Cytoplasmic FUS triggers early behavioral alterations linked to cortical neuronal hyperactivity and inhibitory synaptic defects

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Gene mutations causing cytoplasmic mislocalization of the RNA-binding protein FUS lead to severe forms of amyotrophic lateral sclerosis (ALS). Cytoplasmic accumulation of FUS is also observed in other diseases, with unknown consequences. Here, we show that cytoplasmic mislocalization of FUS drives behavioral abnormalities in knock-in mice, including locomotor hyperactivity and alterations in social interactions, in the absence of widespread neuronal loss. Mechanistically, we identified a progressive increase in neuronal activity in the frontal cortex of Fus knock-in mice in vivo, associated with altered synaptic gene expression. Synaptic ultrastructural and morphological defects were more pronounced in inhibitory than excitatory synapses and associated with increased synaptosomal levels of FUS and its RNA targets. Thus, cytoplasmic FUS triggers synaptic deficits, which is leading to increased neuronal activity in frontal cortex and causing related behavioral phenotypes. These results indicate that FUS mislocalization may trigger deleterious phenotypes beyond motor neuron impairment in ALS, likely relevant also for other neurodegenerative diseases characterized by FUS mislocalization.
myotrophic lateral sclerosis (ALS) is the major adult motor neuron disease, with onset usually in the 6th and 7th decade of life and death due to respiratory insufficiency and progressive paralysis typically occurring 3–5 years after onset of motor symptoms.3–5 Mutations in the Fused in Sarcoma gene (FUS), encoding an RNA-binding protein from the FET family4,5, are associated with the most severe forms of ALS6,7, clinically presenting with a very early onset and rapid disease progression5–8. ALS associated mutations in FUS are clustered in the C-terminal region of the FUS protein that includes the atypical PY nuclear localization sequence, and is required for protein entry into the nucleus.6,7,10–12. The severity of the disease correlates with the degree of impairment of FUS nuclear import11,12, and the most severe cases of ALS known to date, are indeed caused by mutations leading to the complete truncation of the PY-NLS8,9.

A number of clinical and pathological studies suggest that FUS mislocalization to the cytoplasm and subsequent aggregation could be relevant beyond the few ALS-FUS cases. First, FUS mutations, although rare in non-ALS cases, have been found in cases with frontotemporal dementia, either isolated13,14 or as an initial presentation of ALS-FTD15,16, as well as in patients with initial chorea17, mental retardation18, psychosis or dementia19, and essential tremor20. In the absence of FUS mutations, FUS mislocalization21, or aggregation22,23 were found to be widespread in sporadic ALS. FUS pathology also defines a subset of cases with FTD (FTD-FUS) with prominent atrophy of the caudate putamen24–26, concomitant pathology of other FET proteins, such as TAF15 and EWSR1 12,27 and frequent psychiatric symptoms28. FUS aggregates have also been observed in spinocerebellar ataxia and Huntington’s disease31,32. While FUS mislocalization appears to be a common feature in neurodegenerative diseases, its pathological consequences have not been thoroughly studied beyond motor neuron degeneration.

Neurons with FUS pathology show decreased levels of FUS in the nucleus, that might compromise a number of processes dependent on proper FUS levels such as transcription and splicing regulation or DNA damage repair4. Interestingly, loss of FUS alters the splicing of multiple mRNAs relevant to neuronal function33,34, such as MAPT, encoding the TAU protein, and alters the stability of mRNAs, encoding relevant synaptic proteins such as GluA1 and SynGAP135–37. However, loss of nuclear FUS levels is very efficiently compensated for by autoregulatory mechanisms as well as by other FET proteins, and loss of nuclear FUS remains limited as opposed to loss of nuclear TDP-43, observed in TDP-43 pathology40. Indeed, heterozygous Fus knock-in mice, which carry one mutant allele leading to cytoplasmic and not nuclear localization of FUS, only show marginal loss of nuclear FUS due to compensatory overexpression38,41. Beyond nuclear loss of function, accumulation of cytoplasmic FUS was found to be a critical event in ALS-FUS in multiple studies in mouse models. For instance, cytoplasmic FUS is necessary to cause motor neuron degeneration in ALS-FUS40,41–46 as heterozygous Fus knock-in mouse models develop mild, late onset muscle weakness and motor neuron degeneration, but not haploinsufficient Fus knockout mice40,41,46. To date, there are few studies investigating whether the accumulation of cytoplasmic FUS might lead to phenotypes beyond motor neuron degeneration. Interestingly, FUS is also found at synaptic and dendritic sites35,38,47–51, and Sahadevan, Hembach et al.52 identify synaptic mRNA targets for FUS that are critical for synaptic formation, function and maintenance.

Here, we show that a partial cytoplasmic mislocalization of FUS in heterozygous Fus knock-in mice is sufficient to drive a panel of behavioral abnormalities, including locomotor hyperactivity and alterations in social interactions, which preceded motor neuron degeneration. Behavioral deficits were accompanied by ventricle enlargement and atrophy of several subcortical structures in the absence of widespread neuronal loss in the cortex. Mechanistically, we could identify a progressive increase in neuronal activity in the frontal cortex of Fus knock-in mice in vivo. Furthermore, we observed a coordinated down-regulation of multiple genes related to synaptic function in the frontal cortex throughout adulthood, which were confirmed by ultrastructural and morphological defects of synapses. These synaptic defects were more profound in inhibitory compared to excitatory synapses and accompanied by increased levels of FUS protein as well as of 3 of its RNA targets (Fus, Nrxn1, and Gabra1) in synaptosomes of heterozygous Fus knock-in mice. Thus, FUS cytoplasmic enrichment is sufficient to trigger synaptic deficits, leading to increased neuronal activity and behavioral phenotypes. These findings suggest that FUS mislocalization could trigger deleterious phenotypes beyond impaired motor function that could be relevant for both ALS-FUS but also for other neurodegenerative diseases based on FUS mislocalization.

Results

Spontaneous locomotor hyperactivity in FusANLS/+ mice. Since FUS mislocalization and aggregation are observed in patients with various neurodegenerative diseases, we hypothesized that partial FUS cytoplasmic mislocalization in FusANLS/+ mice could be sufficient to cause a number of behavioral phenotypes. Two independent cohorts of mice were analyzed at 4 months of age, before the appearance of motor impairment41 and 10 months of age. Evaluation of basal motor activity in a familiar environment showed significantly increased locomotor activity in FusANLS/+ mice over the 3 consecutive days of observation (Fig. 1a, b). Interestingly, this hyperactivity was observed throughout the entire night in 4-months-old FusANLS/+ mice (Fig. 1a), but only during late night hours in older FusANLS/+ mice (Fig. 1b). In the open field, ambulatory distance, duration of ambulation, mean speed and preference for peripheral quadrants over central quadrants were similar in 10-months-old FusANLS/+ and wild-type littersmates, indicating the absence of hyperactivity in a novel environment (Supplementary Fig. 1a, b). To further study potential anxiety-related phenotypes in FusANLS/+ mice, we used the dark/light box test, based on the preference of mice for dark compartments over illuminated places. In this test, 10-months-old FusANLS/+ and Fus+/+ mice showed a similar latency to enter, similar frequency of transitions and similar duration to explore illuminated compartment (Supplementary Fig. 1c). Thus, FusANLS/+ mice are hyperactive, but do not show evidence of anxious behaviors, at least at the ages tested.

Mildly compromised consolidation of spatial memory in FusANLS/+ mice. To explore the possibility that behavioral phenotypes of FusANLS/+ mice included spatial memory defects, we performed the Morris water maze test. This task requires hippocampal function, at least during acquisition and memory formation, but relies on a proper cortico-hippocampal dialog for longer retention times or remote memory retrieval (Fig. 1c)53. At 10 months of age, FusANLS/+ mice performed similarly well to their Fus+/+ littermates regarding the distance travelled and latency to find the hidden platform over training days (Fig. 1d, e). We then performed a probe trial 18 days after the last training and observed that, although both genotypes searched significantly in the target quadrant compared to nontarget areas, FusANLS/+ mice displayed a slightly decreased performance to retrieve memory at this timepoint (Fig. 1f). Furthermore, FusANLS/+ mice lost their previous memory significantly faster than wild-type
Fig. 1 FusΔNLS/+ mice display increased nocturnal spontaneous locomotor activity and cognitive defects. a, b Line graphs represent mice home cage activity—actimetry over three consecutive days at 4 months (a) and 10 months (b) of Fus+/+ (black) and FusΔNLS/+ (orange) male mice N = 11 for Fus+/+ and N = 10 for FusΔNLS/+ mice at 4 months and N = 15 for Fus+/+ and N = 14 for FusΔNLS/+ mice at 10 months. Repeated measures Two-way ANOVA followed by Sidak for multiple comparisons, with time and genotype as variables. P = 0.0027 at 4 months and p = 0.038 at 10 months for genotype effect. Data are presented as mean ± SEM values of activity score per hour. c Schematic illustration of the Morris water maze (MWM) experimental strategy (paradigm). Mice were subjected to a five-day training period and tested for spatial memory retention in a probe trial (60 seconds) 18 days after the last acquisition. The probe trial was then followed by two extinction tests, performed at 2 h intervals. d, e Line graphs represent latency (in seconds) (d) and total distance swam (in meters) (e) to find the hidden platform during acquisition of 10-months-old Fus+/+ (black) and FusΔNLS/+ (orange) male mice. Both genotypes improved similarly their performance between day 1 and 5. N = 10 for Fus+/+ and N = 11 for FusΔNLS/+ mice. Data are presented as mean ± SEM values of four trials per day of training. A two-way repeated measure analysis of variance (ANOVA) (genotype × days) was conducted to determine the effect of genotype on learning over time. No significant effect of genotype is observed. f Bar graphs represent the time spent in the target quadrant (Target) and the average of the time spent in the other three quadrants (Others) during probe trial. Dashed line indicates chance level (15 seconds per quadrant; i.e., 25%). N = 10 for Fus+/+ and N = 11 for FusΔNLS/+ mice. Data are presented as mean ± SEM. Both genotypes were significantly above random but FusΔNLS/+ mice performed significantly worse than Fus+/+ littermates ($, p < 0.01). One sample t-test was used to compare to a chance level, Target quadrant: p = 0.0008 for Fus+/+ and p = 0.006 for FusΔNLS/+). Genotype comparison was made using One-way ANOVA; F(1,19) = 6.33, p = 0.0208. g, h Bar graphs represent the time spent in quadrants (Target vs Others) during the first (g) and the second (h) extinction test ($, p < 0.05 vs chance levels). One-way ANOVA for genotype effect (F(1,19) = 0.56, p = 0.46) (g), F(1,19) = 0.27, p = 0.6) (h) and One sample t-test was used to compare to a chance level, (Target quadrant: p = 0.025 for Fus+/+ and p = 0.22 for FusΔNLS/+ (g), (Target quadrant: p = 0.08 for Fus+/+ and p = 0.09 for FusΔNLS/+ (h). N = 10 for Fus+/+ and N = 11 for FusΔNLS/+ mice, with same mice as panel f. Data are presented as mean ± SEM. Source data are provided as a Source Data file.

mice, as they were searching randomly in a first extinction test performed 2 h after the probe trial, while wild-type mice still showed a significant more directed searching behaviour and preferred the target area over others (Fig. 1g). This suggests that consolidation of long-term memory was mildly compromised in FusΔNLS/+ mice. Lastly, both genotypes did not distinguish the target over the other quadrants in a second extinction test (Fig. 1h). Altogether, these data show that FusΔNLS/+ mice were able to learn but displayed impaired long-term memory in agreement with a dysfunction of cortical regions.

Social disinhibition in FusΔNLS/+ mice. Marked changes in personality and social behavior, such as social withdrawal or social disinhibition, obsessive-compulsive behaviors, euphoria or apathy are common in subjects with behavioral variant (bv)FTD, a disease with pronounced FUS mislocalization. Social
At 22 months, resident-intruder test in home cage for 4 (for Fus), 10 (b), and 22 (c) months-old Fus+/+ (black) and FusΔNLS/+ (orange) male mice. Note that, young FusΔNLS/+ mice demonstrated a trend towards an increased social interest for intruder mouse (a) while older mice interacted with intruders significantly longer than Fus+/+ (b, c) showing an age-dependent impairment of social behavior-disinhibition. All values are represented as mean ± SEM. At 4 months, N = 9 for Fus+/+ and N = 8 for FusΔNLS/+ mice; At 10 months, N = 14 for Fus+/+ and N = 14 for FusΔNLS/+ mice; At 22 months, N = 8 for Fus+/+ and N = 10 for FusΔNLS/+ mice. Two-way repeated measures ANOVA followed by Sidak post-hoc test (b, c) for evaluating sociability in mice. Interests were also reported in progranulin haploinsufficient mice, a mouse model of FTD57. To determine whether FusΔNLS/+ mice have social behavioral deficits, we first performed the resident-intruder test specific for evaluating sociability in mice. Interestingly, 4-months-old FusΔNLS/+ mice showed a trend towards longer interaction with the intruder mouse as compared with Fus+/+ mice (p = 0.07) (Fig. 2a), that was significant at 10 months of age (Fig. 2b) and persisted until 22 months of age (Fig. 2c). Aggressive behavior was only observed at 4 months of age, and not affected by the Fus genotype (attack duration: 0.0001 - p < 0.001; Fig. 2d). Structured by: a) resident (test) and b; empty (control) (Fig. 2e and f). Interaction time between resident and intruder is represented as mean ± SEM. Three-way ANOVA with Newman Keuls post-hoc test (p = 0.0072 (22 months) for genotype effect). Source data are provided as a Source Data file.
13.0 ± 1.4 s in Fus+/+ mice vs 11.6 ± 1.0 s in FusANLS/+ mice, p = 0.88 (two-sided unpaired Student’s t-test). To further characterize the social behavioral impairment, we used a modified version of the three-chamber social paradigm. After a first trial of habituation using an empty setup, a novel mouse is introduced in a side compartment. The interactions initiated by the test mouse with either the novel mouse or the empty cage was quantified. Of most relevance, across the three consecutive trials (Trial 2, 3, and 4), we observed that 10-months-old FusANLS/+ mice consistently interacted more with the novel mouse than Fus+/+ mice, in line with social disinhibition (Fig. 2e). This was not observed at 4 or 22 months of age (Fig. 2d–f). Importantly, mice of both genotypes spent more time interacting with the novel mouse than with the empty cage, indicating that mice could recognize its conspecific. The interaction time gradually decreased in later trials, suggesting progressive loss of social interest in the novel mouse, while it became familiar (Fig. 2d–f). Similar findings of social disinhibition in both resident-intruder test and three-chamber paradigms as a novel environment exclude the possibility that the observed increased social interactions resulted from locomotor hyperactivity in the home cage. Importantly, the olfactory function of FusANLS/+ mice was preserved, since results showed no differences between genotypes at 22 months of age in the time spent sniffing filter paper, covered with either attractive scent (vanilla) or an aversive scent (2-methyl butyrate) (Supplementary Fig. 2). These findings together with absence of major motor phenotype at that age (Supplementary Fig. 1a, b) indicated that social behavior is specifically affected in FusANLS/+ mice. Taken altogether, behavioral analyses of FusANLS/+ mice uncovered locomotor hyperactivity, cognitive deficits, and altered memory consolidation as well as selective impairment in sociability.

**Increased spontaneous neuronal activity in FusANLS/+ mice in vivo.** As the behavioral changes observed are highly reminiscent of frontal lobe dysfunction, we next asked whether neuronal activity is altered within that brain area. We thus examined spontaneous neuronal activity using in vivo two-photon calcium imaging (Fig. 3a–c). We studied neurons in cortical layer II/III of the frontal cortex expressing the genetically encoded calcium indicator GCaMP (delivered through an AAV vector) in mice at the age of 4 and 10 months (Fig. 3b). Indeed, we observed a significant increase in spontaneous activity, which worsened with age. While in 4-month-old mice the fraction of active neurons did not differ between Fus+/+ and FusANLS/+ mice (Fig. 3d), there was a decrease in transient amplitudes (Fig. 3e) and an increase in transient frequency (Fig. 3f) in FusANLS/+ mice. In 10-month-old animals, this increase in activity was already evident at the level of the fraction of active cells in FusANLS/+ (Fig. 3g). Moreover, we observed an increase in the transient amplitudes (Fig. 3h) and also in the transient frequency (Fig. 3i) in FusANLS/+ mice compared to their Fus+/+ littermates. Taken together, our data demonstrate an age dependent, strong increase in neuronal activity in vivo within the upper layers of frontal cortex of FusANLS/+ mice.

**FusANLS/+ mice show ventricle enlargement and atrophy of subcortical structures but preserved cortical neurons.** We next sought to understand the structural basis of behavioral and electrophysiological abnormalities in FusANLS/+ mice by employing MR imaging. FLASH MRI datasets for FusANLS/+ mice and Fus+/+ littermates were processed for volumetric quantification using an in-house developed script, aimed at registering the MRI images to a template derived from the Allen Brain Atlas reference and then at parcellating the cerebral structures into hierarchically arranged volumes of interest, which can be interrogated for the volume of any region or group of regions (Fig. 4a, b). The overall intracranial volume (ICV) was comparable in FusANLS/+ and Fus+/+ mice (Fig. 4c). However, upon normalization for the ICV, the volume of the brain parenchyma was significantly decreased in FusANLS/+ (by ~1.5%; average normalized volume was 98.52% for Fus+/+ and 97.14% for FusANLS/+; Fig. 4d). Visual inspection of the MRI images revealed a substantial increase in the volume of lateral ventricles, which was confirmed by the registration algorithm and quantitated as an almost doubling of ventricular volumes (Fig. 4e). The ventriculomegaly was not associated with neocortical atrophy (Fig. 4f), but we identified a significant atrophy of the medial septum (Fig. 4g) and of the structures corresponding to the cortical subplate (including claustrum, endopiriform cortex and lateral, basomedial, basolateral, and posterior amygdalar nuclei; Fig. 4h). Only a nonsignificant trend for reduced volume was detected for hippocampus (9.06% for Fus+/+ vs. 8.62% for FusANLS/+; p = 0.15; Two-sided Unpaired Student’s t-test) and striatum (9.76% for Fus+/+ vs. 9.89% for FusANLS/+; p = 0.56, Two-sided Unpaired Student’s t-test). Interestingly, we also detected a significant degree of atrophy in the non-neocortical olfactory areas of the piriform cortex (2.48% for Fus+/+ vs. 2.18% for FusANLS/+; p = 0.0006, Two-sided Unpaired Student’s t-test). The lack of a prominent cortical atrophy phenotype was further confirmed by brain histology in FusANLS/+ mice at both 10 and 22 months of age. Cortical cytoarchitecture appeared preserved in FusANLS/+ mice, with normal lamination and no cortical thinning. The density of NeuN positive neurons in the frontal cortex was similar between FusANLS/+ mice and their wild-type littermates at 10 and 22 months of age (Fig. 4i, j).

Taken together, these data demonstrate a significant hydrocephalus ex vacuo in FusANLS/+ due to the atrophy of subcortical structures, such as the medial septum, several amygdalar nuclei, piriform areas, and tentatively the hippocampus.

**Transcriptome of FusANLS/+ cortex points to defects in inhibitory neurotransmission and synapses.** To understand the molecular basis of altered behavior in FusANLS/+ mice, we performed RNAseq on frontal cortex of 5- and 22-months-old FusANLS/+ mice and their wild-type littermates. Principal component analysis showed a clear separation between FusANLS/+ mice and their wild-type littermates at 22 months of age, while clustering was imperfect at 5 months of age, suggesting an exacerbation of the transcriptional differences between genotypes with age (Supplementary Fig. 3a).

Using a stringent analytical pipeline (FDR < 0.05), we did not identify differentially expressed genes between FusANLS/+ and Fus+/+ mice at 5 and 22 months (Supplementary Fig. 3b). To ensure that the absence of differentially expressed genes was not due to the stringent calibration of p-values, we compared the 5-months and 22-months-old Fus+/+ mice RNAseq datasets to probe age-related alterations. We were able to detect more than 2000 genes differentially expressed between 5- and 22-months-old wild-type mice, at a 5% false discovery rate, demonstrating that this approach can reliably detect changes in gene expression (Supplementary Fig. 3b).

To place gene expression changes in a systems-level framework, we performed weighted-gene coexpression network analysis (WGCNA) across all available FusANLS/+ and Fus+/+ datasets, including 5 and 22-months RNAseq, as well as 1 and 6 months RNAseq datasets from Sahadevan et al. Potential batch effects were removed using a negative binomial regression model to estimate batch effects based on the count matrix (Supplementary Fig. 3c) and allowed clustering between genotypes (Supplementary Fig. 3d). WGCNA analysis allowed us to
identify two mRNA modules significantly correlated with the genotype condition in cortex and labeled as turquoise and yellow modules according to the WGCNA conventions (Bonferroni-corrected \( P < 0.05 \); Fig. 5a, Supplementary Data 1). Cell-type enrichment analysis demonstrated that the turquoise module, but not the yellow module, was enriched in neuronally expressed genes (Fig. 5b). Indeed, the Turquoise module, downregulated in \( \text{Fus}^{\Delta NLS/+} \) mice (Fig. 5c–e), was enriched in genes related to synaptic physiology and development, most notably of GABAergic and glutamatergic synapses (Fig. 5d). Hub genes of the turquoise module included one GABA receptor encoding genes such as \( \text{Gabrb1} \), one glutamate receptor gene (\( \text{Grid2} \)) and genes
for VGAT, GABAAR density was associated with a decrease in the size of the clusters 4 months after implantation. c Representative Examples of average projections of field of views (FOV) imaged in FusANLS/+ and FusANLS/+ mice at 4 months (N = 8 Fus+/+ mice and N = 3 FusANLS/+ mice, left) and at 10 months (N = 5 Fus+/+ mice and N = 6 FusANLS/+ mice, right) are shown together with fluorescence calcium traces of selected regions of interest (ROIs). d The fraction of active cells per FOV was not affected in 4-month-old FusANLS/+ mice. N = 13 FOVs in 3 FusANLS/+ and N = 25 FOVs in 8 Fus+/+ mice. Data are presented as mean ± SEM, p = 0.1627, Two-sided Unpaired Student’s t-test. e, f The calcium transient frequencies (e) increased while the average transient amplitudes (f) were decreased in FusANLS/+ mice. N = 1107 ROIs in 3 FusANLS/+ and N = 2264 ROIs in 8 Fus+/+ mice, superimposed by the median (e). Kolmogorov-Smirnov test, *p < 0.0001 for both panel e and f. g-i The fraction of active cells per FOV (g) as well as (h) the frequencies and (i) the average amplitudes of calcium transients of each ROI were increased in 10-month-old FusANLS/+ mice. Data are individual FOVs (g: N = 14 FOVs in 6 FusANLS/+ and N = 10 FOVs in 5 Fus+/+ mice) or individual ROIs (h, i: N = 855 ROIs in 6 FusANLS/+ and N = 631 ROIs in 5 Fus+/+ mice) superimposed by the mean ± SEM (g) or the median (h, i). panel g: Two-tailed Unpaired Student’s t-test, *p = 0.0126; panel h and i: Kolmogorov-Smirnov test, **p < 0.0001 for both panels. Source data are provided as a Source Data file.

Synaptic defects in in FusANLS/+ mice. To independently validate potential synaptic defects in FusANLS/+ frontal cortex, we performed quantitative ultrastructural analysis of inhibitory (Fig. 6a) and excitatory (Fig. 6b) synapses in this brain region. Inhibitory synapses in layers II/III of the frontal cortex, identified by the presence of mitochondria on both sides of the synapse, showed major ultrastructural alterations in FusANLS/+ mice, with increased bouton sizes (Fig. 6c), longer active zones (Fig. 6d), prominently increased vesicle numbers (Fig. 6e), and increased distance of vesicles to the active zone as compared to wild-type synapses (Fig. 6f). Excitatory synapses, identified as asymmetrical, with a pronounced postsynaptic density, also showed ultrastructural alterations; however, in the opposite direction: excitatory synapses showed overall decreased bouton size, decreased length of the active zone, and decreased vesicle number in FusANLS/+ cortex (Fig. 6d–i). Importantly, ultrastructural alterations of excitatory synapses were less pronounced than those of inhibitory synapses.

To further explore morphological changes occurring at inhibitory synapses, we quantified the density and the cluster size of three inhibitory synaptic markers: the GABA transporter (GAT1), the GABA A receptor α3 (GABAARα3), and gephyrin (Gephyrin) (Fig. 6m), suggesting a functional impairment of the remaining synapses.

We then sought to determine whether these defects in inhibitory synapses were caused or associated with the loss of inhibitory neurons and focused on parvalbumin-positive (PV) interneurons as the largest group of inhibitory interneurons in the cortex. Using immunohistochemistry, we did not detect differences in the number of PV neurons in the frontal cortex of FusANLS/+ mice neither at 10 nor at 22 months of age (Supplementary Fig. 4a–c). As a result of the ANLS mutation, FUS would be expected to accumulate in the cytoplasm of PV neurons as previously shown in other cell types. We thus performed double immunostaining for FUS and parvalbumin and determined the nuclear/cytoplasmic ratio selectively in PV neurons. As shown in Supplementary Fig. 4d, e, cytoplasmatic FUS staining was increased in PV neurons of FusANLS/+ compared to Fus+/+ mice. Intriguingly, FUS cytoplasmic staining increased with age in wild-type PV interneurons, but remained significantly lower than in FusANLS/+ neurons. Altogether, these results demonstrate the existence of defects in cortical FusANLS/+ synapses, affecting inhibitory synapses more prominently, which could underlie the observed neuronal hyperexcitability (Fig. 3).

Synaptic accumulation of FUS and its RNA targets in FusANLS/+ cortex. To determine whether the observed phenotypes could be linked to a disrupted function of FUS at the synapse, we performed synaptosomal fractionation of the frontal cortex from 5-months-old FusANLS/+ mice. Obtained fractions were enriched in the synaptophysin protein (Fig. 7a–c, and Source data for uncropped western blots) and depleted in the nuclear IncRNA Malat (Fig. 7d), consistent with synaptic enrichment. In synaptosomes of FusANLS/+ mice, we observed an almost ten-fold increase in FUS content compared to wild-type synaptosomes, while the total or cytoplasmic FUS contents only increased 2–3 times (Fig. 7a–d). This increased FUS content was mostly due to mutant FUS synaptosomal accumulation, since it was not observed when using an antibody targeting the NLS of FUS (and thus not the mutant FANLS protein) (Fig. 7a–d). FUS is known to bind a number of mRNAs, including Fus mRNA itself, as well as mRNAs important for (inhibitory) synaptic function such as Nrnx1 or Gabrb1. Consistently, we observed increased levels of these 3 mRNAs in synaptosomal fractions of FusANLS/+ mice (Fig. 7e). This enrichment was relatively selective as 3 mRNAs encoding genes from the Turquoise module showed distinct patterns of synaptosomal enrichment: Gabbr1 and Grid2, but not Ctnnd2, mRNAs showed clear synaptosomal enrichment, while only Gabbr1 mRNA showed slightly elevated levels in FusANLS/+ synaptosomes. Collectively, our data show that defects in synapses, which are more pronounced in inhibitory synapses, and are related to synaptic FUS accumulation, likely causing the increased spontaneous neuronal activity and subsequent widespread behavioral abnormalities in FusANLS/+ mice.
**Discussion**

In this study, we show that knock-in mice with cytoplasmic accumulation of FUS display widespread behavioral alterations, beyond motor symptoms. We further determine that FUS mislocalization leads to increased spontaneous neuronal activity in the cortex, indicative of neuronal hyperexcitability, that is associated with structural and ultrastructural alterations of inhibitory synapses. Last, we show that the FUS mutation alters FUS synaptic content and modifies synaptic levels of a subset of its RNA targets, possibly underlying the observed phenotypes. The
timelines of the different experimental studies are summarized in Supplementary Fig. 5.

The notion that FUS mislocalization is a widespread pathological event in sporadic ALS, but also in many other neurological diseases, prompted us to investigate the behavioral phenotype of FusALS+/+ mice. While motor defects can be detected as early as 6 months of age and motor neuron degeneration is not detected before 18–22 months of age, we observed an early spontaneous locomotor hyperactivity in FusALS+/+ mice. In addition, we observed various defects in executive functions, including impaired remote long-term memory, and abnormal social interactions. Hyperactivity and social and executive dysfunctions have been previously documented in other mouse models of ALS/FTD. As such the transgenic overexpression of mutant FUS can e.g., cause hyperactivity and cognitive deficits64. Similar abnormalities are also observed in TDP-43 knock-in mice65, C9ORF72 BAC transgenic mice66, or Chmp2b transgenic mice57, suggesting that ALS mutations commonly lead to various behavioral alterations in mouse models, that are dominant over motor dysfunction. These phenotypes seen in mouse models nicely recapitulate widespread cognitive and executive dysfunction typical of ALS65,66 and support the clinical overlap between ALS and FTD70.

The deficits in executive functions and social behavior that we observe in FusALS+/+ mice are particularly relevant for FTD. Increased ventricular volume71–73 as well as atrophy of subcortical structures74,75 were found in FTD patients and presymptomatic mutation carriers, strengthening the analogy to FusALS+/+ mice. Pathology of FUS and other FET proteins (TAF15 and EWSR1) is a hallmark of a subset of FTD cases (FTD-FET cases). In FTD-FET cases, FUS pathology is associated with nuclear clearance of the FUS protein in neurons with FUS aggregates, although this nuclear clearance is not as pronounced as in cases with TDP-43 pathology40. Importantly, the FUS protein is accompanied by several other proteins in FTD-FET pathological aggregates, including TAF15 and EWSR1, two other FET proteins, as well as Transportin112,27–30. Thus, the disease in FTD-FET patients could be driven by several non-mutually exclusive mechanisms, including cytoplasmic accumulation and/or aggregation of FUS, nuclear clearance of FUS and/or aggregation of co-deposited pathological proteins. Previous studies indicate that complete loss of FUS could be sufficient to lead to FTD like symptoms in mice, and this was consistent with the role of FUS in controlling the splicing of mRNAs relevant to FTD, such as MAPT, encoding the Tau protein, or in the stability of mRNAs encoding relevant synaptic proteins such as GluA1 and SynGAP35–39. In FusALS+/+ mice, there is, however, a limited loss of nuclear FUS immunoreactivity10,44 and no obvious FUS aggregates, ruling out that these pathological events might play a major role in the observed behavioral alterations. The quasi-normal levels of FUS in the nucleus are explained by the existence of potent autoregulatory mechanisms, which are able to largely buffer the effect of the mutation on nuclear FUS levels. Mislocalization of either TAF15 or EWSR1 is also unable to account for behavioral abnormalities as both of these proteins show normal localization in FusALS+/+ neurons, as well as ALS-FUS patients56. Together, our results show that FUS mislocalization alone is sufficient to trigger behavioral symptoms and suggest that this might be a major driver of disease pathophysiology in FTD-FET patients. Importantly, our findings do not exclude that at later stages of disease progression, loss of nuclear FUS function might occur as a result of collapsed autoregulatory mechanisms, thereby exacerbating neurological symptoms.

A major finding of this study is that FusALS+/+ mice develop morphological and ultrastructural synaptic defects. The combination of locomotor hyperactivity with social deficits, as observed in FusALS+/+ mice, is commonly observed in various mouse models with synaptic defects. For instance, mouse models of autism spectrum disorders, such as mice lacking the ProSAP/Shank proteins75,76, display similar behavioral alterations. Our results point to a major defect in synapses, primarily affecting inhibitory synapses. This conclusion is supported by at least three main results: First, transcriptome analyses of the cerebral cortex show that genes related to synapses are affected. Second, the density of inhibitory synapses as well as the clusters size of three typical markers of inhibitory synapses (VGAT, GABAARa3, and gephyrin) are decreased. Third, inhibitory synapses are ultrastructurally abnormal, with increased size, increased number of vesicles and increased distance between vesicles and the active zone, which could be compensatory to their decreased density. Excitatory synapses were also abnormal, but their defects were minor compared to inhibitory synapses. Our data suggest that both the pre- and postsynaptic compartment of inhibitory synapses are affected by the Fus mutation. Indeed, the decrease in the density of Gephyrin positive puncta could reflect a disorganization of the postsynaptic density77, potentially caused by decreased GABAR activity78–80. Decreased VGAT density, as well as increased bouton size or vesicle disorganization further suggest impairment of presynaptic GABAergic terminals. On its own, decreased VGAT density might reflect an overall reduction of inhibitory synapses throughout the cortical layer81, and lead to impaired loading of GABA in the presynaptic vesicles60. Importantly, Sahadevan, Hembach and collaborators performed studies in FusALS+/+ mice at earlier ages and observed defects of inhibitory synapses, as early as 1 month of age, worsening at 6 months of age82. It is important to note that the disruption of inhibitory synapses can explain most of the detected behavioral and electrophysiological phenotypes observed in FusALS+/+ mice. Illustrating this, loss of Gabra182, or of Gabra383 are sufficient to lead to locomotor hyperactivity and the FUS target Nrxn1 (encoding a key factor in the formation of GABAergic and glutamatergic synapses84), is critical in regulating locomotor activity and social behavior in mice85,86. Indeed, the deletion of all three neurexins from PV neurons is causing a decrease in the number of synapses of this neuronal type87, in a manner similar to what is observed in FusALS+/+ mice.

Our current results do not allow to determine whether a specific subpopulation of inhibitory neurons would be more

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**Fig. 4 Structural and histological brain analysis of Fus+/+ and FusALS+/+ mice.** a Representation of the workflow used to determine volumes of corresponding brain structures from MRI slices per each mouse by the custom-made Fiji macro plugin (upper row). b Representative MRI slice images of Fus+/+ (upper row) and FusALS+/+ (lower row) male mice. c-h Bar graph showing intracranial volume (ICV) (c), normalized volume of the brain parenchyma (d), of lateral ventricles (e), cortex (f), medial septum (g), and cortical subplate (h) in FusALS+/+ vs Fus+/+ mice. For panels c-h, N = 5 for Fus+/+ and N = 5 for FusALS+/+ mice. Data are presented as mean ± SEM. Two-tailed Unpaired Student’s t-test; p = 0.0438; d = 0.0249; e = 0.0249; f = 0.9489; g = 0.0153; h = 0.0051. i Representative image of NeuN immunohistochemistry at 22 months of age in Fus+/+ (N = 3 mice) or FusALS+/+ (N = 5) male mice in the anterior region of the M1/M2 cerebral cortex. Scale bar: 100 μm, j Distribution of NeuN+ neurons in Fus+/+ (black) or FusALS+/+ (orange) male mice, in anterior and posterior regions of the M1/M2 cerebral cortex. N = 3 for Fus+/+ and N = 5 for FusALS+/+ mice. Source data are provided as a Source Data file.
Fig. 5 mRNA coexpression network analysis pinpoints defects in inhibitory and excitatory synapses in FusΔNLS/+ mice. a Signed association (Pearson correlation) of the mRNA MEs with transgenic condition. Modules with positive values indicate increased expression in transgenic mice; modules with negative values indicate decreased expression in transgenic mice. The red dotted lines indicate Bonferroni-corrected P < 0.05 for multiple comparisons (n = 12 modules, n = 16 mice per group). b Cell-type enrichment of modules (average n = 200 genes) using mouse genes in mRNA modules (Fisher’s two-tailed exact test, ***FDR = 2 × 10^-5). c Coexpression network plot of the synaptic (turquoise) module. The top 12 hub genes are indicated by name. d Gene ontology term enrichment of the synaptic module using 1791 synaptic module genes. e Trajectory of the synaptic module in the cortex of FusΔNLS/+ mice across time. Boxplot show median and quartile distributions, the upper and lower lines representing the 75th and 25th percentiles, respectively. Two-way ANOVA, F(1,24) = 14.55, p = 0.0008; n = 4–6 mice per group. f Coexpression network plot of the splicing/translation module. The top 12 hub genes are indicated by gene name. g GO term enrichment of the splicing/translation module using 1112 splicing/translation module genes. h Trajectory of the splicing/translation module in the cortex of FusΔNLS/+ mice across time. Boxplot show median and quartile distributions, the upper and lower lines representing the 75th and 25th percentiles, respectively. Two-way ANOVA, F(1,24) = 11.92, p = 0.002; n = 4–6 mice per group. The center line represents the median.
selectively affected in $Fus^{\Delta NLS/+}$ mice. PV interneurons are, however, a strong candidate according to the results of our studies, but also their involvement in TDP-43 knock-in mice$^{65}$, and in TDP-43 transgenic mice that display degeneration of hippocampal PV positive interneurons$^{68}$. Functional impairment of PV interneurons might represent a unifying theme in ALS pathophysiology, as multiple electrophysiological studies demonstrate hypexcitability of PV neurons in SOD1 and TDP-43 transgenic mouse models of ALS$^{89-92}$. Others, however, found PV interneurons to be unaltered presymptomatically and to turn hyperexcitable during the symptomatic phase in the same SOD1$^{G93A}$ mouse model$^{93}$. In either case, those changes in PV excitability were always accompanied by hyperexcitability of layer V pyramidal neurons$^{89-91,93}$. These findings in mouse models nicely
Fig. 6 Defects in synapses in 22-months-old Fus^{ΔNLS/+} mice. a, b Representative image of transmission electron microscopy in Fus^{+/−} or Fus^{ΔNLS/+} layer II/III of the motor cortex at 22 months of age showing inhibitory synapses (a) (as containing 21 mitochondrion on each side of the synapse) and excitatory synapses (b). Pre: presynaptic compartment; active zone is shown with an arrowhead. N = 4 Fus^{+/−} mice (1 male and 3 females), and N = 4 Fus^{ΔNLS/+} mice (1 male and 3 females) have been analyzed. c−f Violin plot showing the distribution of bouton sizes (c), the length of active zones (d), the number of vesicles per synapse (e), and the distance of individual vesicles to the active zone (f) in inhibitory synapses of Fus^{+/−} (black) or Fus^{ΔNLS/+} (orange) mice. For panels c−f, N = 379 synapses from 1 male and 3 female Fus^{+/−} mice and N = 387 synapses from 1 male and 3 female Fus^{ΔNLS/+} mice were analyzed. Kolmogorov-Smirnov test: p = 0.0016; d, p < 0.0001; e, p = 0.0010; f, p < 0.0001. g−j Violin plot showing the distribution of bouton size (g), the length of active zone (h), the number of vesicles per synapse (i), and the distance of individual vesicles to the active zone (j) in excitatory synapses of Fus^{+/−} (black) or Fus^{ΔNLS/+} (cyan) mice. For panels g−j, N = 463 synapses from 1 male and 3 female Fus^{+/−} mice and N = 490 synapses from 1 male and 3 female Fus^{ΔNLS/+} mice were analyzed. Kolmogorov-Smirnov test: g, p = 0.0038; h, p < 0.0001; i, p = 0.0362; j, p = 0.2182. k Representative images of GABAARα3, Gephyrin and VGAT intensity in 22-months male mice, coded by area size (Imaris). N = 3 Fus^{+/−} mice and N = 4 Fus^{ΔNLS/+} mice have been analyzed. k Bar graphs representing the density analysis for VGAT, GABAARα3, and Gephyrin comparing Fus^{+/−} vs Fus^{ΔNLS/+} mice. (Fus^{+/−} vs Fus^{ΔNLS/+}, Mann-Whitney test, VGAT, p = 0.0464; GABAARα3, p = 0.0217; Gephyrin, p = 0.0043). N = 8 FOVs from 3 Fus^{+/−} mice and N = 9 FOVs from 4 Fus^{ΔNLS/+} mice were analyzed for VGAT; N = 8 FOVs from 3 Fus^{+/−} mice and N = 10 FOVs from 4 Fus^{ΔNLS/+} mice were analyzed for GABAARα3 and Gephyrin. Data are presented as mean ± SEM. Mann-Whitney, One tailed, VGAT: p = 0.0464; GABAARα3: p = 0.0217; Gephyrin: p = 0.0043. m Violin plot representing the analysis of the clusters size for VGAT, GABAARα3, and Gephyrin comparing Fus^{+/−} vs Fus^{ΔNLS/+} mice. N = 142,416 synapses from 3 Fus^{+/−} mice and N = 115,151 synapses from 4 Fus^{ΔNLS/+} mice were analyzed for VGAT; N = 202,302 synapses from 3 Fus^{+/−} mice and N = 99,464 synapses from 4 Fus^{ΔNLS/+} mice were analyzed for GABAARα3; N = 169,036 synapses from 3 Fus^{+/−} mice and N = 68,422 synapses from 4 Fus^{ΔNLS/+} mice were analyzed for Gephyrin. Kolmogorov-Smirnov test. VGAT: p < 0.0001; GABAARα3: p < 0.0001; Gephyrin: p < 0.0001. Source data are provided as a Source Data file.

recapitulate human ALS pathology, in which cortical hyper-excitability is a frequent and, most importantly, early finding in familial and sporadic cases, including Fus mutation carriers. In line with these findings, we also observed a pronounced increase in spontaneous neuronal activity in vivo, which is highly indicative of hyperexcitable pyramidal neurons. While we cannot rule out cell autonomous alterations affecting the intrinsic excitability of pyramidal neurons, our histological, ultrastructural, and transcriptomic data strongly argue for defective inhibitory neurotransmission by interneurons. In summary, our results, along with others, support the notion that dysfunction of cortical PV interneurons contribute to neural circuit defects in ALS and FTD. Importantly, while we observe molecular and structural defects in inhibitory neurons, we did not observe a loss of PV cell bodies in Fus^{ΔNLS/+} mice, suggesting that the major defect resembles a synaptopathy rather than frank neuronal loss, consistent with other studies. Altogether, our results identify a role for Fus in regulating GABAAergic synapse structure and function. Since other major classes of inhibitory interneurons were not investigated, we cannot exclude that somatostatin positive (SST) or HTR3A expressing interneurons are also affected, although to a lesser extent than PV neurons. Furthermore, our work also shows that this Fus mutation alters glutamatergic synapses, as judged from both WGCNA analysis of RNAseq (Fig. 5) and electron microscopy (Fig. 7). This is consistent with results from Sahadevan, Hembach et al., providing evidence that Fus is also critically involved in glutamatergic synaptogenesis, at least during development, and is in line with previous studies. Further work is required to disentangle the causes and consequences of GABAAergic and glutamatergic impairment, and their respective mechanisms.

How can mutant FUS regulate inhibitory synaptic structure? We observe that the loss of the FUS NLS leads to an increased level of the mutant protein in purified synaptosomes. These results are consistent with results from Sahadevan, Hembach et al., where the authors identified a number of Fus synaptic RNA targets, and a subset of these were also increased in synaptosomes of Fus^{ΔNLS/+} mice. Interestingly, in both studies, several Fus synaptic targets are not modified in Fus^{ΔNLS/+} synaptosomes, including some related to GABAAergic neurons. Sahadevan, Hembach and collaborators further demonstrate that at least a subset of these Fus synaptic RNA targets show increased stability in Fus^{ΔNLS/+} neurons. It seems thus reasonable to hypothesize that accumulation of synaptic FUS compromises synaptic homeostasis through altered stability of key synaptic RNAs, either through direct binding, or indirectly. This does not exclude additional mechanisms of toxicity for synaptic FUS, in particular effects on local synaptic translation, that could affect synaptic protein levels. Further work should focus on determining whether FUS might also regulate synaptic translation of specific proteins involved in inhibitory transmission, and whether rescuing synaptic defects in inhibitory neurons might translate into an efficient therapeutic strategy.

Methods

Mouse models and behavioral analyses. Wild-type (Fus^{+/−}) and heterozygous (Fus^{ΔNLS/+}) mice on a pure genetic background (C57BL/6J), have been described previously, were bred and housed in the central animal facility of the Faculty of medicine of Strasbourg, with a regular 12 h light and dark cycle (light on at 7:00 am) under constant conditions (21 ± 1 °C; 60% humidity). Standard laboratory rodent food and water were available ad libidum throughout all experiments. Mice were genotyped by PCR of genomic DNA from tail biopsies using oligonucleotide primers (sequence provided in Supplementary Data 2). Mouse experiments were performed in compliance with all relevant ethical regulations for animal testing and researcher. All experiments were approved by local ethical committee from Strasbourg University (CREMEAS) under reference number AL/27/34/02/13 (behavior), by the Government of upper Bavaria (license number Az 55.2-1.54-2532-11-2016, two-photon microscopy) and by „Regierungspräsidium Tübingen“ (animal license number 1431, MRI). Behavioral tests were done during the light phase (between 9 am and 5 pm) of their light/dark cycle except for indicated experiments. Until the mice reached the age when the behavioral tests were performed mice were group-housed. Once mice were single housed for the behavioral task they were kept individually for only a period necessary to finalize the set of behavioral experiments and in order to minimize possible negative effects of isolation, afterwards cohorts were sacrificed and processes for downstream analyses. Male mice of 4, 10, and 22 months of age were subjected to behavioral studies and data were analyzed blind to genotypes. The sex of the animals studied is indicated in each figure legend.

Spontaneous locomotor activity in the home cage–actimetry. Home cage activity was assessed according to previously published protocols. Mice were placed individually in large transparent Makrolon cages (42 x 26 x 15 cm) adapted to the shelves of the testing device (eight cages/shelve). Two infrared light beams, passing through each cage, were targeted onto two photocells, 2.5 cm above the cage floor.
Fig. 7 FUS accumulates in synaptosomes of FusΔNLS/+ mice and alters synaptosomal levels of a subset of its targets. a, b Representative western blot images (a) and respective quantifications (b) of cytoplasmic (a, left) or synaptosome (a, right) extracts from Fus+/+ (+/+) or FusΔNLS/+ (Δ+/+) mice (4 months of age) using two antibodies recognizing the N-terminal part of the FUS protein (FUS N-ter1 and FUS N-ter2), the C-terminal part of FUS (encoding the NLS, FUS C-ter) or synaptophysin protein to show enrichment in synaptic proteins in the synaptosome fraction. N = 6 Fus+/+ mice and N = 6 FusΔNLS/+ mice were analyzed. Data are presented as mean ± SEM. One-Way ANOVA with Tukey post-hoc test. ***p < 0.0001 Please note that the FUS western blots were run on independent gels, to avoid stripping and reprobing on the same membrane for the same protein. Each of these gels were controlled for equal loading using StainFree markers, that are provided in the source data.

c, d Representative western blot images (c) and respective quantifications (d) of total extracts (c) from Fus+/+ (+/++) or FusΔNLS/+ (Δ+/+) mice (4 months of age) using the same antibodies as in panel a. N = 5 Fus+/+ mice and N = 5 FusΔNLS/+ mice were analyzed. Data are presented as mean ± SEM. Two-tailed Unpaired Student’s t-test. Fus: ***p < 0.0001 vs corresponding wild-type fraction; ###p < 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Malat: ###p = 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Nrxn1: *p = 0.0140 vs corresponding wild-type fraction; ###p < 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Gabra1: **p = 0.0012 vs corresponding wild-type fraction; ###p < 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Gabrb1: **p = 0.0029 vs corresponding wild-type fraction; ###p < 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Grid2: ###p < 0.0001 vs corresponding cytoplasmic fraction of the same genotype. Ctnnd2: no significant differences observed (p > 0.05). Source data are provided as a Source Data file.
level and 28 cm apart. The number of cage crossing was recorded automatically and was used to determine or score the spontaneous locomotor activity. The experiment began at 0:00 pm and after 2 h of habituation continued for 3 consecutive days for a complete 24 h nocturnal cycle (12 h dark and 12 h light).

Open field. The general exploratory locomotion and anxiety in a novel environment were tested during 15 min long sessions in the open field arena (72 x 72 x 36 cm) located in a test room and lit by a 600 lux for background lighting, according to published protocols49. The open maze was divided by lines into sixteen squares (18 x 18 cm). Each mouse was placed in the center of the arena and allowed to freely move while being video recorded. The recorded data were analyzed offline with EthoVision XT software system (Noldus Information Technology). The time spent in the center (four central quadrants) vs. the perimeter (12 peripheral quadrants) was used to measure anxiety, while the total distance traversed in the arena and average moving speed (mean velocity) was used to evaluate locomotor activity. For each mouse a movement heat map and trajectory tracking map that are representing a corresponding locomotor activity were made independently.

Dark/light box test. The light/dark box apparatus consisted of two Poly-Vinyl-Chloride (PVC) compartments of equal size (18.5 x 18.5 x 15 cm) one opaque and the other transparent, connected through an opaque tunnel (5 x 5.5 x 5 cm). The illumination of the transparent compartment was set at 400 lux. Each mouse was placed alone in the dark compartment and the mouse’s behavior was recorded during 5 min with a video camera located 15 cm above the center of the box. Test time began at 17.00 pm and after 2 h of habituation continued for 3 consecutive days in the closed compartment and the time spent in each compartment were tested to assess for anxiety level and exploratory behavior, as published previously49.

Object preference test. This test is designed to identify specific detection deficiencies and/or odor preference, namely the ability to sense attractive or aversive scents. After 30 min of habituation to empty cage with no bedding, each mouse was challenged with a filter paper embedded with two strong scents (vanilla and 2-methyl butyrate) or a neutral scent (water) was video recorded over 3 min. A 1-h pause was made between exposure to different scents was applied to each mouse using a procedure adapted from previously reported protocols67,100. The time the mouse spent sniffing the filter paper—the exploration time, is calculated post-hoc by an examiner blind to mouse genotype and condition. Those scents with the exploration time greater than water were designated as “attractive” while those with times less than water were termed “aversive”.

Social interaction in the home cage (resident-intruder test). Social interaction was assessed in the home cage by a standard protocol67,101. Briefly, both resident and intruder mice were isolated and housed individually for 1 week before the test. After 30 min of habituation to the test room resident mouse was allowed to freely roam in his home cage without the cage top for 1 min. A novel male intruder mouse (nonlittermate of same background, same age, and similar weight) was then introduced in the opposite corner as the resident and allowed to interact for 5 min while videotaped. The total physical interaction, defined as the time during which the resident mouse actively explores the intruder was analyzed post-hoc. Only social activities, such as time spent investigating, grooming, following, sniffing etc. were quantified separately for each minute and for the whole time of the test and were differentiated from nonsocial/aggressive activities such as attacks, bites, and tail rattles.

Three-chamber social task. Specific social behaviors such as sociability and social recognition were analyzed by using three-chamber social task. The experimental procedure is adapted from Gason E et al.67. The three-chamber box (59 x 39.5 x 21.5 cm) is made of transparent Plexiglas (Noldus Information Technology, Wageningen, The Netherlands) and is divided into three chambers (one middle and two side chambers) of equal size (18.5 x 39.5 cm) by the walls with a square opening (7 x 7 cm) that could be closed by a slide door. Each of the two side chambers contains a mobile wire cylinder shaped cage (20 x 10 cm diameter) that is made of transparent Plexiglas bars placed 6 mm apart. Cage is closed by the upper and lower lids. Mice of both genotypes (Fus+/+ and FusANLS−/−) that were experimentally tested are referred to as test mice and adult male unfamiliar mice of same background, age and weight used as the social stimulus are called novel mice. All mice were housed individually for 1 week before the test and were habituated to the testing room for at least 1 h before the start of behavioral tasks. One day prior to the testing, the novel mice were habituated to mobile wire cage for 5 min. The keeping of the novel mouse separated in a wire cage prevents aggressive and sexual activities, and in the same time ensures that any social interaction is initiated by the test mouse. Sessions were videotaped and visually analyzed post-hoc. The experimental procedure was carried out in four trials of 5 min each. After each trial, the mouse was returned to his home cage for 15 min. Trials were grouped into two consecutive parts.

Trial 1 (habituation): the test mouse was placed in the middle chamber and left to freely explore each of the three chambers: the empty middle or two sides’ arenas containing the empty wire cages for 5 min.

Trials 2–4 (sociability, social recognition, social learning acquisition): the mouse was placed in the middle chamber, but an unfamiliar mouse (novel mouse) was placed into a wire cage in one of the side chamber (the cage in the other side-chamber remains empty). The test mouse had free access to all three chambers. The position of novel mouse and empty wired cage were alternated between trials. We quantified the time spent actively exploring a novel mouse or an empty cage by the test mouse as a social interaction time or an object exploration time, respectively. We measured the time that test mouse spent in the close proximity of the novel mouse while actively interacting with it (staring, sniffing) compared to the empty cage—object, indicates social preference or social recognition as a result of the capability to differentiate a conspecific from an object. A total of the test mouse to spontaneously interact with novel mouse is considered as sociability which gradually decreased over trials as a result of social learning acquisition.

Water maze task. The water maze consisted of a circular pool (diameter 160 cm; height 60 cm) filled with water (21 ± 1 °C) made opaque by addition of a powdered milk (about 1.5 g/L). The habituation day consisted in a 4-trial session using a visible platform (diameter 11 cm, painted black, protruding 1 cm above the water surface and located in the South-East quadrant of the pool), starting randomly from each of the four cardinal points at the edge of the pool. During this habituation trial, a blue curtain surrounded the pool to prevent the use of distal cues and thus incidental encoding of spatial information. For the following days, the platform was removed. Mice were allowed to freely roam in the 5-day training period (1 trial per day, maximum duration of a trial 60 seconds, inter-trial interval = 10–15 seconds) with a hidden platform located at a fixed position in the North-West quadrant. Animals were starting randomly from each of the four cardinal points at the edge of the pool and the sequence of starting points was randomized. Mice were tested for retention in a 18-days delay probe trial and two extinction tests: the first 2 h after probe trial and the second 2 h after the first. For the probe trial, the platform was removed; the mice were introduced in the pool from the North-East (a starting point never used during acquisition) and allowed a 60-s seconds swimming time to explore the pool. Data were collected and computed by a video-tracking system (SMART: AnyMaze software). For the video platform and training trials following parameters were used: the distance traveled and the latency time before reaching the platform and the average swimming speed. For the probe trial and extinction tests the time (in seconds) spent in the target quadrant (i.e., where the platform was located during acquisition) was analyzed102.

Assessing neuronal activity by in vivo two-photon imaging

Cranial window implantation and virus injection. Mice of both sexes were implanted with a cranial window at 3 and 9 months of age (+/−10 days), respectively and received a stereotaxic injection of the genetically encoded calcium indicator (AAV2/1.BhyGCaMP6m.WPRE.SV40 diluted 1:6 in saline—10-month-old cohort or AAV9.syn.GCaMP7s.WPRE diluted 1:6 in saline—4-month-old cohort (Pgp-Aav-syn.GCaMP7s.WPRE: a gift from Douglas Kim & GENIE Project; Addgene viral prep #104487-AAA9; RRID:Addgene_104487)103 into the primary motor cortex (MI)104. In brief, mice were first anesthetized with Fentanyl (0.05 mg/kg), Midazolam (0.5 mg/kg), and Metodomidin (0.5 mg/kg). A circular craniotomy with a 2 mm radius, centered at 1.7 mm lateral and 0.8 mm anterior to bregma, was performed, followed by the slow injection of a total of ~1 μL of the calcium indicator into three sites (~300 nl per site at 600 μm cortical depth). A 4 mm round glass coverslip (Warner Instruments) was placed over the cortex and sealed with UV-curable dental acrylic (Venus Diamond Flow, Heraeus Kulzer GmbH). A metal head bar was attached to the skull using dental acrylic (Paludur, Heraeus Kulzer GmbH), allowing for stable positioning during two-photon imaging.

Two-photon imaging in anesthetized mice. Four mice were used following the cranial window implantation, in vivo two-photon imaging was performed within cortical layer II/III using a two-photon microscope (Hyperscope, Scientifica, equipped with an 8 kHz resonant scanner) at a frame rate of 30 Hz and a resolution of 512 x 512 pixels. A x16 water-immersion objective (Nikon), measure consisting of 15,000 frames (equivalent to ~8 min) were acquired, covering a field of view (FOV) of 300 x 300 μm. Light source was a Ti:Sapphire laser with a Deepsee pre-chip unit (Spectra Physics MaiTai eHP)49. GCaMP was excited at 910 nm, with a laser power not exceeding 40 mW (typically 10–40 mW). In each mouse, two to five FOVs at cortical depth of 140–310 μm were imaged, yielding 2264 cells in Fus+/+ (n = 25 experiments, 8 mice) and 1107 cells in FusANLS−/− (n = 13 experiments, 3 mice) at 400 μm of area; and 631 cells in Fus+/+ (n = 10 experiments, 5 mice) and 855 cells in FusANLS−/− mice (n = 14 experiments, 6 mice) at 10 months of age. During imaging, mice were anesthetized with 1.0–1.5 volume % isoflurane in pure O2 at a rate between 0.5–0.5 mL/kg/min, yielding 130 breathing cycles per minute. Body temperature was maintained at 37 degrees using a physiological monitoring system (Harvard Apparatus).

Image processing and data analysis. All image analyses were performed in Matlab (Math Works) using custom-written routines105. In brief, full frame images were corrected for baseline, exposure, gain, displacement, and age. ROIs were semi-automatically selected based on the maximum and mean projections of all frames. Fluorescence signals of all pixels within a selected ROI were averaged,
The intensity traces were low pass filtered at 10 Hz. Contamination from neuropil signals was accounted for using the following Eq. (1)\(^{10}\),

\[
F_{ROI_{comp}} = F_{ROI} - 0.7 \times F_{neuropil} + 0.7 \times \text{median}(F_{neuropil})
\]

(1)

\(F_{ROI_{comp}}\) stands for neuropil-compensated fluorescence of the ROI, \(F_{ROI}\) and \(F_{neuropil}\) being the initial fluorescence signal of the ROI and the signal of the neuropil, respectively. A neuron was defined as ‘active’ if it displayed at least one prominent calcium transient over 20 frames (corresponding to ~0.7 seconds). The overall difference in the fraction of active cells between 4- and 10-month-old mice could be due to both age as well as the usage of the more sensitive calcium indicator GCaMP6m. To impact the extent of the indicator alone, we also investigated control 4 m age cohort expressing GCaMP6m, in which case the fraction of active cells was 81% and not different from the average observed in the Fuo/+/+ control cohort used here (ranksum test, \(p = 0.87\), 7 experiments in 3 mice).

**Histological techniques.** Male mice were anesthetized with intraperitoneal injection of 100 mg/kg ketamine chloride (Imalgene 1000®, Merial) and 5 mg/kg xylazine (Rompun 2%, Bayer), and then transcardially perfused with cold PFA 4% in 0.01 M phosphate buffered saline (PBS). After dissection, brains were post-fixed for 24 h and then included in agar 4% and serial cuts of 40 µm thick were made using vibratome (Leica Biosystems, S2000).

**Peroxidase immunohistochemistry.** For peroxidase immunohistochemistry, sections were incubated 10 min with H2O2 3%, rinsed with PBS x2 and incubated with blocking solution (8% Horse serum, 0.3% Bovine Serum Albumin, 0.3% Triton, PBS-0.02% Thimerosal). Sections were rinsed, and then incubated with anti-mouse NeuN or anti-mouse parvalbumin (Millipore, MAB377, 1:100 and Sigma, P3885, 1:1000, respectively) overnight at room temperature. The second day, sections were rinsed and incubated for 1 h at room temperature with donkey anti-mouse antibody (Jackson, 715-067-003, 1:500). After sections were rinsed, they were incubated for 1 h in horseradish peroxidase ABC kit (Vectorstain ABC kit, PK-6100, Vector Laboratories Inc.), rinsed and incubated with DAB (Sigma, D9905). The enzymatic reaction was stopped by adding PBS 1X, rinsed with water and sections were mounted with DPX mounting medium (Sigma, 06522).

**Quantification.** Images were quantified using a homemade ImageJ plugin. A Region Of Interest (ROI) was first defined by the user as the M1/M2 regions of the cerebral cortex as defined by the Paxinos Atlas\(^{106}\) using the following coordinates: interaural 4.06 mm; Bregma 0.26 mm. For NeuN immunohistochemistry, a second, more anterior region of M1/M2 cortex was also quantified with the following coordinates: Interaural: 5.74 mm, Bregma: 1.94 mm.

In this region, a semi-automated segmentation led to the identification of the labelled structures (cells or nuclei). Finally, the plugin subdivided the previous ROI into 10 subregions and measured either the number of objects per subregion or the proportion of each subregion that is covered by labelled structures.

**Immunofluorescence.** Sections were rinsed with PBS 1X then incubated with blocking solution (8% Goat serum, 0.3% Bovine Serum Albumin, 0.3% Triton, PBS-0.02% Thimerosal) overnight at 4 °C in primary antibody: rabbit anti-FUS antibody (ProteinTech, 11570-70-AP, 1:100) and mouse anti-parvalbumin antibody (Sigma, P3885, 1:1000). After sections in PBS x2 were incubated for 1 h at room temperature with H2O2 3%, goat anti-mouse Alexa-488 secondary antibody (Invitrogen, A11034, 1:500) and goat anti-mouse Alexa-647 secondary antibody (Invitrogen, A12245, 1:500), final sections were subsequently washed with PBS (3 x 10 min) and mounted in Aqua polymeze (Roth, 806).

Images were acquired along the Z axis (Z stacking) using a Zeiss Axiolab.M2 microscope equipped with a Plan-Apochromat x20/0.8 objective, high performance B/W camera (Orca Flash4, Hamamatsu) and run by the Zeiss Zen2 software. Images were quantified using the ImageJ freeware. First, the user defined ROIs corresponding to the cytoplasm and nucleus or several PV positive cells at several Z positions. Then a homemade macro was used to calculate the ratio, in the green channel, of the cytoplasm intensity divided by the nucleus one.

**Electron microscopy.** Mice from both sexes were used for electron microscopy. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine chloride and 5 mg/kg xylazine and transcardially perfused with glutaraldehyde (2.5% in 0.1 M cacodylate buffer at pH 7.4). Brains were dissected and immersed in the same fixative overnight. After three rinses in cacodylate buffer (EMS, 11650), serial cuts of 80 µm thick were made with vibratome. Slides were then post-fixed in 1% osmium in cacodylate buffer 1 h at room temperature. Finally, tissues were dehydrated in ethanol graded series and embedded in Embed 812 (EMS, 13940). The ultrathin sections (50 nm) were cut with an ultramicrotome (Leica, EM UC7), dehydrated in graded ethanol series and embedded in Embed 812 (EMS, 13940). Osmium in Cacodylate buffer 1 h at room temperature. Finally, tissues were ultrathin sections (50 nm) were cut with an ultramicrotome (Leica, EM UC7), dehydrated in graded ethanol series and embedded in Embed 812 (EMS, 13940). Osmium in Cacodylate buffer 1 h at room temperature. Finally, tissues were

\[\text{ROI} = \frac{F_{neuropil}}{F_{neuropil} + F_{cytoplasm}}\]

(2)

The enzymatic reaction was stopped by adding PBS 1X, rinsed with water and sections were mounted with DPX mounting medium (Sigma, O6522).

**Synaptic density in brain sections.** Male mice were anesthetized by CO2 inhalation before perfusion with PBS containing 4% paraformaldehyde and 4% sucrose. Brains were harvested and post-fixed overnight in the same fixative and then stored at 4 °C in PBS containing 30% sucrose. Sixty micrometers thick coronal sections were cut on a cryostat and processed for free-floating immunofluorescence staining. Brain sections were incubated with the indicated primary antibodies (Rabbit GABA antibody Synaptic Systems 1:500; Mouse Gephyrin antibody Synaptic Systems, 1:500, Guinea pig VGAT antibody, Synaptic Systems, 1:500) for 48 h at 4 °C followed by secondary antibodies (1:1000) for 24 h at 4 °C. The antibodies were diluted in 1× Tris Buffer Saline solution containing 10% donkey serum, 3% BSA, and 0.25% Triton-X100. Sections were then mounted on slides with applying a gradient (FLASH) television camera (Leica microscope, Germany) equipped with a cryogenically cooled two-element surface (MRI CryoProbe, Bruker Biospec, Ettlingen, Germany) transmit/receive coil. Anatomical brain images were acquired in coronal slice orientation (30 slices) applying a gradient (FLASH) sequence with imaging parameters as: TE/TR 2.95/400 ms (TE = echo time, TR = repetition time), matrix 30 x 340 x 340, resolution 250 x 50 x 50 µm\(^3\).

**Structural MRI scans.** Male mice were used for MRI studies. All data were acquired on a dedicated small bore animal scanner (Biowspc 117/16, Bruker, Ettlingen, Germany) equipped with a cryogenically cooled two-element surface (MRI CryoProbe, Bruker Biospec, Ettlingen, Germany) transmit/receive coil. Anatomical brain images were acquired in coronal slice orientation (30 slices) applying a gradient (FLASH) sequence with imaging parameters as: TE/TR 2.95/400 ms (TE = echo time, TR = repetition time), matrix 30 x 340 x 340, resolution 250 x 50 x 50 µm\(^3\).

**RNaseq.** RNAseq on frontal cortex was performed as previously described\(^{104,105}\). Briefly, RNA from cortex of 22-months-old male Fus\(^{FUS\text{Sel}}\) mice and their control littermates were extracted with TRIzol (Invitrogen). RNA quality was measured using the Agilent Bioanalyzer system or RNA screenTape (Agilent technologies) according to the manufacturer’s recommendations. Samples were processed using the Illumina TruSeq single Stranded mRNA Sample Preparation Kit according to manufacturer’s instructions. Generated cDNA libraries were sequenced using an Illumina HiSeq 2000 sequencer with 4–5 biological replicates sequenced per condition using single read, 50 cycle runs. RNA from the cortex of 5-months-old male Fus\(^{FUS\text{Sel}}\) and control littermate mice was extracted, and libraries were generated using the Illumina TruSeq single Stranded mRNA Sample Preparation Kit. The cDNA library was sequenced on the Illumina HiSeq 4000 with three biological replicates per condition using single-end 50 bp read. Total reads sequenced varies from 35 to 45 million reads. Complete QC report will be made publicly available.
Raw reads were mapped to the mouse reference genome GRCh38 with STAR version 2.7.5 (12) and default parameters using Ensembl gene annotations (version 87). Gene-level abundance estimates were estimated using the option--quantMode geneCount in STAR. We filtered the lowly expressed genes wherein each gene was required to have at least 15 counts across all samples and used both exonic and intronic reads. The filtered set of genes was used for the PCA plot and differential expression analysis. Differential gene expression analysis was performed with the ARMMOR workflow (43) and a cut off FDR value of 0.05 was set in both datasets. RNA samples sequenced in the present study from 5–6-months-old mice (n = 10) were pooled to the samples from Sahadevan et al., 2020, (n = 6).

Weighted-gene coexpression network analysis. Coexpression network analysis was performed using a user-friendly R WGCNA library (14). We wanted to investigate mouse brain coexpression networks that are disease specific in PznSND/s mice. Bioweighted correlations were calculated for all pairs of genes, and then a signed similarity matrix was created. In the signed network, the similarity between genes reflects the sign of the correlation of their expression profiles. The signed similarity matrix was then raised to the power β to emphasize strong correlations and reduce the emphasis of weak correlations on an exponential scale. The resulting adjacency matrix was then transformed into a topological overlap matrix as described. After scaling the network (consensus scaling quantile 0.2), a threshold of power 5 was chosen (because it was the smallest threshold that resulted in a scale-free R2 fit of 0.9) and the consensus network was created by calculating the component-wise minimum values for topological overlap. Using 1 – TOM (distance TOM) as the distance measure, genes were hierarchically clustered. Initial module assignments were determined using the blockwiseModules function as follows: blockwiseModules(datExpr, power = 2, minModuleSize = 30, networkType = “signed”, deepSplit = 2, reassignThreshold = 0, mergeCutHeight = 0.35, numericLabels = TRUE, pamRespectsDendro = FALSE, saveTOM = TRUE, verbose = 3). The resulting modules or groups of coexpressed genes was used to calculate the MEs or the first principal component of the module. MEs were correlated with different biological and technical traits like transgenic condition and batch to find disease-specific modules. Module hubs were defined by calculating module membership (kME) values, which are the Pearson correlations between each gene and each ME. Genes with a kME ≥ 0.7 were removed from the module. Network visualization was done with the igraph package in R. Module definitions from the network analysis were used to create synthetic eigengenes for the 1-month, 6 months and 22-months timepoint and were used to understand the trajectory of various modules across timespoints.

Enrichment analyses using single-cell experiment data. To reduce false positives, we used FDR-adjusted P-values for multiple hypergeometric test comparisons. For cell-type enrichment analysis, we used an already published single-cell mouse brain dataset. Finally, genes in network modules were characterized using Enrichr (version 1.2.5) (17).

Synaptosomal enrichment followed by RT-qPCR and western blotting. Frontal cortex was removed from the brains of 4-months-old female mice by micro-dissection, as previously described, harvested, rapidly frozen in liquid nitrogen and stored at −80 °C until use. Synaptosomal fractionation was isolated using Syn-PET, a Synaptic Protein Extraction kit (Thermo Scientific). RNA was reverse transcribed using SuperScript Ready-to-use cDNA supermix (Bio-Rad, 1708841). Quantitative PCR (qPCR) was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 172574) and quantified with Bio-Rad CFX Manager software. Gene expression was normalized by computing a normalization factor by Genorm software using three standard genes P0L2, Tbp, and Actin for nervous tissue. Primer sequences are provided in Supplementary Data 2.

For western blotting cytosolic and synaptosomal fractions were prepared using the same protocol, and protein concentration was quantitated using the BioRad protein assay kit (Pierce). Fifteen micrograms of proteins were loaded into a gradient 4–20% SDS-PAGE gel (Bio-Rad, 5678094) and transferred on a 0.45 μm nitrocellulose membrane (Bio-Rad) using a semi-dry Transblot Turbo system (Bio-Rad). Membranes were saturated with 10% nonfat milk in PBS and then probed with the following primary antibodies: Anti-Synaptophysin (Abcam, ab14692, 1:1000), Anti-FUS N-ter1 (ProteinTech, 11570, 1:1000), Anti-FUS N-ter2 (Bethyl, A309-293A, 1:2000), and Anti-FUS (Bethyl, A309-294A, 1:2500) all diluted in 3% nonfat milk in PBS. Blots were washed and incubated with anti-Rabbit secondary antibody conjugated with HRP (P.A.R.I.S, B12407, 1:5000) for 2 hours. Membranes were washed several times and analyzed with chemiluminescence using ECL Lumina Forte (Millipore, WBFLU0500) using the Chemidoc XRS Imager (Bio-Rad). Total proteins were detected with a stain-free gel capacity and normalized. Uncropped western blot images and stain-free images are provided in supplementary figures.

Statistics. If not stated otherwise, data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 8 (GraphPad, CA). Unpaired t-test was used for comparison between two groups, one-way or two-way analysis of the variance (ANOVA), followed by Tukey’s multiple comparison post-hoc test and two-way repeated measures (RM) ANOVA, followed by Sidak multiple comparison post-hoc test were applied for three or more groups. Distributions were compared using the Kolmogorov–Smirnov (KS) test. Results were considered significant when p < 0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw reads are provided with this paper as supplementary information. The RNAseq datasets that support the findings of this study have been deposited in GEO with the accession codes GSE166615. Source data are provided with this paper.

Code availability

The code used for MRI analysis is provided as supplementary files.

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