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Specific regulation of transsynaptic nanoalignment and postsynaptic assembly by a novel NCAM family synaptic adhesion molecule

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9 **Specific regulation of transsynaptic nanoalignment and postsynaptic assembly by a**
10 **novel NCAM family synaptic adhesion molecule**
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Abstract

Synapse formation underlies the organization of neurons into functional circuits and requires precise alignment and coordinated maturation of pre- and postsynaptic compartments. Many synaptogenic adhesion molecules have been identified that drive target recognition and sustained adhesion between appropriate synaptic partners. Yet the degree to which individual molecules serve distinct functions in synapse formation and maturation remains poorly understood. In particular, how exquisite nanoalignment of pre- and postsynaptic specializations flows from micron-scale adhesive interactions between synaptic partners remains a key unanswered question. Here we shed new light on this question by establishing a specialized set of synaptic functions for *Epithelial limiter of Fasciclin II function (Elff)*, an NCAM family member with previously unknown roles in the nervous system. Our structural and functional studies at the glutamatergic *Drosophila* NMJ of both sexes indicate that Elff is required for postsynaptic function and maturation as well as transsynaptic alignment; however, it is not required for presynaptic function, bouton formation, or developmental expansion of the NMJ. NMJs in *elff* null mutants display reduced glutamate receptor clusters beginning at the embryonic stage when NMJ synapses first form. Using super-resolution STED microscopy, we demonstrate that these poorly defined postsynaptic specializations are frequently out of register with presynaptic release sites. Surprisingly, the striking defects in *elff* nulls occur in the context of both normal active zone number and developmental expansion of the NMJ. These findings suggest an unexpected degree of specialization among transsynaptic adhesion complexes and demonstrate that Elff-mediated signaling is critical for transsynaptic nanoarchitecture of glutamatergic synapses.

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Significance Statement

This work reveals a surprisingly selective role for a novel NCAM family member called Elff in coordinating postsynaptic assembly and precise transsynaptic nanoalignment without altering NMJ formation, expansion or presynaptic active zone number. This separation of phenotypes is new and provides crucial insight into molecular specialization of adhesion complexes during synaptogenesis. Because NCAM family member dysfunction linked to multiple neurological disorders, most notably schizophrenia, illuminating the molecular mechanisms by which they orchestrate synaptic organization and function is an important goal.

Introduction

A dizzying array of transsynaptic adhesion molecules build, maintain, and remodel synaptic connections. In *Drosophila*, loss of Neurexin, Neuroligin, or Teneurin results in small neuromuscular junctions (NMJs) with too few boutons (Li et al., 2007; Banovic et al., 2010; Chen et al., 2010; Mosca et al., 2012). The gross morphological defects at these mutant NMJs

101 has complicated efforts to assign fine-scale subsynaptic functions to individual adhesion
102 molecules. In particular, it is unclear how the essential transsynaptic nanocolumnar
103 architecture emerges from larger scale adhesive interactions between pre- and postsynaptic
104 cells (Tang et al., 2016; Biederer et al., 2017).

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106 Neural Cell Adhesion Molecule (NCAM) homologs are conserved synaptic adhesion molecules
107 with widespread roles in synapse structure and function (Lin et al., 1994a; Rafuse et al., 2000;
108 Ashley et al., 2005; Shetty et al., 2013). Fasciclin II (Fas II) is the *Drosophila* homolog of
109 mammalian NCAMs (Lin et al., 1994a). Both murine NCAM1 and *Drosophila* Fas II are
110 homophilic adhesion molecules with five immunoglobulin domains (IgSF) and two fibronectin
111 type 3 (FN3) domains. In mice lacking all NCAM1 isoforms, NMJs form but are small and fail to
112 mature (Polo-Parada et al., 2001; Rafuse et al., 2000). Similarly, NMJs form in *fas II* nulls but
113 fail to expand, and the animals die at the first-instar larval stage (Schuster et al., 1996a).
114 NCAM family members are also linked to pre- and postsynaptic maturation (Sytnyk et al.,
115 2006; Kohsaka et al., 2007; Polo-Parada et al., 2001).

116

117 Lending clinical significance to the study of NCAM family members, their dysfunction is well-
118 linked to neurological disorders. Multiple studies implicate NCAM1 or its polysialylated form
119 PSA-NCAM1 to aberrant synaptic plasticity observed in Autism Spectrum Disorder and
120 Schizophrenia (Aonurm-Helm et al., 2016; Williams et al., 2020; Yu et al., 2025). Strikingly,
121 injection of anti-NCAM1 antibodies from individuals with schizophrenia leads to schizophrenia-
122 like synapse and behavior changes in mice (Shiwaku et al., 2022). Loss of human NCAM2 is
123 linked to a neurodevelopmental disorder caused by 21q21 deletion (Petit et al., 2015), while its
124 overexpression is associated with Down Syndrome (Parcerisas et al., 2020, 2021).

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126 NCAM orthologs are alternatively spliced, with transmembrane and GPI-linked isoforms
127 (Cunningham et al., 1987; Neuert et al., 2019). Clear evidence exists for cell type-specific roles
128 of distinct isoforms (Hata et al., 2007, 2018; Neuert et al., 2019). Introducing further
129 complexity, additional NCAM family members also regulate neuronal function. In *C. elegans*,
130 the IgSF-FN3 domain GPI-linked protein Rig-3 collaborates with the Wnt pathway to
131 antagonize activity-induced changes in neurotransmitter receptor abundance (Babu et al.,
132 2011). In *Drosophila*, two additional NCAM family members, CG15630/Factor of interpulse
133 interval (Fipi) and CG33543/Epithelial Limiter of Fasciclin II function (Elff) bind Fas II when
134 overexpressed in cultured cells (Özkan et al., 2013). By sequence, they are closely related to
135 the GPI-linked isoforms of NCAM1/2. Fipi is required in olfactory sensory neurons for courtship
136 song (Fedotov et al., 2014, 2018). Neuronal roles for Elff have not previously been reported,
137 though its function in wing development was recently described (Garcia-Alonso, 2024). We
138 initially became interested in Elff due to its extracellular domain structure, physical interaction
139 with Fas II, and widespread CNS expression profile leading us to hypothesize roles in synapse
140 structure or function.

141

Here we generate novel genetic reagents to undertake a comprehensive analysis of Elff function at glutamatergic synapses at the fly NMJ. Surprisingly, we do not detect defects in target recognition, active zone formation or NMJ expansion in *elff* mutants; however, we find clear defects in the assembly and maturation of the postsynaptic compartment. These phenotypes are present from late embryogenesis onward and are accompanied by misalignment of the diminished postsynaptic glutamate receptor clusters to presynaptic release sites. These structural phenotypes are reinforced by an electrophysiological analysis, which reveals reduced postsynaptic responsiveness in *elff* nulls. We propose that Elff is a critical component of adhesion complexes with essential and specific roles in postsynaptic assembly and maturation as well as transcellular nanoalignment at glutamatergic synapses.

Materials and Methods

Drosophila stocks

Flies were reared on standard medium in incubators set to 29°C (for Gal4-UAS experiments) or 25°C (all other experiments). Males and females were used for all experiments. The following fly lines were used: iso31 (gift of Masashi Tabuchi, Case Western Reserve University), *w*¹¹¹⁸, D42Gal4, 24BGal4, ElavGal4, *Elff*^{MI02791-GFSTF.1} (BDSC #60531), UAS-*elff* RNAi (BDSC #64879), *vasa*>Cas9 (BDSC#51324), and piggyBac transposase (BDSC #8283). Lines generated for this study include *elff*^{JR1}, *elff*^{ST1}, *elff*^{sfGFP-N}, and UAS-Elff (see below). For Gal4-UAS experiments, progeny of respective driver lines crossed to iso31 served as controls. For all other experiments, iso31 flies (Figures 1, 2, 3, 5) or progeny of iso31 flies outcrossed to *w*¹¹¹⁸ (Figures 4, 6, 7, 8, 9) were used as controls.

Generation of *elff* alleles

The *elff*^{JR1} and *elff*^{ST1} alleles were generated via published CRISPR/Cas9 genome engineering strategies (Gratz et al., 2013, 2015). Briefly, for *elff*^{JR1}, ~1kb targeting homology arms flanking the coding region of *elff* were synthesized and subcloned (GenScript, Inc.) into the pHG-DsRed-attP-w+ plasmid (Addgene 80898) to generate a donor template for homology-directed repair. A CG33543-targeting gRNA was generated by inserting a target sequence (5'-TTGCTGGCGATGGGGCAACA-3') into the pU6-3-gRNA plasmid (DGRC 1362) via site-directed mutagenesis (NEB Cat #E0554S). The homology-directed repair and gRNA plasmids were then injected into *vasa*>Cas9 embryos (BDSC 51324; BestGene, Inc.) to create CG33543 deletions, in which 9.4kb of the endogenous CG33543 gene (2Lcomplement:1996930..2006339) were replaced with an attP-loxP-DsRed-loxP cassette. Endogenously tagged *elff* was generated using a scarless CRISPR/piggyBac-based approach (flycrispr.org; (Gratz et al., 2019)). Because SignalP 6.0 predicted a possible signal peptide cleavage site between residues 23 and 24, sequences coding for sfGFP and a visible marker (piggyBac-3xP3-DsRed) flanked by flexible GGS-based linkers were inserted into the second exon of the endogenous *elff* locus, downstream of the predicted cleavage site (insertion site between bp 2,002,900 and 2,002,901 (D. melanogaster genome release r6.53). *elff* is on the minus strand of chromosome 2L, so all positions are given as genomic coordinates. A gRNA (5'-ACCAAGTGGCGCGATCCCCGCGG-3'; target site bp 2,002,722-2,002,744, PAM bolded)

was selected using the CRISPR Optimal Target Finder (targetfinder.flycrispr.neuro.brown.edu/) and used to target the endogenous locus. Two silent point mutations were introduced into the donor within the gRNA target site (genomic positions 2,002,730 and 2,002,733, spanning the codon pair at bp 2,002,730–2,002,735: CGCGAT→CGTGAC (underlined in sequence above), encoding the same Arg-Asp residues) to prevent re-cutting of the donor and resulting allele. Precise editing was molecularly confirmed. The unexcised insertion, *elff[ST1]* (DsRed+), disrupts the locus and was used as the mutant allele. *piggyBac* transposase was subsequently used for removal of the marker to generate the in-frame sfGFP-tagged allele (DsRed-) used in Fig. 1.

Generation of UAS lines

CG33543 cDNA was synthesized and subcloned into the pACU UAS transformation vector (Chun Han, Addgene 58373; GenScript, Inc.). This donor vector was then injected into embryos of an attP transposable element insertion stock (BDSC 36304; BestGene, Inc.) to generate a stable *Drosophila* line expressing CG33543 cDNA downstream of UAS regulatory regions.

Immunohistochemistry

The following commercially available primary antibodies were used: anti-Bruchpilot (DSHB, nc82) [1:100]; anti-Discs large (DSHB, 4F3) [1:500 third instar, 1:100 second instar, 1:100 embryo]; anti-alpha Spectrin (DSHB, 3A9) [1:100 third instar, 1:50 second instar]. Primary antibodies for GluR subunits were provided by Dion Dickman (University of Southern California): guinea pig anti-GluRIIA [1:1000]; rabbit anti-GluRIIB [1:1000]; rabbit anti-GluRIIC [1:2500], rabbit anti-GluRIIE [1:2000 third instar, 1:500 second instar, 1:500 embryo, 1:100 STED]. Secondary antibodies used in this work were goat anti-mouse Alexa Fluor 488-, 568-, 594-, and 647-conjugated IgG (Invitrogen, A-11029; A-11031; A-21125; A-32728), goat anti-rabbit Alexa Fluor 488-conjugated IgG (Invitrogen, A-11034), goat anti-rabbit STAR RED (Abberior), and goat anti-guinea pig Alexa Fluor 488-conjugated IgG (Invitrogen, A-11073). All secondary antibodies were used at a concentration of 1:300 for standard confocal and Airyscan preparations and a concentration of 1:100 for STED preparations. HRP staining was performed by incubating samples with Alexa Fluor 647-conjugated, Alexa Fluor 488-conjugated or DyLight 594-conjugated anti-HRP (Jackson ImmunoResearch 123-585-021, 123-605-021) at 1:200 alongside secondary antibodies. GFP staining was performed by incubating samples with GFP Polyclonal Antibody, Alexa Fluor 488 (Invitrogen, A-21311) [1:500 third instar, 1:400 adult brain].

Adult brain dissections

Adult flies were anesthetized on ice and decapitated. Brains were then rapidly dissected from the heads in ice-cold PBS and fixed for 20 min with 4% PFA in PTX10 (1% Triton X-100 in PBS). Fixed brains were washed with PTX10 and incubated for 2 hr in blocking buffer (5% normal goat serum, 0.3% Triton X-100 in PBS), followed by an overnight incubation at 4°C with

225 conjugated GFP antibody diluted in PTX (0.1% Triton X-100 in PBS). The following day, brains
226 were washed 3x30 min in PTX10, then placed in SlowFade Gold mountant (Invitrogen
227 S36936) for approximately 30 min (stationary). Brains were then mounted as previously
228 described (Kelly et al., 2017). Unless otherwise noted, all fixation, incubation, and washing
229 steps were performed at room temperature (RT) while nutating.

230

231 Larval NMJ dissections

232 Second- and third-instar larvae were dissected in PBS and fixed for 10 minutes in Bouin's
233 fixative. Larval fillets were then blocked at RT in either 1% (third instar) or 5% (second instar)
234 NGS in PTX and incubated overnight with primary antibodies at 4°C. Fillets were then washed
235 in 1% NGS and incubated with secondary antibodies for 2-4 hours at RT. Following a final
236 wash in PTX, fillets were mounted in Pro-Long Gold (Thermo Fisher #P36930).

237

238 Embryo dissections

239 Overnight collections of *Drosophila* embryos were dechorionated in 50% bleach for 4 minutes.
240 After dechorionation, stage 17 embryos were selected, fixed in 2 ml methanol and 2 ml
241 heptane in a glass test tube with gentle rocking for 2 minutes, and devitellinized by 1 minute of
242 shaking in 6 ml methanol. Devitellinized embryos were partially dissected in a droplet of PBS
243 on a glass microscope slide by making a small opening at the midline of the ventral cuticle with
244 sharpened tungsten wires. Partially dissected embryos were then transferred to a watch glass
245 and fixed again in cold methanol on ice for 5 minutes. Fully fixed embryos were blocked in 5%
246 NGS for 3 washes of 5 minutes followed by 1 wash of 30 minutes in the watch glass, then
247 transferred to a 0.2 ml Eppendorf tube and incubated with primary antibodies and conjugated
248 HRP antibody diluted in 5% NGS overnight at 4°C and an additional 4 hr at RT the following
249 day. Embryos were then washed in 5% NGS for 3 washes of 5 minutes and incubated with
250 secondary antibodies overnight at 4°C. After staining, embryos were fully filleted in 50%
251 glycerol on a glass microscope slide by extending the original midline opening down the length
252 of the ventral cuticle with sharpened tungsten wires. Fully filleted embryos were then mounted
253 in Pro-Long Gold directly on the glass slide used for dissection.

254

255 Electrophysiology

256 Electrophysiological recordings were performed as previously described (Zhang et al., 2010)
257 with minor modifications. Male third-instar larvae were dissected in low-Ca²⁺ hemolymph-like
258 saline (HL3; in mM: 70 NaCl, 5 KCl, 10 MgCl₂, 10 NaHCO₃, 115 sucrose, 5 trehalose, 5
259 HEPES, pH 7.2)(Zhang and Stewart, 2010) containing 0.2 mM Ca²⁺. Sharp intracellular
260 recordings were performed in HL3 saline containing 0.6 mM Ca²⁺ from muscle 6 of abdominal
261 segments A3 and A4 using borosilicate electrodes (resistance 5–12 MΩ) filled with 3 M KCl.
262 Recordings were conducted using a Nikon FN1 microscope with a 40X/0.80 NA water-dipping
263 objective, and acquired with an AxoClamp 900A amplifier, Digidata 1550B digitizer, and
264 pClamp 11 software (Molecular Devices). Signals were digitized at 10 kHz and filtered at 1
265 kHz. Miniature excitatory junctional potentials (mEJPs) were recorded in the absence of

stimulation and cut motor axons were stimulated to elicit evoked EJPs. For each cell, at least 60 mEJPs were analyzed using Mini Analysis (Synaptosoft) to calculate mean mEJP amplitude. EJPs were elicited using an Isolated Pulse Stimulator (Model 2100, A-M Systems), at a frequency of 0.2 Hz with intensity adjusted per cell to consistently activate compound responses from both type Ib and Is motor neurons innervating muscle 6. At least 30 consecutive EJPs were recorded and analyzed in Clampfit to obtain mean EJP amplitude. Quantal content was estimated by dividing mean EJP amplitude by mean mEJP amplitude. Muscle input resistance (R_{in}) and resting membrane potential (V_{rest}) were monitored throughout. Recordings were included in the analysis only if V_{rest} was between -50 and -80 mV and R_{in} was ≥ 5 M Ω .

Larval Crawling

Prior to behavioral testing, 100mm x 20mm Petri dishes were prepared with a layer of 1% agarose dyed with 0.6% brilliant black fountain ink (Pelikan). On testing day, three third-instar larvae were transferred to the center of a black-dyed agarose plate containing a thin layer of dH₂O for moisture. Larvae were recorded for 5 minutes using a 48MP phone camera. All movies were then converted to 0.2 frames per second in AVI format and uploaded to ImageJ (v2.16.0). Using the Manual Tracking plugin, larval position was indicated every 5 s (60 frames total) to measure total distance crawled and average velocity. Independent experimental trials were performed around the same time of day.

Confocal Microscopy and Airyscan

Standard confocal images were acquired with a Zeiss LSM 800 laser scanning microscope using a 20x objective for adult brains, a 40x objective for third-instar larvae, a 63x objective for second-instar larvae, and a 100x objective for stage 17 embryos. The Zeiss Airyscan software was used with a 63x objective for obtaining images in Figure 8. For intensity quantification, laser intensity and gain were maintained across an experiment. For nascent bouton quantification, laser intensity and gain were optimized for each Z-stack.

Bouton Number: Bouton number was manually counted from muscle 4 in segments A2-A5 in third instar larvae, from muscle 6/7 in segments A3-A4 in second instar larvae, and from muscle 6/7 in segments A2-A5 in embryos. Bouton number was manually quantified as rounded structures outlined by HRP, considering only Type Ib NMJs. **Nascent Boutons:** NMJs were manually scored from muscles 6/7, segment A2. Nascent boutons were quantified as those having HRP signal with no appreciable Dlg signal present.

Quantification of Fluorescence Intensity and Puncta Size/Density

Quantification of fluorescence intensity and puncta density was done using Imaris 9.7.1 (Bitplane). For third instar NMJs, intensity measurements of synaptic alpha-Spectrin and Dlg were made by generating Surfaces from the respective channels. For fluorescence intensity

measurements of GluR and Fas II, the Surfaces function was used on the HRP channel to outline neuronal membranes. In each case, the resulting surface was manually processed by an experimenter to isolate the NMJ being analyzed, and the mean fluorescence intensity value of the channel of interest within the generated surface was captured. To quantify Brp puncta density in third instar NMJs, the Surfaces function was used on the HRP channel and manually processed to isolate the NMJ. The resulting HRP surface was then used to mask Brp within the NMJ. Using the Spots function, Brp puncta within the masked channel were identified and quantified under the following parameters: algorithm set to different Spot sizes (region growing), background subtraction enabled, estimated diameter of 0.2 μm , and Spots region from absolute intensity with automatic region thresholding enabled. To calculate puncta density, the number of puncta per NMJ was divided by the surface area of the HRP surface. For embryonic NMJs, Brp puncta number and Dlg intensity were quantified using slightly modified versions of the third instar pipelines, while GluR intensity was measured by identifying GluR puncta via a Spots function on GluR signal within the masked HRP surface and capturing mean fluorescence intensity within those spots. Brp puncta size was calculated in ImageJ from STED images by drawing a line across the widest point of each complete planar Brp ring, using the "Plot Profile" function to identify 2 local maxima of Brp intensity, and calculating the distance between the X-values of the local maxima.

Stimulated emission depletion (STED) microscopy and image acquisition

Two-color, pulsed, time-gated STED microscopy was performed on an inverted Leica TCS SP8 STED 3X microscope using an HC PL APO CS2 100 $\times$ /1.40 NA oil-immersion objective (immersion oil refractive index 1.518). Fluorophores were excited with a pulsed white-light laser and depleted with a 775-nm pulsed STED laser using a vortex phase mask to achieve lateral (2D) super-resolution. Optical sectioning was achieved using a confocal pinhole of 151.5 μm (approximately 1 Airy unit). Brp was labeled with Alexa Fluor 594, excited at 586 nm, detected between 596 and 620 nm with time-gated detection from 0.2 to 10.2 ns, and depleted with the 775-nm STED laser at 50% instrument power. GluRIIE was labeled with Abberior STAR RED, excited at 633 nm, detected between 647 and 736 nm with time-gated detection from 1.0 to 4.5 ns, and depleted with the 775-nm STED laser at 10% instrument power. Emission was collected using gated HyD detectors in standard acquisition mode. The two channels were acquired sequentially over the same field of view, and chromatic channel registration was verified using 0.1- μm TetraSpeck fluorescent microspheres imaged under identical acquisition conditions.

Images of boutons from muscle 6/7 NMJs were acquired as z-stacks with a pixel size of 21 $\times$ 21 nm and a z-step of 156 nm using a 400-Hz unidirectional scan speed (0.95- μs pixel dwell time), 3 $\times$ line averaging, and 2 $\times$ frame accumulation. Experimental groups within each experiment were imaged side by side using identical acquisition settings. Samples were mounted in ProLong Glass (refractive index $\sim$ 1.52) beneath #1.5H coverslips (170 $\pm$ 5 μm thickness, 18 mm diameter). Raw STED image stacks were deconvolved in Huygens Professional (Scientific Volume Imaging) using a theoretical point-spread function and a standardized deconvolution template. The same deconvolution template and parameters were

348 applied to all image stacks. Deconvolved images were used for visualization and subsequent
349 quantitative analysis.

350

351 Quantification of Pre and Postsynaptic Apposition

352 To quantify apposition of Brp and GluRIIE in both third instar and embryonic NMJs for images
353 taken via standard confocal microscopy (embryos) or Airyscan microscopy (third instar), Brp
354 spots and GluRIIE fields not colocalized with their respective synaptic partner marker were
355 manually counted and divided by the total number of Brp spots or GluRIIE clusters,
356 respectively, at each NMJ. For this analysis, we defined non-colocalized spots/fields as Brp
357 spots or GluRIIE fields not overlapping with any signal of their respective synaptic partner
358 marker. Quantification of apposition in STED images was done using ImageJ on complete
359 planar Brp rings (oriented parallel to the field of view) and their proximal associated GluRIIE
360 fields from one bouton per NMJ. A straight line of approximately 1.0 μm was drawn across
361 each planar ring so as to capture the GluRIIE signal in closest proximity to the Brp ring, and
362 the “plot profile” function was used to generate intensity values along the line for both Brp and
363 GluRIIE. From these profiles, up to 2 local maxima for both the Brp intensity plots and the
364 GluRIIE intensity plots were identified for each ring, and the distance between Brp and GluRIIE
365 peaks was found by calculating the difference between the X-values for each pair of Brp-
366 GluRIIE peaks in closest proximity to one another. Calculating the fraction of GluR signal
367 within the immediate vicinity of Brp was performed using Imaris 9.7.1 on STED images. Using
368 the Spots function, Spots of fixed 0.6 μm (600 nm) diameter were placed at the center of each
369 Brp punctum. Given that the lateral diameter of a Brp ring is approximately 200 nm, each Spot
370 encapsulated a region spanning the entire Brp ring and extending past its outer edge by about
371 200 nm. GluRIIE signal was then masked within these Spots. Using the masked GluRIIE
372 signal, a Surface was generated to represent the portion of GluRIIE within the mask. In
373 parallel, a surface was generated from the original GluRIIE signal, representing the total
374 GluRIIE in the bouton of interest. Areas for both surfaces were extracted from Imaris, and the
375 ratio of masked GluRIIE surface area to total GluRIIE surface area was calculated.

376

377 Statistics

378 Statistical analyses were performed with Prism 10.2.1 (GraphPad). All data is from at least two
379 independent experiments. For microscopy experiments, each data point represents one
380 neuromuscular junction, unless otherwise noted. For electrophysiology and behavior
381 experiments, each data point represents recordings from one animal. Shapiro-Wilks normality
382 testing was performed on all data sets. On those demonstrating a normal distribution, an
383 unpaired t-test for two groups or a one-way ANOVA followed by Tukey’s multiple comparisons
384 test for three or more groups were performed. For non-normal distributions, nonparametric
385 tests were used: a Mann-Whitney test for comparisons of two groups or a Kruskal-Wallis
386 followed by Dunn’s multiple comparisons test for three or more groups. For categorical data, a
387 chi-square test was used. p-values, statistical tests, ‘n’, and number of animals used are
388 reported in each figure legend.

389

Results

The adhesion protein CG33543/Elff localizes to synapses throughout the CNS

The *Drosophila* genome codes for more than 200 unique adhesion proteins (Özkan et al., 2013), many of which are expressed in the developing and/or adult nervous system. Of the major structural families, IgSF proteins are the most numerous; moreover, their binding partners have been systematically assessed (Özkan et al., 2013), providing insight into the pathways through which they regulate nervous system structure and function. We noted that two relatively uncharacterized IgSF proteins, CG33543 (Elff) and CG15630 (Fipi), are putative extracellular heterophilic binding partners of the quintessential neuronal adhesion protein Fasciclin II (Fas II). Interestingly, neither Fipi nor Elff are expressed during embryonic stages when Fas II promotes axon outgrowth and guidance (Graveley et al., 2011), arguing for at least some degree of independence among Fas II, Elff, and Fipi. Here, we focus on CG33543/Elff, given its widespread neuronal expression pattern at later developmental stages and in adults (see below).

While Fas II undergoes extensive alternative splicing generating both transmembrane and GPI-linked isoforms, the CG33543 locus is predicted to encode a single GPI-linked isoform containing three IgSF domains and a single Fibronectin 3 (FN3) domain (Figure 1A) (Öztürk-Çolak et al., 2024). CG33543 was recently named *elff* (epithelial limiter of Fasciclin 2 function) on the basis of an RNAi phenotype in the wing (Garcia-Alonso, 2024). To assess Elff function, we generated two independent null alleles via CRISPR-mediated genome engineering. *elff^{JR1}* is a 9.4 KB deletion in which the entire *elff* locus is replaced by an attP site (Figure 1B). *elff^{ST1}*, on the other hand, contains an insertion immediately downstream of the signal sequence that includes sfGFP and a visible marker (DsRed) flanked by piggyBac inverted terminal repeat sequences. Prior to DsRed removal, the insertion results in early transcriptional termination and is a predicted null allele (Figure 1B). Both alleles were validated via PCR and sequencing (see Methods) and are homozygous viable and fertile.

Following PiggyBac transposase-mediated removal of the visible marker, *elff^{ST1}* generates an endogenous N-term epitope tagged protein (Elff^{sfGFP-N}). We characterized Elff expression in the nervous system using both Elff^{sfGFP-N} and a preexisting MIMIC protein trap, Elff^{MIMIC{PT-GFSTF.1}} (Nagarkar-Jaiswal et al., 2015). Both Elff^{MIMIC{PT-GFSTF.1}} and Elff^{sfGFP-N} are strongly expressed in synaptic neuropil in third-instar larvae and the adult brain (Figure 1C-D). Additionally, we observe specific GFP expression at the third-instar larval NMJ in animals carrying either the protein trap or the endogenous tag (Figure 1 E-G). Both are expressed in a similar punctate pattern often observed at the edge of HRP-positive boutons, consistent with the presence of Elff in the synaptic cleft. To test whether Elff is present at active zones, we co-labeled Elff-tagged NMJs with GFP and the cytomatrix protein Bruchpilot (Brp) (Wagh et al., 2006). We find that GFP-positive puncta surround Brp-positive active zones (Figure 1H), indicating that Elff is not active zone-localized and may instead be a component of the periaxial zone. Together, these data indicate that Elff is broadly synapse-associated during development and

in adults. At the NMJ, its expression appears predominantly presynaptic, though given its close association with the membrane, we cannot rule out postsynaptic expression.

As a first test of a functional requirement, we investigated if Elff is required for NMJ growth. We characterized the phenotype of animals transheterozygous for the two independent alleles (*elff^{JR1/ST1}*) to minimize potential off-target effects of CRISPR-mediated mutagenesis. We find no difference in the number or size of synaptic boutons in *elff^{JR1/ST1}* mutant NMJs relative to controls (Figures 1J-K and S1 D), arguing that Elff does not control baseline NMJ growth. Both the viability and the lack of a gross morphological phenotype in these mutants are in stark contrast to the early larval lethality and NMJ growth defects in Fas II mutations (Lin et al., 1994b; Schuster et al., 1996a, 1996b; Davis et al., 1997; Yoshihara et al., 1997), arguing that Elff does not partner with Fas II to control motor axon targeting or overall NMJ adhesion. Moreover, loss of Elff does not alter overall Fas II levels at the NMJ (Figure S1), indicating that Elff is not essential for Fas II expression at the NMJ.

Presynaptic Elff promotes postsynaptic maturation at the NMJ

The *Drosophila* equivalent of the postsynaptic density (PSD) at the NMJ is the subsynaptic reticulum (SSR), which corresponds to a weblike structure of filamentous components (Atwood et al., 1993). In the preceding analysis, we co-labeled NMJs with HRP and the commonly used SSR marker Dlg (*Drosophila* PSD95) to distinguish Type 1b and 1s boutons (Zito et al., 1999; Menon et al., 2013) and noticed that *elff* mutant NMJs display an apparent loss of postsynaptic Dlg (Figure 1I-J). Indeed, quantification revealed an approximate 50% decrease in mean Dlg intensity in third-instar *elff^{JR1/ST1}* homozygotes (Figure 2A-B, E). We find a similar decrease in Dlg intensity in homozygous *elff^{sfGFP-N}* animals (Figure 2E), indicating that the N-terminal GFP tag interferes with Elff function. In contrast, Dlg expression levels are normal in animals carrying the MIMIC protein trap (Figure 2E), indicating that Elff function is preserved in this background. While Dlg is widely used as a marker for the PSD, it is also present in presynaptic terminals, albeit at much lower levels (Astorga et al., 2016). To assess if Elff also promotes presynaptic Dlg expression, we compared Dlg intensity inside (presynaptic) and outside (postsynaptic) an HRP membrane mask in *elff* mutants using an Imaris imaging pipeline. We find a clear reduction in postsynaptic Dlg intensity and no change in presynaptic Dlg intensity in *elff* mutants (Figure S2A-B), indicating a specific role for Elff in promoting postsynaptic Dlg expression.

To probe specific cellular requirements of *elff*, we employed an existing *elff* RNAi line for cell-type specific knockdown. Motoneuron-specific, but not muscle-specific, *elff* knockdown results in loss of Dlg (Figure 2A, C-E), arguing that Elff is required in the presynaptic motoneuron for Dlg accumulation. To test if cell type-specific Elff overexpression is sufficient to rescue *elff* null phenotypes, we generated UAS-Elff transgenic animals. Consistent with a presynaptic requirement for Elff, presynaptic expression of Elff driven by D42Gal4 fully rescues Dlg abundance in *elff* nulls (Figure S2C-D).

473 The reduction of Dlg in *elff* mutants suggests Elff is required for postsynaptic maturation. If so,
474 we hypothesized it would be accompanied by an increase in nascent boutons in *elff*
475 homozygotes. Nascent (or ghost) boutons are newly budded boutons lacking a corresponding
476 postsynaptic compartment. They are typically identified as individual HRP-positive presynaptic
477 boutons in the absence of corresponding Dlg-positive postsynaptic specializations. They occur
478 with low frequency in wild-type strains and with higher frequency in mutants with impaired
479 postsynaptic maturation (Ataman et al., 2008; Mosca and Schwarz, 2010; McLaughlin et al.,
480 2016). Because Dlg levels are reduced in *elff* mutants, we scaled up Dlg fluorescence in *elff*
481 LOF mutants in order to visualize whether any Dlg is present in apposition to nascent boutons.
482 In line with our hypothesis, loss of *elff* results in a roughly four-fold increase in nascent boutons
483 (Figure 2F-H). We employed an RNAi-mediated approach to determine cell-type specific
484 requirements. Presynaptic *elff* knockdown also results in significantly increased numbers of
485 nascent boutons (Figure 2H), while postsynaptic *elff* knockdown does not alter nascent bouton
486 number (Figure 2H). Together, these findings argue that presynaptic Elff is necessary for
487 postsynaptic maturation.

488

489 Elff acts transsynaptically to promote alpha-Spectrin accumulation

490 To investigate the extent of the defect in postsynaptic maturation, we assessed alpha-Spectrin.
491 The Spectrin cytoskeleton is composed of an ordered array of alpha and beta Spectrin
492 heterodimers and is an evolutionarily conserved component of pre- and postsynaptic
493 specializations (Malchiodi-Albedi et al., 1993). At the *Drosophila* NMJ, the postsynaptic
494 Spectrin scaffold is particularly apparent and is observed as a halo surrounding HRP-positive
495 presynaptic boutons (Figure 3A). Strikingly, we find a greater than 75% reduction in alpha-
496 Spectrin levels in third-instar larval *elff^{JR1/ST1}* homozygotes (Figure 3A-B, E). Because alpha-
497 Spectrin is also present at low levels in the presynaptic compartment (Featherstone et al.,
498 2001; Pielage et al., 2005), we wanted to test if Elff is exclusively required to promote
499 postsynaptic levels of alpha-Spectrin. Thus, as above for Dlg, we compared alpha-Spectrin
500 intensity inside (presynaptic) and outside (postsynaptic) of an HRP membrane mask. In *elff*
501 nulls, we find a modest, but significant, decrease in presynaptic alpha-Spectrin levels (Figure
502 S3A) compared to a near complete loss of postsynaptic alpha-Spectrin (Figure S3B). Next, we
503 employed RNAi and rescue approaches to address cell type-specific requirements for Elff. We
504 find that motoneuron-specific knockdown, but not muscle-specific knockdown, leads to
505 reduced alpha-Spectrin (Figure 3A, C-E). Moreover, presynaptic overexpression of Elff via
506 D42Gal4 fully rescues alpha-Spectrin levels in *elff* nulls (Figure S3C-D). Together, these
507 findings indicate a presynaptic requirement for Elff in promoting alpha-Spectrin accumulation.

508

509 Before undertaking cell-type specific rescue experiments, we overexpressed Elff pre- or
510 postsynaptically in an otherwise wild-type background. Presynaptic Elff overexpression was
511 not sufficient to alter bouton number or synaptic maturation, permitting the rescue experiments
512 described above. However, we found dramatic phenotypes associated with postsynaptic Elff
513 overexpression. Unexpectedly, postsynaptic Elff overexpression via 24BGal4 has widespread

effects on NMJ morphology. At roughly 75% of NMJs, it disrupts normal terminal morphology and drives large, lamellipodial-shaped HRP-positive axon terminals, here referred to as NMJ webs (Figure 3F-I). We quantified this phenotype at NMJ4, but it is not NMJ-specific and occurs at NMJs on all body wall muscles (data not shown). The normal pattern of motor axon guidance is not altered; the webbing appears over the surface of individual muscles where individual NMJs normally form. Indeed, within the large web-like axon terminals, strings of round boutons are present (arrowheads in Figure 3G). This phenotype is not specific to our UAS-Elff transgene because it is also observed at high frequency with an independently generated UAS-Elff construct (Garcia-Alonso, 2024) (data not shown). Notably, presynaptic Elff overexpression does not have comparable effects (Figure 3I).

The frequent conversions of terminal strings of boutons to enlarged lamellipodial-shaped webs suggests that high levels of postsynaptic Elff are sufficient to drive pervasive cytoskeletal rearrangements on the presynaptic side. Given that presynaptic loss of Elff results in reduced postsynaptic alpha-Spectrin, we wondered whether postsynaptic Elff overexpression results in increased presynaptic alpha-Spectrin. In line with our hypothesis, we find that the enlarged presynaptic HRP-positive webbing contains robust alpha-Spectrin staining (Figure 3G). This broad accumulation of protein throughout both synaptic boutons and webbing is specific to alpha-Spectrin, as Dlg is more concentrated in the boutons and accumulates only weakly in the webbing upon postsynaptic overexpression of Elff (Figure S3E-F). We conclude that the alpha-Spectrin signal is largely presynaptic both because it is contiguous with axonal alpha-Spectrin and because it is contained within the HRP signal (Figure 3G). Whereas alpha-Spectrin is predominantly postsynaptic around the small strings of boutons found within the large web, alpha-Spectrin appears entirely within the web itself (orthogonal views in Figure 3H). These results indicate that postsynaptic Elff overexpression promotes a large-scale morphological transformation of NMJ terminal arbors from strings of round boutons to enlarged webbed axonal terminals rich with alpha-Spectrin. They further imply that Elff is not normally expressed at high levels on the postsynaptic side (see Discussion). Taken together, the loss-of-function and gain-of-function phenotypes argue that Elff plays a particularly central role in promoting alpha-Spectrin expression.

Elff is necessary for baseline synaptic transmission and larval locomotion

We next tested if impaired postsynaptic maturation observed in *elff* loss-of-function mutants is accompanied by deficits in synaptic function by measuring spontaneous (mEJPs) and evoked (EJPs) junctional potentials from muscle 6 in third-instar *elff* null mutants. mEJP amplitude, a measure of the response of the postsynaptic muscle to fusion of a single glutamatergic vesicle, is reduced by 32% in *elff* null mutants, while mEJP frequency is reduced by 61% (Figure 4A-C). The decrease in quantal size is consistent with a defective postsynaptic response to the fusion of a single glutamatergic vesicle. To assess whether reduced mEJP frequency can be explained by small mEJPs that fall beneath our detection threshold, we plotted mEJP amplitude distributions (Figure S4A) and mEJP amplitude against frequency per cell (Figure S4B). While mEJP amplitude and frequency are positively correlated in *elff* mutants (Figure S4B), the left tail of the mEJP amplitude histogram is not appreciably truncated in *elff* mutants

(Figure S4A), suggesting that we are not missing a large number of very small events in the mutants. Thus, while we cannot exclude the possibility that reduction in mEJP amplitude contributes somewhat to the observed decrease in mEJP frequency, it is unlikely to be the primary cause.

The impairment in mEJP frequency could result from either a decrease in presynaptic release probability or decreased postsynaptic responsiveness to the fusion of single neurotransmitter vesicles. To begin to discriminate between these two possibilities, we assessed quantal content, or the number of vesicles released per action potential, which is calculated as the ratio of EJP amplitude to mEJP amplitude. We observed no difference in quantal content (Figure 4E), indicating that similar numbers of synaptic vesicles are released in response to a stimulus in control and *elff* LOF animals. This together with reduced quantal size suggest a largely postsynaptic origin of the physiological defect, consistent with impaired postsynaptic maturation in *elff* mutants as described above (Figures 2, 3) and below (Figure 5-7).

To ascertain whether the defect in neurotransmission is associated with impaired motor function, we tested whether loss of *elff* results in altered locomotion of third instar larvae. We find that *Elff* is required for normal motility since its loss leads to significant reductions in both the mean velocity and the total distance travelled by larvae over a five minute period (Figure 4F-I). Together, functional and behavioral analyses indicate that *Elff* is specifically required for neurotransmission at the NMJ, and more generally, for robust organismal locomotion.

Elff is required for glutamate receptor localization at the NMJ

The diminished mEJP amplitude and frequency in *elff* mutants is reminiscent of phenotypes observed in *Drosophila* ionotropic glutamate receptor (GluR) subunit mutants (Petersen et al., 1997; DiAntonio et al., 1999; Marrus et al., 2004; Featherstone et al., 2005), suggesting that *Elff* promotes proper GluR levels or localization. At the *Drosophila* NMJ, glutamate receptors are heterotetrameric complexes comprised of one of two alternate subunits: GluRIIA or GluRIIB, as well as one subunit each of the three essential subunits: GluRIIC, GluRIID, and GluRIIE (Marrus et al., 2004; Qin et al., 2005).

We measured synaptic levels of individual GluR subunits in *elff* mutants using a collection of subunit-specific antibodies (Perry et al., 2017, 2022; Goel and Dickman, 2018; Qiu et al., 2025). We saw a significant loss of both GluRIIA and GluRIIB from *elff* null NMJs, with a greater than 50% reduction in GluRIIA levels (Figure 5A-F). The reduction in both alternative GluR subunits raised the possibility that overall GluR levels are reduced at *elff* mutant NMJs. To investigate this idea, we quantified synaptic levels of GluRIIC and GluRIIE, two of the essential subunits, at control and *elff* mutant NMJs. In line with our hypothesis, intensities of GluRIIC and GluRIIE are also reduced by greater than 50% in *elff* mutants (Figure 5G-L), with individual receptor clusters appearing smaller and difficult to resolve. The decrease in both

597 alternative and essential GluR subunits indicates that overall levels of heterotetrameric
598 complexes are significantly reduced in *elff* null mutants.

599

600 To test whether Elff is required pre- or postsynaptically, we assessed levels of the essential
601 GluR subunit GluRIIE with cell type-specific RNAi-mediated knockdown. We focused on
602 GluRIIE since it is an essential subunit and particularly clearly reduced in *elff* nulls. We found
603 modest, non-significant, decreases in GluRIIE with either motoneuron-specific or muscle-
604 specific knockdown (Figure 5M-O, Q). These data raise the possibility that Elff expression in
605 either the pre- or postsynaptic cell is sufficient to promote GluR complex localization or
606 stabilization. To test this idea, we simultaneously knocked down *elff* in neurons and muscle
607 cells and found a near 50% reduction in GluRIIE levels at NMJ synapses (Figure 5M, P-Q).
608 These data imply that Elff can be supplied from either the presynaptic motoneuron or the
609 postsynaptic muscle to promote proper GluR clusters. Together, these findings argue that
610 reduced GluR accumulation is likely to be a leading cause of impaired electrophysiological
611 responses in *elff* mutants.

612

613 We next probed a possible glial requirement for Elff in synaptic maturation. As a predicted GPI-
614 linked FN3-IgSF protein, Elff is closely related to Fas II and NCAM GPI-linked isoforms. In both
615 *Drosophila* and mammalian models, the secreted GPI-linked isoforms are expressed
616 predominantly by glial cells (Maness and Schachner, 2007; Neuert et al., 2019). Indeed,
617 expression of a GPI-linked isoform of Fas II in glia rescues *fas* II null mutants to viability in
618 *Drosophila* (Neuert et al., 2019). To test whether Elff likewise functions in glia, we tested
619 whether pan-glial *elff* knockdown results in reduced Dlg or GluRIIE expression— two sensitive
620 read-outs of *elff* function. We find that expression of *elff* RNAi via RepoGAL4 fails to alter
621 either Dlg levels or GluRIIE clustering (Figure S5 A-B). Thus, Elff is not required by glia to drive
622 postsynaptic maturation.

623

624 Elff promotes initial assembly of the postsynaptic compartment

625 The preceding analysis demonstrates that Elff is necessary for multiple facets of postsynaptic
626 maturation at the wandering third instar larval stage, corresponding to roughly five days after
627 egg laying (AEL). The analysis could not distinguish whether Elff is required for the initial
628 formation of GluR clusters or rather for their stabilization since NMJ synapses are assembled
629 at the end of embryogenesis, at 24 hours AEL. Thus, an assessment of Elff function in
630 synapse formation demands a phenotypic analysis at earlier developmental stages. Initial
631 synapse formation at stage 17 embryonic NMJs is not often studied in part because standard
632 embryo staining protocols do not effectively stain very late-stage embryos due to the presence
633 of the embryonic cuticle.

634

635 We started with the more straightforward task of assessing postsynaptic markers at 48 h AEL,
636 or mid second-instar stage. Strikingly, loss of Elff results in an almost complete loss of alpha-
637 Spectrin and Dlg in L2 stage NMJs (Figure 6A-D, G-H) as well as a near complete absence of

GluR complexes marked by anti-GluRIIE (Figure 6E-F, I) in young larvae. The finding that loss of Elff results in more pronounced phenotypes at L2 vs L3 hints that Elff may be required for synapse formation rather than later steps of synapse stabilization or maturation. These data are also consistent with a large body of literature arguing that robust homeostatic mechanisms are in place that can compensate for impaired NMJ function (Dickman and Davis, 2009; Davis, 2013; Perry et al., 2017; Goel and Dickman, 2018; Goel et al., 2019).

These strong second instar phenotypes prompted us to interrogate initial embryonic synapse formation. To this end, we developed a new stage 17 embryonic staining protocol to better preserve embryonic NMJ morphology and enable a quantitative analysis of active zone and postsynaptic receptor levels and apposition (see Materials and Methods). We analyzed mid-stage 17 embryonic NMJs since this is after motor axon terminals elaborate on their target muscles, form boutons, and initiate synapse formation. At this stage, there are on average six boutons at NMJ 6/7 in both control and *elff* LOF embryos (Figure 7A-C). Thus, Elff does not regulate initial bouton formation during embryogenesis, consistent with the observation that *elff* mutants display normal bouton number at the L3 stage (Figure 1F-H).

We next assayed embryonic synapse formation using Brp as a presynaptic marker and GluRIIE as a postsynaptic marker. In control embryos, we find clear evidence of ongoing synapse formation with larger, more mature, presynaptic Brp puncta in close apposition to postsynaptic GluR clusters (Figure 7A). In *elff* mutants, while we do not find a difference in presynaptic Brp relative to controls, we detect a greater than 60% decrease in postsynaptic GluRIIE levels (Figure 7A-E). In addition, while in control embryos larger GluRIIE puncta are closely apposed to Brp puncta, in *elff* mutants this tight apposition is lost (arrows in Figure 7A-B). Together, these data indicate a requirement for *elff* in the formation of postsynaptic GluR complexes, and furthermore, in the critical role of aligning these complexes with presynaptic active zones for rapid neurotransmission.

Finally, we investigated whether Elff promotes postsynaptic assembly beyond the formation of GluR clusters. In particular, since Elff is required for both alpha-Spectrin and Dlg localization at the L2 and L3 stages, we wanted to test whether this function is for initial localization of these proteins to the postsynaptic specialization, or rather for their maintenance. While we were not able to achieve consistent alpha-Spectrin labeling in controls at embryonic stage 17, we found robust Dlg expression in controls at this early stage (Figure 7H). In contrast, we observe a roughly 50% reduction in Dlg levels at embryonic *elff* mutants (Figure 7H-J). Together, these findings argue that Elff is required for multiple essential features of postsynaptic assembly. These embryonic phenotypes imply that Elff is expressed at late embryonic stages, which is supported by *elff* transcript expression beginning between 16-18 hours of embryonic development via the ModENCODE RNAseq database (Flybase).

Elff is required for apposition of pre- and postsynaptic specializations

Our physiological analysis indicated that *elff* mutants do not exhibit reduced quantal content, but do exhibit reductions in both mini amplitude and frequency (Figure 4), suggesting that postsynaptic function is selectively impaired. Interestingly, our embryonic analysis revealed a defect in apposition of pre- and postsynaptic specializations in stage 17 *elff* nulls, raising the possibility that synapse architecture is aberrant. To test this hypothesis at larval stage NMJs, we employed high-resolution Airyscan confocal imaging and an Imaris analysis pipeline to quantify transsynaptic alignment in *elff* mutants using the presynaptic marker Brp and the postsynaptic marker GluRIIE. As previously described, *elff* mutant NMJs display diminished GluR levels. Thus, the GluR signal was scaled up in this analysis to enable clear visualization of GluR clusters (see Methods).

Focusing first on the presynaptic side, we find that active zone density is unaltered in *elff* nulls (Figure 8A-B, D). We then investigated whether Elff is required for alignment of pre- and postsynaptic compartments at the L3 stage. In control NMJs, the vast majority of individual Brp and GluR dyads are observed as overlapping puncta when viewed in single slices of Airyscan images (Figure 8A, E-F). In stark contrast, we find an 8-fold increase in unapposed Brp puncta in *elff* nulls (white arrowheads in Figure 8B, E) and a 7-fold increase in unapposed GluRIIE puncta in *elff* nulls (yellow arrowheads in Figure 8B, F) resulting in approximately 20% unopposed Brp and GluRIIE clusters in the mutants. In particular, the presence of large GluR fields unopposed to presynaptic specializations (yellow arrowheads in 8B) in *elff* mutants argues that this phenotype is not merely the result of an inability to detect GluR clusters in the mutants, but rather a combination of reduced and misaligned receptor clusters.

The widespread misalignment of pre- and postsynaptic compartments in *elff* mutants argues that this extracellular synaptic adhesion molecule acts in the synaptic cleft to bring the pre- and postsynaptic compartments into register. To define the specific cellular requirement for Elff in the alignment of pre- and postsynaptic specializations, we employed cell-type specific RNAi-mediated knockdown. Similar to our GluR analysis, neither *elff* knockdown in motoneurons nor muscle results in overt defects in synapse apposition (Figure S6). However, simultaneous knockdown of *elff* in both pre- and postsynaptic compartments leads to phenotypes comparable to that observed in the nulls (Figure 8A, C-F). Specifically, combined pre- and postsynaptic *elff* knockdown results in a five-fold increase in unopposed Brp puncta (white arrowheads in Figure 8C, E) and a four-fold increase in unopposed GluR puncta (yellow arrowheads in Figure 8C, F) relative to driver-only controls. These data argue that Elff is sufficient when expressed in either the motoneuron or the muscle. Moreover, while Elff has a predicted GPI linkage, the absence of a cell-type specific requirement raises the possibility that Elff can act cell non-autonomously in this context (see Figure 10 and Discussion).

Elff is essential for transcellular nanoalignment at the *Drosophila* NMJ

Although overall apposition of active zone components and postsynaptic receptor fields is observable at the level of standard confocal microscopy, this alignment is organized sub-synaptically, with densities of pre- and postsynaptic components aligning within a single

synapse to form structures termed nanocolumns (Biederer et al., 2017, Muttathukunnel et al., 2022). The apposition defects we observe in *elff* mutants between Brp puncta and GluRIIE receptor fields using Airyscan imaging raised the question of whether *elff* is also required for the alignment of pre- and postsynaptic components at the sub-synaptic level. At the Drosophila larval NMJ, stimulated emission depletion (STED) microscopy has been employed to resolve sub-synaptic organization of both Brp and several GluR subunits into closely co-localized ring-shaped structures with aligned densities (Kittel et al., 2006; Muttathukunnel et al., 2022). Because the lateral resolution limit of Airyscan-enhanced microscopy is ~140 nm, imaging with Airyscan alone was not sufficient to establish whether *elff* is required for nanocolumnar alignment of pre- and postsynaptic markers. To address this question, we used STED microscopy to assess nanoscale alignment of the pre- and postsynaptic markers Brp and GluRIIE at sufficient resolution in *elff* mutants. To account for the possibility that the unopposed GluRIIE fields we observed with Airyscan imaging (Figure 8B, F) were related to raising the confocal gain to image GluRIIE in *elff* mutants, we maintained consistent laser settings to image *elff* mutants and controls with STED.

We observe no difference in Brp ring architecture between controls and *elff* mutants (Figure 9A-E). Indeed, the diameter of Brp rings in both groups is in line with previous estimates (Kittel et al., 2006; Hong et al., 2020; Muttathukunnel et al., 2022). Alongside our electrophysiological analysis, these findings indicate that *Elff* is not required for presynaptic structure or function. Conversely, we find clear differences between controls and mutants in the postsynaptic compartment. In controls, GluRIIE signal is robust and forms a largely continuous structure of interconnected rings across boutons (Figure 9A). At individual synapses, the GluRIIE signal is closely associated with the Brp ring around most of its circumference (Figure 9B). In contrast, GluRIIE in *elff* mutants is more punctate, with fewer ring-like conformations (Figure 9C-D). Consistent with our observations using Airyscan microscopy, the residual GluRIIE signal in *elff* mutants is less closely associated with Brp rings relative to controls (Figure 9C-D). At control NMJs, about 50% of the total surface area of GluRIIE is located within the vicinity of Brp, which we define as the region spanning the diameter of the Brp ring and extending approximately 200 nm beyond the outer edge of the ring in all directions (Figure 9E, see Methods). This observation is consistent with previous observations of other GluR subunits at the larval NMJ (Muttathukunnel et al., 2022). Strikingly, in *elff* mutants, the proportion of total GluRIIE adjacent to Brp is about half that of controls (Figure 9F). Since we do not observe any differences in Brp ring size or puncta density between *elff* mutants and controls, this result indicates that GluRIIE is less aligned with Brp-labeled release sites in *elff* mutants.

To assess this alignment defect in *elff* mutants at the level of individual synapses, we plotted line intensity profiles of Brp and GluRIIE across single synapses in *elff* mutants and controls, excluding synapses with non-planar or partial Brp rings. Plot profiles of planar Brp rings and GluRIIE in controls indicate clear signal peaks with local maxima slightly offset from one another by about 97 nm (Figure 9G-I). In *elff* mutants, the average distance between Brp and GluRIIE peaks is about 123 nm, a distance about 25% larger than in controls (Figure 9I).

Together, these data indicate that *elff* promotes transsynaptic nanoscale alignment of the pre- and postsynaptic architecture.

Discussion

The number of adhesion molecules expressed on growth cones and at synapses begs the question of the degree to which they have unique versus redundant functions. While unique functions for some were discovered in pioneering axon guidance screens (Seeger et al., 1993; Lin et al., 1994a; Tessier-Lavigne and Goodman, 1996), redundancy among others is proposed to ensure robustness of synapse formation and plasticity (Piechotta et al., 2006; Shen and Scheiffele, 2010). For example, single mutants of many synaptic adhesion molecules display only modest defects in axon targeting or NMJ growth (Nose, 2012; McLaughlin et al., 2016; Ashley and Carrillo, 2024). The prevalence of subtle phenotypes in mutations of conserved synapse organizers raises the possibility of widespread molecular redundancy. In contrast to this prevailing model, here we demonstrate a clear requirement for the IgSF protein Elff in postsynaptic assembly and function in the absence of defects in presynaptic function or NMJ expansion.

Elff serves specific functions in subsynaptic organization

While Elff is not required for NMJ growth, it is critical for postsynaptic assembly and maturation. Specifically, GluR subunit abundance is strongly reduced at *elff* mutant NMJs. To determine if this reflects a failure of postsynaptic assembly or maintenance, we analyzed earlier developmental stages. We drew two main conclusions from these studies. First, Elff promotes initial assembly of the postsynaptic compartment since we find defects from embryonic late stage 17 onward. Second, our L2 analysis indicates a more stringent requirement for Elff at the L2 versus L3 stage. These findings are consistent with others demonstrating the existence of homeostatic pathways at the NMJ (Dickman and Davis, 2009; Davis, 2013; Perry et al., 2017; Goel and Dickman, 2018), and argue that in the absence of Elff, alternative mechanism(s) compensate. More broadly, the relative strength of the *elff* L2 phenotype suggests that the expressivity of mutant phenotypes related to synapse organization (as opposed to bouton number) may be diminished by analyses focused solely on L3 animals.

The viability and modestly impaired motility of *elff* mutants was initially surprising to us given the dramatic loss of GluR clusters. In fact, our data are consistent with studies indicating that even 5% of normal GluR cluster abundance supports larval motility and viability due to the presence of a safety factor ensuring muscle contraction (Marrus et al., 2004; Qin et al., 2005; Schmid et al., 2006). In addition, our finding that active zone density and quantal content are normal in *elff* mutants is in line with work establishing that total, or near-total, GluR loss does not disrupt presynaptic organization (Marrus et al., 2004; Schmid et al., 2006; Kim et al., 2012).

Using high-resolution and super-resolution imaging, we uncovered apposition defects in *elff* mutants. We handled the decrease in GluR accumulation in *elff* mutants differently in these two analyses. In the Airyscan analysis, we scaled up the GluR fluorescence in the mutants to ensure that we did not miss weak GluR clusters. We found that roughly 20% of Brp and GluRIIE clusters are unopposed in *elff* backgrounds relative to less than 5% in controls. The penetrance of these phenotypes is similar to that observed in Neuroligin and Teneurin mutants (Banovic et al., 2010; Mosca et al., 2012), indicating that Elff is a key synapse organizer (Figure 10). They also suggested that Elff might be required for transsynaptic nanoalignment as defined by superresolution techniques (Tang et al., 2016; Biederer et al., 2017). We employed STED microscopy to explore this possibility and here maintained consistent laser settings to directly compare the fraction of localized GluR. To the best of our knowledge, STED has been used only once previously to look at transcellular nanoalignment at the Drosophila NMJ (Muttathukunnel et al., 2022). These authors found that a significant fraction of GluR is not closely associated with Brp rings in wild type, a pool they refer to as ambient GluR. Similarly, we find that nearly 50% of GluRIIE is part of this pool at control NMJs, while in *elff* mutants the ambient pool increases to 74% of total GluR (Figure 9F). Thus, Elff preferentially maintains active zone-localized GluRIIE. The little GluRIIE that remains associated with Brp rings in *elff* mutants is found in discrete clusters. Importantly, these GluRIIE clusters are located farther away from Brp rings in *elff* mutants than in controls (Figure 9I), indicating that Elff drives precise nanoalignment of pre- and postsynaptic specializations.

Is the loss of GluRs in *elff* mutants likely to underlie the breakdown of apposition? Loss of GluRs at the Drosophila NMJ does not necessarily lead to extensive defects in apposition (Schmid et al., 2006; Kim et al., 2012). For example, hypomorphic alleles of the auxiliary GluR subunit Neto display reduced GluR localized to Brp rings (Muttathukunnel et al., 2022), though unlike *elff* mutants, the distance of the remaining GluR clusters to Brp rings is unchanged. While it is possible that *neto* nulls would display alignment defects akin to those observed in *elff* nulls, it is alternatively possible that Elff's function in nanoalignment is distinct. Identification of extracellular Elff binding partners at the NMJ is essential to elucidate Elff-dependent signaling mechanisms. Both the Elff MIMIC protein trap and Elff^{sfGFP-N}, an endogenous N-term epitope tagged protein described here, display punctate expression at the NMJ. Interestingly, Elff-positive puncta do not colocalize with Brp-positive puncta. Thus, although Elff promotes GluR localization and alignment to active zones, Elff itself is not localized to active zones or GluR clusters. We propose that Elff acts within periaxial zones in a center-surround fashion to promote proper alignment of presynaptic release sites with neurotransmitter receptor clusters.

We undertook RNAi experiments to validate phenotypes observed in the *elff* nulls and to address its cell-type specific requirements. Simultaneous knockdown of *elff* pre- and postsynaptically leads to defects in apposition and GluR localization comparable to that observed in our nulls, while *elff* knockdown in either the pre- or postsynaptic cell does not. These findings are consistent with the identity of Elff as a secreted/GPI-linked protein deposited in the synaptic cleft from both pre- and postsynaptic cells. While these RNAi results support the existence and function of postsynaptic Elff, the dramatic morphological effects of

postsynaptic Elff overexpression argue that its postsynaptic expression levels are normally low. Importantly, the requirement for pre- and postsynaptic Elff in GluR clustering/apposition is distinct from its strictly presynaptic requirement in regulating alpha-Spectrin and Dlg, arguing that Elff is a member of distinct protein complexes (Figure 10).

Identifying Elff-mediated signaling pathways

The set of NMJ phenotypes described here is unique among *Drosophila* mutants. Specifically, the combination of defects in apposition and postsynaptic maturation, alongside normal NMJ growth, argues that Elff does not influence the activity of key regulators of synapse adhesion including NrX, Nlg, and the BMP pathway, since all regulate baseline growth (Marqués et al., 2002; Li et al., 2007; Banovic et al., 2010; Hoover et al., 2019). Might Elff homologs provide insight into the identity of Elff-mediated signaling pathways? Structurally, Elff is closely related to the *C. elegans* IgSF protein Rig-3, which regulates Acetylcholine receptor levels via inhibiting the Ror receptor tyrosine kinase CAM-1 and the Wnt pathway (Babu et al., 2011). In *Drosophila*, loss of Wnt pathway components results in phenotypes overlapping that of *elff* nulls (Packard et al., 2002; Restrepo et al., 2022), suggesting a possible relationship. It will also be important to probe possible functional relationships among Elff and the three *Drosophila* Ror family members (NrX, Ror, and Drl-2).

Elff shares homology with Fipi, and both were identified as extracellular binding partners of Fasciclin II in a systematic cataloging of pairwise extracellular interactions among LRR and IgSF proteins (Özkan et al., 2013). Fipi's molecular function in the CNS has not been reported, though a behavioral phenotype is reported in courtship song (Fedotov et al., 2018). Recently, Fipi and Elff have been shown to act in parallel in the wing disk to restrain EGFR activity. In this capacity, they act independently and in conjunction with Fas II (Garcia-Alonso, 2024). We do not favor the model that Fipi functions analogously to Elff at the NMJ since neither knockdown nor overexpression of Fipi display phenotypes akin to that described here (MNW and HTB, unpublished data).

Finally, it is critical to test if Elff regulates aspects of Fas II-dependent signaling at the NMJ. Elff is not an obligate partner of Fas II since loss of Fas II results in early larval lethality, and mutant NMJs exhibit growth defects (Schuster et al., 1996b, 1996a; Neuert et al., 2019). Consistent with the phenotypic differences between *elff* and *fas II* mutants, we do not detect a change in overall Fas II levels at the NMJ in *elff* nulls. Notably, in addition to the function of Fas II in growth regulation and adhesion, it promotes postsynaptic specification at embryonic stages (Kohsaka et al., 2007). Indeed, loss of Fas II results in phenotypes overlapping those observed with loss of Elff, raising the possibility that Elff sculpts Fas II function. Moving forward, defining Elff-dependent signaling mechanisms will elucidate the molecular pathways driving transsynaptic nanoarchitecture and postsynaptic assembly at the NMJ.

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References

- Aonurm-Helm A, Jaako K, Jürgenson M, Zharkovsky A (2016) Pharmacological approach for targeting dysfunctional brain plasticity: Focus on neural cell adhesion molecule (NCAM). *Pharmacol Res* 113:731–738.
- Ashley J, Carrillo RA (2024) The Drosophila Larval Neuromuscular Junction: Developmental Overview. *Cold Spring Harb Protoc* 2025:pdb.top108449.
- Ashley J, Packard M, Ataman B, Budnik V (2005) Fasciclin II Signals New Synapse Formation through Amyloid Precursor Protein and the Scaffolding Protein dX11/Mint. *J Neurosci* 25:5943–5955.
- Astorga C, Jorquera RA, Ramírez M, Kohler A, López E, Delgado R, Córdova A, Olguín P, Sierralta J (2016) Presynaptic DLG regulates synaptic function through the localization of voltage-activated Ca²⁺ Channels. *Sci Rep* 6:32132.
- Ataman B, Ashley J, Gorczyca M, Ramachandran P, Fouquet W, Sigrist SJ, Budnik V (2008) Rapid Activity-Dependent Modifications in Synaptic Structure and Function Require Bidirectional Wnt Signaling. *Neuron* 57:705–718.
- Atwood HL, Govind CK, Wu C -F. (1993) Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in Drosophila larvae. *J Neurobiol* 24:1008–1024.
- Babu K, Hu Z, Chien S-C, Garriga G, Kaplan JM (2011) The Immunoglobulin Super Family Protein RIG-3 Prevents Synaptic Potentiation and Regulates Wnt Signaling. *Neuron* 71:103–116.
- Banovic D, Khorramshahi O, Oswald D, Wichmann C, Riedt T, Fouquet W, Tian R, Sigrist SJ, Aberle H (2010) Drosophila Neuroligin 1 Promotes Growth and Postsynaptic Differentiation at Glutamatergic Neuromuscular Junctions. *Neuron* 66:724–738.

954 Biederer T, Kaeser PS, Blanpied TA (2017) Transcellular Nanoalignment of Synaptic Function.
955 Neuron 96:680–696.

956

957 Chen K, Gracheva EO, Yu S-C, Sheng Q, Richmond J, Featherstone DE (2010) Neurexin in
958 Embryonic Drosophila Neuromuscular Junctions. PLoS ONE 5:e11115.

959

960 Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM (1987)
961 Neural Cell Adhesion Molecule: Structure, Immunoglobulin-Like Domains, Cell Surface
962 Modulation, and Alternative RNA Splicing. Science 236:799–806.

963

964 Davis GW (2013) Homeostatic Signaling and the Stabilization of Neural Function. Neuron
965 80:718–728.

966

967 Davis GW, Schuster CM, Goodman CS (1997) Genetic Analysis of the Mechanisms
968 Controlling Target Selection: Target-Derived Fasciclin II Regulates the Pattern of Synapse
969 Formation. Neuron 19:561–573.

970 DiAntonio A, Petersen SA, Heckmann M, Goodman CS (1999) Glutamate Receptor
971 Expression Regulates Quantal Size and Quantal Content at the Drosophila Neuromuscular
972 Junction. J Neurosci 19:3023–3032.

973

974 Dickman DK, Davis GW (2009) The Schizophrenia Susceptibility Gene dysbindin Controls
975 Synaptic Homeostasis. Science 326:1127–1130.

976

977 Featherstone DE, Davis WS, Dubreuil RR, Broadie K (2001) Drosophila α - and β -Spectrin
978 Mutations Disrupt Presynaptic Neurotransmitter Release. J Neurosci 21:4215–4224.

979

980 Featherstone DE, Rushton E, Rohrbough J, Liebl F, Karr J, Sheng Q, Rodesch CK, Broadie K
981 (2005) An Essential Drosophila Glutamate Receptor Subunit That Functions in Both Central
982 Neuropil and Neuromuscular Junction. J Neurosci 25:3199–3208.

983

984 Fedotov SA, Bragina JV, Besedina NG, Danilenkova LV, Kamysheva EA, Kamyshev NG
985 (2018) Gene CG15630 (fipi) is involved in regulation of the interpulse interval in Drosophila
986 courtship song. J Neurogenet 32:15–26.

987

988 Fedotov SA, Bragina JV, Besedina NG, Danilenkova LV, Kamysheva EA, Panova AA,
989 Kamyshev NG (2014) The effect of neurospecific knockdown of candidate genes for locomotor
990 behavior and sound production in *Drosophila melanogaster*. *Fly* 8:176–187.

991

992 Garcia-Alonso L (2024) Fasciclin 2 functions as an expression-level switch on EGFR to control
993 organ shape and size in *Drosophila*. *PLOS ONE* 19:e0309891.

994

995 Goel P, Dickman D (2018) Distinct homeostatic modulations stabilize reduced postsynaptic
996 receptivity in response to presynaptic DLK signaling. *Nat Commun* 9:1856.

997

998 Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J,
999 O'Connor-Giles KM (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided
1000 Cas9 nuclease. *Genetics* 194:1029–1035.

1001

1002 Gratz SJ, Goel P, Bruckner JJ, Hernandez RX, Khateeb K, Macleod GT, Dickman D,
1003 O'Connor-Giles KM (2019) Endogenous Tagging Reveals Differential Regulation of Ca²⁺
1004 Channels at Single Active Zones during Presynaptic Homeostatic Potentiation and Depression.
1005 *J Neurosci* 39:2416–2429.

1006

1007 Gratz SJ, Rubinstein CD, Harrison MM, Wildonger J, O'Connor-Giles KM (2015) CRISPR-
1008 Cas9 Genome Editing in *Drosophila*. *Curr Protoc Mol Biology* 111:31.2.1-31.2.20.

1009

1010 Graveley BR et al. (2011) The developmental transcriptome of *Drosophila melanogaster*.
1011 *Nature* 471:473–479.

1012 Hata K, Maeno-Hikichi Y, Yumoto N, Burden SJ, Landmesser LT (2018) Distinct Roles of
1013 Different Presynaptic and Postsynaptic NCAM Isoforms in Early Motoneuron–Myotube
1014 Interactions Required for Functional Synapse Formation. *J Neurosci* 38:498–510.

1015

1016 Hata K, Polo-Parada L, Landmesser LT (2007) Selective Targeting of Different Neural Cell
1017 Adhesion Molecule Isoforms during Motoneuron–Myotube Synapse Formation in Culture and
1018 the Switch from an Immature to Mature Form of Synaptic Vesicle Cycling. *J Neurosci*
1019 27:14481–14493.

1020

1021 Hoover KM, Gratz SJ, Qi N, Herrmann KA, Liu Y, Perry-Richardson JJ, Vanderzalm PJ,
1022 O'Connor-Giles KM, Broihier HT (2019) The calcium channel subunit $\alpha 2\delta$ -3 organizes

synapses via an activity-dependent and autocrine BMP signaling pathway. Nat Commun
10:5575.

Kelly SM, Elchert A, Kahl M (2017) Dissection and Immunofluorescent Staining of Mushroom
Body and Photoreceptor Neurons in Adult *Drosophila melanogaster* Brains. J Vis Exp :
JoVE:56174.

Kim Y-J, Bao H, Bonanno L, Zhang B, Serpe M (2012) *Drosophila* Neto is essential for
clustering glutamate receptors at the neuromuscular junction. Genes Dev 26:974–987.

Kohsaka H, Takasu E, Nose A (2007) In vivo induction of postsynaptic molecular assembly by
the cell adhesion molecule Fasciclin2. J Cell Biol 179:1289–1300.

Li J, Ashley J, Budnik V, Bhat MA (2007) Crucial Role of *Drosophila* Neurexin in Proper Active
Zone Apposition to Postsynaptic Densities, Synaptic Growth, and Synaptic Transmission.
Neuron 55:741–755.

Lin DM, Fetter RD, Kopczynski C, Grenningloh G, Goodman CS (1994a) Genetic analysis of
Fasciclin II in *drosophila*: Defasciculation, refasciculation, and altered fasciculation. Neuron
13:1055–1069.

Lin DM, Fetter RD, Kopczynski C, Grenningloh G, Goodman CS (1994b) Genetic analysis of
Fasciclin II in *drosophila*: Defasciculation, refasciculation, and altered fasciculation. Neuron
13:1055–1069.

Malchiodi-Albedi F, Ceccarini M, Winkelmann JC, Morrow JS, Petrucci TC (1993) The 270 kda
splice variant of erythrocyte β -spectrin (β i Σ 2) segregates in vivo and in vitro to specific
domains of cerebellar neurons. J Cell Sci 106:67–78.

Maness PF, Schachner M (2007) Neural recognition molecules of the immunoglobulin
superfamily: signaling transducers of axon guidance and neuronal migration. Nat Neurosci
10:19–26.

Marqués G, Bao H, Haerry TE, Shimell MJ, Duchek P, Zhang B, O'Connor MB (2002) The
Drosophila BMP Type II Receptor Wishful Thinking Regulates Neuromuscular Synapse
Morphology and Function. Neuron 33:529–543.

1059

1060 Marrus SB, Portman SL, Allen MJ, Moffat KG, DiAntonio A (2004) Differential Localization of
 1061 Glutamate Receptor Subunits at the Drosophila Neuromuscular Junction. *J Neurosci* 24:1406–
 1062 1415.

1063

1064 McLaughlin CN, Nechipurenko IV, Liu N, Broihier HT (2016) A Toll receptor–FoxO pathway
 1065 represses Pavarotti/MKLP1 to promote microtubule dynamics in motoneurons. *J Cell Biology*
 1066 214:459–474.

1067

1068 Menon KP, Carrillo RA, Zinn K (2013) Development and plasticity of the Drosophila larval
 1069 neuromuscular junction. *Wiley Interdiscip Rev Dev Biol* 2:647–670.

1070

1071 Mosca TJ, Hong W, Dani VS, Favaloro V, Luo L (2012) Trans-synaptic Teneurin signalling in
 1072 neuromuscular synapse organization and target choice. *Nature* 484:237–241.

1073

1074 Mosca TJ, Schwarz TL (2010) The nuclear import of Frizzled2-C by Importins- β 11 and α 2
 1075 promotes postsynaptic development. *Nat Neurosci* 13:935–943.

1076

1077 Muttathukunnel P, Frei P, Perry S, Dickman D, Müller M (2022) Rapid homeostatic modulation
 1078 of transsynaptic nanocolumn rings. *Proc Natl Acad Sci* 119:e2119044119.

1079

1080 Nagarkar-Jaiswal S, Lee P-T, Campbell ME, Chen K, Anguiano-Zarate S, Gutierrez MC,
 1081 Busby T, Lin W-W, He Y, Schulze KL, Booth BW, Evans-Holm M, Venken KJ, Levis RW,
 1082 Spradling AC, Hoskins RA, Bellen HJ (2015) A library of MiMICs allows tagging of genes and
 1083 reversible, spatial and temporal knockdown of proteins in Drosophila. *Elife* 4:e05338.

1084

1085 Neuert H, Deing P, Krukkert K, Naffin E, Steffes G, Risse B, Silies M, Klämbt C (2019) The
 1086 Drosophila NCAM homolog Fas2 signals independently of adhesion. *Development*
 1087 147:dev181479.

1088 Nose A (2012) Generation of neuromuscular specificity in Drosophila: novel mechanisms
 1089 revealed by new technologies. *Front Mol Neurosci* 5:62.

1090

1091 Özkan E, Carrillo RA, Eastman CL, Weiszmann R, Waghray D, Johnson KG, Zinn K, Celniker
 1092 SE, Garcia KC (2013) An Extracellular Interactome of Immunoglobulin and LRR Proteins
 1093 Reveals Receptor-Ligand Networks. *Cell* 154:228–239.

1094

1095 Öztürk-Çolak A et al. (2024) FlyBase: updates to the Drosophila genes and genomes
1096 database. GENETICS 227:iyad211.

1097

1098 Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V (2002) The Drosophila
1099 Wnt, Wingless, Provides an Essential Signal for Pre- and Postsynaptic Differentiation. Cell
1100 111:319–330.

1101

1102 Parcerisas A, Ortega-Gascó A, Pujadas L, Soriano E (2021) The Hidden Side of NCAM
1103 Family: NCAM2, a Key Cytoskeleton Organization Molecule Regulating Multiple Neural
1104 Functions. Int J Mol Sci 22:10021.

1105 Parcerisas A, Pujadas L, Ortega-Gascó A, Perelló-Amorós B, Viais R, Hino K, Figueiro-Silva J,
1106 Torre AL, Trullás R, Simó S, Lüders J, Soriano E (2020) NCAM2 Regulates Dendritic and
1107 Axonal Differentiation through the Cytoskeletal Proteins MAP2 and 14-3-3. Cereb Cortex
1108 30:3781–3799.

1109

1110 Perry S, Han Y, Das A, Dickman D (2017) Homeostatic plasticity can be induced and
1111 expressed to restore synaptic strength at neuromuscular junctions undergoing ALS-related
1112 degeneration. Hum Mol Genet 26:4153–4167.

1113

1114 Perry S, Han Y, Qiu C, Chien C, Goel P, Nishimura S, Sajani M, Schmid A, Sigrist SJ,
1115 Dickman D (2022) A glutamate receptor C-tail recruits CaMKII to suppress retrograde
1116 homeostatic signaling. Nat Commun 13:7656.

1117

1118 Petersen SA, Fetter RD, Noordermeer JN, Goodman CS, DiAntonio A (1997) Genetic Analysis
1119 of Glutamate Receptors in Drosophila Reveals a Retrograde Signal Regulating Presynaptic
1120 Transmitter Release. Neuron 19:1237–1248.

1121

1122 Petit F, Plessis G, Decamp M, Cuisset J-M, Blyth M, Pendlebury M, Andrieux J (2015) 21q21
1123 deletion involving NCAM2: Report of 3 cases with neurodevelopmental disorders. Eur J Méd
1124 Genet 58:44–46.

1125

1126 Piechotta K, Dudanova I, Missler M (2006) The resilient synapse: insights from genetic
1127 interference of synaptic cell adhesion molecules. Cell Tissue Res 326:617–642.

1128

1129 Pielage J, Fetter RD, Davis GW (2005) Presynaptic Spectrin Is Essential for Synapse
1130 Stabilization. *Curr Biol* 15:918–928.

1131

1132 Polo-Parada L, Bose CM, Landmesser LT (2001) Alterations in Transmission, Vesicle
1133 Dynamics, and Transmitter Release Machinery at NCAM-Deficient Neuromuscular Junctions.
1134 *Neuron* 32:815–828.

1135

1136 Qin G, Schwarz T, Kittel RJ, Schmid A, Rasse TM, Kappei D, Ponimaskin E, Heckmann M,
1137 Sigrist SJ (2005) Four Different Subunits Are Essential for Expressing the Synaptic Glutamate
1138 Receptor at Neuromuscular Junctions of *Drosophila*. *J Neurosci* 25:3209–3218.

1139

1140 Qiu C, Perry S, Chen C, Chen J, Zhuang J, Han Y, Goel P, Dickman D (2025) Nonionic
1141 signaling rapidly remodels postsynaptic DLG to induce retrograde homeostatic plasticity. *Proc*
1142 *Natl Acad Sci* 122:e2502997122.

1143

1144 Rafuse VF, Polo-Parada L, Landmesser LT (2000) Structural and Functional Alterations of
1145 Neuromuscular Junctions in NCAM-Deficient Mice. *J Neurosci* 20:6529–6539.

1146

1147 Restrepo LJ, DePew AT, Moese ER, Tymanskyj SR, Parisi MJ, Aimino MA, Duhart JC, Fei H,
1148 Mosca TJ (2022) γ -secretase promotes *Drosophila* postsynaptic development through the
1149 cleavage of a Wnt receptor. *Dev Cell*.

1150 Schmid A, Qin G, Wichmann C, Kittel RJ, Mertel S, Fouquet W, Schmidt M, Heckmann M,
1151 Sigrist SJ (2006) Non-NMDA-Type Glutamate Receptors Are Essential for Maturation But Not
1152 for Initial Assembly of Synapses at *Drosophila* Neuromuscular Junctions. *J Neurosci*
1153 26:11267–11277.

1154

1155 Schuster CM, Davis GW, Fetter RD, Goodman CS (1996a) Genetic Dissection of Structural
1156 and Functional Components of Synaptic Plasticity. I. Fasciclin II Controls Synaptic Stabilization
1157 and Growth. *Neuron* 17:641–654.

1158

1159 Schuster CM, Davis GW, Fetter RD, Goodman CS (1996b) Genetic Dissection of Structural
1160 and Functional Components of Synaptic Plasticity. II. Fasciclin II Controls Presynaptic
1161 Structural Plasticity. *Neuron* 17:655–667.

1162

1163 Seeger M, Tear G, Ferres-Marco D, Goodman CS (1993) Mutations affecting growth cone
1164 guidance in drosophila: Genes necessary for guidance toward or away from the midline.
1165 Neuron 10:409–426.

1166

1167 Shen K, Scheiffele P (2010) Genetics and Cell Biology of Building Specific Synaptic
1168 Connectivity. Annu Rev Neurosci 33:473–507.

1169

1170 Shetty A, Sytnyk V, Leshchyns'ka I, Puchkov D, Haucke V, Schachner M (2013) The Neural
1171 Cell Adhesion Molecule Promotes Maturation of the Presynaptic Endocytotic Machinery by
1172 Switching Synaptic Vesicle Recycling from Adaptor Protein 3 (AP-3)- to AP-2-Dependent
1173 Mechanisms. J Neurosci 33:16828–16845.

1174

1175 Shiwaku H, Katayama S, Kondo K, Nakano Y, Tanaka H, Yoshioka Y, Fujita K, Tamaki H,
1176 Takebayashi H, Terasaki O, Nagase Y, Nagase T, Kubota T, Ishikawa K, Okazawa H,
1177 Takahashi H (2022) Autoantibodies against NCAM1 from patients with schizophrenia cause
1178 schizophrenia-related behavior and changes in synapses in mice. Cell Rep Med 3:100597.

1179

1180 Sytnyk V, Leshchyns'ka I, Nikonenko AG, Schachner M (2006) NCAM promotes assembly and
1181 activity-dependent remodeling of the postsynaptic signaling complex. J Cell Biol 174:1071–
1182 1085.

1183

1184 Tang A-H, Chen H, Li TP, Metzbower SR, MacGillavry HD, Blanpied TA (2016) A trans-
1185 synaptic nanocolumn aligns neurotransmitter release to receptors. Nature 536:210–214.

1186

1187 Tessier-Lavigne M, Goodman CS (1996) The Molecular Biology of Axon Guidance. Science
1188 274:1123–1133.

1189

1190 Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle
1191 M-C, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E (2006) Bruchpilot, a
1192 Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of
1193 Synaptic Active Zones in Drosophila. Neuron 49:833–844.

1194

1195 Williams SE, Mealer RG, Scolnick EM, Smoller JW, Cummings RD (2020) Aberrant
1196 glycosylation in schizophrenia: a review of 25 years of post-mortem brain studies. Mol
1197 Psychiatry 25:3198–3207.

1198

1199 Yoshihara M, Rheuben MB, Kidokoro Y (1997) Transition from Growth Cone to Functional
1200 Motor Nerve Terminal in *Drosophila* Embryos. *J Neurosci* 17:8408–8426.

1201

1202 Yu M, Tan Q, Dong W, Xiang B (2025) Identification and prioritization of gene sets associated
1203 with schizophrenia risk by network analysis. *Psychopharmacology*:1–11.

1204

1205 Zhang B, Stewart B (2010) Electrophysiological Recording from *Drosophila* Larval Body-Wall
1206 Muscles. *Cold Spring Harb Protoc* 2010:pdb.prot5487.

1207

1208 Zito K, Parnas D, Fetter RD, Isacoff EY, Goodman CS (1999) Watching a Synapse Grow
1209 Noninvasive Confocal Imaging of Synaptic Growth in *Drosophila*. *Neuron* 22:719–729.

1210

1211

1212

1213

1214

1215

1216

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Figure Legends

Figure 1. The adhesion protein CG33543/Elff localizes to synapses throughout the CNS.
(A) Schematic depicting the predicted protein domain organization of Fas II (left) and CG33543/Elff (right). Fas II has seven known isoforms (PA-PH) containing five IgSF-like domains and two FN3 domains. These Fas II isoforms are predicted to be anchored to the membrane via GPI linkage or a transmembrane (TM) domain with variable cytoplasmic regions. Elff has one known isoform predicted to be GPI-linked with three IgSF domains and one FN3 domain. (B) Schematic view of *elff* allele designs. *elff^{JR1}* features a deletion of the entire endogenous coding region, which is replaced with attP and loxP docking sites and a DsRed visible marker. *elff^{ST1}* harbors an insertion immediately downstream of the predicted signal sequence, which causes an early stop. (UTR, untranslated region; TR, PiggyBac terminal repeats). (C-D) Representative Z-projections of Drosophila larval ventral nerve cords (top) and adult brains (bottom) from *Elff^{MI{PT-GFSTF.1}}* and *Elff^{sfGFP-N}* stained for GFP (grayscale). Scale bars: 20 μ m. (E-F) Representative Z-projections of Drosophila muscle 4 NMJ boutons from *Elff^{MI{PT-GFSTF.1}}*, *Elff^{sfGFP-N}*, and control larvae stained for GFP (grayscale) and HRP (magenta). Scale bar: 5 μ m. (H) Representative Airyscan Z-projection of a single bouton from *Elff^{sfGFP-N}* stained for GFP (green) and Brp (magenta). Single channels shown in grayscale above merged image for clarity. Scale bar: 1 μ m. (I-J) Representative Z-projections of Drosophila NMJs at muscle 4 from the indicated genotypes stained for Dlg (green) and HRP (magenta). Scale bar: 5 μ m. (K) Quantification of bouton number. Data are mean $\pm$ SD (control: 19.6 ± 3.2 , *elff^{JR1/ST1}*: 19.6 ± 3.6 , $t(58) = 0.975$, $p = 0.9277$). Significance determined by unpaired t-test. $n \geq 30$, Animals ≥ 8 .

Figure 2. Presynaptic Elff promotes postsynaptic maturation at the NMJ.
(A-D) Representative Z-projections of NMJ boutons from the indicated genotypes stained for Dlg (green) and HRP (magenta). For clarity, single channels are shown in grayscale next to or below merged images across all figures. For all figures, drivers used for presynaptic (Pre) and postsynaptic (Post) knockdown are D42Gal4 and 24BGal4, respectively. Scale bar: 5 μ m. (E) Quantification of Dlg fluorescence intensity. Data are mean values normalized to control $\pm$ SD (control: 100.0 ± 22.9 , *elff^{JR1/ST1}*: 47.5 ± 8.7 , *Elff^{sfGFP-N}*: 45.1 ± 7.5 , *elff^{MI{PT-GFSTF.1/JR1}}*: 99.8 ± 7.8 ,

1269 H(3) = 121.5, $p < 0.0001$); control: 100.0 ± 15.5 , Pre > *elff* RNAi: 65.6 ± 12.4 , $t(70.63) = 10.35$,
 1270 $p < 0.0001$; control: 100.0 ± 5.54 , Post > *elff* RNAi: 100.2 ± 13.67 , $U = 421.5$, $p = 0.4108$). (F-
 1271 G) Representative Z-projections of boutons at NMJ 6/7 from the indicated genotypes stained
 1272 for Dlg (green) and HRP (magenta). Dlg intensity in *elff* mutants was scaled up to visualize any
 1273 weak signal. Asterisks indicate nascent boutons. Scale bar: 5 μm ; inset scale bar: 2 μm . (H)
 1274 Quantification of nascent boutons. Data are mean $\pm$ SD (control: 1.6 ± 1.2 , *elff*^{JR1/ST1}: 5.5 ± 3.2 ;
 1275 control: 0.7 ± 0.9 , Pre > *elff* RNAi #1: 3.7 ± 2.2 ; control: 1.4 ± 1.8 , Post > *elff* RNAi #1: $2.4 \pm$
 1276 2.0 , H(5) = 35.98, $p < 0.0001$). Significance determined by Kruskal-Wallis test with Dunn's
 1277 multiple comparisons test [ns, not significant, ** $p < 0.01$, **** $p < 0.0001$]. $n \geq 10$, Animals ≥ 8 .

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1279 Figure 3. Elff acts transsynaptically to promote alpha-Spectrin accumulation.

1280 (A-D) Representative Z-projections of boutons from the indicated genotypes stained for alpha-
 1281 Spectrin (green) and HRP (magenta). Scale bar: 5 μm . (E) Quantification of alpha-Spectrin
 1282 intensity. Data are mean values normalized to control $\pm$ SD (control: 100.0 ± 18.8 , *elff*^{JR1/ST1}:
 1283 23.1 ± 4.5 ; control: 100.0 ± 16.0 , Pre > *elff* RNAi: 50.8 ± 18.4 ; control: 100.0 ± 17.0 , Post > *elff*
 1284 RNAi: 94.1 ± 12.4 , H(5) = 134.5, $p < 0.0001$). (F-G) Representative Z-projections of muscle 4
 1285 NMJs of the indicated genotypes stained for alpha-Spectrin (green) and HRP (magenta).
 1286 Arrows indicate synaptic boutons. Scale bars: 5 μm . (H) Orthogonal projections from synaptic
 1287 boutons (left) and expanded HRP webbing (right) in Post > Elff. Dotted lines in G indicate the
 1288 areas used to generate orthogonal projections. Scale bars: 5 μm . (I) Quantification of NMJ
 1289 webbing phenotype (control: 0% of NMJs with webbing, Pre > Elff: 6.25% of NMJs with
 1290 webbing, Post > Elff: 77.1% of NMJs with webbing). Significance determined by Kruskal-Wallis
 1291 test with Dunn's multiple comparisons (E) or Fisher's exact test (I, $p = 0.4921$ for WT vs. Pre >
 1292 Elff, $p = 2.275 \times 10^{-9}$ for WT vs. Post > Elff) [ns, not significant, **** $p < 0.0001$]. $n \geq 27$, Animals $\geq$
 1293 8.

1294

1295 Figure 4. Elff is necessary for baseline synaptic transmission and larval locomotion.

1296 (A) Representative traces of evoked (top) and spontaneous (bottom) excitatory junction
 1297 potentials (EJPs) from the indicated genotypes. Recordings obtained from larval muscle 6 of
 1298 abdominal segments A3 and A4. (B-E) Quantification of (B) mean mEJP amplitude (control:
 1299 $0.92 \text{ mV} \pm 0.23$, *elff*^{JR1/ST1}: $0.62 \text{ mV} \pm 0.21$), (C) mean mEJP frequency (control: $3.1 \text{ Hz} \pm 1.3$,
 1300 *elff*^{JR1/ST1}: $1.2 \text{ Hz} \pm 0.49$), (D) mean EJP amplitude (control: $31.4 \text{ mV} \pm 6.3$, *elff*^{JR1/ST1}: 21.6 mV
 1301 ± 6.3), and (E) mean quantal content (control: 35.21 ± 7.5 , *elff*^{JR1/ST1}: 36.65 ± 12.6). Data are
 1302 mean $\pm$ SD. Each data point represents one animal ($n \geq 23$). (F-G) Representative traces of
 1303 third-instar larval crawling behavior following a 5 min tracking period for animals of the
 1304 indicated genotypes. Scale bar: 1 cm. (H-I) Quantification of total distance crawled (H) and
 1305 average velocity (I) obtained from manual tracking (H, control: $19.0 \text{ cm} \pm 6.4$, *elff*^{JR1/ST1}: 15.8
 1306 $\text{cm} \pm 5.8$); (I, control: $1.6 \text{ cm/s} \pm 0.5$, *elff*^{JR1/ST1}: $1.3 \text{ cm/s} \pm 0.5$). Each data point represents one
 1307 animal ($n \geq 42$). Significance determined by Mann-Whitney test (B) or unpaired t-test (C-E, H-I)
 1308 [ns, not significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$].

1309

Figure 5. Elff is required for glutamate receptor localization at the NMJ.

(A-B, D-E, G-H, J-K) Representative Z-projections of boutons from the indicated genotypes stained for (A-B) GluRIIA, (D-E) GluRIIB, (G-H) GluRIIC, or (J-K) GluRIIE (green) and HRP (magenta). Scale bar: 5 μ m. (C, F, I, L) Quantification of fluorescence intensity for (C) GluRIIA (control: 100.0 ± 30.1 , *elff*^{JR1/ST1}: 44.1 ± 11.4 , U = 52, p < 0.0001), (F) GluRIIB (control: 100.0 ± 18.5 , *elff*^{JR1/ST1}: 81.4 ± 21.2 , U = 277, p = 0.0001), (I) GluRIIC (control: 100.0 ± 23.4 , *elff*^{JR1/ST1}: 41.6 ± 12.3 , U = 10, p < 0.0001), and (L) GluRIIE (control: 100.0 ± 23.0 , *elff*^{JR1/ST1}: 31.1 ± 10.4 , t(59) = 15.15, p < 0.0001). Data are mean values normalized to control $\pm$ SD. (M-P) Representative Z-projections of boutons from the indicated genotypes. For all figures, drivers for knockdown on both sides of the synapse (Pre + Post) are *elavGal4* and *24BGal4*. Scale bar: 5 μ m. (Q) Quantification of GluRIIE intensity (control: 100.0 ± 15.8 , Pre > *elff* RNAi #1: 85.5 ± 24.6 ; control: 100.0 ± 22.9 , Post > *elff* RNAi: 90.7 ± 22.8 ; control: 100.0 ± 21.6 , Pre+Post > *elff* RNAi: 63.5 ± 14.3 , F(5, 195) = 3.436, p < 0.0001). Significance determined by Mann-Whitney test (C, F, I), unpaired t-test (L) or one-way ANOVA with Tukey's multiple comparisons test (Q) [ns, not significant, ***p<0.001, ****p<0.0001]. n $\geq$ 21, Animals $\geq$ 6.

Figure 6. Elff is required for the differentiation of the postsynaptic compartment during mid-larval development.

(A-F) Representative Z-projections of boutons in second instar larvae of the indicated genotypes, stained for HRP (magenta) and (A-B) alpha-Spectrin, (C-D) Dlg, or (E-F) GluRIIE (grayscale). Scale bar: 2 μ m. (G) Quantification of alpha-Spectrin intensity. Data are mean values normalized to control $\pm$ SD (control: 100 ± 48.80 , *elff*^{JR1/ST1}: 6.665 ± 8.820 , U = 10, p < 0.0001). (H) Quantification of Dlg intensity (control: 100 ± 48.42 , *elff*^{JR1/ST1}: 10.81 ± 10.57 , U = 22.50, p < 0.0001). (I) Quantification of GluRIIE intensity (control: 100 ± 29.30 , *elff*^{JR1/ST1}: 5.311 ± 5.195 , U = 0, p < 0.0001). Significance determined by Mann-Whitney test [****p<0.0001]. n $\geq$ 20, Animals $\geq$ 6.

Figure 7. Elff promotes initial assembly of the postsynaptic compartment.

(A-B) Representative Z-projections of embryonic stage 17 NMJs from the indicated genotypes stained for Brp (orange), GluRIIE (green), and HRP (magenta). Scale bar: 2 μ m. (C) Quantification of bouton number per NMJ. Data are mean $\pm$ SD (control: 6.386 ± 2.505 , *elff*^{JR1/ST1}: 6.417 ± 2.395 , U = 1337, p = 0.8424). (D) Quantification of GluRIIE intensity. Data are mean values normalized to control $\pm$ SD (control: 100.0 ± 52.1 , *elff*^{JR1/ST1}: 37.9 ± 21.4 , U = 233, p < 0.0001). (E) Quantification of Brp puncta number per NMJ (control: 31.51 ± 13.14 , *elff*^{JR1/ST1}: 32.72 ± 17.64 , t(91) = 0.6752, p = 0.5013). (F) Quantification of the percentage of Brp puncta unopposed to GluR. Data are mean $\pm$ SD (control: 63.35 ± 21.26 , *elff*^{JR1/ST1}: 87.55 ± 9.002 , U = 221.5, p < 0.0001). (G) Quantification of the percentage of GluRIIE puncta unopposed to Brp (control: 29.45 ± 17.0 , *elff*^{JR1/ST1}: 64.38 ± 21.70 , U = 226, p < 0.0001). (H-I) Representative Z-projections of stage 17 NMJs from the indicated genotypes stained for Dlg (green) and HRP (magenta). Scale bar: 2 μ m. (J) Quantification of Dlg intensity. Data are mean values normalized to control $\pm$ SD (control: 100.0 ± 54.1 , *elff*^{JR1/ST1}: 62.4 ± 45.3 , U = 718, p < 0.0001). Significance determined by Mann-Whitney test [ns, not significant, ****p<0.0001]. n $\geq$ 45, Animals $\geq$ 15.

1353

1354 Figure 8. Elff is essential for transcellular apposition of pre- and postsynaptic specializations.

1355 (A-C) Representative Airyscan Z-projections of boutons from the indicated genotypes stained
1356 for Brp (magenta) and GluRIIE (green). White arrowheads indicate unapposed Brp. Yellow
1357 arrowheads indicate unopposed GluRIIE. Scale bars: 2 μ m. (D) Quantification of Brp density
1358 per square micron. Data are mean $\pm$ SD (control: 0.35 ± 0.098 , *elff*^{JR1/ST1}: 0.32 ± 0.1 ; control:
1359 0.37 ± 0.057 , Pre+Post > *elff* RNAi: 0.38 ± 0.016 , $F(3, 53) = 1.712$, $p = 0.1757$). (E)
1360 Quantification of the percentage of unapposed Brp (control: 2.8 ± 2.0 , *elff*^{JR1/ST1}: 22.3 ± 8.6 ;
1361 control: 4.8 ± 2.6 , Pre+Post > *elff* RNAi: 20.0 ± 8.2 , $F(3,53) = 9.552$, $p < 0.0001$). (F)
1362 Quantification of the percentage of unapposed GluRIIE (control: 3.1 ± 4.2 , *elff*^{JR1/ST1}: $21.3 \pm$
1363 6.8 ; control: 5.8 ± 4.8 , Pre+Post > *elff* RNAi: 19.3 ± 9.0 , $H(3) = 36.36$, $p < 0.0001$). Significance
1364 determined by one-way ANOVA with Tukey's multiple comparisons test (D, E) or Kruskal-
1365 Wallis test with Dunn's multiple comparisons (F) [ns, not significant, ** $p < 0.01$, *** $p < 0.001$,
1366 **** $p < 0.0001$]. $n \geq 12$, Animals ≥ 6 .

1367

1368 Figure 9. Elff is required for nanoscale alignment of pre- and postsynaptic architecture.

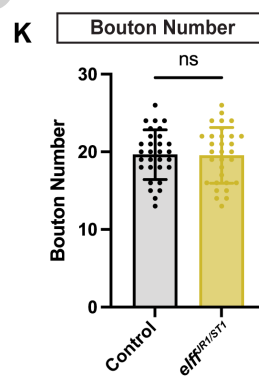
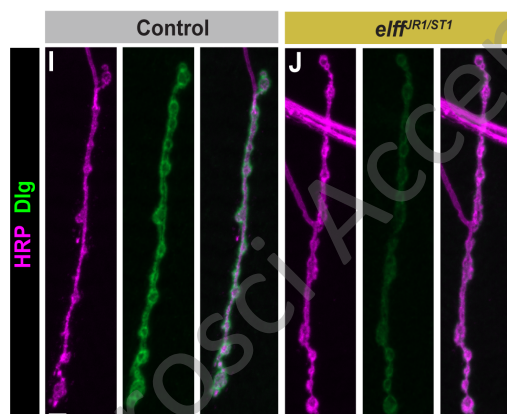
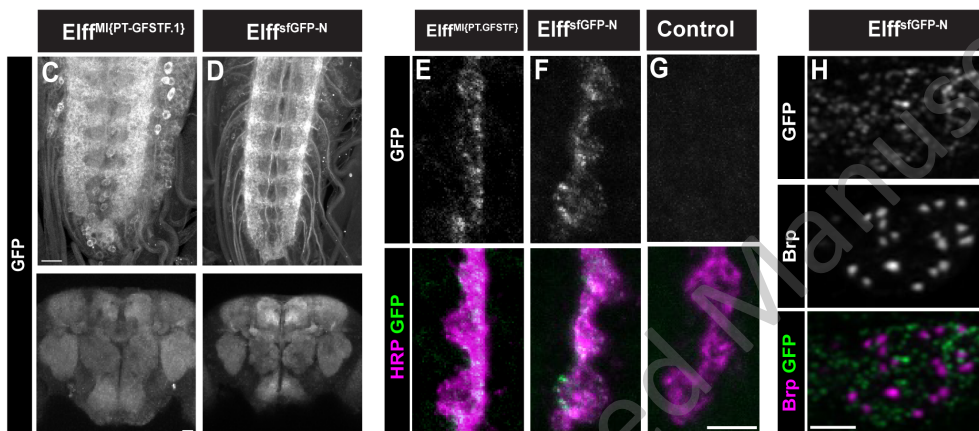
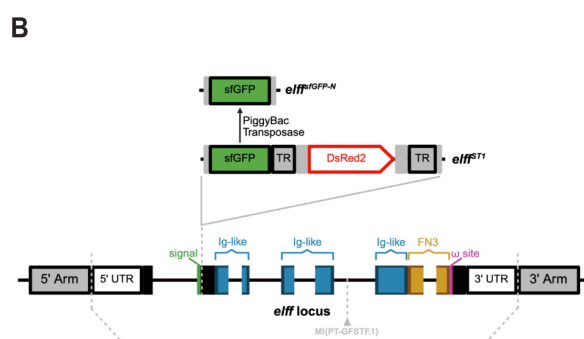
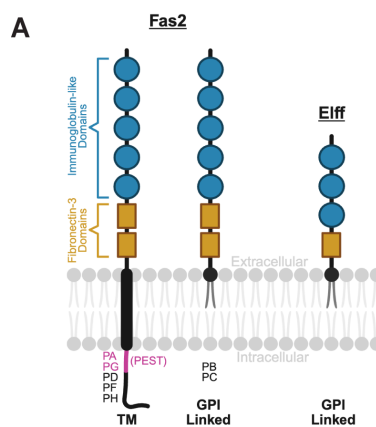
1369 (A, C) Representative Z-projections of single boutons from muscle 6/7 NMJs of the indicated
1370 genotypes stained for Brp (magenta) and GluRIIE (green). Scale bar: 1 μ m. (B, D) Single
1371 synapses from panels A and C, respectively (indicated by white arrowheads) shown at higher
1372 magnification. (E) Quantification of Brp ring diameter. Data are mean $\pm$ SD (control: $198.0 \pm$
1373 0.043 , *elff*^{JR1/ST1}: 197.0 ± 0.043 , $U = 10,810$, $p = 0.6049$). (F) Quantification of the percentage
1374 of GluRIIE surface area within 200 nm of a Brp punctum (control: 50.61 ± 10.5 , *elff*^{JR1/ST1}:
1375 26.22 ± 7.1 , $t(25) = 7.200$, $p < 0.0001$). (G-H) Representative plot profiles of GluRIIE and Brp
1376 intensity along the lines shown in Figures A and C. (I) Quantification of the lateral distance
1377 between Brp and GluRIIE local maxima (control: 97.5 ± 64.6 , *elff*^{JR1/ST1}: 122.9 ± 88.0 , $U =$
1378 46685 , $p = 0.0022$). Significance determined by Mann-Whitney test (E, I) or unpaired t-test (F)
1379 [ns, not significant, ** $p < 0.01$, **** $p < 0.0001$] $n \geq 12$, Animals ≥ 6 .

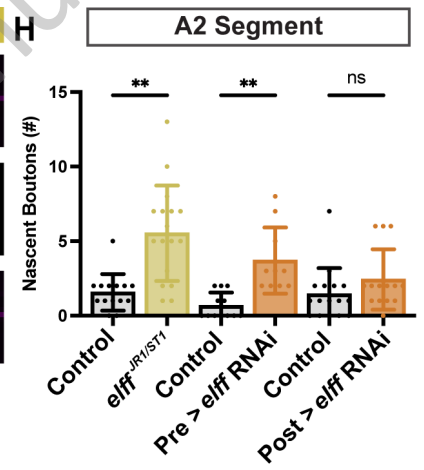
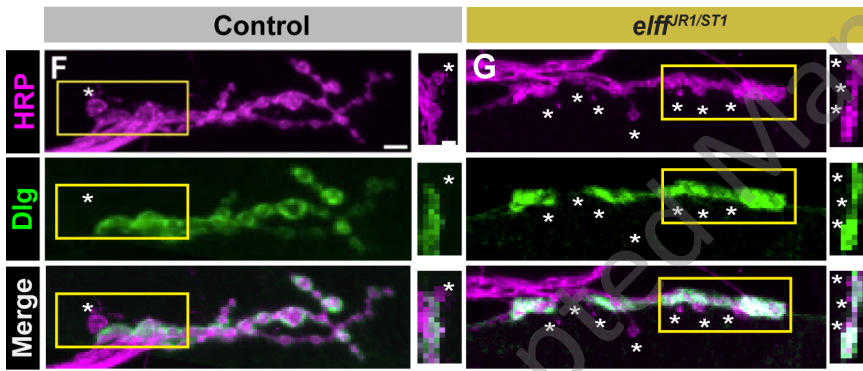
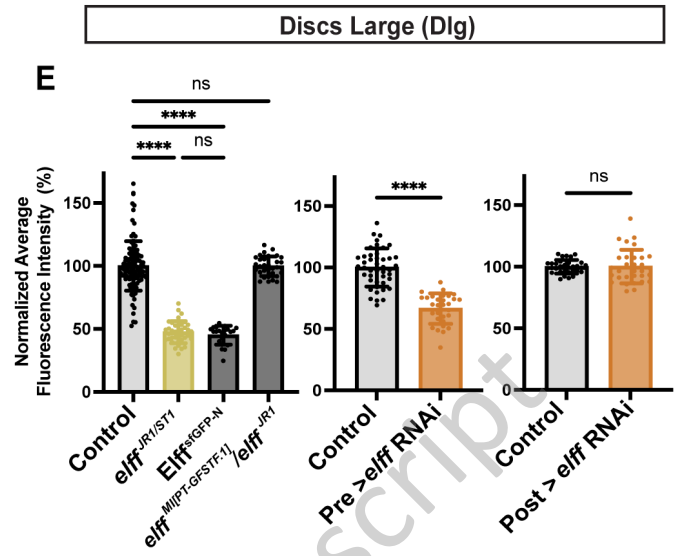
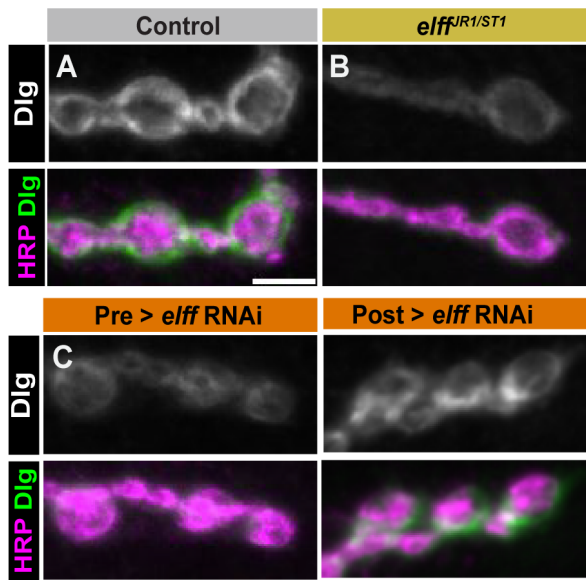
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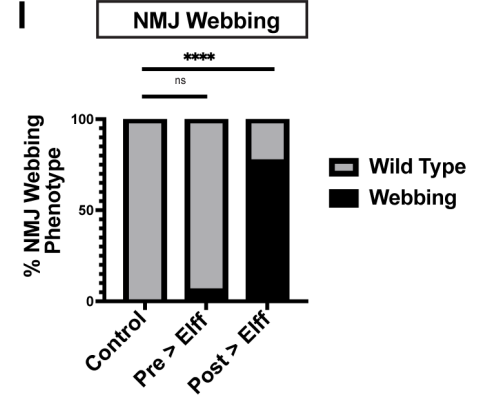
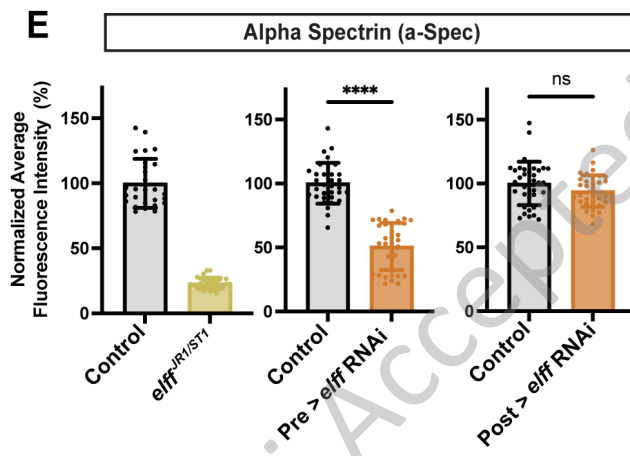
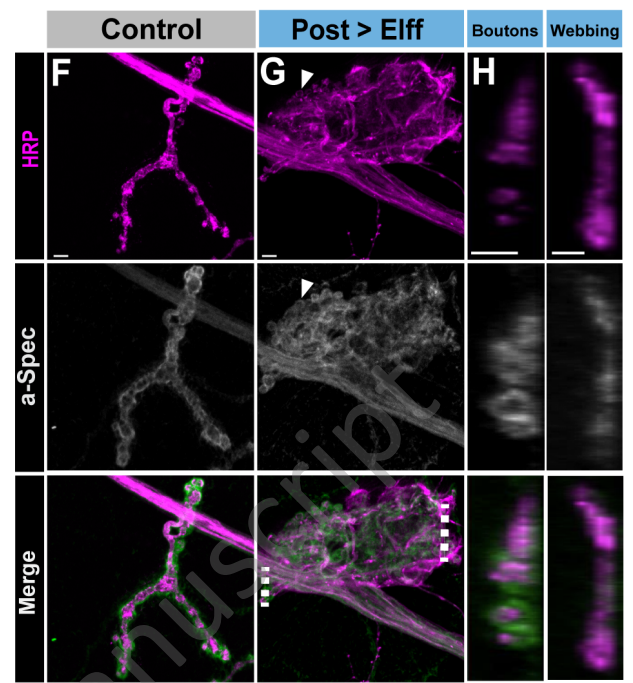
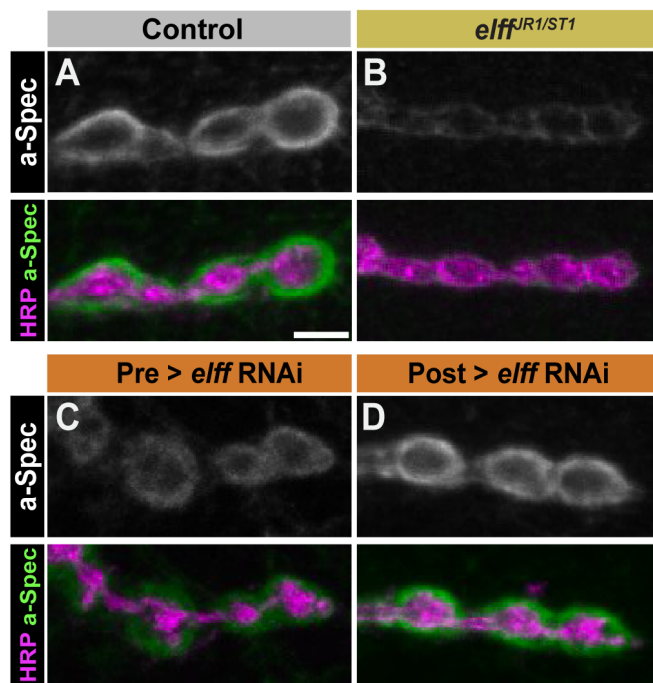
1381 Figure 10. A model of distinct mechanisms for Elff's functions in postsynaptic maturation and
1382 glutamate receptor clustering. Left: Elff is required in trans for postsynaptic accumulation of
1383 spectrin andDlg. Its postsynaptic partner(s) and other possible interactors are not known.
1384 Right: Elff facilitates glutamate receptor clustering and synaptic apposition in cis and/or in trans
1385 by stabilizing transsynaptic interactions through an unknown mechanism. In this context, Elff
1386 may act as a membrane-bound protein and/or as a secreted adaptor for other adhesion
1387 proteins in the extracellular matrix of the synaptic cleft.

1388

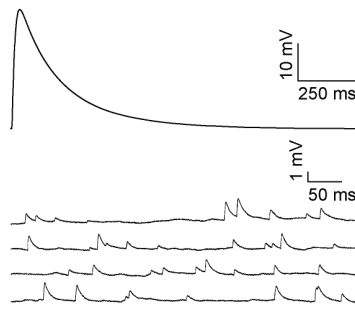
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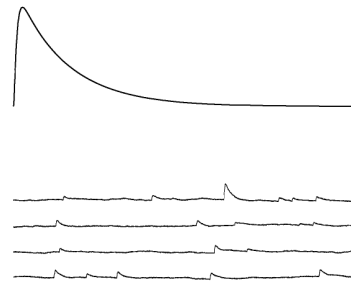




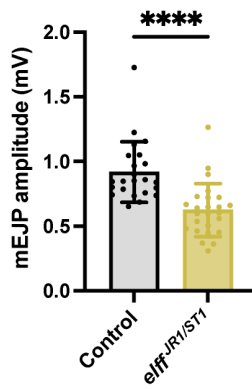
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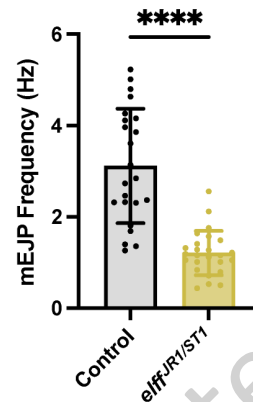
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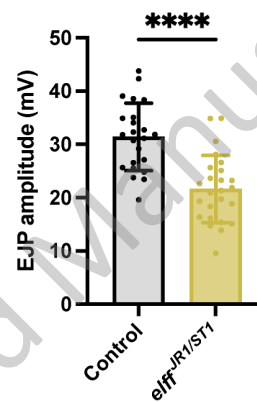
B mEJP Amplitude



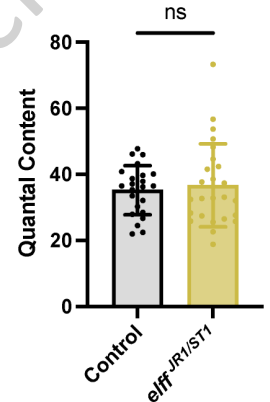
C mEJP Frequency



D EJP Amplitude



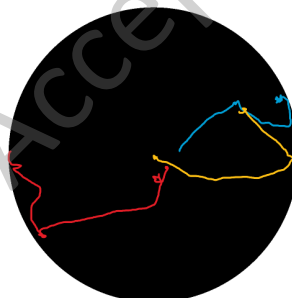
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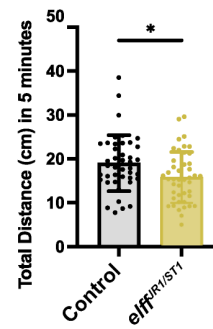
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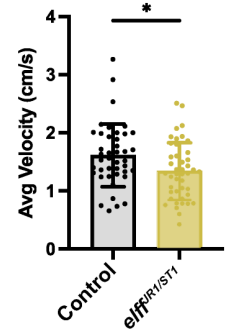
G *elff*^{JR1/ST1}

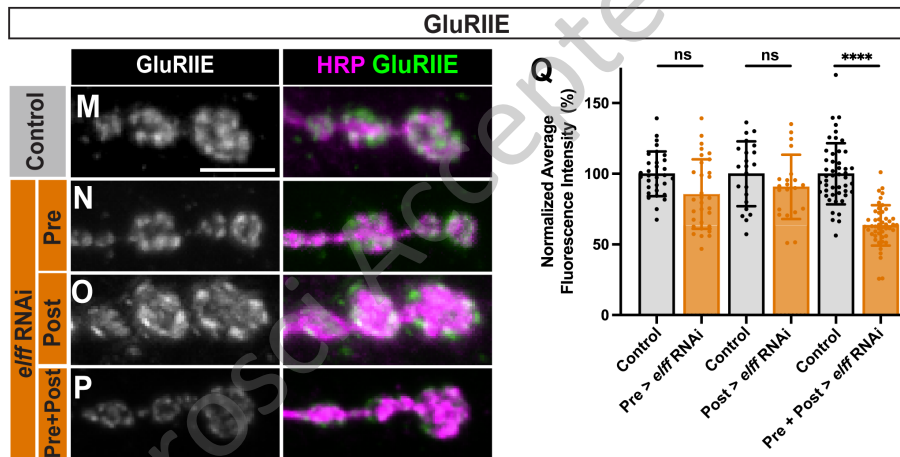
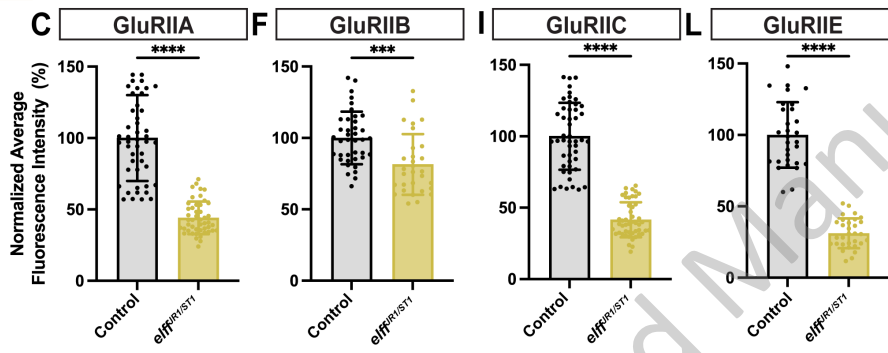
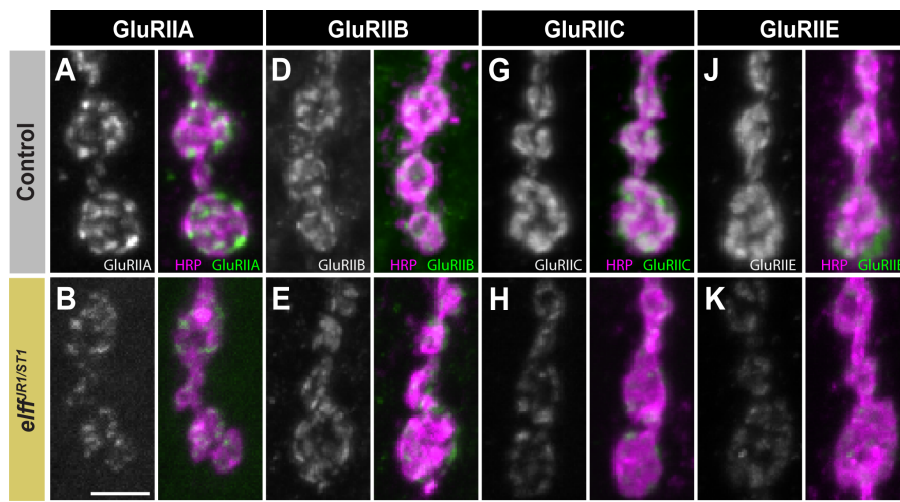


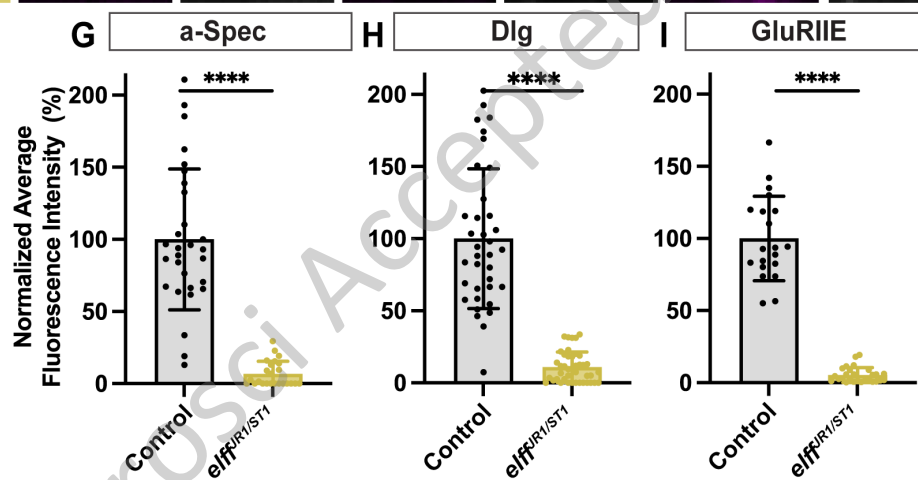
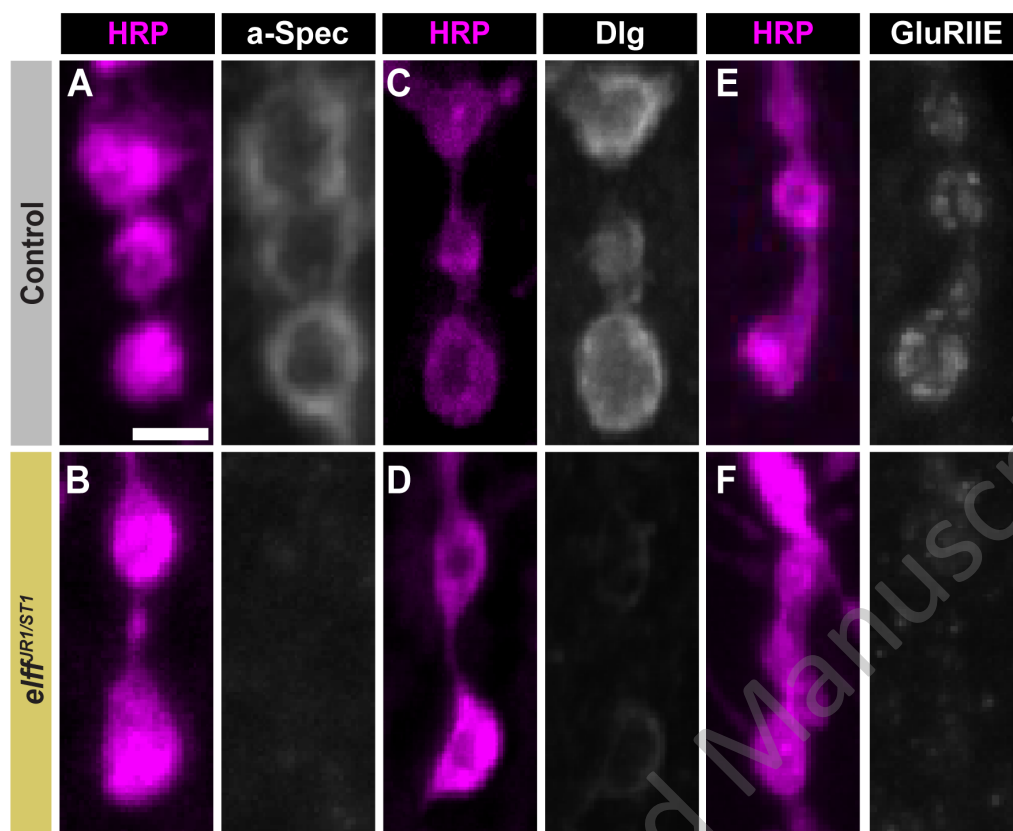
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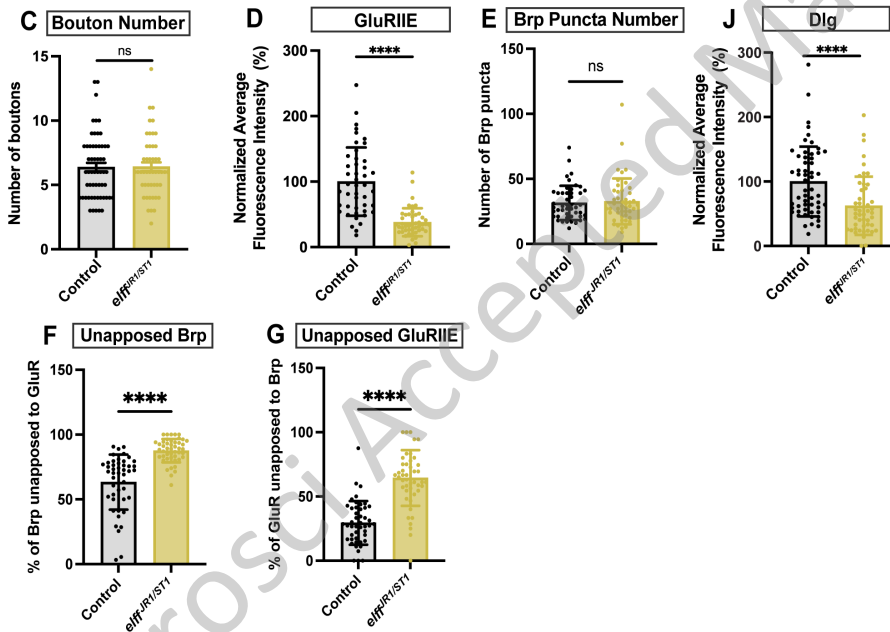
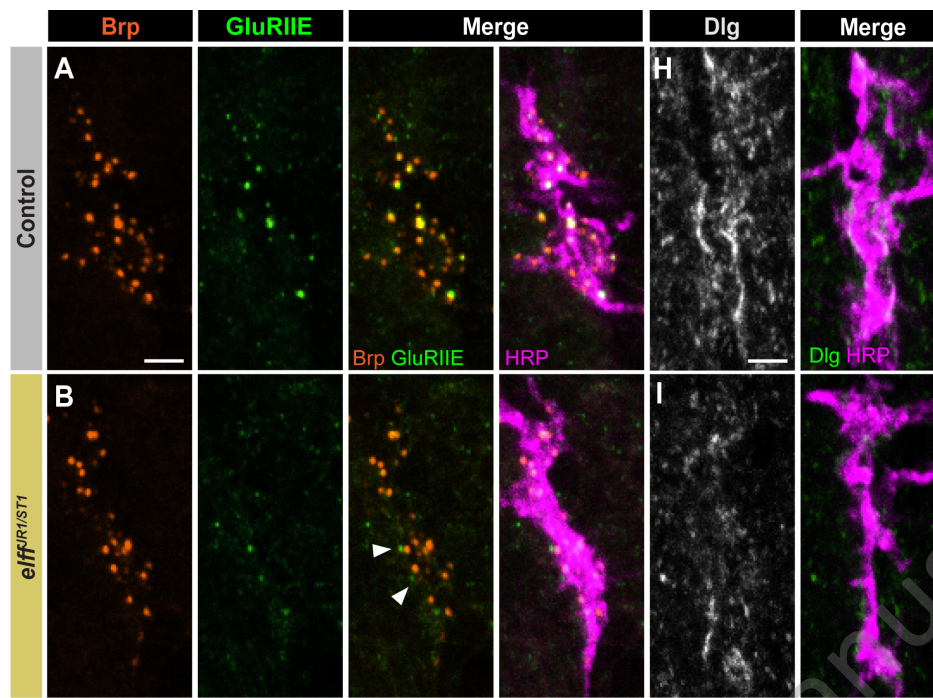


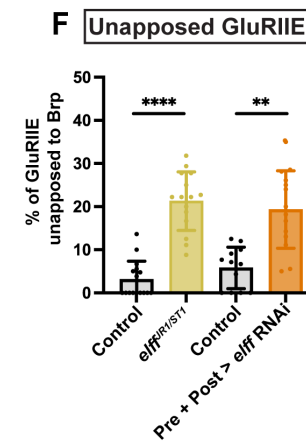
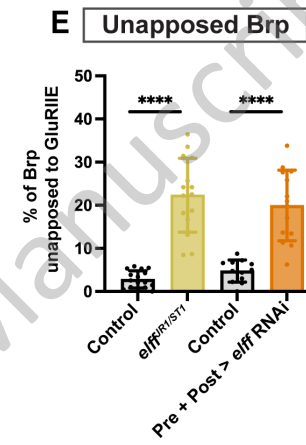
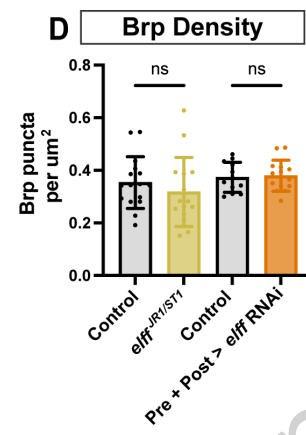
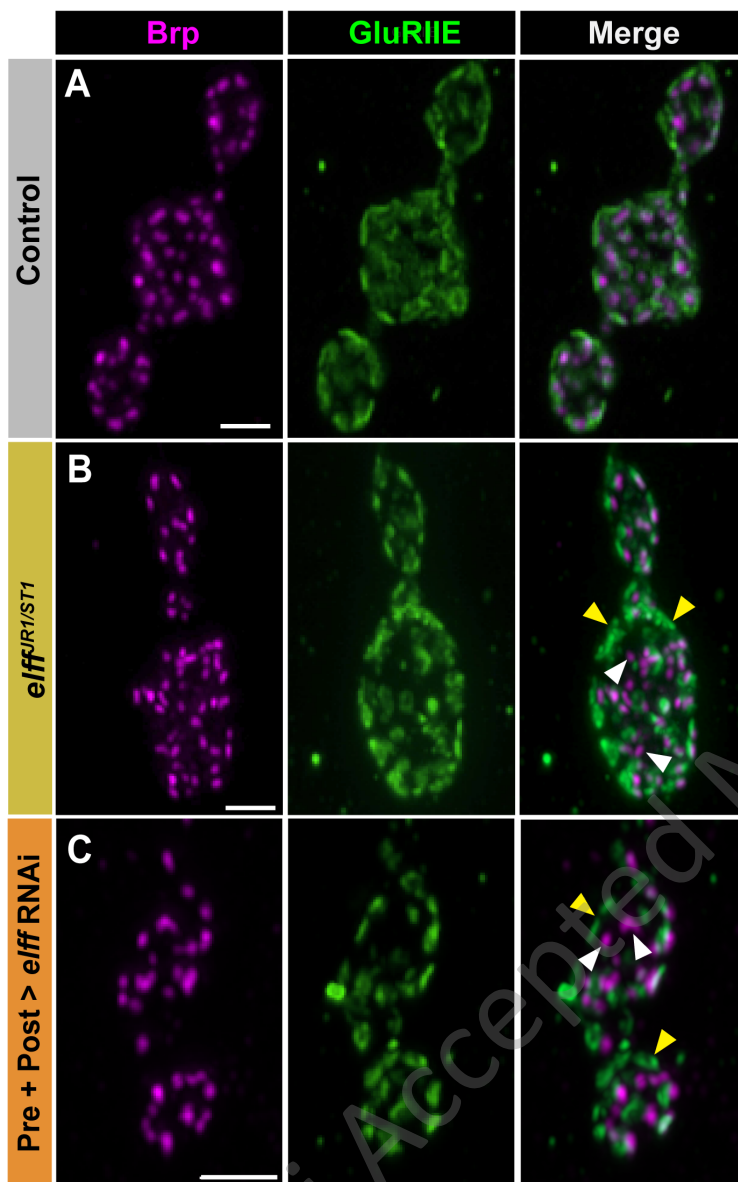
I Average Velocity

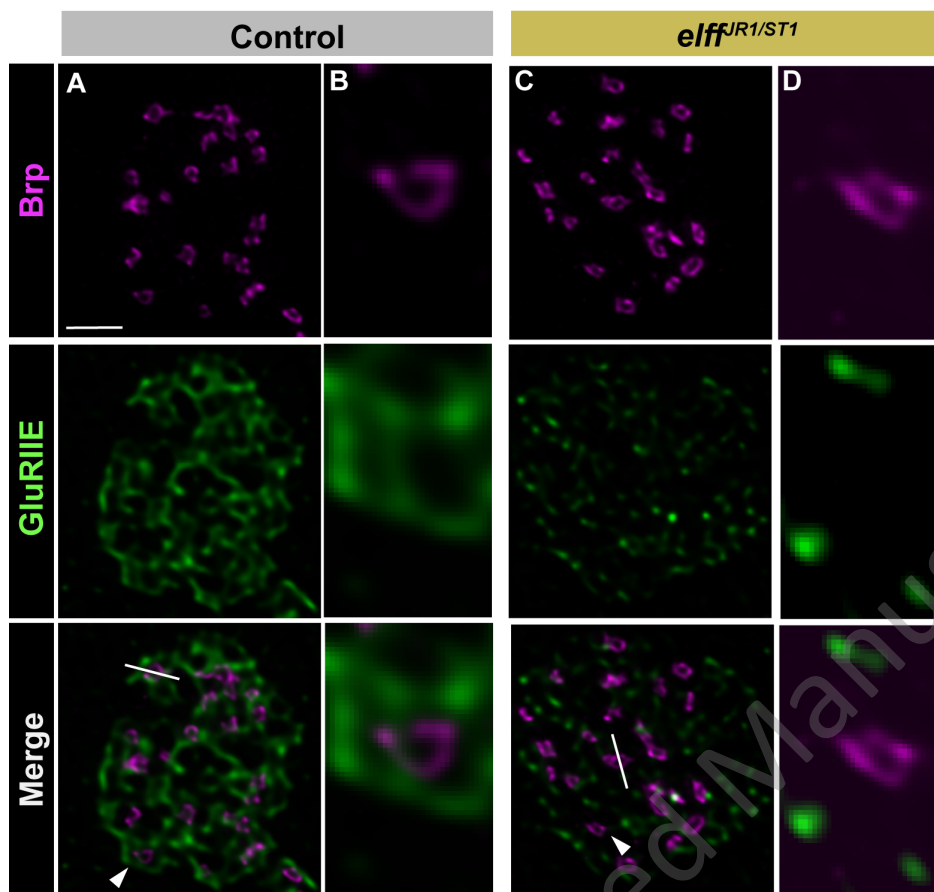




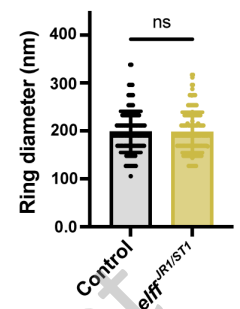




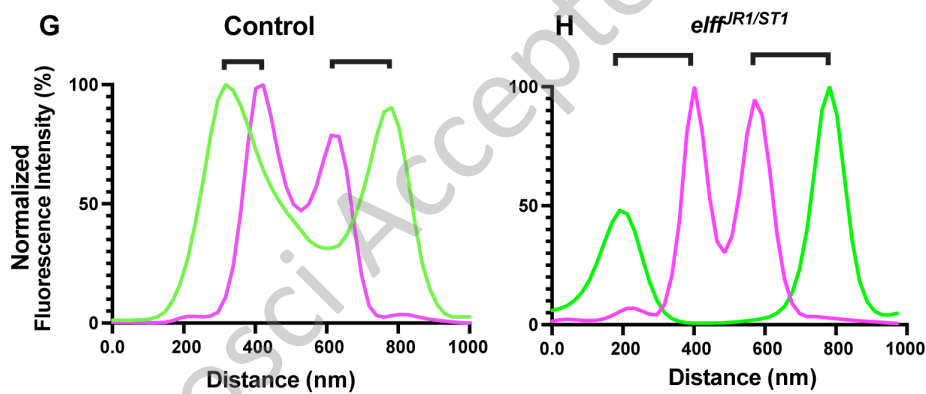
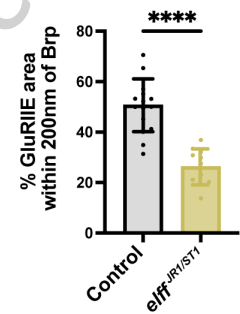




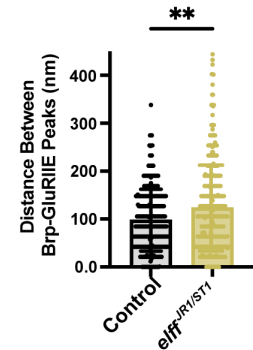
E Brp Diameter



F AZ-Adjacent GluRIIE Area



I Brp-GluRIIE Peak-to-Peak Distance



Postsynaptic maturation

Synaptic apposition and GluR clustering

