

# The ALS gene *FUS* regulates synaptic transmission at the *Drosophila* neuromuscular junction

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Mutations in the RNA binding protein *Fused in sarcoma* (*FUS*) are estimated to account for 5–10% of all inherited cases of amyotrophic lateral sclerosis (ALS), but the function of *FUS* in motor neurons is poorly understood. Here, we investigate the early functional consequences of overexpressing wild-type or ALS-associated mutant *FUS* proteins in *Drosophila* motor neurons, and compare them to phenotypes arising from loss of the *Drosophila* homolog of *FUS*, *Cabeza* (*Caz*). We find that lethality and locomotor phenotypes correlate with levels of *FUS* transgene expression, indicating that toxicity in developing motor neurons is largely independent of ALS-linked mutations. At the neuromuscular junction (NMJ), overexpression of either wild-type or mutant *FUS* results in decreased number of presynaptic active zones and altered postsynaptic glutamate receptor subunit composition, coinciding with a reduction in synaptic transmission as a result of both reduced quantal size and quantal content. Interestingly, expression of human *FUS* downregulates endogenous *Caz* levels, demonstrating that *FUS* autoregulation occurs in motor neurons *in vivo*. However, loss of *Caz* from motor neurons increases synaptic transmission as a result of increased quantal size, suggesting that the loss of *Caz* in animals expressing *FUS* does not contribute to motor deficits. These data demonstrate that *FUS/Caz* regulates NMJ development and plays an evolutionarily conserved role in modulating the strength of synaptic transmission in motor neurons.

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and uniformly fatal neurodegenerative disease primarily affecting motor neurons. About 10% of ALS cases are familial (fALS), of which the majority shows autosomal dominant inheritance (1). Mutations in several RNA binding proteins have recently been identified that cause fALS, and increasing evidence suggests that alterations in RNA metabolism may be critical to pathogenesis (2–7). The remaining 90% of ALS cases are sporadic (sALS); however, motor neurons of these patients have cytoplasmic neuronal inclusions containing the RNA binding protein TDP-43 (8), suggesting that alterations in RNA metabolism are also present in sALS.

Fused in sarcoma (*FUS*) is an RNA binding protein of the FET family that has been implicated in many aspects of RNA biology, both in the nucleus and in the cytoplasm, including splicing, transcription, translation and noncoding RNA biogenesis (9–11). Over 20 mutations in *FUS* have been identified in fALS (12), and most of these mutations cluster within the C-terminal

nuclear localization signal, resulting in a loss of *FUS* from the nucleus and the formation of cytoplasmic aggregates containing *FUS* in ALS patients and in animal models of *FUS*-ALS (13–17). Notably, two other proteins of the FET family, *EWS* and *TAF15*, have also been linked to ALS (7,18), highlighting the importance of these RNA binding proteins in the pathogenesis of ALS. The normal function of *FUS* in motor neurons is poorly understood, and it remains unclear whether ALS-associated mutations in *FUS* cause a loss-of-function due to depletion of *FUS* from the nucleus, a toxic gain-of-function due to increased cytoplasmic localization and/or aggregation, or a combination of both. In *Drosophila*, either a null mutation of the fly homolog of *FUS*, termed *cabeza* (*caz*) (19,20) or overexpression of human *FUS* (19,21–24) in flies causes locomotor defects and disruption of eye morphology. Furthermore, overexpression of either ALS-associated mutant or wild-type *FUS* results in progressive loss of locomotion and early lethality (19,22,23).

In this study, we investigated the effects of either loss of endogenous *caz* or overexpression of wild-type or mutant human *FUS* on the structure and function of the neuromuscular junction

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(NMJ) in *Drosophila* third-instar larvae. As alterations in synaptic transmission at the NMJ are some of the earliest changes observed in ALS mouse models and patients (25–27), we focused our efforts on the highly accessible larval NMJ in order to investigate the normal function of FUS/Caz at the NMJ and to determine the early physiologic changes in neurons before they begin to degenerate. Here, we find that expression of either wild-type or mutant human FUS reduces the level of endogenous *Drosophila* Caz in motor neurons, alters the structure of the NMJ, and inhibits synaptic transmission. Conversely, we find that the loss of Caz results in enhanced synaptic transmission without overt changes in NMJ structure. Our findings suggest that FUS/Caz has a conserved function in modulating synaptic structure and function, and identifies deficits in synaptic transmission that precede the onset of neurodegeneration.

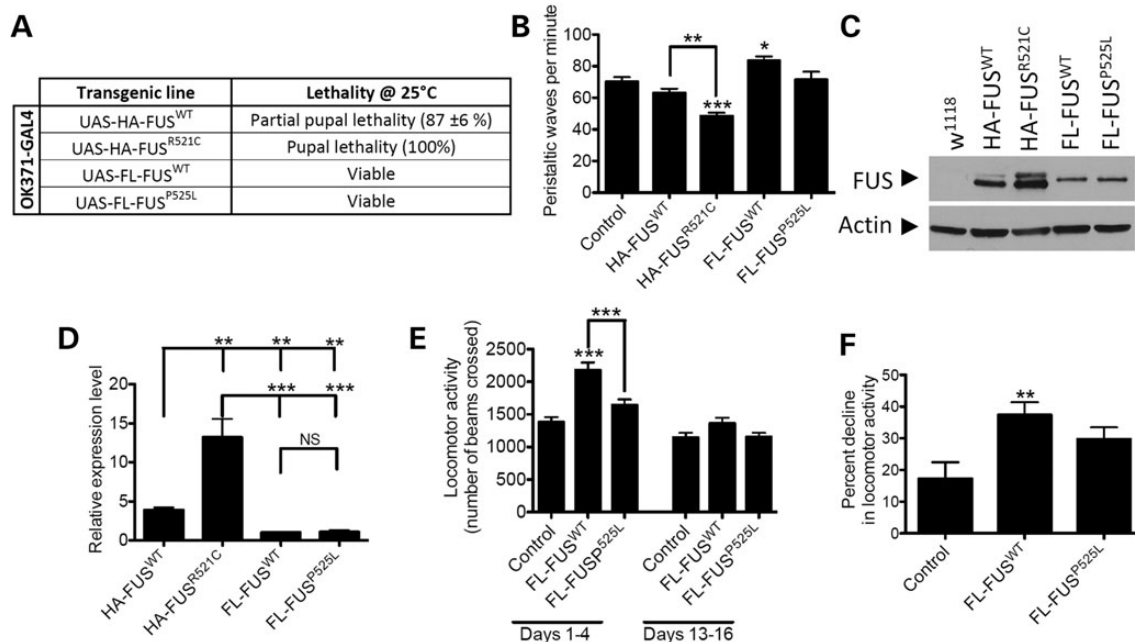
## RESULTS

### Lethality and locomotor deficits correlate with FUS expression levels

To investigate the consequences of FUS overexpression in motor neurons, we used two independently generated sets of transgenic *UAS-FUS* lines expressing under control of *OK371-GAL4*. Lanson *et al.* (23) generated randomly inserted transgenic lines

expressing HA-tagged FUS (HA-FUS) utilizing the *pUAST* vector and concluded that the ALS-associated mutations caused a toxic gain-of-function. In contrast, Wang *et al.* (19) generated site-directed insertions expressing flag-tagged FUS (FL-FUS) using the *pBid* vector and concluded that ALS-associated mutations cause loss-of-function. Consistent with these prior reports, we find that expression of HA-FUS in motor neurons results in pupal lethality (HA-FUS<sup>R521C</sup> or HA-FUS<sup>R521H</sup>) or partial pupal lethality (HA-FUS<sup>WT</sup>) (23), whereas expression of either FL-FUS<sup>WT</sup> or FL-FUS<sup>P525L</sup> (19) is viable to the adult stage without gross phenotypes (Fig. 1A). Next, we assayed larval motility in animals expressing FUS in motor neurons and found that HA-FUS<sup>WT</sup> overexpression has no significant effect, whereas HA-FUS<sup>R521C</sup> causes a decrease in motility (Fig. 1B), consistent with previous studies demonstrating larval locomotor defects in FUS<sup>R521C</sup>-expressing animals (23). In contrast, animals expressing FL-FUS<sup>WT</sup> but not FL-FUS<sup>P525L</sup> demonstrate a slight increase in motility, suggesting that the P525L mutation may cause a loss of FUS function.

Next, we compared protein and RNA expression level in these transgenic lines to determine if this could explain the marked difference in lethality and larval motility. We took advantage of the temperature-dependence of the UAS/GAL4 system to decrease expression levels by raising animals at 18°C to avoid cell death during development (28), allowing viability of HA-FUS lines to



**Figure 1.** FUS toxicity correlates with expression level. (A) Expression of HA-FUS<sup>WT</sup> in motor neurons using the *OK371-GAL4* driver results in 87 ± 6% of pupae dying as pharate adults, whereas HA-FUS<sup>R521C</sup> causes 100% pupal lethality at 25°C. This is in contrast to FL-FUS transgenic lines which are completely viable at 25°C. (B) Expression of HA-FUS<sup>R521C</sup> but not HA-FUS<sup>WT</sup> results in impaired larval motility as assayed by the number of peristaltic muscle waves per minute, whereas expression of FL-FUS<sup>WT</sup> but not FL-FUS<sup>P525L</sup> increases motility. For *OK371-GAL4*, *N* = 13; *OK371>HA-FUS<sup>WT</sup>*, *N* = 7; *OK371>HA-FUS<sup>R521C</sup>*, *N* = 7; *OK371>FL-FUS<sup>WT</sup>*, *N* = 7; *OK371>FL-FUS<sup>P525L</sup>*, *N* = 7. (C) Western blot analysis of FUS protein expression from flies expressing FUS under the control of *OK371-GAL4* raised at 18°C to prevent pupal lethality shows markedly increased expression of HA-FUS<sup>R521C</sup> protein relative to HA-FUS<sup>WT</sup> protein and lower but equivalent protein levels of FL-FUS<sup>WT</sup> and FL-FUS<sup>P525L</sup>. (D) Transcript expression level was quantified using qRT-PCR from RNA extracted from the heads of adult flies expressing indicated FUS transgenes under the control of the *OK371-GAL4* driver. (E) Adult locomotor activity as determined by single beam activity monitors at two time points: days 1–4 and days 13–16. A significant increase in activity is seen in *OK371>FL-FUS<sup>WT</sup>* but not *OK371>FL-FUS<sup>P525L</sup>*. (F) The rate of activity decline expressed as a fraction of initial activity was determined by dividing the number of beam crossings for days 13–16 by the number of beam crossings for days 1–4. Expression of FL-FUS<sup>WT</sup> but not FL-FUS<sup>P525L</sup> resulted in a significant decrease in motility over time as compared with *OK371-GAL4/+* controls. *N* = >45 for all genotypes. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Bonferroni posttest for multiple comparisons. \*\*\**P* < 0.001, \*\**P* < .01 and \**P* < 0.05.

adulthood. Surprisingly, under these conditions, we find that the *HA-FUS<sup>R521C</sup>* line expresses FUS protein at much higher levels than the *HA-FUS<sup>WT</sup>* line, whereas the site-directed *FL-FUS<sup>P525L</sup>* and *FL-FUS<sup>WT</sup>* lines express equivalent low levels of FUS protein (Fig. 1C). Furthermore, the *HA-FUS<sup>WT</sup>* line expresses increased protein compared with the *FL-FUS<sup>WT</sup>* line, consistent with the increased severity of phenotypes observed with *HA-FUS<sup>WT</sup>* as compared with *FL-FUS<sup>WT</sup>*. These changes in FUS protein expression are due to differences in message level, as the *HA-FUS<sup>R521C</sup>* line expresses  $\sim 3\times$  higher RNA levels than *HA-FUS<sup>WT</sup>*, and the *HA-FUS<sup>WT</sup>* line expresses  $\sim 4\times$  higher RNA levels than *FL-FUS<sup>WT</sup>* (Fig. 1D). Thus, we conclude that when expressed in motor neurons, *HA-FUS<sup>R521C</sup>* transgenic lines express at higher levels than *HA-FUS<sup>WT</sup>*, and that the severity of toxicity caused by FUS overexpression (Fig. 1A) most strongly correlates with levels of FUS expression rather than the presence of the disease-associated mutation.

Given the marked toxicity of *HA-FUS<sup>WT</sup>* overexpression as well as the differences between levels of *HA-FUS<sup>WT</sup>* and *HA-FUS<sup>R521C</sup>* expression in these transgenic lines, we reasoned that the lower expressing *FL-FUS* lines are likely better suited to model the human disease. A previous study found that pan-neuronal expression of *FL-FUS<sup>P525L</sup>* has no effect on lifespan (19), so we sought to determine whether expression of *FL-FUS* in motor neurons alters locomotor behavior with aging using a *Drosophila* activity monitoring (DAM) system (29). Interestingly, overexpression of *FL-FUS<sup>WT</sup>* but not *FL-FUS<sup>P525L</sup>* in motor neurons leads to significantly increased locomotor activity in young flies (1–4 days old, Fig. 1E). A similar increase in locomotor activity was observed in third-instar larvae with *FL-FUS<sup>WT</sup>* expression, but not with *FL-FUS<sup>P525L</sup>* (Fig. 1B). Furthermore, *FL-FUS<sup>WT</sup>* expression leads to a greater decline in locomotor activity with age when compared with controls or to *FL-FUS<sup>P525L</sup>*-expressing flies (Fig. 1E and F). These data are

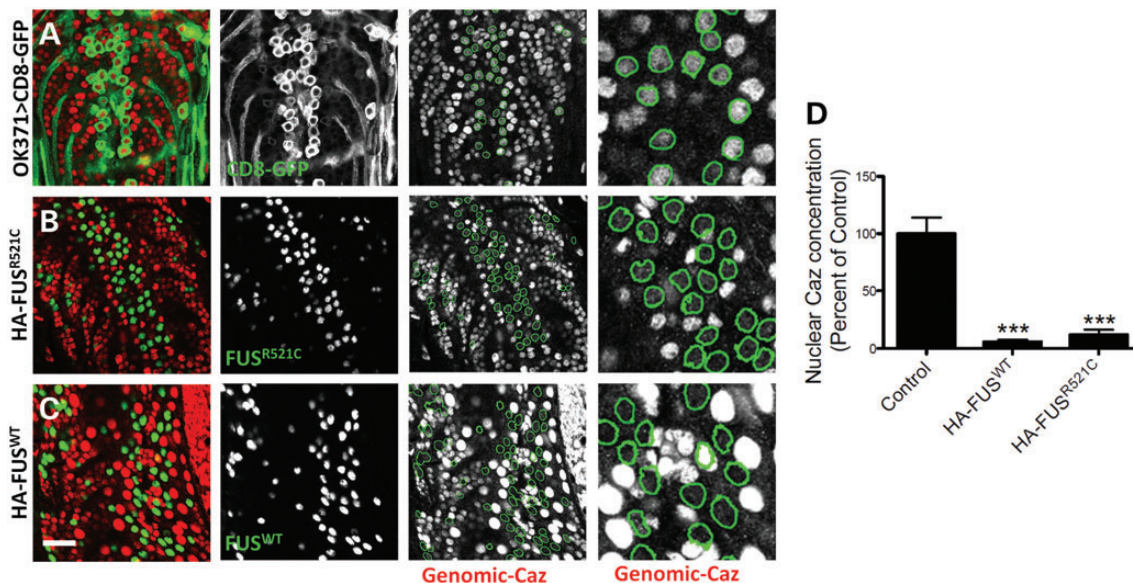
consistent with the larval locomotor phenotypes and suggest that the P525L mutation causes a loss-of-function of FUS.

### FUS overexpression depletes nuclear Caz

The toxicity associated with high-level expression of wild-type FUS suggests that a narrow range of FUS activity is normally maintained. Precise expression levels of FUS and TDP-43 are important for normal cellular function (1), and both TDP-43 and FUS have been shown to autoregulate their expression (30–32), suggesting that exogenous FUS expression might alter the expression of its *Drosophila* homolog, *caz*. Indeed, *caz* contains a consensus GUGGU FUS binding site (33). Consistent with previous studies (19), we confirm that endogenous Caz protein (labeled with a Flag-tagged genomic construct) (19) is normally present in all motor neuron nuclei, identified with *OK371-GAL4* driving expression of the membrane marker mCD8-GFP (Fig. 2A) and colocalization with 4',6-diamidino-2-phenylindole (DAPI) (Supplementary Material, Fig. S1). Interestingly, when either *HA-FUS<sup>R521C</sup>* (Fig. 2B) or *HA-FUS<sup>WT</sup>* (Fig. 2C) is overexpressed, endogenous Caz is reduced by  $\sim 90\%$  in motor neuron nuclei (outlined in green, quantified in Fig. 2D). These data indicate that expression of human FUS leads to the downregulation of the endogenous expression of its *Drosophila* ortholog Caz, demonstrating that FUS autoregulation occurs in motor neurons *in vivo* and is evolutionarily conserved.

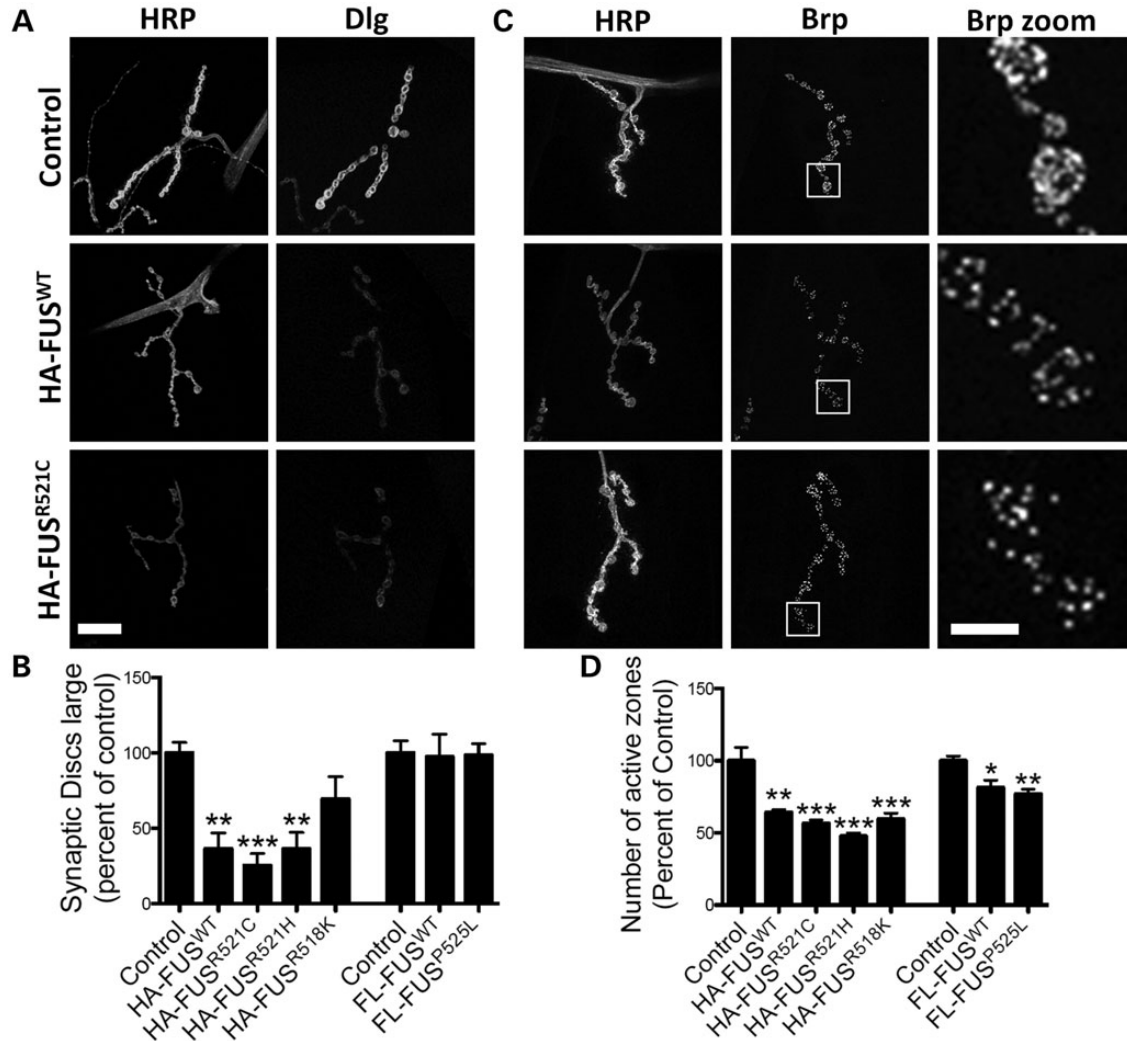
### FUS overexpression results in loss of presynaptic active zones and postsynaptic Discs large (Dlg)

To investigate the mechanism of motor neuron dysfunction as a consequence of FUS expression, we assayed the morphology and development of synaptic terminals of the larval NMJ. Similar to what has been previously reported (23), we find that



**Figure 2.** FUS expression downregulates nuclear Caz concentration. (A) Expression of mCD8-GFP under the control of the *OK371-GAL4* driver in animals expressing Flag-tagged Caz (red) under the control of the endogenous enhancer (*gen-FL-Caz*) confirms that Caz is expressed in motor neurons and localizes to nuclei. Expression of either *HA-FUS<sup>R521C</sup>* (B) or *HA-FUS<sup>WT</sup>* (C) with *OK371-GAL4* results in the loss of Caz protein (detected with anti-Flag staining) from the nucleus specifically in cells expressing *HA-FUS* transgenes (outlined in green), as quantified in (D). *OK371-GAL4/UAS-mCD8-GFP* was used as the control.  $N = 4$  for all genotypes. Scale bar = 25  $\mu\text{m}$ . Statistical analysis was performed using one-way ANOVA with a Bonferroni posttest for multiple comparisons. \*\*\* $P < 0.001$ .





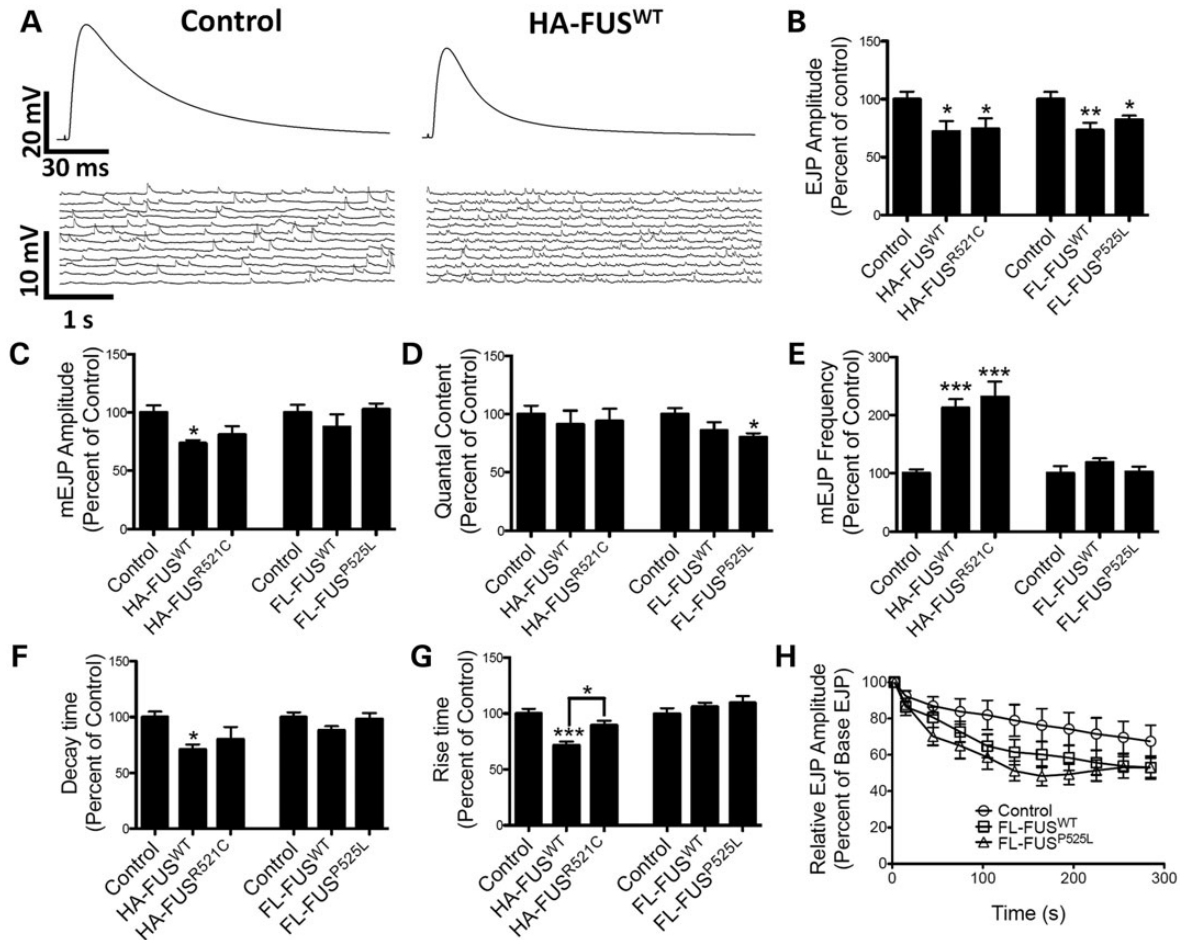
**Figure 3.** FUS expression disrupts the expression of pre- and postsynaptic structural proteins. (A) Representative images of Dlg and HRP stainings of type Ib boutons of animals overexpressing indicated FUS transgenes. (B) Expression of HA-FUS<sup>R521C</sup>, HA-FUS<sup>R521H</sup>, or HA-FUS<sup>WT</sup>, but not HA-FUS<sup>R518K</sup> or FL-FUS transgenes results in a significant reduction in the level of postsynaptic Dlg (HA-FUS lines,  $N = 5$ ; FL-FUS lines,  $N = 7$ ). (C) Active zone component Bruchpilot (Brp) was labeled with HRP counter stain in the indicated genotypes. (D) Overexpression of all HA-FUS transgenic lines and FL-FUS transgenic lines result in a significant reduction in the number of active zones (HA-FUS lines,  $N = 3$ ; FL-FUS lines,  $N = 7$ ). *OK371-GAL4/+* was used for the genotype of control larvae in all of the experiments. Scale bar = 20  $\mu\text{m}$  for (A) and 5  $\mu\text{m}$  for (C, last column of panels, 'Brp zoom'). A one-way ANOVA with a Bonferroni posttest for multiple comparisons was performed to determine statistical significance. \*\*\* $P < 0.001$  and \*\* $P < 0.01$ .

expression of wild-type or mutant FUS does not significantly affect synaptic terminal size relative to muscle size (Supplementary Material, Fig. S2A). However, we found a  $\sim 70\%$  reduction in the level of the postsynaptic scaffolding protein Dlg, the invertebrate homolog of PSD-95, in animals expressing HA-FUS<sup>WT</sup>, HA-FUS<sup>R521C</sup> or HA-FUS<sup>R521H</sup> (Fig. 3A and B). Furthermore, we noted in these same animals a  $\sim 3$ -fold increase in the number of interbouton regions that label with anti-HRP but are devoid of Dlg (Supplementary Material, Fig. S2B and C). Such long intervening sections of motor axons between boutons have been described in Neurexin (*nrx1*) and Neuroligin (*nlg1*) mutants (34,35). Since expression of FUS in motor neurons results in altered Dlg expression in muscle cells, these data indicate that FUS overexpression has non-cell autonomous effects at the NMJ.

Next, we investigated the effect of FUS expression on pre-synaptic active zones by assaying the level and localization of Bruchpilot (Brp) (36,37), a major structural component of active zones. Interestingly, we find that both HA-FUS<sup>WT</sup> and HA-FUS<sup>R521C</sup> expressing animals display a  $\sim 50\%$  reduction in the number of presynaptic active zones (Fig. 3C and D). Similar phenotypes are observed in FUS<sup>R521H</sup> and FUS<sup>R518K</sup> expressing lines, and to a lesser extent, in lower expressing FL-FUS lines (Fig. 3D). Thus, overexpression of FUS in presynaptic motor neurons results in changes in both pre- and postsynaptic scaffolding proteins required for normal synaptic transmission.

#### FUS overexpression impairs synaptic transmission

To address functional consequences of FUS overexpression, we assayed synaptic transmission at the larval NMJ. We find that



**Figure 4.** FUS expression impairs synaptic transmission. (A) Representative electrophysiological traces of two genotypes quantified in B, C, D and E. Upper traces—EJP; lower traces—mEJP. (B) Expression of either HA-FUS or FL-FUS results in decreased EJP amplitude, independent of the presence of mutations. (C) Expression of HA-FUS<sup>WT</sup> results in decreased mEJP amplitude, demonstrating that quantal size is reduced. (D) Expression of FL-FUS<sup>P525L</sup> results in decreased quantal content. (E) Expression of either HA-FUS<sup>WT</sup> or HA-FUS<sup>R521C</sup> causes increased spontaneous mEJP frequency. (F) Measurement of the 90–10% decay time of EJPs indicates that expression of HA-FUS<sup>WT</sup> decrease decay time, but other FUS transgenes do not. Similarly, the 10–90% EJP rise time in HA-FUS<sup>WT</sup> expressing animals is decreased compared with HA-FUS<sup>R521C</sup> animals (G). (H) Expression of FL-FUS transgenes results in altered motor neuron fatigue response to 10 Hz stimuli. *OK371-GAL4/+* was used for the genotype of control larvae in all of the experiments. For *OK371>FL-FUS<sup>WT</sup>*, *N* = 8; *OK371>FL-FUS<sup>P525L</sup>*, *N* = 11, *OK371>HA-FUS<sup>WT</sup>*, *N* = 7; *OK371>HA-FUS<sup>R521C</sup>*, *N* = 9. For *OK371-GAL4/+* (controls), *N* = 10 or 11. Statistical analysis was performed using one-way ANOVA with Dunnett's posttest for multiple comparisons. \*\*\**P* < 0.001, \*\**P* < 0.01 and \**P* < 0.05.

both HA-FUS- and FL-FUS-expressing animals display a ~25% reduction in evoked excitatory junction potential (EJP) amplitude independent of the presence of ALS-associated mutations (Fig. 4A and B). EJP amplitude is a measure of the product of the quantal size (mEJP amplitude) and the quantal content (vesicles fused per EJP), and thus a reduction in EJP amplitude is due to decreased quantal size, decreased quantal content, or a combination of both. We find that FUS-expressing animals trend towards a reduction in both quantal size (Fig. 4C) and quantal content (Fig. 4D), and that each of these trends reaches statistical significance in one genotype: reduced quantal size in *OK371>HA-FUS<sup>WT</sup>* animals and reduced quantal content in *OK371>FL-FUS<sup>P525L</sup>* animals. Thus, we conclude that the reduction of EJP amplitude in FUS-expressing animals is likely due to a combination of both reduced quantal size and reduced quantal content. Interestingly, we observed a greater than 2-fold increase in mEJP frequency in HA-FUS<sup>WT</sup> and HA-FUS<sup>R521C</sup>-expressing animals compared

with control, whereas expression of FL-FUS transgenes had no effect (Fig. 4A and E).

Given the alterations in pre- and postsynaptic scaffolding proteins Brp andDlg, we hypothesized that FUS overexpression may disrupt transsynaptic cell adhesion complexes. To investigate functional coupling between the pre- and postsynaptic active zones, we investigated the kinetics of the EJP waveform. Interestingly, HA-FUS<sup>WT</sup> causes a significant reduction in both rise and decay time, whereas HA-FUS<sup>R521C</sup>, FL-FUS<sup>WT</sup>, and FL-FUS<sup>P525L</sup> did not cause a significant alteration in EJP kinetics (Fig. 4F and G). We also looked for subtle differences between FL-FUS<sup>WT</sup> and FL-FUS<sup>P525L</sup> by analyzing synaptic fatigue at 10 Hz (Fig. 4H). While animals expressing either FL-FUS<sup>WT</sup> or FL-FUS<sup>P525L</sup> fatigue faster than control animals, there was not a significant difference between animals expressing FL-FUS<sup>WT</sup> and FL-FUS<sup>P525L</sup>. Together, these physiological data demonstrate that presynaptic expression of FUS inhibits

evoked neurotransmitter release and increases the frequency of spontaneous release, and the disease-associated mutations have little effect or cause loss-of-function.

### Presynaptic FUS overexpression disrupts postsynaptic glutamate receptor subunit composition

Dlg is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that regulate the trafficking and clustering of glutamate receptors (GluRs) (38,39). Given the reduction in postsynaptic Dlg expression seen with FUS overexpression, we asked whether the reduction in mEJP amplitude was due to altered GluR expression. The *Drosophila* NMJ expresses both 'A' and 'B'-type ionotropic heterotetrameric GluRs that contain either GluRIIA (A-type) or GluRIIB (B-type) subunits in addition to nonvariable GluRIIC (also called GluRIII), GluRIID and GluRIIE subunits (40,41). Since A-type receptors are more sensitive to glutamate than B-type receptors (42), if the reduction in mEJP amplitude seen with FUS expression is due to reduced GluRII levels, we would predict that we would see either a reduction of total GluRII expression (measured by GluRIIC levels) or a decrease in the ratio of A:B type receptors. In contrast, we do not see a significant reduction in total GluRIIC levels in any genotype (Fig. 5A and B), but rather find an increase in the levels of GluRIIA in FUS<sup>WT</sup> expressing animals (Fig. 5A and C) and a reduction of GluRIIB in all FUS-expressing lines (Fig. 5A and D). This translates into an increase in the ratio of A:B type glutamate receptors in FL-FUS<sup>P525L</sup> and HA-FUS<sup>R521C</sup>-expressing larvae (Fig. 5E). These data demonstrate that the reduction in quantal size seen with FUS overexpression is not due to alterations in GluRII protein levels, and suggest that the relative increase in type A GluRs is a compensatory response.

Efficient synaptic transmission requires close apposition of pre- and postsynaptic active zones. Given the altered kinetics of evoked release and loss of Dlg from the postsynaptic density, we asked whether there was an alteration in the clustering of glutamate receptors opposite the presynaptic release sites. Indeed, there is a significant reduction in the fraction of GluRIIB and GluRIIC apposed to Brp-labeled active zones in HA-FUS<sup>R521C</sup>-expressing animals (Supplementary Material, Fig. S3). These data suggest that mutant FUS may reduce quantal size by altering GluRII clustering at the postsynaptic density.

### Loss of Caz enhances synaptic transmission

If FUS overexpression disrupts synaptic transmission through a dominant-negative mechanism or by downregulation of endogenous Caz, we would predict that we would see similar phenotypes with *caz* loss-of-function. In contrast, we find that *caz*<sup>1</sup> null alleles display increased EJP amplitude compared with controls (Fig. 6A and B), and this increase is due to a ~25% increase in mEJP amplitude (Fig. 6A and C) without affecting quantal content (Fig. 6D). We confirmed that these phenotypes are due to the loss of Caz by rescuing all electrophysiological defects of *caz*<sup>1</sup> animals with one copy of a *caz* genomic construct (Fig. 6B–E). In contrast to animals expressing FUS (Fig. 3C–D), we do not see an alteration of active zones in *caz*<sup>1</sup> animals (Supplementary Material, Fig. S4), suggesting that the alterations in

Brp morphology seen in FUS-expressing animals are not due to loss of *caz*.

To determine if the phenotypes seen in *caz*<sup>1</sup> null mutants are due to a presynaptic loss of Caz, we knocked down endogenous Caz specifically in motor neurons using a transgenic line expressing Caz<sup>RNAi</sup> under control of UAS (43). We find that targeted loss of Caz from motor neurons causes a ~20% increase in EJP amplitude compared with control (Fig. 6B), similar to that seen in *caz*<sup>1</sup> animals. This increase in EJP amplitude is due to a ~25% increase in mEJP amplitude without any significant change in quantal content (Fig. 6C and D). In contrast to what was observed with high-level FUS overexpression, *caz*<sup>1</sup> animals have normal mEJP frequency (Fig. 6E). These results suggest that FUS/Caz normally functions to inhibit evoked synaptic transmission. Because the loss of Caz has the opposite effect of FUS overexpression on synaptic transmission, these data strongly argue that wild-type and mutant FUS overexpression do not inhibit synaptic transmission by disrupting endogenous Caz.

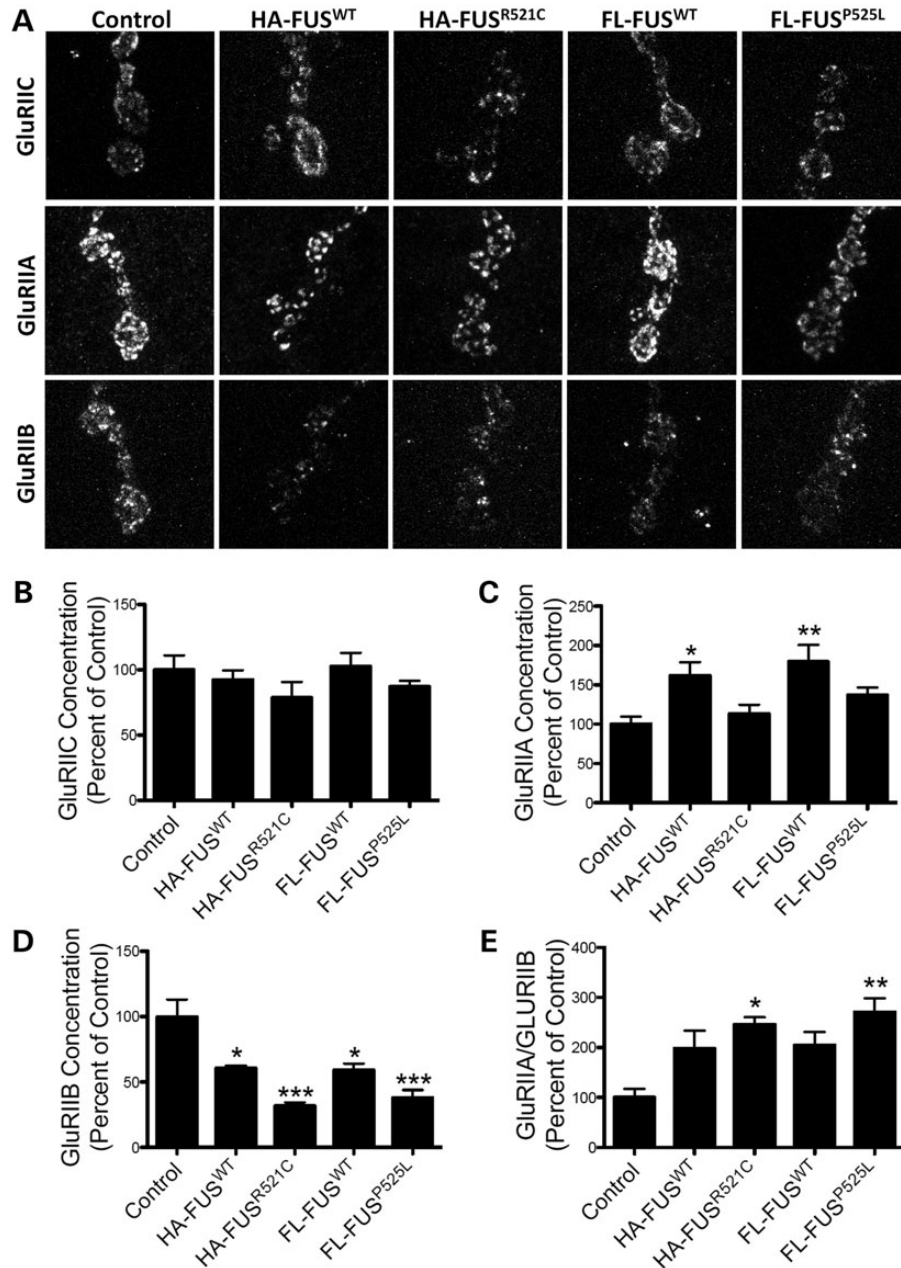
## DISCUSSION

### Overexpression of FUS in motor neurons causes toxicity independent of disease-associated mutations

Several models of FUS-mediated neurodegeneration have been developed in *Drosophila* (19–23) and other organisms (44–46), but the pathogenic mechanisms underlying their phenotypes are poorly understood. Toxicity in these models can arise from overexpression of wild-type FUS alone, independent of any disease-associated mutations. Dissecting the effects of disease-associated mutations when wild-type expression alone confers toxicity is difficult and requires carefully controlled levels of gene expression (47,48). A previous study concluded that ALS-linked mutations in FUS resulted in a toxic gain-of-function in *Drosophila*, and this conclusion was based on the finding that the wild-type and mutant UAS-HA-FUS transgenic lines expressed equivalent levels of protein in the eye (23). However, our analysis of these same UAS-HA-FUS lines demonstrates that when expressed in motor neurons, message and protein expression are 3- to 4-fold higher in the R521C mutant line than the wild-type line. Thus, these results suggest that the increased severity of phenotypes seen in the mutant line relative to wild-type is due to increased expression level rather than mutation-specific toxicity. Therefore, we do not find evidence of a gain-of-function effect of ALS-associated mutations in FUS in this model. These results are further supported by another study using independently generated transgenic lines that demonstrated that FUS-mediated overexpression toxicity in the adult eye was independent of ALS pathogenic mutations (22).

There are two non-mutually exclusive explanations for these dramatic differences in relative transgene expression levels observed in different tissues. First, given that HA-FUS<sup>R521C</sup> overexpression in eyes causes degeneration, the simplest explanation is that HA-FUS<sup>R521C</sup> lines express at higher levels than wild-type in the eyes during development, and this leads to cell loss or dysfunction that causes a reduction in protein expression in the adult, whereas expression in glutamatergic neurons with OK371-GAL4 does not have these effects. Indeed, morphological analysis of GMR>FUS<sup>R521C</sup> fly eyes demonstrates severe cell loss (23). An alternative possibility is



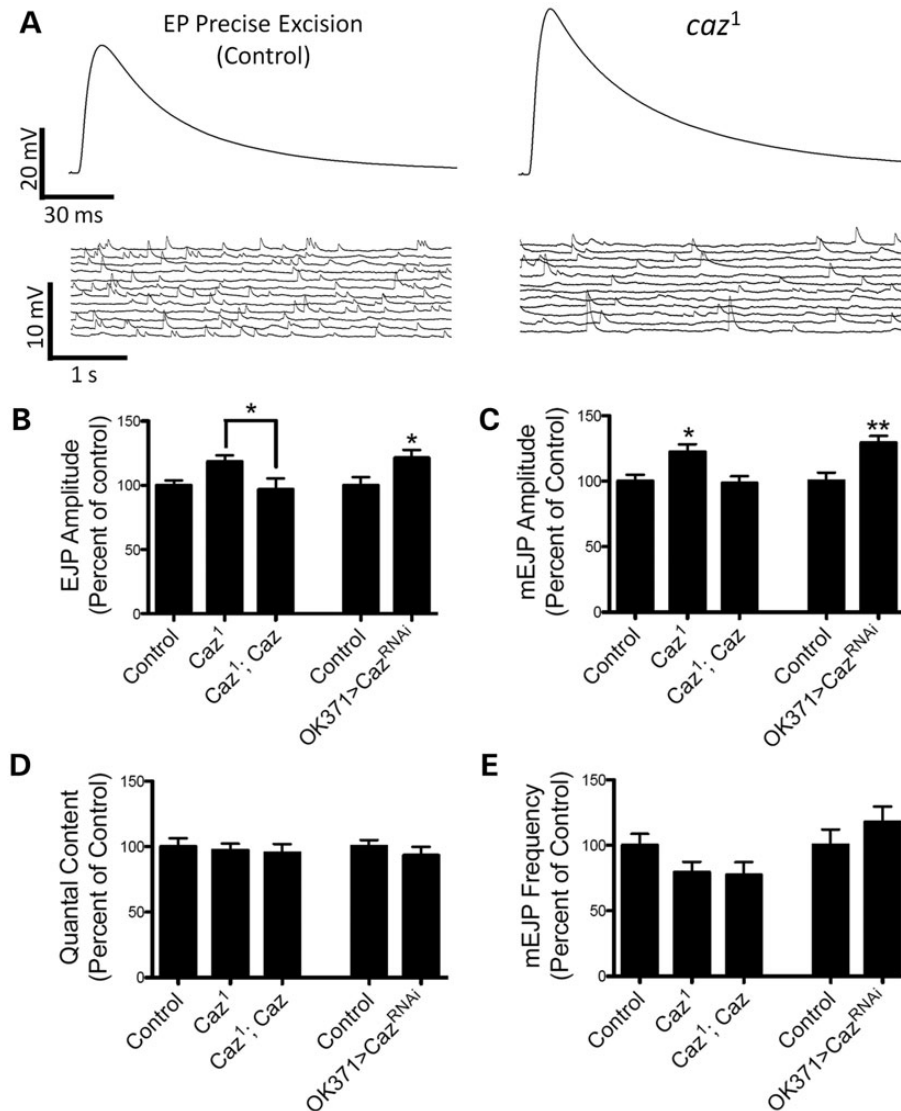


**Figure 5.** Presynaptic FUS expression disrupts GluR subunit composition. (A) Representative images of GluRIIC, GluRIIA and GluRIIB in type Ib boutons of animals overexpressing either *HA-FUS* or *FL-FUS* transgenes. (B–D) GluR subunit level concentrations were measured in ImageJ in the masked area surrounding the synaptic terminal created from dilating the Brp channel six times (Illustration of the analytical method can be found in Supplementary Material, Fig. S3). (B) Total GluRIIC levels are not significantly affected. (C) GluRIIA levels are increased in both *FL-FUS*<sup>WT</sup> and *HA-FUS*<sup>WT</sup> animals. (D) GluRIIB levels are decreased in all FUS-expressing animals. (E) Both *HA-FUS*<sup>R521C</sup> and *FL-FUS*<sup>P525L</sup> significantly increased the GluRIIA/GluRIIB ratio. Scale bar = 5  $\mu$ m. For GluRIIC,  $N = 4$ , GluRIIA,  $N = 7$ , GluRIIB,  $N = 3$ . A one-way ANOVA with a Bonferroni posttest for multiple comparisons was performed to determine statistical significance. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$ .

that tissue-specific enhancers may differentially regulate gene expression from P elements located at different genomic loci, as these position effects are well known in *Drosophila* (49,50). These observations suggest two important guidelines for analyzing overexpression disease models in *Drosophila*. First, given the widely available technologies for site-specific transgenesis, comparisons of gain-of-function phenotypes between wild-type and mutant proteins should utilize transgenic lines inserted at identical genomic sites. Indeed, when we analyze lines

generated in this manner (*FL-FUS*) (19), we see identical expression levels between the wild-type and mutant proteins in motor neurons. Second, when comparing protein expression levels between wild-type and mutant transgenic lines, expression levels should be compared in the absence of cell loss and in the tissue most relevant to the disease.

Recently, FUS has been shown to autoregulate its expression levels in HeLa cells by binding to exon 7 and flanking introns of its own pre-mRNA (32). Interestingly, ALS-associated



**Figure 6.** Presynaptic Caz inhibits synaptic transmission. (A) Representative electrophysiological traces of indicated genotypes. Upper traces—EJP; lower traces—mEJP. Quantification of EJP amplitude (B), mEJP amplitude (C), quantal content (D) and mEJP frequency (E) reveals that loss of *caz* results in increased mEJP amplitude. For *caz*<sup>1</sup> animals, precise excision of the P-element used to generate the null allele was used as a control, *N* = 10. For the *Ca<sup>2+</sup>*<sup>RNAi</sup> line, *OK371-GAL4/+* was used as a control, *N* = 10. For *caz*<sup>1</sup>, *N* = 20; for *caz*<sup>1</sup>; *gen-Caz*, *N* = 10; for *OK371>Ca<sup>2+</sup>*<sup>RNAi</sup>, *N* = 9. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest for multiple comparisons or Student's *t*-test. \*\*\**P* < 0.001, \*\**P* < 0.01 and \**P* < 0.05.

mutations in the C-terminus of FUS have been suggested to disrupt autoregulation (32), and mutations in the 3'UTR of human FUS that cause FUS overexpression have been shown to cause ALS (51). Together, these data suggest that disruption of FUS autoregulation leading to overexpression of wild-type FUS is sufficient to cause disease. In this study, we show that wild-type or mutant FUS overexpression in *Drosophila* motor neurons leads to severe (~90%) downregulation of fly FUS. This suggests that an autoregulatory mechanism is conserved in *Drosophila* and occurs in motor neurons *in vivo*. This autoregulation likely explains why in many cases, we do not see stronger phenotypes in higher-expressing transgenic lines. Future studies will investigate the mechanism of this autoregulation and the effect of ALS-causing disease mutations in this model.

#### Effects of ALS-associated mutations on FUS function in *Drosophila*

Whether disease-associated mutations in FUS and other ALS genes cause disease through a gain- or loss-of-function is a matter of debate. Simple genetic model systems such as *Drosophila* are ideal for interpreting the effect of mutations on protein function; however, one must be cautious in interpreting results gained from overexpression studies, particularly when overexpressing human proteins in the fly. However, because low-level expression of human FL-FUS in neurons rescues phenotypes caused by loss of *Drosophila* FUS (i.e. *Caz*) (19), this strongly argues for evolutionary conservation of FUS as well as a requirement for FUS in neurons. Furthermore, because FUS<sup>P525L</sup> fails to rescue *Caz* phenotypes (19), this strongly argues that the P525L mutation causes a loss-of-function.



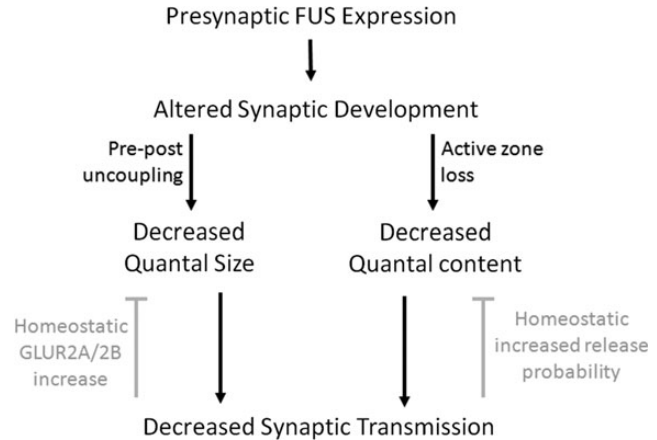
Consistent with this interpretation, our analyses of disease-associated mutations in FUS are most consistent with a partial loss-of-function, given that (a) low-level FUS<sup>WT</sup> but not FUS<sup>P525L</sup> expression causes increased larval and adult locomotor activity (Fig. 1B and E), and (b) expressing higher level FUS<sup>R521C</sup> in some instances causes less severe phenotypes than lower level FUS<sup>WT</sup> expression (e.g. Caz downregulation) (Fig. 2D), altered EJP rise time (Fig. 4G), and GluRIIA upregulation (Fig. 5C). Nonetheless, we cannot exclude the possibility that FUS mutations do exhibit some gain-of-toxic effects, particularly given that we observe a greater reduction in GluRIIB levels with mutant FUS expression than with wild-type expression (Fig. 5D–E). Since mutations in the 3'UTR autoregulatory domain are sufficient to cause ALS in patients by upregulating wild-type FUS protein, ALS may be caused by increased levels of wild-type protein. In this context, low-level overexpression of FUS or Caz in aging flies may be a reasonable way to model the disease.

### Role of FUS in regulation of synaptic transmission

FUS has been implicated in a wide range of processes in many cell types both within the nucleus and cytoplasm (9–11). Since we are unable to detect significant wild-type FUS or Caz protein within motor axons or at the NMJ (data not shown), we postulate that FUS/Caz normally functions in the nucleus to regulate the expression of genes that modulate synapse function. Importantly, FUS/Caz appears to be required within neurons to regulate synaptic transmission, as *caz*<sup>1</sup> loss-of-function phenotypes are mimicked by presynaptic *caz* knockdown, and overexpression of FUS in motor neurons leads to altered synaptic transmission.

Our study shows that FUS expression inhibits evoked release due to both a reduction of quantal size (mEJP amplitude) and quantal content (number of quanta released per stimulus). A reduction in mEJP amplitude can be due to a decrease in synaptic vesicle size, glutamate concentration or postsynaptic currents due to alterations in glutamate receptor levels, localization or composition (40). As shown in Figure 7, we postulate that the reduction in quantal size is due to a disruption of the spatial coupling of synaptic vesicle release sites with glutamate receptors given the disruption in the number and morphology (52) of active zones (labeled with Brp) and the postsynaptic density (labeled with Dlg). Importantly, we demonstrate that reduced GluR levels are not responsible for the decrease in mEJP amplitude observed in FUS overexpressing animals, but rather that GluR clustering at active zones may be altered. Furthermore, we see an increase in relative expression of A-type GluRs which would be expected to have the opposite effect on mEJP amplitude. This increase in ratio of A- to B-type GluRs is seen when glutamate release is blocked at larval NMJs and is a homeostatic response to reduction in glutamate-mediated synaptic transmission (53).

We also observed a striking reduction in the number of active zones in FUS-expressing animals (Fig. 3C and D). As shown in Figure 7, these changes likely contribute to the reduction in quantal content, as *bruchpilot* mutations in *Drosophila* have reduced synaptic transmission due to a reduction in the readily releasable pool (54). The relatively subtle reduction in quantal content in FUS-expressing animals may be due to a homeostatic



**Figure 7.** Model of FUS regulation of synaptic transmission. We propose that presynaptic FUS expression leads to altered development of the NMJ resulting in improper apposition of pre- and postsynaptic machinery at the NMJ and a decrease in the number of presynaptic active zones. As a consequence, quantal size and content are reduced leading to a reduction in synaptic transmission as a result of the synaptic terminal containing fewer sites of vesicle fusion and decreased postsynaptic response to vesicle fusion. Compensatory feedback mechanisms act to mitigate the loss of synaptic transmission by increasing the number of vesicles released per active zone and increasing the GluRIIA/GluRIIB subunit ratio of glutamate receptors.

increase in the probability of vesicle fusion at any given active zone. Surprisingly, the reduction in active zone number is associated with a marked increase in the frequency of spontaneous release, suggesting that the remaining active zones have a marked increase in release probability. Consistent with this hypothesis, superresolution microscopic imaging of Brp demonstrates abnormal morphology of active zones in FUS-expressing animals (52).

The results of our study contrast with those of a recent report analyzing larval NMJ physiology of *HA-FUS*<sup>R521C</sup>-expressing and *caz*<sup>1</sup> animals using discontinuous single electrode voltage clamp recordings (52). This study did not observe a physiologic phenotype with *FUS*<sup>WT</sup> overexpression, and they observed a reduction in evoked release in *caz*<sup>1</sup> animals; their findings were consistent with findings in zebrafish with overexpression of mutant FUS and with morpholino-mediated knockdown of wild-type FUS (46). Consistent with our analysis, Shahidullah *et al.* see a reduction in amplitude of evoked release and decreased decay time with *FUS*<sup>R521C</sup> expression. Although the electrophysiological techniques in these two studies are different, the reported genotypes and synapses analyzed are identical, and the reasons for the different findings are unclear. Since FUS protein redistributes to cytoplasmic stress granules with various stressors (55), one possibility is that alterations in animal rearing or recording conditions may have large effects.

### FUS may disrupt coupling of the pre- and postsynaptic active zones

We report here the occurrence of cell nonautonomous loss of Dlg from muscle postsynaptic compartments and alterations in GluRII subunit composition at the NMJ as a result of FUS overexpression in motor neurons. The mechanism of Dlg loss induced by FUS overexpression is unclear, and it is unknown

whether the reduction in Dlg expression is accompanied by morphological alterations in the subsynaptic reticulum of muscle cells (56). Although the increase in the ratio of A- to B-type GluRs is consistent with homeostatic compensation for impaired synaptic transmission (53), Dlg is not known to be regulated by homeostatic feedback at the *Drosophila* NMJ. Thus, we speculate that non-cell autonomous effects on Dlg are mediated through alterations in transsynaptic adhesion molecules. This notion is supported by the altered kinetics of EJP rise and decay times (Fig. 4F and G) and disrupted apposition of GluRIIB/IIC with the active zone (Supplementary Material, Fig. S3). Furthermore, several of the morphological and electrophysiological phenotypes caused by FUS overexpression overlap with loss-of-function alleles of the neuroligin (*nrx1*)/neuroligin (*nlg1* and *nlg2*) transsynaptic adhesion complex in *Drosophila* (34, 35, 57–60). For example, both *nrx1* and *nlg1* animals show expanded interbouton regions that lack post-synaptic markers (34, 35), a phenotype observed with FUS overexpression (Supplemental Material, Fig. S2). *nrx1* mutants show decreased quantal content, increased mEJP frequency, and an increase in ratio of A to B-type glutamate receptors, phenotypes we have observed with FUS overexpression (34, 58). Interestingly, *nlg2* mutants show an increase in ratio of A to B-type receptors and a decrease in EJP rise and decay times (59), as we have observed in FUS overexpressing animals. Neuroligin/neuroligin and other neuronal cell adhesion complexes form transsynaptically to mediate the proper development and function of both the pre- and postsynaptic active zone, and alterations in these interactions result in changes in synaptic architecture, active zone formation and localization, post synaptic density formation, DLG levels, and synaptic transmission (34, 35, 57–62).

As the best described function of FUS is regulation of transcription and splicing (9–11), alterations in transcription and/or splicing may underlie the changes seen in synaptic function. Many cell adhesion molecules are highly alternatively spliced, and this splicing alters their function in synaptic development and differentiation (63–65). For example, mutations in *beag* alter splicing of *fasciclin II* (*fasII*), the *Drosophila* homologue of neural cell adhesion molecule (NCAM), and altered splicing leads to fewer synaptic boutons and decreased neurotransmitter release (66). Transcriptional profiling experiments in mice indicate FUS regulates the expression level and splicing of several synaptic adhesion molecules necessary for normal synaptic development (33). Thus, we hypothesize that the changes in synaptic structure and function may be partially explained by altered expression and/or splicing of transsynaptic adhesion molecules.

## METHODS AND MATERIALS

### Fly rearing and genetics

Flies were raised on standard cornmeal-agar fly food at 25°C in a humid incubator. The following fly lines were used in experiments: *w<sup>1118</sup>*, OK371-GAL4, UAS-mCD8-GFP, UAS-Caz<sup>RNAi</sup> (BL 34839) from Bloomington *Drosophila* Stock Center. UAS-HA-FUS flies were a gift from Pandey and coworkers (23) and UAS-FL-FUS, *caz<sup>1</sup>*, FL-genomic-Caz, and P{EP}cazEP1564 precise excision flies were a gift from Brian McCabe (19).

### Motility assays

Larval motility was determined by measuring the number of peristaltic body movements performed per animal in 1 min. Individual larvae were placed within a 10 cm Petri dish containing a moist Kimwipe insert spread completely on the bottom of the dish. Larvae were allowed to acclimate for 15 s and then allowed to explore the dish for 1 min.

Adult motility was assayed using single beam DAM system (Trikinetics). The number of beam crossings per fly per day in the first 4-day-periods post eclosion (days 1–4) and days 13–16 was calculated for the indicated genotypes. The fraction of original activity was determined by dividing the number of beam crossings from days 13–16 by the number of beam crossing from days 1–4.

### Immunohistochemistry

Larval dissections and staining were performed as previously described (48). Briefly, third-instar larvae were partially dissected in Ca<sup>2+</sup>-free HL-3, and fixed in PBS with 4% PFA for 20 min, with the exception of the GluR stainings. For GluR stainings, larvae were fixed in Bouin's fixative for 5 min. The following probes and antibodies were used: goat- $\alpha$ -HRP-Dylight 549 (Pierce), 1:500; mouse- $\alpha$ -Flag (Sigma F1804), 1:1000; mouse- $\alpha$ -HA (Santa Cruz HA-7), 1:200; rabbit- $\alpha$ -GFP (Invitrogen), 1:250; mouse- $\alpha$ -DLG (DSHB), 1:200; mouse- $\alpha$ -Brp (DSHB), 1:100; mouse  $\alpha$ -GluRIIA (DSHB), 1:100; rabbit  $\alpha$ -GluRIIB (gift from DiAntonio Lab), 1:2500; rabbit  $\alpha$ -GluRIII (gift from DiAntonio Lab), 1:2500; goat- $\alpha$ -mouse-A488 (Molecular Probes), 1:250; goat- $\alpha$ -mouse-A549 (Molecular Probes), 1:250; goat- $\alpha$ -rabbit-A488 (Molecular Probes), 1:250; goat- $\alpha$ -mouse IgG2a-Cy3 (Jackson ImmunoResearch), 1:250; goat- $\alpha$ -mouse IgG1-A488 (Jackson ImmunoResearch), 1:250 and DAPI.

### Biochemistry

For western blots, 10 adult fly heads were homogenized using a pellet pestle in SDS sample buffer and run on a Bio-Rad precast 4–15% gradient polyacrylamide gel. After transferring to polyvinylidene difluoride membrane, the blots were probed using rabbit  $\alpha$ -FUS (Bethyl Laboratories A300–302A, 1:000), and mouse  $\alpha$ -actin (Millipore, MAB1501R, 1:1000) antibodies. Secondary antibodies used were goat  $\alpha$ -rabbit-HRP (Pierce 1:5000) and goat  $\alpha$ -mouse-HRP (Pierce 1:5000) and the blot was developed with the ThermoScientific Super Signal West Pico chemiluminescent substrate.

### Quantitative real time PCR

Three replicates of 10 adult fly heads, age 0–2 days old, were collected for each genotype. The heads were homogenized using a pellet pestle and total RNA was extracted with TRIzol (Invitrogen). Following genomic DNA degradation using DNase I (NEB), 1  $\mu$ g of total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (ABI). qRT-PCR was performed in triplicate, using the TaqMan Assay (Invitrogen) on an Applied Biosystems 7900HT machine. A TaqMan probe for human FUS (Hs01100224\_m1) and a control probe for *Drosophila* Rp1140 (Dm02134593\_g1) were

used. Relative quantification compared with the FL-FUS<sup>WT</sup> samples was performed using the delta–delta CT method (67).

### Image quantification and analyses

Immunohistochemical stainings were imaged on a LSM 510 laser scanning confocal microscope using the appropriate filters and laser excitation wavelength for a given fluorophore. Images were taken using either 40× or 63× oil immersion objectives. Quantification of active zone numbers and Dlg levels was performed using surface creation in Imaris imaging software (Bitplane). Analyses of both active zones and Dlg were performed on motor neurons forming type Ib synapses on muscle 4 from two hemisegments per animal, either from abdominal segment A2 or A3. For measurement of synaptic terminal size, bouton number was counted and muscle size was measured using an Olympus BX50 epifluorescent microscope with a scaled eyepiece. Nuclear Caz levels were quantified by measuring the levels of Caz in motor neuron nuclei of slices after background subtraction in Image J. GluRII subunit synaptic densities were measured in Image J in a collapsed stack by segmenting an area of the image using a mask created from the dilated Brp channel and normalized to Brp area.

### Basic electrophysiological analysis

Third-Instar larvae of the appropriate genotype were dissected in 0.8 mM Ca<sup>2+</sup> HL3 as previously described (68). Recordings were performed using an Axoclamp 900A amplifier and digidata 1440A (Molecular Devices). Spontaneous activity from muscle six was recorded for 2 min followed by at least 10 EJPs elicited from each muscle. Recordings were made in up to two muscles per animal in abdominal segments A3 and A4. Muscle stimulation was performed on severed segmental nerves using a stimulus of 0.3 ms at an amplitude large enough to elicit maximal EJP amplitude. EJP amplitude and miniature EJP (mEJP) amplitude and frequency, as well as EJP 10–90% rise time and 10–90% decay time were quantified using Clampfit 10.2 (Molecular Devices).

### Larval fatigue electrophysiological analysis

Fatigue analysis was performed using similar techniques as those employed for basic electrophysiological analysis. Larvae were dissected in 2.0 mM Ca<sup>2+</sup> HL3 and stimulated at 10 Hz for 5 min. The amplitudes of the EJPs from the first 5 s were averaged to determine base line synaptic transmission, represented as the earliest time point. Each successive time point represents the amplitudes of EJPs from 30 s periods normalized to this baseline EJP amplitude.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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