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Drosophila Homolog of the Human Carpenter Syndrome Linked Gene, *MEGF8*, is Required for Synapse Development and Function

Abbreviated Title: *dMegf8* in synapse development and function

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ABSTRACT

Drosophila Multiple Epidermal Growth Factor-like Domains 8 (dMegf8) is a homolog of human MEGF8. *MEGF8* encodes a multi-domain transmembrane protein which is highly conserved across species. In humans, *MEGF8* mutations cause a rare genetic disorder called Carpenter syndrome, which is frequently associated with abnormal left-right patterning, cardiac defects and learning disabilities. *MEGF8* is also associated with psychiatric disorders. Despite its clinical relevance *MEGF8* remains poorly characterized, and though it is highly conserved, studies on animal models of Megf8 are also very limited. The presence of intellectual disabilities in Carpenter syndrome patients and association of *MEGF8* with psychiatric disorders indicate that mutations in *MEGF8* cause underlying defects in synaptic structure and functions. In this study, we investigated the role of *Drosophila dMegf8* in glutamatergic synapses of the larval neuromuscular junctions (NMJ) in both males and females. We show that dMegf8 localizes to NMJ synapses and is required for proper synaptic growth. *dMegf8* mutant larvae and adults show severe motor coordination deficits. At the NMJ, *dMegf8* mutants show altered localization of pre- and post-synaptic proteins, defects in synaptic ultrastructure and neurotransmission. Interestingly, *dMegf8* mutants have reduced levels of the type II BMP receptor Wishful thinking (Wit). *dMegf8* displays genetic interactions with *neurexin-1* (*dnrx*) and *wit*, and in association with Dnrx and Wit plays an essential role in synapse organization. Our studies provide insights into human MEGF8 functions and potentially into mechanisms that may underlie intellectual disabilities observed in Carpenter syndrome as well as MEGF8-related synaptic structural and/or functional deficits in psychiatric disorders.

SIGNIFICANCE STATEMENT:

Carpenter Syndrome, known for over a century now, is a genetic disorder linked to mutations in *Multiple Epidermal Growth Factor-like Domains 8* (*MEGF8*) gene and associated with intellectual disabilities among other symptoms. *MEGF8* is also associated with psychiatric disorders. Despite the high genetic conservation and clinical relevance, the functions of *MEGF8* remain largely uncharacterized. Patients with intellectual disabilities and psychiatric diseases often have an underlying defect in synaptic structure and function. This work defines the role of the fly homolog of human *MEGF8*, *dMegf8*, in glutamatergic synapse growth, organization and function and provide insights into potential functions of *MEGF8* in human central synapses and synaptic mechanisms that may underlie psychiatric disorders and intellectual disabilities seen in Carpenter Syndrome.

Introduction

Carpenter Syndrome (CS), a rare genetic disorder with multiple-congenital-anomalies, was first described by George Carpenter (Carpenter, 1901). CS patients often present a variety of developmental defects at a very young age, including craniosynostosis, musculoskeletal abnormalities (mostly in fingers and toes), congenital heart disease and intellectual disabilities (Hidestrand et al., 2009; Taravath and Tonsgard, 1993). Other clinical features like hypogenitalism, umbilical hernia and obesity have also been reported (Alessandri et al., 2010; Haye et al., 2014). Genome-wide sequence analyses identified three single nucleotide polymorphisms in the *Multiple Epidermal Growth Factor-like Domains 8* (*MEGF8*) gene that showed association with the CS (Twigg et al., 2012). *MEGF8* has also been implicated in neuropsychiatric disorders such as schizophrenia (Bersani et al., 2003; Cox and Butler, 2015; Giacopuzzi et al., 2017).

Human *MEGF8* is clinically relevant, and its homologs are highly conserved across species and encode a multi-domain transmembrane protein (Fig. 1). Murine *Megf8* is involved in bone morphogenetic protein (BMP) signaling and mutations in mice *Megf8* disrupt axon guidance and lead to defects that phenotypically resemble human CS, including deformities of the limb, heart, and abnormal left-right patterning (Engelhard et al., 2013). *Megf8*, together with *Mgrn1*, catalyzes the ubiquitination and degradation of the Hedgehog pathway signaling molecules that coordinate cell-cell communication required for spinal cord and heart development (Jenkins et al., 2007; Kong et al., 2020; Pusapati et al., 2018). These findings suggest that *Megf8* functions in multiple intracellular signaling pathways and may modulate different cellular processes during development. Despite these observations on the role of Megf8 in different developmental events and in neuronal function, it has remained unclear

whether Megf8 has any role in synaptic development and function. Most importantly, intellectual disabilities seen in CS patients as well as the association of human MEGF8 with psychiatric disorders like schizophrenia, point towards a potential role of Megf8 in synaptic processes as many of the learning disabilities and psychiatric disorders have underlying defects in synaptic development and/or function (Giacopuzzi et al., 2017; Obi-Nagata et al., 2019; Valnegri et al., 2012).

The *Drosophila* neuromuscular junctions (NMJ) have served as an ideal model for studying synapse development and functions (Wu et al., 2010), and have similarities with the mammalian central synapses. Since dMegf8 shows high amino acid sequence and domain homology with vertebrate *Megf8* (Lloyd et al., 2018), studies on dMegf8 will be informative about the role of vertebrate Megf8 proteins. While not much is known about *dMegf8*, several genome-wide RNAi-screens found that *dMegf8* knockdown resulted in cell death or reduced cell viability phenotype (Chittaranjan et al., 2009; Mummery-Widmer et al., 2009). In a machine-learning study, *dMegf8* was found to be one of the putative synaptic genes indicating a potential involvement of dMegf8 in synapse assembly and function (Pazos Obregon et al., 2015). A recent report showed that *dMegf8* mutants have disrupted larval denticle belts and bristle formation, and a delayed transition to 3rd instar larvae associated with larval lethality (Lloyd et al., 2018). There are no published reports on any role of Megf8 in synapse organization or function in any species.

Here we report the generation of *dMegf*8 null mutants and show that *dMegf8* is expressed both pre- and post-synaptically. dMegf8 is required for synaptic growth and loss of *dMegf8* leads to synaptic ultrastructural defects and reduced synaptic transmission. Additionally, genetic rescue experiments support that *dMegf8* is necessary pre-synaptically for proper NMJ growth. However, synaptic ultrastructural

defects resulting from dMegf8 loss are significantly rescued by dMegf8 pre- and/or post-synaptic expression. Importantly, *dMegf8* shows genetic interactions with *Drosophila neurexin* (*dnrx*) and the BMP receptor *wishful thinking* (*wit*), and these proteins exist in a biochemical complex to coordinate proper NMJ development. Together, our studies establish the role of *dMegf8* in NMJ synaptic development and function, and provide key insights into potential *Megf8* functions in vertebrate synaptic mechanisms that underlie intellectual disabilities seen in CS patients.

Materials and Methods

Fly stocks

The *Drosophila* lines used in this study include *w1118* Canton-S line (used as wild type control; kind gift from Dr. Vivian Budnik), $dMegf8^{\Delta 8}$ (Lloyd et al., 2018), $dnrx^{273}$, *UAS-dnrx* (Li et al., 2007), *UAS-wit-GFP* (a gift from Dr. Michael O'Connor). To generate *UAS-dMegf8* flies, we obtained a partial *dMegf8* cDNA clone from DGRC (clone # *LD09511*) that encodes a polypeptide containing only the C-terminal amino acids 2089 to 2892 of the full-length dMegf8RB protein. The remaining 2088 N-terminal amino acids were obtained by RT PCR using PolyA+ mRNA isolated from adult fly brains. A full-length clone was assembled containing 10201 nucleotides representing the largest dMegf8 isoform (*dMegf8 RB*), which was confirmed by sequencing and cloned into *pUAS-attB* vector to create transgenic flies. All other fly stocks including *Df(2L)7147,* wit^{A12} *and* wit^{B11} and all *Gal4* lines were obtained from the Bloomington Drosophila Stock Center, Indiana. All flies were maintained at 25^{o}C, 50% humidity and with a 12-hour light/dark cycle.

Generation of *dMegf8* mutants

To generate a targeted deletion in the *dMegf8* locus that would produce a *dMegf8* null allele, we used CRISPR/Cas9 methodology and procedures established at the GenetiVision company based on the previously reported strategies (Zhang et al., 2014). The targeting construct contained 5' and 3' homology arms flanking a GFP expression cassette that replaced the deleted sequences in the *dMegf8* locus. The targeting construct was injected into embryos and the transgenic flies were tested by PCR analysis using a combination of primers that distinguished the wild type and *dMegf8* null alleles (primer 1- 5'-GCACGCTTCAGGTAAGTCGTA-3', primer 2- 5'-GCGGCTTGTGATCCGTAACCT-3' and primer 3- 5'-GATGGGACAAGTCGCCATGT T-3'). Further characterization of the *dMegf8* alleles used standard methodologies.

Larval Locomotion Assays

The larval locomotion assay was performed as described previously (Banerjee and Riordan, 2018). Briefly, larvae were first washed in distilled water to remove any traces of food before performing various assays. Each larva was acclimatized to the test plate for 1 minute prior to testing. 5 trials per larvae were conducted and the total number of larvae analyzed per genotype was 50.

For the *grid crossing assay* individual larvae were placed in the center of a 145-mm diameter Petri Dish, with 2% non-nutritious agar previously poured and allowed to harden covering a graph paper at the bottom with 0.5 cm^2 marked grids. The number of grid line crossings within a 30 second time window was recorded five times per larva.

For the *central zone release assay* a circular white card 1.5 cm in diameter was taped

to the bottom of the dish to mark the central release zone. 5 animals were placed at the center of the release zone. The time taken for each animal to exit the release zone was recorded.

For the *peristalsis contraction assay* full body peristalsis contractions (full posterior to anterior movement = 1 contraction) were counted for each larva in one minute while observing under a dissection microscope.

For the *righting assay* larvae were turned on their dorsal surfaces with a fine brush and the time taken to return to their ventral crawling position was recorded.

Adult Locomotion Assay

To determine the locomotor ability of the flies, adult climbing assay was performed as described previously (Gargano et al., 2005; Xie et al., 2021). Briefly, 10 freshly eclosed male flies were collected in individual vials and a total of 50 flies for each genotype were analyzed. The assay was started 24 h after CO_2 anesthesia. Flies were gently tapped down to the bottom of an empty clear vial and the number of flies crossing a 10 cm mark drawn from the base of the vial within 10 seconds were recorded. Each assay was repeated 6 times with a recovery time of 1 min in between, and the mean was calculated. The climbing ability is shown as the percentage of mean number of flies crossing the mark.

Production of dMegf8 Antibodies

To generate antibodies against the dMegf8 protein, a partial cDNA clone of *dMegf8* that contained 2/3rd of the coding sequences of dMegf8 from the 3'-end was obtained from the *DGRC Center* and further confirmed by DNA sequencing. A portion of the dMegf8 was subcloned (cDNA nucleotide numbers 6706 to 7323) into *pET28 a(+)*

vector and expressed in *E. coli BL21DE3* followed by His-column affinity purification. The recombinant polypeptide was used as an antigen to generate rabbit and guinea pig polyclonal antibodies, which were further tested by immunostaining and immunoblotting methods.

Immunohistochemistry

Wandering third-instar larvae from various genotypes were dissected and fixed in either Bouin's fixative or 4% paraformaldehyde for 15 minutes and processed as previously described (Banerjee et al., 2017; Chen et al., 2012). Dnrx signal at NMJ was enhanced using previously described protocols (Li et al., 2007). Primary antibodies used were rabbit anti-dMegf8 (1:500, this study), FITC-conjugated anti-HRP (1:250, Jackson ImmunoResearch laboratories), mouse anti-GluRIIA (1:250), (Chen et al., 2012; Marrus et al., 2004), guinea pig anti-Dnrx (1:250) (Li et al., 2007), rabbit anti-PS1 (p-Mad) (1:500; a gift from P. ten Dijke), rabbit anti-Smad (1:200, ab52903, Abcam). Anti-PS1 recognizes pMad at the NMJ and anti-Smad antibody, which is a recombinant anti-Smad3 [phospho S423 + S425], recognizes pMad in the VNC as well as detects pMad on immunoblots (Banerjee and Riordan, 2018; Banerjee et al., 2017). Mouse monoclonal anti-Dlg (1:500, 4F3), anti-BRP (1:250; NC82) and anti-Wit (1:25, 23C7) were obtained from Developmental Studies Hybridoma Bank (DSHB), University of Iowa. Secondary antibodies conjugated to Alexa 488, 568 and 647 (Invitrogen-Molecular Probes) were used at 1:400 dilution. Confocal images of all dissected larval tissues belonging to the same experimental group were imaged under identical settings with a Zeiss LSM710 confocal microscope and all image processing was done using Adobe Photoshop software.

Immunoblotting and Immunoprecipitations

The immunoblotting and immunoprecipitation (IP) experiments were performed as previously described (Banerjee et al., 2017). Briefly, for immunoblotting of dMegf8, fly heads of desired genotypes were homogenized using a glass homogenizer in a weight/volume ratio of 1:3 in ice-cold lysis buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40 and 0.5% Deoxycholate with protease inhibitors. The lysates were kept on ice for 10 min and centrifuged at 20,000xg for 15 min at 4°C. The resultant supernatant was centrifuged at 100,000×g for 30 min at 4°C. The membrane pellet was further solubilized in 1×PBS and used subsequently for IP and immunoblot analyses. For the immunoblotting of 3rd instar larval musculature or VNC and brain lobes without any attached imaginal discs, tissues were homogenized in ice-cold RIPA buffer. The supernatants with equal amounts of proteins from each genotype were separated on SDS-PAGE followed by immunoblotting with appropriate antibodies. For IP studies, fly heads of the appropriate genotypes were processed according to previously described protocols (Banerjee et al., 2017). Each experiment was performed independently three times and the most representative blots are presented. Primary antibodies used for immunoblotting were anti-dMegf8 (1:1000), anti-Dnrx (1:250) (Li et al., 2007), anti-Wit (1:1000, DSHB), anti-Smad (1:150, Abcam), anti-Trio (1:250, DSHB) and anti-β Actin (1:10,000, 4967S, Cell Signaling).

Electron Microscopy and Morphometric Analysis

Ultrastructural analyses of third-instar larval NMJs were processed for TEM as previously described (Banerjee et al., 2017). Briefly, third-instar larval fillets were dissected in ice-cold, Jan's 0.1mM Ca^{2+} saline, pH 7.2, and subsequently fixed in 4%

paraformaldehyde/1% glutaraldehyde/0.1 M cacodylic acid, pH 7.2 for 30 minutes at room temperature followed by overnight fixation at 4°C. The fixed fillets were rinsed 3X in 0.1M cacodylic acid, pH 7.2 and postfixed in 2% aqueous osmium tetroxide for 1 hour, followed by rinsing and dehydration in increasing ethanol concentrations. Samples were incubated for an hour in propylene oxide and gradually infiltrated in increasing resin to propylene oxide ratio (1:2, overnight; 2:1, at least 6 hours; and full resin for 36 hours with constant agitation). Samples were embedded in flat silicone molds with Polybed resin and cured in the oven at 55°C for at least 36 hours. 5 larvae were processed for EM analysis from each of the genotypes. The number of boutons (n) analyzed for each genotype is indicated in respective figure legends. Image J was used for morphometric analysis of EM images of only Type Ib boutons from A2 and A3 as previously described (Banerjee et al., 2017; Chen et al., 2012).

Electrophysiology

Electrophysiological analysis of larval NMJ was performed as previously described (Shi et al., 2019). All third instar larvae were grown in an incubator at 25^{o}C (65% humidity). All electrophysiological experiments were performed at room temperature and all recordings were made only from abdominal segment A3, muscle 6 of third-instar larvae, in HL-3 solution (0.5mM Ca^{2+} and 20mM $MgCl_2$) (Stewart et al., 1994). Electrophysiological signals were amplified with an Axoclamp 900A, under the control of Clampex 10 (Molecular Devices). Data were collected only when resting potential was below -63mV. Excitatory junction potentials (EJPs) were evoked by applying currents of 6 $\pm$ 3 mA with fixed stimulus duration at 0.3 ms with 0.2 Hz of stimuli rate. Twenty evoked EJPs were recorded for each muscle for analysis. Miniature EJP (mEJP) events were collected for 2 min. The evoked EJP amplitude

was corrected by using non-linear summation (Feeney et al., 1998). The quantal content was calculated from individual muscles by ratio of the averaged EJP and averaged mEJP amplitudes. Statistical analyses of EJP and mEJPs between genotypes were done using Student's t-test (Sigma- Plot 10.0, Systat Software). Error bars represent mean ± SEM (****p < 0.0001; ns − not significant).

Quantification

Bouton number quantifications (n = number of larvae analyzed) were performed from muscles 6/7 of abdominal segment 3 (A3) by staining of the body wall musculature preparations with anti-HRP and anti-Dlg. Fluorescence intensity measurements for dMegf8, Dnrx, Wit and pMad were quantified from confocal slices of Z-stack images compressed using maximum projection functions, which were stained in combination with either NC82 or anti-Hrp antibodies. Same regions of interest were selected for each channel and used for assessment and quantification of fluorescence intensity using Image J. At least 30 NMJ branches from 8 larvae were analyzed for various genotypes. All genotypes listed under the same quantification groups were stained and processed for imaging and quantified under identical parameters and settings. Image J (NIH, USA) was used for quantification of band intensities of immunoblots from three independent experiments. The intensity of the bands of interest was divided by their respective Actin protein blots to control for any possible unequal loading.

Experimental Design and Statistical Analysis

All experiments described above, including microscopy, image processing, and quantification were performed by trained researchers. All experiments used both

sexes, except for adult locomotion assay, in which only male flies were used to exclude the influence of female's oviposition. Larvae and adult flies from the control and experimental groups were reared in the same media and maintained at the same temperature (25^0C) and humidity (50%) and were processed identically.

All statistical analyses were performed using the GraphPad PRISM software and data are presented as mean ± SEM. Statistical significance was determined by one way ANOVA followed by post hoc Tukey's multiple comparison test and Student's t-test. Specifically, the adult locomotion assay used two-way ANOVA as there were two independent variables (age and climbing index, Fig. 1J). Error bars represent mean ± SEM (****$p < 0.0001$, ***$p < 0.001$, **$p \leq 0.01$, *$p \leq 0.05$, ns – not significant). Wherever possible exact p values are provided in figure legends. For Fig.1F, we analyzed the data using Welch's student t-test, assuming unequal variances and unequal sample sizes. For Fig. 4G, instead of using normal one-way ANOVA, we used Brown-Forsythe and Welch ANOVA, which takes care of unequal sample size issues. Additional information about each statistical test including degrees of freedom and other statistic-specific values are included in the figure legends. For all quantifications, the statistical significance immediately above the bars is with respect to the control genotype for that experimental group.

Results

Generation of *dMegf8* null mutants

The primary amino acid sequence of human MEGF8 protein revealed it as a transmembrane protein with a complex domain structure in its extracellular region including CUB domains, multiple EGF repeats, KELCH domains and laminin-EGF-like domains (Twigg et al., 2012). The mouse Megf8 protein also

revealed a conserved domain structure similar to the human MEGF8 (Engelhard et al., 2013). The *Drosophila* Megf8, referred as dMegf8 (Lloyd et al., 2018) also revealed a complex domain structure with similar domains as the vertebrate Megf8 with some variations, but is the closest homolog of the vertebrate Megf8 family (Fig. 1A). dMegf8 shares more than 2,400 amino acid sequence homology with human MEGF8 and close to 33% amino acid identity (Lloyd et al., 2018). In our studies, the *dMegf8* locus was identified in an ethyl methane sulphonate (EMS) chemical mutagenesis screen that was designed to uncover behavioral mutants with motor coordination deficits (*Venkatesan and Bhat, unpublished data*). To explore the *in vivo* functions of *dMegf8*, and whether it has a role in neuronal and synaptic functions, we proceeded to generate a *dMegf8* null allele. The *dMegf8* locus (Fig. 1B) is composed of 6 exons with the ATG codon in exon 1 and the termination codon in exon 6. We used the *CRISPR-Cas9* gene editing strategy (Zhang et al., 2014) and inserted a *GFP* cassette to replace approximately 4062 base pairs (bp) starting from nucleotide sequence 5'-AAGCTATGGGGTCTG-3' in exon 3 and ending in nucleotide sequence 5'-GATCGAATGCCTCTT-3' in exon 4 (*for details see* Fig. 1B). We carried out PCR amplification using fly genomic DNA with a primer combination that distinguished the wild type allele (Primers 1+2= 407 bp) (Fig. 1B, C) from the *dMegf8* mutant allele (Primers 1+3= 505 bp) (Fig. 1B, D). The PCR amplification confirmed the deletion of the sequences and this *dMegf8* allele is referred as $dMegf8^{HSC}$. Next, we generated polyclonal antibodies against dMegf8 polypeptide to determine the relative molecular weight of dMegf8 and also to establish that $dMegf8^{HSC}$ is indeed a protein null allele. We also generated *UAS-dMegf8* transgenic flies to express dMegf8 using cell-specific *Gal4* drivers. We performed immunoblot analysis of adult head lysates from wild type (+/+), $dMegf8^{HSC}$ and *elav-Gal4;UAS-dMegf8 (elav>dMegf8)* using

anti-dMegf8 antibodies. As shown in Fig. 1E, wild type and *elav>dMegf8* lysates showed three major protein bands ranging from 250 kDa to higher than 300 kDa which were absent from $dMegf8^{HSC}$ head lysates confirming the specificity of the dMegf8 antibodies and $dMegf8^{HSC}$ as a protein null allele. Using pan-neuronal expression of *dMefg8* (*elav>dMegf8*), the dMegf8 protein is expressed at higher levels than in the wild type control adult heads indicating overexpression from the *dMegf8* transgene. The $dMegf8^{HSC}$ null allele reported here showed no significant embryonic lethality and only had <10% larva to adult lethality. We next assayed 3rd instar larvae for various larval behavioral and coordination defects that included general motor activity using grid crossing assay (Fig. 1F), central zone release assay (Fig. 1G), peristalsis assay which analyses the muscle contraction waves (Fig. 1H) and larval righting assay (Fig. 1I). In all these assays $dMegf8^{HSC}$ larvae showed significant deficits compared to wild type control larvae. We also carried out adult locomotor assay that uses negative geotaxis (Gargano et al., 2005), as part of motor coordination and climbing activity. $dMegf8^{HSC}$ adult flies showed a significant weakness in their climbing activity which declined progressively as flies aged (Fig 1J) indicating that loss of dMegf8 leads to severe motor deficits in 3rd instar larvae as well as adults. Another *dMegf8* null allele ($dMegf8^{\Delta 8}$) was reported previously which showed larval lethality with denticle belt formation defects (Lloyd et al., 2018). It is not known whether variable genetic background contributes to early developmental lethality or whether there are any second site mutations associated with the $dMegf8^{\Delta 8}$ allele (Lloyd et al., 2018). Together, these data show that $dMegf8^{HSC}$ is indeed a null allele that is viable and that loss of dMegf8 is associated with severe motor deficits in both the larvae and adult flies.

dMegf8 Localizes to Synaptic Terminals and is Required for Synaptic Growth

Since mutations in human *MEGF8* have been associated with developmental malformations (Giacopuzzi et al., 2017; Pan et al., 2014; Twigg et al., 2012), and the murine *Megf8* null allele showed developmental malformations and embryonic lethality (Engelhard et al., 2013), we wanted to examine where dMegf8 was expressed and whether loss of dMegf8 had any consequences on synaptic development at the NMJ, as these synapses are generally considered to have a functional relevance to mammalian glutamatergic central synapses (Ruiz-Canada and Budnik, 2006; Xue et al., 2009). We first examined the endogenous expression of dMegf8 at the 3rd instar larval NMJs by carrying out immunostaining for dMegf8 in combination with antibodies against the presynaptic active zone protein, Bruchpilot (Brp). We found that dMegf8 localized to the larval NMJ synapses in wild type (Fig. 2A-A") when labeled with anti-dMegf8 (Fig. 2A, A") with respect to Brp (Fig. 2A', A") (Wagh et al., 2006; Weyhersmuller et al., 2011). dMegf8 was not detected in the larval NMJ synapses in *dMegf8*^{HSC} mutants (Fig. 2B, B"), which were clearly highlighted by Brp immunostaining (Fig. 2B', B") indicating that dMegf8 localizes to larval NMJ synapses. We next investigated the consequences of dMegf8 loss on the NMJ synaptic growth. We immunostained the larval NMJ of specified genotypes including several alleles of *dMegf8* (*dMegf8*^{F1} -from the EMS screen, *dMegf8*^{HSC}, and *dMegf8*$^{\Delta 8}$) (Lloyd et al., 2018) (Fig. 2C-K) using anti-HRP (green, Fig. 2C-J) to label neuronal membranes and anti-Discs large (Dlg) (red, Fig. 2C-J) to label type I boutons (Budnik et al., 1996; Lahey et al., 1994). Compared to wild type (Fig. 2C, quantified in 2K), all *dMegf8* mutant alleles showed synaptic undergrowth with fewer boutons (Fig. 2D-F, K). *dMegf8*^{F1} also showed reduced synaptic growth comparable to *dMegf8*^{HSC} (Fig. 2D, K). To ensure the *dMegf8*^{HSC} homozygous phenotype was not caused by any

other mutations in *cis*, we analyzed the *dMegf8*^{HSC} allele over a deficiency chromosome (*Df(2L)7147*) that uncovered the *dMegf8* locus. Mutant larvae of *dMegf8*^{HSC}*/Df(2L)7147* revealed NMJ growth phenotype that was not significantly different from *dMegf8*^{HSC}*/dMegf8*^{HSC} homozygous mutants (data not shown) indicating that *dMegf8*^{HSC} allele can be used for NMJ phenotypes in a homozygous state. While the *dMegf8*^{HSC} homozygous null flies were viable and fertile, an independently generated *dMegf8* allele, also thought to be null, was reported to be larval lethal (Lloyd et al., 2018). We utilized one of the reported *dMegf8* mutant alleles, *dMegf8*$^{\Delta 8}$ (Lloyd et al., 2018), to analyze the synaptic growth in trans-allelic combination with *dMegf8*^{HSC}. *dMegf8*^{HSC}*/dMegf8*$^{\Delta 8}$ larvae showed similar synaptic undergrowth as the *dMegf8*^{HSC} homozygous null mutants (Fig. 2F, K). Since *dMegf8*$^{\Delta 8}$ allele was also generated using *CRISPR/Cas9* deletion, it remains to be determined whether there is a second site mutation associated with this allele. Together these data show that dMegf8 is expressed at the larval NMJ and that loss of dMegf8 causes a significant reduction in bouton growth at the NMJ.

On the contrary, presynaptic expression of dMegf8 in *elav>dMegf8* larvae led to increased growth of synaptic boutons (Fig. 2G, K) suggesting that dMegf8 overexpression caused enhanced bouton growth at the NMJ. Next, we wanted to assess if the reduced synaptic growth seen in *dMegf8*^{HSC} mutants could be rescued by pre- or post-synaptic expression of dMegf8. We found that presynaptic expression of dMegf8 in *dMegf8*^{HSC} mutants (*dMegf8*$^{HSC/HSC}$*;elav-Gal4/UAS-dMegf8*) restored synaptic growth to wild type levels (Fig. 2H, K), however, the postsynaptic expression of dMegf8 using *MHC-Gal4* (*MHC>dMegf8*) (Fig. 2I, K) did not show any changes in synaptic bouton growth compared to wild type (Fig. 2C, K). The postsynaptic expression of dMegf8 in *dMegf8*^{HSC} mutants (*dMegf8*$^{HSC/HSC}$; *MHC-Gal4>dMegf8*)

failed to rescue the reduced bouton growth in *dMegf8*^{HSC} mutants (Fig. 2I, K). These data show that pre-synaptic dMegf8 expression is able to promote synaptic growth at NMJ and that the synaptic growth function of dMegf8 is mostly presynaptic as the postsynaptic expression neither promoted nor rescued the synaptic growth at the NMJ.

Loss of dMegf8 Affects the Distribution of Pre- and Postsynaptic Proteins

Since NMJ bouton growth was affected in *dMegf8* mutants, we wanted to determine whether loss of dMegf8 would have any consequences on the proper assembly of pre- and post-synaptic proteins which display a stereotypic localization at the NMJ synapses with respect to assembly and distribution. We first studied the localization of the well characterized active zone protein, Brp (Wagh et al., 2006) (green, Fig. 3A, A" and 3B, B") with respect to the postsynaptic glutamate receptor subunit, GluRIIA (Marrus et al., 2004) (red, 3A', A" and 3B', B") in wild type (3A-A") and *dMegf8*^{HSC} mutant (3B', B") larval NMJ. In wild type larvae (Fig. 3A-A"), active zones labeled with anti-Brp (Fig. 3A, A") were juxtaposed to the GluRIIA punctae (Fig. 3A', A") which was similar in *dMegf8*^{HSC} mutant synapses (Fig. 3B-B") indicating that the alignment of the pre-synaptic BRP and post-synaptic GluRIIA was not significantly affected. Next, we analyzed the number of Brp puncta in the wild type (Fig. 3A-A") and *dMegf8*^{HSC} mutants (Fig. 3B-B") to determine whether there were any differences in the number of active zones per bouton area. *dMegf8*^{HSC} mutants showed a significant increase in the number of Brp-positive puncta as quantified and normalized to the bouton area in *dMegf8*^{HSC} mutants compared to wild type (Fig. 3C). GluRIIA puncta also showed similar increase as the Brp puncta in *dMegf8* mutants compared to the wild type (data not shown). These data indicate that there is increase in the number of Brp-positive

active zones in *dMegf8*^{HSC} mutant NMJ synapses.

Next, we determined if *dMegf8*^{HSC} mutants caused any aberrations in the organization or differentiation of postsynaptic specializations. We studied the localization of Discs large (Dlg, red, Fig. 3D-E'), which has been shown to function both pre- and post-synaptically in the proper assembly of the subsynaptic reticulum (SSR) in Type Ib boutons (Budnik et al., 1996; Lahey et al., 1994) together with the neuronal membrane marker, Hrp (green, Fig. 3D', E'). In wild type (Fig. 3D, D'), Dlg localization is typically in the periphery of the bouton circumference and mostly excluded from the core of the boutons. However, in *dMegf8*^{HSC} mutants (Fig. 3E, E'), there was a more diffuse Dlg distribution throughout the bouton and there was no clear separation in the pre-synaptic HRP areas, as is observed in the wild type boutons (Fig. 3D, D') suggesting that loss of dMegf8 affected the localization of Dlg at the NMJ. Next, we tested whether the mislocalization of Dlg in the *dMegf8*^{HSC} mutant boutons would be rescued by pre- or post-synaptic expression of dMegf8. As shown in Fig. 3F and at a higher magnification 3F', *elav>dMegf8;dMegf8*^{HSC} NMJs showed improved Dlg localization that was more in the bouton perimeter similar to the wild type boutons (Fig. 3D) than that was displayed by *MHC>dMegf8;dMegf8*^{HSC} NMJs (Fig. 3G, higher magnification 3G') indicating that pre-synaptic expression of dMegf8 restored Dlg localization better than the post-synaptic dMegf8 expression. Together, these data show dMegf8 is required for proper pre- and post-synaptic protein assembly and distribution at the NMJs, and that pre-synaptic expression was able to restore Dlg localization at the NMJ compared to *dMegf8*^{HSC} mutants.

Pre- and post-synaptic ultrastructural abnormalities in *dMegf8* mutants

The reduced synaptic growth and altered subcellular localization of Dlg observed in

485 *dMegf8* mutants led us to analyze the ultrastructure of the synaptic boutons in these
486 mutants to examine the organization of the overall synaptic architecture and how that
487 compared with wild type controls. The wild type boutons are characterized by
488 morphologically distinct and closely apposed pre- and postsynaptic membranes (Fig.
489 4A). The presynaptic compartment also contains synaptic vesicles, apart from
490 organelles such as mitochondria. The presynaptic membrane has active zones that
491 are composed of electron dense structures called T-bars (Fig. 4A) (Wagh et al., 2006).
492 The type Ib boutons at the postsynaptic muscle are surrounded by elaborate
493 membrane invaginations, the SSR, which occupies a large area on the post-synaptic
494 side (Fig. 4A, B) (Budnik et al., 1996; Jia et al., 1993). We performed serial sectioning
495 of boutons from wild type, $dMegf8^{F1}$ and $dMegf8^{HSC}$ mutants and subjected them to
496 morphometric analyses to determine any pre- and/or post-synaptic defects. In
497 addition, we also assessed if any of the synaptic defects displayed by $dMegf8^{HSC}$
498 mutants would be rescued by expressing *dMegf8* either pre- or post-synaptically.

499 We did not observe any significant changes in the overall area of the boutons
500 in $dMegf8^{HSC}$ (Fig. 4B, quantified in F) and $dMegf8^{F1}$ mutants (Fig. 4C, F) compared
501 to wild type (Fig. 4A, F) or any of the other genotypes analyzed (Fig. 4D-E, F). It is
502 important to note that although many d$Megf8^{HSC}$ mutant boutons had larger bouton
503 areas but there was also a significant variability. The number of active zones (arrows)
504 and total PSD length (arrowheads) showed a significant increase in $dMegf8^{HSC}$ (Fig.
505 4B, quantified in G and H, respectively) and $dMegf8^{F1}$ mutants (Fig. 4C, G, H)
506 compared to wild type controls (Fig. 4A, G, H). Both increased active zone numbers
507 and total PSD length in $dMegf8^{HSC}$ mutants were rescued to wild type levels in the
508 pre-synaptic rescue as seen in *elav>dMegf8;*$dMegf8^{HSC}$ (Fig. 4D, G, H), while the
509 postsynaptic expression of *dMegf8* in $dMegf8^{HSC}$ mutants as seen in

MHC>dMegf8;dMegf8^{HSC} (Fig. 4E, G, H) did not show any changes in these parameters compared to *dMegf8*^{HSC} mutants (Fig. 4B, G, H). Postsynaptic SSR morphology was severely compromised in *dMegf8*^{HSC} (Fig. 4B, B') and *dMegf8*^{F1} (Fig. 4C, C') with thinner folds compared to wild type controls (Fig. 4A, A'). Morphometric analysis of normalized SSR width (Fig. 4I) showed a significant reduction in *dMegf8*^{HSC} mutants compared to wild type controls (Fig. 4I). *dMegf8*^{F1} mutants also showed reduced normalized SSR width (Fig. 4I). Interestingly, reduction in the normalized SSR width was fully rescued when *dMegf8* was expressed post-synaptically in *dMegf8*^{HSC} mutants as seen in *MHC>dMegf8;dMegf8*^{HSC} (Fig. 4I) but not by the pre-synaptic expression of *dMefg8* as observed in *elav>dMegf8; dMegf8* ^{HSC} (Fig. 4I). Taken together, the EM and morphometric analyses indicate that loss of *dMegf8* leads to defective ultrastructural organization of both the pre- and postsynaptic areas at the NMJ and that dMegf8 functions both pre- and post-synaptically to organize the proper synaptic apparatus.

Synaptic transmission is reduced in *dMegf8* mutants

As shown in the preceding sections, loss of dMegf8 results in reduced synaptic growth at the larval NMJ (Fig. 2) and causes ultrastructural abnormalities at the synapse (Fig. 4). We next examined the consequences of loss of dMegf8 on synaptic transmission at the NMJs, as well as whether pre- or post-synaptic expression of *dMegf8* in *dMegf8* mutants will restore the synaptic transmission in *dMegf8* mutants. We performed electrophysiological analyses on muscle 6 of third-instar larval body walls of all relevant genotypes and recorded the EJPs in 0.5 mM $[Ca^{2+}]_o$ at 0.2 Hz under identical conditions (Feeney et al., 1998; Shi et al., 2019). Representative EJP graphs from the control (*wCS*), (Fig. 5A), *dMegf8*^{HSC} mutants (Fig. 5B), pre-synaptic

dMegf8 expression in *elav>dMegf8;dMegf8^{HSC}* mutants (Fig. 5C) and post-synaptic dMegf8 expression in *MHC>dMegf8;dMegf8^{HSC}* mutants (Fig. 5D). *dMegf8^{HSC}* mutants exhibited a reduction in EJP amplitude (Fig. 5B, quantified in Fig. 5E). The pre-synaptic expression of dMegf8 was able to fully rescue the EJP amplitude of *dMegf8^{HSC}* mutants (Fig. 5C, E), whereas the pos-synaptic *dMegf8* expression did not rescue the EJP amplitude in *dMegf8^{HSC}* mutants (Fig. 5D, E). Interestingly, the mEJP amplitudes did not show any significant differences in *dMegf8^{HSC}* mutants, in *elav>dMegf8;dMegf8^{HSC}* mutants and in *MHC>dMegf8;dMegf8^{HSC}* mutants compared to *wCS* controls suggesting that the synaptic vesicle contents were not altered by loss of dMegf8 (Fig. 5F). *dMegf8^{HSC}* mutants and post-synaptic *dMegf8* expression in *MHC>dMegf8;dMegf8^{HSC}* mutants revealed severely decreased quantal contents compared with *wCS* controls, and the pre-synaptic *dMegf8* expression in *elav>dMegf8;dMegf8^{HSC}* mutants was similar to *wCS* controls (Fig. 5G). The paired pulse ratio (Fig. 5H) and the mEJP frequency (Fig. 5I) in *dMegf8^{HSC}* mutants, pre-synaptic *dMegf8* expression in *elav>dMegf8;dMegf8^{HSC}* mutants and post-synaptic *dMegf8* expression in *MHC>dMegf8;dMegf8^{HSC}* mutants did not show any significant differences compared to *wCS* controls. Together our data show that dMegf8 functions pre-synaptically for synaptic transmission at the NMJs.

Synaptic Proteins Regulating NMJ Growth and Structure show reduced expression in *dMegf8* Mutants

Since dMegf8 is a transmembrane protein and dMegf8 mutants display synaptic growth defects as well as deficits in synaptic ultrastructure and synaptic transmission, we next wanted to examine whether dMegf8 has any association or interactions with proteins that regulate synaptic growth and architecture. Synaptic transmembrane

proteins are critical for trans-synaptic adhesion and signaling for proper synaptic organization and function (Banerjee and Riordan, 2018; Banerjee et al., 2017; Sun and Xie, 2012). Since most phenotypes of *dMegf8*^{HSC} mutants were rescued pre-synaptically, *dMegf8* likely functions in association with other pre-synaptic proteins to coordinate synaptic growth and organization. Two key proteins in the synaptic machinery that are well known in regulating synaptic growth, organization and function are the trans-membrane proteins, *Drosophila neurexin* (*Dnrx*) (Li et al., 2007; Zeng et al., 2007) and the type II receptor of the BMP signaling pathway *wishful thinking* (*wit*) (Aberle et al., 2002; Banerjee and Riordan, 2018; Banerjee et al., 2017; Guangming et al., 2020; McCabe et al., 2003). We next set out to investigate if dMegf8 coordinates synaptic growth with Dnrx and/or Wit and might be part of a synaptic membrane protein complex. We first wanted to test whether the endogenous dMegf8 localization was affected in larval NMJ of *dnrx* and *wit* mutants. Similar to data presented in Fig. 2, endogenous dMegf8 (green, Fig. 6A-D") localized to the NMJ synaptic terminals in wild type (Fig. 6A, A") and absent in *dMegf8*^{HSC} mutants (Fig. 6B, B"). Compared to the wild type dMegf8 localization (Fig. 6A, A"), the dMegf8 localization in *dnrx* (Fig. 6C, C") and *wit* mutants (Fig. 6D, D") was diffuse with a significant decrease in the fluorescence intensity levels in both *dnrx* and *wit* mutants (quantified in Fig. 6J). Brp was used as a pre-synaptic marker (red, Fig. 6A', A", B', B", C', C", D', D"). Next, we wanted to examine any alterations in the localization and/or fluorescence intensities of Dnrx and Wit in *dMegf8*^{HSC} mutants by immunolocalization of Dnrx in wild type, *dMegf8*^{HSC} mutants and *dnrx* mutants NMJs. We observed that Dnrx localization in *dMegf8*^{HSC} mutants (Fig. 6F, F') was also significantly reduced compared to wild type controls (Fig. 6E, E'), quantified in Fig. 6K. We also tested for Wit localization, however, the endogenous Wit levels in the larval NMJs were

undetectable using standard immunohistochemistry protocols with anti-Wit antibodies as reported previously (Banerjee et al., 2017; Nahm et al., 2013). Therefore, in order to study Wit localization, we followed a strategy of overexpressing Wit pre-synaptically using the *elav-Gal4* driver in wild type (*elav-Gal4; UAS-wit*, Fig. 6H, H') and in $dMegf8^{HSC}$ mutant background (*elav-Gal4/UAS-wit;*$dMegf8^{HSC}$, Fig. 6I, I'). The expression of Wit in *elav-Gal4;UAS-wit;dMegf8* NMJs (red, Fig. 6I, I') was dramatically reduced compared to Wit expression in *elav-Gal4; UAS-wit* (red, Fig. 6H, H', quantified in 6L) in the wild-type background. The immunolocalization and levels of *dnrx* and *wit* were not significantly different between $dMegf8^{HSC}/dMegf8^{HSC}$ homozygous mutants when compared to $dMegf8^{\Delta 8}/dMegf8^{HSC}$ trans-allelic mutants (data not shown). Together these data demonstrate that dMegf8 is necessary for the proper localization and clustering of pre-synaptic Dnrx and Wit, and that these proteins may be interdependent for their proper assembly at the NMJ synaptic terminals.

dMegf8 mutants show normal levels pMad and Trio, and _dMegf8_ shows genetic interactions with _dnrx_ and _wit_

Since Wit localization was significantly reduced by loss of dMegf8, we next determined whether any downstream effectors of the BMP signaling pathway would also get impacted by loss of dMegf8. At the *Drosophila* NMJ, BMP signaling is essential for synaptic growth and homeostasis (Bayat et al., 2011). In retrograde BMP signaling pathway, the ligand glass bottom boat (Gbb) from postsynaptic muscles binds to presynaptic type I receptors Thickveins (Tkv), Saxophone (Sax) and type II receptor Wit leading to increased phosphorylation of BMP transcription factor, Mothers against dpp (Mad), and its subsequent accumulation in the nucleus (Aberle

et al., 2002; Ball et al., 2010; Dudu et al., 2006; Marques et al., 2002; McCabe et al., 2003). pMad binds directly to *Trio* promoter and enhance the transcription of *Trio*. Trio activates other downstream effectors in the neuronal soma or at the synapses leading to alterations in the actin cytoskeleton and regulating synaptic growth (Awasaki et al., 2000; Ball et al., 2010). Immunolabeling of NMJs with anti-pMad (PS-1, red) and Hrp (green) in wild type (Fig. 7A, A') and *dMegf8*^{HSC} mutants (Fig. 7B, B') did not show any difference in fluorescence intensities of pMad (quantification in Fig. 7E). Similarly, pMad (SMAD, red) fluorescence levels in wild type (Fig. 7C, quantified in 7F) and *dMegf8*^{HSC} mutant ventral nerve cord (VNC) (Fig. 7D, quantified in 7F) also did not show any significant differences. Total levels of pMad (Smad) analyzed by immunoblots (Fig. 7G, quantified in 7I) from VNC of wild type and *dMegf8*^{HSC} mutants showed no significant difference while pMad levels in *wit-/-* showed severe reduction as reported before (Fig. 7G, I) (Banerjee et al. 2017). Trio levels assessed by immunoblotting analysis of VNC also showed no significant difference between wild type and *dMegf8*^{HSC} mutants (Fig. 7H, quantified in 7J). These data indicate that loss of dMegf8 does not impact the levels of downstream BMP effectors, pMad and Trio.

Given the interdependency of dMegf8, Dnrx and Wit localization as shown in Fig. 6 and a common synaptic undergrowth phenotype seen in *dMegf8*, *dnrx* and *wit* mutants (Fig. 2) (Aberle et al., 2002; Banerjee et al., 2017; Li et al., 2007; Marques et al., 2002), we were interested in determining whether *dMegf8, dnrx* and *wit* displayed any genetic interactions to coordinate synaptic growth. Thus, for our genetic interaction studies, we used the synaptic undergrowth as our phenotypic readout and generated various genetic combinations of *dMegf8* and *dnrx* as well as *dMegf8* and *wit* (Fig. 7K-R). First, we examined the synaptic growth of *dMegf8*^{HSC}+/- (Fig. 7K, S) and *dnrx*+/- (Fig. 7S) heterozygotes in comparison with wild type and found no

significant differences (Fig. 7S). Next, we analyzed the double heterozygous combination of *dMegf8*^{HSC}*+/-; dnrx+/-* (Fig. 7L, S) and found a significant decrease in bouton numbers compared to the single heterozygotes of *dMegf8*^{HSC}*+/-* and *dnrx+/-* (Fig. 7S). We further compared the bouton numbers in single mutants of *dMegf8*^{HSC}*-/-* (Fig. 7S) and *dnrx-/-* (Fig. 7M, S) and found no significant differences between the two genotypes (Fig. 7S). Similarly, double mutants of *dMegf8*^{HSC}*-/-; dnrx-/-* (Fig. 7N, S) showed similar reduction in bouton growth when compared to *dMegf8*^{HSC}*-/-* or *dnrx-/-* single mutants (Fig. 7S). These data suggest that *dMegf8* and *dnrx* loss affects synaptic growth similarly and that they may function together in regulating NMJ synaptic growth.

We next tested genetic interactions between *dMegf8* and *wit* utilizing a similar approach as presented above. NMJ bouton counts of *dMegf8*^{HSC}*+/-* and *wit+/-* (Fig. 7O, T) showed no differences compared to wild type (Fig. 7T), while the double heterozygous combination of *dMegf8*^{HSC}*+/-;wit+/-* (Fig. 7P, T) displayed a significant reduction. Next, the single mutants of *dMegf8*^{HS}*-/-* and *wit-/-* (Fig. 7Q, T) showed undergrowth phenotypes similar to *dMef8*^{HSC}*-/-;wit-/-* double mutants (Fig. 7R, T). These data indicate that *dMegf8* and *wit* display genetic interactions and are involved in coordinating synaptic growth at NMJ. Together, our data show that *dMegf8*, *dnrx* and *wit* show genetic interactions and function in synaptic growth and organization at the NMJ synapses without severely impacting the BMP signaling pathway.

dMegf8, Dnrx and Wit Function in a Biochemical Complex

Given that the dMegf8 fluorescence intensity was reduced in *dnrx* and *wit* mutants and the localization of Dnrx and Wit were also affected in *dMegf8* mutants, we wanted to examine the total levels of these proteins in the mutant backgrounds of one

another (Fig. 8). In addition, since *dMegf8* displayed genetic interactions with *dnrx* and *wit*, we also wanted to determine if dMegf8, Dnrx and Wit existed as an *in vivo* biochemical complex and potentially function together. Therefore, we performed immunoblots and co-immunoprecipitations (co-IPs) to resolve the question whether they formed a molecular complex. Immunoblots were performed both from adult fly heads (Fig. 8A, B, D) and third instar larval musculature that contains NMJs (Fig. 8F, H), mostly because the expression levels of dMegf8 and Dnrx in the larval musculature were too low to be detected. All co-IP analyses were performed using fly head lysates due to large amount of protein needed for these experiments.

First, we examined the total levels of dMegf8 in the *dMegf8* point mutation allele isolated from the EMS screen, $dMefg8^{F1}$ (Fig. 8A, quantified in 8C). dMegf8 levels were reduced by ~25% in $dMefg8^{F1}$ mutants compared to wild type control (Fig. 8A, quantified in 8C). Immunoblotting analyses showed that dMegf8 levels (Fig. 8B, quantified in 8C) were not significantly affected in *dnrx* mutants (Fig. 8B, quantified in 8C). Likewise, Dnrx levels (Fig. 8D, quantified in 8E) levels were not significantly altered in *dMegf8* mutants compared to the wild type in the fly head lysate preparations indicating that in the adult head lysates the protein levels of dMegf8 and Dnrx were not affected in each other's mutants. We next tested the levels of Wit in wild type and loss- and gain-of *dMegf8* backgrounds using the larval musculature (Fig. 8F, H). The total levels of Wit were significantly decreased in $dMegf8^{HSC}$ mutants compared to wild type (Fig. 8F, quantified in 8G). Wit levels were significantly elevated in presynaptic *dMegf8* overexpression (Fig. 8F, quantified in 8G). Also, consistent with the finding that Wit fluorescence levels were reduced at NMJ (Fig. 6), total levels of Wit from lysates of Wit overexpression in *dMegf8* mutant background (e*lav-Gal4;UAS-wit;dMegf8-/-*) were also significantly decreased when compared to

Wit overexpression in the wild type background (Fig. 8H, quantified in 8I) indicating that dMegf8 loss led to a significant reduction in total Wit levels. Actin was used as loading control for all immunoblots (Fig. 8A, B, D, F, H).

For co-immunoprecipitation analyses, due to the extremely low level of dMegf8 expression, no association with dMegf8 and Dnrx/Wit was observed with co-IPs using antibodies to endogenous proteins at normal levels. To overcome this, we used adult head lysates from presynaptic *dMegf8* overexpression to test whether Dnrx and Wit would co-precipitate dMegf8. As shown in Fig. 8J, immunoblots of *dMegf8* show IP with Dnrx antibodies co-precipitated dMegf8 from dMegf8 overexpression lysates which was absent from *dnrx* mutant IPs confirming the association between dMegf8 and Dnrx. Co-IP with anti-Wit monoclonal antibodies also detected dMegf8 from dMegf8 overexpression lysates which was absent in the Protein-A bead controls (Fig 8K). IP using Dnrx and Wit antibodies efficiently precipitated Dnrx and Wit, respectively (data not shown) (Banerjee et al., 2017). Together, these results demonstrate that dMegf8, Dnrx and Wit exist *in vivo* as a molecular complex and may function together in synaptic organization and function.

Discussion

Many neurodevelopmental and psychiatric disorders have been associated with disturbances in synaptic organization and function (Batool et al., 2019; Nanou and Catterall, 2018; Parenti et al., 2020). Since Carpenter syndrome subjects show intellectual disabilities (Giacopuzzi et al., 2017; Taravath and Tonsgard, 1993; Twigg et al., 2012), it has remained unknown whether human MEGF8 will have a role in synaptic function. Our characterization of *dMegf8* in the neuromuscular synapses provides first evidence that Megf8 proteins have synaptic functions. Our findings

reveal that *dMegf8* mutants have multiple abnormalities in synaptic development, ultrastructural organization and physiological functions, and that dMegf8 forms a biochemical complex with two well-characterized synaptic proteins Dnrx and Wit. Our studies provide insights into possible human MEGF8 functions and lay the groundwork for further characterization of the mechanisms underlying the intellectual disabilities associated with the Carpenter Syndrome.

Megf8 in Synaptic Development and Function

Megf8 has been linked to various developmental abnormalities including congenital heart defects as well as neurodevelopmental and axon guidance defects with early lethality associated with the murine homozygous null allele (Engelhard et al., 2013; Kong et al., 2020; Wang et al., 2020). These phenotypes have pointed to defects in Hedgehog and BMP signaling pathways (Engelhard et al., 2013; Kong et al., 2020; Lloyd et al., 2018; Twigg et al., 2012; Wang et al., 2020). The $dMegf8^{HSC}$ mutants reported here produced viable and fertile adults with motor coordination deficits (Fig. 1), unlike the *dMegf8* null reported in Lloyd et al. (2018), showing a late larval lethality. Although both alleles were generated using CRISPR/Cas9 methodology, it remains to be seen whether genetic background or a second site mutation caused $dMegf8^{\Delta 8}$ lethality (Lloyd et al., 2018). Our studies provide evidence that dMegf8 plays an important role during *Drosophila* larval NMJ development as dMegf8 localizes in synaptic terminals both pre- and post-synaptically, and loss of *dMegf8* leads to synaptic undergrowth, while overexpression of *dMegf8* in neurons causes synaptic overgrowth pointing to a presynaptic dMegf8 requirement. The synaptic undergrowth in *dMegf8* mutants is fully rescued by pre- and not by the post-synaptic expression of *dMegf8*, further demonstrating that *dMegf8* functions primarily in the presynaptic

735 compartment.

736 While we did not see any disruption in the apposition of the presynaptic active
737 zone protein, BRP, with the postsynaptic GluRIIA, the number of BRP-positive
738 puncta/bouton area was significantly increased in *dMegf8* mutants, which was
739 consistent with the ultrastructural analyses revealing increased active zones in
740 *dMegf8* mutant synapses (see below). These findings are suggestive of a loss of
741 dMegf8 impacting the proper organization of synaptic active zones. *dMegf8* mutants
742 displayed diffuse Dlg distribution throughout the boutons unlike the peripheral rim of
743 the boutons where Dlg normally localizes. Pre-synaptic expression of dMegf8 in
744 $dMegf8^{HSC}$ mutants was able to restore Dlg localization better than the post-synaptic
745 expression of dMegf8. Dlg has been previously shown to function both pre- and
746 post-synaptically and loss of Dlg affects the SSR at the boutons (Budnik et al., 1996).
747 The synaptic structural abnormalities are often accompanied by corresponding
748 functional abnormalities. We observed significant reduction in the EJP amplitude and
749 the quantal content in *dMegf8* mutants which was restored by *dMegf8* pre-synaptic
750 and not by the post-synaptic expression. dMegf8 loss also affected the ultrastructural
751 organization of the synapses with specific defects in the pre-synaptic elements as
752 well as the post-synaptic specializations. Interestingly, restoration of dMegf8
753 expression either pre- or post-synaptically revealed that most functions of dMegf8 are
754 pre-synaptic except the SSR specialization which was rescued by dMegf8
755 post-synaptic expression suggesting that dMegf8 also has post-synaptic functions.
756 Our studies are consistent with many of the synaptic phenotypes previously observed
757 in mutations associated with genes involved in trans-synaptic functions, e.g., *dnrx,*
758 *dnlg1, dnlg2* and *wit* (Aberle et al., 2002; Banerjee et al., 2017; Banovic et al., 2010;
759 Chen et al., 2012; Li et al., 2007; McCabe et al., 2003) indicating that dMegf8 may be

involved or functions in close association with these proteins. Our data thus provide insights that vertebrate Megf8 may also play an essential role in synaptic function as human MEGF8 has been linked to neurodevelopmental and psychiatric disorders. As our studies highlight the synaptic role of dMegf8 at the NMJs, it is possible that dMegf8 also functions in proper axonal growth, guidance, maturation and fasciculation during neuronal development. As reported previously, CS patients present multiple developmental anomalies in addition to the intellectual disabilities (Engelhard et al., 2013), it is plausible that human MEGF8 might be involved in a plethora of functions in the developing nervous system ranging from neuronal survival, axon growth, guidance, maturation, fasciculation, target innervation and synapse formation. Future studies on dMegf8 will explore its functions in some of these neural development processes.

dMegf8 and BMP Signaling at the NMJ

The dMegf8 primary structure and its synaptic localization and the NMJ synaptic defects observed in *dMegf8* mutants suggested that it could potentially interact with other known synaptic proteins. Our immunohistochemical analyses showed that the absence of dMegf8 or Dnrx leads to their diffuse synaptic localization as well as protein instability in each other's mutant backgrounds (refer Fig. 6). However, the immunoblot analysis of dMegf8 or Dnrx did not show any variation in overall levels in each other's mutant backgrounds (Fig. 8) suggesting that despite the total protein levels remaining unchanged, dMegf8 and Dnrx fail to properly localize and cluster at the NMJ terminals and remain diffuse indicating that dMegf8 and Dnrx are mutually required for their proper synaptic localization. This is also reflected by the reduced levels of Wit in *dMegf8* mutants indicating that loss of dMegf8 affects Wit localization and/or its stability at the NMJ. Together these data underscore the important role that

dMegf8 plays in the proper localization of other synaptic proteins and suggest interdependency of dMegf8, Dnrx and Wit for their proper subcellular localization and/or stability at the NMJ.

Previously, Dnrx was shown to be essential for proper synaptic growth (Li et al., 2007; Sudhof, 2008), and regulation of the BMP signaling pathway in coordination with BMP type II receptor, Wit (Banerjee and Riordan, 2018; Banerjee et al., 2017). Interestingly, the murine Megf8 has also been shown to functions as a modifier of BMP4 signaling in the trigeminal ganglion neurons (Engelhard et al., 2013). These observations suggest that dMegf8 along with other proteins is involved in BMP signaling. This is further strengthened by our genetic analysis which revealed that *dMegf8* displays genetic interactions *dnrx* and *wit*, and that trans-heterozygous combinations of *dMegf8*^{HSC}*+/-;dnrx+/-* and *dMegf8*^{HSC}*+/-;wit+/-* displayed a significant reduction in bouton growth compared to the single heterozygotes supporting the possibility that these genes function together in a dose-dependent manner. Furthermore, double mutants of *dMegf8*^{HSC}*-/-;dnrx-/-* and *dMegf8*^{HSC}*-/-,wit-/-* showed no significant differences in the bouton counts compared to their single mutants. These findings strongly support that *dMegf8*, *dnrx* and *wit* function cooperatively to coordinate synaptic growth. Interestingly, however, there was no significant difference in pMad localization either in the VNC or at the NMJ or Trio levels in *dMegf8* mutants compared to wild type (Fig. 7). One of the possibilities might be that dMegf8 works with Dnrx and Wit in a large complex structurally to regulate synaptic development, but not participate directly in signaling functions as part of the BMP pathway (Fig. 9). Another possibility is, even though the proper localization and stability of Wit requires dMegf8, other BMP receptors such as Tkv and Sax could still function effectively to activate downstream signaling of the BMP pathway. Thus, dMegf8 may function

pre-synaptically to coordinate BMP signaling to ensure normal synaptic bouton growth and also post-synaptically to organize the SSR and other postsynaptic structures which are both necessary for proper synaptic function. A more detailed analyses of some of these questions will be addressed as part of our future investigations.

dMEGF8 Molecular Complex

Previous studies on Megf8 have not reported any biochemical interactions with other proteins that would link Megf8 to neuronal functions. Our biochemical analyses of dMegf8 showed that dMegf8, Dnrx and Wit exist in a large biochemical complex. While the overall levels of dMegf8 and Dnrx did not seem to change in each other's mutant backgrounds using adult head lysates, the levels of these proteins at the NMJ containing musculature are too low to detect any changes in their levels. Interestingly, the total levels of Wit using the musculature lysates were affected by the presence or absence of dMegf8, as pre-synaptic expression of dMegf8 increased Wit levels at the NMJ. As the endogenous dMegf8 levels were extremely low for the immunoprecipitation analysis, we used dMegf8 overexpression for immunoprecipitation which revealed that dMegf8, Dnrx and Wit form a large protein complex. How these proteins may interact with each other and what the stoichiometry of these interactions is remains to be fully investigated. Given the complex domain structure and large size of dMegf8, it is likely that dMegf8 interacts with a host of other pre- and post-synaptic proteins. It will be of significant interest to know what these proteins are and how they function in a macromolecular complex at the NMJ or other

synapses. Further elucidation of the dMegf8 genetic and molecular functions and the signaling complexes that dMegf8 engages in will uncover the relevant functions of the human MEGF8 that is associated with deficits seen in the Carpenter Syndrome or other psychiatric disorders.

FIGURE LEGENDS

Figure 1. Generation of *dMegf8* mutants.

(A) Protein domain structure of human MEGF8, mouse Megf8 and *Drosophila* dMegf8. The antibody region in dMegf8 is highlighted as the green bar.

(B) Genomic structure of *dMegf8* showing exons 1 to 5. The targeting construct using CRISPR/Cas9 for recombination and the final targeted allele of $dMegf8^{HSC}$ is shown. The blue boxes next to *loxP* sites on both sides represent phage C31 integration sites referred to as *attP* sites in the targeting vector. Location of the primers for genotyping that differentiate the wild type and the $dMegf8^{HSC}$ mutants are shown as red arrows with numbers.

(C, D) PCR confirmation of the targeted deletion using primer combinations 1+2 for wild type and 1+3 for $dMegf8^{HSC}$ mutants in (B).

(E) Immunoblot analysis of dMegf8 showing presence of dMegf8 in wild type, loss of dMeg8 in $dMegf8^{HSC}$ mutants and over-expression of dMegf8 in *elav>dMegf8*. The blot was probed for Actin as loading control.

(F-I) WT (+/+) and $dMegf8^{HSC}$ larval locomotor behaviors assayed by measuring the number of 0.5 cm^2 grids crossed in 30 seconds (F), time taken in seconds for larvae to exit a circle of 1.5 cm in diameter (G), number of full body peristaltic contractions in 1 minute (H) and time taken in seconds for larvae to right themselves when turned on their dorsal surface (I). n = 50 larvae. The data is shown as the mean ± SEM. $t(46.49)=18.22$, ****$p<0.0001$ using Welch's Student's t-test (F), $t(78)=4.833$, ****$p<0.0001$ using unpaired Student's t-test (G), $t(78)=5.753$, ****$p<0.0001$ using unpaired Student's t-test (H) and $t(78)=6.863$, ****$p<0.0001$ using unpaired Student's t-test (I).

(J) Adult locomotion assay using climbing ability of WT (+/+) (black) and $dMegf8^{HSC}$

mutants (red) flies. n = 50 flies. The data is shown as the mean ± SEM, interaction F(9, 80)=4.579, ****p<0.0001 using two-way ANOVA test.

Figure 2. dMegf8 is expressed in synaptic terminals and required for synaptic bouton growth.

(A-B'') Confocal images in (A) +/+ and (B) *dMegf8*^{HSC} mutant third instar larvae NMJ type Ib boutons at muscles 6/7 labeled with anti-dMegf8 (green) and anti-Brp (red). (C-J) Confocal images in (C) *dMegf8*^{HSC}, (D) *dMegf8* point mutant *dMegf8*^{F1}*/Df*, (E) *dMegf8* null mutant (*dMegf8*^{HSC}), (F) *dMegf8*^{HSC}*/dMegf8*$^{\Delta 8}$ trans-allelic combination, (G) presynaptic overexpression of *dMegf8* (*elav>dMegf8*), (H) presynaptic rescue (*elav>dMegf8;dMegf8*^{HSC}), (I) postsynaptic overexpression of dMegf8 (*MHC>dMegf8*) and (J) postsynaptic rescue (*MHC>dMegf8;dMegf8*^{HSC}) third instar larvae NMJ at muscles 6/7 labeled with the presynaptic marker Hrp (green) and the postsynaptic marker Dlg (red).

(K) Quantification of total bouton numbers in indicated genotypes. The data in (K) is shown as the mean ± SEM using one-way ANOVA test with Tukey's multiple comparisons: F(8,160)=22.3064, +/+ *vs. F1/Df*: ***p=0.0001, +/+ *vs. dMegf8*^{HSC}: ****p<0.0001, +/+ *vs. dMegf8*^{HSC}*/*$^{\Delta 8}$: ****p<0.0001, +/+ *vs. MHC>dMegf8*: p=0.7496, +/+ *vs. elav>dMegf8*: **p=0.0014, +/+ vs. Genomic Rescue: p=0.9467, +/+ *vs. MHC>dMegf8; dMegf8*^{HSC}: ****p<0.0001, +/+ *vs. elav>dMegf8; dMegf8*^{HSC}: p=0.9992, *dMegf8*^{HSC} *vs. F1/Df*: p=0.9915, *dMegf8*^{HSC} *vs. dMegf8*^{HSC}*/*$^{\Delta 8}$: p=0.9989, *dMegf8*^{HSC} vs. genomic Rescue: **p=0.0027, *dMegf8*^{HSC} *vs. MHC>dMegf8; dMegf8*^{HSC}: p>0.9999, *dMegf8*^{HSC} *vs. elav>dMegf8; dMegf8*^{HSC}: ****p<0.0001. Scale bars: (A-B'') 5 µm, (C-J) 20 µm. ns= not significant.

Figure 3. *dMegf8*^{HSC} mutants show altered localization of pre/postsynaptic proteins.

(A-B'') Confocal images in (A) +/+ and (B) *dMegf8*^{HSC} mutant third instar larval NMJ type Ib boutons at muscles 6/7 labeled with the presynaptic protein Brp (green) and the postsynaptic protein GluRIIA (red).

(C) Quantification of Brp puncta/bouton area (μm^2) in WT and *dMegf8*^{HSC} mutants.

(D-E') Confocal images in (D) +/+ and (E) *dMegf8*^{HSC} mutant third instar larvae NMJ labeled with the presynaptic marker Hrp (green) and the postsynaptic protein Dlg (red). The quantification data in (C) is shown as the mean ± SEM; $t(62)=11.14$, ****$p<0.0001$ using unpaired Student's t-test.

(F-G') Dlg localization in *dMegf8*^{HSC} mutants that have either pre-synaptic expression (*elav>dMegf8*) (F-F') or post-synaptic expression (*MHC>dMegf8*) (G-G'). Scale bars: (A-B', F, G) 5 µm, (D-E', F', G') 20 µm.

Figure 4. Loss of dMegf8 causes synaptic ultrastructural defects.

(A-E') TEM images of cross sections through type Ib boutons in (A) +/+, (B) *dMegf8*^{HSC} mutant, (C) *dMegf8* point mutant *dMegf8*^{F1}*/Df*, (D) presynaptic rescue (*elav>dMegf8;dMegf8*^{HSC}) and (E) postsynaptic rescue (*MHC>dMegf8;dMegf8*^{HSC}) at low magnification (A-E) and high magnification (A'-E'). Arrows represent the active zones (AZs) and arrowheads represent the PSDs in (A-E).

(F-I) Quantification in (F) total bouton area, (G) number of AZs, (H) total PSD length/perimeter (%) and (I) normalized SSR width in represented genotypes. The data in (F-I) are shown as mean ± SEM using one-way ANOVA test with Tukey's

multiple comparisons. ns = not significant. (F) F(4, 183)=0.4529, +/+ *vs. dMegf8^{HSC}*: *p*=0.7990, +/+ *vs. F1/Df*: *p*>0.9999, +/+ *vs. elav>dMegf8; dMegf8^{HSC}*: *p*=0.9994, +/+ *vs. MHC>dMegf8; dMegf8^{HSC}*: *p*>0.9999. (G) F(4.000, 159.4)=25.97, +/+ *vs. dMegf8^{HSC}*: ****p<0.0001, +/+ *vs. F1/Df*: ****p<0.0001, +/+ *vs. elav>dMegf8; dMegf8^{HSC}*: *p*=0.9999, +/+ *vs. MHC>dMegf8; dMegf8^{HSC}*: ****p<0.0001 using Brown-Forsythe and Welch ANOVA with Games-Howell's multiple comparisons specifically. (H) F(4, 179)=18.18, +/+ *vs. dMegf8^{HSC}*: ****p<0.0001, +/+ *vs. F1/Df:* *p=0.0292, +/+ *vs. elav>dMegf8; dMegf8^{HSC}*: *p*>0.9999, +/+ *vs. MHC>dMegf8; dMegf8^{HSC}*: ****p<0.0001. (I) F(4, 169)=16.03, +/+ *vs. dMegf8^{HSC}*: ****p<0.0001, +/+ *vs. F1/Df*: *p=0.0136, +/+ *vs. elav>dMegf8; dMegf8^{HSC}*: ****p<0.0001, +/+ *vs. MHC>dMegf8; dMegf8^{HSC}*: *p*=0.1136, *dMegf8^{HSC} vs. elav>dMegf8; dMegf8^{HSC}*: *p*=0.2102, *dMegf8^{HSC} vs. MHC>dMegf8; dMegf8^{HSC}*: ****p<0.0001. Scale bars: (A-E) 600 nm, (A'-E') 200 nm.

Figure 5. dMegf8 is required for proper synaptic transmission.

(A-D) Representative electrophysiological traces showing EJPs from (A) wild type (*wCS*), (B) *dMegf8^{HSC}* mutant, (C) presynaptic rescue (*elav>dMegf8;dMegf8^{HSC}*) and (D) postsynaptic rescue (*MHC>dMegf8;dMegf8^{HSC}*).

(E-I) Quantification of (E) EJP amplitude, (F) mEJP amplitude, (G) quantal contents, (H) paired pulse ratio and (I) mEJP frequency in respective genotypes. The data in (E-I) are shown as the mean ± SEM using one-way ANOVA test with Tukey's multiple comparisons. ns = not significant. (E) F(4, 58)=11.53, +/+ *vs. dMegf8^{HSC}*: ****p<0.0001, +/+ *vs. elav>dMegf8; dMegf8^{HSC}*: *p*=0.9980, +/+ *vs. MHC>dMegf8;*

934 *dMegf8*^{HSC}: **p=0.0026. (F) F(4, 58)=4.311, +/+ *vs.* *dMegf8*^{HSC}: p=0.9658, +/+ *vs.*
935 *elav>dMegf8; dMegf8*^{HSC}: p=0.9936, +/+ *vs. MHC>dMegf8; dMegf8*^{HSC}: p=0.0861. (G)
936 F(4, 43)=13.95, +/+ *vs. dMegf8*^{HSC}: ****p<0.0001, +/+ *vs. elav>dMegf8; dMegf8*^{HSC}:
937 p=0.9503, +/+ *vs. MHC>dMegf8; dMegf8*^{HSC}: ****p<0.0001. (H) F(4, 58)=3.195, +/+
938 *vs. dMegf8*^{HSC}: p=0.1143, +/+ *vs. elav>dMegf8; dMegf8*^{HSC}: p=0.2908, +/+ *vs.*
939 *MHC>dMegf8; dMegf8*^{HSC}: p=0.0800. (I) F(4, 58)=6.865, +/+ *vs. dMegf8*^{HSC}:
940 p=0.9998, +/+ *vs. elav>dMegf8; dMegf8*^{HSC}: p=0.9088, +/+ *vs. MHC>dMegf8;*
941 *dMegf8*^{HSC}: p=0.2920.
942
943 **Figure 6. Interdependency of dMegf8, Dnrx and Wit in their localization and**
944 **stability.**
945 (A-D'') Confocal images in (A) +/+, (B) *dMegf8*^{HSC} mutant, (C) *dnrx* mutant and (D) *wit*
946 mutant third instar larval NMJ labeled with anti-dMegf8 (green) and anti-Brp (red).
947 (E-G') Confocal images in (E) +/+, (F) *dnrx* mutant and (G) *dMegf8*^{HSC} mutant third
948 instar larval NMJ labeled with anti-Dnrx (green) and anti-Brp (red).
949 (H-I') Confocal images of (H) *elav>wit* and (I) *elav>wit; dMegf8*^{HSC} larval NMJ labeled
950 with anti-Hrp (green) and anti-Wit (red).
951 (J) Quantification of dMegf8 fluorescence intensity/bouton area in WT (+/+),
952 *dMegf8*^{HSC}, *dnrx* and *wit* mutants. Data shown as mean ±SEM; F(7, 37)=18.25, +/+ vs.
953 *dMegf8*^{HSC}: ****p<0.0001, +/+ vs. *dnrx-/-*: ***p=0.0005, +/+ vs. *wit-/-*: ****p<0.0001
954 using one-way ANOVA test with Tukey's multiple comparisons.
955 (K) Quantification of Dnrx fluorescence intensity/bouton area in WT (+/+), *dnrx* and
956 *dMegf8*^{HSC} mutants. Data shown as mean ±SEM; F(2, 21)=46.54, +/+ vs. *dnrx-/-*:

****p<0.0001, +/+ vs. *dMegf8*^{HSC}: ****p<0.0001 using one-way ANOVA test with Tukey's multiple comparisons.

(L) Quantification of Wit/Hrp fluorescence intensity ratio in *elav>wit* and *elav>wit; dMegf8*^{HSC} mutants. Data is shown as mean ± SEM; t(24)=11.02, ****p<0.0001 using unpaired Student's t-test. Scale bars: (A-D'') 10 µm, (E-G') 5 µm, (H-I') 5 µm.

Figure 7. BMP downstream effectors in *dMegf8* mutants and genetic interactions between *dMegf8, dnrx* and *wit*

(A-B') Confocal images in (A) +/+ and (B) *dMegf8*^{HSC} mutant third instar larval NMJ labeled with anti-PS-1 (red) and anti-Hrp (green).

(C, D) Confocal images of (C) +/+ and (D) *dMegf8*^{HSC} mutant larval VNC labeled with anti-Smad (red).

(E) Quantification of PS-1 fluorescence intensity/bouton area in the specified genotypes (in A, B). Data shown as mean ±SEM; t(34)=0.1063, p=0.9159 using unpaired Student's t-test.

(F) Quantification of Smad fluorescence intensity/bouton area in the specified genotypes (C, D). Data shown as mean ±SEM; t(34)=1.063, p=0.2951 using unpaired Student's t-test.

(G-J) Representative immunoblots showing total levels of Smad (G) and Trio (H), and corresponding quantification of ratio of band intensities of Smad (I) and Trio (J). Data in (I) shown as mean ±SEM; F(2, 6)=31.42, +/+ *vs. dMegf8*^{HSC}: p=0.7653, +/+ *vs. wit-/-*: **p= 0.0011 using one-way ANOVA with Tukey's multiple comparisons. ns = not

significant. Data in (J) shown as mean ±SEM; t(4)=0.5412, p=0.6171 using unpaired Student's t-test.

(K-R) Confocal images in (K) *dMegf8* heterozygote (*dMegf8*^{HSC}+/−), (L) *dMegf8, dnrx* trans-heterozygote (*dMegf8*^{HSC}+/−;*dnrx*+/−), (M) *dnrx*−/− mutant, (N) *dMegf8, dnrx* double mutant (*dMegf8*^{HSC}−/−;*dnrx*−/−), (O) *wit* heterozygote (*wit*+/−), (P) *dMegf8, wit* trans-heterozygote (*dMegf8*^{HSC}+/−;*wit*+/−), (Q) *wit*−/− mutant and (R) *dMegf8, wit* double mutant (*dMegf8*^{HSC}−/−;*wit*−/−) third instar larval NMJ labeled with anti-Hrp (green) and anti-Dlg (red).

(S, T) Quantification of total bouton numbers in indicated genotypes. The data in (S) is shown as the mean ± SEM; F(6, 107)=21.47, +/+ *vs. dMegf8*+/-: p>0.9999, +/+ *vs. dnrx*+/-: p=0.7589, +/+ *vs. dMegf8*+/-;*dnrx*+/-: ****p<0.0001, +/+ *vs. dnrx*-/-: ****p<0.0001, +/+ *vs. dMegf8*-/-: ****p<0.0001, +/+ *vs. dMegf8*-/- ; *dnrx*-/-: ****p<0.0001, *dMegf8*+/- ; *dnrx*+/- *vs. dnrx*-/-: p=0.5614, *dnrx*-/- *vs. dMegf8*-/-: p=0.9379, *dnrx*-/- *vs. dMegf8*-/- ; *dnrx*-/-: p=0.8967 using one-way ANOVA test with Tukey's multiple comparisons. ns = not significant. Data in (T) is shown as mean ± SEM; F(7, 120)=21.32, +/+ *vs. dMegf8*+/-: p=0.9211, +/+ *vs. wit*^{A12}/-: p=0.9985, +/+ *vs. dMegf8*+/-; *wit*^{A12}/-: ***p=0.0009, +/+ *vs. dMegf8*+/-; *wit*^{B11}/-: ***p=0.0007, +/+ *vs. dMegf8*-/-: ****p<0.0001, +/+ *vs. wit*$^{A12/B11}$: ****p<0.0001, +/+ *vs. dMegf8*-/-; *wit*$^{A12/B11}$: ****p<0.0001, *dMegf8*-/- *vs. wit*$^{A12/B11}$: p=0.9612, *dMegf8*-/- *vs. dMegf8*-/-; *wit*$^{A12/B11}$: p=0.9994 using one-way ANOVA test with Tukey's multiple comparisons. Scale bars: (A-H) 20 μm.

Figure 8. Biochemical interactions between dMegf8, Dnrx and Wit.

(A-C) Representative immunoblots from adult head lysates showing total levels of dMegf8 in wild type (+/+), *dMegf8*$^{F1/Df}$ (A) and in *dnrx* mutant (B), and quantification of the normalized ratio of protein band intensities (C). +/+ *vs. dMegf8*^{F1}*/Df*: t(4)=6.815, ***p*=0.0024 using unpaired Student's t-test; +/+ *vs. dnrx-/-*: t(6)= 0.1961, *p*=0.8510 using unpaired Student's t-test.

(D, E) Representative immunoblots from adult head lysates showing total levels of Dnrx in wild type (+/+), *dMegf8*^{HSC} and *dnrx* mutants (as a negative control), and quantification of the normalized ratio of protein band intensities (E). +/+ *vs. dMegf8*^{HSC}: t(6)=0.3921, *p*=0.7085 using unpaired Student's t-test.

(F, G) Representative immunoblots from larval musculature lysates showing levels of Wit in specified genotypes, and quantification of the normalized ratio of protein band intensities (G). F(2, 11)=45.91, +/+ *vs. dMegf8*^{HSC}: **p*=0.0107, +/+ *vs. elav>dMegf8*: ****p*=0.0003 using one-way ANOVA test with Tukey's multiple comparisons.

(H, I) Representative immunoblots from larval musculature lysates showing levels of Wit in specified genotypes, and quantification of the normalized ratio of protein band intensities (I). F(2, 12)=37.98, +/+ *vs. elav>wit;dMegf8*^{HSC}: **p*=0.0396, +/+ *vs. elav>wit*: *****p*<0.0001 using one-way ANOVA test with Tukey's multiple comparisons. Actin was used as the loading control in (A, B, D, F, H).

(J, K) dMegf8 co-immunoprecipitated with anti-Dnrx (J) and anti-Wit (K) antibodies, respectively and probed with anti-dMegf8. The data in C, E, G and I are shown as the mean ± SEM; **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 using unpaired Student's t-test (C, E) and one-way ANOVA test with Tukey's multiple comparisons (G, I). ns = not significant.

Figure 9. Schematic model of dMegf8 function at the NMJ synapse.

A schematic model depicting dMegf8 and other known pre- and post-synaptic proteins at the NMJ. Based on our immunohistochemical, ultrastructural, electrophysiological, biochemical and genetic rescue analyses, we propose that dMegf8 functions pre- synaptically to coordinate BMP signaling for the synaptic bouton growth and post-synaptically to organize the SSR and other synaptic structures that are necessary for proper synaptic function. While dMegf8 interactions with Dnrx and Wit are established, uncovering potential molecular interactions between dMegf8 and Dnlg1, Tkv and Sax, or other synaptic proteins would shed further light on the functions of dMegf8 at the synapse.

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