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PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 111(44)

0027-8424

Sephton, CF
Tang, AA
Kulkarni, A
et al.

2014-11-04

10.1073/pnas.1406162111

Peer reviewed
Activity-dependent FUS dysregulation disrupts synaptic homeostasis

Chantelle F. Sephton1,a,b,1, Amy A. Tang1, Ashwinikumar Kulkarni1,b,d, James Westa, Mieu Brooksa, Jeremy J. Stubblefieldb, Yun Liu1, Michael Q. Zhanga,e, Carla B. Greena, Kimberly M. Hubera, Eric J. Huangf, Joachim Herza,f, and Gang Yua1

Departments of aNeuroscience and fMolecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390; bInstitut Universitaire en Sante Mentale de Quebec, Department of Psychiatry and Neuroscience, University Laval, Quebec City, QC, Canada G1L 2G3; cDepartment of Pediatric Neuropathology and Developmental Neurobiology, University of California, San Francisco, CA 94143; dDepartment of Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX 75080; and eCenter for Synthetic and System Biology, Tsinghua National Laboratory of Information Science and Technology, Tsinghua University, Beijing 100084, China

Edited by Thomas C. Südhof, Stanford University School of Medicine, Stanford, CA, and approved September 17, 2014 (received for review April 2, 2014)

The RNA-binding protein fused-in-sarcoma (FUS) has been associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), two neurodegenerative disorders that share similar clinical and pathological features. Both missense mutations and overexpression of wild-type FUS protein can be pathogenic in human patients. To study the molecular and cellular basis by which FUS mutations and overexpression cause disease, we generated novel transgenic mice globally expressing low levels of human wild-type protein (FUSWT) and a pathological mutation (FUSR521G). FUSWT and FUSR521G mice that develop severe motor deficits also show neuroinflammation, denervated neuromuscular junctions, and premature death, phenocopying the human diseases. A portion of FUSR521G mice escape early lethality; these escapers have modest motor impairments and altered sociability, which correspond with a reduction of dendritic arbors and mature spines. Remarkably, only FUSR521G mice show dendritic defects; FUSWT mice do not. Activation of metabotropic glutamate receptors 1/5 in neocortical slices and isolated synaptoneurosomes increases endogenous mouse FUS and FUSWT protein levels but decreases the FUSR521G protein, providing a potential biochemical basis for the dendritic spine differences between FUSWT and FUSR521G mice.

FUS | frontotemporal lobar degeneration | amyotrophic lateral sclerosis | metabotropic glutamate receptors | synaptic homeostasis

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of upper and lower motor neurons, leading to muscle weakness, paralysis, and death within 3–5 y of onset. Interestingly, ~10–15% of ALS patients have clinical features of frontotemporal lobar degeneration (FTLD), marked by a decline in decision-making, behavioral control, emotion, and language, and as many as half have mild-to-moderate cognitive or behavioral abnormalities (1). FTLD comprises a group of heterogeneous diseases characterized by progressive neurodegeneration of the frontal and temporal lobes and clinically by frontotemporal dementia (FTD) with or without motor neuron disease. There is no cure or effective therapy for those who suffer from ALS or FTLD, and the mechanisms by which these diseases occur are not well understood.

The clinical, pathological, and genetic overlap between ALS and FTLD suggests that there are mechanisms shared by these diseases. The RNA-binding proteins fused in sarcoma (FUS) and transactive response DNA-binding protein-43 (TDP-43) are the major protein components of inclusions that are characteristic of ALS and FTLD-U (FTLD with ubiquitinated inclusions) (2). More than 50 genetic FUS mutations have been identified in these related neurodegenerative disorders (3). Similarly, more than 40 dominant mutations in the TDP-43 gene have been linked to ALS cases and, to a lesser extent, to FTLD (4). The identification of mutations in the FUS and TDP-43 genes has provided insights for uncovering the disease mechanisms for ALS and FTLD.

FUS is a ubiquitously expressed RNA-binding protein that exists in dynamic ribonucleoprotein complexes involved in pre-mRNA splicing, mRNA stability, and mRNA transport. FUS is a member of the FET family of proteins that bind RNAs (5) and contains an RNA recognition motif, three arginine-glycine-glycine (RGG) boxes, and a zinc finger (ZnF) (6). RGG2-ZnF-RGG3 is the major RNA-binding domain, which has a preference for GU-rich sequences (7, 8). The N terminus of FUS contains a low-complexity sequence domain involved in RNA granule formation (9). Nucleocytoplasmic shuttling of FUS occurs by a nonclassical proline-tyrosine nuclear localization signal (PY-NLS) and a nuclear export signal (NES) (10). Methylation of the C-terminal RGG3 domain of FUS is necessary for transportin 1 interaction and nuclear localization (11).

The majority of clinical ALS/FTLD-associated FUS mutations occur in its C-terminal PY-NLS sequence (12), which is believed to enhance the cytoplasmic localization and aggregation propensity of the protein and reduce its ability to bind nuclear RNAs. In response to various stressors, FUS localizes into cytoplasmic stress granules (13). In neurons, there is more immunodetectable FUS at dendritic spines in response to metabolic glutamate receptor (mGluR) agonists (14). Moreover, neurons cultured from

Significance

Both overexpression of wild-type fused in sarcoma (FUS) protein and missense mutations can be pathogenic in a group of related neurodegenerative disorders that includes amyotrophic lateral sclerosis and frontotemporal lobar degeneration. It is unclear how FUS overexpression and missense mutations cause disease in human patients. In this work, we generated novel transgenic mouse models expressing low levels of wild-type and mutant human FUS, both of which recapitulate aspects of the human diseases. We found a profound difference in the underlying mechanisms by which missense mutation and wild-type overexpression cause disease. Overexpression of wild-type FUS protein alters its nuclear function at the level of gene expression. In contrast, missense mutation disrupts activity-dependent synaptic homeostasis to gain a toxic function at dendritic spines.


This article is a PNAS Direct Submission.

This paper contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1406162111/-/DCSupplemental.
FUS-knockout mice have abnormal spine morphology and spine density (14). It is unclear whether pathological FUS protein mutations disrupt activity-dependent synaptic structure or function.

Besides missense mutations at the C terminus of FUS protein, overexpression of wild-type FUS caused by mutations in its 3′ UTR also has been linked to ALS (15), suggesting that overexpression of wild-type FUS is pathogenic under certain circumstances. Indeed, pathogenic effects of increased levels of wild-type proteins are common in other neurodegenerative disorders, as exemplified by increased gene dose or overexpression of wild-type TDP-43, α-synuclein, and amyloid β precursor protein (APP) in ALS/FTLD, Parkinson disease, and Alzheimer’s disease (16–18). However, in all these cases (including FUS), it is unclear whether protein overexpression and missense mutations contribute to neurodegenerative disorders via common or distinct mechanisms. In this work, we developed novel FUS transgenic mice expressing low levels of human wild-type FUS (FUSWT) and an ALS-associated missense mutation (FUSR521G), which is located in the PY-NLS, to investigate the pathological consequences and molecular mechanisms of FUS overexpression and missense mutation.

Results

Development of Cre-Inducible Transgenic Mice Globally Overexpressing Low Levels of Human FUSWT and FUSR521G. We generated transgenic mice expressing human wild-type FUS or the R521G mutation under the control of the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter (Fig. 1A), referred to as “CAG-FUSWT” and “CAG-FUSR521G,” respectively. In this study, we chose to overexpress the FUS transgenes globally, starting from germ line and at low levels to recapitulate more closely the expression profile and FUS levels in patients with ALS and FTLD, because FUS is ubiquitously expressed in human tissues, and human patients carry FUS mutations (or overexpressed wild-type protein) all their lives, starting from germ line. Accordingly, two mouse lines harboring CAG-Z-FUSWT-IRE-EGFP or CAG-Z-FUSR521G-IRE-EGFP were crossed with the germ-line Moex2Cre mice. Cre recombinase excises the LacZ DNA sequence flanked by loxP sequences, allowing translation of FUS and GFP (Fig. 1B and C). Transgenic CAG-FUSWT mice (lines 629 and 638) and CAG-FUSR521G mice (lines 673 and 682) were born at normal Mendelian ratios (SI Appendix, Table S1). Analysis of total brain lysates from CAG-FUSWT (line 638) and CAG-FUSR521G (line 682) mice showed that the level of human FUS expression was similar to that of endogenous mouse FUS (Fig. 1D).

Mice from CAG-FUSWT (629 and 638) and CAG-FUSR521G (673 and 682) transgenic lines were found to have reduced lifespan (Fig. 1E and F). Nearly 100% of CAG-FUSWT mice (lines 629 and 638) die before postnatal day (P)30, and ~70% of the CAG-FUSR521G (line 673) and ~50% of CAG-FUSR521G (line 682) mice have early lethality before P30. In monitoring the body weights of these mice from birth, we observed that the body weights of CAG-FUSWT mice are significantly different from their littermates starting at ~P4 (Fig. 1G and SI Appendix, Fig. S2A). The weight differences between CAG-FUSWT mice and their littermates are less obvious (Fig. 1H and SI Appendix, Fig. S2B). CAG-FUSWT mice developed gait abnormalities at P10. By P14 their grip strength and righting ability were reduced, and hindlimb clasp was present (Fig. 1I and J and SI Appendix, Table S2). Animals at this stage either died or were euthanized. Compared with the CAG-FUSWT mice, the CAG-FUSR521G mice that die early display similar but less severe impairments in locomotion in terms of gait, grip strength, righting ability, and hindlimb clasp (Fig. 1K and L and SI Appendix, Table S2). The CAG-FUSR521G mice that escaped early lethality had somewhat reduced body mass and displayed subtle motor impairment (SI Appendix, Fig. S2 C–E).

Defective Neuromuscular Synapses and Neuroinflammation in Juvenile CAG-FUSWT and CAG-FUSR521G Mice with Severe Motor Impairment. Inflammation, degeneration of motor neurons, and FUS aggregation are present in patients with ALS-FUS (3, 19, 20). We examined brains and spinal cords of end-stage CAG-FUSWT and CAG-FUSR521G mice and found no detectable cytoplasmic FUS localization or protein aggregates (SI Appendix, Fig. S1 E–H). Using immunohistochemistry, we then examined the activation of microglia and astrocytes as markers for neuroinflammation in the brains and spinal cords of end-stage CAG-FUSWT and CAG-FUSR521G mice. We found evidence of activation of astrocytes and microglia in all regions of the brain and spinal cord (Fig. 2 A and B and SI Appendix, Fig. S3 A and B). In contrast, CAG-FUSR521G mice that escaped early lethality did not have these markers of neuroinflammation (SI Appendix, Fig. S3 C and D).

ALS patients develop muscle atrophy caused by the degeneration of spinal motor neurons, together with axonal degeneration and sclerosis of the later columns of the spinal cord, which contain the corticospinal tracts. Examination of the lumbar region of the spinal cord revealed no degeneration of axons in the dorsal corticospinal tract or lateral columns or in the dorsal or ventral roots (SI Appendix, Figs. S4 B and C), indicating that descending motor axons were not altered in CAG-FUSWT or CAG-FUSR521G mice. Muscle histology from end-stage CAG-FUSWT and CAG-FUSR521G mice showed scattered and grouped atrophic muscle fibers (Fig. 2C), a characteristic of denervation in muscle from patients with ALS. CAG-FUSWT muscle showed more severe abnormalities, as observed by the presence of pyknotic myofibers (Fig. 2C, Left), whereas muscle abnormalities in CAG-FUSR521G mice were less severe (Fig. 2C, Right). Quantification of spinal motor neuron numbers in the cervical spinal cord of CAG-FUSWT and CAG-FUSR521G mice showed no evidence of neuron loss as compared with control littermates (Fig. 2 D and E). Importantly, there were abnormalities in the neuromuscular junctions (NMJs) of end-stage animals (SI Appendix, Fig. S5), and analysis of the NMJs revealed significant denervation (Fig. 2 F–H). Our results indicate that degeneration of NMJs and muscle atrophy contribute to loss of motor function in CAG-FUSWT and CAG-FUSR521G mice.

FUS has been implicated in transcriptional and posttranscriptional regulation of gene expression (21–26). We therefore asked whether changes in gene-expression patterns in CAG-FUSWT and CAG-FUSR521G mice explain both the similarities and differences in the behavioral and cellular phenotypes of the wild-type and mutant transgenic mice. To do so, we generated paired-end RNA sequencing (RNA-seq) libraries from total RNA isolated from spinal cords of CAG-FUSWT and CAG-FUSR521G mice and their littermate controls. To avoid secondary effects of end-stage mice on gene expression, we selected transgenic mice that had not yet shown severe deficits in motor function and did not meet our end-stage criteria. To this end, we used P20 mice with a health score between 1 and 2 (as described in Materials and Methods). We carefully selected these mice to be phenotypically similar. Additionally, the samples for each RNA-seq library (n = 2 for each genotype) were pooled from three individual mice, to take into account any phenotype variability (see SI Appendix, Supplemental Experimental Procedures for details). The analysis from CAG-FUSWT mice revealed 185 differentially expressed genes (with adjusted P value <0.05) (SI Appendix, Fig. S6 A and Table S3). Genes with increased expression are enriched with Gene Ontology (GO) terms related to immune response: “DNA replication, recombination and repair” and “regulation of cell proliferation.” Genes with decreased expression show GO terms related to lipid and steroid biosynthesis. In contrast, CAG-FUSR521G mice had very few genes that were differentially expressed (with adjusted P value <0.05) (SI Appendix, Fig. S6B and Table S3), yielding no significant GO terms. The transcriptome profiles of
these mice are consistent with the phenotypic differences observed between the CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} transgenic models, wherein altering wild-type FUS levels is more deleterious than expression of FUS\textsuperscript{R521G}.

**Impaired Motor Function and Sociability in Adult FUS\textsuperscript{R521G} Transgenic Mice.** CAG-FUS\textsuperscript{R521G} mice that escape early lethality were monitored further. They showed persistently lower body weight (SI Appendix, Fig. S2 C and D) with no obvious deficits in locomotion (Fig. 3D and SI Appendix, Fig. S7A) and displayed subtle behavioral differences as compared with their littermates. The motor function of CAG-FUS\textsuperscript{R521G} mice was assessed on a rotordor over a 2-d period. On day 1 of rotordor testing, CAG-FUS\textsuperscript{R521G} mice performed as well as their littermate controls, but on day 2 they had impaired motor function (Fig. 3A). CAG-FUS\textsuperscript{R521G} mice were monitored on voluntary running wheels and showed less activity over a 9-d period (Fig. 3B). Despite the reduction in overall locomotor activity, food intake was not altered significantly (SI Appendix, Fig. S7 B and C). Gait analysis of the CAG-FUS\textsuperscript{R521G} mice revealed that the braking phase was greater in the forelimbs (Fig. 3C and SI Appendix, Table S4), and the swing phase was reduced in the hindlimbs (Fig. 3C and SI Appendix, Table S5). Results from the ladder-walking test indicate that the forelimbs have more errors in stepping with few deficits in the hindlimbs (SI Appendix, Fig. S7 E–H). These data indicate that deficits in the motor function of the CAG-FUS\textsuperscript{R521G} mice are modest and more prominent when their motor function is challenged.

We also examined the social interactions of CAG-FUS\textsuperscript{R521G} mice with intruder/novel juvenile and adult mice. We found that the interaction with juvenile mice was significantly reduced at 4 mo of age (SI Appendix, Fig. S7). When introduced to intruder adult mice, CAG-FUS\textsuperscript{R521G} mice did not show any significant deficits before 8 mo of age (Fig. 3E and Movies S1 and S2). We analyzed the types of social interactions of 8-mo-old CAG-FUS\textsuperscript{R521G} mice with an intruder adult and found that chasing behavior was reduced in CAG-FUS\textsuperscript{R521G} mice (Fig. 3F). No alterations in cognitive function or olfaction were detected in the CAG-FUS\textsuperscript{R521G} mice (SI Appendix, Fig. S7 J and K).
Altered Dendritic Branching in Spinal Motor Neurons and Sensorimotor Neurons of CAG-FUS<sub>R521G</sub> Mice. FUS is found in RNA granules at dendritic spines, and immunodetectable FUS at synapses increases in response to group 1 mGluR stimulation (14, 27). Hippocampal cultures from FUS-knockout mice have altered dendritic branching and reduced mature spines (14), suggesting that FUS has an important role at the synapse. We hypothesized that deficits in motor function and sociability in the CAG-FUS<sub>R521G</sub> escapers could be caused by alterations in dendrites or dendritic spines in the motor neurons and/or sensory motor cortex. We first examined the dendrites of spinal motor neurons in P18 FUS transgenic mice and found no reduction in the number and density of mature spines (Fig. 4A and D). In contrast, the dendritic intersections and cumulative area of dendrites were reduced significantly in spinal motor neurons in CAG-FUS<sub>R521G</sub> mice of the same age (Fig. 4 B and D). We then examined the CAG-FUS<sub>R521G</sub> escapers at age 2 mo (P60). Even though the distribution of the numbers of intersections and cumulative area of dendrites were slightly different in P18 and P60 mice, we found significant and persistent deficits in the dendritic branches in spinal motor neurons (Fig. 4 C and D).

Moreover, analysis of apical and basal dendrites in neurons in sensorimotor cortex layers IV–V in CAG-FUS<sub>R521G</sub> mice showed fewer intersections and reduced cumulative area in the apical and basal dendrites of P18 and P60 mice (Fig. 4 E and F).

Activity-Dependent Reduction of FUS<sub>R521G</sub> Protein Levels at Synapses. Cultured neurons from FUS-knockout mice have abnormal spine morphology as well as spine density (14). Therefore we decided to examine whether our transgenic models had alterations in the number of mature spines. We found that there was no difference in the total number of mature spines in the CAG-FUS<sub>WT</sub> mice, but CAG-FUS<sub>R521G</sub> mice had a significant decrease in the number and density of mature spines (Fig. 5 A and B).

Activation of group 1 mGluRs in hippocampal neurons has been shown to affect spine shape in a protein synthesis-dependent manner (28). Given what is known about the existence of FUS at synapses and its response to mGluR signaling, we hypothesized that deficits in dendritic branching and spine formation may stem from altered responses of FUSR521G protein to mGluR activation. To test this hypothesis, we determined whether FUS<sub>R521G</sub> protein displayed an altered synaptic expression upon activation of mGluRs.

Fig. 2. Gliosis, muscle atrophy, and denervated NMJs in CAG-FUS<sub>WT</sub> and CAG-FUS<sub>R521G</sub> mice. (A and B) Immunofluorescence staining of the CA3 region of the hippocampus (A) and of the ventral horn of the spinal cord (B) for Iba1 (microglia; red) and GFAP (astrocytes; green) of end-stage mice. Images are representative of three animals per genotype. (C) H&E staining of the gastrocnemius muscle of CAG-FUS<sub>WT</sub> and CAG-FUS<sub>R521G</sub> mice at end stage show wasting of the muscle and scattered and grouped muscle atrophy, characteristic of motor axon degeneration. Arrowheads indicate pyknotic myofibers. Images are representative of three animals per genotype. (D) Immunostaining for ChAT highlights motor neurons in the cervical spinal cord of CAG-FUS<sub>WT</sub> and CAG-FUS<sub>R521G</sub> mice. (E) Quantification of spinal motor neuron numbers in the cervical spinal cord of CAG-FUS<sub>WT</sub> and CAG-FUS<sub>R521G</sub> mice show no evidence of neuron loss compared with control littermates. n = 3 CAG-FUS<sub>WT</sub> mice and littermate controls, and n = 4 CAG-FUS<sub>R521G</sub> mice and littermate controls. ns, not significant (Student t test). (F) Costaining for presynaptic terminals (nerve; red) and with bungarotoxin for postsynaptic terminals (AchR; green) shows that NMJs are denervated in P20–P24 CAG-FUS<sub>WT</sub> and CAG-FUS<sub>R521G</sub> mice at end stage compared with littermate controls. (G and H) Quantification of innervated NMJs. a, P < 0.05; b, P < 0.01 (Student t test). Error bars represent SD of the mean.
(R,S)-3,5-dihydroxyphenylglycine (DHPG) (Fig. 5 C and D). This result is consistent with an increase in immunodetectable FUS at synapses in response to mGluR stimulation previously reported in dissociated hippocampal cultured neurons (14, 27). In contrast, FUSR521G protein levels were reduced in response to DHPG treatment in acute cortical slices (Fig. 5 C and D). These results indicate that mutant FUS does not respond properly to mGluR activation, and the reduced FUS levels may lead to the altered dendritic branching and spines.

The decrease in FUSR521G could be caused by a deficit in the synthesis, trafficking, and/or degradation of FUS proteins. To test whether FUS’s response to DHPG stimulation is a local event at synapses, we isolated synaptoneurosomes using a discontinuous Percoll–sucrose gradient and treated them with DHPG in vitro. We found that FUS expression is induced significantly in the synaptoneurosomes of control mice (Fig. 5 E and F), suggesting that local synthesis of the protein does occur. We then performed these same in vitro experiments in synaptoneurosomes from CAG-FUSWT and CAG-FUSR521G mice. We found that both endogenous mouse FUS and exogenous human FUSWT increase in the CAG-FUSWT samples and decrease in the CAG-FUSR521G samples (Fig. 5G), as is consistent with our observation in the acute cortical tissue slice model (Fig. 5 C and D). We then tested whether the decrease in FUSR521G could be blocked using a proteasome inhibitor, MG132, and found that inhibiting the proteasome does not prevent a decrease in FUS expression (Fig. 5H). Together, these observations suggest that the alterations of FUS levels in response to mGluR activation are local synaptic events, likely related to protein synthesis.

**Discussion**

**Cre-Inducible Transgenic Mice Expressing Low Levels of FUS as Novel Models of ALS and FTLD.** The pathological and genetic association of FUS with ALS and FTLD suggests that dysregulation of FUS may lead to neurodegenerative diseases. However, the mechanism by which FUS aggregation or mutations cause ALS and FTLD is not known. To study the role of FUS in neurodegeneration, we generated Cre-inducible FUS transgenic mice that express low levels of wild-type (FUSWT) or mutant (FUSR521G) proteins. Under control of the CAG promoter, the human FUS transgene is expressed ubiquitously in the germ line of CAG-FUSWT and CAG-FUSR521G mice (Fig. 1). CAG-FUSWT and CAG-FUSR521G mice that develop severe deficits in motor function have denervation of the NMJs, muscle atrophy, neuroinflammation, and early lethality (Figs. 1 and 2). The phenotypes observed in our transgenic models phenocopy aspects of adult cases of ALS. However, the onset of phenotypes in the mouse models is earlier, more closely reflecting FUS-linked neurodegeneration, we generated Cre-inducible FUS transgenic mice that express low levels of wild-type (FUSWT) or mutant (FUSR521G) proteins. Under control of the CAG promoter, the human FUS transgene is expressed ubiquitously in the germ line of CAG-FUSWT and CAG-FUSR521G mice (Fig. 1). CAG-FUSWT and CAG-FUSR521G mice that develop severe deficits in motor function have denervation of the NMJs, muscle atrophy, neuroinflammation, and early lethality (Figs. 1 and 2). The phenotypes observed in our transgenic models phenocopy aspects of adult cases of ALS. However, the onset of phenotypes in the mouse models is earlier, more closely reflecting FUS-linked juvenile ALS (29–31).

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A portion of CAG-FUSWT mice that escape early lethality have impairments in motor function and sociability (Figs. 1 I–L and 3), which are likely linked to the alterations in dendritic branches and spines in the upper and lower motor neurons (Figs. 4 and 5). Adult CAG-FUSR521G mice do not perform as well on the rotorod and they are less active on a running wheel (Fig. 3 A and B). Specifically, the forelimbs of these mice are impaired (SI Appendix, Fig. S7 E and F). Upper or lower limb weakness is common in both ALS and FTLD with motor function deficits (32–35). Changes in social interactions also are
a common clinical feature of patients with FTLD and in ALS patients with dementia (1). Similar to progranulin (Gmn)-knockout mice, a model of familial FTLD that has deficits in social interaction (36), CAG-FUS<sup>R521G</sup> mice have deficits in social interactions with intruder/novel juvenile and adult mice (Fig. 3 E and F and SI Appendix, Fig. S7I).

**Differences and Commonalities in FUS Overexpression and Missense Mutations.** The CAG-FUS<sup>WT</sup> and CAG-FUS<sup>R521G</sup> mouse models demonstrate that increased expression of FUS alone can cause cellular toxicity. This result is consistent with the recent finding that mutations in the 3’ UTR of FUS increase FUS expression levels and cause ALS (15). This result is also in agreement with

**Fig. 4.** Reduced dendritic branching in spinal motor neurons and sensorimotor neurons of CAG-FUS<sup>R521G</sup> mice. (A) Sholl analyses show no reduction in the number of dendritic intersections or cumulative area of dendrites in spinal motor neurons in CAG-FUS<sup>WT</sup> mice. (B and C) In contrast, the dendritic intersections and cumulative area of dendrites show significant reductions of spinal motor neurons in P18 (B) and in 2-mo-old (P60) (C) CAG-FUS<sup>R521G</sup> mice. (D) Representative images of Neurolucida tracing of the dendrites of spinal motor neurons in control (CTL), CAG-FUS<sup>WT</sup>, and CAG-FUS<sup>R521G</sup> mice. A total of 36 spinal motor neurons were analyzed in CAG-FUS<sup>WT</sup> and CAG-FUS<sup>R521G</sup> mice and corresponding littermate controls. (E, Upper) Sholl analyses show reduced intersections and cumulative area in the apical dendrite within 50–250 μm from the cell body of cortical neurons of P18 and P60 CAG-FUS<sup>R521G</sup> mice. (Lower) Similar reductions in the dendritic intersections and cumulative surface areas are also identified in the basal dendrites of CAG-FUS<sup>R521G</sup> neurons. A total of 24 neurons from cortical layers IV–V were analyzed in CAG-FUS<sup>R521G</sup> mice and corresponding littermate controls. (F) Representative images of Neurolucida tracing of the apical and basal dendrites in neurons from layers IV–V in the sensorimotor cortex in control and CAG-FUS<sup>R521G</sup> mice. For each group three or four animals were analyzed. (A–C and E) P < 0.0001 (two-way repeated measures ANOVA). Error bars represent SEM.
the observations that increased gene dose or overexpression of APP, α-synuclein, and TDP-43 can cause Alzheimer’s disease, Parkinson disease, and ALS/FTLD (16–18). On the other hand, we did not detect overt motor neuron loss or apparent ubiquitin-positive aggregation and mislocalization of FUS in neurons and glia of either of our transgenic mouse models (SI Appendix; Fig. S1 E–H), suggesting that permanent FUS mislocalization, aggregation, and motor neuron loss are not necessary for disease onset but might be
end-stage pathological markers or outcomes in human patients. Moreover, our studies suggest that peripheral and central synapses are more vulnerable than axons and cell bodies and that synaptic defects precede axonal and neuronal degeneration.

The cellular phenotypes observed in our animal models may represent cellular events occurring before FUS mislocalization, aggregation, and neuronal death that are the key neuropathological features of ALS/FTD. Although FUSRS521G is not overtly mislocalized in our animals, it is likely that the shuttling dynamics of FUSRS521G are altered, because FUS R521 is located at PY-NLS. On the other hand, PY-NLS has very high binding affinity (kd = 9.5 nM) for karyopherinβ2 (Kapβ2, also known as “transportin”), which mediates FUS nuclear shuttling (37). Although ALS mutations in PY-NLS reduce Kapβ2-binding affinities by several fold (37), the mutant FUS proteins still have nanomolar affinity and thus are expected to be translocated efficiently to the nucleus unless the nuclear import machinery is overwhelmed (such as when wild-type or mutant FUS proteins are massively overexpressed). Therefore it is not surprising that there is no overt FUS mislocalization in our transgenic mice with low FUS expression.

Some of our CAG-FUSRS212G mutant mice escaped early lethality, but none of the CAG-FUSWT mice survived to adulthood. The similar phenotypes we observe in both of the CAG-FUSWT and CAG-FUSRS212G founding lines reduces the possibility that these observations are caused by insertional effects. Also, these transgenic lines have very low copy numbers of transgenes with single genomic insertion (SI Appendix, Fig. S1C). Interestingly, in ALS patients the age of disease onset and clinical phenotypes are variable, with incomplete penetrance for TDP-43 and FUS mutations. It is possible that other factors, such as genetics and environment, have an impact on whether an individual can escape the consequences of these autosomal dominant mutations. Incomplete penetrance has also been observed in carriers of the APOE4 allele, 50% of whom develop Alzheimer’s disease but the remainder do not. Another argument for incomplete penetrance of FUS mutations has to do with the possibility that mutations in the PY-NLS are also partial loss-of-function mutations, presumably in gene expression. This is supported by the finding that the ALS-associated FUS mutant proteins R521G and H517Q have reduced binding to intronic sequences of its nuclear RNA targets (24).

We note that the CAG-FUSWT mice readily recapitulated human diseases caused by increased levels of wild-type FUS. Although CAG-FUSRS212G mice can model the toxic gain of functions of FUS (which is highly relevant to the studies of ALS/FTLD), modeling loss of function is more difficult in the mutant mice. Nevertheless, results from our parallel studies of the wild-type and mutant animals are consistent with a model wherein overexpression of FUSWT alters the nuclear function of endogenous FUS at the level of gene expression, but FUSR521G mutation has both a partial loss of function in RNA regulation and gene expression and a partial toxic gain of function in disrupting synapses. This model is supported by our transcriptome analysis of the spinal cords of the transgenic mice, which revealed that the gene-expression pattern is altered in CAG-FUSWT mice but not in CAG-FUSRS212G mice (SI Appendix, Fig. S6). This result also potentially explains why an increase in wild-type FUS level is more deleterious than the overexpression of FUSRS212G (Fig. 1 and SI Appendix, Fig. S6 and Table S2). At the steady state, both FUSWT and FUSR521G stay mainly in the nucleus, but FUSR521G has no apparent effect on gene expression. This result supports the view that overexpressed FUSR521G has a diminished ability to alter the nuclear function of endogenous FUS in gene expression. As discussed in more detail in the next section, although FUSR521G does not affect gene expression, it has a toxic gain of function disrupting synaptic homeostasis at dendritic spines. In contrast, FUSWT does not affect synaptic homeostasis.

In other published animal models (38–40), overexpression of wild-type FUS has been reported as being less toxic than overexpression of mutant FUS. In addition, a recently developed FUSWT transgenic mouse line showed no deficits until crossed to homozygosity, wherein these mice displayed progressive hindlimb paralysis and neuromuscular denervation (41). These studies contrast with our observation that overexpression of FUSRS212G is more toxic than overexpression of FUSR521G in terms of early lethality. The reason for the different degree of toxicity in different FUS transgenic models needs to be examined further and will yield important insight into disease progression. In this regard, we note that in our models FUS proteins are expressed globally at low levels and during early embryonic development. Because ALS is thought to be a non-cell-autonomous disease (42), it will be important to examine the contribution of different cell types, particularly astrocytes and microglia, to the phenotypes observed in our transgenic models. The Cre-inducible transgenic system developed herein will allow temporal and spatial expression of FUS proteins in glial cells to test the hypothesis that ALS and FTLD are non-cell-autonomous. Similarly, it will be informative to use Cre-lines specific for motor neurons and cortical neurons to examine separately FUS’s contributions to ALS and FTLD.

Mutant-Specific Disruption of mGluR-Dependent Synaptic Homeostasis. Primary hippocampal cultures from FUS-knockout mice have altered dendritic branching and reduced mature spines (14). Based on this information and the behavioral phenotypes in the CAG-FUSRS212G mice, we examined dendritic branching in the motor neurons and sensorimotor cortex and found significant reductions in dendritic intersections and in the cumulative area of dendrites. Moreover, the density of mature dendritic spines is reduced in the apical and secondary dendrites in the mutant mice (Fig. 5). These data are consistent with transgenic mice harboring the R521C mutation under control of the Syrian hamster prion promoter (43). However, Qiu et al. (43) reported only transgenic mice for FUS mutant R521C, without comparable wild-type transgenic animals. Therefore it was unclear whether the phenotypes in their studies were caused by simple overexpression of FUS protein or were specific to the FUS mutation. In our studies we observed persistent dendritic defects in the spinal motor neurons and cortical neurons in FUSR521G mice at P18 and P60, suggesting that the negative impacts of FUSR521G on dendritic morphology can occur at young age. Importantly, we did not see the same alternations in the CAG-FUSWT mice, indicating that although certain aspects of the CAG-FUSWT and CAG-FUSR521G models are similar, the alterations in synaptic homeostasis resulting in alternations in dendritic branches and spines are specific to the R521G mutation. It is likely that disruption of synaptic homeostasis at dendritic spines contributes to the alterations in motor function and social interaction of the mutant transgenic animals.

Interestingly, Gm-knockout mice display similar alterations in dendritic branching and spine maturation, which correspond with deficits in social interaction (36). FTLD and ALS share common clinical and pathological features including loss of cognition, motor impairment, and TDP-43- or FUS-positive inclusions. Although the FUS R521G mutation is associated with familial ALS, the rare FUS mutations P106L, G206S, and M254V are linked to familial FTLD (3). Indeed, our CAG-FUSRS212G mice phenocopy aspects of the loss of motor function observed in ALS. They also show changes in social interactions resembling those observed in FTLD. On the other hand, alterations in dendritic branching and spines have not been documented for ALS or FTLD, but the findings from our CAG-FUSRS212G mice suggest that these alterations might exist in human patients.

FUS also localizes to RNA granules at the synapse (27) and copurifies with the NMDA receptor (44). In response to mGluR
stimulation, there is more immunodetectable FUS at dendritic spines (14). Fujii et al. (14) did not assess whether the increase in immunodetectable FUS at the synapse was caused by local translation of FUS mRNA or by localization to the synapse. Using an in vitro assay to assess local protein translation in isolated synaptoneurosomes, we demonstrate that the increase in FUS expression at the synapse in response to mGluR activation is a local event (Fig. 5 E and F). The acute increase in synaptic FUS expression in response to mGluR activation as demonstrated by Fujii et al. (14) and in our study strongly suggests that FUS participates in the regulation of mRNAs important to synaptic function and serves as an important synaptic RNA-binding protein. This finding is reproducible in our studies using acute cortical tissue slices, where FUSWT protein is increased but FUSR521G protein is reduced in response to mGluR activation (Fig. 5 C and D). We also found that inhibiting the proteasome does not prevent the activity-dependent decrease of FUS expression in synaptoneurosomes isolated from CAG-FUSR521G mice. Together, these observations suggest that the alterations of FUS levels in response to mGluR activation are local synaptic events that are likely to be related to protein synthesis. Future studies will test whether dysregulation of synaptic FUS in response to mGluR activation contributes to the altered dendritic branching and maturation of spines in the CAG-FUSR521G mice and perhaps also in human patients with ALS or FTLD.

Activity-dependent down-regulation of the FUSR521G protein at the synapses and its potential role in disrupting the formation or maintenance of dendritic spines provides a tantalizing mechanism for FUS regulation at the synapse. In this context, we note that our finding that FUS dysfunction disrupts synaptic homeostasis at dendritic spines somewhat parallels observations for another RNA-binding protein, fragile X mental retardation protein (FMRP). FMRP has been shown to regulate spine shape in a protein synthesis-dependent manner. In response to mGluR signaling, FMRP regulates local translation of mRNAs at the synapse (45). Loss-of-function mutations in the FMR1 gene cause fragile X mental retardation syndrome, in which a deficit in spine maturation is thought to underlie the autism-like symptoms in individuals with the syndrome (46). In the future, it would be important to test how deficits in the synthesis of mutant FUS proteins leads to the disruption of synaptic homeostasis. Moreover, it would be of interest to examine whether disruption of synaptic homeostasis caused by dysfunction of RNA metabolism represents a common theme of brain disorders.

Materials and Methods

For more details, see SI Appendix, Supplemental Experimental Procedures.

Generation of FUS Transgenic Mice. Wild-type or mutant RS21G human FUS cDNAs were inserted into the CAG-Z-ires-EGFP vector (provided by Yuji Mishina, University of Michigan, Ann Arbor, MI). The CAG-Z-FUS-ires-EGFP construct was digested with AvFl and SpeI to remove the vector sequence and then was injected into fertilized oocytes from C57BL/6 female mice and implanted into pseudopregnant ICR mice. Mice carrying the transgene were identified by PCR analysis and then was injected into fertilized oocytes from C57BL/6 mice and generated as previously described (47). Immunodetected proteins were quantified by densitometry using the NIH ImageJ software. We were able to estimate the amounts of human and mouse FUS in the transgenic animals visually by Western Blot Analysis.

Histology Staining. Tissues stained with H&E were fixed in 10% (wt/vol) formalin fixative for 48 h, paraffin embedded, and sectioned to 8-μm thickness.

Weblinks. Western Blot Analysis. Lysates from tissues were processed, and equal proteins were resolved by SDS-PAGE as previously reported (47). Primary antibodies used were glutamate receptor 1 (GluR1; MAB2263; Millipore), GADPH (G9545; Sigma), FUS (HPA008784; Sigma), FUS (sc-47711; Santa Cruz), GPDH (1020; Aves); human FUS antibody was a gift from Hongxia Zhou and Xu-Gang Xia, Thomas Jefferson University, Philadelphia (40) and human FUS peptide antibody B272D (SYGGQPSQGSSYQGPS) was generated in rabbits as previously described (47). Immunodetected proteins were quantified by densitometry using the NIH ImageJ software. We were able to estimate the amounts of human and mouse FUS in the transgenic animals visually by using larger SDS-PAGE gels (Fig. 1D) but were unable to quantify the human FUS level accurately because of the similar molecular weights of human FUS (526 amino acids) and mouse FUS (518 amino acids) (SI Appendix, Fig. S1D).

Choline Acetyltransferase Staining of Spinal Cord and Quantification of Spinal Motor Neurons. Tissues from the cervical spinal cord were processed, immunostained, and analyzed as previously reported (43). Anti-choline...
acetytransferase (CHAT) (1:300; Millipore), biotinylated rabbit anti-goat IgG antibody (1:100; Vector Labs); and the VECTASTAIN Elite ABC Kit (Vector Labs) were used for staining motor neurons. CHAT-positive cells in the ventral horn region were quantified using at least 12 images per animal. CAG-FUSWT (n = 3) and CAG-FUSVT (n = 4) transgenic mice and their littermate controls (n = 3 or 4) were analyzed.

Acute Treatment of Cortical Tissue Slices with DHPG. After DHPG treatment of acute cortical tissue slices, total cell lysates were collected, and the remaining homogenate was passed through two 100-μm filters and then through one 10-μm filter. Synaptoneurosomes were pelleted after 10-min centrifugation at 1,000 x g. Total cell lysates, supernatants, and synaptoneurosomes were lysed as previously reported (50). Experimental replicates (n = 3) were analyzed for each genotype.

Synaptoneurosomes isolation, in vitro Treatment with DHPG. Synaptoneurosomes used for in vitro DHPG stimulation experiments were isolated as previously reported (51). Synaptoneurosomes were equilibrated to room temperature for 10 min before stimulation with DHPG (100 μM). Pretreatment with DMSO vehicle (or 25 μM MG132 (Toxic Biosciences) was performed at room temperature for 10 min before DHPG stimulation.

ACKNOWLEDGMENTS. We thank Jim Richardson and John Shelton of the University of Texas Southwestern (UTSW) Histology Core Facilities for assistance with histology staining and analysis; Shari Birmbaum and Laura Peca of the UTSW Behavior Core Facility for assistance with rotodors, Didigait, social interaction, olfactory, and Y-test testing; Erik Plautz and Sherry Rovinsky of the UTSW Neuro-Models Facility, with support from the Haggerty Center for Brain Injury and Repair, for assistance with ladder-running testing and analysis; Leighton Stein of the Roswell Park Cancer Institute for assistance with FISH analysis of copy number in mouse embryonic fibroblasts; Weichun Lin for advice on NMI studies; Vincent Zinnem (Ecole Polytechnique Federale de Lausanne) and Pradipita Ray (University of Texas at Dallas) for contributions to the initial RNA-Seq analysis; and Paul A. Dutchak for experimental discussion and critical feedback on the manuscript. This work was supported by Alzheimer’s Association, Consortium for Frontotemporal Dementia Research, Friends of the Alzheimer’s Disease Center of UT Southwestern Medical Center, National Institutes of Health, National Natural Science Foundation of China, Ministry of Science and Technology of China, US Department of Veterans Affairs Biomedical Laboratory Research and Development Merit and Pilot Awards, and Muscular Dystrophy Association.
SUPPLEMENTAL INFORMATION

Dysregulation of FUS Disrupts Synaptic Homeostasis

Chantelle F. Sephton, Amy A. Tang, Ashwinikumar Kulkarni, James West, Mieu Brooks, Jeremy J. Stubblefield, Yun Liu, Michael Q. Zhang, Carla B. Green, Kimberly M. Huber, Eric J. Huang, Joachim Herz, Gang Yu

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunostaining. Mice (ages P18-P25) were anesthetized and were transcardially perfused with 4% paraformaldehyde (PFA, wt/vol) dissolved in 1X phosphate-buffered saline (PBS) and post-fixed in 4% PFA (wt/vol) overnight at 4°C. Tissues were washed extensively in 1X PBS, dehydrated in 30% sucrose (wt/vol) and frozen in OCT. Tissue sections of 15 μM were immunostained with primary antibodies GFAP (Millipore, AB5541), IBA1 (Wako, 019-19741), GFP (Aves 1020), human FUS (B327D) FUS (Santa Cruz, sc-47711), FUS (Sigma, HPA008784), Ubiquitin (Abcam, Ab7780) or To-Pro3 (LifeTechnologies T3605) followed by Alexa Fluor®-conjugated secondary antibodies (Invitrogen). Primary antibodies were incubated overnight at 4°C and Alexa Fluor®-conjugated secondary antibodies (Invitrogen) were incubated for 2 hours at room temperature.

Whole mounts of triangularis sterni muscles of mice (P18-P25) were fixed in 2% PFA (wt/vol) in 0.1 M phosphate buffer (pH 7.3) for 1 hr at room temperature. The samples were blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% bovine serum albumin (wt/vol) and 0.01% thimerosal, then incubated for 30 min with Alexa Fluor® 647 α-bungarotoxin (Invitrogen) followed by overnight incubation at 4°C with antibodies: GFP (Aves 1020), Syntaxin 1, or S100 (Dako, Z0311). After extensive washes, muscle whole mounts were incubated with Alexa Fluor®-conjugated secondary antibody (Invitrogen). Samples were then washed with 1X PBS and mounted in VECTASHIELD mounting medium (Vector Laboratories). Images were acquired using a Zeiss LSM 510 confocal microscope. Three animals from each genotype (n=3) were analyzed and a minimum of 400 NMJs per genotype were assessed.

Western blot analysis. Tissues were homogenized in lysis buffer (10mM HEPES, pH 7.4, 4M Urea, 1% LDS (wt/vol), 1X protease cocktail inhibitor (Roche) in lysing matrix D tubes, using the FastPrep homogenizer (Millipore). Lysates were clarified by centrifugation at 20,000 x g for 30 min at 4°C. Protein concentration was determined by BCA assay (Themo Scientific), and equivalent amounts were resolved by SDS-PAGE and immunoblotted by a standard protocol. GAPDH (Sigma, G9545), FUS (Sigma, HPA008784), FUS (Santa Cruz (SC), sc-47711) and GFP (Aves, 1020) were used as primary antibodies. Quantification of western blots by densitometry was done using the NIH ImageJ software. Each sample was normalized to GAPDH. Affinity purified Human FUS antibodies were a gift from Hongxia Zhou at Thomas Jefferson University (1). Human FUS peptide antibodies B327D (SYGQPQSGSYSQPS) were generated in rabbits as previously described (2). Note that although we were able to visually estimate the amounts of human and mouse FUS in the transgenic animals by using larger SDS-PAGE gels (Figure 1D), we were unable to accurately quantify human FUS level.
due to the similar molecular weights between human FUS (526 amino acids) and mouse FUS (518 amino acids) (Figure S1D).

**Li-Cor Odyssey.** Equal protein lysates were resolved by SDS-PAGE and transferred to Immobilon® FL PVDF membrane (Millipore IPFL00010). Blots were then rinsed with MiliQ water and blocked using Odyssey blocking buffer (Li-Cor P/N: 927-40000). Blots were probed with following primary antibodies in Odyssey blocking buffer: Arc (Synaptic Systems, 156003), CamKII (Santa Cruz, sc-5391), GluR1 (Millipore, MAB2263), and Psd-95 (Thermo, MA1-0145). Following primary antibody incubation, blots were probed with IR Dye 800CW goat anti-rabbit (Li-Cor P/N: 827-08365), IR Dye 800CW donkey anti-goat (Li-Cor P/N: 926-32214), IR Dye 800CW goat anti-mouse (Li-Cor P/N: 827-08364) and IR Dye 680RD goat anti-mouse (Li-Cor P/N: 926-68170) respectively. Blots were imaged using Li-Cor Odyssey imaging system and quantified using Li-Cor Image Studio software.

**Golgi staining for the analysis of dendrites in cortical neurons and cervical spinal motor neurons.** Both male and female CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} transgenic mice and their littermate controls were used for this analysis. Golgi staining on brains and cervical spinal cords from postnatal day 18 (P18) wild-type and CAG-FUS\textsuperscript{WT} or CAG-FUS\textsuperscript{R521G} littermate mice was performed using the Rapid GolgiStain Kit (FD Neurotechnologies) following the manufacturer's instructions. Briefly, brains and spinal cords from P18 mice were removed and immersed in solutions A and B in the dark for 2 weeks at room temperature. Brains were then transferred into solution C for at least 48 h at 4°C, sectioned at 100 μm thickness using a cryostat, mounted onto 3% gelatin-coated slides (wt/vol) and developed following the manufacturer's protocol (3). Dendritic tracing was performed using Neurolucida software (MicroBrightField, Williston, VA) with Olympus BX51 and a 60X objective. Neurons were traced with the center of the soma as a focal point. Three animals from each genotype (n=3) were analyzed, with 12 cervical spinal motor neurons (from ventral horn region) and 10 cortical neurons (from layers IV-V in the sensorimotor cortex) randomly selected and analyzed from regions of interest. Neurolucida Explorer 10 software (MicroBrightField, Williston, VA) was used to perform Sholl analysis to determine the number of intersections, cumulative surface area and to generate representative Golgi-tracing neurons (3).

Counting of the dendritic spines in the apical dendrites of the cortical motor neurons was performed using Neurolucida and analyzed with NeuroExplorer software (3). Briefly, beginning with a radius of 30 μm away from the center of the soma, a total distance of 100 μm from the primary apical dendrite was traced and analyzed. The entire length of the immediate secondary apical dendrite attached to the primary dendrite was also traced and analyzed for the study. Three animals from each genotype (n=3) were analyzed, with 10 primary and secondary branches from each animal traced and analyzed.

**ChAT staining of spinal cord and quantification of spinal motor neurons.** Tissues were fixed with 4% PFA (wt/vol) and sectioned at 40 μm thickness and free-floated in 1X PBS. Free-floating sections were treated with antigen retrieval solution (10 mM sodium citrate buffer) at 95°C for 10 min, washed three times in 1X TBS, and
then incubated in blocking solution (5% goat serum (wt/vol), 0.1% Triton X-100 in 1X TBS) for 1 hour at room temperature. DAB staining of the floating sections was then performed. The following antibodies and reagents were used: anti-choline acetyltransferase (ChAT)(Millipore), biotinylated rabbit anti-goat IgG antibody (Vector Labs); VECTASTAIN Elite ABC Kit (Vector Labs). DAB stained sections were mounted onto slides with Permount. Bright-field images of the ventral horns were captured using a 10X objective on an Olympus BX53 and on an Olympus DP72 digital camera. ChAT positive cells were quantified in each image field of the ventral horn region, and a minimum of 12 images for each animal were examined. CAG-FUS<sup>R521G</sup> (n=3) and CAG-FUS<sup>WT</sup> (n=4) transgenic mice and their littermate controls (n=3-4) were analyzed.

**Acute cortical tissue slices, treatment with DHPG and synaptoneurosome isolation.** Treatment of acute cortical tissue slices and isolation of synaptoneurosomes (SNs) from P18 mice were performed similar to previously reported (4). P18 mice were anesthetized with pentobarbital, whole brains were dissected out and immersed into ice cold oxygenated dissection buffer (110 mM choline Cl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 25 mM D-glucose, 3.1 Na pyruvate, 11.6 Na ascorbate, 14 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>). Acute slices of neocortex were taken at 400 μm thickness and recovered in normal artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM dextrose, 2 mM MgSO<sub>4</sub>-anhydrous, 2 mM CaCl<sub>2</sub>-2H2O) for 35 minutes in a 35 °C, oxygenated water bath. Slices were then transferred to SN recovery buffer containing AMPA (20 μM DNQX) and NMDA (5 μM CPP) inhibitors and pretreated for 30 minutes before stimulation with DHPG (100 μM, 10 minutes) (Tocris, Biosciences, US). Cortical slices were then transferred to 1 ml ice-cold homogenization buffer (10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 1X Roche Protease inhibitors) and homogenized using a Dounce homogenizer (10 strokes with A and 10 strokes with B). Total cell lysates (TLC) were collected and the remainder was passed through two 100 μm filters followed by one 10 μm filter. SNs were pelleted after a 10 min centrifugation at 1000 x g. TCL, Supernatants, and SNs were lysed in lysis buffer as previously described (5). Experimental replicates (n=4) were analyzed for each genotype CAG-FUS<sup>WT</sup>, CAG-FUS<sup>R521G</sup>.

**Synaptoneurosome isolation, in vitro treatment with DHPG.** Brain cortices from P16 mice were removed, washed in ice-cold gradient medium (GM buffer: 0.25 M sucrose, 5 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA), transferred to a glass Dounce homogenizer containing ice cold GM buffer, and gently homogenized with ten strokes of the loose pestle followed by ten strokes of the tight pestle. Cellular debris and nuclei were pelleted from the homogenate by centrifugation at 1000 x g for 10 min at 4°C. The supernatant was applied to percoll gradients (layers 2 ml each of 23%, 15%, 10%, and 3% isosmotic percoll, vol/vol) and spun at speed (32,500 x g) for 5 min at 4°C. The third band from the top of the gradient (the 23%/15% interface) containing intact SNs was removed and pooled for the experiments. The salt concentration of the SNs was adjusted by adding one-tenth volume of 10X stimulation buffer (100 mM Tris-HCl, pH 7.5, 5 mM Na2HPO4, 4mM KH2PO4, 40 mM NaHCO3, 800 mM NaCl). To suppress nonspecific excitation, tetrodotoxin (Tocris, Biosciences, US) to 1 μM was added. SNs were equilibrated to room temperature by rotation on a nutator mixer for 10 minutes, samples
were then placed at 37°C and stimulated with DHPG (100 μM) for the times indicated. All samples were incubated at 37°C for the same total time. Pretreatment of DMSO (vehicle) or 25 μM MG132 (Tocris, Biosciences, US) occurred at room temperature for 10 minutes prior to DHPG stimulation.

**Toluidine blue staining.** Mice were anesthetized and transcardially perfused with 4% PFA (wt/vol) and 1% glutaraldehyde (wt/vol) dissolved in 0.1M cacodylate, pH7.4. Tissues were post-fixed in 2.5% glutaraldehyde dissolved (wt/vol) in 0.1M cacodylate, pH7.4. Tissues were then post-fixed in buffered 1% osmium tetroxide (wt/vol) for 2 changes of 90 minutes each. Tissues were rinsed with dH2O, en bloc stained in 4% uranyl acetate (vol/vol) in 50% ethanol, dehydrated with a graded series of ethanol, and embedded in EMbed-812 resin. 1 μm semi-thin sections of the L4 spinal cord and dorsal and ventral roots were taken and stained with 1% toluidine blue (wt/vol).

**Counting Alpha Motor Neurons.** Alpha motor neurons were counted in spinal cord sections prepared from mice after perfusion with 4% PFA (wt/vol). Samples were paraffin-embedded, sectioned serially (10 μm) onto 10 slides, and stained with cresyl violet. Motor neurons were counted in every 10th section through each population examined. Alpha motor neurons were chosen based on the criteria: 1) located in the ventral horns (right and left) of the spinal cord; 2) 80-100 μm in size; 3) containing large soma; 4) containing a clear nucleus with intact nuclear membrane; and 5) having at least one clump of nucleolar material.

**Juvenile Social Interaction.** Adult mice were placed into a clean, empty mouse cage for approximately 15 min to habituate to the cage. A novel juvenile mouse (3-4 weeks, same sex as the test mouse) was then introduced into the cage and the total time that the adult mouse interacted with the juvenile was recorded. Trial duration was 2 min. All tests were conducted under red light in order to minimize any stress and anxiety. CAG-FUSR521G (Tg/+;Cre/+, n=18) and littermate controls (Tg/+;+/+, n=19 and +/+;Cre/+, n=19) were tested at 2, 4, 6 and 8 months of age.

**Ladder Walking Test.** This task was used to evaluate fine motor skills involved in performing accurate stepping behavior (6, 7). The task apparatus and scoring system were adapted from Farr et al. (2006) and Tennant & Jones (2009). The horizontal ladder (Plexiglas walls, 81 cm long, 15 cm tall, elevated 25 cm from ground) was composed of 0.15 cm diameter metal rungs spaced evenly 1.5 cm apart. Animals performed 3 trials (crossings) on a single test day (inter-trial interval at least 10 min). Video was analyzed frame-by-frame for step quality according to a 0-6 point scale. Scores of 0-2 indicated varying severity of slips, with scores of 3-5 indicating lesser types of missteps, and a score of 6 indicating an ideal paw placement. Two values were derived from this analysis: a step score (average of all scored steps) and an error rate (count of steps scored 0-2 divided by total step count). Forelimb and hindlimb scores were tallied separately; scores from right/left limbs were pooled. CAG-FUSR521G (Tg/+;Cre/+; n=10) and littermate controls (Tg/+;+/+, n=9 and +/+;Cre/+, n=11) were tested at 4 months of age.
**Water Y-maze.** Mice were tested in a Y-shaped maze (arms 34 cm long and 10 cm wide) filled with water (21°C) and a small amount of white paint. The submerged (1 cm) escape platform was located at one end of the arms of the maze. The location of the platform was alternated between cages. Mice were given 5 blocks of trials to learn the platform location. Each block consisted of 5 trials separated by approximately 30 sec – 2 min. Each block was separated by approximately 1 hr. 24 hours after the training, mice were given another 2 blocks of trials with the platform in the same location to assess whether they had learned the location. Mice that did not score 80% or better were excluded from analysis. 24 hrs. after the test the platform was moved to the arm opposite the location they were trained and the mice were given another 5 blocks of trials to learn this new location. Once the mouse entered an arm, the data were scored as either correct (the arm which contained the platform) or an error (the arm which did not contain the platform). CAG-FUS\textsuperscript{R521G} (Tg/+;Cre/+, n=18) and littermate controls (Tg/+;+/+, n=19 and +/+;Cre/+, n=19) were tested at 2 months of age.

**Olfactory Discrimination Test.** Mice were placed individually into a clean mouse cage with bedding and allowed to habituate for 15-45 min. During this time a dry, long-handled cotton-tipped applicator was placed through the lid into the center of the cage and lowered to the height of the mice. For the test, the cage was moved into a quiet, dimly lit room to minimize any anxiety. The applicator was replaced by new cotton tipped applicator that had been dipped into water. The time that the mouse sniffed this applicator during a 2 min period was recorded. This process was repeated a total of three times with a new applicator used for each test. The test was then repeated with an applicator which had been run through the dirty bedding of another mouse cage. This test was also repeated 3 times with a new applicator dipped into the dirty bedding. Sniffing was defined as the mouse’s nose pointed in the direction of the applicator and within approximately 1 cm. CAG-FUS\textsuperscript{R521G} (Tg/+;Cre/+, n=18) and littermate controls (Tg/+;+/+, n=19 and +/+;Cre/+, n=19) were tested at 4 months of age.

All behaviour testing were performed on CAG-FUS\textsuperscript{WT}, CAG-FUS\textsuperscript{R521G} and their littermate controls. There were no sex differences observed for any behavioral tests performed and sexes were evenly distributed for each genotype tested.

**Paired-end RNA-seq.** Spinal cords were dissected from control and transgenic mice at postnatal day P20 and stored at -80°C until total RNA was extracted using RNA Stat 60 reagent (Amsbio). Selected mice were between a health score of 1-2 as described in the material and methods in the main text. The mice were carefully selected to be phenotypically similar. Additionally, each paired-end RNA-Seq library was generated using equal amounts of RNA pooled from 3 animals, to take into account phenotypic variability. Quality of RNA was assessed with a Bioanalyzer using a nanochip. RNA samples with RIN (RNA integrity number) > 7 were used for RNA-Seq and qRT-PCR. Paired-end RNA-Seq libraries were generated for CAG-FUS\textsuperscript{WT} (n=2), CAG-FUS\textsuperscript{R521G} (n=2) and their littermate controls (n=2) using the Illumina TruSeq RNA Sample Preparation Kit v2 (RS-122-2001). A total of ~630 million paired-end RNA-Seq reads (2 x 100nt) were obtained using the Illumina HiSeq GAII sequencing platform. Reads were mapped to reference mouse genome (mm10) using TopHat (8-
10) (v 2.0.4) with default parameters (read alignment with up to 2 mismatches allowed, using a known mouse reference annotation (UCSC genes), etc.). Post read-mapping DESeq (11) was implemented to identify the differentially expressed genes (DEG).

Properly paired mapped reads were used to identify differentially expressed genes using read DESeq (11). DESeq (11) is an R/Bioconductor package based method which employs a negative binomial distribution method to quantify differential gene expression between transgenic samples and control samples, using count data from mapped RNA-Seq reads. HTSeq (12) (a python based tool) was used to generate the count data for each condition. DESeq (11) identified differentially expressed genes (with adjusted P-value < 0.05) which were assessed for functional annotation using the DAVID (13) functional annotation tool.

**Statistical Analysis.** Results are expressed as the mean ± SEM (standard error of the mean) or ± SD (standard deviation) where indicated. Three-way statistical comparisons use one-way ANOVA (GraphPad Prism version 6). We utilized a two-tailed, unpaired Student’s t-test for all pair-wise comparisons (GraphPad Prism version 6). P values less than 0.05 were considered significant.

**Genotyping.** Genomic DNA from ear biopsies were lysed in Quick Lysis Buffer (50 mM NaCl, 10 mM Tris-HCl pH 8.3, 0.2% Tween 20 and 0.4 mg/ml proteinase K) at 55°C for 1 hour and then 95°C for 10 min. The PCR contained genomic DNA, genotyping primers (listed below) and standard Taq buffer supplemented with 1 M betaine, 3.3% DMSO (vol/vol), 1.5 mM MgCl₂, 0.1 mg/ml BSA, 0.2 mM deoxynucleoside triphosphates and 1.25 units of Taq polymerase (New England Biolabs, NEB). After enzymatic amplification for 35 cycles, the PCR products were resolved on 2% agarose gel (wt/vol) in 1X Tris acetate-EDTA buffer.

**Genotyping Primers:**

<table>
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<tr>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Cre</td>
<td>GCATAACCAGTGAAACAGCATTGC</td>
<td>GGACATGTTCAAGGATCGCCAGGC</td>
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<td>GFP</td>
<td>CTGACCCTGAAGTTCATGCACC</td>
<td>TGGCTGTTGTAGTTGTACTCCAGC</td>
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<tr>
<td>Human FUS</td>
<td>GACCAGGTGGCTCTCACATG</td>
<td>GTCGCTACAGACGTTGTTGTC</td>
</tr>
<tr>
<td>Internal control (Pin1)</td>
<td>ATCATCCTGCGCACAGAATG</td>
<td>TCAATTCCTCCAGAAGGAGC</td>
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**SUPPLEMENTAL FIGURE LEGENDS**

**Figure S1.** (A,B) Genotyping results from Meox2Cre crosses from three founders from the CAG-Z-FUS<sup>WT</sup>-EGFP (lines: 629 and 638) and CAG-Z-FUS<sup>RS21G</sup>-EGFP (lines: 673 and 682) transgenic lines. PCR products using primers for FUS, GFP, Cre and Pin1 (internal control) are shown. (C) FISH (fluorescent in situ hybridization) of chromosomes isolated from CAG-FUS<sup>WT</sup> (629) and CAG-FUS<sup>RS21G</sup> (673) MEF cells. CAG-FUS<sup>WT</sup> (629) and CAG-FUS<sup>RS21G</sup> (673) founders show single insertion of transgenes. (D) Immunoblot of HeLa total cell lysates (human) and whole mouse brain lysates (mouse) showing molecular weight differences for human and mouse FUS protein. (E,F) Immunostaining for GFP (green) shows specific staining in brain and spinal cord of CAG-FUS<sup>WT</sup> and CAG-FUS<sup>RS21G</sup> mice (P0) and increased staining for FUS (red, Sigma,
Spinal cord sections from end-stage CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} mice are co-stained with (G) anti-hFUS (red) and anti-GFP (green) or (H) Ubiquitin (red) and FUS (green, Santa Cruz). No mislocalization or ubiquitination of human FUS\textsuperscript{WT} or FUS\textsuperscript{R521G} are observed. (G,H) Shown is the ventral horn of the lumbar region of the spinal cord.

**Figure S2.** (A-B) Body weights of CAG-FUS\textsuperscript{WT} (638) and CAG-FUS\textsuperscript{R521G} (682) mice from P0-P20. (C-D) Body weights of female and male CAG-FUS\textsuperscript{R521G} (682) mice from 5-14 weeks of age. (E) Grip test of CAG-FUS\textsuperscript{R521G} (682) mice, postnatal stages (P14-30), n=17 litters. Red circles (o) indicate CAG-FUS\textsuperscript{R521G} mice that had loss of motor function and early lethality. (A-E) Error bars represent SD of the mean.

**Figure S3.** ImageJ quantification of integrated density of GFAP and Iba1 staining in (A) CAG-FUS\textsuperscript{WT} and (B) CAG-FUS\textsuperscript{R521G} mice. Immunofluorescence staining of CAG-FUS\textsuperscript{R521G} mice that escape early lethality in the CA3 region of the hippocampus (C) and of the ventral horn of the spinal cord (D) for Iba1 (microglia;red) and GFAP (astrocytes;green) showing no neuroinflammation. (A,B) Quantification of microglia and astrocytes a, \(P < 0.05\); b, \(P < 0.01\); c, \(P < 0.005\) (Student \(t\) test). Error bars represent SEM of the mean.

**Figure S4.** H&E staining of the hippocampus and cortex (A) showing no loss of cells. Toluidine blue staining of dorsal and ventral roots (L4-5) (B), dorsal cortical (DCST) and lateral spinal tracts (LST) (C) of CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} mice (P20-23) showing no changes in myelinated axons. (D) Cresyl violet (top panel) and H&E (bottom panel) staining of cervical spinal cord from aged CAG-FUS\textsuperscript{R521G} mice (2 years old). (E) Quantification of cervical motor neurons from CAG-FUS\textsuperscript{R521G} mice (2 years old). Student \(t\) test shows no significant differences between groups. ns, not significant. Error bars represent SEM of the mean.

**Figure S5.** (A) Neuromuscular junctions (NMJ) from CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} mice at end-stage are costained for presynaptic terminals (nerve;red) and bungarotoxin for postsynaptic terminals (AchR;green) showing abnormal morphology compared with littermate controls. (B,C) NMJs in CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} mice (P20) stain positive for terminal myelinating Schwann cells (S100B;red), GFP;green, and bungarotoxin for postsynaptic terminals (AchR;blue), although their morphology is not typical of the pretzel shape observed in control (CTL) mice.

**Figure S6.** MA plot showing differentially expressed genes (DEG) in (A) CAG-FUS\textsuperscript{WT} (638) transgenic mice against control wild-type mice, and (B) CAG-FUS\textsuperscript{R521G} (682) transgenic mice against control wild-type mice. All genes are shown in grey and DEG are shown in black. CAG-FUS\textsuperscript{WT} transgenic mice show more genes affected compared to CAG-FUS\textsuperscript{R521G}.

**Figure S7.** (A) Open field test from 2 and 4 month old CAG-FUS\textsuperscript{R521G} mice and littermates show no differences in total distance travelled. (B) Total daily food intake and (C) food intake per body weight of 2 month old CAG-FUS\textsuperscript{R521G} (682) mice during running wheel testing. (D) Digigait trace for a control animal showing parameters that are measured for gait analysis. Ladder walking test shows the forepaws have a lower step score (E) and more errors per step (F). Hindpaws show no deficits (G,H). Social interactions of CAG-FUS\textsuperscript{R521G} mice were
reduced with juveniles at 2 months and significantly decreased by 4 months of age (I). All mice performed equally in a Y-test which measures learning and decision making (J). Shown are the results from reversal testing that measures the ability of the mice to find the platform in the opposite arm to which they were entrained (J). Olfaction testing showing no alterations in CAG-FUS\textsuperscript{R521G} mice (K). Studies were conducted with littermate controls (+/+;Cre/+ and Tg/+;+/+), which showed no statistical impairments. (A,E-K) Statistical comparisons uses one-way ANOVA. a, \( P < 0.05 \); b, \( P < 0.01 \). (‘ compares +/+;Cre/+ with Tg/+;Cre/+). (B,C) Uses Student t test. ns, not significant. Error bars represent SEM of the mean.

**Figure S8.** Li-Cor Odyssey quantification for synaptic proteins Arc, CamKII, GluR1 and Psd-95 from (A,C) total cell lysates (TCL) and (B,D) synaptoneurosomes (SN). The graphs represent the average of 3-5 independent experiments. Student’s t test shows no statistical differences in protein expression between CAG-FUS\textsuperscript{WT} and CAG-FUS\textsuperscript{R521G} (682) compared to their littermate controls (CTL). Error bars represent SEM of the mean.

**Movie S1.** Video recording of a control resident mouse from the 8-month resident-intruder test. The video clip is representative of the mean interaction of the control groups. The control resident “test” mouse (#3935) shows normal social behaviors towards the “novel” intruder mouse. The video shown is taken 4 min after the intruder mouse is introduced into the home cage.

**Movie S2.** Video recording of a CAG-FUS\textsuperscript{R521G} resident mouse from the 8-month resident-intruder test. The video clip is representative of the mean interaction of the CAG-FUS\textsuperscript{R521G} group. The CAG-FUS\textsuperscript{R521G} resident “test” mouse (#3890) spends less time chasing the “novel” intruder mouse and displays less active social behavior overall. The video shown is taken 4 min after the intruder mouse is introduced into the home cage.

**REFERENCES**


Figure S1
Figure S2
Figure S3
Figure S5
Figure S6
Figure S7
Figure S8
Table S1. Genotypes of P0 offspring from Tg/+;+/+ x Meox2Cre intercrosses

| Line | CAG-FUS\textsuperscript{WT} | | | | | Total |
|------|----------------------------|----------------------------|----------------------------|----------------------------|-----------|
|      | +/++;Cre/+                 | +/++;+/+                   | Tg/+;+/+                   | Tg/+;Cre/+                 |           |
| 629  | 24%                        | 24%                        | 25%                        | 26%                        | 95        |
| 638  | 23%                        | 22%                        | 21%                        | 34%                        | 120       |

| Line | CAG-FUS\textsuperscript{R521G} | | | | | Total |
|------|--------------------------------|----------------------------|----------------------------|----------------------------|-----------|
|      | +/++;Cre/+                 | +/++;+/+                   | Tg/+;+/+                   | Tg/+;Cre/+                 |           |
| 673  | 24%                        | 25%                        | 22%                        | 28%                        | 99        |
| 682  | 27%                        | 17%                        | 29%                        | 26%                        | 66        |

Expected ratio: 25% 25% 25% 25%
<table>
<thead>
<tr>
<th>Age (days)</th>
<th>CTL</th>
<th>CAG-FUS&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>CAG-FUS&lt;sup&gt;RS21G&lt;/sup&gt;</th>
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<td>Mean (sec)</td>
<td>% Complete</td>
<td>Mean (sec)</td>
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<tr>
<td>14</td>
<td>24.9 ± 4.0</td>
<td>3.1</td>
<td>3.8 ± 1.1&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>16</td>
<td>39.2 ± 4.6</td>
<td>28.8</td>
<td>1.6 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>18</td>
<td>45.8 ± 4.0</td>
<td>47.9</td>
<td>0.6 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>20</td>
<td>50.8 ± 3.6</td>
<td>61.5</td>
<td>0.4 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<table>
<thead>
<tr>
<th>Age (days)</th>
<th>CTL</th>
<th>CAG-FUS&lt;sup&gt;RS21G&lt;/sup&gt;</th>
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<td>14</td>
<td>20.1 ± 2.7</td>
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<tr>
<td>18</td>
<td>38.0 ± 4.2</td>
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<td>22</td>
<td>47.2 ± 3.5</td>
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<td>26</td>
<td>55.0 ± 2.2</td>
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<td>30</td>
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Student’s t-test, <sup>a</sup>p<0.05; <sup>b</sup>p<0.01; <sup>d</sup>p<0.001
± SE (standard error of mean)
<table>
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Note: For two samples, the number of properly paired reads/quality passed reads is larger than total reads for those samples. This is due to the fact that Tophat allows reads to map to more than one place in the genome (multihits) as its default parameter, which causes such reads to be counted more than once, leading to increased number of reads in the BAM file after mapping compared to total reads in the FASTQ file.
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<th>Parameter</th>
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<th>Mean (n=19)</th>
<th>Mean (n=18)</th>
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<tr>
<td>Swing Stride (%)</td>
<td>38.4 ± 0.5</td>
<td>38.9 ± 0.5</td>
<td>37.5 ± 0.7</td>
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<td>Brake Stride (%)</td>
<td>30.1 ± 1.4</td>
<td>29.3 ± 1.5</td>
<td>34.8 ± 1.2</td>
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<td>Propel Stride (%)</td>
<td>31.4 ± 1.5</td>
<td>31.8 ± 1.6</td>
<td>27.7 ± 1.4</td>
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<td>Brake Stance (%)</td>
<td>49.1 ± 2.3</td>
<td>48.1 ± 2.6</td>
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<td>Propel Stance (%)</td>
<td>50.9 ± 2.3</td>
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<td>Stride Length (cm)</td>
<td>5.72 ± 0.10</td>
<td>5.82 ± 0.05</td>
<td>5.65 ± 0.08</td>
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<tr>
<td>Stance Width (cm)</td>
<td>1.46 ± 0.05</td>
<td>1.51 ± 0.05</td>
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<td>Midline Distance (cm)</td>
<td>-2.08 ± 0.09</td>
<td>-2.20 ± 0.09</td>
<td>-2.17 ± 0.09</td>
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One-way ANOVA, a,p<0.05; b,p<0.01; ns,not significant, ' compares +/-;Cre/+ with Tg/+;Cre/+ ± SE (standard error of mean)
Table S5. Hindpaw gait measurements of CAG-FUS<sup>R521G</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/-;Cre/+ (n=19)</th>
<th>Tg/+;+/- (n=19)</th>
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<td>Swing Stride (%)</td>
<td>35.4 ± 0.6</td>
<td>34.9 ± 0.5</td>
<td>32.8 ± 0.8&lt;sup&gt;a,b'&lt;/sup&gt;</td>
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<td>Brake Stride (%)</td>
<td>18.1 ± 0.8</td>
<td>18.8 ± 0.8</td>
<td>17.8 ± 0.8</td>
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<tr>
<td>Propel Stride (%)</td>
<td>46.4 ± 0.8</td>
<td>46.3 ± 0.8</td>
<td>49.4 ± 1.2</td>
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<tr>
<td>Brake Stance (%)</td>
<td>28.0 ± 1.2</td>
<td>28.1 ± 1.1</td>
<td>26.6 ± 1.3</td>
</tr>
<tr>
<td>Propel Stance (%)</td>
<td>72.0 ± 1.2</td>
<td>71.2 ± 1.1</td>
<td>73.4 ± 1.3</td>
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<td>Stride Length (cm)</td>
<td>5.74 ± 0.10</td>
<td>5.86 ± 0.06</td>
<td>5.73 ± 0.09</td>
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<tr>
<td>Stance Width (cm)</td>
<td>2.66 ± 0.05</td>
<td>2.55 ± 0.06</td>
<td>2.62 ± 0.05</td>
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<td>Midline Distance (cm)</td>
<td>1.62 ± 0.04</td>
<td>1.61 ± 0.06</td>
<td>1.42 ± 0.07&lt;sup&gt;a,a'&lt;/sup&gt;</td>
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One-way ANOVA, <sup>a</sup>p<0.05; <sup>b</sup>p<0.01, ' compares +/-;Cre/+ with Tg/+;Cre/+ ± SE (standard error of mean)
Supporting Information

Sephton et al. 10.1073/pnas.1406162111

**Movie S1.** Video recording of a control resident mouse from the 8-month resident-intruder test. The video clip is representative of the mean interaction of the control groups. The control resident “test” mouse (#3935) shows normal social behaviors towards the “novel” intruder mouse. The video shown is taken 4 min after the intruder mouse is introduced into the home cage.

**Movie S2.** Video recording of a CAG-FUS<sup>G521G</sup> resident mouse from the 8-month resident-intruder test. The video clip is representative of the mean interaction of the CAG-FUS<sup>G521G</sup> group. The CAG-FUS<sup>G521G</sup> resident “test” mouse (#3890) spends less time chasing the “novel” intruder mouse and displays less active social behavior overall. The video shown is taken 4 min after the intruder mouse is introduced into the home.

**Other Supporting Information Files**

[SI Appendix (PDF)](#)