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Excitotoxicity induces nuclear egress of FUS/TLS

The RNA-binding protein FUS/TLS undergoes calcium-mediated nuclear egress during excitotoxic stress and is required for GRIA2 mRNA processing

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ABSTRACT

Excitotoxic levels of glutamate represent a physiological stress that is strongly linked to amyotrophic lateral sclerosis (ALS) and other neurological disorders. Emerging evidence indicates a role for neurodegenerative disease-linked RNA-binding proteins (RBPs) in the cellular stress response. However, the relationships between excitotoxicity, RBP function, and disease have not been explored. Here, using primary cortical and motor neurons, we found that excitotoxicity induced the translocation of select ALS-linked RBPs from the nucleus to the cytoplasm within neurons. RBPs affected by excitotoxicity included TAR DNA-binding protein 43 (TDP-43) and, most robustly, fused in sarcoma/translocated in liposarcoma (FUS/TLS). We noted that FUS is translocated through a calcium-dependent mechanism and that its translocation coincides with striking alterations in nucleocytoplasmic transport. Further, glutamate-induced up-regulation of glutamate ionotropic receptor AMPA type subunit 2 (GRIA2) in neurons depended on FUS expression, consistent with a functional role for FUS in excitotoxic stress. These findings reveal molecular links among prominent factors in neurodegenerative diseases, namely excitotoxicity, disease-associated RBPs, and nucleocytoplasmic transport.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the central nervous system. Upon release from pre-synaptic terminals, relatively low levels of glutamate activate metabotropic glutamate receptors as well as the ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate and kainate, for normal neurotransmission. However, excessive
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glutamate exposure overstimulates neurons. This causes a massive influx of calcium, which triggers an excitotoxic cascade involving oxidative damage as well as mitochondrial and ER dysfunction(1). Excitotoxicity has been implicated in neuronal death and degeneration for various neurological conditions, including the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS). Pathological evidence for excitotoxicity includes elevated levels of glutamate in patient cerebrospinal fluid as well as aberrant processing of the AMPA subunit that controls calcium influx at both the transcript (Gria2) and protein (Glutamate Receptor 2; GluA2) level in patient tissue and disease models (2). Further, ALS-causing mutations are present in D-amino acid oxidase, an enzyme that regulates the degradation of the N-methyl-D-aspartate co-agonist, D-serine (3). Riluzole, the first FDA approved treatment for ALS, is thought to reduce glutamate signaling through anti-excitotoxic effects (4). Despite this wealth of knowledge and profound disease relevance, the biological mechanisms underlying the cellular response to excitotoxicity have not been fully elucidated.

RNA-binding proteins (RBPs) have emerged as relevant factors in neurodegenerative disease pathogenesis, particularly in the context of ALS and the related disorder, frontotemporal dementia (FTD) (5). RBPs belong to a unique class of biomolecules that undergo nucleocytoplasmic shuttling in response to various stimuli, including stress. For instance, the disease-linked RBPs fused in sarcoma/translocated in liposarcoma (FUS/TLS or FUS), TAR DNA-binding protein 43 (TPD-43) and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) all exhibit nuclear egress during hyperosmotic stress (6–8). The purpose of this translocation is unclear, and may represent a functional response to cellular stress (6, 9). In support of this notion, cell viability under hyperosmotic stress is compromised when FUS expression is reduced (6). However, cell stress also represents a non-genetic factor that likely contributes to neurodegenerative disease pathogenesis (9). Indeed, chronic stress may contribute to the pathological accumulation of TDP-43 and FUS in some cases of ALS and FTD (10–14). For example, TDP-43 partitions into the insoluble fraction of cultured cells following oxidative stress or heat shock (15, 16) and disease-linked RBPs have been found to aggregate in vivo following cerebral ischemia (17). Intriguingly, the effects of stress on RBP translocation appear selective. While ER stress, oxidative stress and heat shock induce the cytoplasmic accumulation of TDP-43 and other RBPs (18, 19), these stressors fail to elicit a response of FUS (6, 20). Given the physiological relevance of excitotoxicity to neurodegenerative disease, an important but unanswered question is whether excitotoxic stress elicits a functional and/or pathological response from disease-associated RBPs.

Here, we demonstrate that excitotoxic levels of glutamate induce the nuclear egress of several ALS- and FTD-linked RBPs, including FUS, TDP-43 and hnRNPA1 into the cytoplasm of neurons. The nucleocytoplasmic equilibrium of FUS was especially sensitive to excitotoxic stress, as FUS was found to rapidly and robustly accumulate within soma and dendrites of cortical and motor neurons under stress. Further, a glutamate-induced increase in dendritic Gria2 was dependent on FUS, consistent with a role for FUS in glutamatergic signaling during the cellular response to excitotoxic stress. Our results also revealed potentially adverse consequences of excitotoxicity, including the translocation of ALS-linked FUS variants and early signs of nucleocytoplasmic transport dysregulation. This study therefore demonstrates that excitotoxicity can trigger neurodegenerative disease-associated phenotypes including cytoplasmic RBP accumulation and nucleocytoplasmic transport decline.

RESULTS

Excitotoxic levels of glutamate shift the nucleocytoplasmic equilibrium of disease-linked RNA binding proteins.

To investigate a potential relationship between excitotoxicity and neurodegenerative disease-linked RBPs, we first examined whether excitotoxicity affects the nucleocytoplasmic equilibrium of a panel of proteins including FUS, TDP-43, hnRNPA1 and TATA-Binding Protein-Associated Factor 15 (TAF15). All four proteins have been linked to ALS (5) and FUS, TDP-43 and TAF15 are also associated with FTD (21).
DIV 14-16 primary cortical neurons, the majority of which are excitatory, were bath treated with excitotoxic and physiologically relevant levels of glutamate (22, 23) (10 $\mu$M; hereon referred to as Glu$^{\text{excito}}$) for 10 minutes followed by a 30-minute washout period (Fig. 1A). Immunofluorescence was then used to assess the effect of Glu$^{\text{excito}}$ on the cytoplasmic to nuclear (C:N) ratio of the endogenous RBPs (Fig. 1B-I). Strikingly, the FUS C:N ratio significantly increased ~15-fold from 0.04±0.05 to 0.6±0.3 in response to Glu$^{\text{excito}}$ (Fig. 1B,F). This increase is likely due to a rapid egress of FUS from the nucleus into the cytoplasm, as a Western analysis revealed total FUS protein levels are unchanged before and after stress (Fig. S1A,B). Glu$^{\text{excito}}$ likewise induced a significant increase in the C:N ratio of TDP-43 (Fig. 1C,G) and hnRNPA1 (Fig. 1D,H), although not to the same extent as FUS. Glu$^{\text{excito}}$ did not significantly alter the C:N ratio of TAF15 (Fig. 1E,I). As for FUS, protein expression levels of TDP-43, hnRNPA1 and TAF15 were unaffected by Glu$^{\text{excito}}$ (Fig. S1C-F).

In light of the robust response of FUS to Glu$^{\text{excito}}$, we focused our attention on the properties of FUS translocation in more detail. First, endogenous FUS translocation in response to Glu$^{\text{excito}}$ was confirmed using a panel of different anti-FUS antibodies (Fig. S2 A,B). We then examined the relationship between FUS translocation and glutamate concentration. With 10 $\mu$M glutamate, the vast majority of neurons (91.3±11.5%) exhibited FUS egress (Fig. 2A,B), whereas <5% neurons exhibited translocation at ≤1 $\mu$M, revealing a dependence of FUS localization on glutamate concentration (Fig. 2B). Within the time course of our experiment (Fig. 1A), a significant accumulation of endogenous FUS was also detected throughout microtubule associated protein 2 (MAP2)-positive dendrites (Fig. 2C,D). The response of FUS to Glu$^{\text{excito}}$ appears to be all-or-none, as lower concentrations of glutamate resulted in fewer neurons responding, but with similar degrees of FUS egress (Fig. S2C).

The toxicity of Glu$^{\text{excito}}$ on neurons (22), we interrogated whether the rapid and robust accumulation of FUS outside the nucleus was simply a consequence of cell death and/or loss of nuclear envelope integrity. extent of cell death was assessed using the LDH cytotoxicity assay, which detects the activity of LDH upon its release into the media from dead or dying cells. In contrast to neurons treated with lysis buffer, there was no evidence of cell death for neurons treated with Glu$^{\text{excito}}$ (Fig. 2E). Further, Lamin A/C staining revealed an intact nuclear envelope in neurons exposed to Glu$^{\text{excito}}$ (Fig. 2F). These observations support the premise that cytoplasmic FUS accumulation represents a cellular response to Glu$^{\text{excito}}$, rather than a non-specific consequence of cell death. Moreover, RBP translocation appears selective, as TAF15 (Fig. 1E,I) and the cytoplasmic protein, fragile X mental retardation protein (FMRP; Fig. S3A), did not change localization following excitotoxic insult. It is noteworthy that Glu$^{\text{excito}}$ affects neuron morphology at 30 minutes, potentially indicative of a stressed state. Anti-MAP2 staining revealed a rearrangement of the cytoskeleton; staining was more pronounced around the nucleus and indicative of dendritic fragmentation (Fig. 1,2). Likewise, the nuclear lamina appeared more concentrated around the nuclear envelope. This is illustrated by the line scan through the nucleus (Fig. 2G). Further, nuclear size was significantly smaller in stressed neurons (Fig. 2H). As expected, neurons exposed to excitotoxic stimuli (10 $\mu$M, but not 1 $\mu$M glutamate) eventually undergo cell death within 24 hours of the initial insult (22) (Fig. S3 B,D).

Excitotoxic stress induces egress of predominately nuclear ALS-linked FUS variants.

Next, we investigated the behavior of ALS-linked FUS variants under conditions of Glu$^{\text{excito}}$. To this end, a series of FLAG-HA-tagged FUS variants were transiently expressed in neurons and the C:N ratio of exogenous FUS was determined in the absence and presence of Glu$^{\text{excito}}$ (Fig. 3). In addition to wildtype (WT) FUS, we examined: H517Q, the only autosomal recessive FUS mutation associated with ALS; R521G, representing a mutational ‘hot spot’ for ALS-FUS (24); and R495X, a particularly aggressive ALS-linked mutation (20). All of these variants have mutations within the nuclear localization sequence (NLS), yet the degree to which FUS H517Q and R521G mislocalize to the cytoplasm depends on their expression level. Near-endogenous levels of
these variants exhibit a predominately nuclear localization in vitro (20) and in vivo (25), whereas overexpression leads to higher levels of cytoplasmic mutant FUS (10). In contrast, the entire NLS is omitted from the FUS R495X variant, which significantly mislocalizes to the cytoplasm regardless of expression level (20). Here, we observed minimal cytoplasmic mislocalization of mutant FUS H517Q and R521G without stress, indicating the expression levels of these exogenous proteins were below the threshold for significant cytoplasmic mislocalization in these cells. As expected, FUS R495X exhibited robust cytoplasmic mislocalization under basal conditions (Fig. 3). In response to Gluexcito, FLAG-HA-FUS WT exhibited a significant translocation to the cytoplasm as observed for endogenous FUS (Fig 1A, F). The C:N ratio of FLAG-HA-FUS H517Q and R521G also increased significantly with Gluexcito, providing evidence that the ALS-linked mutations did not interfere with the response of FUS to Gluexcito. In the case of FLAG-HA-FUS R495X, the C:N ratio was the same with and without Gluexcito, potentially indicative of a ‘ceiling effect’ in that the normal nucleocytoplasmic distribution of R495X-FUS is equivalent to that of ‘maximally’ redistributed endogenous FUS following excitotoxic stress.

Nucleocytoplasmic transport is disrupted in response to excitotoxic stress.

To understand the mechanism(s) underlying endogenous FUS egress in response to Gluexcito, we began with an examination of nucleocytoplasmic transport factors. FUS contains two predicted chromosome region maintenance 1 (CRM1)-dependent nuclear export sequences (NES) within the RNA-recognition motif (26). CRM1 is a major protein export factor, although whether this receptor controls nuclear FUS export is controversial (26, 27). To determine if excitotoxic FUS egress is CRM1-dependent, we pretreated neurons with the CRM1 inhibitor, KPT-330, prior to treatment with Gluexcito (28). The CRM1-dependent NLS-ttdTomato-NES shuttling reporter was used as a positive control (29). As expected, NLS-ttdTomato-NES exhibited both a nuclear and cytoplasmic localization under basal conditions (Glu−, KPT), whereas the localization of this reporter was significantly restricted to the nucleus in the presence of KPT-330 (Glu+, KPT; Fig. 4A,B). In contrast, KPT-330 had no effect on nuclear FUS egress in response to Gluexcito (Gluexcito +/− KPT; Fig. 4A,C). Surprisingly, KPT-330 also failed to fully restrict NLS-ttdTomato-NES to the nucleus under conditions of Gluexcito (Fig. 4A,B). Although there was a significant decrease in the percentage of cells with cytoplasmic NLS-ttdTomato-NES in the presence of both KPT-330 and Gluexcito (60.1±8.0%) compared to Gluexcito alone (98.3±2.6, p=<0.0001), these results suggest that CRM1-mediated export is dysregulated under conditions of stress (Fig. 4B). Moreover, while endogenous CRM1 predominately localizes to the nucleus, significantly more cells exhibited altered CRM1 localization with Gluexcito. CRM1 alterations included enhanced cytoplasmic localization and a concentration of CRM1 signal around the nuclear membrane (Fig. 4D-F). This finding prompted us to examine another critical nucleocytoplasmic transport factor, Ras-related nuclear protein (Ran). Ran is a GTPase that shuttles between the nucleus and cytoplasm and, depending on its nucleotide bound state, facilitates nuclear export and import (30). Indeed, Gluexcito also induced a significant change in the nucleocytoplasmic distribution of Ran in that a higher percentage of cells exhibited enhanced cytoplasmic Ran expression under stress (Fig. 4G-I). These changes to CRM1 and Ran localization appear to be independent from changes in total protein expression as determined by a Western blot analysis of both proteins in the presence and absence of Gluexcito (Fig. S3D-F). Taken together, Gluexcito caused the redistribution of critical transport factors and attenuated the effects of KPT-330 on CRM1 export.

Excitotoxic FUS egress is calcium dependent.

Knowing that calcium influx is a critical component of excitotoxicity (1), we sought to determine whether this signaling chemical is required for the response of FUS to excitotoxicity. To this end, the calcium chelator, EGTA, was included in the neuronal media during the experimental time course. Indeed, EGTA completely prevented Gluexcito-induced FUS egress in primary cortical neurons (Fig. 5A,B). Further, application of the calcium ionophore, ionomycin, was sufficient to induce FUS translocation in the vast majority (89.0±5.6%) of neurons (Fig. 5C,D). In light of our previous finding that hyperosmotic
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stress induces nuclear FUS egress (6), we wondered whether calcium also mediated this response. In contrast to Gluexcito, there was no effect of EGTA on FUS egress in neurons treated with hyperosmotic levels of sorbitol (Fig. 5E,F), indicative of distinct mechanisms for FUS egress under these stress conditions.

Next, we investigated the effect of calcium on FUS localization in primary motor neurons, the neuronal cell type predominately affected in ALS. Consistent with cortical neurons, the application of Ionomycin to DIV 6-8 motor neurons shifted the nucleocytoplasmic equilibrium of FUS towards the cytoplasm (Fig. S4). Application of the glutamatergic agonist, kainic acid, to motor neurons also induced a significant increase in the C:N ratio of FUS (Fig. 5G,H). Kainic acid is known to induce motor neuron excitotoxicity (31) and was used here to avoid confounding effects of glutamate uptake by astroglia present in the motor neuron cultures (32). As observed for MAP2 staining in cortical neurons under Gluexcito (Fig. 2A), the cytoskeleton in motor neurons was affected by stress. For example, signals corresponding to neurofilaments (detected by SMI-32; Fig. S4A) and microtubules (detected by TUJ1; Fig. 5G and Fig. S4C) were altered and/or diminished under stress. Importantly, these treated motor neurons were viable as determined by the lack of pyknosis (Fig. 5G and Fig. S4). Further, cleaved Caspase-3 staining was not detected in cells with kainic acid-induced FUS translocation but was present in apoptotic cells treated with the proteasome inhibitor MG132 (Fig. S4C).

There was a relatively wide range in the C:N ratio of FUS in kainic acid treated neurons. For example, a sub-population of cells exhibited near complete egress of nuclear FUS (Fig. 5G,H), a result that was not observed in cortical neurons treated with glutamate. Efforts to characterize the properties of neurons that have different degrees of FUS translocation were hampered by an inability to perform live-cell imaging, as a GFP-tagged version of FUS did not undergo cytoplasmic translocation under excitotoxic stress (data not shown). We did notice a trend towards smaller nuclear area for kainic acid-treated motor neurons, as observed for glutamate-treated cortical neurons (Fig. 2H), however the former analysis did not reach statistical significance (Fig. S4D). Addition of EGTA to the media prior to kainic acid resulted in only a partial rescue of the FUS response; overall there were fewer cells with high levels of cytoplasmic FUS (i.e., high C:N ratios), yet some cells were insensitive to EGTA under these conditions. Nevertheless, our collective findings are consistent with a role for calcium in modulating the cytoplasmic egress of FUS under conditions of excitotoxicity.

**Excitotoxic stress represses translation independent of FUS expression and stress granule formation.**

Translational repression and stress granule formation are common cellular responses to stress (33, 34). Given that cytoplasmic FUS has been linked to both translational regulation (35, 36) and stress granule formation (6, 20, 37, 38), we investigated both of these processes during excitotoxic stress. In contrast to neurons treated with sodium arsenite, a stressor known to induce the formation of stress granules (20), Gluexcito did not induce the formation of Ras GTPase-activating protein-binding protein 1 (G3BP1)-positive stress granules in neurons (Fig. 6A). Next, protein translation was assessed by pulse labeling neurons with puromycin, a small molecule that incorporates into elongating peptides (39) (Fig. 6C-E). As observed by others, Gluexcito treatment inhibited global translation (40–42) and lead to a reduction in puromycin incorporation (Fig. 6C-E), likely through a mechanism involving inhibition of the translation elongation factor, eukaryotic elongation factor 2 (eEF2) (41, 43). Immunofluorescence detection of puromycin revealed a strong correlation between neurons exhibiting FUS translocation and translational repression; neurons with obvious translocated FUS were puromycin-reduced, and vice versa (Fig. 6C-F). The degree of translational repression induced by Gluexcito was comparable to treatment with the translational inhibitor, cycloheximide (Fig. 6C-F), which did not promote FUS egress (Fig. 6C). Given the strong correlation between FUS localization and puromycin intensity, we investigated whether FUS expression was required for translational repression (Fig. 7). Knockdown of endogenous FUS with two different shRNAs (44) that target FUS (Fig. 7A,B and Fig. S5) had no effect on puromycin intensity in the absence or
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presence of Gluexcito (Fig. 7C-F). Further, there was no net difference in puromycin intensity as a function of FUS expression between stressed and unstressed conditions (determined by comparing puromycin intensities between stressed and unstressed conditions, for cells with FUS expression or with FUS knock-down) (Fig. 7G).

Following the same FUS knock-down protocol, we did not detect a change in cell viability after Gluexcito treatment as a function of FUS expression (data not shown); FUS knock-down was neither protective nor detrimental to the cell under these conditions.

**Gria2 mRNA is elevated in dendrites following excitotoxic insult in a FUS-dependent manner.**

RBPs such as FUS play crucial roles in mRNA processing (9). Although FUS expression did not affect global protein synthesis (Fig. 7), this analysis would not necessarily detect differences in the translation of specific transcripts, especially those targeted to dendrites for local translation (45). Therefore, we investigated whether FUS modulates mRNA metabolism following excitotoxic insult and focused on Gria2, a transcript that is reportedly bound by FUS (46). Gria2 mRNA encodes the GluA2 protein subunit of the AMPA receptor and has been implicated in calcium dyshomeostasis in both ALS (1) and FTD (47). Following depolarization, dendritic GluA2 levels are enhanced (48). Under excitotoxic conditions, we uncovered a significant increase in Gria2 transcript density by FISH in both the soma and dendrites of cortical neurons (Fig. 8, Fig. S6A-C). To examine whether this increase in Gria2 mRNA density is FUS dependent, endogenous FUS levels were knocked down using two shRNAs targeting distinct sequences within FUS as described for Figure 7 prior to excitotoxic treatment. Consistent with previous findings (46), reduced FUS expression did not have a significant effect on the levels of Gria2 under basal conditions, as determined by FISH within the neuronal soma and dendrites (Fig. 8B,C). In contrast, Gluexcito-induced changes to Gria2 were significantly attenuated upon FUS knockdown. Dendritic expression of Gria2 was particularly sensitive to FUS levels under Gluexcito, as knockdown of FUS restored dendritic Gria2 levels to baseline (Fig. 8C, D). Within the time course of the analysis, we were unable to detect significant changes in GluA2 protein levels by Western blot analysis of whole cell lysates (Fig. S6D,E). Nevertheless, these data show that FUS expression is required for Gluexcito-induced changes to Gria2 localization in neuronal dendrites (Fig. 8). Indeed, Gria2 mRNA was found associated with FUS upon FUS immunoprecipitation from neurons under both unstressed (Glu) and stressed (Gluexcito) conditions (Fig. 8E). Although FUS was known to interact with Gria2 under unstressed conditions, this interaction had not been investigated under conditions of stress. To investigate the effects of ALS-linked mutations on FUS-mediated Gria2 processing and localization, FLAG-HA-FUS constructs were transiently transfected into neurons as described for Figure 7. However, in contrast to non-transfected neurons (Fig. 8), dendritic levels of Gria2 were unaffected by Gluexcito in FLAG-HA-FUS WT expressing cells (data not shown), possibly a consequence of overexpressed FUS WT exerting a dominant negative effect. Together, our data suggest that wild-type FUS directly affects the subcellular localization of Gria2 under Gluexcito, and that the role of mutant FUS should be investigated in systems with endogenous levels of protein (25).

**DISCUSSION**

This study uncovered an association between disease-linked RBPs and excitotoxicity, a stress that has particularly profound effects on the nucleocytoplasmic distribution of FUS in both cortical (Fig. 1, 2) and motor neurons (Fig. 5). There is a compelling body of evidence linking glutamate-induced excitotoxicity to neurodegenerative diseases, including ALS (1, 2, 49). For instance, elevated levels of glutamate were detected in biological samples from ALS patients (50–52). Cell death caused by glutamate and calcium dysregulation has also been documented in multiple animal and cellular models (1, 2, 50, 53–55). The outcomes of this study shed new light on the excitotoxicity cascade and implicate a role for the ALS/FTD-linked protein FUS in this process.

Our results are consistent with a functional role for FUS in response to glutamatergic signaling (56) rather than a non-specific effect of cell death. First, FUS egress precedes cell death (Fig. 2 and
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Second, there is selectivity with respect to proteins that undergo a change in cellular localization; the response of FUS is particularly robust compared to the other proteins assessed in this study (Fig. 1). Third, the effects of excitotoxicity on Gria2 depend on FUS expression (Fig. 8). FUS binds Gria2 mRNA within introns and the 3' untranslated region (UTR), and Gria2 splicing is affected by FUS expression under basal conditions (46). FUS binding to the 3'UTR of Gria2 is thought to occur synergistically with microRNAs that modulate Gria2 expression (57). Here, we confirmed that FUS binds Gria2 under unstressed conditions and show that FUS remains associated with Gria2 under conditions of Gluexcito (Fig. 8E). Subsequently, Gria2 density was enhanced in neuronal dendrites in a FUS-dependent manner under Gluexcito (Fig. 8B,D). These data are consistent with a role for FUS in Gria2 nucleocytoplasmic transport. In the absence of stress, FUS binds Gria2 (Fig. 8E) and we suspect this interaction takes place predominately in the nucleus (Fig. 9). Upon exposure to glutamate stress, we posit that Gria2 is transported into the dendrites as part of a complex with FUS (Fig. 9). Gria2 encodes the GluA2 protein subunit of the AMPA receptor. Normally, GluA2 is post-transcriptionally edited and GluA2-containing AMPA receptors are calcium impermeable. As such, the calcium permeability of AMPA receptors and the susceptibility of neurons to excitotoxicity is dependent on GluA2 (1, 58). We speculate that the enhanced dendritic density of Gria2 may serve to increase the number of calcium impermeable (GluA2-containing) AMPA receptors and thereby offset calcium influx caused by existing calcium permeable (GluA2-lacking) receptors (Fig. 9). Accordingly, we expect FUS to serve a protective role under conditions of Gluexcito. We suspect that the acute toxicity imparted by Gluexcito was too strong to detect additional toxicity from FUS knock-down, and that this relationship between FUS expression and cellular viability could be better addressed using in vivo models of chronic stress (59).

In ALS, a putative role for FUS under excitotoxic stress could be compromised as a result of dysregulated Gria2 editing and/or GluA2 expression (58, 60) particularly in motor neurons that rely heavily on AMPA receptor signaling (1, 2). Although we were unable to determine if Gria2 processing is affected by exogenous expression of ALS-linked FUS, altered expression of genes involved in glutamate receptor signaling were recently found in humanized mutant FUS mouse models. Subsequent bioinformatics analyses predict an inhibition of this pathway within mutant FUS spinal cord tissues (25). A “loss of nuclear FUS function” may also impact gene expression under conditions of Gluexcito. For example, FUS egress under conditions of hyperosmotic stress coincided with altered splicing of a known FUS target, sodium voltage-gated channel alpha subunit 4 (SCN4A) (61). Further, endogenous expression of a severely mislocalized FUS variant correlated with reduced levels of Gria2 in motor neurons, a phenotype that was attributed to both loss of nuclear and gain of cytoplasmic functions of the mutant protein (57). However, the relationship between FUS and Gria2 was not examined under conditions of stress in this prior study, nor was the effect of FUS expression on Gria2 sub-cellular localization. Our data suggests that FUS directly influences the localization and expression of specific transcripts such as Gria2, but that FUS expression does not augment or attenuate global protein translation under either stressed or unstressed conditions (Fig. 7). It will be of interest to identify other genes that are influenced by FUS translocation under Gluexcito or hyperosmotic stress, and as a function of disease (25, 35, 62).

While investigating the mechanism(s) underlying excitotoxic FUS egress, we uncovered changes to the CRM1 nuclear export pathway (Fig. 4). Inhibition of CRM1-mediated export by KPT-330 failed to restrict both NLS-ttdTomato-NES (Fig. 4A,B) and FUS (Fig. 4A,C) within the nucleus under Gluexcito. Further, CRM1 localization was significantly shifted towards the cytoplasm (Fig. 4D,E). Despite these changes, nucleocytoplasmic transport was not completely dysregulated, as a partial inhibitory effect of KPT-330 on the shuttling reporter was observed (Fig. 4B). Our KPT-330 studies suggest that Gluexcito-induced FUS egress occurs through a mechanism other than active CRM1 export, and could entail passive diffusion (26) or alternative transport factors (63). Selectivity of RBP egress following Gluexcito may stem from differences in nucleocytoplasmic shuttling dynamics, which are influenced by
Excitotoxicity induces nuclear egress of FUS/TLS multiple factors including binding interactions and post-translational modifications (64). An interesting area of future study could be to elucidate these factors and determine whether they are modulated by stress.

Alterations to CRM1 and Ran localization (Fig. 4) under Glu\textsuperscript{excito} may represent early signs of nucleocytoplasmic transport decline. Indeed, previous studies show that various forms of stress (e.g., excessive calcium influx, oxidative, and hyperosmotic stress) cause damage to nuclear pores and impair nucleocytoplasmic transport (65–69). Mice deficient in key astroglial glutamate transporters exhibited both nuclear pore degradation and motor neuron degeneration (59). Moreover, the nucleocytoplasmic transport pathway has been implicated in age-related neurodegeneration, particularly in the context of ALS and FTD (70). While most ALS/FTD-associated studies have focused on the role of mutant proteins in dysregulating nucleocytoplasmic transport (30, 70), ALS/FTD-associated forms of cellular stress (e.g., excitotoxicity) may also contribute to nucleocytoplasmic transport defects in both inherited and sporadic forms of disease. In fact, nucleocytoplasmic transport is an emerging area of therapeutic development and the CRM1 inhibitor KPT-350 is advancing towards ALS clinical trials. Partial inhibition of CRM1 is expected to offset defects in nuclear import. CRM1 inhibitors have had a therapeutic effect in some (70, 71), but not all (59, 63), models of neurodegeneration. Collectively, the available data support CRM1-mediated nucleocytoplasmic transport as a viable therapeutic target for neurodegenerative disorders. However, a combination therapy addressing additional effects of stress-induced nuclear pore degradation (i.e., calpain inhibitors (59)) may be required for a significant therapeutic outcome.

The calcium-mediated response of FUS to Glu\textsuperscript{excito} has additional implications for neurodegeneration, including cases of FUS-mediated ALS. For instance, motor neurons derived from human ALS-FUS induced pluripotent stem cells are intrinsically hyperexcitable (72). Further, the effects of ALS-linked FUS on calcium-mediated motor neuron toxicity is exacerbated by expression of the mutant protein in astrocytes (55, 58). Most ALS-linked FUS mutations are located within the NLS (24) and induce a shift in the nucleocytoplasmic equilibrium of the protein toward the cytoplasm, where it is believed to exert a gain of toxic function (20, 73). As ALS-linked variants R521G and H517Q translocate further into the cytoplasm under Glu\textsuperscript{excito} (Fig. 3), we predict these and other variants with impaired binding to nuclear import factors will accumulate in the cytoplasm under conditions of chronic stress \textit{in vivo} (36, 65). Moreover, chronic stress may result in nuclear depletion and cytoplasmic aggregation of wild-type FUS and TDP-43 in sporadic cases as well (13, 14). We propose a model whereby FUS and related RBPs play a functional role in response to normal stimulation and moderate degrees of stress, but that excessive or chronic stress severely disrupts their nucleocytoplasmic equilibrium and contributes to disease pathology (Fig. 9).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stress Application**

HEK293-T cells were cultured as described (6). All research involving animals for the primary neuron cultures described below was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. Dissociated primary cortical neuron cultures were prepared using cortices from C57BL/6 embryonic day 14-15 mice. Embryos were isolated in ice-cold Hanks Buffered Saline Solution (Corning 21-023-CV, Corning, NY, USA) and the meninges removed. Cells were dissociated for 12 minutes in 0.05% Trypsin (Invitrogen 25300-054, Carlsbad, CA, USA) at 37˚C, diluted in Dulbecco’s Modified Eagle Medium (Invitrogen 11965-092) containing 10% Fetal Bovine Serum (MilliporeSigma F4135, Burlington, MA, USA) and strained with a cell strainer before gently pelleting. Cells were then resuspended in Neurobasal media (Invitrogen 21103-049), supplemented with 1% Glutamax (Invitrogen 35050-061), 1% Pen-strep (Invitrogen 15140-122) and 2% B-27 (Invitrogen 00800-01), and plated at 1-10^5 cells/mL on polyornithine (final concentration of 1.5 µg/mL; MilliporeSigma P4957) coated plates or coverslips. Neuronal cultures were grown under standard culture conditions (37°C, 5% CO2/95% air) fed every 3-4 days by adding half volumes of
supplemented neurobasal media to each well/dish, with additional half changes of media occurring every other feeding. Unless indicated, during the first feeding (DIV 2 or 3) neuron cultures were also treated with a final concentration of 0.5-1µM Cytosine β-D-arabinofuranoside hydrochloride (MilliporeSigma C6645) to inhibit non-neuronal cell growth. Experiments were performed day in vitro (DIV) 14-16.

Primary motor neurons were isolated from embryonic day 12.5 murine spinal cords as described (73). Briefly, after dissociation in 0.1% trypsin (Worthington LS003707, Columbus, OH, USA) at 37°C for 12 minutes, primary motor neurons were purified using a 6% Optiprep (MilliporeSigma D1556) density gradient and plated on glass coverslips coated with 0.5g/L polyornithine and natural mouse laminin (Thermo Fisher 23017015, Waltham, MA, USA). Cells were grown in glia-conditioned Neurobasal medium and supplemented with 2% B27, 2% horse serum (MilliporeSigma H1270), and 10ng/ml BDNF (PeproTech 450-02, Rocky Hill, NJ, USA), GDNF (PeproTech 450-44), and CNTF (PeproTech 450-50). Primary motor neurons were treated on DIV 6-8 with Ionomycin or dimethyl sulfoxide and on DIV 8 with kainic acid. For glutamate experiments, 100mM glutamate (MilliporeSigma G5889) was freshly prepared in neurobasal media and diluted using primary neuron cultured media to achieve 0.1-10µM solutions. To apply stress, neuronal media was replaced with glutamate-containing primary cultured media or primary cultured media alone (glutamate-free control) for 10 minutes. After 10 minutes, treatment media was replaced with primary cultured media for 30 minutes or longer depending on the experiment prior to fixation or lysate collection. Kainic acid (Abcam ab144490) was diluted from 10 mM/ml to 300µM/ml in primary cultured media and added to motor neurons for 10 minutes followed by a replacement with glia-conditioned media for one hour. Stock solutions of 5mM Ionomycin (MilliporeSigma I9657) or 1M sodium arsenite (MilliporeSigma 71287) prepared in prepared in dimethyl sulfoxide (Corning 25-950-CQC) or water were diluted to 10µM or 1 mM in primary cultured media, respectively, prior to addition to neurons for one hour. MG132 was prepared in DMSO and diluted to 1µM in neuronal media prior to adding to primary motor neurons for 24 hours. Sorbitol (MilliporeSigma S6021) was directly dissolved in primary cultured media to obtain a final concentration of 0.4M and applied to cells for one hour. For experiments in which ethylene glycol tetraacetic acid (EGTA; MilliporeSigma E3889) was added, a 100mM stock was prepared in water, diluted to 2mM in primary cultured media, and allowed to incubate for 30 minutes prior to use during the experimental time course. Translation was inhibited with 2µM cycloheximide (MilliporeSigma C7698). Neurons were treated with 500mM KPT-330 (Cayman Chemical 18127) dissolved in water on DIV 13 for 48 hours prior to treatment with glutamate as well as during the experimental time course.

**Immunofluorescence Analysis**

Primary cortical and motor neurons were fixed with 4% paraformaldehyde (Fisher Scientific AAA1131336, Waltham, MA, USA) at room temperature for 15 minutes and permeabilized with 0.1-0.2% Triton X-100. Cortical neuron immunofluorescence experiments were conducted as described (6, 20) using antibodies described in Table 1. Primary motor neuron samples were blocked in 4% bovine serum albumin for 45 minutes and hybridized overnight at 4°C with primary antibodies (Table 1) and AlexaFluor-conjugated secondary antibodies (73).

**Image Acquisition and Processing**

Primary motor neuron images were imaged using a widefield fluorescence microscope (Nikon TiE, Melville, NY, USA) equipped with a cooled CMOS camera (Andor, South Windsor, CT, USA). Primary motor neurons images were acquired as Z-stacks (0.2µm step size) using a 60x lens. As indicated, fixed primary cortical neurons were imaged using a Leica TCS SP5 II laser scanning confocal (Leica Microsystems, Buffalo Grove, IL, USA) or Leica DMI6000B microscope as described (6). For confocal images of whole cells, 12-bit stacks (∆z = 0.25µM steps, zoom = 3x, n = 23-30 planes) were acquired at 40x with a pixel size of 126nm (1024x1024 pixels; 1000Hz). For dendrites, 12-bit stacks (∆z = 0.08µM steps, zoom = 3x, n = 40-50 planes) were acquired at 63x using a pixel size of 80nm (1024x1024 pixels; 1000Hz). For fluorescence in situ hybridization
Excitotoxicity induces nuclear egress of FUS/TLS (FISH), mFUS and somatic puromycin analyses, widefield stacks of the entire cell were acquired (z=0.2-2.5µm) and deconvolved using the LAS AF One Software Blind algorithm (10 iterations). All neuron images were analyzed using MetaMorph software (Molecular Devices Inc., San Jose, CA, USA). The background and shading of stacks were corrected as described (20). Sum or maximum projections were created from corrected stacks for downstream analyses.

For the quantification of cytoplasmic to nuclear (C:N) ratios, a 20x20 pixel region was applied to the nucleus and perinuclear area in the soma for each cell (visualized by DAPI and MAP2, neuronal nuclei (NeuN), TUJ1 or SMI-32, respectively) as well as an area within each image that contained no cells (representing background fluorescence). Using MetaMorph or ImageJ, the integrated intensity for the signal of interest was obtained for each region and a ratio of the cytoplasmic:nuclear signal was then generated following subtraction of background signal. For each experiment, the statistical comparison of C:N ratios with or without excitotoxic stress was completed using average C:N ratios collected from ≥three independent, biological experiments. For the analysis of FUS levels in neuronal dendrites, Microtubule-associated protein 2 (MAP2) was used to visualize neuronal dendrites and create a dendritic mask using MetaMorph. Using the MAP2-defined mask, the integrated intensity of FUS staining was obtained and used to quantify the relative amount of FUS staining in dendrites. For the quantification of nuclear area, DAPI images were thresholded using MetaMorph and the area of individual nuclei per each cell obtained. For the quantification of total neurons and neurons exhibiting FUS, CRM1 or RAN cytoplasmic translocation by immunofluorescence, ≥10 fields of view were imaged at 40x for each condition tested. As indicated by MAP2 or NeuN staining, neurons were quantified from images with computer assistance from the ‘Cell Count’ feature in MetaMorph. To assess the percent neurons with protein translocation, cells were scored for the presence of cytoplasmic protein divided by the total neuron number to generate the percent population exhibiting a response. All line scan analyses were completed using the Intensity Profile feature in ImageJ and plotted using GraphPad Prism.

Western Analysis
Neurons were treated, washed twice with phosphate buffered saline and lysed using RIPA buffer (Boston BioProducts BP-115-500, Ashland, MA, USA) supplemented with protease (Roche 11836170001, Basel, Switzerland) and phosphotase inhibitors (Roche 4906837001). Lysates were centrifuged at 19,357 x g for 15 minutes, after which the supernatant was collected and its protein concentration determined using a bicinchoninic acid assay (Thermo Scientific Pierce 23227, Rockford, IL, USA). Lysates were subsequently used for Western and densitometry analysis as described (44). Gels were loaded with 8-20µg lysate and GAPDH was used as a loading standard to determine relative protein levels. Primary antibodies used for analysis are described in Table 1; LI-COR (Lincoln, NE, USA) secondary antibodies were used as described (44).

Lactate Dehydrogenase (LDH) Analysis
Neuron toxicity to glutamate was analyzed by the LDH assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega G1782, Madison, WI, USA).

Plasmids and Cloning
Human cDNA for FLAG-HA-tagged wildtype, H517Q, R521G or R495X FUS were cloned into the lentiviral vector, pLenti-CMV-TO-Puro-DEST (Addgene 670-1, Cambridge, MA, USA) using the In-Fusion HD Cloning Plus kit (Clontech 638909, Mountain View, CA). To achieve FUS knockdown, shRNA sequences (44) were packaged using In-Fusion HD cloning into the lentiviral backbone, CSGW2 (a generous gift from Dr. Miguel Sena-Esteves, UMMS), which contains a green fluorescent protein (GFP)-reporter expressed under a separate CMV promoter. The shRNA targeting sequences were: 5’-GCAACAAAGCTACGGACAA-3’ (shFUS1) and 5’-GAGTGGAGGTTATGGTCAA-3’ (shFUS2) as well as the scrambled control sequence, 5’-AATTCTCCGAACGTGTCACGT-3’ (shSC). The shuttling reporter, NLS-tdTomato-NES (a generous gift from Dr. Martin Hetzer, Salk Institute (29)) was cloned into the pLenti-CMV-TO-Puro-DEST vector backbone (Addgene 670-1).
Excitotoxicity induces nuclear egress of FUS/TLS using Gateway BP and LR Clonase reactions (Invitrogen 11789020 and 11791020, respectively). The shuttling reporter contained an NLS sequence (PPKKKRKVQ) and NES sequence (LQLPPLERLTL) attached to tdTomato by a GGGG linker at the N and C termini, respectively.

**Transient Expression of ALS-Mutant FUS**

For transient transfection experiments, neurons were fed at DIV 6 and transfected with FLAG-HA-FUS constructs on DIV 7 using NeuroMag (Oz Biosciences NM51000, Marseille, France) transfection reagents. DNA (1.0 µg) and 1.75 µL NeuroMag (for one 24-well well; 500 µL volume) were combined in an eppendorf tube and the volume adjusted to 50 µL with neurobasal media. The DNA mixture was allowed to incubate for 20 minutes before addition to neurons. Neuron cultures were then placed on a NeuroMag magnet plate (Oz Biosciences MF10096) within the tissue culture incubator for 15 minutes to complete transfection. Transfected neurons were collected for experimental analyses on DIV 14-16.

**Lentiviral Production and Application**

High-titer lentivirus was prepared as described (74). Briefly, HEK-293T cells were individually transfected using calcium phosphate with the shRNA or NLS-tdTomato-NES constructs described along with the packing plasmids: CMVdR8.91 plasmid and VSV-G. DNA constructs were prepared using EndoFree Maxi Prep (Qiagen 12362, Germantown, MD, USA). Three hours after transfection, cell media was replaced with Opti-MEM (Invitrogen 31985-070) and virus was collected in open-top Beckman tubes (Beckman Coulter 344058, Brea, CA, USA) by ultracentrifugation at 133907 rcf in an SW32Ti rotor 72 hours following transfection. Lentivirus titer was obtained by transduction of HEK cells with serially diluted lentivirus. Upon titer determination, virus was added to DIV 6 non-cytosine β-D-arabinofuranoside hydrochloride treated neurons at an approximate titer of 1.2-1.8 \(10^8\) tu/ml. For all transduction experiments except fluorescence *in situ* hybridization, neurons were cytosine β-D-arabinofuranoside hydrochloride treated on DIV 7. Transduced neurons were collected 9 days post-transduction (DIV 15) for analysis.

**Puromycin Analysis**

Based on previously described experiments (35), 4mM stocks of puromycin (Invitrogen, A11138-03) were prepared in water. Neurons were treated with glutamate as described, except that a final concentration of 2µM puromycin was added to the primary cultured media during the last 15 minutes of the ‘washout’ period. As a positive control of translational inhibition, 100µg/ml cycloheximide (MilliporeSigma C7698) was included throughout the experimental time course. Neurons were then analyzed by Western or Immunofluorescence using an anti-puromycin antibody (Table 1). For the analysis of puromycin immunostaining upon FUS knockdown, a 20x20 pixel region was placed in the soma of GFP-positive cells. Using MetaMorph, the integrated intensity of this region was obtained and used to quantify relative puromycin levels as described for the C:N signal intensity analyses above. Similarly, a 20x20 pixel region was applied to the neuronal soma to obtain integrated intensity values for cytoplasmic FUS and puromycin staining. Obtained values for a given cell were used for the correlation analysis of these variables.

**Fluorescence *in situ* Hybridization (FISH) Analysis**

Non-cytosine β-D-arabinofuranoside hydrochloride treated neurons were plated on coverslips and transduced with shFUS or shSC-expressing lentivirus on DIV 6 and harvested on DIV 15. Following stress application, neurons were fixed with fresh 4% paraformaldehyde (Fisher Scientific F79-500) diluted in RNAse free water (Corning 46-000-CM) for 30 minutes at ambient temperature. FISH labeling was completed using a QuantiGene ViewRNA ISH Cell Assay Kit (Affymetrix QVC0001, Santa Clara, CA, USA) according to the manufacturer’s instructions. One exception to the protocol was that samples were dehydrated after fixation with two-minute incubations in 50%, 70%, and 100% ethanol at ambient temperature followed by a second addition of 100% ethanol and stored at -20°C for five days before processing. The Gria2-Cy3 probe was designed and tested for specificity by Affymetrix. For post-FISH immunofluorescence staining, after probe labeling, coverslips were washed in phosphate buffered
Excitotoxicity induces nuclear egress of FUS/TLS

saline for five minutes and then blocked and processed for immunofluorescence as described (75). Coverslips were probed with MAP2 and GFP to visualize neurons and transduced cells, respectively. For analysis, neurons with at least 2 dendrites of >50 µm in length that did not excessively overlap with other cells were selected. Max projections of the imaged z-stacks were analyzed using MetaMorph software. For each neuron analyzed, 2-3 dendrites and the cell body were assessed for their area and the number of mRNA puncta present. Average dendrite data were reported for each cell and 10 cells were analyzed per construct/condition. Images were prepared for visualization in the figures based upon methods previously described (76).

RNA-immunoprecipitation and Quantitative PCR.

Primary cortical neurons were plated on 1.5 µg/mL poly-ornithine coated 10 cm plates and stressed as described above at DIV 13-15. Cells were harvested with polysome lysis buffer (100 mM KCl, 5 mM MgCl$_2$, 10 mM HEPES pH=7, 0.5% NP40, freshly supplemented with protease inhibitors and 200 units RNaseOUT, Invitrogen #10777019). Lysates were cleared through centrifugation (16,000 xg) for 15 min at 4°C. Protein concentrations were determined using the BCA protein assay kit (ThermoFisher, #23227). Lysates (1 mg total protein) were subjected to immunoprecipitation (IP) and 100 µg (10%) of the lysates were reserved for ‘input’ samples. Magnetic Surebeads protein A (100 µL/per condition; BioRad, #1614013) were washed with NT2 buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM MgCl$_2$, 0.05% NP40) and charged with 40 µg rabbit anti-FUS antibody (Genscript) or Chrompure rabbit IgG whole molecule (Jackson ImmunoResearch, #011-000-003) for 2 hr at 4°C by rotation. Charged beads were washed with NT2 buffer and 1 mg lysates were incubated with charged beads at 4°C overnight by rotation. Supernatants were discarded and beads were thoroughly washed six times with NT2 buffer. Beads were resuspended in 100 µL NT2 buffer supplemented with 0.1% SDS and 30 µg Proteinase K (Invitrogen, AM2546) and incubated for 30 min at 55°C. RNA from both IP and input were extracted using TRIzol reagent (Invitrogen, #15596026) following the manufacturer’s protocol. Reverse transcription was performed using iScript Reverse Transcription Supermix (BioRad, #170-8841) as per the manufacturer’s recommendation. Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (BioRad, #1725122) with PrimePCR SYBR mouse GRIA2 probe (BioRad, #qMmuCED0047098) on a CFX384 Real-Time PCR thermal cycler (BioRad). Ct values were acquired through CFX Manager™ Software and data were presented as % input, which is calculated as followed: ∆Ct [normalized IP]= Ct [IP]- (Ct [input]-log2 [Input Dilution Factor]); % input= 2-∆Ct [normalized IP].

Experimental Design and Statistical Analysis.

At least n=3 independent biological experiments were performed for every statistical analysis with GraphPad Prism software; this includes having an independent primary neuron preparation for each biological experiment. The number of independent biological experiments and the statistical test(s) performed are explicitly described in the figure legends as follows. A Student’s t-test at 95% confidence was used when comparing two conditions. For more than two conditions, either a one-way or two-way ANOVA was used followed by the appropriate post-hoc analyses (Tukey’s or Dunnett’s). A Pearson correlation coefficient was used for correlations analyses.
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Table 1. Primary Antibodies Used for Immunofluorescence and Western Analyses.

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<tr>
<th>Antibody</th>
<th>Species*</th>
<th>Company</th>
<th>Dilution/Incubation**</th>
</tr>
</thead>
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*R = rabbit host; M= mouse host
**IF = immunofluorescence; WB = Western blot; O/N = overnight
Figure 1. Endogenous FUS robustly translocates to the cytoplasm in response to excitotoxic stress. (A) DIV 14-16 primary cortical neurons were bath treated with 10μM glutamate (Glu\textsuperscript{excito}) for 10 minutes, after which the glutamate-containing media was ‘washed out’ and replaced with cultured neuronal media for an additional 30 minutes. (B-E) Immunofluorescence and confocal microscopy revealed the cellular localization of FUS, TDP-43, hnRNPA1 and TAF15 (green) in the absence and presence of Glu\textsuperscript{excito}. Endogenous RBP staining (green) visualized by a 16-color intensity map (Int) further demonstrates the cytoplasmic presence of these proteins. Neurons and dendrites were identified with anti-MAP2 staining (red), and nuclei with DAPI (blue). Scale bars = 10μm. (F-I) Quantification of the cytoplasmic to nuclear ratio (C:N) from (B-E) was based on fluorescence intensities of the signal in each compartment as described in the Methods. A significant nuclear egress of FUS (F), TDP-43 (G) and hnRNPA1 (H), but not TAF15 (I) was observed following Glu\textsuperscript{excito} treatment (n = 3-4 biological replicates). Black squares represent the C:N ratio of individual cells, and error bars correspond to SEM. Experimental means were calculated from the average C:N ratio across the individual biological replicates and significant comparisons were determined with a Student’s T-test (FUS **p = 0.0013, hnRNPA1 *p = 0.0107, TDP-43 *p = 0.0185, n.s. = non-significant).
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Figure 2. Cell viability and nuclear membrane integrity are intact under conditions of Glu\textsuperscript{excito} that promote FUS translocation. (A) Following excitotoxic insult, FUS egress and cytoskeletal rearrangements were detected by anti-FUS (green) and -MAP2 (red) staining, respectively. Scale bar = 40µm. (B, C) Quantification of FUS translocation revealed a dose dependent response to glutamate in neurons using a one-way ANOVA and Tukey’s post-hoc test (10µM compared to 1µM ***p = 0.0002, to 0.1µM ***p = 0.0001, to 0.01µM ***p = 0.0002, and to Glu\textsuperscript{−} ***p = 0.0002; 5µM compared to 0.1µM *p = 0.0404, to 0.01µM *p = 0.0426, and to Glu\textsuperscript{−} *p = 0.0451; n = 3 biological replicates). (C) Increased dendritic FUS staining (green) was observed by confocal microscopy following excitotoxic stress. Dendrites were labeled with anti-MAP2 (red). Scale bar = 10µm. (D) Quantification of (C). Black squares represent the intensity of dendritic FUS staining per cell. Means represent the average of n = 4 biological replicates (Student’s T-test; *p = 0.0142) normalized to the control (Glu\textsuperscript{−}). (E) Cytotoxicity induced by Glu\textsuperscript{excito} was assessed after the washout period (Fig. 1A) with the LDH assay. In contrast to the positive control (neurons treated with lysis buffer; lysed neurons), membrane permeabilization was not detected for neurons exposed to Glu\textsuperscript{excito}. Neurons cultured in the absence of Glu\textsuperscript{excito} (Glu\textsuperscript{−}) served as a negative control. Wells containing only primary neuron cultured medium (PCM) served as a background control. Results reflect n = 3 biological replicates analyzed with a one-way ANOVA and Tukey’s post-hoc test (for all statistical comparisons ****p<0.0001, n.s. = non-significant). (F) Immunofluorescence with anti-Lamin A/C staining (red) and confocal microscopy revealed the nuclear envelope was thickened yet still intact within neurons exhibiting translocated FUS (green) after Glu\textsuperscript{excito}. 

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Excitotoxicity induces nuclear egress of FUS/TLS exposure. The time point is the same as (E). Scale bar = 25 µm. (G) Representative line scan analyses of Lamin staining demonstrates enhanced lamin intensity at the nuclear periphery in neurons exposed to excitotoxic insult (scale bars = 10 µm). For (B), (D), (E) and (H), error bars represent SEM. (H) Quantification of nuclear size using the nuclear counterstain, DAPI, revealed a significant decrease in nuclear area following excitotoxic insult. Black squares represent the area of individual neurons. Means represent the average of n = 4 biological replicates (Student’s T-test; *p = 0.0154).
Figure 3. The effect of Glu\textsuperscript{excito} on ALS-linked FUS variants. (A) Cortical neurons transfected with the indicated FLAG-HA-tagged FUS variants were exposed to Glu\textsuperscript{excito} and nuclear FLAG-HA-FUS egress was assessed by immunofluorescence. Exogenous FUS was detected using an anti-HA antibody (green) within MAP2-positive neurons (red). Nuclei were stained with DAPI (blue). Scale bar = 10µm. (B) Quantification of the C:N ratio for FLAG-HA-FUS variants in (A) revealed a significant shift in equilibrium towards the cytoplasm for FLAG-HA-FUS WT, H517Q and R521G, but not R495X (Student’s T-test; WT **p = 0.0003, H517Q ***p = 0.0003, R521G *p = 0.0197, n.s. = not significant, n=3-5 biological experiments). However, the C:N ratio of H517Q, R521G and R495X was not significantly different from FLAG-HA-FUS WT following Glu\textsuperscript{excito} treatment, (Glu\textsuperscript{−}; one-way ANOVA and Dunnett’s post-hoc test; n.s. = non-significant, n = 3-5 biological replicates). Black squares represent individual, cellular C:N measurements. Error bars represent SEM.
Figure 4. Nucleocytoplasmic transport is disrupted by Glu<sup>excito</sup>. (A-C) Cortical neurons expressing the shuttling reporter, NLS-tdTomato-NES, were treated with or without 500 nM of the exportin 1 inhibitor KPT-330 (KPT) prior to Glu<sup>excito</sup> exposure. Neurons were identified with anti-MAP2 staining (A; red). The percentage of MAP2-positive cells expressing cytoplasmic NLS-tdTomato-NES (A; white) or FUS (A; green) was quantified in (B) and (C), respectively (n = 3 biological experiments). KPT-330 effectively prevents NLS-tdTomato-NES from localizing to the cytoplasm in the absence of stress (Glu<sup>-</sup>; two-way ANOVA and Tukey’s post-hoc test; for all statistical comparisons ****p<0.0001), as expected. Conversely, in the presence of stress (Glu<sup>excito</sup>), KPT-330 fails to restrict NLS-tdTomato-NES and FUS localization to the nucleus, indicative of dysregulated nucleocytoplasmic transport (in B and C, compare Glu<sup>-</sup> to Glu<sup>excito</sup> in the presence of KPT-330, two-way ANOVA and Tukey’s post-hoc test; for all significant statistical comparisons ****p<0.0001, n.s. = non-significant). The localization of nuclear transport factors CRM1 (D-F) and RAN (G-I) were significantly altered under conditions of Glu<sup>excito</sup> in MAP2-positive neurons (red); cytoplasmic CRM1 and RAN (green in D and E, respectively) were more apparent in stressed cells. The percentage of neurons with CRM1 or RAN mislocalization were quantified in (E) and (H), respectively (Student’s T-test; CRM1 ***p = 0.0006, RAN ***p = 0.0003; n = 3 biological replicates). Error bars represent SEM. (F, I) Representative line scan analyses of CRM1 and RAN staining demonstrates an increase in the cytoplasmic presence of these proteins. Specific to CRM1, an increase in staining intensity at the nuclear periphery was observed in neurons exposed to excitotoxic insult. Scale bars = 10 µm.
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Figure 5. Calcium is necessary and sufficient for FUS translocation in primary cortical and motor neurons. (A) Reducing extracellular calcium levels with 2mM EGTA attenuates FUS egress (green) in MAP2-positive neurons (red) following excitotoxic insult. Nuclei were stained with DAPI (blue). (B) Quantification of confocal microscopy findings in (A) confirmed the effect of EGTA treatment (two-way ANOVA and Tukey’s post-hoc test; for all statistical comparisons ****p<0.0001; n = 4 biological replicates). (C,D) Application of 10µM of the calcium ionophore, Ionomycin (Iono), for 1 hour induced FUS translocation relative to the dimethyl sulfoxide control (Student’s T-test; ****p<0.0001; n=3 biological replicates). (E,F) FUS translocation induced by hyperosmotic stress (HOS) was not significantly attenuated by EGTA treatment (two-way ANOVA and Tukey’s post-hoc test; for all significant statistical comparisons ****p<0.0001, n.s. = non-significant; n=3 biological replicates). (G,H) A 10-minute treatment of 300µM kainic acid (KA) followed by a 1-hour recovery induced FUS egress in primary motor neurons. Neurons were identified using the neuronal marker, TUJI (red) and nuclei were stained with DAPI (blue). (H) Kainic acid (KA) induced FUS egress was statistically significant relative to the washout control, however the addition of EGTA did not significantly restore FUS localization (two-way ANOVA and Tukey’s post-hoc test; *p<0.0190, n.s. = non-significant; Control and KA n = 8 biological replicates, EGTA and KA + EGTA n = 3 biological replicates). Black squares indicate
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individual cell measurements normalized to the average of the replicate control. Accordingly, means represent the normalized average of biological replicates. Error bars represent SEM. Scale bars = 10µm.
Figure 6. FUS translocation coincides with translational repression in neurons exposed to Glu\textsuperscript{excito}.

(A) Immunofluorescence staining of stress granule marker, G3BP1 (red), shows neurons treated sodium arsenite (NaAsO\textsubscript{2}) form stress granules unlike Glu\textsuperscript{excito} or Glu\textsuperscript{−} conditions where G3BP1 signal remains diffuse. Scale bar = 20 µm. (B) Cellular translation in neurons was monitored by pulse-treatment and incorporation of the small molecule, puromycin, into nascent peptides during excitotoxic and/or cycloheximide treatment (CHX; inhibitor of protein translation). (C) The localization of FUS (green) and incorporated puromycin (magenta) in MAP2-positive neurons (red) was assessed by immunofluorescence. Relative to Glu\textsuperscript{−}, protein translation was reduced upon application of cycloheximide or Glu\textsuperscript{excito}, however cycloheximide did not induce FUS egress from nuclei (DAPI; blue). The white arrowhead marks a neuron with predominately nuclear FUS and high puromycin staining under Glu\textsuperscript{excito}, whereas most neurons under this condition have cytoplasmic FUS and reduced puromycin staining. White boxes denote higher magnification details (right) to highlight neurons with representative levels of translation, as observed by anti-puromycin staining. Scale bars = 10 µm. (D,E) Western and densitometry analysis of puromycin incorporation confirms a significant reduction in translation following cycloheximide or Glu\textsuperscript{excito} treatment relative to Glu\textsuperscript{−} (one-way ANOVA and Tukey’s post-hoc test, for all statistical comparisons ****p<0.0001, n = 3 biological replicates). Puromycin signal was normalized to total protein levels. Error bars represent SEM. (F) Plotting cytoplasmic FUS intensity (x-axis) with puromycin intensity (y-axis) for
neurons treated with glutamate (blue) or control conditions (grey), revealed a significant, inverse correlation between cytoplasmic FUS and puromycin following excitotoxic insult. Statistical analysis of (D) was completed using data from n = 3 biological replicates (Pearson’s correlation coefficient r = -0.3214, ****p < 0.0001, n = 144 neurons).
Figure 7. Reduced protein translation following excitotoxic stress is independent of FUS levels.
Primary neurons were transduced with shRNAs against mouse FUS (shFUS1, shFUS1) or a scrambled control (shSC) to induce FUS knockdown. (A, B) Western and densitometry analysis confirms FUS knockdown relative to non-transduced (NT) and shSC conditions. A modest increase in FUS levels was observed upon expression of shSC relative the loading standard, GAPDH (GAP; n=3; one-way ANOVA and Tukey’s Post Hoc test, ****p<0.0001, **p = 0.0020; n=3 biological replicates). (C, D) Neurons were pulse-chased labelled with puromycin (Puro; magenta) to assess nascent protein translation in transduced cells (as in B-D). The intensity of puromycin staining (Puro) for each condition was normalized to the respective stressed or unstressed non-transduced (NT) control. Scale bar = 10 µm. (E,F) Quantification of puromycin (Puro) staining from (G,H) reveals no statistical difference in the somatic levels of translation following FUS knockdown (shFUS1, shFUS2) relative to shSC (one-way ANOVA and Dunnett’s post-hoc test, n.s. = not significant, n = 3 biological replicates). (G) Further, there was no significant difference in the relative amount of puromycin intensity between stress and non-stress conditions (Glu^excito/Glu^-) as a result of FUS levels (shSC, shFUS1 and shFUS2). For stress and non-stress conditions, puromycin intensities were normalized to the respective, non-transduced control; black squares represent the means represent from n = 3 biological replicates (one-way ANOVA and Tukey’s Post Hoc test, n.s. = non-significant). Error bars = SEM.
Figure 8. Elevation of Gria2 mRNA in dendrites following Glu^{excito} requires FUS expression. (A) Lentivirus expressing a GFP reporter and scrambled control shRNA (shSC) or shRNA against FUS (shFUS1, shFUS2) were used to reduce FUS levels in neurons in order to evaluate Gria2 mRNA distribution in soma and dendrites. (B, C) Following excitotoxic insult, the density of Gria2 was increased in both (B) soma and (C) dendrites of shSC transduced neurons; upon FUS knockdown dendritic Gria2 did not increase following treatment with Glu^{excito} using a two-way ANOVA and Dunnett’s post-hoc test (Soma: Glu^{−} shSC vs. Glu^{excito} shSC *p = 0.0368, Glu^{−} shFUS2 vs. Glu^{excito} shSC **p = 0.0050, Glu^{excito} shSC vs. Glu^{excito} shFUS2 *p = 0.0476; Dendrites: ****p<0.0001, Glu^{excito} shSC vs. Glu^{excito} shFUS1 ****p = 0.0001, Glu^{excito} shSC vs. Glu^{excito} shFUS2 ****p = 0.0002; n = 3 biological replicates). Black squares indicate individual cell measurements. Error bars represent SEM. Scale bars = 10µm. (D) Representative images of (B,C). Gria2 mRNA was detected by FISH (white) in neurons outlined in green (raw images shown in Fig. 7-1B; image processing described in Fig. 7-1C). (E) The amount of Gria2 mRNA bound by immunoprecipitated neuronal FUS is not significantly different between stress and non-stress conditions (Student’s t-test, n.s. = non-significant, n = 3 biological replicates).
Figure 9. A model depicting the impact of excitotoxic stress on neuronal homeostasis and disease pathogenesis. Under homeostatic conditions, shuttling RBPs such as FUS are predominately localized within the nucleus (top). Excitotoxic levels of glutamate (bottom) induce a massive influx of calcium, which is sufficient to induce the robust nuclear egress of FUS into the neuronal soma and dendrites. Excitotoxic stress also leads to translational repression, a re-distribution of nucleocytoplasmic transport factors, and increased levels of Gria2 transcript within dendrites. The expression of FUS is required for enhanced levels of dendritic Gria2 in response to excitotoxic stress, implicating an RNA-processing role for FUS under these conditions. Enhanced levels of edited Gria2 transcript may represent a mechanism to offset the toxic effects of calcium influx. We speculate that prolonged or severe stress could manifest in the pathological aggregation of RBPs, including FUS, in neurodegenerative diseases such as ALS and FTD. Aberrant processing of Gria2 and/or GluA2 can occur through several mechanisms (e.g., expression of mutant FUS in astrocytes, loss of FUS function due to aggregation, and other means as described in the text), and contributes to calcium dyshomeostasis during disease.
The RNA-binding protein FUS/TLS undergoes calcium-mediated nuclear egress during excitotoxic stress and is required for GRIA2 mRNA processing

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