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The translational regulator Cup controls NMJ presynaptic terminal morphology

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ABSTRACT

During oogenesis and early embryonic development in *Drosophila*, translation of proteins from maternally deposited mRNAs is tightly controlled. We and others have previously shown that translational regulatory proteins that function during oogenesis also have essential roles in the nervous system. Here we examine the role of Cup in neuromuscular system development. Maternal Cup controls translation of localized mRNAs encoding the Oskar and Nanos proteins and binds to the general translation initiation factor eIF4E. In this paper, we show that zygotic Cup protein is localized to presynaptic terminals at larval neuromuscular junctions (NMJs). *cup* mutant NMJs have strong phenotypes characterized by the presence of small clustered boutons called satellite boutons. They also exhibit an increase in the frequency of spontaneous glutamate release events (mEPSPs). Reduction of eIF4E expression synergizes with partial loss of Cup expression to produce satellite bouton phenotypes. The presence of satellite boutons is often associated with increases in retrograde bone morphogenetic protein (BMP) signaling, and we show that synaptic BMP signaling is elevated in *cup* mutants. *cup* genetically interacts with four genes (*EndoA*, *WASp*, *Dap160*, and *Synj*) encoding proteins involved in endocytosis that are also neuronal modulators of the BMP pathway. Endophilin protein, encoded by the *EndoA* gene, is downregulated in a *cup* mutant. Our results are consistent with a model in which Cup and eIF4E work together to ensure efficient localization and translation of endocytosis proteins in motor neurons and control the strength of the retrograde BMP signal.

1. Introduction

Most key developmental regulatory proteins are regulated by multiple mechanisms. These include transcriptional regulation, mRNA localization, translational control, protein localization, and protein lifetime. In *Drosophila*, many proteins involved in oogenesis and early development are translated from maternal mRNAs. Because these mRNAs are deposited in the egg, their translation must be precisely regulated in order to allow production of their protein products at the appropriate times and places. Most genes encoding maternally expressed regulators are also zygotically transcribed later in development, and some of these genes function during nervous system development. The translational control mechanisms used to regulate expression and localization of these maternal gene products are also operative in the nervous system. However, translational regulatory circuits in neurons can have different organizations from those that operate during early development.

Pumilio (Pum) and Nanos (Nos) are key maternal translational regulators. They form a complex that binds to the 3′ UTR of *hunchback (hb)* mRNA and represses its translation in the posterior part of the embryo (Sonoda and Wharton, 1999). We and others have shown that zygotic Pum and Nos are also required for neural development and function (Baines, 2005; Menon et al., 2009; Menon et al., 2004; Muraro et al., 2008; Ye et al., 2004). Pum, Nos, and the general translational initiation factor eIF4E are components of a regulatory circuit in the neuromuscular system that controls postsynaptic translation of glutamate receptor (GluR) mRNAs. In postsynaptic muscles, Pum binds to the 3′ UTRs of the *eIF4E* (Menon et al., 2004), *GluRIIA*, and *nos* mRNAs (Menon et al., 2009) and represses their translation. Postsynaptic Nos represses expression of the alternate GluR subunit, GluRIIB, by an unknown mechanism that is not dependent on Pum (Menon et al., 2009). In neurons, the Pum/Nos complex binds to and represses translation of *paralytic (para)* mRNA, which encodes a voltage-gated sodium channel (Muraro et al., 2008). Pum and Nos are also required for normal development of neuromuscular junction (NMJ) presynaptic terminals (Menon et al., 2009; Menon et al., 2004), and they regulate branching of the dendrites of peripheral sensory neurons (Ye et al., 2004).

Since Pum and Nos function in the nervous system, we wished to investigate molecules that interact with these translational regulators during oogenesis or early embryonic development and define their roles at the larval NMJ. In this paper, we examine the zygotic functions of Cup, which is a maternal regulator of *nos* mRNA translation in oocytes. Cup also binds to eIF4E (Nakamura et al., 2004; Nelson et al.,

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2004; Wilhelm et al., 2003; Zappavigna et al., 2004), and eIF4E expression is controlled by Pum in the neuromuscular system (Menon et al., 2004). Thus, we were interested in determining whether Cup is also important for neuromuscular system development.

Cup is encoded by the female-sterile gene *fs(2)cup*, which is required for production of functional oocytes (Keyes and Spradling, 1997; Schupbach and Wieschaus, 1991). Cup's major role during oogenesis is to regulate translation of mRNAs that are localized to specific domains within the oocyte. *oskar (osk)* mRNA is localized to the posterior pole of the oocyte and is required for the establishment of the germ line and for posterior patterning (Ephrussi et al., 1991). Cup is required for *osk* mRNA localization, and it represses translation of *osk* mRNA until it reaches its final location. Translation of *osk* mRNA is prematurely derepressed in *cup* mutants, resulting in the expression of Osk protein at the wrong pole of the oocyte (Wilhelm et al., 2003). Cup itself does not bind to mRNAs, but engages with *osk* mRNA by forming a complex with Bruno, a sequence-specific RNA-binding protein (Nakamura et al., 2004). Cup also represses translation of *nos* mRNA that is not localized at the posterior pole of the embryo. It engages with *nos* mRNA through its interactions with Smaug, another sequence-specific RNA-binding protein (Nelson et al., 2004). Cup was also identified as a binding partner of Nos in a yeast two-hybrid screen (Verrotti and Wharton, 2000).

Cup represses translation through a variety of mechanisms. One proposed mechanism is to obstruct the formation of the elongation initiation factor 4F (eIF4F) complex. The eIF4F complex includes an RNA helicase, eIF4A, a scaffolding protein, eIF4G, and the cap-binding protein, eIF4E. eIF4E is the target for translational repressors known as eIF4E-binding proteins (4E-BPs). By competing with eIF4G for binding to eIF4E, 4E-BPs inhibit the recruitment of the 43S preinitiation complex and block translation (Wilhelm and Smibert, 2005). Cup is a 4E-BP, and contains two eIF4E-binding motifs located within its N-terminal domain (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004). In addition to binding each other directly in vitro and in cell culture assays, *eIF4E* and *cup* genetically interact to regulate ovary development. Cup is also required for localizing eIF4E to the posterior pole in developing oocytes (Zappavigna et al., 2004).

Since Nos and Cup interact and function together in the germline, and Cup regulates *nos* mRNA translation, we anticipated that if zygotically expressed Cup has a function at the NMJ, zygotic *cup* mutants might have phenotypes that resembled either loss-of-function (LOF) or gain-of-function (GOF) *nos* phenotypes (Menon et al., 2009). In the present study, we show that Cup is indeed expressed by motor neurons and localized to NMJ presynaptic terminals. However, we found that the *cup* zygotic phenotype is quite distinct from those of *nos* LOF or GOF mutants. There was also no obvious change in Nos protein levels in *cup* mutants.

Given these results, we decided to perform a broader analysis of the functions of Cup and eIF4E at the NMJ. We find that *cup* mutant NMJs contain many small, clustered boutons. These are known as satellites (reviewed by Menon et al., 2013; O'Connor-Giles and Ganetzky, 2008; Oh and Robinson, 2012). The frequency of spontaneous glutamate release events (minis) is altered in *cup* mutants. *cup* and *eIF4E* genetically interact, as in the ovary (Zappavigna et al., 2004), and the nature of these interactions suggest that Cup is a positive regulator of eIF4E function in motor neurons. We also show that, like other genes for which mutations produce satellite bouton phenotypes, Cup is a negative regulator of the retrograde Bone Morphogenetic Protein (BMP) signaling pathway, in which Glass Bottom Boat (Gbb), a BMP ligand secreted by the muscle, activates BMP receptors on the presynaptic terminal and thereby increases expression of genes required for synaptic growth (Marques and Zhang, 2006). Cup appears to indirectly affect BMP signaling via positive regulation of endocytic proteins that are neuronal modulators of the pathway.

Our data are consistent with a model in which Cup and eIF4E facilitate localization and translation of endocytic proteins at the proper time(s) and place(s). Cup is known as a translational repressor and competes with eIF4G for binding to eIF4E (Nelson et al., 2004). However, Cup's binding to eIF4E is not necessarily a repressive mechanism. Based on studies in cultured cells, it was recently proposed that eIF4E binding to Cup tethers eIF4E to mRNAs destined for specific locations, so that translation can begin promptly when Cup dissociates from the mRNA complex (Igreja and Izaurralde, 2011). In this way, Cup can both repress translation of mRNAs via a non-eIF4E-dependent mechanism, and positively control translation by facilitating rapid assembly of the eIF4F complex when repression is relieved. We suggest that the second mechanism operates in motor neurons. It is consistent with our findings that Cup is a positive regulator of direct and indirect modulators of the BMP signaling pathway and works in concert with eIF4E to facilitate normal development and function of NMJ presynaptic terminals.

2. Results

2.1. Cup protein localizes to NMJ presynaptic terminals

To evaluate Cup's functions during NMJ development, we first determined whether Cup is expressed in the nervous system. Third-instar larvae were dissected and stained with antibodies against Cup and visualized by confocal microscopy. Three polyclonal antibodies made against different regions of the protein by different research groups were tested for immunoreactivity. Rabbit and mouse anti-Cup antibodies were made against amino acids 595–975 of Cup, while the rat anti-Cup antibody was generated against amino acids 201–499 (Keyes and Spradling, 1997; Nakamura et al., 2004). We found that all three antibodies stained the larval NMJ (Figs. 1 and S1). The postsynaptic marker Discs-Large (Dlg) was used to outline the boutons (Fig. 1A2). Rabbit (Fig. 1A1) and rat and mouse (Fig. S1) Cup antibodies all showed punctate Cup staining within the presynaptic bouton and to a lesser extent on the postsynaptic muscle membrane. In addition to staining at the NMJ, Cup is also found in the cytoplasm of neuronal cell bodies in the ventral nerve cord (VNC) (Figs. 1A4 and S1). These cells were identified as neurons using antibodies against horseradish peroxidase (HRP), which selectively stain the surfaces of insect neurons and are commonly used as neuronal markers.

Presynaptic localization of Cup at the NMJ was confirmed by double staining with antibodies for the synaptic markers Fasciclin 2 (Fas2), a cell adhesion molecule in the periactive zone, and Bruchpilot (Brp), an active zone protein (Fig. 1B and C, respectively). Anti-Fas2 stains in a network-like pattern within the presynaptic bouton, and also stains postsynaptically around the bouton (Fig. 1B2). Brp shows punctate staining marking active zones (Fig. 1C2). Cup puncta inside presynaptic boutons show partial overlap with both Fas2 and Brp (insets in B3 and C3). Cup is enriched in the type 1b boutons and localizes faintly at type 1 s boutons (asterisk in Fig. S1). Anti-Cup does not stain the axon trunks.

To confirm that the presynaptic staining we observed is due to Cup protein, we next examined Cup immunoreactivity at NMJs in *cup* mutants and in larvae where Cup was knocked down using transgenic RNAi. The *cup* alleles used in this study are $cup^{\Delta 212}$ (Nakamura et al., 2004), a P-element imprecise excision line that produces an N-terminally truncated Cup protein, two ethyl methane sulfonate (EMS)-induced alleles (cup^{20} and cup^{8}), and two deficiencies that remove *cup*, *Df(2L)BSC7* (26D10-27C1) and *Df(2L)BSC187* (26F3-27A1). The two EMS-induced mutations are not protein nulls, and their sequences are not known (Keyes and Spradling, 1997). During oogenesis, cup^{20} and cup^{8} have stronger defects in egg chamber development than does $cup^{\Delta 212}$ (Nakamura et al., 2004).

We observed a decrease in Cup protein levels at the NMJ relative to wild-type, as visualized with the rabbit antibody, in cup^{20}/*Df(2L)BSC7* transheterozygotes (Fig. S2). Wild-type and *cup* NMJs exhibited similar staining intensities with anti-HRP (Fig. S2). The residual Cup antibody immunoreactivity seen in the mutant is likely due to expression of

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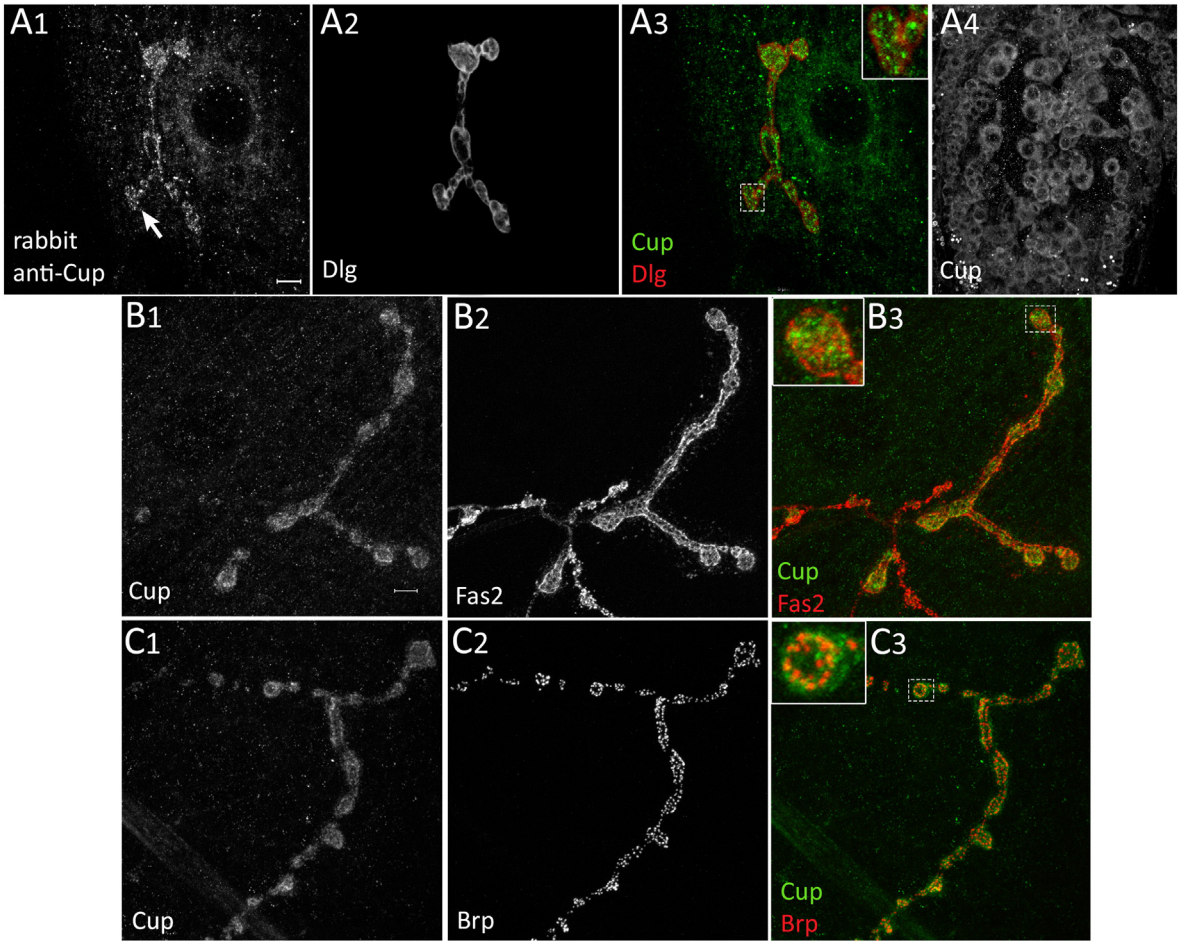

Fig. 1. Cup protein is localized to NMJ presynaptic terminals. (A1–A4) A wild type third instar larva stained with rabbit anti-Cup and mouse anti-Dlg antibodies. (A1) Cup is found in punctate structures in NMJ presynaptic terminal and is also expressed at much lower levels in muscles. (A2) Anti-Dlg staining of the same NMJ as in A1. (A3) Merged images of A1 and A2 with the inset showing a zoomed in view of the highlighted bouton to display the Cup puncta (green). (A4) Cup also localizes to cell bodies in the ventral nerve cord. (B1–B3) Colocalization of Fas2 and Cup at the muscle 4 NMJ in a wild type larva. (B1) Anti-Cup staining, (B2) Anti-Fas2 staining, and (B3) overlap of Cup (green) and Fas2 (red) from B1 and B2, respectively. Inset is a zoomed-in view of the highlighted bouton to show Cup puncta within the presynaptic terminal. (C1–C3) Colocalization of Cup and Brp at the muscle 4 NMJ in a wild type larva. (C1) Anti-Cup staining, (C2) Anti-Brp staining, and (C3) overlap of Cup (green) and Brp (red) staining from C1 and C2, respectively. Inset is a magnified view of the highlighted bouton. Scale bar: 5 μm.

mutant protein from the cup^{20} allele, since cup^{20} is not a protein null (Keyes and Spradling, 1997). Complete elimination of Cup protein at NMJs was not observed with any *cup* allelic combination. We also knocked down Cup presynaptically by expressing *cup* RNAi using a pan-neuronal driver, *C155-Gal4*. NMJs in *cup* RNAi larvae recapitulated the decreased staining seen in *cup* mutants relative to wild-type (Fig. S2).

2.2. Loss of Cup function causes alterations in NMJ morphology

To evaluate *cup* phenotypes, we stained larvae bearing transheterozygous combinations of the various *cup* alleles. Third-instar larvae from controls and *cup* mutants were dissected and stained with anti-HRP, and NMJs were analyzed by confocal microscopy. Our previous studies of Nos showed that it regulates NMJ growth. In *nos* mutants, there was an increase in bouton number (Menon et al., 2009), and neuronal Nos overexpression decreased the number of boutons. In *cup* transheterozygotes, however, there was no statistically significant change in the total number of full-size NMJ boutons (data not shown). Thus, although loss of maternal Cup produces patterning defects due to Nos mislocalization and Cup was identified as a Nos interactor using the yeast two-hybrid assay, loss of zygotic Cup does not recapitulate *nos* LOF or GOF phenotypes in the nervous system.

cup mutants, however, displayed a strong NMJ phenotype affecting bouton organization. Wild-type muscle 4 NMJs show a simple pattern of type 1b boutons connected to each other in a linear chain (Fig. 2A). In *cup* transheterozygotes, there are multiple small boutons budding from the parent type 1b boutons. These are rarely seen in control animals. Clustered small boutons were most commonly observed at the terminal parent bouton (arrows and insets, Fig. 2B, C). Small boutons were also seen emerging from regions connecting two boutons, or from a branching bouton, although this was less frequent (Fig. 2B, arrowhead). These small, clustered boutons have been observed in many other studies, and are known as satellite boutons.

The number of satellite boutons per muscle 4 NMJ from segments A2–A5 was quantitated in different mutant combinations and controls (Fig. 2D). Due to the non-normal distribution of the satellite bouton numbers, the non-parametric Kolmogorov–Smirnov (K–S) test was used to calculate the D statistic and evaluate the statistical significance (see Experimental methods). Box plots were used to represent the data and significance is indicated above each genotype. Satellite bouton numbers in *cup* mutants were significantly higher than in wild type (*WT*) or heterozygous controls. The number of satellite boutons in $cup^{20}/cup^{\Delta 212}$ was 4-fold higher than in the corresponding heterozygous controls (K–S test, $cup^{20}/+$ D = 0.34, $p < 0.001$; $cup^{\Delta 212}/+$ D = 0.40, $p < 0.001$; Fig. 2D). The differences in satellite bouton numbers between mutants and controls were highly statistically significant for other

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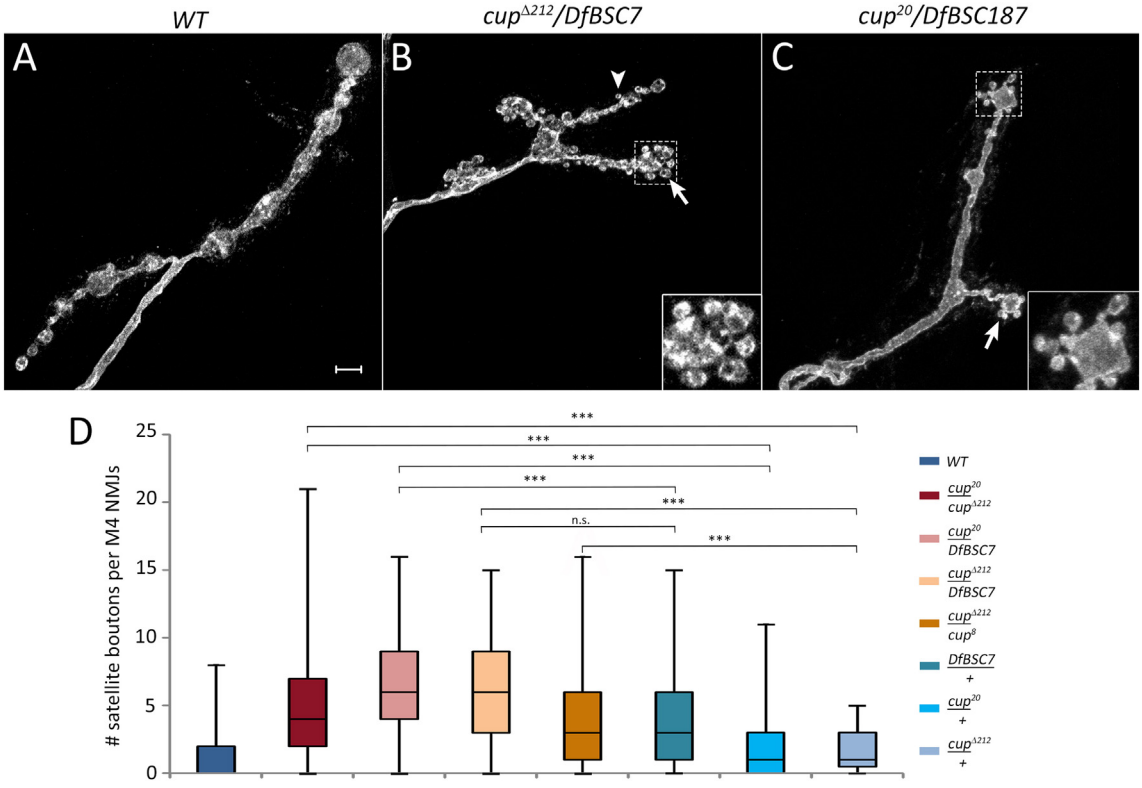

Fig. 2. Loss of Cup function produces NMJ satellite bouton phenotypes. Muscle 4 NMJs in wild type larvae (A), $cup^{\Delta 212}/DfBSC7$ (B), and $cup^{20}/DfBSC187$ (C). Arrows in (B) and (C) highlight satellite boutons sprouting from terminal parent boutons. Arrowhead in (B) shows a satellite bouton on the axon trunk. Insets in (B) and (C) are blowups of the outline areas. (D) Box plots showing number of satellite boutons per muscle 4 NMJs in different genotypes. Wild type (WT; n = 157), $cup^{20}/cup^{\Delta 212}$ (n = 228); $cup^{20}/DfBSC7$ (n = 124); $cup^{\Delta 212}/DfBSC7$ (n = 46); $cup^{\Delta 212}/cup^{8}$ (n = 55); $DfBSC7/+$ (n = 80); $cup^{20}/+$ (n = 45); $cup^{\Delta 212}/+$ (n = 43). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. Significance of satellite bouton numbers as determined by the Kolmogorov–Smirnov (K–S) non-parametric test is shown with asterisks. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. $p > 0.05$. Scale bar: 5 μm.

transheterozygous combinations as well, including $cup^{20}/DfBSC7$ and $cup^{\Delta 212}/cup^{8}$ (compared to heterozygous controls ($cup^{20}/+$ D = 0.59, $p < 0.00$; $DfBSC7/+$ D = 0.31, $p < 0.001$; $cup^{\Delta 212}/+$ D = 0.42, $p < 0.001$)). However, satellite bouton numbers in $cup^{\Delta 212}/DfBSC7$ did not differ significantly from those in $DfBSC7/+$ (D = 0.22, p = 0.09). All heterozygous controls, especially the $DfBSC7/+$, showed an increase in satellite bouton numbers compared to *WT* ($cup^{20}/+$ D = 0.22, $p > 0.05$; $cup^{\Delta 212}/+$ D = 0.25, $p < 0.05$; $DfBSC7/+$ D = 0.46, $p < 0.001$) suggesting that satellite bouton formation is very sensitive to Cup levels.

Cup staining at NMJs suggests that it functions in the motor neurons, since it is localized predominantly presynaptically, although there are a few puncta within the postsynaptic muscles (Fig. 1). To evaluate whether Cup function is required pre- or postsynaptically to facilitate normal synaptic growth, we expressed a *cup* cDNA in *cup* mutant backgrounds using *24B-Gal4* (postsynaptic) and *elav-Gal4* (panneuronally). Expression of Cup postsynaptically in a $cup^{20}/DfBSC7$ background did not rescue the satellite bouton phenotype (Fig. 3C; $cup^{20}/DfBSC7$ D = 0.08, $p > 0.05$). However, when Cup was expressed panneuronally in both $cup^{\Delta 212}/DfBSC7$ and $cup^{20}/DfBSC7$ backgrounds, this produced significant rescue of satellite bouton phenotypes ($cup^{\Delta 212}/DfBSC7$ D = 0.33, $p < .01$; $cup^{20}/DfBSC7$ D = .32, $p < 0.001$, as compared to their corresponding rescue genotypes). When the presynaptic rescues were compared to *elav-Gal4/+* controls, there was no significant difference, suggesting a complete rescue of the mutant phenotype ($cup^{\Delta 212}/DfBSC7$; *elavGal4/UAS-cup* D = 0.19, $p > 0.05$; $cup^{20}/DfBSC7$; *elavGal4/UAS-cup* D = 0.21, $p > 0.05$, as compared to *elav-Gal4/+*). To further confirm the presynaptic function of cup, we used RNAi to knock down Cup expression pre- and postsynaptically (Fig. 3D). Knockdown of Cup presynaptically resulted in a 3-fold increase in satellite bouton number compared to the *cup* RNAi control (D = 0.24, $p < 0.01$), whereas postsynaptic Cup knockdown (*cup RNAi (GL)/24B-Gal4*) did not produce a phenotype (*cup RNAi (GL)/+* D = 0.05, $p > 0.05$; *24B-Gal4/+* D = 0.17, $p > 0.05$; Fig. 3D). This provides further evidence that Cup functions in the presynaptic neuron rather than in the postsynaptic muscle.

2.3. Electrophysiological analysis of cup mutants shows an increase in spontaneous release frequency

To determine whether Cup is important for synaptic function, we recorded evoked and spontaneous activity from muscle 6/7 NMJs of *cup* mutants and controls (Fig. 4). The K–S test for non-parametric distributions was used to compare the mutant genotypes to their respective controls (see Experimental methods). None of the *cup* transheterozygote combinations revealed a change in evoked excitatory postsynaptic potential (EPSP) amplitudes (Fig. 4B). Representative EPSP traces are shown in Fig. 4A. The resting membrane potentials were also similar among all the genotypes and controls (data not shown).

Miniature (mini) EPSPs (mEPSPs) are due to spontaneous release of single vesicles of neurotransmitter. Analysis of mEPSP frequency showed an approximate 1.5-fold increase over heterozygous controls for all *cup* mutant combinations (Fig. 4D). All values were statistically significant ($p < 0.001$) when analyzed by the K–S test ($cup^{20}/cup^{\Delta 212}$ vs. $cup^{20}/+$ D = 0.41, $cup^{\Delta 212}/+$ D = 0.38; $cup^{20}/DfBSC7$ vs. $cup^{20}/+$ D = 0.34, $DfBSC7/+$ D = 0.45; $cup^{\Delta 212}/DfBSC7$ vs. $cup^{\Delta 212}/+$ D = 0.32, $DfBSC7/+$ D = 0.46). Representative mEPSP traces are shown in Fig. 4C for wild type and two *cup* transheterozygotes. These results are consistent with a role for *cup* in motor neurons, since changes in frequencies of spontaneous release events are normally associated with presynaptic function. mEPSP amplitudes did not show significant changes for any of the genotypes tested (Fig. 4E).

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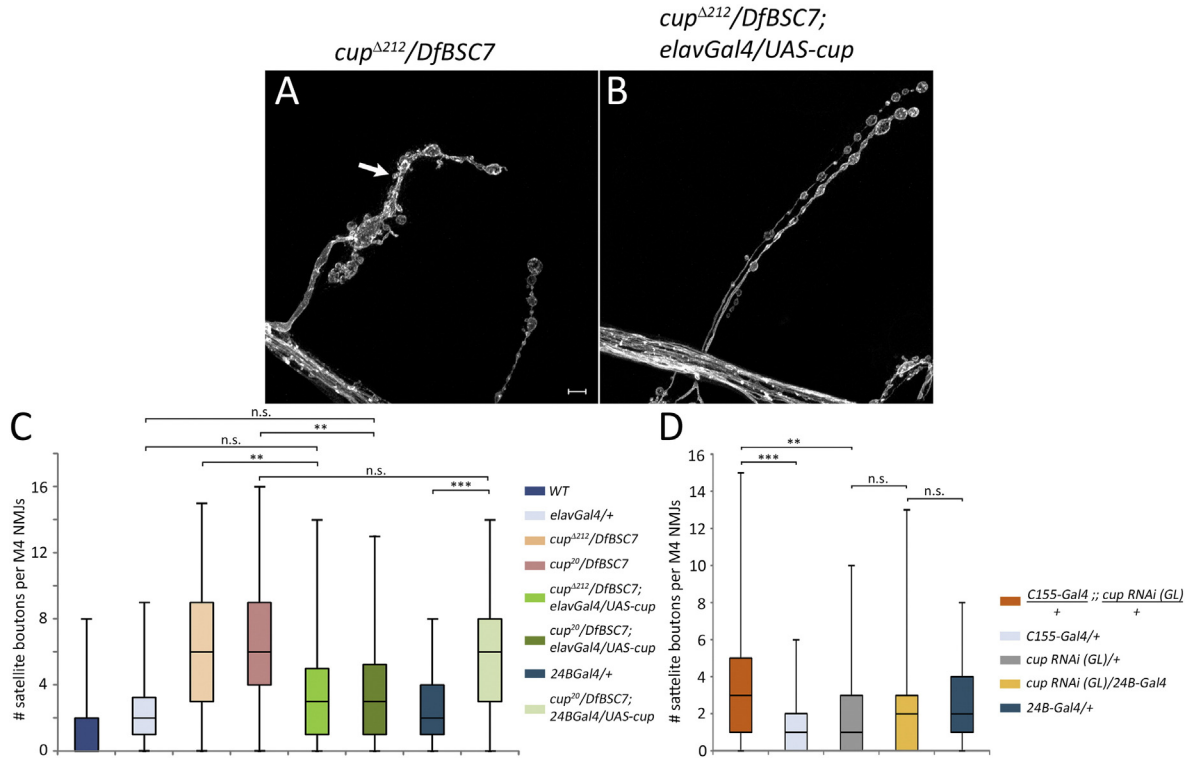

Fig. 3. Cup is required in motor neurons. (A) A $cup^{\Delta 212}/DfBSC7$ mutant shows an elevated number of satellite boutons (arrow). (B) Expression of a Cup transgene in neurons in a $cup^{\Delta 212}/DfBSC7$ mutant background rescues the satellite bouton phenotype. (C) Box plots showing number of satellite boutons per muscle 4 NMJs in control, mutant, and rescued genotypes. Wild type (WT; n = 157), *elav-Gal4* (n = 80), $cup^{\Delta 212}/DfBSC7$ (n = 46); $cup^{20}/DfBSC7$ (n = 124); $cup^{\Delta 212}/DfBSC7$; *elav-Gal4/UAS-cup* (n = 56); $cup^{20}/DfBSC7$ (n = 124); $cup^{20}/DfBSC7$; *elav-Gal4/UAS-cup* (n = 48), *24B-Gal4* (n = 80), $cup^{20}/DfBSC7$; *24B-Gal4/UAS-cup* (n = 68). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. (D) Box plots showing number of satellite boutons per muscle 4 NMJs in panneuronal expression of *cup RNAi* (genotype *C155-Gal4/+;; cup RNAi (GL)/+*), muscle expression of *cup RNAi* (genotype *cup RNAi (GL)/24B-Gal4 cup*) and their corresponding controls. *C155-Gal4/+;;cup RNAi (GL)/+* (n = 94), *C155-Gal4/+* (n = 56), *cup RNAi (GL)/+* (n = 95), *cup RNAi (GL)/24B-Gal4* (n = 88), *24B-Gal4/+* (n = 80). Significance of satellite bouton numbers as determined by the K–S test is shown with asterisks. ***p < 0.001, **p < 0.01, *p < 0.05, n.s. p > 0.05. Scale bar: 5 μm.

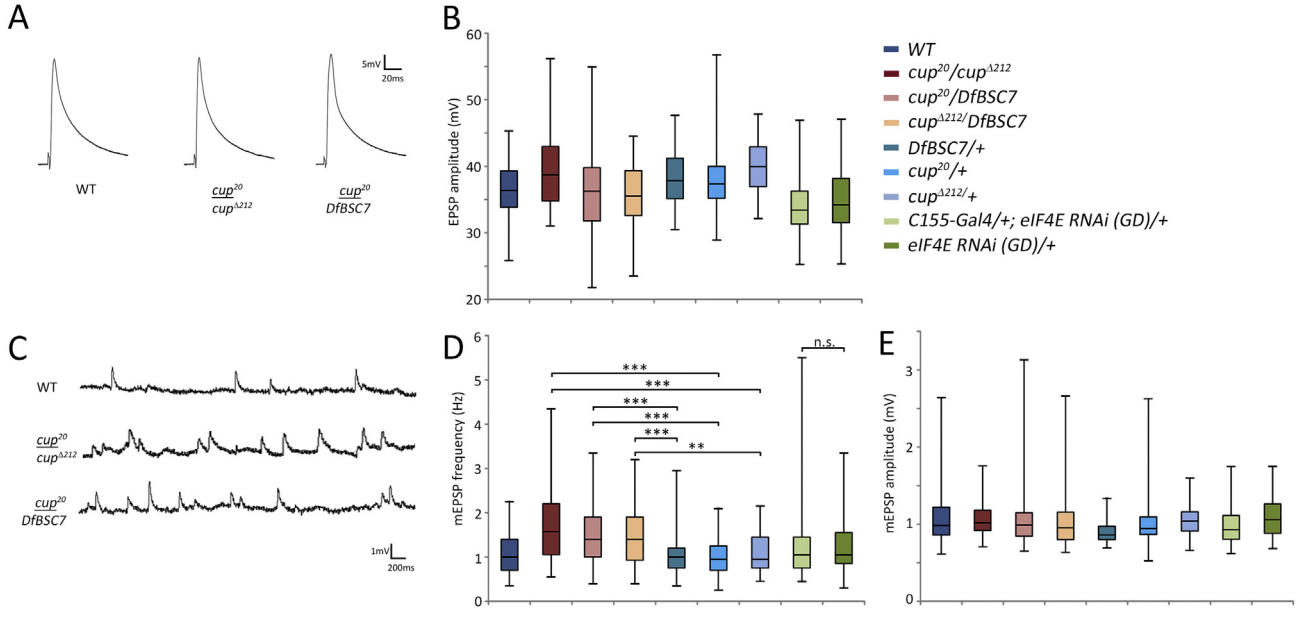

Fig. 4. Cup mutants have an elevated frequency of spontaneous glutamate release events. (A) Representative EPSPs in wild type, $cup^{20}/cup^{\Delta 212}$, and $cup^{20}/DfBSC7$. (B) Box plots showing EPSP amplitudes in wild type, various *cup* alleles, and eIF4E knockdown larvae. Wild type (WT; n = 112), $cup^{20}/cup^{\Delta 212}$ (n = 77); $cup^{20}/DfBSC7$ (n = 106); $cup^{\Delta 212}/DfBSC7$ (n = 85); *DfBSC7/+* (n = 59); $cup^{20}/+$ (n = 60); $cup^{\Delta 212}/+$ (n = 46); *C155-Gal4/+; eIF4E RNAi (GD)/+* (n = 91); *eIF4E RNAi (GD)/+* (n = 77). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. No statistically significant differences were observed. (C) Representative traces of miniature EPSPs (mEPSPs) in wild type, $cup^{20}/cup^{\Delta 212}$, and $cup^{20}/DfBSC7$. (D) Box plots showing the mEPSP frequencies in the various *cup* mutants and *eIF4E RNAi (TRiP)* lines. Significance of mEPSP frequencies as determined by the K–S test is shown with asterisks. ***p < 0.001, **p < 0.01, *p < 0.05, n.s. p > 0.05. Wild type (WT; n = 89), $cup^{20}/cup^{\Delta 212}$ (n = 72); $cup^{20}/DfBSC7$ (n = 97); $cup^{\Delta 212}/DfBSC7$ (n = 71); *DfBSC7/+* (n = 58); $cup^{20}/+$ (n = 61); $cup^{\Delta 212}/+$ (n = 45); *C155-Gal4; eIF4E RNAi (GD)/+* (n = 76); *eIF4E RNAi (GD)/+* (n = 73). (E) Box plots showing mEPSP amplitudes in wild type, various *cup* mutants, and eIF4E knockdown larvae. No statistically significant differences as determined by the K–S test were observed. The number of samples was the same as in panel D.

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2.4. Cup interacts with eIF4E to regulate synaptic growth at the larval NMJ

eIF4E is part of the eIF4F translation initiation complex. Cup has been shown to directly interact with eIF4E, and there are two eIF4E-binding sites in Cup (Nakamura et al., 2004; Nelson et al., 2004; Zappavigna et al., 2004). There is a single *eIF4E* gene in *Drosophila*, which is expressed in all cells and is required for cell viability. There are also 5 *eIF4E*-related genes, all of which are primarily expressed in the testis. Their functions are unknown. None of them are detectably expressed in the larval CNS (see Flybase for details). Maternal *cup* and *eIF4E* interact genetically, and these interactions suggest that they function collaboratively in the developing oocyte (Zappavigna et al., 2004).

To determine if Cup-eIF4E interactions are required for NMJ development, we carried out a genetic interaction test and quantitated satellite bouton formation in *cup*/+, *eIF4E*/+ transheterozygotes (Fig. 5). We used $cup^{\Delta 212}$, which eliminates the primary eIF4E binding site, 4E-BM1 (Nakamura et al., 2004), and a deficiency, *DfBSC113*, that removes *eIF4E* and thus represents a null mutation. NMJs of $cup^{\Delta 212}$/+; *DfBSC113 (eIF4E)*/+ transheterozygotes showed a 3.5-fold increase in satellite boutons compared to *DfBSC113 (eIF4E)*/+ controls (D = 0.54, $p < 0.001$; Fig. 5D).

We used two *eIF4E RNAi* lines (GD from VDRC collection, Vienna, and TRiP from the TRiP RNAi collection, Harvard) to confirm the role of eIF4E, since *DfBSC113* also removes other genes. Because eIF4E is required for cell viability, strong knockdown of eIF4E causes pleiotropic phenotypes and early lethality. Many UAS transgenes, however, have leaky, low level expression even without a Gal4 driver. The GD and TRiP *eIF4E RNAi* lines both showed satellite boutons, although to different extents, in the absence of any driver (Fig. 5D). We used this low level knockdown of eIF4E to our advantage and examined if satellite bouton numbers changed when the *eIF4E RNAi (GD)* line was combined with two heterozygous *cup* alleles. Indeed, satellite bouton numbers increased (*eIF4e RNAi (GD)*/+ vs. $cup^{\Delta 212}$/+; *eIF4E RNAi (GD)*/+ D = .34, $p < 0.05$ and *eIF4e RNAi (GD)*/+ vs. cup^{20}/+; *eIF4E RNAi (GD)*/+ D = 0.44, $p < 0.001$; Fig. 5D).

The specificity of the *cup*/*eIF4E* interaction led us to more closely investigate eIF4E itself. Even though leaky eIF4E RNAi expression showed a slight increase in the number of satellite boutons, we wanted to further reduce eIF4E levels in neurons to see if the phenotype would become stronger. The neuronal driver *C155-Gal4* was crossed to the *eIF4E RNAi (TRiP)* line, because this line showed less leaky expression as compared to the *GD RNAi* line. *C155*-driven *eIF4E RNAi* did not cause larval lethality or obvious larval motility phenotypes. We observed that there was a 2-fold increase in the number of satellite boutons when *eIF4E RNAi* was expressed presynaptically, as compared to the non-driven RNAi line (*C155-Gal4; eIF4E RNAi (TRiP)* vs. *eIF4E RNAi (TRiP)*/+ D = 0.33, $p < 0.001$; Fig. 5D).

2.5. BMP signaling is upregulated in cup mutants

Most mutations that produce satellite boutons have been found to upregulate the retrograde BMP signaling pathway. To examine whether *cup* mutations also do this, we employed two tests that are standard in the field (O'Connor-Giles et al., 2008; reviewed by O'Connor-Giles and Ganetzky, 2008). BMP signaling acts through Mothers against decapentaplegic (Mad). When Mad is phosphorylated, it translocates to the nucleus where it serves as a transcription factor. To evaluate if BMP signaling is involved in the *cup* satellite bouton phenotype, we

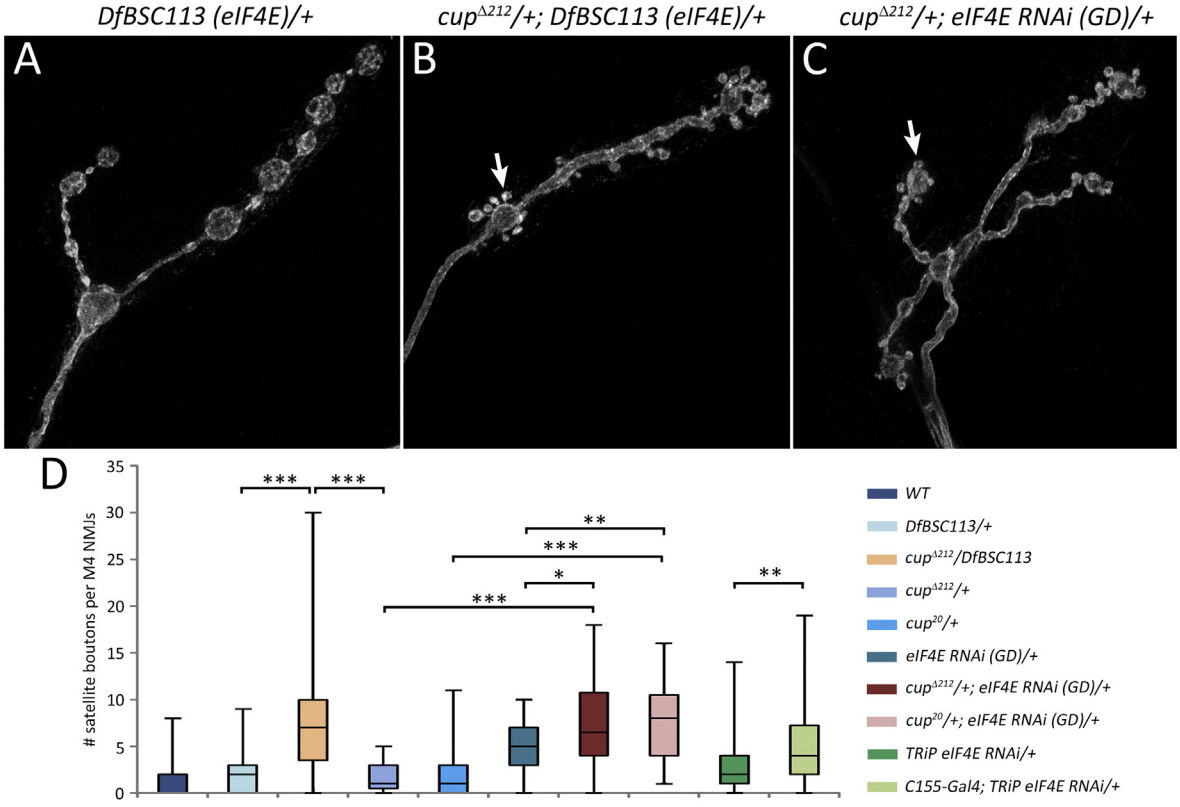

Fig. 5. *cup* genetically interacts with *eIF4E*. (A) *DfBSC113 (eIF4E)*/+ heterozygotes have normal NMJs. (B) A transheterozygous $cup^{\Delta 212}$/+; *DfBSC113 (eIF4E)*/+ mutant has numerous satellite boutons. (C) RNAi knockdown of eIF4E in a $cup^{\Delta 212}$/+ background also shows the satellite bouton phenotype. (D) Box plots showing number of satellite boutons per muscle 4 NMJs in various genotypes. Partial knockdown of both *cup* and *eIF4E* together greatly increases the number of satellite boutons. Wild type (WT; n = 157), *DfBSC113 (eIF4E)*/+ n = 48; $cup^{\Delta 212}$/+ (n = 43); $cup^{\Delta 212}$/+; *DfBSC113 (eIF4E)*/+ (n = 55); cup^{20}/+ (n = 45); *eIF4E RNAi (GD)*/+ (n = 31); $cup^{\Delta 212}$/+; *eIF4E RNAi (GD)*/+ (n = 30); *cup20*/+; *eIF4E RNAi (GD)*/+ (n = 39); *eIF4E RNAi (TRiP)*/+ (n = 72); *C155-Gal4; eIF4E RNAi (TRiP)* (n = 64). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. Significance of satellite bouton numbers as determined by the K–S test is shown with asterisks. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. $p > 0.05$. Scale bar: 5 μm.

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examined synaptic phosphorylated Mad (pMad), which is a marker of pathway activation at presynaptic terminals (Aberle et al., 2002; Marques et al., 2002). We stained wild-type and *cup* mutant larvae with antibody against pMad. We found that pMad immunoreactivity was increased in $cup^{\Delta 212}/DfBSC7$ as compared to control NMJs (Fig. 6A1, B1). The levels of anti-HRP reactivity were unchanged between mutant and control animals (Fig. 6A2, B2).

The increase in pMad levels in *cup* mutants suggests that Cup and BMP signaling may function together to regulate satellite bouton formation. We investigated this further using genetic interaction analysis. We made transheterozygotes for *Daughters against decapentaplegic* (*Dad*), a negative regulator of BMP signaling (Sweeney and Davis, 2002), and *cup*. We reasoned that if BMP signaling is involved in Cup's function, then the allelic combination of *cup* and *dad* should have a greater number of satellite boutons, as compared to single heterozygote controls. This was in fact observed: satellite boutons are increased by ~5-fold in $cup^{\Delta 212}/+; Dad^{1jE4}/+$ as compared to heterozygotes, $cup^{\Delta 212}/+$ ($D = 0.50$, $p < 0.001$) or $Dad^{1jE4}/+$ ($D = 0.56$, $p < 0.001$; Fig. 6C).

2.6. The endocytic protein Endophilin may be a Cup target

During oogenesis, Cup is required for localization and translational repression of *osk* mRNA prior to when it reaches its final posterior location in the oocyte (Nakamura et al., 2004). Cup is also required for efficient translation of posteriorly localized *osk* mRNA (Ottone et al., 2012). In order to identify potential targets in the neuromuscular system, we reasoned that partial loss of Cup might result in abnormal expression of proteins encoded by target mRNAs. As an initial step towards identifying targets of Cup in the nervous system, we searched the literature on satellite boutons to see which genes produced strong satellite bouton phenotypes when mutated. Some of these genes encode proteins that are involved in endocytosis and/or in the organization of the actin cytoskeleton in the presynaptic terminal (Dickman et al., 2006; Koh et al., 2004; Marie et al., 2004; Rodal et al., 2011). We tested four of these (*Endophilin A (EndoA*; also referred to as *Endo*), *Synaptojanin (Synj)*, *Dap160*, and *Wiskott–Aldrich Syndrome protein (WASp)*) for genetic interactions with *cup* by quantitating satellite boutons in heteroallelic combinations involving *cup* and each of the potential target genes. The most statistically significant interaction was with *Endo*, which showed approximately a 3-fold increase when combined with *cup* as compared to $endo^{\Delta 4}/+$ ($D = 0.60$, $p < 0.001$) or $cup^{\Delta 212}/+$ ($D = 0.64$, $p < 0.001$) alone (Fig. 7D). $cup^{\Delta 212}/+$,*DfBSC302 (dap160)*/+ transheterozygotes also had a statistically significant increase in satellite bouton numbers compared to $cup^{\Delta 212}/+$ ($D = 0.36$, $p < 0.01$) and *DfBSC302 (dap160)*/+ ($D = 0.29$, $p < 0.05$). Transheterozygotes of $cup^{\Delta 212}$ and *synj* or *WASp* did not show any statistically different number of satellite boutons when compared to their heterozygous controls.

Since *EndoA* and *cup* showed the strongest interaction, and a good antibody against EndoA was available (Verstreken et al., 2002), we next investigated if EndoA protein levels were altered in *cup* mutants. Indeed, EndoA NMJ staining in $cup^{20}/cup^{\Delta 212}$ is decreased when compared to wild type (Fig. 7E1, F1). This reduction in EndoA immunoreactivity at the NMJ was statistically significant ($D = 0.72$, $p < 0.01$, Fig. 7G).

3. Discussion

Maternal translational regulators that function during oogenesis and early embryo development, including Pum, Nos, FMRP (dFmr1), Staufen, and Brat, are also zygotically expressed at the larval NMJ and have functions in the neuromuscular system (Gardiol and St Johnston, 2014; Menon et al., 2009; Menon et al., 2004; Shi et al., 2013; Zhang et al., 2001). Here we show that Cup, another of these maternal regulators, controls larval NMJ structure and function. Cup is localized to the cytoplasm of CNS neurons and in punctate structures within presynaptic NMJ terminals (Fig. 1). *cup* mutants have strong NMJ phenotypes characterized by the accumulation of small satellite boutons that bud from parent boutons or axonal segments (Fig. 2). Satellite bouton phenotypes are also observed when *cup* is knocked down with *cup* RNAi in presynaptic neurons, but not in postsynaptic muscles (Fig. 3D). Moreover, the satellite boutons phenotype in *cup* mutants is only rescued by presynaptic, and not by postsynaptic, expression of Cup (Fig. 3). *cup* mutant NMJs also display an increase in the frequency of spontaneous release events (minis), but have no change in evoked responses (Fig. 4).

Cup interacts physically and genetically with eIF4E, the cap-binding protein, which is required for translation in all cells (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004). We tested whether the Cup-eIF4E interaction is important for NMJ development by examining the phenotypes of transheterozygotes

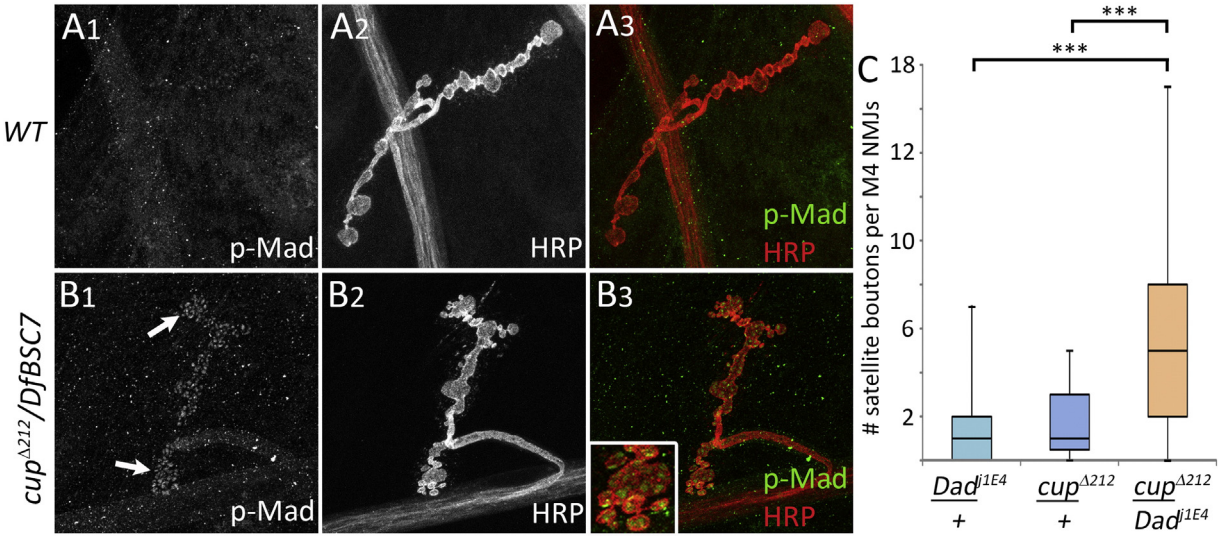

Fig. 6. BMP signaling is elevated in cup mutants. (A1–A3) Wild type larva, muscle 4 NMJ. (A) pMad staining at the NMJ is almost undetectable (NMJ outline is not visible with anti-pMad in A1). (A2) Anti-HRP staining of neuronal membranes. (A3) Overlay of pMad and anti-HRP staining from A1 and A2. (B1–B3) A $cup^{\Delta 212}$/DfBSC7 mutant. (B1) pMad staining is greatly increased compared to (A1); the NMJ outline is clearly visible. (B2) Anti-HRP staining. (B3) Overlap of pMad and anti-HRP staining. Anti-HRP was normalized between A2 and B2 in order to accurately compare pMad intensities. Inset, pMad puncta within satellite boutons. (C) Box plots showing number of satellite boutons per muscle 4 NMJs in $cup^{\Delta 212}/+$, $Dad^{j1E4}/+$, and $cup^{\Delta 212}/+; Dad^{j1E4}/+$ transheterozygotes. $Dad^{j1E4}/+$ ($n = 55$); $cup^{\Delta 212}/+; Dad^{j1E4}/+$ ($n = 69$); $cup^{\Delta 212}/+$ ($n = 43$). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. Significance of satellite bouton numbers as determined by the K–S test is shown with asterisks. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. $p > 0.05$.

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lacking one copy of each gene. We also reduced eIF4E expression in neurons using RNAi in a *cup* heterozygous background. In both cases, the resulting larvae with reductions in Cup and eIF4E had strong satellite bouton phenotypes. Neuronal knockdown of eIF4E alone also produced the phenotype (Fig. 5).

The presence of satellite boutons is often associated with elevation of retrograde BMP signaling from the muscle to the presynaptic terminal. To evaluate whether *cup* behaves genetically as a regulator of the BMP pathway, we examined transheterozygotes missing one wild-type copy of *cup* and one copy of *Dad*, a negative regulator of BMP signaling. *cup*/+, *Dad*/+ transheterozygotes displayed strong satellite bouton phenotypes. NMJs in cup mutants also showed elevated levels of synaptic pMad, a marker for activation of BMP signaling (Fig. 6).

A number of genes for which mutations produce satellite boutons encode proteins involved in endocytosis and the organization of the presynaptic actin cytoskeleton. It is thought that endocytic mutants have satellite bouton phenotypes because they have excessive levels of BMP receptors on the plasma membrane of presynaptic terminals (O'Connor-Giles and Ganetzky, 2008). We examined whether genes encoding endocytic/cytoskeletal proteins might be Cup targets by making transheterozygotes between *cup* mutations and mutations in four of these genes: *EndoA*, *Synj*, *Dap160*, and *WASp*. The strongest interaction was with the *EndoA* gene, which encodes an Endophilin protein (Fig. 7).

During oogenesis, Cup regulates translation of localized *osk* and *nos* mRNAs. Cup represses translation of *osk* mRNA until it arrives at its appropriate posterior location within the oocyte (Wilhelm et al., 2003). *nos* mRNA is concentrated at the posterior pole of the oocyte, but is also present in the remainder of the oocyte cytoplasm. However, unlocalized *nos* mRNA is not translated. In the absence of Cup, Osk and Nos proteins are translated from unlocalized mRNAs and are present in the anterior regions of the oocyte or embryo, resulting in pattern formation defects (Nakamura et al., 2004; Nelson et al., 2004; Verrotti and Wharton, 2000; Wilhelm et al., 2003). Cup does not bind to mRNA directly, but is recruited to *osk* and *nos* mRNAs by sequence-specific RNA-binding proteins (Nakamura et al., 2004; Nelson et al., 2004).

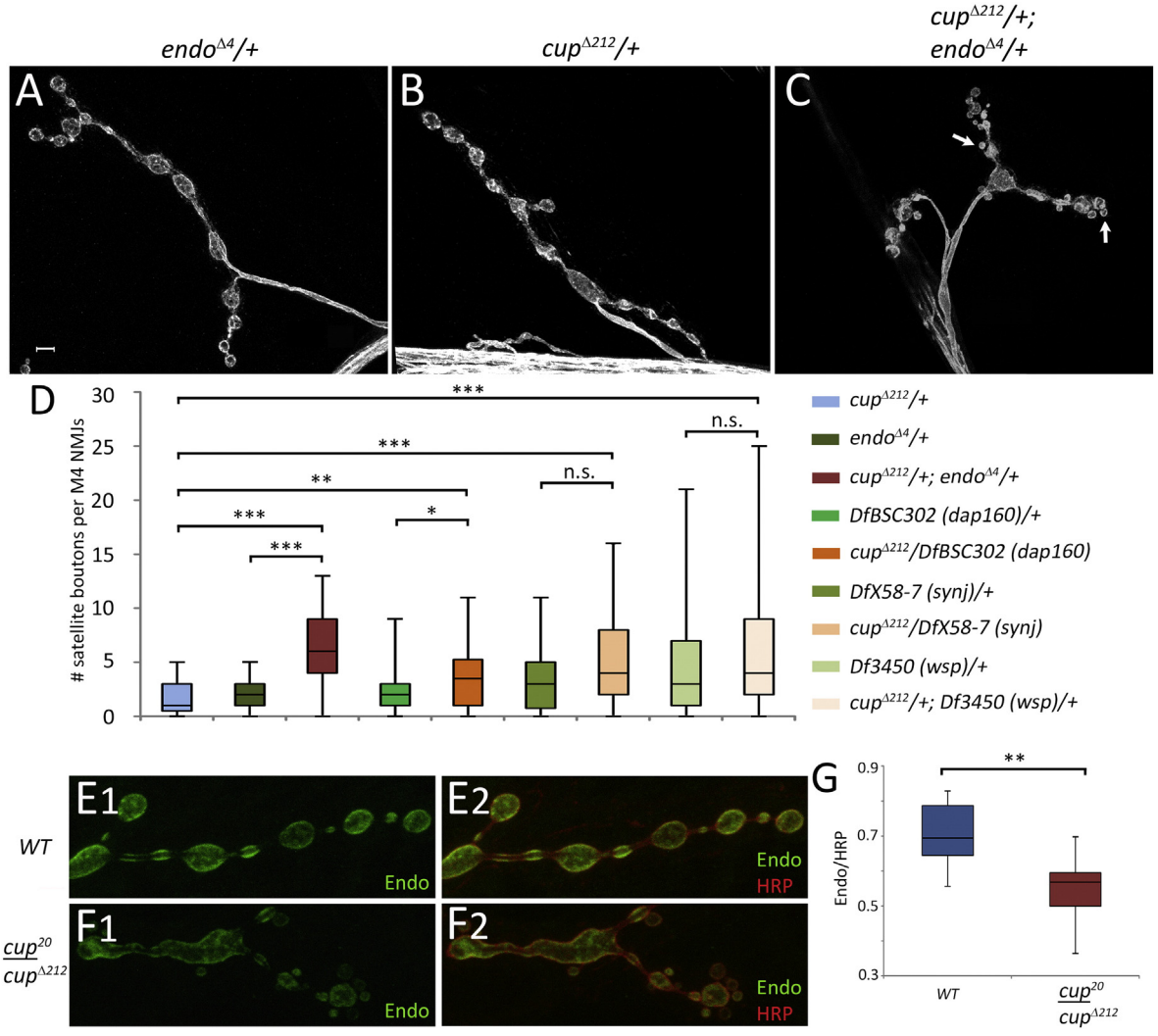

Fig. 7. Genetic interactions between *cup* and genes encoding modulators of synaptic BMP signaling. *endo* is another name for EndoA, and *wsp* is another name for *WASp*. (A) $endo^{\Delta4}/+$ muscle 4 NMJs look similar to wild type (see Fig. 3). (B) $cup^{\Delta212}/+$also shows no phenotype. (C) A $cup^{\Delta212}/+$; $endo^{\Delta4}/+$ transheterozygote has excess satellite boutons (arrows). (D) Box plots showing number of satellite boutons per muscle 4 NMJs in *cup* and heterozygotes of candidate genes and $cup^{\Delta212}/+$, *candidate gene*/+ transheterozygotes. $cup^{\Delta212}/+$; $endo^{\Delta4}/+$ (n = 40); $cup^{\Delta212}$/*DfBSC302 (Dap160)*/+ (n = 40); $cup^{\Delta212}$/*DfX58-7 (Synj)* (n = 69); $cup^{\Delta212}/+$; *Df3450 (wsp)*/+ (n = 53); $endoA^{\Delta4}/+$ (n = 40); *DfBSC302 (Dap160)*/+ (n = 96); *DfX58-7 (Synj)*/+ (n = 48); *Df3450 (wsp)*/+ (n = 125); $cup^{\Delta212}/+$ (n = 43). 25th to 75th percentiles (boxes), medians (line in boxes) and ranges (whiskers, 1.5 times the interquartile range extended from both ends of the box) are shown for each genotype. (E, F) Endophilin expression at the NMJ in wild-type (E) and in a *cup* mutant (F). Endophilin staining (green) is brighter in wild-type (E1) than in $cup^{20}/cup^{\Delta212}$ (F1). Endophilin staining was normalized to anti-HRP staining intensity (red in (E2, F2)). (G) Box plots showing that Endophilin staining intensity differs significantly between wild-type and mutant NMJs. *WT* (n = 14); $cup^{20}/cup^{\Delta212}$ (n = 18). Significance of all data sets as determined by the K–S test is shown with asterisks. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. $p > 0.05$. Scale bar: 5 μm.

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Cup is a component of a macromolecular complex that includes eIF4E and a variety of proteins involved in mRNA dynamics (Nakamura et al., 2004; Wilhelm et al., 2003). Cup binds to eIF4E through two binding motifs, denoted as 4E-BM1 and 4E-BM2. 4E-BM1 is required for eIF4E binding, and it interacts with the same parts of eIF4E as eIF4G and 4E-BPs. 4E-BM2 contributes to binding but is not required, and interacts with a different part of eIF4E (Kinkelin et al., 2012). Because Cup, like other 4E-BPs, can compete with eIF4G for binding to eIF4E and thereby prevent formation of the eIF4F complex, it was thought that this was the mechanism by which Cup represses translation of *osk* and *nos* mRNAs. However, *cup* and *eIF4E* genetically interact during oogenesis in a manner that suggests that eIF4E function is reduced, not increased, in a *cup* mutant (Zappavigna et al., 2004). Consistent with this, eIF4E is depleted from the posterior pole in *cup* mutants (Wilhelm et al., 2003; Zappavigna et al., 2004). Also, even correctly localized *osk* mRNA may be translated less efficiently in *cup* mutants than in wild-type, because Osk staining at the posterior pole is decreased in the mutants (Nakamura et al., 2004; Wilhelm et al., 2003).

Igreja and Izaurralde (2011) showed that eIF4E binding is not required for translational repression by Cup. Rather, Cup promotes mRNA deadenylation and represses translation via eIF4E-independent mechanisms. Based on these findings, these authors proposed that Cup's binding to eIF4E brings eIF4E to mRNAs, so that translation can begin as soon as Cup repression is relieved, and thereby increases the efficiency of translation of localized mRNAs. This is consistent with our findings in the neuromuscular system, where we show that eIF4E function is reduced when one copy of the *cup* gene is mutant (Fig. 5). Furthermore, *cup* interacts genetically with potential target genes in a manner that suggests that it is a positive rather than a negative regulator of function (Fig. 7).

What is Cup's function in motor neurons? Our genetic data suggest that one function of Cup is to positively regulate endocytic proteins that are modulators of retrograde BMP signaling. Consistent with this, there is a small but statistically significant decrease in Endophilin protein levels at the NMJ in *cup* mutants (Fig. 7). We suggest that the *cup* satellite bouton phenotype is the consequence of simultaneous down-regulation of expression of a number of BMP modulators, and that the amount of any single modulator protein is not dramatically altered in *cup* mutants. Like Endophilin, correctly localized Osk is reduced but not eliminated in *cup* mutants (Nakamura et al., 2004; Wilhelm et al., 2003), so the increase in translation efficiency that Igreja and Izaurralde (2011) propose is contributed by Cup-eIF4E interactions may be a relatively subtle effect.

Since Cup's function during oogenesis is to regulate translation of localized mRNAs, this raises the question of whether Cup's mRNA targets in motor neurons are also subcellularly localized, and, if so, to which compartment(s). Cup localizes to punctate structures in the NMJ presynaptic terminal. An epitope-tagged version of eIF4E expressed in motor neurons is also detected in puncta at presynaptic terminals (data not shown). This overexpression experiment, however, does not prove that endogenous eIF4E is normally localized at the NMJ. It is not possible to use anti-eIF4E staining to evaluate whether endogenous eIF4E is in presynaptic terminals, since eIF4E is present at very high levels in the underlying muscle fibers. Localization of epitope-tagged eIF4E was not visibly altered in a *cup* mutant.

Zucker and colleagues (Beaumont et al., 2001) showed that local translation occurs within presynaptic NMJ terminals in crayfish, so it is possible that this also could occur in *Drosophila*. We and others have also observed that a variety of translational regulators, including Pum, Nos, Staufen, FMRP, and Brat, localize to presynaptic terminals. If translation of localized mRNA does occur in presynaptic terminals, it might be controlled by Cup, in a similar manner to Cup's selective regulation of translation of mRNAs localized to the posterior pole of the oocyte. However, we have no evidence for this model. An alternative, and perhaps more likely, model is that mRNAs controlled by Cup are in a compartment in the motor neuron cell body that is dedicated to production of proteins that are destined for the presynaptic terminal (Fig. 8).

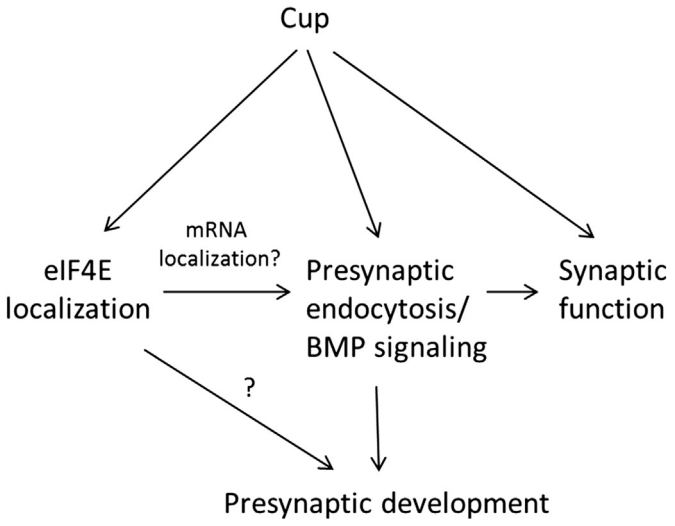

Fig. 8. Summary diagram of Cup's actions at the NMJ. Based on its functions in ovaries and on the data presented here, we suggest that Cup affects eIF4E localization in neurons and thereby regulates localization and translation of mRNAs encoding endocytic proteins that modulate BMP signaling and thereby affect presynaptic terminal development. Cup may also have other effects on these mRNAs that do not involve eIF4E. Cup and the endocytic proteins also regulate mEPSP frequency.

4. Experimental methods

4.1. Drosophila stocks

"Wild type" controls were w^{1118} crossed to *Canton S.* cup^{8} and cup^{20} alleles were provided by J. Wilhelm (University of California, San Diego, CA) (Keyes and Spradling, 1997), and $cup^{\Delta 212}$ was obtained from A. Nakamura (Institute of Molecular Embryology and Genetics, Kumamato University, Japan). $EndoA^{\Delta 4}$, *Df(Dap160)*, *Synj*, *WASp*, dad^{1jE4}, *DfBSC7(cup)*, *DfBSC113(eIF4E)*, and *DfBSC187(cup)* were obtained from the *Drosophila* Stock Center (Bloomington, IN). The Gal4 lines used were *C155* (*Elav*)-Gal4 and *elav-Gal4* (on first and third chromosome, respectively) for neuronal expression and 24B-Gal4 for muscle expression (3rd chromosome). The *cup RNAi (GL)* line was obtained from the Harvard TRiP collection (Boston, MA). The *eIF4E RNAi (GD)* line was obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007) and the *eIF4E RNAi (TRiP)* line was obtained from the Harvard TRiP collection (Boston, MA). Both of the *eIF4E* RNAi lines have leaky expression and the non-driven lines were used to cross to *cup* alleles in order to perform the genetic interaction tests. The control and experimental crosses for *eIF4E RNAi* were done at room temperature since elevated temperature seemed to restrict satellite bouton growth.

The UAS-cup transgenic line was constructed with UAS-*cup* cDNA clone kindly provided by C. Smibert (University of Toronto, Ontario, Canada). The DNA was sent to Rainbow Transgenic Flies (Camarillo, CA) for injection. We used a transgenic line with UAS-Cup on the third chromosome.

4.2. Antibodies

The primary antibodies used in this study are below. The rabbit and mouse Cup antibodies were obtained from Akira Nakamura (Institute of Molecular Embryology and Genetics, Kumamato University, Japan) and used at a dilution of 1:2000 and 1:1000, respectively (Nakamura et al., 2004). Rat anti-Cup was obtained from Allan Spradling (Carnegie Institution for Science, Baltimore, MD). Guinea pig anti-EndoA was obtained from Hugo Bellen and was used at 1:200. The Discs-Large (4F3;

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1:100) and Bruchpilot (nc82; 1:100) antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA). Mouse anti-Fas2 was used at 1:3. The rabbit pMad antiserum was kindly provided by Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) and P. ten Dijke (Leiden University, Leiden, The Netherlands) and used at a dilution of 1:100. Tetramethylrhodamine isothiocyanate-horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories) was used at 1:50. The secondary antibodies, Alexa-Fluor 488 anti-mouse, anti-rabbit, and anti-rat were used at 1:500 and Alexa-Fluor 568 anti-mouse was used at 1:500 also.

4.3. Immunohistochemistry

Third instar larvae were dissected, stained, and processed as described previously (Menon et al., 2009). For EndoA and p-Mad staining experiments, 4% paraformaldehyde was used instead of Bouin's fix (Sigma-Aldrich, St. Louis, MO) and incubated for 30 min. Larvae were then incubated with the appropriate primary and secondary antibodies and imaged on a Zeiss LSM 510 confocal microscope.

In larval preps where EndoA staining intensity was compared between *cup* mutants and controls, the HRP signal was used for normalization. Average intensity projections were created from z-stacks collected on Zeiss LSM510 and processed in Image J (NIH, Bethesda, MD). NMJs were outlined to create a region of interest and corresponding red and green channel intensities were measured. The green/red ratio was then calculated and used to compare the genotypes.

4.4. Electrophysiology

Third instar larvae were dissected as described in Carrillo et al. (2010). The dissection was performed in HL3.1 solution containing (in mM): 70 NaCl, 5 KCl, 4 $MgCl_2$, 12.5 $CaCl_2$, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.2. For recording, HL3.1 solution was also used with 1 mM $CaCl_2$. Excitatory postsynaptic potentials (EPSPs) were recorded using 20–30 MΩ microelectrodes filled with 3 M KCl. Evoked EPSPs were recorded from ventral longitudinal muscles by using a 2–4 μm suction electrode to stimulate nerve branches. Recordings were obtained from muscles 6 and 7 in segments 3–5. The average EPSP amplitude was calculated from 10 sequential EPSPs per muscle at a frequency of 0.5 Hz. Miniature EPSPs were recorded for 20 s and the number of events and amplitudes was analyzed using Synaptosoft (by J. Lee; Synaptosoft). Both evoked and spontaneous EPSPs were recorded using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA). The K–S test was used to determine significance.

4.5. Statistics

We used the Kolmogorov–Smirnov (K–S) test to evaluate the statistical significance of all data sets. The K–S test is a non-parametric test that calculates a D statistic, which is the maximum difference between the empirical distribution functions of two data sets. The p value significance is as follows: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, n.s. $p > 0.05$.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2015.06.010.

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