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

Maeve Tischbein[1‡], Desiree M. Baron[1§], Yen-Chen Lin[1§], Katherine V. Gall[1#], John E. Landers[1], Claudia Fallini[1], Daryl A. Bosco[1]*

From the [1]Department of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts, MA 01605

Running title: *Excitotoxicity induces nuclear egress of FUS/TLS*

[‡]Present address: Wave Life Sciences, 733 Concord Avenue, Cambridge, MA 02138

[#]Present address: Homology Medicines, Inc., 1 Patriots Park, Bedford, MA 01730

*To whom correspondence should be addressed: Daryl A. Bosco: Department of Neurology, University of Massachusetts Medical Center, Worcester, MA 01605;
Daryl.Bosco@umassmed.edu; Tel. (774) 455-3745; Fax. (508) 856-6750

[§] These authors contributed equally.

Supporting Information

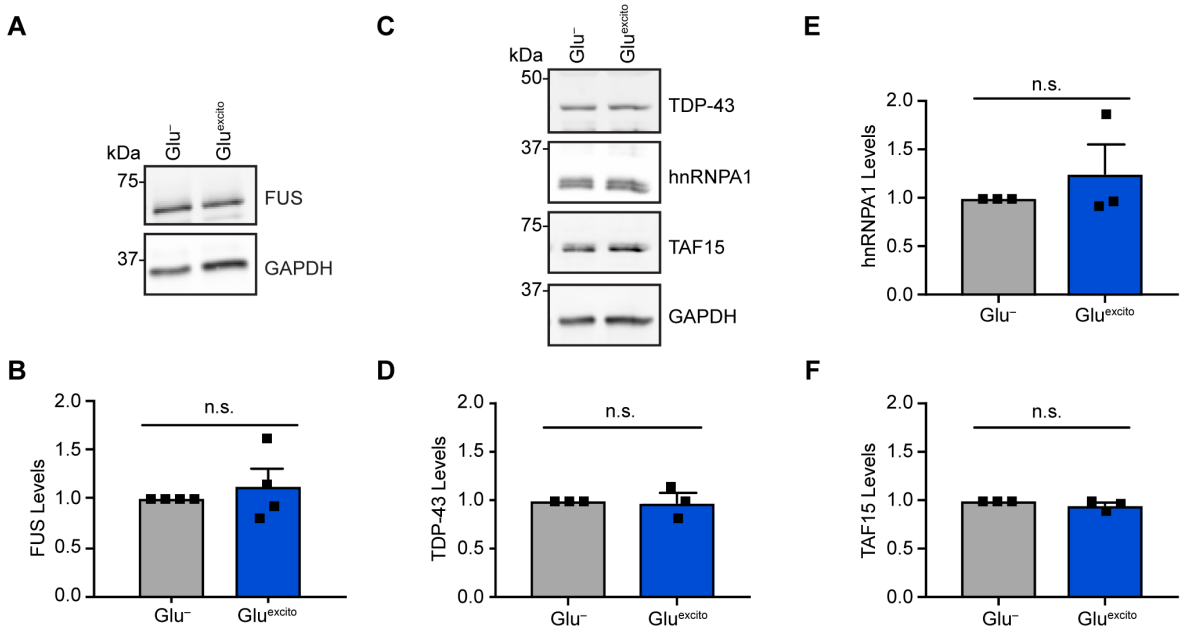


Figure S1. RBP protein levels do not change in response to Glu^{excito}. (A) Western analysis of cortical neurons demonstrate that FUS, TAF15, hnRNPA1 and TDP-43 protein levels do not change in response to Glu^{excito}. **(B)** This observation was confirmed using densitometry. For quantification, FUS levels were first normalized to the loading standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and then the control condition, Glu^{-} (Student's t-test, n.s. = non-significant, n=3 biological replicates). **(C-F)** As in (A), no change in protein levels were observed for TAF15, hnRNPA1 and TDP-43 by Western analysis and subsequent quantification by densitometry (Student's t-test, n.s. = non-significant, n=3 biological replicates). Error bars = SEM.

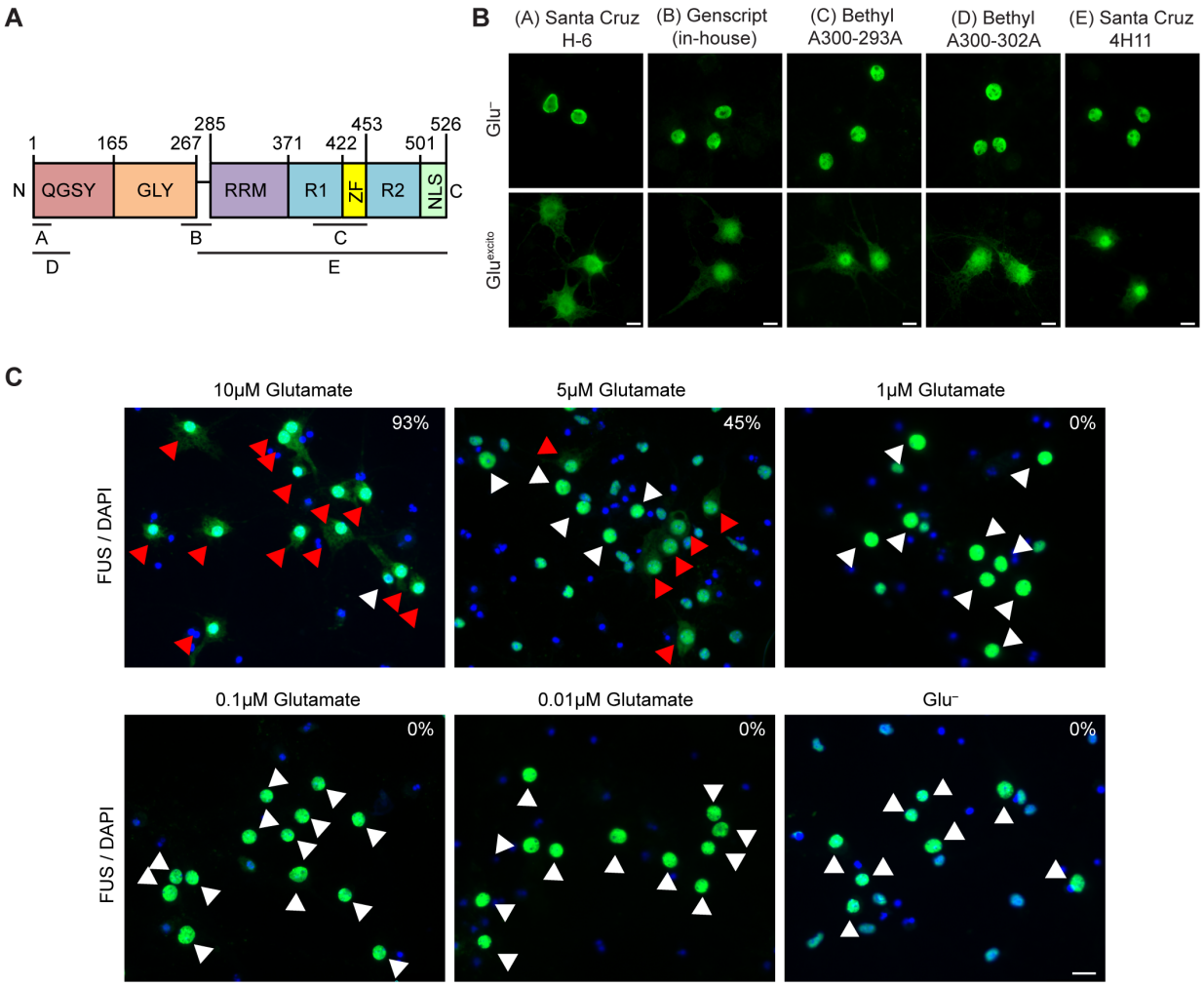


Figure S2. Additional analyses specific to FUS translocation under glutamate-induced stress. (A) Anti-FUS antibody epitopes mapped to the domain structure of human FUS (QGSY = glycine-serine-tyrosine rich region, GLY = glycine-rich region, RRM = RNA recognition motif, R1 and R2 = arginine-glycine-glycine-rich region, ZF = zinc-finger domain and NLS = nuclear localization sequence). **(B)** Immunofluorescence staining of endogenous FUS (green) using antibodies with epitopes described in (A) consistently demonstrates FUS translocation following treatment with. **(C) (C)** Representative images from Figure 2B demonstrate that the response of FUS (green) to Glu^{excito} is all-or-none. NeuN-positive neurons are indicated by an arrow-head (NeuN is not shown for clarity of the FUS signal). Red arrow-heads indicate a neuron with detectable cytoplasmic FUS translocation, whereas white arrow-heads indicate a neuron without detectable FUS translocation. The percentage of neurons with FUS translocation (red triangles) is indicated in the top right of each micrograph, and decreases with decreasing concentrations of glutamate. Scale bar = 20μm.

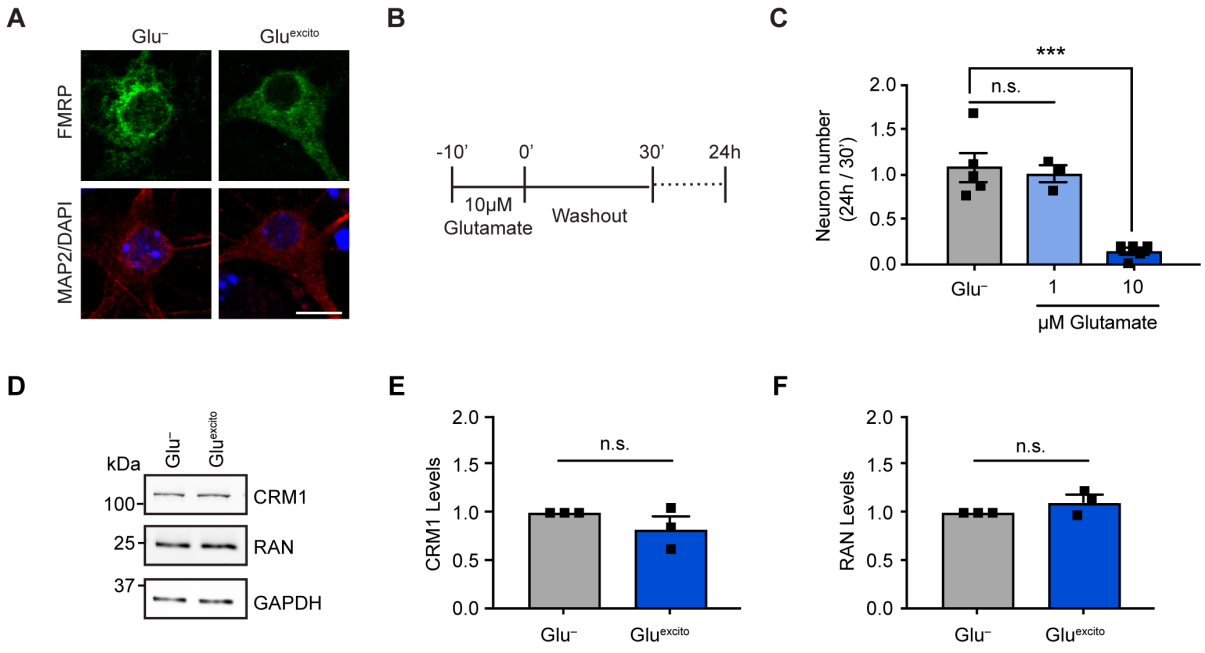


Figure S3. Additional analyses of cortical neurons exposed to excitotoxic levels of glutamate. (A) Confocal analysis of anti-FMRP staining (green) demonstrates that the cytoplasmic localization of this protein is retained in neurons following excitotoxic stress (n = 2 biological replicates). Neurons were identified using a MAP2 antibody (red) and nuclei with DAPI (blue). **(B,C)** Quantification of MAP2-postive neurons at 24 hours relative to 30 minutes shows a significant reduction in neuron number following treatment with 10 but not 1μM glutamate relative to Glu^{-} (one-way ANOVA and Tukey's post-hoc test, ***p = 0.0003, n.s. = non-significant, n = 3 biological replicates). Scale bars = 10μm. Error bars = SEM. **(D-F)** Western analysis of cortical neurons demonstrate that CRM1 and RAN protein levels do not change in response to Glu^{excito}. **(E,F)** For quantification, CRM1 and RAN levels were first normalized to the loading standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and then the control condition, Glu^{-} (Student's t-test, n.s. = non-significant, n=3 biological replicates). Error bars = SEM.

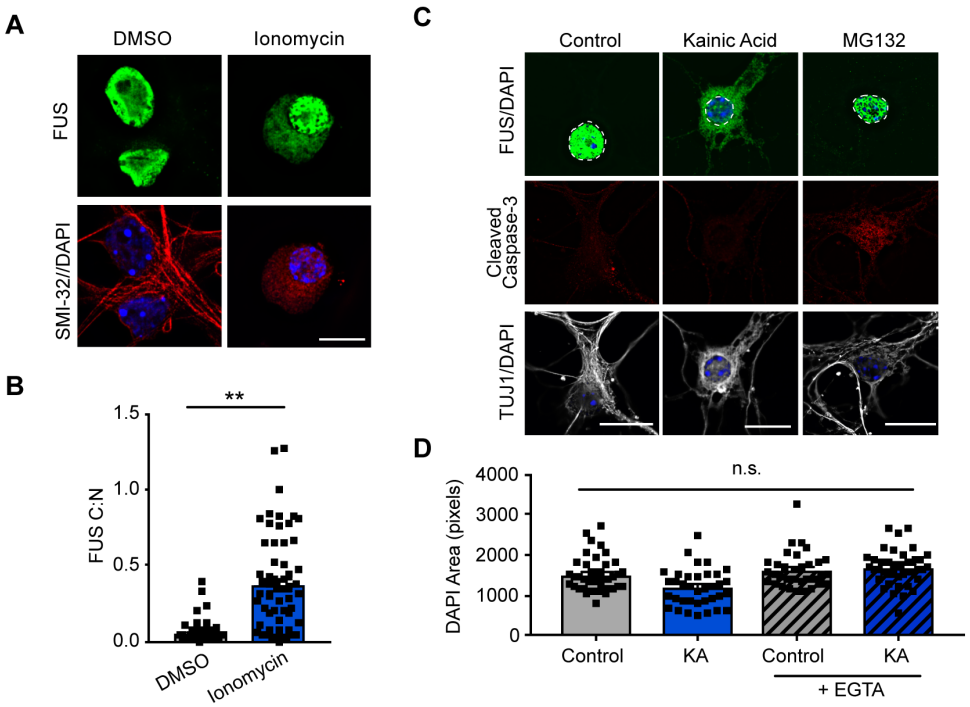


Figure S4. Additional analyses of primary motor neurons exposed to excitotoxic levels of Kainic acid. (A,B) Primary motor neurons treated with Ionomycin also exhibit FUS egress (green) and a significant increase in FUS C:N ratio (Student's T-test, **p = 0.0094, n=3 biological replicates). Motor neurons were identified using the motor neuron marker SMI-32 (specific for neurofilament H) and nuclei were stained with DAPI (blue). Note that the integrity of the neurofilament becomes diminished under stress, yet the nucleus is still intact and not pyknotic. **(C)** Primary motor neurons treated for 10-minutes with 300 µM kainic acid followed by a 1-hour recovery resulted in FUS egress in TUJ1-positive motor neurons (TUJ1 is specific for the neuron-specific class III beta-tubulin). Neuronal nuclei are indicated by DAPI and further highlighted using a white dotted circle. Elevated levels of cleaved Caspase-3 (red), indicative of cells undergoing apoptosis, were not detected Kainic acid treated neurons (representative image shown). In contrast, motor neurons treated with 1µM of the proteasome inhibitor, MG132, as a positive control for 24 hours exhibited elevated levels of cleaved Caspase-3 without FUS translocation. Note that the intensity of the tubulin appears diminished under both Kainic acid and MG132 compared to untreated control cells, yet is still intact. **(D)** Kainic acid (KA) and/or EGTA treatment did not significantly decrease nuclear size as indicated by DAPI area (two-way ANOVA and Tukey's post-hoc test; n.s. = non-significant; Control and KA n = 8 biological replicates, EGTA and KA + EGTA n = 3 biological replicates).

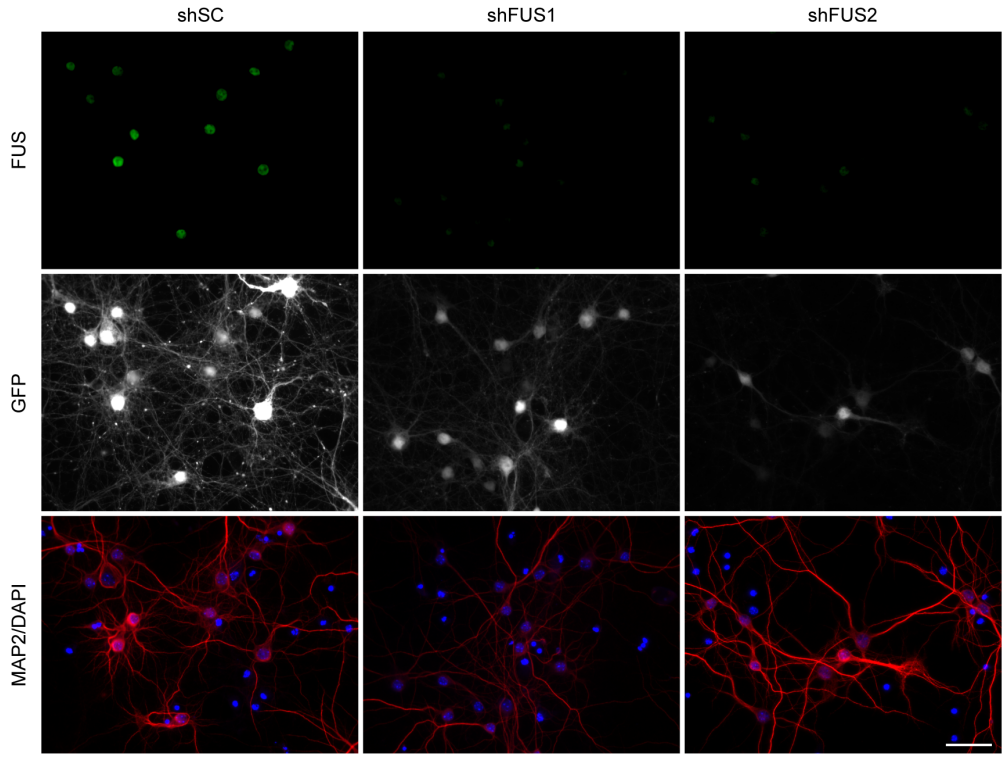
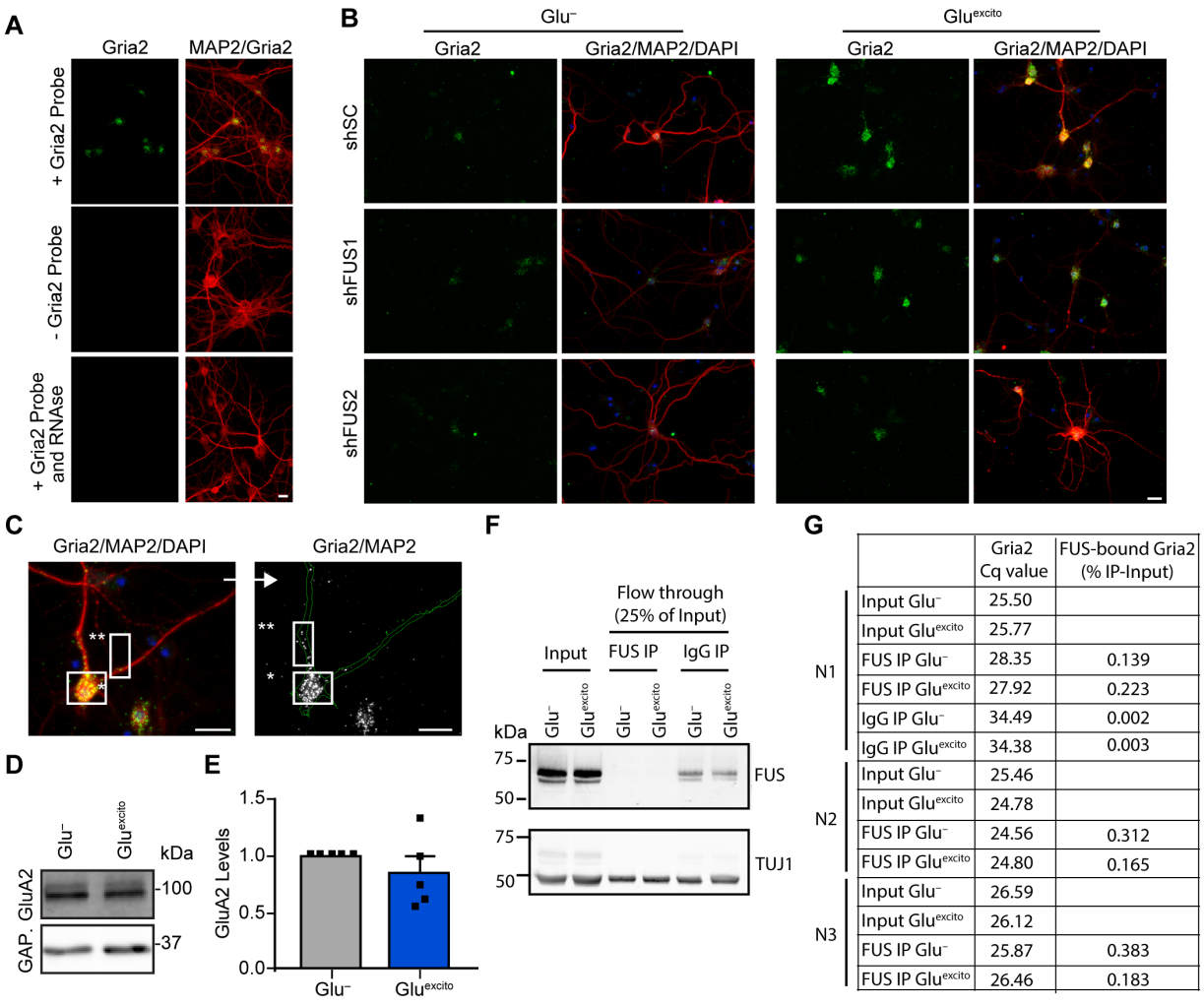


Figure S5. Fluorescence microscopy images demonstrate FUS knockdown upon lentiviral transduction of shRNAs against FUS. (A) Primary neurons were transduced with shRNAs against mouse FUS (shFUS1, shFUS1) or a scrambled control (shSC) to induce FUS knockdown. Transduced neurons were identified by expression of a GFP reporter (white). Immunofluorescence staining of FUS (green) reveals FUS knockdown in transduced neurons identified using a MAP2 antibody (red). Scale bar = 50μm.



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		Gria2 Cq value	FUS-bound Gria2 (% IP-Input)
N1	Input Glu⁻	25.50	
	Input Gluexcito	25.77	
	FUS IP Glu⁻	28.35	0.139
	FUS IP Gluexcito	27.92	0.223
	IgG IP Glu⁻	34.49	0.002
	IgG IP Gluexcito	34.38	0.003
N2	Input Glu⁻	25.46	
	Input Gluexcito	24.78	
	FUS IP Glu⁻	24.56	0.312
	FUS IP Gluexcito	24.80	0.165
N3	Input Glu⁻	26.59	
	Input Gluexcito	26.12	
	FUS IP Glu⁻	25.87	0.383
	FUS IP Gluexcito	26.46	0.183

Figure S6. Additional analyses of Gria2 and GluA2 in cortical neurons exposed to Glu^{excito}. (A) Detection of the Gria2 transcript by FISH (green) was confirmed by the absence of signal in 'no probe' and 'RNAse' controls in MAP2-postive neurons (red). **(B)** Unprocessed images of Gria2 FISH in neurons represented in (**Fig 8D**). **(C)** To generate the images used in (**Fig 8D**), Gria2 puncta (green) were digitally dilated and converted to white. Images of MAP2 staining used to indicate neurons and dendrites (red) were converted to a binary signal and then used as a mask (green) to outline the dendrites. White boxes highlight somatic (*) and dendritic (**) areas used for analysis and depiction in (Fig. 8). (A-C) Scale bars = 25µm. **(D, E)** Western and densitometry analysis of steady-state GluA2 protein levels reveal no statistical difference following Glu^{excito} relative to Glu^{-}. Bands corresponding to GluA2 were normalized to the loading standard, GAPDH (GAP; Student's T-test, n.s. = not significant, n = 5 biological replicates). Error bars = SEM**. (F)** A representative western analysis of the FUS/RNA-immunoprecipitation (IP) reaction confirms similar levels of FUS in the input (lysate used for the IP). The entire elution was used for qPCR analysis and therefore not shown in the Western blot. The flow through represents the lysate leftover after FUS IP, and shows that FUS was depleted from reactions with anti-FUS antibody but not from reactions with the negative IgG control, as expected. TUJ1 was used as a loading control. **(G)** Gria2 qPCR quantitation cycle (Cq) values and the calculated percentage of FUS-bound Gria2 from individual biological replicates (n = 3) are shown.