The Extracellular-Regulated Kinase Effector Lk6 is Required for Glutamate Receptor Localization at the *Drosophila* Neuromuscular Junction

Nizar A. Hussein, Taylor L. Delaney, Brittany L. Tounsel and Faith L.W. Liebl

Department of Biological Sciences, Southern Illinois University Edwardsville, Edwardsville, IL, USA.

ABSTRACT: The proper localization and synthesis of postsynaptic glutamate receptors are essential for synaptic plasticity. Synaptic translation initiation is thought to occur via the target of rapamycin (TOR) and mitogen-activated protein kinase signal-integrating kinase (Mnk) signaling pathways, which is downstream of extracellular-regulated kinase (ERK). We used the model glutamatergic synapse, the *Drosophila* neuromuscular junction, to better understand the roles of the Mnk and TOR signaling pathways in synapse development. These synapses contain non-NMDA receptors that are most similar to AMPA receptors. Our data show that Lk6, the *Drosophila* homolog of Mnk1 and Mnk2, is required in either presynaptic neurons or postsynaptic muscle for the proper localization of the GluRIIA glutamate receptor subunit. Lk6 may signal through eukaryotic initiation factor (eIF) 4E to regulate the synaptic levels of GluRIIA as either interfering with eIF4E binding to eIF4G or expression of a nonphosphorylatable isoform of eIF4E resulted in a significant reduction in GluRIIA at the synapse. We also find that Lk6 and TOR may independently regulate synaptic levels of GluRIIA.

KEYWORDS: synapse, glutamate receptors, ERK signaling, Lk6, translation initiation

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CORRESPONDENCE: fliebl@siue.edu

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Introduction

Glutamate mediates the majority of neurotransmission in the central nervous system (CNS).[1] Once a synapse is established, its strength is modulated by changes in presynaptic glutamate release, synaptic size, and number of postsynaptic receptors.[2,3] The capacity of the synapse to change as a result of synaptic activity, or synaptic plasticity, requires the translation of proteins that modulate synapse function.[4–7] Although synaptic plasticity is initially mediated by changes in protein trafficking, translation of synaptic mRNAs is required for structural changes to the synapse and to maintain the altered neurotransmission.[8] The importance of local translation for synaptic plasticity is illustrated by aberrant translation initiation, which is thought to occur in fragile X syndrome as a result of the loss of function of the translational repressor, fragile X mental retardation protein.[9]

Protein synthesis is primarily regulated at the step of translation initiation,[10] which requires the binding of eukaryotic initiation factor (eIF) 4E to the 5′7-methylguanosine cap (5′ cap) of mRNA.[11,12] This binding recruits eIF4G to eIF4E in a process that facilitates ribosome binding. eIF4G provides a scaffold for the binding of eIF4A to form the eIF4F complex.[13] Two growth factor-activated pathways, including the mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) pathways, regulate translation initiation by influencing the capacity of eIF4E to bind to the 5′ cap.

MAPKs are involved in a variety of cellular processes, including, but not limited to, cellular proliferation, apoptosis, differentiation, transformation, and cell movement.[14,15] MAPK signaling can occur via three divergent pathways, including extracellular-regulated kinase (ERK), p38, and c-Jun N-terminal kinase signaling.[16] Each of the MAPKs is a Ser/Thr kinase activated by phosphorylation via upstream MAPK kinases.[14,15] ERK is activated by synaptic depolarization, calcium influx, and neurotrophin signaling.[17,18] MAPK signal-integrating kinases (Mnks), including Mnk1 and Mnk2, are subsequently phosphorylated and activated by ERK.[19,20] Once phosphorylated, Mnks regulate translation initiation by phosphorylating eIF4E to influence translation initiation.[21–23]

Similar to the Mnks, mTOR is a highly conserved Ser/Thr kinase involved in several cellular processes, such as cellular homeostasis and proliferation. mTOR forms two different complexes, one of which, mTORC1, phosphorylates S6 kinase 1 to promote the synthesis of ribosomal proteins[24] and phosphorylates 4E-binding protein (4E-BP) to inhibit its

binding to eIF4E.[25] Thus, the mTOR pathway represses the actions of a translational inhibitor, 4E-BP, thereby promoting the interaction between eIF4E and eIF4G.[26]

Both of these pathways regulate synaptic plasticity by influencing translation initiation. Mnk activation downstream of brain-derived neurotrophic factor (BDNF) signaling enhances long-term potentiation (LTP) using distinct mechanisms at two different time points to enhance translation in dentate gyrus cells.[17] BDNF signaling through Mnk1 was subsequently shown to increase the levels of proteins required for vesicle trafficking and exocytosis in cortical neurons.[27] Rapamycin, an inhibitor of mTORC1,[28] suppresses BDNF-induced late LTP in rat hippocampal neurons.[29] These data indicate that both mTOR and Mnk signaling enhance synaptic plasticity, but the mechanisms utilized by these pathways are unclear.

We used the *Drosophila* neuromuscular junction (NMJ) to investigate the mechanisms by which Mnk and TOR regulate the synaptic levels of the glutamate receptor (GluR) subunit, GluRIIA. *Drosophila* expresses a single Mnk homolog, Lk6, which phosphorylates eIF4E[30] and regulates growth.[31] The *Drosophila* NMJ is a glutamatergic synapse, which is structurally and functionally similar to mammalian CNS glutamatergic synapses, and contains AMPA-like, non-NMDA receptors.[32] One NMJ postsynaptic GluR subunit, GluRIIA, is locally translated in an eIF4E-dependent manner as a result of synaptic activity.[33] We show, for the first time, that Lk6 regulates glutamatergic neurotransmission and synaptic localization of GluRIIA. Lk6 is required in either presynaptic neurons or postsynaptic muscles for proper GluRIIA localization. Inhibiting the interaction between eIF4E and eIF4G pharmacologically or overexpressing a nonphosphorylatable isoform of eIF4E in these tissues also resulted in the loss of GluRIIA from the NMJ indicating that Lk6 may signal through eIF4E to regulate GluRIIA localization. Lk6 may regulate the synaptic levels of GluRIIA independent of the TOR complex as simultaneous inhibition of Lk6 and TOR signaling resulted in an additive loss of synaptic GluRIIA.

Materials and Methods

Fly stocks. Fly stocks were maintained at 25°C in vials containing Jazz-Mix *Drosophila* food (Fisher Scientific). The *UAS-GAL4* system was used for tissue-specific expression.[34] All fly stocks were obtained from the Bloomington *Drosophila* Stock Center except $lk6^{RNAi}$, which was obtained from the Vienna *Drosophila* RNAi Center, and *Dcr2;;elav-Gal4* and *Dcr2;;24B-Gal4*, which were gifts from Aaron DiAntonio's Lab. w^{1118} and outcrossed controls were used as controls for each experiment as appropriate.

Immunohistochemistry and confocal microscopy. Third-instar larvae were dissected on Sylgard plates in Roger's Ringer solution (135 mM NaCl, 5 mM KCl, 4 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5 mM TES, and 72 mM sucrose) supplemented with 2 mM glutamate.[35] Larvae were fixed for 30–45 minutes in either Bouin's fixative (for GluR and Bruchpilot [Brp] antibodies) or 4% paraformaldehyde in phosphate buffered saline (PBS) (for all other antibodies). After fixation, larval dissections were washed in PTX (PBS + 0.1% Triton) and PBTX (PBS + 0.1% Triton + 1% bovine serum albumin [BSA]). Primary antibodies were applied overnight at 4°C in PBTX. Mouse α-Brp (aka nc82, 1:50), mouse α-discs large (DLG) (1:1000), mouse α-synaptotagmin (Syt) (1:100), and mouse α-GluRIIA (1:100) were acquired from the Developmental Studies Hybridoma Bank. Rabbit α-GluRIIB (1:2000) and rabbit α-GluRIIC (1:5000) were generous gifts from Aaron DiAntonio.[36] Mouse α-acetylated tubulin (1:1000) and phalloidin (1:200) were obtained from Sigma-Aldrich and Invitrogen, respectively. After washing the larval preparations with PBTX, additional antibodies including horseradish peroxidase (HRP) (1:125, Jackson ImmunoResearch) and species-specific fluoroscein isothiocyanate (FITC) (1:400, Jackson ImmunoResearch) were applied for two hours at room temperature. Larvae were washed with PBTX before mounting on slides with Vectashield (Vector Laboratories).

Images of third-instar larval 6/7 NMJs from left or right hemisegments at A3 or A4 were acquired using the 60× objective of an Olympus FV1000 confocal microscope. Imaging parameters were established for controls and subsequently used for all experimental animals. Approximately equal numbers of control and experimental animals were imaged during each imaging session.

Electrophysiology. Third-instar larvae were filet dissected at room temperature on Sylgard-coated coverslips and glued down with Vetbond Tissue Adhesive (World Precision Instruments). Dissections and recordings were performed in Roger's Ringer solution. Muscle 6 of hemisegments 3 or 4 was clamped at −60 mV using an Axoclamp 900A amplifier (Molecular Devices). Current injecting and recording electrodes were filled with 3 M KCl and were used provided their resistances were 10–20 MΩ. Segmental nerves were stimulated with an electrode filled with bath saline. A 0.5 Hz, 10 V stimulus was delivered using a Grass S88 stimulator with a SIU5 isolation unit (Grass Technologies). Recordings were digitized using a Digidata 1443 digitizer (Molecular Devices). pClamp software (version 10.4) was used for data analyses. Quantal content was calculated by dividing the eEJC area (nA × ms) by the mEJC area (nA × ms) for each animal. An equal number of control and experimental recordings were acquired each day.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). RNA was extracted from 8 to 12 third-instar larvae using TRIzol (Invitrogen) as previously described.[37] Single-plex reactions using gene-specific primers for *gluRIIA*, *gluRIIB*, *gluRIIC*, or *GAPDH* and the iTaq Universal SYBR Green One-Step Kit (Bio-Rad) were performed using a Stratagene Mx3000P qPCR System (Agilent Technologies). 100 ng of total RNA was added to each reaction. Three technical replicates and two biological replicates

were performed for each reaction. ΔC(t) values were obtained by subtracting the *GAPDH* C(t) value from the *gluR* C(t) value.

4EGI-1 and rapamycin treatments. Control and *lk6* mutant larvae were reared for four days in vials as previously described. After four days, wandering third-instar larvae were placed on nutrient-rich apple juice agar plates containing yeast supplemented with 10 µM 4EGI-1 (Fisher Scientific) or yeast containing an equal volume of dimethyl sulfoxide (DMSO). Larvae remained on plates for 24 hours and then were used for dissection and immunohistochemistry. 10 µM 4EGI-1 was previously shown to inhibit cap-dependent translation when fed to flies.[38] We also found that 10 µM 4EGI-1 did not significantly affect larval viability (Supplementary Fig. 1).

Parental genotypes, including w^{1118} and $lk6^{RNAi}$, and flies containing the tissue-specific drivers *Actin5c-Gal4* (for expression in all tissues), *Dcr2;;elav-Gal4* (for expression in neurons), and *Dcr2;;24B-Gal4* (for expression in postsynaptic muscles), were placed on apple juice agar plates[39] with a yeast mix for 24 hours. After 24 hours, the parental generation was removed and F1 offspring remained on the plates. After 96 hours, the yeast mix was replaced with a yeast mix containing either 0.1 mM rapamycin (Fisher Scientific) dissolved in ethanol or yeast mix containing an equal volume of ethanol for an additional 24 hours. This concentration of rapamycin was selected based on the previous experiments[40,41] and to standardize the size and optimize the viability of the animals. Third-instar larvae were used 24 hours after changing the yeast mix for dissection and immunohistochemistry.

Image analyses and statistics. Compressed images of confocal micrograph z-series were used for data analyses. GluRIIA cluster sizes were obtained by measuring the area of GluRIIA puncta[42] localized immediately adjacent to or in direct opposition of the presynaptic motor neuron (as determined by HRP immunolabeling) using National Institutes of Health's ImageJ. Brp densities were calculated by counting the number of Brp puncta and dividing by the area of the presynaptic motor neuron. Relative fluorescence intensities were quantified by manually tracing around the NMJ, recording the mean fluorescence for the area and fluorophore-specific channel using the Adobe Photoshop (CS6 version 13), and then subtracting the background from a nonsynaptic area of equal size. For tubulin and phalloidin quantifications, a nonsynaptic and nonmuscle area was used to determine the background. Morphology of the presynaptic motor neuron was determined by manually counting the boutons and branches. Branches were defined as bifurcations of the motor neuron containing at least two boutons.

Statistical analyses of the data were performed using GraphPad Prism (version 5.00). Student's *t*-tests were used to analyze two data sets. One-way analysis of variance using Tukey's (equal variance between data sets) or Holm-Sidak's (significant difference in variance between data sets) post hoc tests were used to analyze the potential differences between more than two data sets. In all figures, the level of statistical significance is as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, and $^{***}P \leq 0.0001$. Error bars represent the standard error of the mean. Summary statistics for all data are reported in Supplementary Table 1.

Results

Lk6 promotes the synaptic localization of postsynaptic GluRs and synaptic transmission. Translation initiation is required for several forms of synaptic plasticity, including LTP,[4,5] long-term depression,[43,44] and memory consolidation.[6,7] We found, through a forward genetic screen, that the *Drosophila* homolog of Mnk1 and Mnk2, Lk6, regulates the localization of GluRs to the NMJ. These glutamatergic synapses are similar to mammalian central glutamatergic synapses both at the subcellular and molecular levels.[45] *Drosophila* NMJ GluRs, similar to AMPA receptors, are tetramers[46] that mediate fast synaptic transmission.[47] Lk6 is 57% identical and 74% similar to Mnk1 (using NP_001272416.1) and 58% identical and 73% similar to Mnk2 (using NP_067437.2) with slightly higher sequence conservation in the Lk6 catalytic domains.[48] Mnks are activated by MAPKs[20] and regulate translation initiation by phosphorylating eIF4E[21–23,49] thereby influencing assembly of the eIF4F cap-binding complex.

In order to better understand the role of Lk6 in glutamatergic synapse development, we characterized the *lk6* mutant phenotype using the $lk6^{2}$ hypomorph. The $lk6^{2}$ mutant contains a deletion of all exons downstream of the first intron caused by the excision of an *EP P*-element producing a loss of function allele.[30] *Drosophila* NMJ GluRs contain either the GluRIIA or GluRIIB subunits along with three essential subunits, including GluRIIC, GluRIID, and GluRIIE.[36,46] Both GluRIIA[50] and GluRIIB[51] are localized exclusively to postsynaptic muscle cells. $Lk6^{2}$ mutants exhibited a significant decrease in GluRIIA cluster size (Fig. 1A and B) but no significant differences in GluRIIB (Fig. 1D) or GluRIIC (Fig. 1E) cluster sizes compared to w^{1118} controls. Cluster size measurements were used because they correlate with the function of the synapse.[42] We confirmed the loss of synaptic GluRIIA in $lk6^{36}$ mutants (GluRIIA cluster sizes, w^{1118}: 1.14 ± 0.06 µm^2, n = 120 clusters from 12 animals; $lk6^{36}$: 0.70 ± 0.05 µm^2, n = 140 clusters from 14 animals; $P < 0.0001$), which contain a His to Arg substitution at amino acid 154.[31] Mutations in *lk6* did not affect NMJ morphology (Fig. 1C).

Mnks may indirectly affect transcription by regulating mRNA localization of transcriptional regulators.[52] To determine whether mutations in *lk6* impact the synaptic localization of GluRs by affecting transcription of *gluR* subunits, we measured *gluR* subunit mRNA levels using qRT-PCR. Interestingly, *gluRIIA* and *gluRIIB* mRNA levels were significantly increased in $lk6^{2}$ mutants compared to controls, while there were no significant differences in *gluRIIC* mRNA levels (Fig. 1F).

The microtubule[53–55] and actin cytoskeletons[56–58] enable GluR trafficking and synaptic localization. Furthermore, Mnk1[59] and the 220 kDa isoform of Lk6[60] are colocalized

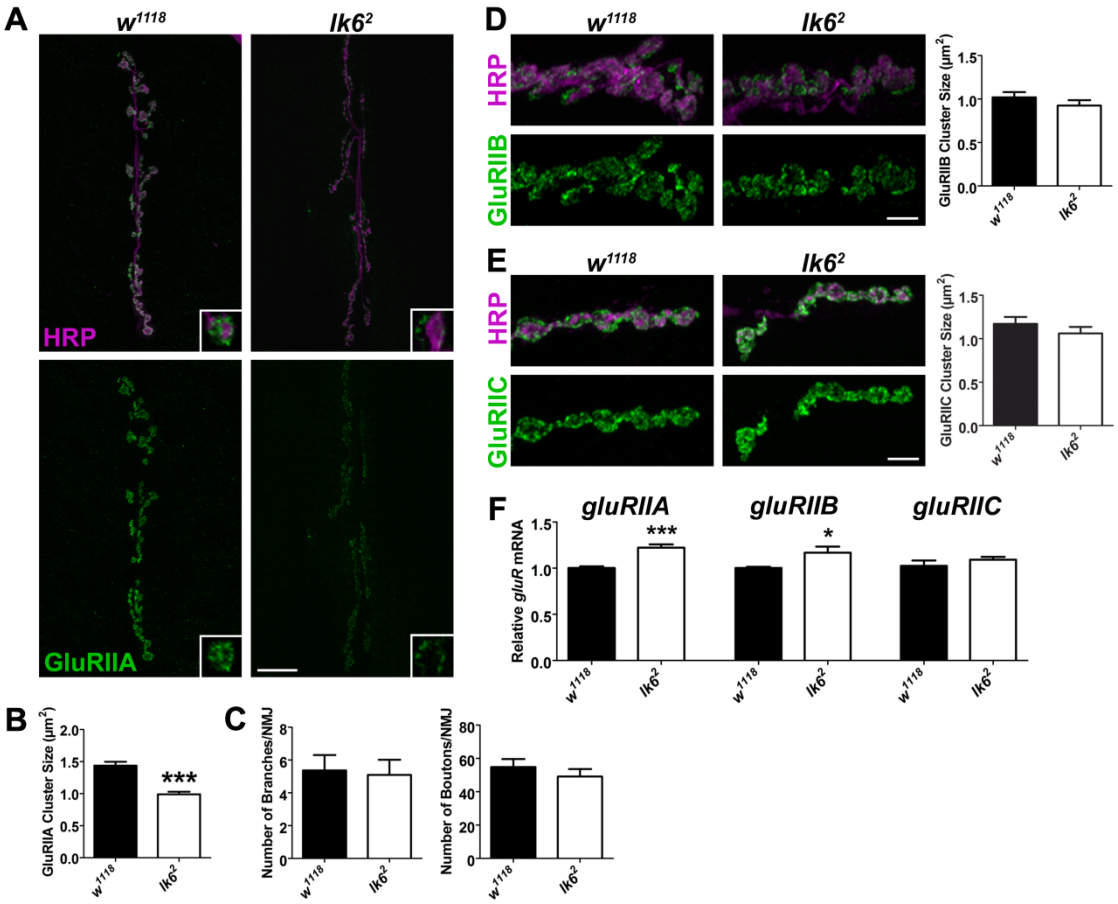

Figure 1. Lk6 promotes the synaptic localization of GluRIIA. (**A**) Third-instar larval NMJs on ventral longitudinal muscles 6 and 7, labeled using antibodies against the neuronal membrane marker HRP (magenta) and GluRIIA (green). Inset panels show anterior terminal boutons at higher magnification. Scale bar = 20 μm. (**B**) Quantification of GluRIIA cluster sizes. (**C**) Quantification of the number of presynaptic boutons (right) and the number of branches (left) at the 6/7 NMJ of the third-instar larvae. High-resolution confocal images of GluRIIB (**D**) and GluRIIC (**E**) along with the quantification of respective cluster sizes (right histograms). Scale bar = 5 μm. (**F**) Normalized ΔC(t) values of *glur* transcript levels in *lk6*^{2} mutants.

with centrosomes, structures composed of microtubules. Therefore, we investigated the possibility that Lk6 may affect GluR trafficking to the synapse by affecting cytoskeletal dynamics. We examined the microtubule cytoskeleton by immunolabeling acetylated tubulin, which demarcates stable microtubules.[61] There were no significant differences in the levels of synaptic or muscle acetylated tubulin in *lk6*^{2} mutants compared to controls (Supplementary Fig. 2A). Similarly, we did not detect any gross morphological differences in the sarcomeric structure of *lk6*^{2} mutant muscles as indicated by labeling F-actin with phalloidin.[62] We also did not observe alterations in axon targeting, muscle patterning, or muscle sizes in *lk6*^{2} mutants compared to controls (Supplementary Fig. 2B).

Reductions in the number of postsynaptic GluRs may occur as a result of changes in presynaptic glutamate release[42] or extracellular glutamate concentrations.[63] To assess the possibility that Lk6 may affect synaptic glutamate availability thereby altering the localization of postsynaptic GluRs, we used two complimentary approaches. First, we used immunocytochemistry to examine synaptic proteins that influence neurotransmitter release and organization of postsynaptic protein complexes. There were no significant differences in the synaptic levels of DLG, a postsynaptic scaffolding protein that regulates synaptic development,[64] or the presynaptic proteins Brp and Syt, which are localized to active zones[65,66] and regulate presynaptic neurotransmitter release,[67] respectively (Supplementary Fig. 3), in *lk6*^{2} mutants compared to controls.

Next, we used two-electrode voltage clamp to further investigate whether Lk6 regulates presynaptic glutamate release. Evoked endplate junction current (eEJC) amplitudes were significantly reduced in *lk6*^{2} mutants (Fig. 2A and B) compared to *w*^{1118} controls. Similarly, spontaneous neurotransmission, as measured by miniature EJC (mEJC) amplitudes, were also significantly reduced in *lk6*^{2} mutants (Fig. 2C–E). There were no significant differences, however, in quantal content or mEJC frequency (Fig. 2B and D). Interestingly, there was a significant reduction in mEJC rise time in *lk6*^{2} mutants suggesting that Lk6 regulates GluR channel kinetics likely by influencing the synaptic localization of the GluRIIA subunit. These data indicate that Lk6 may primarily

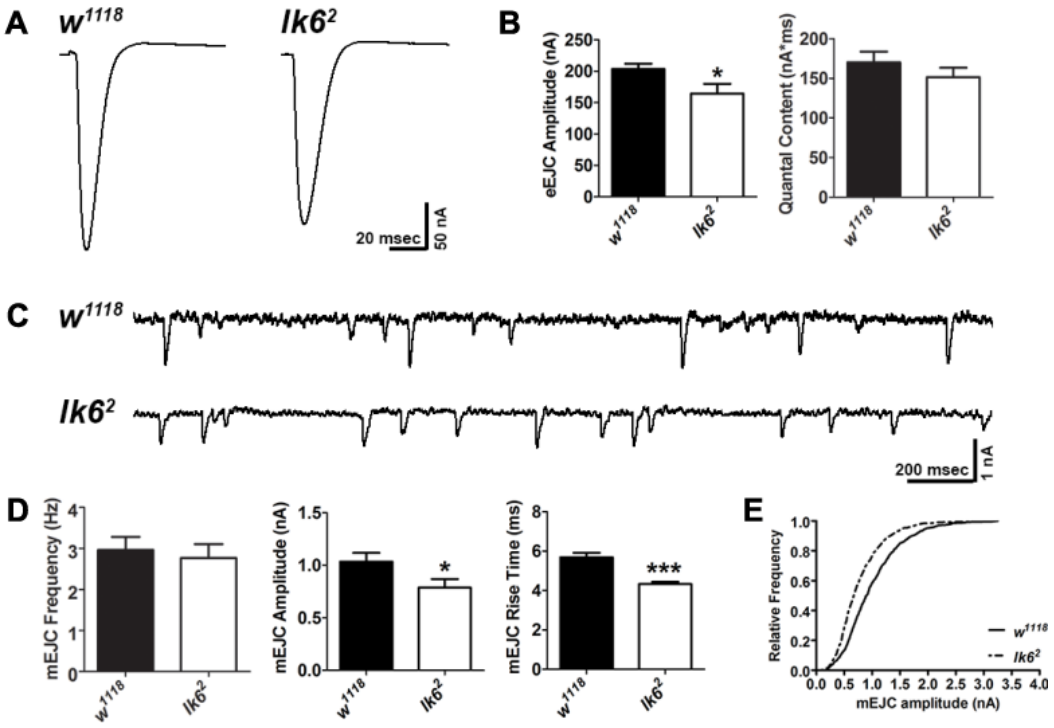

Figure 2. Lk6 positively regulates synaptic transmission. Spontaneous miniature (mEJCs) and evoked endplate junctional currents (eEJCs) were recorded from muscle 6 of third-instar larvae after the muscle was clamped at –60 mV. (**A**) Representative eEJCs from control and *lk6*^{2} mutants. (**B**) Quantification of eEJC amplitudes and quantal content. (**C**) Representative traces showing mEJCs. (**D**) Quantification of mEJC frequencies, mEJC amplitudes, and mEJC rise times. (**E**) Cumulative frequency distribution of mEJC amplitudes.

affect postsynaptic GluRs without affecting presynaptic glutamate release. Collectively, our data are consistent with a loss of synaptic GluRs in *lk6* mutant synapses resulting in an attenuated response to evoked stimuli and spontaneous neurotransmission.

Lk6 regulates GluRIIA localization by both pre- and postsynaptic mechanisms. Our efforts to detect Lk6 at the NMJ using an antibody[60] were unsuccessful. Therefore, we assessed the tissue-specific contributions of Lk6 to GluR localization using a combination of knock down and rescue experiments. We knocked down *lk6* by expressing an *UAS-lk6*^{RNAi} transgene under the control of the *Actin5c-Gal4* (all tissues), *Dcr2;;elav-Gal4* (neurons), or *Dcr2;;24B-Gal4* (muscle) drivers. Knock down of *lk6* in all tissues, neurons, or muscles produced a significant reduction in GluRIIA cluster sizes compared to the outcrossed controls as determined by a one-way analysis of variance (Supplementary Table 1). There was also a significant reduction in relative GluRIIA fluorescence intensity when *lk6* was knocked down in all tissues or in postsynaptic muscles compared to the outcrossed controls. Although there was a trend toward reduced GluRIIA fluorescence when *lk6* was knocked down in neurons, it was not significant (Fig. 3A and B). This may indicate that, while the individual GluRIIA clusters are smaller, there may be a slight increase in the number of clusters. Knock down of *lk6* in any tissue type did not alter the morphology of presynaptic motor neurons (Fig. 3C). These data indicate that Lk6 is important in both presynaptic neurons and postsynaptic muscle for the proper localization of GluRIIA-containing receptors.

We next performed rescue experiments to determine whether Lk6 is required in pre- or postsynaptic cells or both cell types for GluR localization. An *UAS-lk6* transgene was expressed in the *lk6*^{2} mutant background specifically in neurons (using the *elav-Gal4* driver) or muscles (using the *24B-Gal4* driver). Expression of *lk6* in either neurons or muscles rescued GluRIIA cluster sizes and GluRIIA fluorescence to near *w*^{1118} control levels (Fig. 4A and B). These data show that the loss of GluRIIA in *lk6*^{2} mutants can be attributed specifically to the loss of Lk6. Consistent with our knock down data, our rescue experiments indicate that Lk6 is required in presynaptic motor neurons or postsynaptic muscles for the synaptic localization of GluRIIA.

Lk6 may signal through eIF4E to regulate synaptic levels of GluRIIA. Lk6 is activated by ERK signaling but not by p38 MAPK signaling. Lk6 phosphorylates eIF4E at Ser251,[48] which is analogous to Ser209 in mammals, the residue phosphorylated by Mnk1.[20] Mnk-mediated phosphorylation of eIF4E reduces its interaction with the 5′ cap,[68,69] but this may enhance translation by liberating eIF4E from eIF4G to enable additional rounds of translation initiation.[70] Our data demonstrate that Lk6, localized in both neurons and muscles, is important for synaptic levels of GluRIIA (Figs. 3 and 4). Given the role of Lk6 in translation initiation, we hypothesized that Lk6 positively regulates translation initiation of

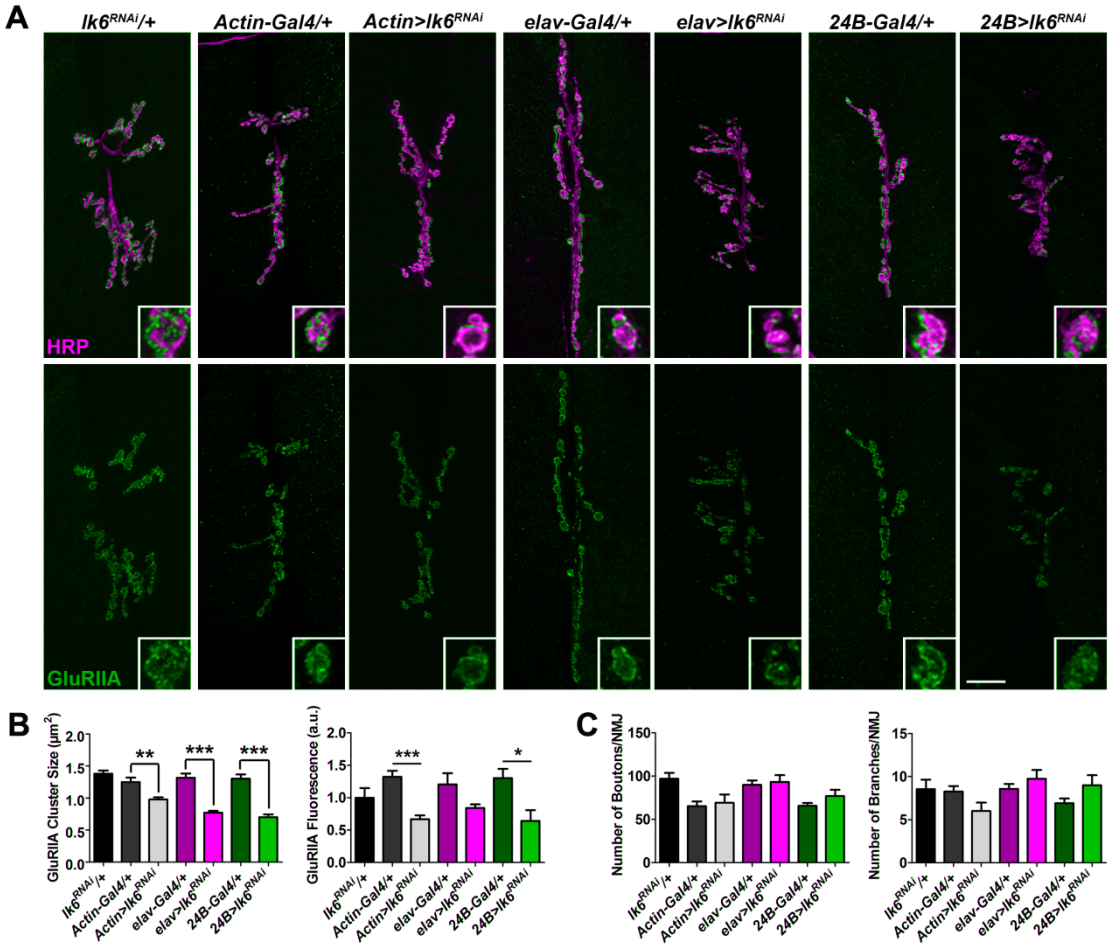

Figure 3. Lk6 is important both pre- and postsynaptically for GluRIIA localization. (**A**) Third-instar larval NMJs on ventral longitudinal muscles 6 and 7, labeled using antibodies against the neuronal membrane marker HRP (magenta) and GluRIIA (green). *Lk6* was knocked down in all tissues using the *Actin5c-Gal4* driver, in neurons using the *Dcr2;;elav-Gal4* driver, or in postsynaptic muscles using the *Dcr2;;24B-Gal4* driver. Scale bar = 20 μm. Inset panels show terminal boutons at high magnification. (**B**) Quantification of GluRIIA cluster sizes and relative GluRIIA fluorescence intensities, which were normalized to the $lk6^{RNAi}$/+ outcrossed control. (**C**) Quantification of presynaptic neuronal morphology including the 6/7 NMJ boutons and branches.

the *gluRIIa* subunit transcript, which has been shown to be locally translated at the NMJ,[33] by phosphorylating eIF4E. To test this hypothesis, we first disrupted the interaction between eIF4E and eIF4G and then expressed a nonphosphorylatable isoform of eIF4E encoded by the *UAS-eIF4E*^{S251A} construct[31] to determine if these manipulations would alter the localization of GluRIIA to the synapse.

Formation of the eIF4F complex is regulated by 4E-BP, which binds to eIF4E preventing the interaction of eIF4E with eIF4G.[25] Similarly, 4EGI-1 is a reversible competitive inhibitor of eIF4E. 4EGI-1 specifically binds to and blocks the eIF4G motif of eIF4E without affecting the binding of 4E-BP.[71] Interfering with eIF4F complex formation by feeding larvae 10 μM 4EGI-1 resulted in a significant reduction in GluRIIA cluster sizes and relative GluRIIA fluorescence intensity in w^{1118} controls but not in $lk6^{2}$ mutants (Fig. 5A and B). Inhibition of the eIF4E–eIF4G interaction, however, did not significantly affect NMJ morphology (Fig. 5C). The loss of GluRIIA in w^{1118} but not in $lk6^{2}$ mutants after inhibiting the binding of eIF4G to eIF4E indicates that Lk6 may promote the functional interaction of eIF4E and eIF4G to positively regulate the synaptic localization of GluRIIA.

Similarly, expression of eIF4E^{S251A} ubiquitously, presynaptically, or postsynaptically resulted in significantly decreased GluRIIA cluster sizes compared to outcrossed controls (Fig. 6A and B). The expression of eIF4E^{S251A} in neurons or muscle resulted in a significant reduction in GluRIIA relative fluorescence intensity (Fig. 6A and B). Although there was a trend toward reduced GluRIIA fluorescence, when the *UAS-eIF4E*^{S251A} transgene was expressed in all tissues, it was not significant (Supplementary Table 1). When eIF4E^{S251A} was expressed ubiquitously, there were no significant differences observed in the morphology of the presynaptic motor neuron (Fig. 6C). The expression of eIF4E^{S251A} either presynaptically or postsynaptically, however, differentially affected the morphology of the presynaptic motor neuron. When eIF4E^{S251A} was expressed in presynaptic neurons but not in postsynaptic muscles, there was a significant increase in bouton number.

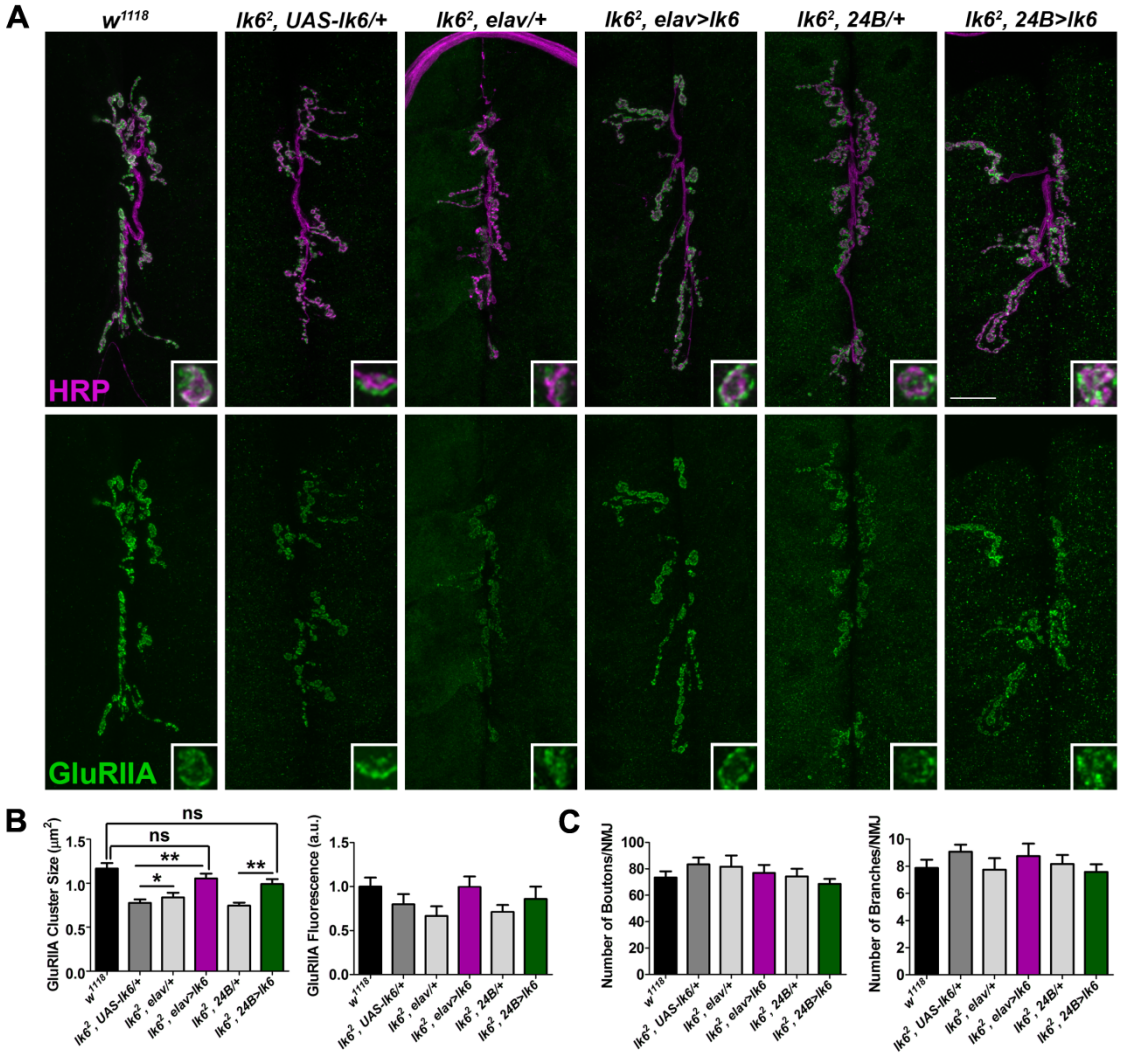

Figure 4. Lk6 is required in either presynaptic neurons or postsynaptic muscle for proper GluRIIA localization. (**A**) Confocal micrographs showing representative third-instar larval NMJs on ventral longitudinal muscles 6 and 7. NMJs were labeled using antibodies against the neuronal membrane marker HRP (magenta) and GluRIIA (green). An *UAS-lk6* transgene was expressed in the *lk6*^{2} mutant background in neurons using the *elav-Gal4* driver or in postsynaptic muscles using the *24B-Gal4* driver. Scale bar = 20 µm. Inset panels show terminal boutons at high magnification. (**B**) Quantification of GluRIIA cluster sizes and relative GluRIIA fluorescence intensities in genotypes as indicated. Fluorescence intensities were normalized to the *w*^{1118} control. (**C**) Quantification of presynaptic neuronal morphology including the 6/7 NMJ boutons and branches.

Conversely, the expression of eIF4E^{S251A} in muscles but not in presynaptic neurons resulted in a significant reduction in the number of NMJ branches (Fig. 6C). Taken together, our data suggest that Lk6 may phosphorylate eIF4E to promote the synaptic localization of GluRIIA and indicate that translation efficiency is important for presynaptic motor neuron morphology.

Lk6 and TOR may independently regulate the synaptic localization of GluRIIA. Translation initiation in *Drosophila* is regulated by both Lk6 and TOR, which phosphorylates 4E-BP thereby increasing the availability of eIF4E for translation initiation.[26] In order to establish a molecular model for GluR localization, we sought to test whether we would observe a similar impact on synaptic GluR localization by inhibiting TOR activity at its FKBP-rapamycin binding domain using rapamycin.[72] Because Lk6 and TOR use different mechanisms to influence eIF4E availability, we hypothesized that either inhibition of Lk6 or TOR function would affect the localization of synaptic GluRs. To test this, we again knocked down *lk6* by expressing *UAS-lk6*^{RNAi} in all tissues using the *Actin5c-Gal4* driver, in presynaptic neurons using the *Dcr2;;elav-Gal4* driver, or in postsynaptic muscle using the *Dcr2;;24B-Gal4* driver and simultaneously inhibited TOR signaling using rapamycin. Knock down of *lk6* in all tissues, presynaptic neurons, or postsynaptic muscles coupled with TOR inhibition resulted in a significant decrease in mean GluRIIA cluster sizes and relative GluRIIA fluorescence compared to knock down only animals (Fig. 7A and B). TOR inhibition coupled with the knock down of *lk6* in all conditions examined resulted in a significant overgrowth of the presynaptic motor neuron (Fig. 7C). Thus, TOR

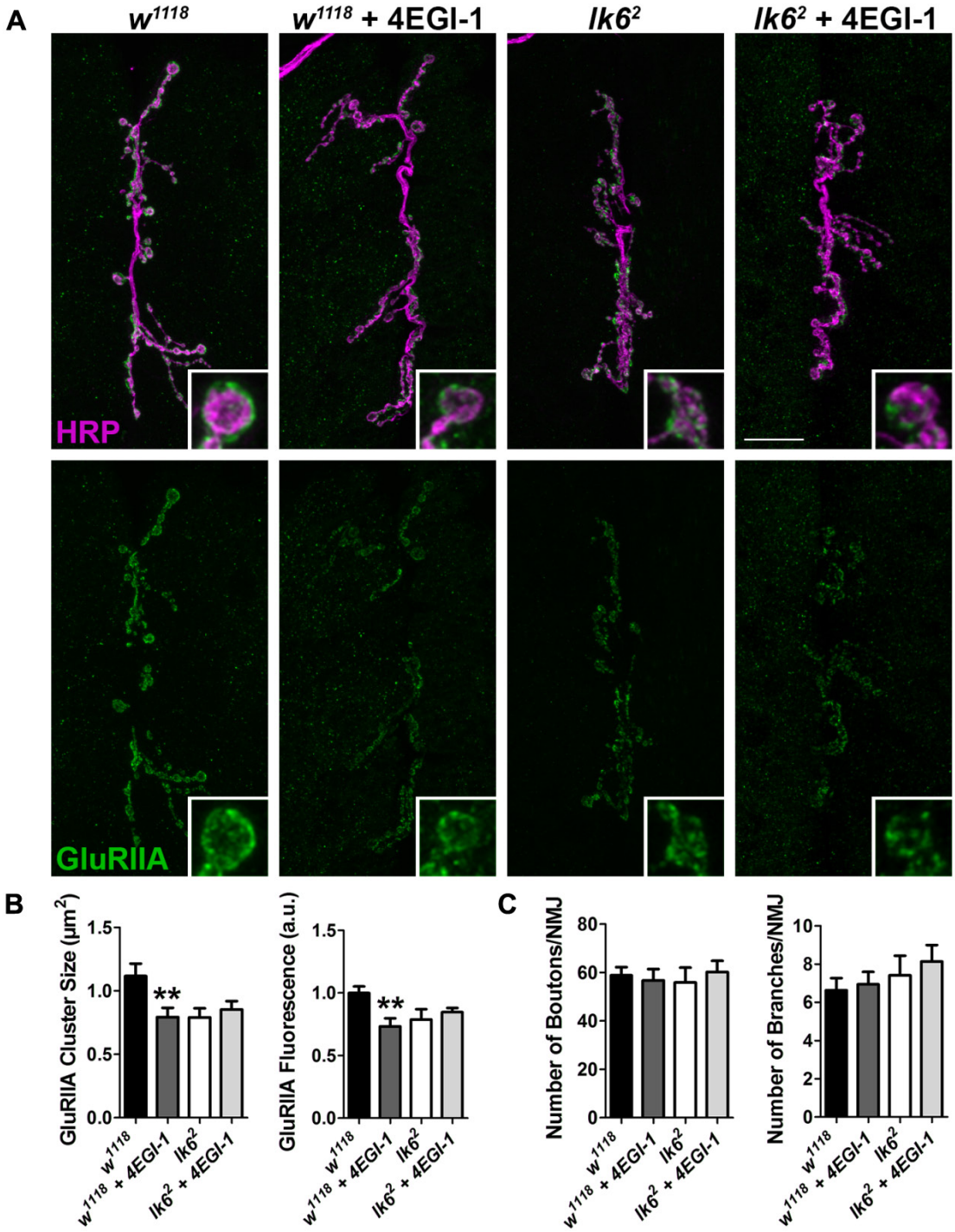

Figure 5. Disrupting the interaction between eIF4E and eIF4G negatively affects synaptic GluRIIA in w^{1118} controls but not in $lk6^2$ mutants. 10 μM 4EGI-1 was fed to larvae to interfere with eIF4G binding to eIF4E. (**A**) Confocal images of representative 6/7 NMJs labeled with antibodies against HRP (magenta), a neuronal membrane marker, and GluRIIA (green). Scale bar = 20 μM. Inset panels show anterior terminal boutons at high magnification. (**B**) Histogram depicting the quantification of GluRIIA cluster sizes and relative GluRIIA fluorescence intensities in the conditions as indicated. Fluorescence intensities were normalized to the w^{1118} control. (**C**) Quantification of presynaptic neuronal morphology as indicated by the number of 6/7 NMJ boutons and branches.

signaling but not Lk6 signaling restrains growth of the NMJ. These data suggest that Lk6 and TOR signaling independently regulate the synaptic localization of GluRIIA, possibly by each regulating different intermolecular interactions required for translation initiation.

Discussion

Translation of synaptic mRNAs is required for synaptic plasticity,[4–7,43,44] but the role of important translation initiators including the Mnks in synaptic development is largely unexplored. We found that the *Drosophila* homolog of Mnk1

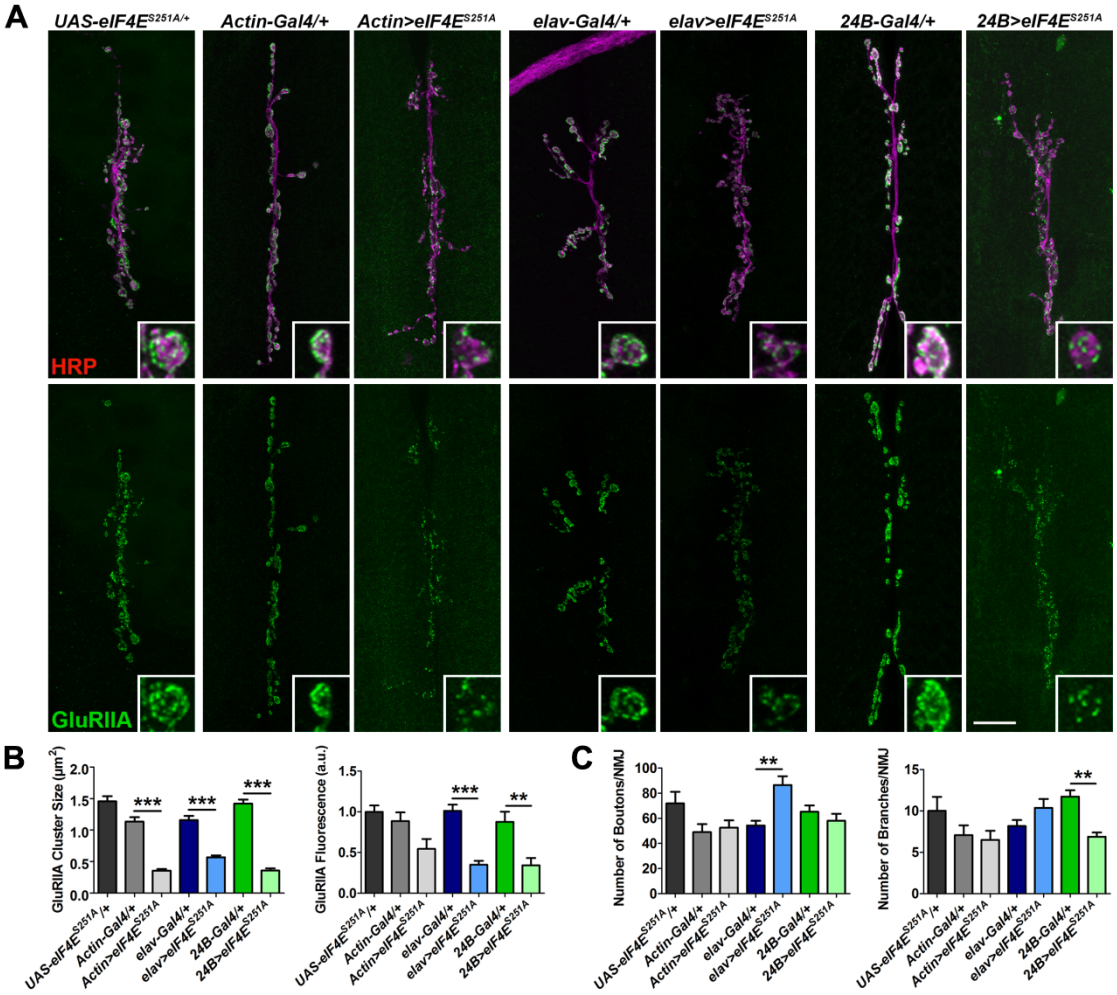

Figure 6. Expression of a nonphosphorylatable eIF4E impairs the synaptic localization of GluRIIA. An *UAS-eIF4E^{S251A}* transgene was expressed in all tissues using the *Actin5c-Gal4* driver, in neurons using the *elav-Gal4* driver, or in postsynaptic muscles using the *24B-Gal4* driver. (**A**) Third-instar larval NMJs on ventral longitudinal muscles 6 and 7, labeled using antibodies against the neuronal membrane marker HRP (magenta) and GluRIIA (green). Scale bar = 20 μm. Inset panels show the representative terminal boutons at high magnification. (**B**) Quantification of GluRIIA cluster sizes and mean relative fluorescence intensities for the listed genotypes. Fluorescence intensities were normalized to the *UAS-eIF4E^{S251}/+* outcrossed control. (**C**) Quantification of 6/7 boutons and branches in genotypes listed.

and Mnk2, Lk6,[30] is required in either pre- or postsynaptic cells for the synaptic localization of GluRs. Lk6 may regulate *gluRIIa* translation as the loss of synaptic GluRIIA also occurs as a result of expression of an isoform of eIF4E that cannot be phosphorylated by Lk6 but does not occur when the interaction between eIF4E and eIF4G is disrupted in *lk6^2* mutants. Lk6 likely works in parallel with TOR signaling to regulate the synaptic localization of GluRIIA (Fig. 8). Our collective data demonstrate that Lk6 is essential for synapse development and function.

Lk6 is required for the synaptic localization of GluRIIA. Postsynaptic GluRs cluster in apposition to presynaptic release sites.[51] The GluRIIA subunit increases the open time of the receptor leading to larger mEJPs[47] and is locally translated as a result of synaptic activity.[33] Synaptic levels of GluRIIA but not GluRIIB or GluRIIC are significantly reduced in *lk6^2* mutants (Fig. 1). The loss of GluRIIA impairs synaptic function as the amplitudes of both evoked and spontaneous neurotransmission are attenuated in *lk6^2* mutants (Fig. 2). Our data do not support a dominant pre- or postsynaptic role for Lk6 at the synapse. The reduction in mEJC amplitudes could be attributed to the postsynaptic loss of GluRIIA-containing receptors at the synapse of *lk6^2* mutants, but it could also be attributed to a reduction in the size of presynaptic vesicles.[73] Although we did not observe a significant difference in quantal content, an indicator of the number of presynaptic vesicles released,[51] in *lk6^2* mutants, this may be due to a compensatory increase in the number of vesicles released as a result of the selective loss of GluRIIA as occurs in *GluRIIA* mutants.[47]

Our data instead suggest that Lk6 is required in either presynaptic neurons or postsynaptic muscle for synaptic GluR localization (Fig. 4). Knock down of *lk6* in either pre- or postsynaptic cells resulted in the mislocalization and/or loss

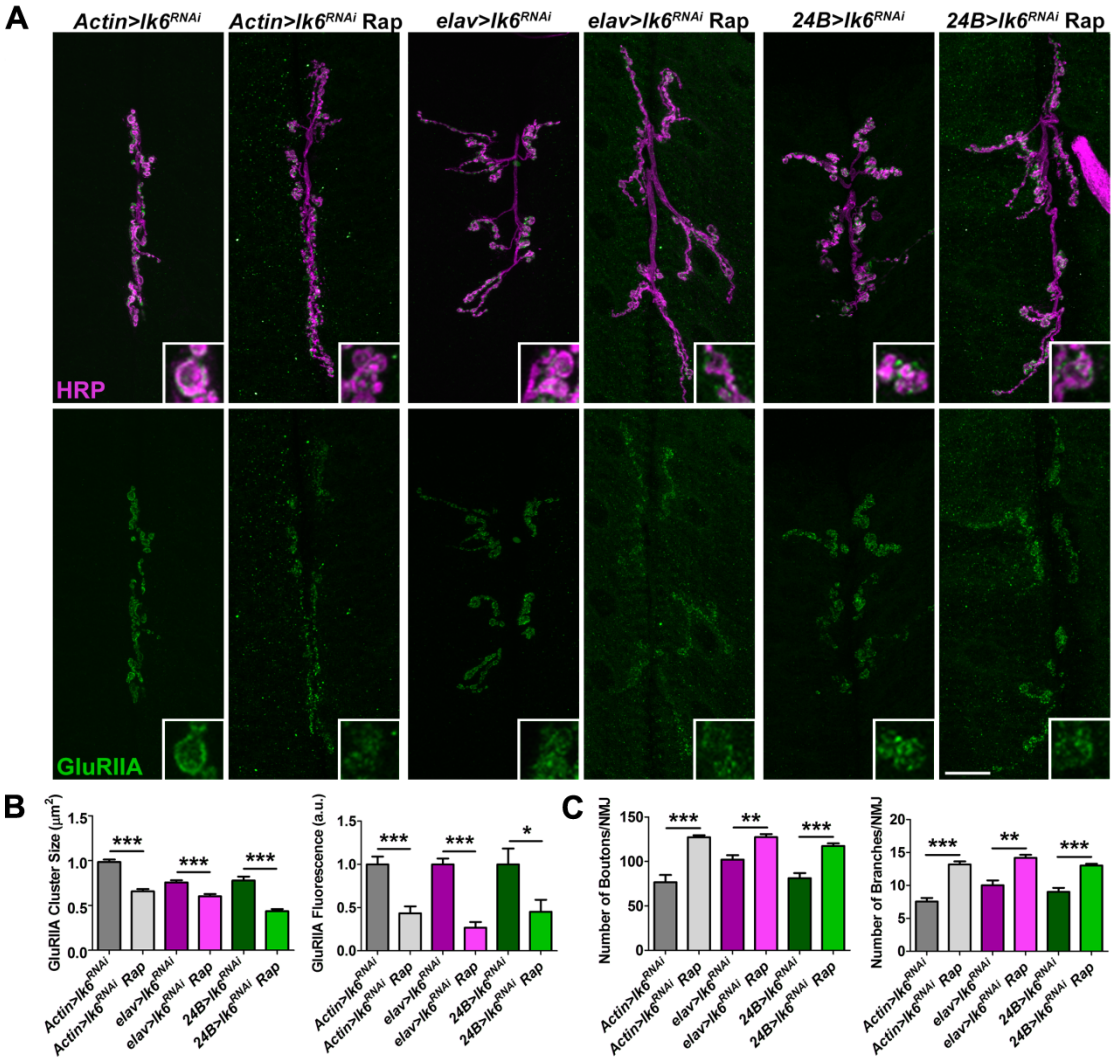

Figure 7. Knock down of *lk6* coupled with the inhibition of TOR signaling results in a greater loss of synaptic GluRIIA. *Lk6* was knocked down in all tissues using the *Actin5c-Gal4* driver, in neurons using the *Dcr2;;elav-Gal4* driver, or in postsynaptic muscles using the *Dcr2;;24B-Gal4* driver. TOR signaling was inhibited by feeding animals 100 µM of rapamycin in a yeast mix for 24 hours. (**A**) Representative third-instar larval NMJs on ventral longitudinal muscles 6 and 7 were immunolabeled using antibodies against the neuronal membrane marker HRP (magenta) and GluRIIA (green). Scale bar = 20 µm. Inset panels show the high-resolution images of terminal boutons. (**B**) Quantification of GluRIIA cluster sizes and mean GluRIIA fluorescence intensities for genotypes and conditions listed. (**C**) Quantification of presynaptic motor neuron morphology as indicated by the number of boutons and branches for genotypes and conditions listed.

of synaptic GluRIIA (Fig. 3). Although we did not observe differences in the density of Brp or synaptic levels of Syt (Supplementary Fig. 3), other synaptic mechanisms influence postsynaptic GluR localization. Bidirectional synaptic signaling may be altered in *lk6*[2] mutants. For example, acute BDNF treatment increased the surface expression of the AMPA receptor subunits, GluA1 and GluA2, as a result of ERK signaling.[74] BDNF is released from both presynaptic neurons and postsynaptic dendrites and binds to tropomyosin-related kinase B receptors localized both pre- and postsynaptically. BDNF signaling enhances presynaptic glutamate release and levels of the postsynaptic proteins GluA1, GluA2, and GluA3, along with the scaffolding proteins SAP97, GRIP1, and PICK1.[75] *Drosophila* expresses two neurotrophins, DNT1 and DNT2, which are similar in sequence and structure to BDNF.[76] DNT1 and DNT2 are secreted from postsynaptic muscles, but the receptors for these neurotrophins have not yet been identified.[77] Therefore, a similar mechanism may exist at the *Drosophila* NMJ where bidirectional neurotrophin signaling positively affects both presynaptic glutamate release and the levels of postsynaptic GluRs and this mechanism may be attenuated in *lk6*[2] mutants.

An additional possibility is that Lk6 may regulate communication between pre- and postsynaptic cells by acting on cell adhesion molecules (CAMs). CAMs are transmembrane proteins that stabilize the connection between the presynaptic neuron, the postsynaptic cell, and/or the glial cell. In the mature synapse, most CAMs are centrally

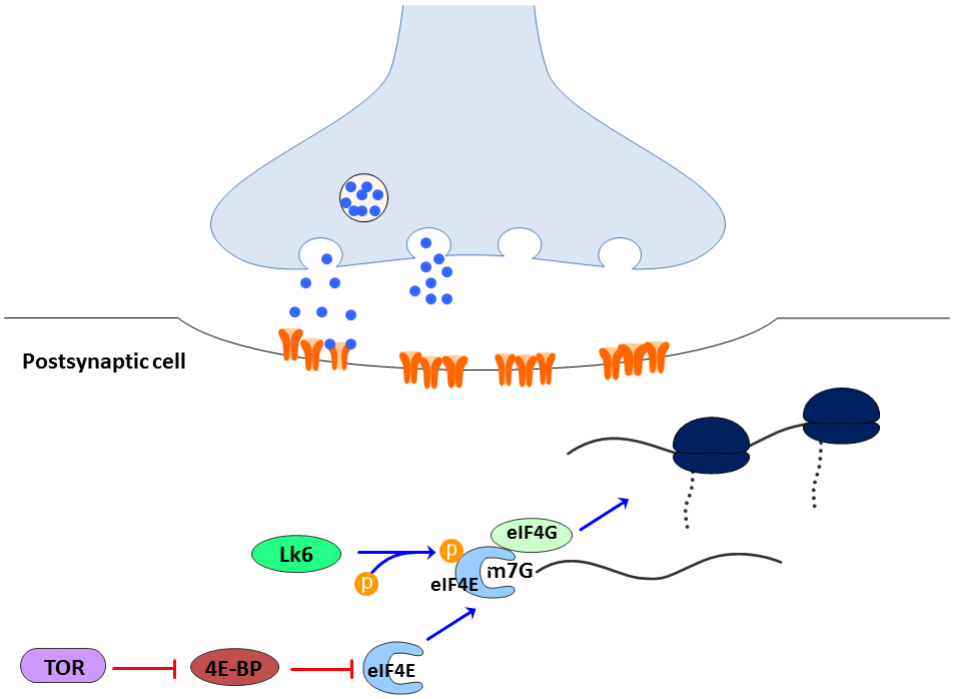

Figure 8. Model for the translation of synaptic mRNAs. Lk6 and TOR signaling converge on eIF4E to regulate translation initiation in postsynaptic cells. These pathways differentially promote GluRIIA expression and/or localization.

localized[78] and help organize the synaptic protein network of their respective cell.[79] CAM signaling is activated by binding to themselves, other CAMs, or the extracellular matrix and is important for synaptic plasticity and the localization of neurotransmitter receptors.[80,81] Attractive potential targets of Lk6 include the neurexin–neuroligin complex, which, at mammalian CNS synapses, recruits GluA2 to the synapse through interactions with PICK1[82] and has been shown to regulate GluRIIA localization in *Drosophila* embryos.[83] Lk6 may also regulate translation of cadherin–catenin complexes, which, in mammals, have been shown to enhance the surface localization of GluA2[84] and the kainate receptor subunit, GluK6.[85] In support of this, Mnk1 has been shown to regulate the translation of laminins, which are extracellular matrix ligands of integrin receptors, and Neurexin-1.[27]

Lk6 may regulate synaptic levels of GluRIIA by a non-canonical mechanism or by directly regulating translation of *gluRIIa* subunit mRNA. Lk6 is phosphorylated by ERK and phosphorylates eIF4E[48] at Ser251, which is analogous to Ser209 in mammals.[86] Exactly how this phosphorylation regulates translation is not well understood. While an early report found that phosphorylation of eIF4E enhanced its affinity for the 5′ cap,[87] other reports indicated that Mnk-induced phosphorylation of eIF4E reduced its interaction with the 5′ cap.[68,69] Disrupting the interaction between eIF4E and the 5′ cap, however, may enhance translation efficiency by increasing the availability of eIF4E to enable additional rounds of translation initiation.[70] Similarly, Mnk-mediated phosphorylation of eIF4E has been shown to either enhance[88,89] or repress[90,91] translation initiation.

Our data indicate that the loss of synaptic GluRIIA in *lk6*^{2} mutants may be translation dependent. Interfering with the association between eIF4E and eIF4G using the competitive inhibitor, 4EGI-1, produced a significant decrease in synaptic GluRIIA (Fig. 5) in controls but not in *lk6*^{2} mutants. Similarly, expression of a nonphosphorylatable mutant isoform of eIF4E (eIF4E^{S251A}) in either presynaptic neurons or postsynaptic muscle resulted in the loss of GluRIIA from the synapse (Fig. 6). Two hypotheses emerge from these data. First, Lk6 and eIF4E may independently regulate the synaptic localization of GluRIIA. The loss of GluRIIA is more pronounced after the expression of *UAS-eIF4E*^{S251A} than *UAS-lk6*^{RNAi} in both presynaptic neurons and postsynaptic muscle (compare the quantification in Figs. 3 and 6). We attempted to test this hypothesis by performing epistatic analyses to delineate the relationship between Lk6 and eIF4E. Consistent with previous reports,[31] however, overexpression of *lk6* or expression of a constitutively active isoform of Lk6 phenocopies *lk6* loss-of-function phenotypes (data not shown).

Lk6 could phosphorylate other substrates in addition to eIF4E. In support of this hypothesis, Mnk1 phosphorylates a variety of substrates, including polypyrimidine tract-binding protein-associated splicing factor (PSF)[92] in addition to eIF4E[20] and eIF4G.[21] Phosphorylation of PSF increases the association between PSF and AU-rich elements (AREs),[92] which are sequences in the 3′-untranslated region

that regulate the stability of mRNAs as a result of interactions with AU-rich element-binding proteins.[93] In addition, Mnk1 phosphorylates phospholipase A2, enhancing its catalytic activity.[94] Phospholipase A2 regulates membrane fusion events[95] and promotes LTP[96,97] and the plasma membrane localization of AMPA receptors.[98]

Second, the loss of GluRIIA in *lk6²* mutants may be the result of deficient translation initiation of *gluRIIa* itself or synaptic proteins that properly localize GluRIIA. In support of this hypothesis, Mnks[23] and Lk6[31] are not global regulators of translation initiation. Synaptic GluRIIA levels are positively correlated with eIF4E levels in the *Drosophila* NMJ postsynaptic subsynaptic reticulum.[33,99] Genheden et al (2015) recently identified 718 proteins whose synthesis was altered after BDNF treatment in Mnk knock out primary neurons compared to wild-type neurons. Gene ontology analysis indicated that proteins important for vesicle release and/or trafficking and synaptic plasticity are translated after BDNF treatment in a Mnk-dependent manner.[27]

The Lk6/Mnk pathway may function in parallel to the TOR signaling pathway to regulate the synaptic localization of GluRIIA. TOR signaling is important for synaptic plasticity[100] and regulates translation initiation by phosphorylating 4E-BP leading to its dissociation from eIF4E.[26] Loss of 4E-BP2 function increases GluA1 and GluA2 levels but not NMDA receptor subunit levels and enhances AMPA receptor-dependent neurotransmission.[101] Thus, TOR signaling may work cooperatively with Lk6 to regulate translation. This model is supported by data from mammalian cells where mTOR phosphorylation of 4E-BP[26] increases the binding of eIF4E to eIF4G and the 5′ cap.[102] Mnks then interact with eIF4G to phosphorylate eIF4E.[21] Components of both the ERK–Mnk–eIF4E and phosphoinositol 3-kinase–Akt–mTOR pathways are localized to synapses with eIF4E enriched in the postsynaptic density.[103] Similarly, eIF4E is localized to the postsynapse at the *Drosophila* NMJ,[33] and Akt specifically regulates GluRIIA localization and mEJC amplitudes.[104]

We observed a greater reduction in the synaptic localization of GluRIIA when TOR signaling was inhibited and *lk6* was knocked down compared to animals with intact TOR signaling but knocked down *lk6* (Fig. 6). These data could indicate that both TOR and Lk6 regulate translation, and this regulation is, at least partly, independent of the other pathway. In agreement with this, Mnk inhibitors in combination with rapamycin more severely attenuate protein synthesis compared to either Mnk inhibition or rapamycin alone.[88] Although we might expect to see a more severe reduction in synaptic GluRs after the inhibition of TOR signaling coupled with *lk6* knock down, there are a few probable explanations for these results. First, a number of studies suggest that rapamycin treatment only partially inhibits 4E-BP phosphorylation.[105–107] Therefore, some level of translational initiation could proceed uninhibited. Second, the inhibition of mTORC1 signaling by rapamycin may activate Mnk2, which then phosphorylates eIF4E.[108,109] Finally, neuronal and synaptic mRNAs have been shown to be translated via both cap-dependent and cap-independent mechanisms.[110,111]

Conclusion

Lk6 is required in presynaptic neurons or postsynaptic muscle cells for the proper synaptic localization of the GluRIIA subunit. Our collective results support a model whereby Lk6 regulates GluRIIA localization through molecular interactions with eIF4E (Fig. 8). Our data suggest that Lk6 and TOR signaling occur in parallel and may converge to regulate the eIF4E activity. Disruptions within these pathways have differential negative effects on GluR localization. These data further our understanding of the mechanisms that regulate GluR localization and provide insight on the contribution of translational regulators to synapse development.

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Author Contributions

Conceived and designed the experiments: NAH and FLWL. Analyzed the data: NAH, TLD, and FLWL. Wrote the first draft of the article: FLWL. Contributed to the writing of the article: NAH, BLT, and FLWL. Agreed the article results and conclusions: NAH, TLD, BLT, and FLWL. Jointly developed the structure and arguments for the article: NAH and FLWL. Made the critical revisions and approved the final version: NAH, TLD, BLT, and FLWL. All authors reviewed and approved the final article.

Supplementary Materials

Supplementary figure 1. Larval viability after 4EGI-1 treatment. Histogram showing the mean per cent viability after 24 hours of feeding third-instar larvae DMSO (control) or 5, 10, or 15 µM of 4EGI-1.

Supplementary figure 2. The loss of GluRIIA at *lk6²* mutant synapses is not likely due to changes in cytoskeletal structure. (**A**) Confocal micrographs of representative third-instar larval NMJs on ventral longitudinal muscles 6 and 7. NMJs were labeled using antibodies against the neuronal membrane marker HRP (magenta) and acetylated tubulin (green). Scale bar = 20 µm. Right histograms show the quantification of synaptic and extrasynaptic acetylated tubulin levels. (**B**) Confocal micrographs of 6/7 NMJs showing presynaptic motor neurons (green) and F-actin (magenta), labeled with phalloidin. Right histogram shows the quantification of muscle 7 area in genotypes listed.

Supplementary figure 3. Mutations in *lk6* do not affect the synaptic localization of Brp, DLG, or Syt. High-magnification confocal images showing NMJ terminals labeled with HRP (magenta) and DLG (**A**), Syt (**B**), and Brp (**C**). Scale bar = 5 µm. Quantification (right histograms) of immunolabeling for genotypes listed.

Supplementary table 1. Summary statistics for all figures. This is an Excel spreadsheet containing summary statistics for all experiments including means, standard error of the mean, and ns for each condition. *P*-values and relevant comparisons are also included.

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