monitored with a digital thermometer was applied to the larval body-wall medial at hemisegments 3 and 4. Behavioral responses were recorded with a Canon Rebel EOS digital camera for 10 seconds from stimulus onset. Movement responses were categorized as fast (≥1 seconds), slow (2–10 seconds) or no response (≥10 seconds). In total, 60 w1118 control and 54 lgl mutant larvae were tested, and the data were analyzed by a 2×3 Chi^2 test of independence.

Immunocytochemistry

Adult flies were induced to lay for 24 hours at 25°C on apple juice/agar plates with nutritional supplementation provided by a yeast and water paste. 30 size-matched larvae of each genotype were selected at 48 hours and transferred to a fresh supplemented agar plate. We observed that lgl mutant larvae grew at rates similar to controls in the absence of their heterozygous cohorts. Wandering 3rd instars were collected at size and matched control and mutant larvae were dissected in physiological saline (see the Electrophysiology section), fixed in ice-cold methanol (GluR studies) or 4% paraformaldehyde (all other markers) for 5 minutes, and then washed 2×20 minutes in 0.3% PBST (PBS+0.3% Tween). Antibody probes used included: affinity-purified LGL antibody (rabbit anti-LGL, 1:500; Liu et al., 2000; Zarnescu et al., 2005); fluorescein-conjugated Horse Radish Peroxidase antibody (goat HRP-cy5 1:50; Dylight 649, Jackson Laboratories); presynaptic active zone Bruchpilot NC82 antibody (mouse anti-DLG, 1:200; DSHB; Parnas et al., 2001); GluRIIA subunit 8B4D2 antibody [mouse anti-GluRIIA, 1:100; DSHB; (Marrus et al., 2004), originally developed by Christoph Schuster (Max Planck Gesellschaft, Tubingen, Germany) and Corey Goodman (Renovis, San Francisco, CA)] and affinity-purified GluRIIB and GluRIBC subunit antibodies (both rabbit, anti-GluRIIB 1:2500, anti-GluRIBC 1:500; Marrus et al., 2004). Primary antibodies were incubated overnight at 4°C.
and washed 3 × 20 minutes at RT. Secondary antibodies were incubated 2 hours at RT and washed 3 × 20 minutes at RT (Alexa 488; goat anti-Mouse 1:250; goat anti-Rabbit 1:250; Alexa 568; goat anti-Mouse, goat anti-Rabbit 1:250; Invitrogen). Experimental and control samples for each trial were dissected and processed identically in the same dish. Ventral-longitudinal muscle 6/7 NMJs of one abdominal hemisegment A2 and A3 per animal were visualized with a Zeiss LSM 510 meta confocal microscope using LSM acquisition software. Fluorescent intensity and structural parameters were measured in ImageJ and statistical significance was assessed using InStat3 statistical software.

Western blotting
The brain and ventral nerve chord of 5–10 wandering 3rd instar larvae were homogenized in Laemmli sample buffer (BioRad), boiled for 4 minutes, and samples were loaded onto a 4–12% Bis–Tris gel, electrophoresed (1 hour at 200 V) and transferred (1 hour at 100 V) to nitrocellulose. Membranes were blocked for 1 hour in Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) and probed for 12–16 hours at 48°C with primary antibodies anti-LGL (1:10,000) and anti-α-Tubulin (1:400,000). Blots were washed with THAM/NaCl/NP-40 buffer and then probed for 1 hour at 25°C with secondary antibodies. Secondary Antibodies used include: Alexa-Fluor-680-conjugated goat anti-mouse (1:10,000) and Alexa-Fluor-680-conjugated goat anti-rabbit (1:10,000) (Invitrogen-Molecular Probes, Carlsbad, CA). Blots were imaged using the Odyssey Infrared Imaging System (Li-Cor).

Electrophysiology
Two-electrode voltage-clamp (TEVC) recordings were performed from muscle of female wandering third instars, as previously described (Rohrbough et al., 1999; Long et al., 2008). Briefly, all recordings were performed at 18°C in physiological recording saline containing 128 mM NaCl, 2 mM KCl, 4 mM MgCl2, 0.5 mM CaCl2, 70 mM Sucrose and 5 mM HEPES. Synaptic currents were recorded from ventral-longitudinal muscle 6 of abdominal hemisegments A23 clamped with a command voltage (Vcom) of ~60 mV (Rohrbough et al., 1999). Evoked excitatory junctional currents (EJCs) were induced with a 0.5 milliseconds suprathreshold stimulation of the severed motor nerve using a glass suction electrode. EJCs were stimulated at 0.2 Hz and sampled at 20 kHz via an Axon Instruments Digidata 1322A acquisition system, filtered with 2 kHz lowpass using Clampex 9.2 recording software. Each EJC recording session consisted of 10 evoked traces (n = 1), with the mean amplitude measured using Clampfit 9.2 analysis software. Minimal excitatory junctional currents (mEJCs) were sampled at 10 kHz in continuous gap-free mode for 180 seconds (n = 1) with a 500 Hz low-pass filter. mEJC frequency and amplitude were analyzed in Clampfit 9.2 using a threshold filter excluding events below 250 pA, a cutoff near background noise level. All physiological records were taken from a minimum of 6 animals per genotype, with recordings from single A2 hemisegments representing an n = 1. Representative electrophysiology traces were exported using Microsoft Excel software, and statistical significance was assessed using InStat3 statistical software.

Lipophilic dye labeling
FM1-43 dye (10 μM; Invitrogen) was used to visualize synaptic vesicle cycling as previously described, with minor modifications (Ihara et al., 2006; Vijayakrishnan et al., 2009). Briefly, control and mutant wandering third instars were dissected in parallel in the same dish in Ca2+-free saline at 18°C to ensure identical experimental conditions. Dissected preparations were incubated for 5 minutes in physiological saline containing 1.8 mM Ca2+, 90 mM KCl (NaCl reduced to 40 mM to preserve osmolarity) and 10 μM FM1-43, to depolarize the presynaptic terminal and stimulate the internalization of the dye. Synaptic vesicle cycling was then blocked by 5× quick washes in Ca2+-free physiological saline. Ventral-longitudinal muscle 6/7 NMJs of one abdominal hemisegments A2 and A3 per animal were visualized using a Zeiss LSM 510 meta confocal microscope (‘loaded’; Vijayakrishnan et al., 2009). The same NMJ terminals were subsequently unloaded by a 2-minute incubation in 90 mM KCl, 1.8 mM Ca2+ physiological saline, and then once again imaged (‘unloaded’; Vijayakrishnan et al., 2009). The fluorescent intensity of FM1-43 loaded and unloaded NMJs was measured in ImageJ and analyzed using InStat3 statistical software.

Statistical analysis
All data obtained from independent experiments were normalized to appropriate controls. Statistical analyses for all datasets of more than two groups were performed using either one-way ANOVA or the non-parametric Kruskal–Wallis test. Assumptions of parametric analysis were tested by the Shapiro–Wilks test for normality, and Levene’s test for equal variance. In some instances, datasets were log-transformed to meet these assumptions. For post-hoc comparisons of three group datasets, the Student–Newman–Keuls pairwise analysis was used. For experiments with more than three groups, Tukey–Kramer or Dunn’s (non-parametric) pairwise comparisons were used. All comparisons between two groups were performed using the Mann–Whitney non-parametric U-test or Wilcoxon matched pairs test for paired data. All data analyses were performed using the Instat3 statistical software package. A table including all pairwise comparison data may be found in supplementary material Table S1.

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Author contributions
J.S. conducted all experiments and analysed all data. K.B. guided experimental design and co-wrote the paper.

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Supplementary material available online at

References
Dickson, B., Dietzel, G., Kelemen, K. and VDRC project members (2007). RNAi construct and insertion data submitted by the Vienna Drosophila RNAi Center.


Fig. S1. Anti-LGL western blot analysis of lgl mutant alleles. Representative anti-LGL western blot comparing the *w1118* genetic background control with a *lgl* mutant allelic series. The single ~110kDa band corresponding to the predicted molecular weight of LGL in the *w1118* control is undetectable in both *lgll*/*lgl* and *lgld*/*lgl* mutants, but a very faint band is present in the *lglu334* homozygous mutant. These data are consistent with the published characterizations of the *lgll* and *lgld* alleles as protein nulls and *lglu334* as a hypomorphic allele (Mechler et al., 1985).

Fig. S2. Null *lgl* mutant larvae exhibit impaired behavioral movement activity. Loss of LGL reduces the coordinated motor response in a well-characterized movement escape behavior. Wandering third instars were stimulated with a 45°C probe, and movement response time assayed (see Materials and Methods). In the *w1118* genetic background control, 42% of larvae exhibited the stereotyped escape response in ≤1 second (fast; Blue), 30% responded between 2–10 seconds (slow; Green) and 28% failed to respond (none; Red). In *lgl* nulls (*lgll*/*lgl*), only 6% initiated a fast response, 48% exhibited a delayed response, and 46% failed to respond. Statistical comparison Chi^2^ test of independence (*χ^2^ = 20.004, P<0.0001 (***) ; N=114, DF=2).

Fig. S3. Anti-LGL western blot analysis of *lgl* mutant alleles. Representative anti-LGL western blot comparing the uh1-Gal4/+ genetic background control with uh1-Gal4 × *lgl* RNAi. The ~110kDa band in the uh1-Gal4/+ control corresponds to the predicted molecular weight of LGL. This band is undetectable following RNAi knockdown.
Fig. S4. High magnification image of panels A–D of Fig. 8. Loss of LGL induces a net increase in total glutamate receptors (GluRs) caused by the selective elevation of GluRIIB-containing receptors at wandering third instar NMJ synapses. (A) Representative muscle 6/7 NMJ images of anti-GluRIIC labeling in control (w^1118) and lgl mutant backgrounds, with ubiquitous, neuronal and muscle driven UAS-lgl expression. (B) Quantification of GluRIIC labeling intensity in w^1118 and UAS-lgl/+; lgl1/u334; uh1-Gal4/+ backgrounds compared to lgl mutants (N = 12, 12, 13 and 13 NMJs, respectively; Kruskal–Wallis P<0.0001). (C) Quantification of GluRIIC intensity in the elav-Gal4/+ control versus UAS-lgl expression condition (N = 12 NMJs each; P=0.03, Mann–Whitney Test). (D) Quantification of GluRIIC intensity in 24b-Gal4/+ control versus UAS-lgl/+; lgl2/u63; 24b-Gal4/+ condition (N=14 NMJs each; P=0.88).

Fig. S5. High magnification image of panels E–G of Fig. 8. (E) Representative NMJ 6/7 images of anti-GluRII A/B labeling in control and lgl mutants. (F) Quantification of GluRIIA intensity in control and lgl mutant backgrounds (N=12 NMJs each; ANOVA P=0.62, not significant (NS)). (G) Quantification of GluRIIB intensity in control and lgl mutants (N=12 NMJs each; ANOVA P=0.005). Significance shown as not significant (NS), P<0.01 (**), and P<0.001 (***)
**Table S1. Post-hoc pairwise comparisons of all experiments analyzed by One-Way ANOVA.** Table showing all pairwise comparisons of individual genotypes and their significance values. (A) Fig. 2: All results of Tukey–Kramer post-hoc tests for branch number (left) and bouton number (right). (B) Fig. 4: All results for Tukey–Kramer post-hoc tests for evoked junctional current responses for lgl mutants, rescue, and controls (left), and Student–Newman–Keuls multiple comparisons for pre- and postsynaptic LGL rescue and RNAi (right). (C) Fig. 5: All pairwise comparisons of lgl mutants, rescue, and control mEJC data. Dunn’s non-parametric pairwise test for mEJC amplitude (left) and Tukey–Kramer pairwise test for mEJC frequency (right). (D) Fig. 6: Results of pairwise comparisons for active zone size (left) and active zone number (right) by Tukey–Kramer. (E) Fig. 7: Results of Student–Newman–Keuls multiple comparisons test for FM1-43 unloaded intensity (left) and unloaded.loaded ratio (right). (F) Fig. 8: Dunn’s Multiple comparisons test of lgl mutants, rescue, and controls for GluRIIC intensity (left) and Student–Newman–Keuls multiple comparisons test for lgl mutants and control for GluRIIB intensity.

(A) Fig. 2. Structure

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<tr>
<td>lgl1/4 vs uhl-Gal4/+</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td>uhl-Gal4/+ vs uhl-Gal4 Rescue</td>
<td>ns P&gt;0.05</td>
</tr>
</tbody>
</table>

(B) Fig. 4. EJC

<table>
<thead>
<tr>
<th>Tukey–Kramer</th>
<th>Student–Newman–Kuels multiple comparisons test</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>24b Rescue vs 24b-Gal4/+ * P&lt;0.05</td>
</tr>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>24b Rescue vs 24b RNAi ns P&gt;0.05</td>
</tr>
<tr>
<td>w1118 vs uhl-Gal4/+</td>
<td>24b RNAi vs 24b-Gal4/+ * P&lt;0.05</td>
</tr>
<tr>
<td>lgl1/4 vs lgl1/u334</td>
<td>24b Rescue vs 24b-Gal4/+ * P&lt;0.05</td>
</tr>
<tr>
<td>lgl1/4 vs uhl-Gal4/+</td>
<td>24b Rescue vs 24b RNAi ns P&gt;0.05</td>
</tr>
<tr>
<td>uhl-Gal4/+ vs uhl-Gal4/+</td>
<td>24b Rescue vs 24b-Gal4/+ * P&lt;0.05</td>
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</tbody>
</table>

(C) Fig. 5. mEJC

<table>
<thead>
<tr>
<th>Dunn’s Multiple comparisons test (non-parametric)</th>
<th>Tukey–Kramer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplitude</strong></td>
<td><strong>Frequency</strong></td>
</tr>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>** P&lt;0.01</td>
</tr>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td>w1118 vs lgl1/u334</td>
<td>*** P&lt;0.001</td>
</tr>
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**D** Fig. 6. Active zones

<table>
<thead>
<tr>
<th>Tukey–Kramer</th>
<th>Tukey–Kramer</th>
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</thead>
<tbody>
<tr>
<td><strong>Active zone size</strong></td>
<td><strong>Active zone number</strong></td>
</tr>
<tr>
<td><em>w</em>&lt;sup&gt;1118&lt;/sup&gt; vs <em>lg</em>&lt;sup&gt;1/4&lt;/sup&gt;</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td><em>w</em>&lt;sup&gt;1118&lt;/sup&gt; vs <em>lg</em>&lt;sup&gt;h334&lt;/sup&gt;</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td><em>w</em>&lt;sup&gt;1118&lt;/sup&gt; vs *uh1-*Gal4/+</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>w</em>&lt;sup&gt;1118&lt;/sup&gt; vs *uh1-*Gal4 Rescue</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;tu334&lt;/sub&gt; vs <em>lg</em>&lt;sup&gt;h334&lt;/sup&gt;</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;tu334&lt;/sub&gt; vs <em>lg</em>&lt;sub&gt;1/1&lt;/sub&gt;</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;tu334&lt;/sub&gt; vs *uh1-*Gal4/+</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;tu334&lt;/sub&gt; vs *uh1-*Gal4 Rescue</td>
<td>** P&lt;0.01</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;h334&lt;/sub&gt; vs <em>lg</em>&lt;sup&gt;1/1&lt;/sup&gt;</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;h334&lt;/sub&gt; vs *uh1-*Gal4/+</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;h334&lt;/sub&gt; vs *uh1-*Gal4 Rescue</td>
<td>** P&lt;0.01</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;h334&lt;/sub&gt; vs *uh1-*Gal4/+</td>
<td>*** P&lt;0.001</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sub&gt;h334&lt;/sub&gt; vs *uh1-*Gal4 Rescue</td>
<td>** P&lt;0.01</td>
</tr>
<tr>
<td>*uh1-*Gal4/+ vs *uh1-*Gal4 Rescue</td>
<td>ns P&gt;0.05</td>
</tr>
</tbody>
</table>

**E** Fig. 7. FM1-43

**Student–Newman–Kuels multiple comparisons test**

<table>
<thead>
<tr>
<th>Unloaded intensity</th>
<th>Unloaded/loaded intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lgf</em>&lt;sup&gt;1/4&lt;/sup&gt; vs <em>w</em>&lt;sup&gt;1118&lt;/sup&gt;</td>
<td>* P&lt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sup&gt;1/4&lt;/sup&gt; vs <em>w</em>&lt;sup&gt;h334&lt;/sup&gt;</td>
<td>ns P&gt;0.05</td>
</tr>
<tr>
<td><em>lgf</em>&lt;sup&gt;h334&lt;/sup&gt; vs <em>w</em>&lt;sup&gt;1118&lt;/sup&gt;</td>
<td>ns P&gt;0.05</td>
</tr>
</tbody>
</table>

**F** Fig. 8. GluR expression

**GluRIIC intensity**

| *w*<sup>1118</sup> vs *lgf*<sup>1/4</sup> | *** P<0.001 |
| *w*<sup>1118</sup> vs *lgf*<sup>h334</sup> | *** P<0.001 |
| *w*<sup>1118</sup> vs *uh1-*Gal4/+ | ns P>0.05 |
| *w*<sup>1118</sup> vs *uh1-*Gal4 Rescue | ns P>0.05 |
| *lgf*<sup>1/4</sup> vs *lgf*<sup>h334</sup> | ns P>0.05 |
| *lgf*<sup>1/4</sup> vs *uh1-*Gal4/+ | ns P>0.05 |
| *lgf*<sup>1/4</sup> vs *uh1-*Gal4 Rescue | * P<0.05 |
| *lgf*<sub>h334</sub> vs *uh1-*Gal4/+ | *** P<0.001 |

**GluRIIB intensity**

| *w*<sup>1118</sup> vs *lgf*<sup>1/4</sup> | *** P<0.001 |
| *w*<sup>1118</sup> vs *lgf*<sup>h334</sup> | *** P<0.001 |
| *w*<sup>1118</sup> vs *uh1-*Gal4/+ | ns P>0.05 |
| *w*<sup>1118</sup> vs *uh1-*Gal4 Rescue | ns P>0.05 |
| *lgf*<sup>1/4</sup> vs *lgf*<sup>h334</sup> | ns P>0.05 |
| *lgf*<sub>h334</sub> vs *uh1-*Gal4/+ | *** P<0.001 |

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*P<0.001, **P<0.01, *P<0.05, ns: not significant*
<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Comparison</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\ell g^{l334}$</td>
<td>vs $uh1$-Gal4 Rescue</td>
<td>* P&lt;0.05</td>
</tr>
<tr>
<td>$uh1$-Gal4/+</td>
<td>vs $uh1$-Gal4 Rescue</td>
<td>ns P&gt;0.05</td>
</tr>
</tbody>
</table>