Online Methods

Drosophila Stocks

All Drosophila strains were raised at 25°C. All mutants and transgenes were examined in a y, w background and maintained over GFP balancer chromosomes. The isogenic parental stock y, w; FRT42D was used as the wild-type control. The following alleles were used: eag1 Sh133, witA12, witB11, dfz2C1; Df(3L)ED4782 uncovering dfz2; wgL114, referred to here as wgTS; wg1; imp-α2D3, imp-α2D14; Df(3L)a1S1 uncovering importin-α1; dl7; imp-β1170; Df(2R)Δm22 uncovering importin-β11. Allele sources and references are detailed in Supplemental Table 1. wgTS larvae were raised at 18°C until early third instar and then shifted to 30°C for 18 hours before dissection. The following transgenic strains were used: 24B-GAL4; Elav-GAL4; BG487-GAL4; UAS-Importin-β11-eGFP; UAS-DFz2-FLAG; UAS-Wingless; UAS-myc-NLS-DFz2-C, UAS-nls-GFP, UAS-Importin-α2.

Immunohistochemistry

Wandering third instar larvae raised at low density were dissected and fixed for 20 minutes in fresh 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS or for 5 minutes in Bouin’s fixative (Sigma-Aldrich, St. Louis, MO). Larvae were incubated overnight at 4°C in primary antibodies and for 2 hours at room temperature in secondary antibodies. The following primary antibodies were used: rabbit anti-DFz2-C 1:200, rabbit anti-DFz2-N 1:100, rat anti-dGRIP 1:200, mouse anti-DLG 1:500, rabbit anti-Dorsal 1:1000, rabbit anti-FLAG 1:500 (Sigma-Aldrich, St. Louis, MO), mouse anti-Futsch 1:50, rabbit anti-Importin-α1 1:100, rabbit anti-Importin-α2 1:100, rabbit anti-Importin-α3 1:100, rabbit anti-Importin-β11 1:750, rabbit anti-Ketel 1:200, mouse anti-Lamin C 1:200, rat anti-Nanos 1:300, mouse anti-nonA 1:100, rabbit anti-Wingless 1:200, mouse anti-Myc 1:300 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-GluRIIA 1:100, rabbit anti-GluRIIB 1:2500, rabbit anti-GluRIIC 1:2000, mouse anti-
dPix 1:25, rabbit anti-dPak 1:500, rabbit anti-PAR-1 1:100, rabbit anti-pDLG$^{S797}$ 1:200, guinea pig anti-Syndapin 1:500, guinea pig anti-WASp 1:1000, mouse anti-α-spectrin 1:50. Antibody sources and references are detailed in Supplemental Table 2. FITC-, Cy3- or Cy5-conjugated secondary antibodies were used at 1:200 (Jackson ImmunoResearch, West Grove, PA). Alexa488-, Alexa546- or Alexa647-conjugated secondaries were used at 1:250 (Invitrogen, Carlsbad, CA). Cy3-, Cy5- or FITC-conjugated anti-HRP antibodies were used at 1:100 (Jackson ImmunoResearch, West Grove, PA). Larvae were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and stored at -20°C until imaging.

Electron Microscopy

Wandering third instar larvae were dissected as above and internal organs gently removed. Following dissection, the body walls, still pinned, were fixed at 4°C overnight in 2.5% paraformaldehyde, 5.0% glutaraldehyde, 0.06% picric acid in 0.1 M cacodylate buffer, rinsed 3x for 20 min in 0.1 M cacodylate buffer on ice, unpinned, and post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 h on ice. They were then rinsed 3 x for 10 min in deionized water and dehydrated in an ethanol series (50%, 70%, 95%, 100%, and 100%), and propylene oxide, and placed overnight in 50% TAAB 712 Resin in propylene oxide. They were transferred to fresh resin for 4 h and then embedded in fresh resin at 65°C for two days or until hard. The 6/7 muscle region was located by eye and the block trimmed around the desired area. Sections were taken parallel to the surface of the muscles: 4-90 nm sections were collected as a series and then 3 µm of thick sections removed before the next series of 90 nm sections. Sections were mounted on formvar coated single slot grids, stained with lead and uranyl acetate, and imaged on a Tecnai G² Spirit BioTWIN (FEI Company, Hillsboro, OR) electron microscope at 11000 x and 30000x magnification.

Imaging and Statistical Analysis
Larvae were imaged with a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and either a 63X 1.4 NA or 40X 1.0 NA objective. Images of NMJs were taken as confocal z-stacks with the upper and lower parameters determined by HRP immunoreactivity. Images of nuclei were taken as confocal z-stacks with the parameters defined by either nonA or Lamin C staining. Images were processed with the LSM software, Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) or Microsoft PowerPoint (Microsoft, Redmond, WA). “Ghost” boutons were analyzed in confocal z-stacks of an entire NMJ from muscles 6 and 7 in segments A2, A3 and A4 on the right and left sides and only counted if connected to the rest of the nerve. For comparisons of immunofluorescence, larvae were processed in the same tube and genotypes were imaged under identical conditions in the same session.

Measurements of bouton diameters and the width of the spectrin-containing zone were determined from α-spectrin and HRP immunoreactivity and were conducted as follows: for each NMJ, four type Ib boutons were measured (two terminal and two internal) from the NMJ on muscle 4. From these boutons, width measurements were conducted on high magnification images using ImageJ (NIH, Bethesda, MD). The width of the spectrin zone was defined as the distance from the margin of the HRP-positive bouton to the outer margin of spectrin immunoreactivity and experimentally it was determined by taking the diameter of spectrin immunoreactivity that surrounded the bouton, subtracting the diameter of the bouton, and dividing by two.

In electron micrographs, subsynaptic reticulum (SSR) thickness was calculated (ImageJ, NIH, Bethesda, MD) only on boutons that contained vesicles, an active zone, subcellular organelles and were > 1 µm in both length and width. An arbitrary center was chosen for each bouton and 8 radii drawn at 45° angles; the SSR was measured along each line and average for each bouton. Bouton area and SSR area were measured by tracing the borders of both the bouton and the entire bouton + SSR in ImageJ. The SSR area was determined by subtracting the bouton area measurement from the full area measurement. Other parameters were
determined as previously. Statistical analysis used GraphPad Prism 5 (Graphpad Software, La Jolla, CA). In cases involving more than two samples, statistical significance was calculated using ANOVA followed by with a Dunnett post-hoc test to the control sample. Where only two samples were compared, an unpaired student's t-test was used. Measurements are given as mean ± SEM. p values are listed as compared to the wild-type sample unless otherwise noted and *** indicates p < 0.0001.

Fz2-C puncta were quantified as previously described. Briefly, puncta were scored live on a Nikon E800 Epifluorescence microscope as distinct spots of Fz2-C immunoreactivity over background staining at muscles 6 and 7 in segments A2 and A3 on both the right and left sides. In all cases, nuclei were positively identified by co-staining with antibodies to nonA, Lamin C, or with DAPI.

Fluorescent intensity for glutamate receptors and postsynaptic molecules was measured in ImageJ as follows: confocal z-stacks of the entire NMJ at muscle 4 were imported and converted into multi-channel composite images. An ROI was drawn based on the anti-HRP channel and the mean fluorescence intensity measured in the other channels. In all genotypes, the average anti-HRP fluorescence did not differ significantly, enabling direct comparison of the experimental labeling. For all comparisons, larvae were processed simultaneously and imaged under identical settings.

**Immunoprecipitation**

For each genotype, larval body walls from 20 wandering third instar larvae were dissected as above, on ice, and drop-frozen in liquid nitrogen prior to homogenization on ice in 400 µL of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1 mM EDTA, 100 µM Na3VO4, 10 mM NaF, complete protease inhibitor tablet, Roche Applied Science, Indianapolis, IN). Debris was pelleted at 13000 RPM for 10 minutes at 4°C. M2 anti-FLAG-conjugated
agarose beads (Sigma-Aldrich, St. Louis, MO) were added to supernatants for 2 hours at 4°C and then washed extensively in lysis buffer. Proteins were then eluted at 95°C.

**Western Blots and SDS-PAGE Analysis**

Proteins were separated on 8% polyacrylamide gels and transferred to nitrocellulose membrane. Primary antibodies were applied overnight at 4°C and secondary antibodies for 1 hour at room temperature, diluted in blocking solution. The following primary antibodies were used: rabbit anti-GFP 1:3000 (Invitrogen, Carlsbad, CA), rabbit anti-GFP 1:5000 (AbCam, Cambridge, MA), rabbit anti-FLAG 1:5000 (Sigma-Aldrich, St. Louis, MO), mouse anti-FLAG 1:5000 (Sigma-Aldrich, St. Louis, MO), rabbit anti-Importin-α2 1:2000, mouse anti-α-Tubulin 1:25000 (Sigma-Aldrich, St. Louis, MO), mouse anti-Myc 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-V5 1:2500 (Invitrogen, Carlsbad, CA). HRP-conjugated secondary antibodies were used at 1:10000 (anti-mouse) or 1:20000 (anti-rabbit) and were obtained from Jackson ImmunoResearch (West Grove, PA). Blots were developed using the SuperSignal West Dura Extended Duration Substrate Kit (Thermo Scientific, Waltham, MA).

**S2 Cell Culture**

*Drosophila* S2 cells were maintained at room temperature in Schneider’s *Drosophila* medium (Invitrogen, Carlsbad, CA) containing 10% FBS, penicillin (1 U / mL), streptomycin (1 µg / mL) and amphotericin B (2.5 ng / mL). The following constructs were used: pUAST-Importin-β11-eGFP\[^{16}\], pAcpA-Actin5c-GAL4\[^{50}\], pAc5.1-Fz2-8xMyc-C\[^{10}\], pAc5.1-dGRIP-V5\[^{11}\]. Cells were transfected at 80% confluence using 1 µg of each plasmid (0.2 µg for pAcpA-Actin5c-GAL4) with the Effectene transfection reagent according to manufacturer’s protocols (QIAgene, Valencia, CA). Cells were lysed in lysis buffer following 48-hour incubation and processed for immunoprecipitation as above, with the following modification: supernatants were
incubated for 2 hours with anti-Myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and then with Protein A-conjugated Affi-Prep beads (Bio-Rad, Hercules, CA) for 1 hour at 4°C.