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Behavioural impairments in mice of a novel FUS transgenic line recapitulate features of frontotemporal lobar degeneration.

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Abstract

Multiple clinical and experimental evidence suggest that ALS and FTLD are members of a disease continuum. Pathological FUS inclusions have been observed in subsets of patients with these diseases but their anatomical distribution is different for two diseases. These structures are present in motor neurons in ALS cases but in cortical neurons in FTLD cases. Expression of a C-terminally truncated form of human FUS causes an early onset and progressive motor neuron pathology in transgenic mice but only when these neurons express a certain level of this protein. Severe motor dysfunction and early lethality of mice with expression above this level prevent their use for studies of FTLD-related pathology caused by expression of this form of FUS. In the present study we used another line of mice expressing the same protein but not developing any signs of motor system dysfunction due to substantially lower level of transgene expression in motor neurons. In a set of tests 5-month old mice displayed certain behavioural abnormalities, including increased impulsivity, decreased anxiety and compromised social interaction, that recapitulate behaviour characteristics typically seen in FTLD patients.

Introduction

Frontotemporal lobar degeneration (FTLD) is a key histopathological feature of frontotemporal dementia (FTD), the second common type of dementia in the presenile age group making up 5 – 15% of cases (reviewed in Ref. 1). Clinical symptoms include progressive deterioration of patients' behaviour, distortion (defacing) the personality, and problems with cognitive domains such as language, speech and memory. The pace of the disease progression varies as well as prevalence and severity of clinical symptoms particularly at the early stages of the disease when predominantly behavioural or predominantly linguistic changes are common [2].

A hallmark of FTLD is the presence of intraneuronal inclusion bodies that vary in morphology and protein composition. Based on *post-mortem* analysis the majority of cases can be classified as those with TDP43-positive or those with tau-positive pathological inclusions however, the less common third type exists with FET protein deposits in frontal and temporal lobes of brain [1].

FET proteins comprise a family of highly conserved predominantly nuclear proteins that include FUS, EWS and TAF15. These proteins are ubiquitously expressed and involved in regulation of gene expression, as well as in RNA processing, transport and metabolism [3, 4].

The role of FUS in FTLD pathology is well established, although mutations in FUS are usually associated with ALS and extremely rarely found in FTLD

patients with only three familial cases have been reported (two FTLD/ALS and one pure FTLD) [5, 6, 7]. So far there is no clear understanding why *FUS* gene mutations virtually inevitably cause ALS rather than FTLD and what defines anatomical localisation of FUS pathology that is restricted to frontal and temporal lobes in idiopathic FTLD.

In healthy neurons FUS is mainly localised in the nucleus but also shuttles to the cytoplasm; in both cell compartments it is involved in various steps of RNA processing and transport. Most common disease-associated mutations of the FUS gene affect the C-terminal nuclear localisation signal of the encoded protein causing its accumulation in the cytoplasm and at least partial depletion of its nuclear pool. It is believed that the amount of FUS accumulated in the cytoplasm should reach a critical level to trigger pathological changes that eventually cause dysfunction and death of the affected neuron, and this level might be different for different types of neurons.

Expression of ALS/FTLD-associated genes in transgenic rodents at a relatively high level produces phenotype resembling ALS, while animals with low levels of transgene expression stay healthy. This makes it difficult to produce rodent models of FTLD based on expression of the disease associated genes and proteins. Nevertheless, in some models, ageing animals display behavioural changes recapitulating certain symptoms observed in FTLD patients. For

example, aged rats with overexpression of wild type human FUS have developed deficit in spatial learning and memory as a result of neuronal loss in the cortex and dentate gyrus [8].

Previously we have produced a transgenic mouse line, S-FUS[1-359], expressing high level of a C-terminally truncated human FUS protein (FUS 1-359), which caused FUSopathy with sudden offset of severe motor phenotype and early lethality [9]. Analysis of another line, L-FUS[1-359], revealed a similar number of tandemly arranged copies of the same transgenic cassette located at Chr 11 but the level of human FUS expression in the nervous system of these mice was substantially lower than in the first line with different genomic location of the cassette at Chr 12. The low level of expression in the spinal cord was not sufficient to trigger the development of motor phenotype in L-FUS[1-359] mice, although RNA sequencing identified a set of genes that change their expression in the spinal cord of these mice [10].

Here we demonstrate that L-FUS[1-359] mice develop behavioural impairments. At the age of 5 months these animals display increased impulsivity, decreased anxiety and compromised social interaction behaviour.

Materials and Methods

Animals and ethics approval

A transgenic mouse line, L-FUS[1-359] with neurospecific expression of a C-terminally truncated human FUS has been produced as described previously [9] and transferred to CD1 genetic background [10] in a specific pathogen free facility of IPAC RAS. Experimental animals were single cage housed and maintained at constant temperature (22°C) on a 12 h light/dark cycle with *ad libitum* access to food and water.

All animal experiments were approved by The Bioethics committee of Institute of Physiologically Active Compounds, Russian Academy of Sciences (Approval No. 20 dated 23.06.2017).

Genotyping

Transgenic cassette has been detected in genomic DNA from ear biopsies by qPCR as described previously [9, 10].

Quantitative RT-PCR

Total RNA was extracted from mouse brain cortex, cerebellum and spinal cord using RNeasy Plus Mini Kit with genomic DNA eliminator columns (Qiagen) and 1 µg of total RNA was used for synthesis of the first-strand cDNA in reverse transcription reaction with random hexamer primers according to manufacturer's instructions (Evrogen, Russia). The first-strand cDNA was used

as a template in real-time qPCR amplification reaction using qPCR HS SYBR mix (Evrogen, Russia) and the CFX96 real-time PCR detection system (Biorad). For detection of human FUS transcript encoded by the transgenic cassette primers 5'-TCTTTGTGCAAGGCCTGGGT-3' and 5'-TAATCATGGGCTGTCCCGTT-3' were used. GAPDH was used as a reference gene (primers 5'-CACTGAGCATCTCCCTCACA-3' and 5'-GTGGGTGCAGCGAACTTTAT-3'). The cycle parameters were as follows: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Protein extraction and Western blot analysis

Total protein samples were prepared by homogenisation of dissected mouse tissues in 2xSDS-PAGE loading buffer followed by incubation at 100ºC for 5 min. Separation of proteins in SDS-PAGE and consequent semidry transfer on PVDF membrane (GE Healthcare) were performed as described elsewhere [10, 11]. Human FUS protein was detected using rabbit polyclonal antibody 14080 (a kind gift from Don Cleveland) specific to the N-terminal epitope of human FUS protein, secondary anti-rabbit HRP-conjugated antibodies (GE Healthcare) and WesternBright TM Sirius chemiluminescent detection system (Advansta). The membranes were re-probed with mouse monoclonal antibody against beta-actin (clone AC-15, Sigma-Aldrich) for loading control.

Immunohistochemistry

Paraffin-embedded parasagittal sections of paraformaldehyde-fixed mouse brains were immunostained with human FUS specific rabbit polyclonal antibody 14080 as described elsewhere [12, 13].

Behavioural tests

Transgenic male L-FUS[1-359] mice (N=12) and their wild type male littermates (N=12) were moved to the testing room one hour prior commencing testing. All behavior tests were performed during the light cycle, between 10:00 a.m. and 3:00 p.m. Tests were performed in the following order: water induced grooming, light-dark box, elevated O-maze, fear conditioning test, resident-intruder test with the 5 days intervals between them.

Dark-light box camera consisted of two compartments: light zone (30 × 20 × 25 cm, 100 lux) and dark zone (15 × 20 × 25 cm). Mice were placed into the dark compartment and their movements were recorded for 5 minutes using Logitech HD 720p camera. The video records were analysed for the latency time for the first exit to the light compartment, total number of exits and total duration of time spent in the light compartment [14].

Elevated O-maze consisted of a black circular path (runway width 5.5 cm, diameter 46 cm) that was placed 40 cm above the floor. Two opposing compartments were upbuild by walls of 10 cm in height. A testing mouse was placed in the middle of closed compartment and recorded for 5 minutes using Logitech HD 720p camera. The video records were analysed for latency time of

the first exit to the anxietygenic open compartments, the number of exits from the closed compartments and total duration of time spent in open (light) compartments. Illumination intensity was kept at 50 Lux [14, 15].

Water induced grooming. Spontaneous grooming was induced by misting the mouse with the room temperature water (25^0C) using standard spray bottle in 'misting' mode. The mouse was gently fixed by the tail and sprayed three times toward the mice head from the 10 cm distance, placed into the observation glass cylinder and its grooming behavior was recorded for 10 minutes. The total time spent grooming and number of grooming episodes (bouts) were registered [16].

Fear conditioning test. On the first training day a mouse was placed on the metal grid floor in the conditioning apparatus chamber and after 118 sec delay a 2-sec electric foot shock (1.5 mA) was applied. The animal was returned to the home cage immediately thereafter. 24 hours after the training, this mouse was re-exposed to the same apparatus chamber and the percentage of time spent freezing was measured for 180 sec, followed by 7 minutes of re-exploring the box under shock-free conditions for memory extinction. 24 hours later, i.e. on the third experimental day, the mouse was exposed to the chamber again and the percentage of time spent freezing was re-measured for 180 sec [17].

Resident-intruder test. Each resident mouse was housed individually, bedding material in resident's cages hasn't been changed for a week before testing. Thereafter, a male mouse of the same strain and a similar weight and age to that of the resident, and housed in the group before the test was introduced as an

intruder. The number of social contacts and acts of aggression were recorded for 8 min.

Statistical analysis was performed using GraphPad 7.0 software (San Diego, USA). For comparing relative mRNA levels Kruskal-Wallis ANOVA and Dunn's multiple comparisons test were used. Nonparametric Mann-Whitney U-test was used when data for two groups were compared. The level of statistical significance was set at $p< 0.05$. Results are presented as mean ± SEM.

Results

Expression of human FUS in the nervous system of L-FUS[1-359] mice.

The levels of human FUS mRNA expressed under control of Thy-1 regulatory elements were compared by real-time quantitative RT-PCR. Total RNA samples were prepared from the cortex, cerebellum and spinal cord of five L-FUS[1-359] aging homozygous males (average age 299 days) and individually reverse transcribed followed by the real-time qRT-PCR with primers specific for human FUS mRNA. The level of expression was lowest in the cerebellum, and in the cortex and spinal cord it was 6.5 and 4.7 times higher, respectively (Fig 1a).

A semi-quantitative Western blot analysis demonstrated that the truncated human FUS protein is more abundant in total protein samples extracted from

the cortex of 2-month old heterozygous L-FUS[1-359] than 2-month old heterozygous S-FUS[1-359] mice (Fig 1b).

Immunohistochemistry with antibody specific to human FUS protein revealed the highest level of human FUS protein in the cytoplasm of large neurons predominantly localised in the layer V of the cerebral cortex of L-FUS[1-359] mice. The protein was evenly distributed in the cytoplasm of these neurons and did not form inclusions (Fig 1c). Consistently with the result of Western blot analysis, substantially weaker signals were detected in cortical neurons of S-FUS[1-359] mice, although these weakly positive neurons were more spread across the cortex layers of these mice (Fig 1c).

Behavioral analysis of L-FUS[1-359] mice

Cognitive function was analysed in 5 months old L-FUS[1-359] male mice homozygous for the transgenic cassette compared to their wild type male littermates.

The dark-light box was employed to study the anxiety. The normal behaviour of wild type mice is characterised by preference of dark compartment of the apparatus towards the open illuminated one. Therefore, the number of exits from the dark compartment to the open illuminated zone and total time that the animals have spent in this zone could reflect the level of their anxiety. L-FUS[1-

359] mice entered the open illuminated compartment of the camera more often than control wild type mice (Fig 2a). The number of light compartment entries for L-FUS[1-359] mice was statistically significantly higher ($U=32$, $p<0.05$) than for the control group. The latency time to the first exit from the dark compartment to the open compartment was also shorter in the L-FUS[1-359] group than in the control group ($U=21$, $p<0.01$). Although the total time spent in the illuminated open compartment by in L-FUS[1-359] mice had a tendency to increase, this difference was not statistically significant ($p=0.08$). These data clearly demonstrate that L-FUS[1-359] mice exhibit a lower level of anxiety and higher level of impulsivity than their wild type littermates.

Decreased anxiety of L-FUS[1-359] mice was even more obvious in the elevated O-maze test (Fig 2b). L-FUS[1-359] mice spent significantly longer time in the open arm compared to the wild type control animals ($U=7$, $p<0.0001$). The number of entries to the open sector of the maze was also much higher ($U=5$, $p<0.0001$). Moreover, the latency time that L-FUS[1-359] mice took for the first exit to the open arm of the maze was shorter than for wild type controls ($U=22$, $p<0.01$).

To study the repetitive behavior that is known to be affected in FTD patients and that may reflect the pathology in the model animals with amygdala and striatal dysfunction, we have analysed the spontaneous grooming in L-FUS[1-

359] mice. Misting a mouse with the room temperature water induces spontaneous self-grooming that consists of specific and highly stereotyped patterns of four sequential movements (bouts). Stress and anxiety in animals is often accompanied by increased self-grooming and disorganized patterning [16].

Neither the number of grooming episodes (full bouts) ($p=0.6$), no total grooming time ($p=0.4$) were different in L-FUS[1-359] mice when compared to wild type controls (Fig 3).

A long-term memory formation has been tested in L-FUS[1-359] mice using the fear conditioning test (Fig 4a). A significant decrease in time spent freezing was found in L-FUS[1-359] mice versus the control group during a recall of fear conditioning 24 h post-training ($U=33.5$, $p<0.05$). 48 hours after the initial training, during the recall of memory extinction, the L-FUS[1-359] mice did not exhibit the decrease of freezing behaviour from the level detected during the recall of fear conditioning 24 h post-training ($p=0.17$), which was obvious for wild type animals ($U=36$, $p< 0.05$).

Social behaviour of resident males were analysed by the number of contacts and number of attacks towards intruder (Fig. 4b). Transgenic L-FUS[1-359] mice shows less social interest ($U=16$, $p<0.001$) compared to wild type mice. There is no statistical difference in aggression behaviour with neither transgenic L-

FUS[1-359] nor wild type mice have shown high level of aggression. However, L-FUS[1-359] mice spent significantly less time ($U=25$, $p<0.01$) exploring intruder mice (Fig. 4c), which can indicate social stress in animals that recapitulates a clinical symptom of FTD.

Discussion

Malfunction of DNA/RNA-binding protein FUS causes certain forms of amyotrophic lateral sclerosis (FUS-ALS) and frontotemporal lobar degeneration (FUS-FTLD) (reviewed in Ref. 18). Mislocalisation of normally nuclear FUS to the cytoplasm is believed to be a key trigger or first-hit in the pathological cascade that leads to dysfunction and eventual death of neurons [19, 20]. The severity of the neurodegenerative changes directly correlates with the degree of FUS mislocalisation in patients' neurons [21, 22, 23]. Therefore, expression of C-terminally truncated FUS, which translocation to the nucleus is substantially compromised due to complete lack of its nuclear localisation signal, appeared to be a suitable approach to recapitulate pathological traits of human diseases in genetically altered mice. Pathological changes are developed in the nervous systems even when truncated FUS is expressed from the edited endogenous *Fus* locus in knockin mice [24, 25, 26]. Similarly, in the spinal cord of S-FUS[1-359] transgenic mice expression of C-terminally truncated human FUS under control of Thy-1 regulatory sequences at the level lower than the level of endogenous mouse FUS causes severe early onset motor neuron pathology [9]. However, mice

of L-FUS[1-359] transgenic line with further reduced expression level of the same truncated variant of FUS in the spinal cord do not develop motor neuron pathology and have normal lifespan [9, 10]. This is consistent with results of studies of other FUS transgenic mice that demonstrated a direct correlation between the level of transgene expression and the severity of pathological changes [27]. It is feasible that the low level of mislocalised pathogenic form of FUS protein can be successfully compensated by certain intrinsic defence mechanisms of motor neurons.

In contrast to much lower level of the truncated FUS expression in the spinal cord of L-FUS[1-359] mice as compared to S-FUS[1-359] mice [10], its expression in the cerebral cortex of L-FUS[1-359] transgenic mice is higher than in the cerebral cortex of S-FUS[1-359] mice. This difference is most probably due to effects of endogenous mouse regulatory elements in the vicinity of transgene integration sites in these two mouse lines. However, this level of expression and mislocalisation of truncated FUS lacking nuclear localisation signal are not sufficient to trigger pathological aggregation in the cytoplasm of cortical neurons. Nevertheless, it is feasible that even in the absence of FUS inclusions, persistent expression of a pathogenic FUS variant in the cerebral cortex neurons might cause their dysfunction and the development of clinical signs relevant to some symptoms of FTLD. Importantly, the absence of motor deficiency in L-FUS[1-359] mice allowed us to test this hypothesis using

behavioural paradigms that require uncompromised movement ability of experimental animals.

Results of these experiments have indeed revealed changes in the behaviour and cognition of young adult homozygous transgenic L-FUS[1-359] male mice.

Decreased anxiety of L-FUS[1-359] when compared to their wild type control littermates has been demonstrated in two tests that are commonly used for the assessment of the anxiety and risk-taking behaviour, namely the dark-light box and elevated O-maze. In both tests, transgenic mice entered earlier and showed increased number of entries into the brighter illuminated compartments. In the elevated O-maze transgenic mice also spent more time exploring the open arm. Short latency time to exit from the dark compartment is a parameter that reflecting impulsivity and the increased exploratory interest in the light compartments is an indicator of decreased anxiety, which could be interpreted as excessive risk-taking behaviour and/or disinhibition or even emotional dysfunction, which are often observed in FTLD patients [28].

The development of a memory deficit is not uncommon in FTLD patients and therefore we studied formation of memory in L-FUS[1-359] mice using the extinction fear conditioning test. In mice, freezing in response to an adverse stimulus, like an electric shock used in this test, reflects their formation of

memory to the context. 24 hours after receiving an electric shock, transgenic L-FUS[1-359] mice showed significantly shorter time remaining motionless than wild type control animals. As this time was already similar to time that wild type spent motionless during assessment of the recall of memory extinction, it was not expected to see further reduction of the freezing time for L-FUS[1-359] mice during this assessment and indeed this has not been observed. The decreased level of freezing behaviour in response to a fear-conditioned stimulus can be interpreted as an impairment of long-term memory formation in transgenic L-FUS[1-359] mice [17], however it could not be excluded that the decreased anxiety demonstrated in other tests affects animal behaviour in this test as well.

Changes in social behaviour is one of the main symptoms of FTLD and L-FUS[1-359] mice recapitulated these changes in the resident-intruder test. Transgenic mice had a decreased number of social interactions, were much less interested in any type of physical contact with intruder mice and showed no signs of aggressive behaviour.

Repetitive behavior is a common symptom in FTLD patients and may be represented in mice by abnormal repetitive grooming [29]. However, the induced self-grooming in L-FUS[1-359] mice was not significantly different from the wild type control animals.

Certain mouse lines that display altered social engagement, reduced anxiety, hyperactivity and memory impairments are regarded as models of frontotemporal dementia. These include GRN knockout mice and transgenic mice expressing mutant tau, TDP-43 lacking the nuclear localisation signal and FUS with a C-terminal shorter than in our mice [30], [31], [32], [33]. Overall, behavioural abnormalities observed in L-FUS[1-359] mice are consistent with those reported for these mouse models.

In conclusion, results of our study suggest that expression of a pathogenic form of human FUS in mouse cortical neurons at a low level that does not trigger formation of FUS inclusions and in the absence of motor deficiency causes decreased anxiety, increased impulsivity and changes in social interaction, which are indicators of FTLD-like pathology.

Conflict of interest

The authors declared no conflict of interest.

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Figure Legends

Figure 1. Expression of C-terminally truncated form of human FUS in the nervous system of L-FUS[1-359] mice.

(a) Relative levels of expression of transgenically expressed human FUS mRNA in the brain regions of aging L-FUS[1-359] mice assessed by real-time qRT-PCR with primers specific for human FUS mRNA. Total RNA samples from five ageing (average age 299 days) homozygous L-FUS[1-359] male mice were analysed in triplicates (Kruskal-Wallis ANOVA with Dunn's multiple comparisons test, $^{**}p<0.01$, $^{*}p<0.05$).

(b) Western blot analysis of total protein samples extracted from the cortex of 2-month old hemizygous L-FUS[1-359], S-FUS[1-359] and wild type (WT) mice with antibody specific to human FUS. For loading control the same membrane was re-probed with anti-beta-actin antibody.

(c) Immunostaining of histological sections through the frontal cortex of L-FUS[1-359], S-FUS[1-359] and wild type (WT) mice with antibody specific to human FUS. Scale bar, 100 μm.

Figure 2. Behaviour tests for anxiety.

(a) In the light dark box test, transgenic L-FUS[1-359] mice display only a tendency to spent longer time in the light compartment ($U=42$, $p=0.08$) as compared to control WT mice. However, there was a significant reduction in the latency to first exit to the light compartment in L-FUS[1-359] mouse group ($U=21$, $p<0.01$). L-FUS[1-359] animals also have a significantly increased number of exits to the light compartment compared to control animals ($U=32$, $p<0.05$).

(b) In the elevated O-maze test L-FUS[1-359] mice displayed dramatically increased time spent in the open arm ($U=7$, $p<0.0001$) and the number of entries to the open arm ($U=5$, $p<0.0001$), as well as decreased latency to first exit to the open arm ($U=22$, $p<0.01$) compared to control WT animals. N=12 for both groups.

Figure 3. Water induced grooming test.

Analysis of induced self-grooming in mice shows no significant changes in grooming behaviour between L-FUS[1-359] and wild type animals in the

grooming time (U=57, p=0.4) and in the number of grooming bouts (U=63, p=0.6).

N=12 for both groups.

Figure 4. Analysis of memory and social function.

(a) In the contextual post-training fear-conditioning test, L-FUS[1-359] animals spent significantly lower time freezing than control animals (U=33.5, $p < 0.05$). No significant differences between groups in the duration of freezing behaviour were observed in the test for extinguishing of contextual fear conditioning (p=0.09). Wild type mice demonstrate significant decrease in the time spent freezing 48 hours after the initial training compared to 24 h post-training because, suggesting that mice have retrained in the post-training session (U=36, $p< 0.05$).

(b) Analysis of social behaviour in resident-intruder test show that L-FUS[1-359] mice demonstrate significantly less social interest (U=16, $p<0.001$) in exploring the intruder mice. Both L-FUS[1-359] and wild type mice has not shown high level of aggression and violence.

(c) Analysis of the duration of social contacts in resident-intruder test also demonstrate that L-FUS[1-359] mice spent significantly less time (U=25, $p<0.01$) exploring intruder mice.

N=12 for both groups.

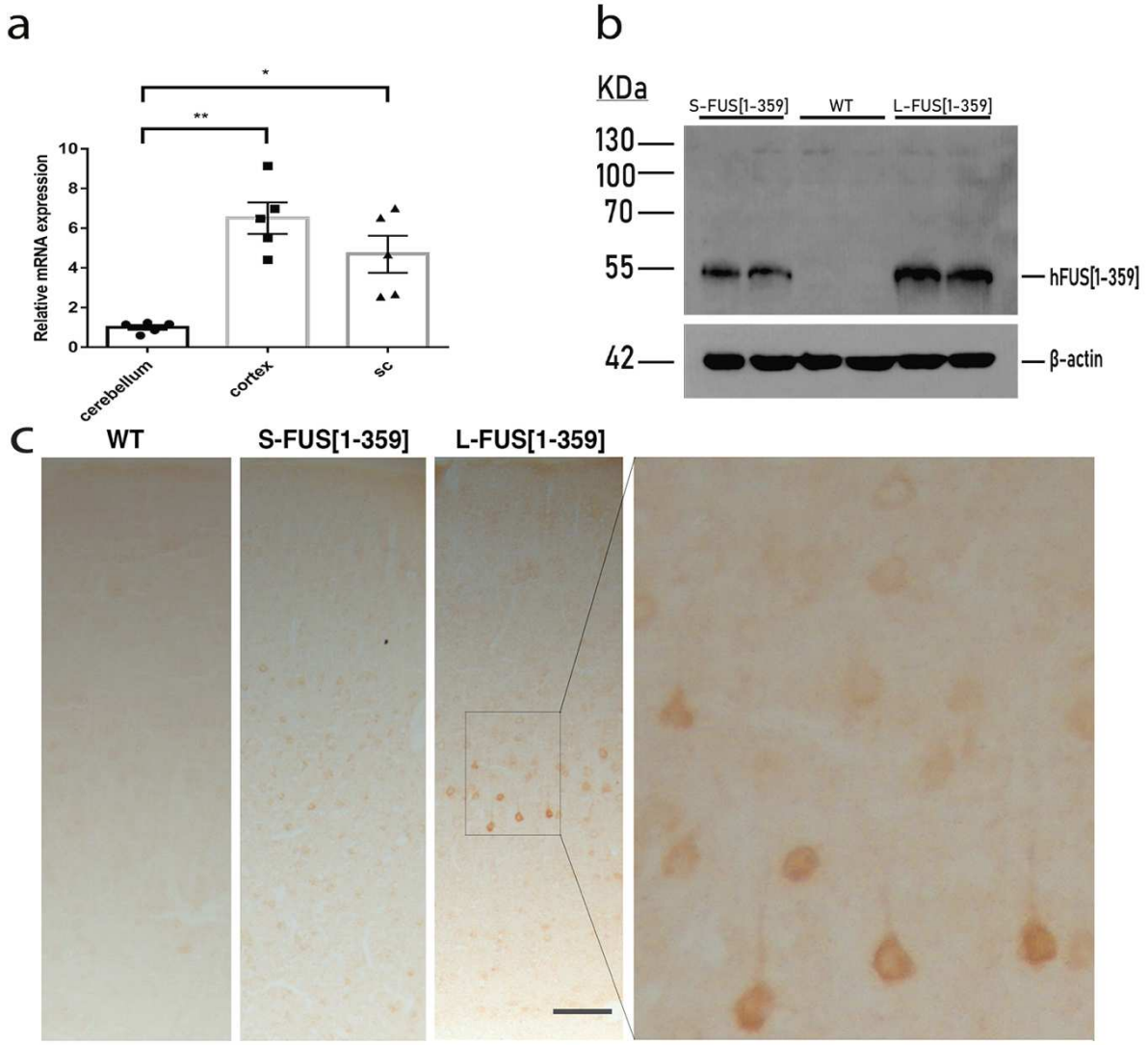

Figure 1.

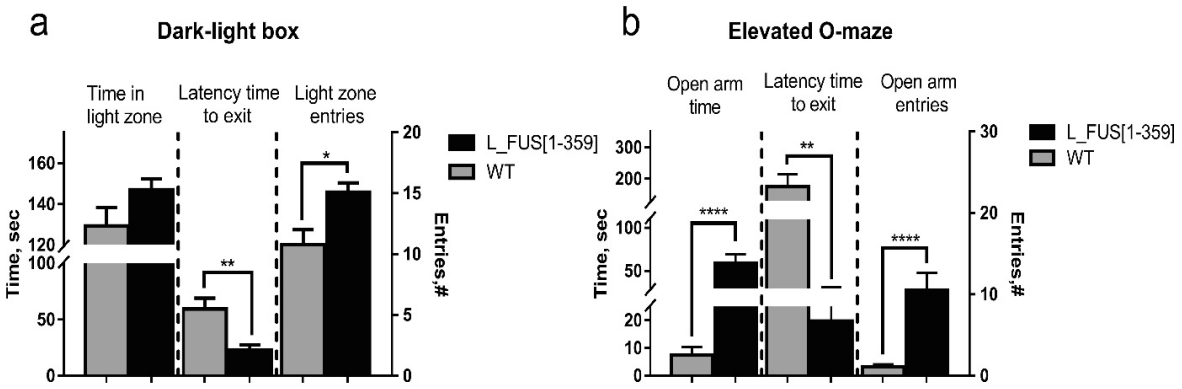

Figure 2.

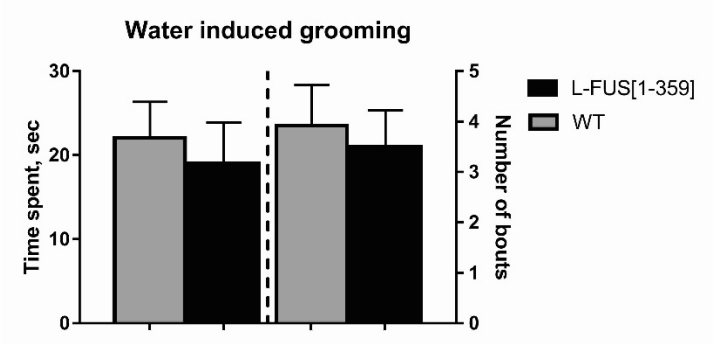

Figure 3.

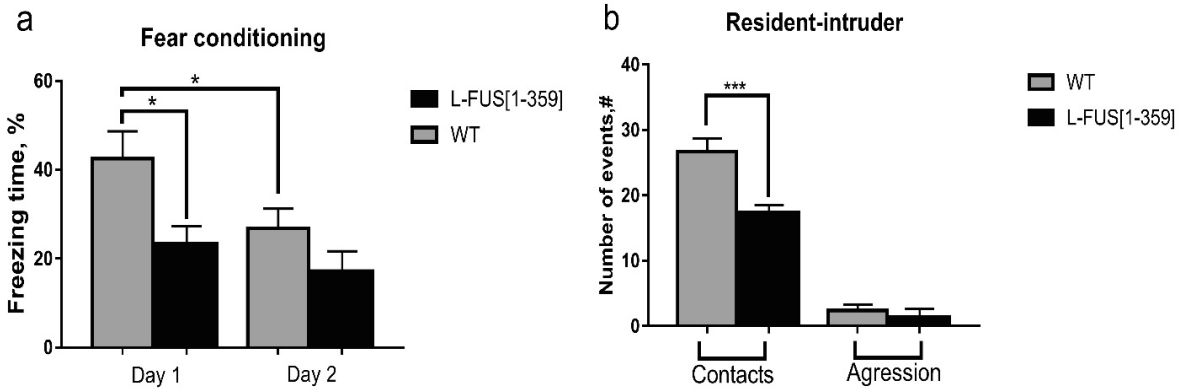

Figure 4.