

Overexpression of HSP70 in mice with mutant FUS protein is accompanied by a mitigated neurodegeneration in limbic system

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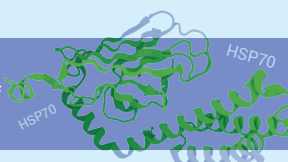
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GRAPHICAL ABSTRACT



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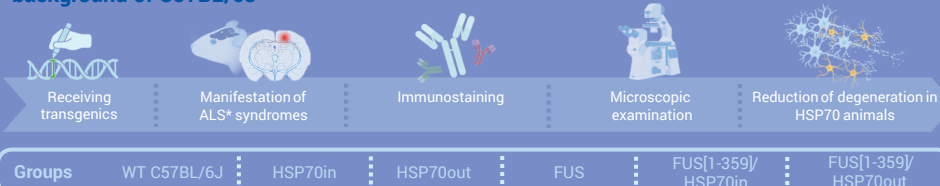
Summary

In all cases, a reduction in neurodegenerative changes was demonstrated with simultaneous overexpression of the cytosolic form of HSPA1A and mutant FUS protein with cytoplasmic mislocalization.



Materials and methods

Study of HSP70 and FUS animal lines and their double transgenes on the genetic background of C57BL/6J



Results

Markers of neurodegeneration

Brain areas	HSP70in / FUS		FUS[1-359]		HSP70out / FUS	
Hippocampus	NeuN ▲	GFAP ▼	NeuN ▼	GFAP ▲	NeuN ▼	GFAP ▲
	SYP ▲	S100 ▼	SYP ●	S100 ▲	SYP ●	S100 ▲
Amygdala	NeuN ▲		NeuN ▼		NeuN ▼	
Striatum	NeuN ▲	GFAP ▼	NeuN ▼	GFAP ▲	NeuN ▼	GFAP ▲
	SYP ▲		SYP ▼		SYP ▼	
Cell types	Neurons	Astroglia	Neurons	Astroglia	Neurons	Astroglia

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*Amyotrophic lateral sclerosis

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Abstract

Aim. To study morphological and developmental changes in the structure of mice's limbic system, which overexpress 70 kDa heat shock proteins (HSP70) and co-express mutant fused-in-sarcoma (FUS) protein with amyotrophic lateral sclerosis (ALS).

Materials and methods. The study was based on mice ($n = 36$; six for each group) of six lines either without FUS mislocalization: C57BL/6 (wild-type); extracellular (*HSP70out*) or intracellular (*HSP70in*) overexpression of HSP70 family 1A protein; or with FUS mislocalization: transgenic mice with ALS-FUS (*FUS[1-359]*); and double transgenic animals (*FUS[1-359]/HSP70out* and *FUS[1-359]/HSP70in*). All FUS expressing mice were symptomatic with myasthenia up to limb paralysis in some animals. When the mice were 20 weeks old, they were killed by staining histological slides of their brain using hematoxylin and eosin, toluidine blue by Nissl, immunofluorescent antibodies for the neuronal nuclear marker (NeuN) of the caudoputamen, septal nuclei, and hippocampus, as well as glial fibrillar

acid protein (GFAP), S100 β protein, and synaptophysin for the hippocampus. The number of neurons and the number of cells with a positive reaction to antibodies were counted. The statistical processing included ANOVA and were compared with the results from the Tukey test for.

Results. Statistically significant differences were observed for the FUS-expressing groups compared to FUS-negative groups: (1) a reduction in the number of neurons and NeuN⁺-cells in the caudoputamen and amygdala, especially for *FUS[1-359]/HSP70out* group; (2) an increase in the number of hyperchromic neurons in the subiculum, cornu Ammonis (CA1), and dentate gyrus, with a significantly greater increase for *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to *FUS[1-359]/HSP70in* group; (3) an increase in the number of GFAP⁺- and S100 β ⁺-cells in the hippocampus, with the most pronounced change for the *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to *FUS[1-359]/HSP70in* group.

Conclusion. An overexpression of HSP70 family 1A protein and co-expression of mutant FUS protein in the cytoplasm is accompanied by mitigated neurodegeneration in the structure of the limbic system compared with the expression of mutant FUS protein alone.

Keywords: molecular chaperones; heat shock proteins; amyotrophic lateral sclerosis; amygdala; caudoputamen; hippocampus; septal nuclei.

MeSH terms:

AMYOTROPHIC LATERAL SCLEROSIS-PATHOLOGY
 AMYOTROPHIC LATERAL SCLEROSIS-PHYSIOPATHOLOGY
 HSP70 HEAT-SHOCK PROTEINS-ANALYSIS
 LIMBIC SYSTEM-PATHOLOGY
 MICE, INBRED C57BL/6J

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Ethics statements. The study complies with the standards of the EU Directive for the Protection of the Vertebrate Animals used for Experimental and other Scientific Purposes. All manipulations with animals were approved by the Local Bioethics Committee of the Sechenov First Moscow State Medical University (Sechenov University), No. 04-23 from 02.03.2023.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.

Conflict of interests. Sergey L. Kuznetsov is a member of the editorial board and did not participate in the editorial review or decision-making on this article.

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Гиперэкспрессия белков теплового шока HSP70 у мышей с мутантным белком FUS сопровождается меньшими нейродегенеративными изменениями в структурах лимбической системы

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Аннотация

Цель. Изучить морфологические изменения в структурах лимбической системы у мышей с гиперэкспрессией белков теплового шока молекулярной массой 70 кДа (70 kDa heat shock proteins, HSP70) и экспрессией мутантного белка fused-in-sarcoma («слитый при саркоме»; FUS) с развитием бокового амиотрофического склероза.

Материалы и методы. Объектом исследования служили мыши ($n = 36$) линии C57Bl/6 (wild-type) и трансгенных линий, разделенные на 6 групп по 6 мышей в каждой. Три группы были FUS-отрицательные: контрольная, с вне и внутриклеточной гиперэкспрессией белка 1A семейства HSP70: *HSP70out* и *HSP70in*; три группы – FUS-положительные: *FUS[1-359]*, *FUS[1-359]/HSP70out* и *FUS[1-359]/HSP70in*. У всех FUS-положительных мышей развивалась мышечная слабость вплоть до паралича. На 20-й неделе жизни мышей выводили из эксперимента, гистологические препараты головного мозга окрашивали гематоксилином и эозином, толуидиновым синим по Ниссля или иммунофлуоресцентными антителами к нейрональному ядерному маркеру (NeuN) для препаратов каудопутамена, септальных ядер и гиппокампа, а также к глиальному фибриллярному кислому белку (GFAP), белку S100 β и синаптофизину для препаратов гиппокампа; подсчитывали количество клеток. Сравнение средних значений между группами проводили при помощи однофакторного дисперсионного анализа и теста Тьюки.

Результаты. В группах с экспрессией FUS наблюдались статистически значимые различия по сравнению с FUS-отрицательными группами: (1) снижение количества нейронов и NeuN⁺-клеток в каудопутамене и миндалевидном теле, наиболее выраженное изменение отмечено в группе *FUS[1-359]/HSP70out*; (2) увеличение количества гиперхромных нейронов в основании гиппокампа, зоне Аммонова рога (CA1) и зубчатой извилине, прирост был значимо больше в группах *FUS[1-359]* и *FUS[1-359]/HSP70out* по сравнению с *FUS[1-359]/HSP70in*; (3) рост количества GFAP⁺- и S100 β ⁺-клеток в гиппокампе, увеличение было значимо больше в группах *FUS[1-359]* и *FUS[1-359]/HSP70out* по сравнению с *FUS[1-359]/HSP70in*.

Закключение. Одновременная гиперэкспрессия белка 1A семейства HSP70 и экспрессия мутантного белка FUS в цитоплазме клеток сопровождается меньшей выраженностью нейродегенеративных изменений в структурах лимбической системы по сравнению с экспрессией только мутантного белка FUS.

Ключевые слова: молекулярные шапероны; белки теплового шока; боковой амиотрофический склероз; миндалевидное тело; каудопутамен; гиппокамп; септальные ядра

Рубрики MeSH:

СКЛЕРОЗ БОКОВОЙ АМИОТРОФИЧЕСКИЙ – ПАТОЛОГИЯ

СКЛЕРОЗ БОКОВОЙ АМИОТРОФИЧЕСКИЙ – ПАТОФИЗИОЛОГИЯ

БЕЛКИ HSP70 ТЕПЛООВОГО ШОКА – АНАЛИЗ

ЛИМБИЧЕСКАЯ СИСТЕМА – ПАТОЛОГИЯ

МЫШИ ИНБРЕДНОЙ ЛИНИИ C57Bl/6J

Для цитирования: Пьявченко Г.А., Покидова К.С., Кузьмин Е.А., Венедиктов А.А., Кузнецов С.Л. Гиперэкспрессия белков теплового шока HSP70 у мышей с мутантным белком FUS сопровождается меньшими нейродегенеративными изменениями в структурах лимбической системы. Сеченовский вестник. 2025; 16(1): 4–19. <https://doi.org/10.47093/2218-7332.2025.16.1.4-19>

КОНТАКТНАЯ ИНФОРМАЦИЯ:

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Соответствие принципам этики. Исследование проведено с соблюдением положений Европейской конвенции о защите позвоночных животных, которые используются для экспериментальных и других научных целей. Все манипуляции с животными проведены в соответствии с разрешением Локального этического комитета ФГАОУ ВО «Первый МГМУ им. И.М. Сеченова» Минздрава России № 04-23 от 02.03.2023.

Доступ к данным исследования. Данные, подтверждающие выводы этого исследования, могут быть получены у автора по переписке по обоснованному запросу.

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Abbreviations:

ALS – amyotrophic lateral sclerosis

BLAa/BLAp – basolateral amygdalar nucleus, anterior and posterior parts

BLAv – basolateral amygdalar nucleus, ventral part

CA – cornu Ammonis

CeL – central amygdalar nucleus, lateral part

CP – caudoputamen

DG – dentate gyrus

FUS – fused-in-sarcoma protein

GFAP – glial fibrillary acidic protein

HSP70 – 70 kDa heat shock proteins

HSPA1A – heat shock proteins member 1A

IF – immunofluorescent study

LA – lateral amygdalar nucleus

LSc – lateral septal nucleus, caudal part

NeuN – neuronal nuclear marker

SUBd – subiculum, dorsal part

SYP – synaptophysin

WT – wild-type animals

HIGHLIGHTS

An excess of molecular chaperones HSP70 in the cytosol may have a beneficial effect on the course of the amyotrophic lateral sclerosis variant associated with the accumulation of FUS protein in the cytoplasm rather than in the nucleus.

Simultaneous expression of both intracellular human HSP70 (HSPA1A exactly) and mutant FUS in mice leads to greater neuronal survival in certain areas of the striatum, amygdala, and hippocampus compared to FUS expression without HSPA1A.

Activation of neuroglia in the structures of murine limbic system in simultaneous mutant FUS and HSPA1A expression is less pronounced than in mice with FUS expression only.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder primarily affecting the motor regions of the nervous system [1]. The disorder is characterized by high rates of disability and mortality, with no effective treatment methods available [2]. The development of ALS treatment principles is challenging due to the fact that ALS can arise from mutations in various genes [3] with different clinical phenotypes [4]. Given these differences, research that examines molecular mechanisms that counteract key pathological changes in the most common genetic variants of ALS is in high demand.

One of the genes that can mutate leading to the development of ALS encodes human RNA-binding protein “fused-in-sarcoma (FUS)” and is normally localized in the nucleus [5]. Aberrant FUS protein leaves the cell nucleus, and its pathological accumulation in the cytoplasm of neurons underlies the FUS variant of ALS, FUS-ALS, and associated cell death [6]. A promising direction in studying this problem is the search for experimental conditions to reduce the severity of neurodegenerative changes related to the cytoplasmic accumulation of FUS.

Thus, pathological protein accumulation in the cytoplasm can be controlled using molecular

² Карточка проекта фундаментальных и поисковых научных исследований, поддержанного Российским научным фондом. Изучение нейроиммунологических эффектов экстра- и внутриклеточного HSP70 при нейродегенеративном повреждении мозга у мышей. <https://rscf.ru/project/23-25-00448/> (дата обращения: 01.12.2024).

chaperones, particularly 70 kDa heat shock proteins (HSP70) [7]. It has been also shown that HSP70 can affect the accumulation of mutant FUS protein in FUS-ALS [7, 8].

It should be emphasized that HSP70 member 1A (HSPA1A) is a constant, homeostatic component of the cytosol [9], which does not prevent the development of FUS-ALS. It is likely that the amount or activity of HSPA1A in patients with FUS-ALS is insufficient to prevent a pathological FUS accumulation. One may therefore assume that overexpression of HSPA1A favorably affects neuronal survival in ALS with cytoplasmic FUS accumulation [7].

In ALS, damage occurs in the motor areas of the cerebral cortex, the limbic system, as well as motor neurons in the spinal cord and motor plaques [1, 10]. However, changes in the structures of the limbic system have been the least studied, although the basal ganglia are involved in the pathogenesis of ALS [10]. For example, as of 01.12.2024, a search in the PubMed database using tags “FUS + ALS + brain”, “FUS + ALS + spinal cord”, “FUS + ALS + muscle” reveals more than 200 publications, while “FUS + ALS + hippocampus” reveals 21 publications, “FUS + ALS + striatum” yields 2 publications, and “FUS + ALS + amygdala” did not appear in any publications.

Aim of the study: to investigate morphological changes in the structures of the limbic system in mice overexpressing HSP70 and expressing mutant FUS protein with ALS development.

MATERIALS AND METHODS

Animal experiments

The study was conducted using 36 wild-type (WT) and transgenic mice weighing 32.0 ± 2.5 g. The mice were divided into six groups ($n = 6$ per group). Only healthy animals (except ALS) were included. The mice were obtained from the vivarium at Sechenov University.

Group 1 included C57Bl/6 mice (WT), selected using the random number method. The randomization for Groups 2 to 6 was based on the genotype of the animals. Group 2 included transgenic animals with the human *FUS*[1-359] gene, expressing mutant FUS protein with the development of FUS-ALS. The FUS expression model described in the work of T.A. Shelkovanikova et al. [11] was implemented. Group 3 (*HSP70out*) included transgenic mice with extracellular expression of HSPA1A, and the fourth group (*HSP70in*) included mice with intracellular expression of HSPA1A. Groups 2 to 4 were formed with respect to polymerase chain reaction data, including only animals with hemizygous genes of *FUS*[1-359] and HSPA1A. Homozygous animals with the phenotype corresponding to Groups 2 to 4 served to breed double transgenic mice: Group

5 (*FUS*[1-359]/*HSP70out*) and Group 6 (*FUS*[1-359]/*HSP70in*).

The mice were kept in the vivarium at a temperature of 20–22 °C and with a humidity of 55–60% with free access to clean water and granulated food *ad libitum*. In groups expressing FUS, starting from 10–12 weeks of age, muscle weakness signs gradually increased, progressing up to paralysis in some animals. These symptoms primarily affected one of the hind limbs sometimes affecting both ones or manifesting in the forelimbs. At the same time, sexual activity decreased, but food intake did not. These changes were not observed in FUS-negative groups.

At Week 20, the animals were killed by decapitation (Fig. 1). Anesthesia was induced with 5 mg/kg of xylazine hydrochloride (Interchemie, Netherlands) and 40 mg/kg of tiletamine/zolazepam (Virbac, France).

Preparation of histological specimens

The brains were placed in 10% neutral buffered formalin (ErgoProduction LLC, Russian Federation). Twenty-four hours later, the organs were put into isopropyl alcohol of increasing concentrations (Biovitrum LLC, Russian Federation) and then finally placed into paraffin. Coronal sections 3 μ m thick were made with HM325 rotary microtome (Thermo Fisher Scientific, USA), transferred onto silane-coated adhesive slides (Minimed LLC, Russian Federation), and dried at 37 °C.

Staining of specimens

For the purposes of a histological study, the specimens were stained with hematoxylin and eosin or toluidine blue by Nissl method. For immunofluorescent (IF) staining, after incubation in a dewaxing retrieval buffer solution (pH 9.0, 20 \times , lot XF05RT4N9592, Elabscience, China), the sections were washed with 10% PBS (Eco-Service LLC, Russian Federation), bovine serum albumin (lot RM-T1725/1000, Biosera, France) was added for 30 min, and then primary antibodies were added with accordance to the manufacturers' recommendations.

In order to identify neurons, monoclonal antibodies to neuronal nuclear marker (NeuN) (1:1000, clone SR45-07, item number ET1602-12, lot H661803001, Huabio, PRC) were used. And in order to detect activated astrocytes, we employed monoclonal antibodies to glial fibrillary acidic protein (GFAP) (1:500, clone SA03-04, item number ET1601-23, lot HO0913, Huabio, PRC) and S100 β protein (1:1000, clone SC57-02, item number ET-1610-3, lot H661380007, Huabio, PRC) were used. To assess the level of synaptic contacts, polyclonal antibodies to synaptophysin (SYP) (1:200, item number O407-2, lot HG0614, Huabio, PRC) were used. After staining with primary antibodies, the samples were washed three times, and polyclonal

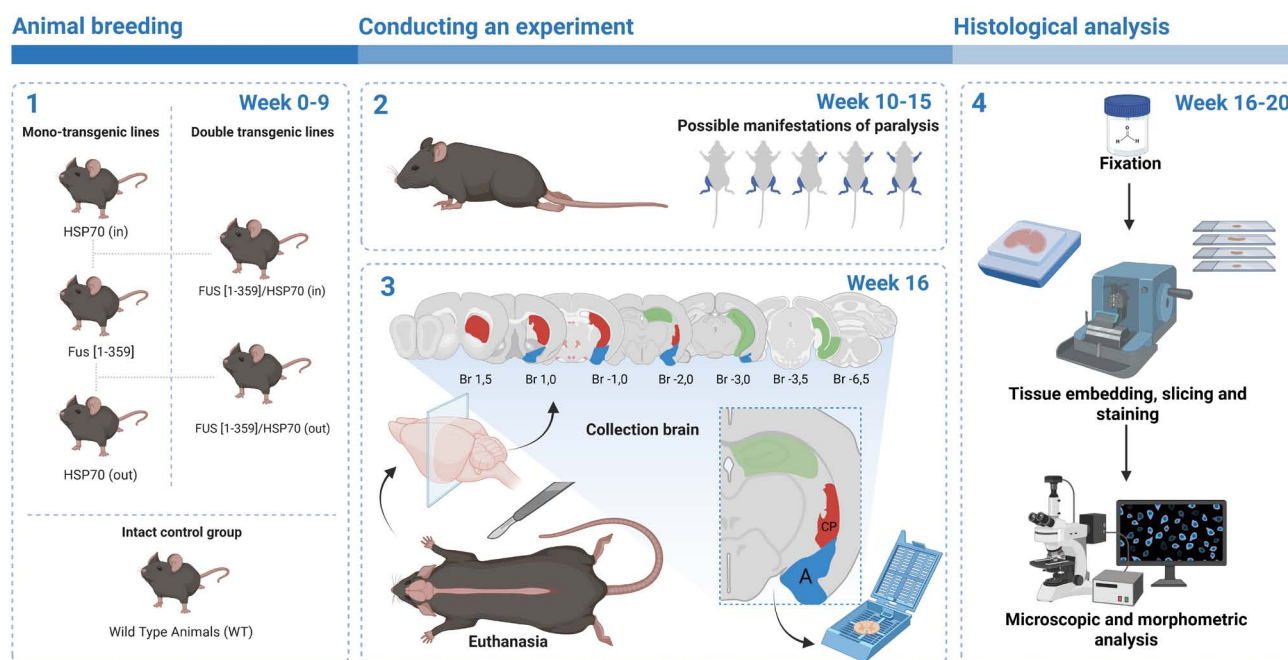


FIG. 1. Design of the experimental study.

Note. WT – wild type.

secondary antibodies (Anti-Rabbit-TRITC, 1:100, item number E-AB-1053, lot 22038, Elabscience, PRC) were applied, followed by DAPI staining (item number E-IR-R103, Elabscience, PRC). We washed the specimens with PBS and mounted them with coverslip.

Morphometric study

On the relevant sections, we identified the structure of rodent limbic system (6 fields of view per animal). According to the stereotaxic atlas [12], the following were identified: caudoputamen (CP); caudal part of the lateral septal nucleus (LSc); hippocampal formations, including dorsal part of the hippocampal subiculum (SUBd), regions of the hippocampus proper, or cornu Ammonis (CA1 and CA3), and dentate gyrus (DG); amygdala nuclei, namely lateral nucleus (LA), ventral part of the basolateral nucleus (BLAv), anterior and posterior parts of the basolateral nucleus (BLAa/BLAp), and lateral part of the central nucleus (CeL). Photographs were taken using the Axio Imager.A1 complex with $\times 40$ and $\times 100$ objective magnification, including the Axiocam 305 color camera and Zen 3.10 software (Zeiss, Germany).

The number of neurons per field of view was counted for CP, LSc, and amygdala structures. For hippocampal formations and LSc, the number of hyperchromic neurons was counted, and in CP, the number of neurons with eosinophilic cytoplasm, too. For antibody staining, microphotographs of hippocampal structures, CP, and LSc were taken at fluorescent wavelengths from 540 to 620 nm. The total number of NeuN-positive cells per field of view in CP and LSc zones and the ratio

of NeuN⁺ cells to DAPI-stained cells in hippocampal structures were counted.

The following changes were considered as neurodegenerative ones: a reduction in the total number of neurons per field of view, the appearance of hyperchromic neurons or neurons with eosinophilic cytoplasm, as well as a reduction in the number of NeuN-positive cells or the NeuN/DAPI ratio compared to the control group (WT). In the hippocampus and LSc zone, the proportion of GFAP-positive cells per field of view, given as percents, and the number of S100 β -positive cells per field of view were also counted. An increase in GFAP and S100 β expression was considered to be indicative of an astrocyte activation, and together with neurodegenerative changes, an indicator of neuroinflammation. For the hippocampus and CP zone, the level of SYP expression was calculated as the ratio of SYP expression area to the field-of-view area, given as percentages. The SYP expression area was assessed using open-source machine learning software QuPath 0.5.0 [13].

Statistical analysis

IF study data were assessed for normality using Shapiro-Wilk test. Mean values of the samples were compared using single-factor analysis of variance (ANOVA) with Tukey's post hoc test. Differences with a p value of less than 0.05 were considered statistically significant, with a sample size sufficient for 80% power and in accordance with the 3R principles. Statistical calculations were performed using OriginPro software (OriginLab, USA).

RESULTS

Histological study

Microphotographs and graphs of cell counts for brain sections in CP and LSc zones are shown in Fig. 2. In the CP zone, the total number of neurons decreased in all groups expressing mutant FUS compared to WT, *HSP70in*, and *HSP70out* groups (Fig. 2E). However, in the *FUS[1-359]/HSP70out* group, the number of neurons was lower than in *FUS[1-359]* and *FUS[1-359]/HSP70in* groups.

Neurons with eosinophilic cytoplasm were found only in the CP zone at the histological study (Fig. 2F). Their number increased significantly in all FUS-expressing groups, reaching maximum values in *FUS[1-359]/HSP70out* group. In *FUS[1-359]* and *FUS[1-359]/HSP70in* groups, the number of neurons with eosinophilic cytoplasm decreased but remained higher than in the groups without aberrant FUS.

In contrast to the CP zone, in the LSc zone, the number of neurons did not decrease in all FUS-expressing groups compared to WT group (Fig. 2G). Such a decrease in the number of neurons was characteristic only for *FUS[1-359]/HSP70out* group. At the same time, the number of hyperchromic neurons in the LSc zone (Fig. 2H) was higher in all FUS-expressing groups compared to WT group. The smallest number of hyperchromic neurons was a feature in *FUS[1-359]/HSP70in* group, compared to *FUS[1-359]* and *FUS[1-359]/HSP70out* groups.

Microphotographs and graphs of cell counts for brain sections in the hippocampal region are shown in Fig. 3. In the SUBd (Fig. 3G), CA1 (Fig. 3H), and DG (Fig. 3I) zones, the number of hyperchromic neurons increased in all FUS-expressing groups compared to the WT, *HSP70in*, and *HSP70out* groups. However, in *FUS[1-359]/HSP70in* group, the number of hyperchromic neurons was lower than in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. In the CA3 zone, no statistically significant differences in hyperchromic neurons between groups were found (Fig. 3I).

Microphotographs and graphs of cell counts per field of view for brain sections in the amygdala region are shown in Fig. 4. In the BLAv (Fig. 4H), BLAa/BLAp (Fig. 4I), and CeL (Fig. 4J) zones, the total number of neurons decreased in the *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to WT, *HSP70in*, and *HSP70out* groups. However, in *FUS[1-359]/HSP70in* group, a decrease in the number of neurons relative to WT group was only revealed in the CeL zone. In the LA zone (Fig. 4G), the pattern of changes at the graph is similar to changes in other amygdala zones, but only a slight decrease in the number of neurons in *FUS[1-359]* group compared to the WT group where the changes were statistically significant.

Immunofluorescent study

The results of IF study for the CP and LSc zones are shown in Fig. 5. In both zones, a decrease in the number of NeuN⁺ cells was observed in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. The level of NeuN⁺ cells in *FUS[1-359]/HSP70in* group was higher than in other FUS-expressing groups, while in the CP zone, it was comparable to WT group, and in the LSc zone, it was significantly lower than in WT group (Fig. 5C, 5D).

Fig. 6 shows the results of the IF study for hippocampal structures. In the DG and CA1 zones, the proportion of GFAP⁺ and the number of S100β⁺ cells increased significantly in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups, and to a lesser extent in *FUS[1-359]/HSP70in* group compared to WT group (Fig. 6H, 6I, 6K, 6L). Minor differences in SYP expression were observed only between FUS-expressing groups (Fig. 6M).

In the CA1 zone, a sharp decrease in the NeuN⁺/DAPI⁺ ratio was noted (Fig. 6J) in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. In contrast, in *FUS[1-359]/HSP70in* group, this ratio was not only higher than in other FUS-expressing groups but also did not differ significantly from the WT group.

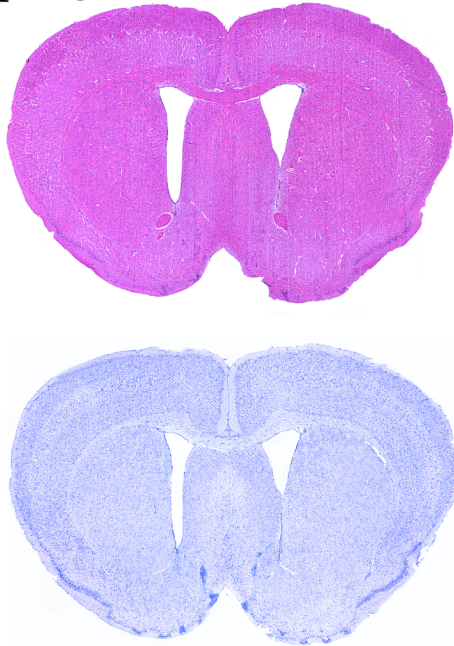
Fig. 7 shows the results of the IF study in the LA zone. In all FUS-expressing groups (*FUS[1-359]*, *FUS[1-359]/HSP70out*, and *FUS[1-359]/HSP70in*), the number of NeuN⁺ cells was lower compared to WT group and groups with monoexpression of HSP70. At the same time, among the groups with mutant FUS, NeuN expression was higher in *FUS[1-359]/HSP70in* group and lower in *FUS[1-359]/HSP70out* group.

DISCUSSION

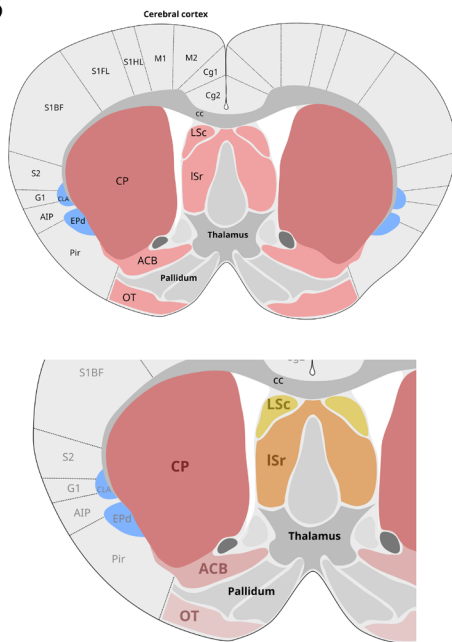
A key component of this work was the appropriate selection of transgenic mouse lines expressing HSP70 and FUS. The ALS model line was chosen based on the following criteria: 1) a model with the effectiveness in developing pathological and clinical manifestations of the FUS variant of ALS is well studied and aberrant FUS expression is proven; 2) there are pronounced neurodegenerative manifestations; 3) the mice survive up to an age where they can be crossed with rodents of other lines to breed double transgenic animals [6, 11]. The line expressing HSPA1A was chosen because this variant of HSP70 is capable of interacting with the FUS protein in its cytoplasmic mislocalization [14].

Our study revealed neurodegenerative changes in mutant FUS expression: a reduction in the number of neurons, an increase in the number of hyperchromic neurons, a decrease in NeuN expression and NeuN⁺/DAPI⁺ ratio, and co-expression of intracellular HSPA1A and mutant FUS in mice that is associated with lower levels of neurodegeneration. In the initial description of this ALS model, a neurodegenerative damage to

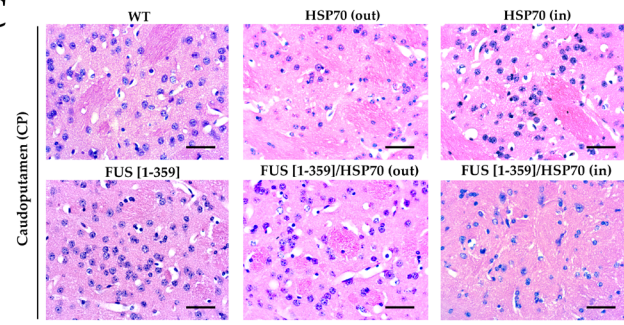
A Bregma +0.5 mm; Lambda +4.3 mm



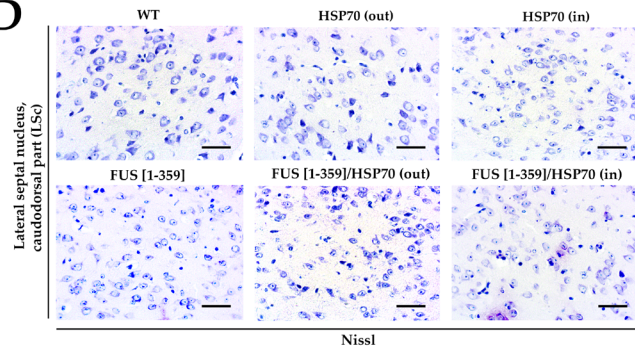
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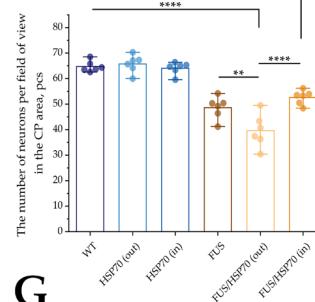
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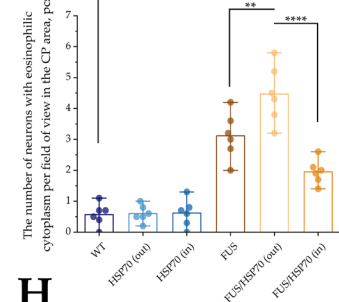
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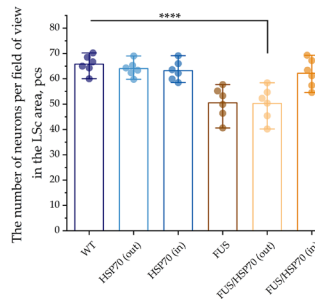
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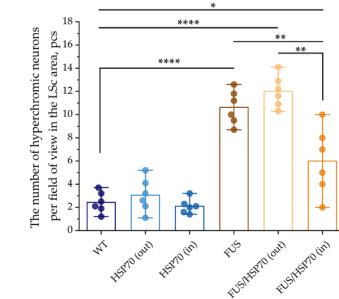


FIG. 2. Histological study of the caudoputamen and the caudal part of lateral septal nucleus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; CP – caudoputamen; LSc – caudal part of the lateral septal nucleus.

A. Coronal brain sections stained with hematoxylin and eosin (above) and Nissl toluidine blue (below).

B. Stereotactic coronal section with localization of CP and LSc zones.

C. Histological slides of murine brains, CP, hematoxylin and eosin, ob. $\times 40$. Scale bar: 20 μm .

D. Histological slides of murine brains, LSc, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .

E. The number of neurons per field of view in CP, hematoxylin and eosin.

F. The number of neurons with eosinophilic cytoplasm in CP, hematoxylin and eosin.

G. The number of neurons per field of view in LSc, Nissl toluidine blue.

H. The number of hyperchromic neurons in LSc, Nissl toluidine blue.

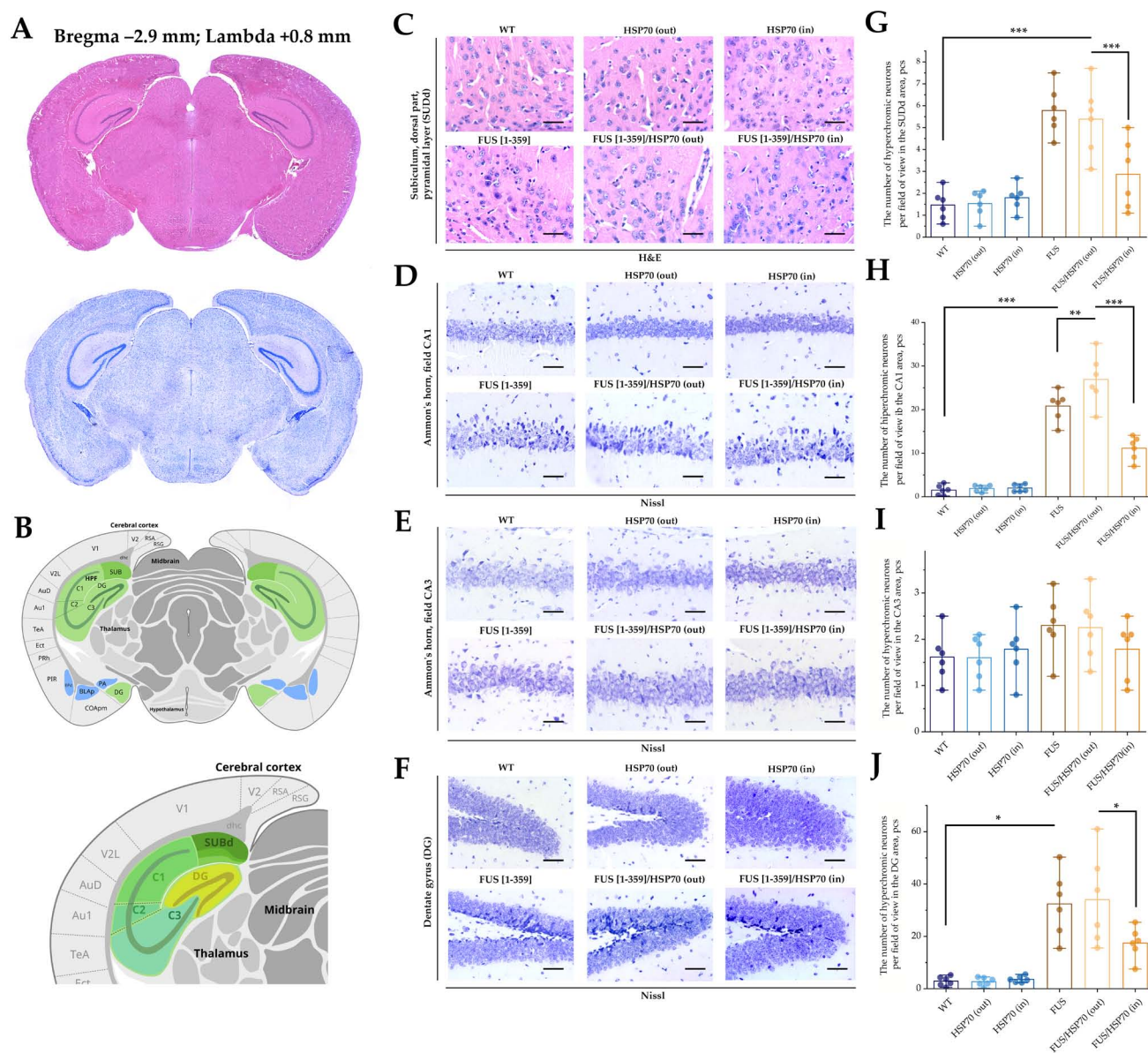


FIG. 3. Histological study of hippocampus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; SUBd – subiculum; CA – cornu Ammonis; DG – dentate gyrus.

- A. Coronal brain sections, stained by hematoxylin and eosin (above) and toluidine blue by Nissl (below).
 B. Stereotaxic coronal section with localization of hippocampal zones: SUBd, CA1 and CA3, DG.
 C. Histological slides of murine brains, SUBd, hematoxylin and eosin, ob. $\times 40$. Scale bar: 20 μm .
 D. Histological slides of murine brains, CA1, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .
 E. Histological slides of murine brains, CA3, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .
 F. Histological slides of murine brains, DG, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .
 G. The number of hyperchromic neurons in SUBd, hematoxylin and eosin.
 H. The number of hyperchromic neurons in CA1, Nissl toluidine blue.
 I. The number of hyperchromic neurons in CA3, Nissl toluidine blue.
 J. The number of hyperchromic neurons in DG, Nissl toluidine blue.

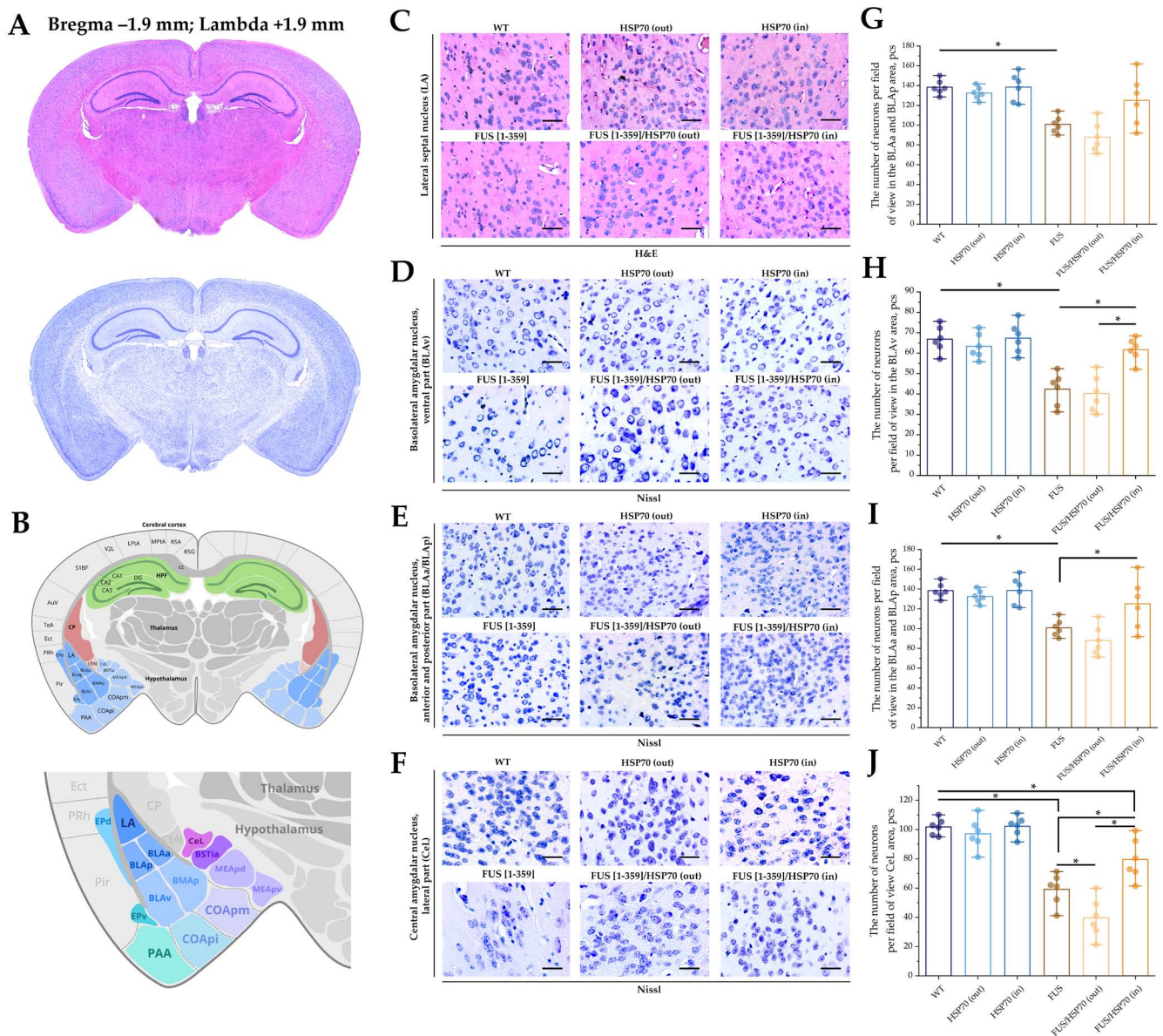


FIG. 4. Histological study of hippocampus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; LA – lateral amygdalar nucleus; BLAv – basolateral amygdalar nucleus, ventral part; BLAa/BLAp – basolateral amygdalar nucleus, anterior and posterior parts; CeL – central amygdalar nucleus, lateral part.

A. Coronal brain sections, stained by hematoxylin and eosin (above) and toluidine blue by Nissl (below).

B. Stereotactic coronal section with localization of amygdalar zones: LA, BLAv, BLAa/BLAp and CeL.

C. Histological slides of murine brains, LA, hematoxylin and eosin, ob. $\times 40$. Scale bar: 20 μm .

D. Histological slides of murine brains, BLAv, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .

E. Histological slides of murine brains, BLAa/BLAp, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .

F. Histological slides of murine brains, CeL, toluidine blue, ob. $\times 40$. Scale bar: 20 μm .

G. The number of neurons per field of view, LA, hematoxylin and eosin.

H. The number of neurons per field of view, BLAv, toluidine blue.

I. The number of neurons per field of view, BLAa/BLAp, toluidine blue.

J. The number of neurons per field of view, CeL, toluidine blue.

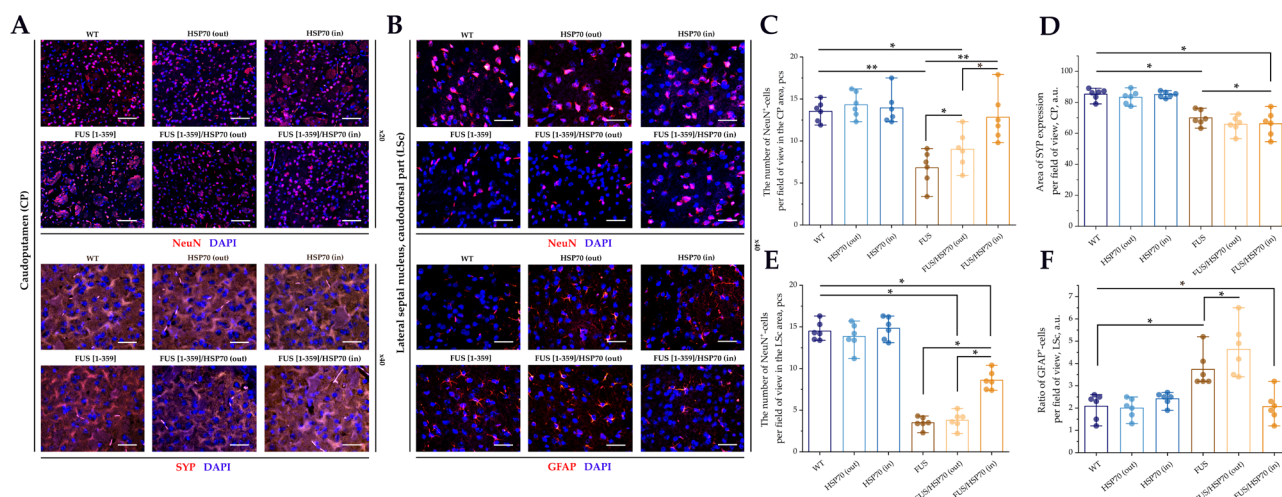


FIG. 5. Immunofluorescent study of caudoputamen and septal nuclei in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; CP – caudoputamen; LSc – caudal part of the lateral septal nucleus; GFAP – glial fibrillary acidic protein; NeuN – neuronal nuclear marker; SYP – synaptophysin.

- A. Brain sections, CP, ob. $\times 100$. Scale bar: 50 μm . Anti-NeuN and DAPI (above), Anti-SYP and DAPI (below).
 B. Brain sections, LSc, ob. $\times 100$. Scale bar: 50 μm . Anti-NeuN and DAPI (above), Anti-GFAP and DAPI (below).
 C. The number of NeuN⁺ cells per field of view, CP.
 D. Area of SYP expression per field of view, CP.
 E. The number of NeuN⁺ cells per field of view, LSc.
 F. Ratio of GFAP⁺-cells per field of view, LSc.

brainstem structures was noted without specifying the nature of changes at the limbic system [11].

This study demonstrated a reduction in the number of neurons in the CP zone in all FUS-expressing animals. According to the literature, CP structures are susceptible to neurodegenerative changes in humans with FUS-associated ALS [15]. It has also been previously shown that electrical activity in the CP zone increases in mice expressing mutant FUS [16]. In this study, the animals with extracellular expression of HSPA1A (group *FUS[1-359]/HSP70out*) had neuronal survival in the CP even lower than in monotransgenic animals expressing mutant FUS (group *FUS[1-359]*), while in animals with intracellular expression (group *FUS[1-359]/HSP70in*), it was higher. Similar data were obtained for the number of cells with a positive reaction to NeuN, a known marker of mature neurons. Since the presence of excessive HSPA1A does not cause neurodegenerative changes in the CNS, as we had showed previously [9], it can be assumed that extracellular expression of HSPA1A exacerbates the pathological changes in the FUS variant of ALS. At the same time, co-expression of HSPA1A and FUS in the cytoplasm of CP neurons positively affects their survival.

In the CP zone, neurons with eosinophilic inclusions in the cytoplasm were also found, and their number increased in mutant FUS-expressing groups, especially

in *FUS[1-359]/HSP70out* group. Similar “cherry spot” inclusions have previously been found in the nuclei of hippocampal neurons in the FUS variant of frontotemporal dementia [17]. A. Murakami et al. also reported the detection of FUS-positive inclusions in the striatum in autopsy material [18]. This finding may represent more evidence of FUS-associated neurodegenerative changes. However, there is insufficient experimental material for comparison to consider eosinophilic inclusions a specific marker.

For limbic system parts (septal nuclei, hippocampus) with a high concentration of neurons per unit area and, consequently, a higher probability of bias during comparison, the study considered not the total number of neurons, but the number of hyperchromic cells. This parameter is commonly used to assess neurodegenerative changes [19]. In the study of the LSc zone, the number of hyperchromic neurons, as well as cells expressing NeuN, increased in all FUS-expressing groups. These data are consistent with the general trend of changes in the CP zone, namely a more pronounced level of neurodegenerative changes in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to *FUS[1-359]/HSP70in* group. To date, no previous studies have been revealed in the accessible databases demonstrating changes in the number of neurons or hyperchromic neurons in the septal nuclei of animals expressing mutant FUS or in ALS-suffered humans.

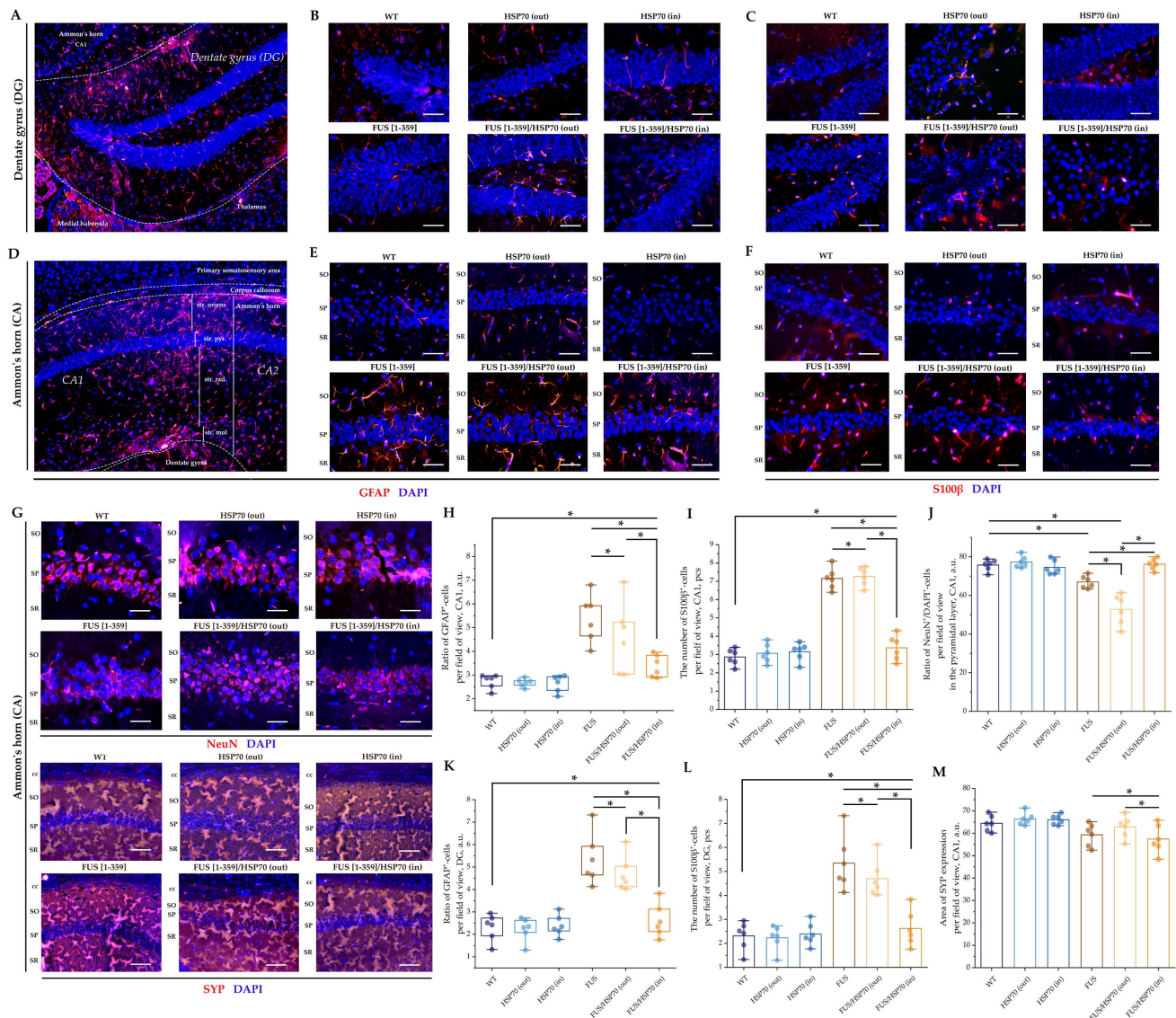


FIG. 6. Immunofluorescent study of hippocampal structure in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; DG – dentate gyrus; CA – Cornu Ammonis; GFAP – glial fibrillary acidic protein; NeuN – neuronal nuclear marker; SYP – synaptophysin.

- A. Brain sections, DG, ob. $\times 40$. Scale bar: 20 μm . Anti-GFAP and DAPI.
- B. Brain sections, DG, ob. $\times 100$. Scale bar: 50 μm . Anti-GFAP and DAPI.
- C. Brain sections, DG, ob. $\times 100$. Scale bar: 50 μm . Anti-S100 β and DAPI.
- D. Brain sections, CA1, ob. $\times 40$. Scale bar: 20 μm . Anti-GFAP and DAPI.
- E. Brain sections, CA1, ob. $\times 100$. Scale bar: 50 μm . Anti-GFAP and DAPI.
- F. Brain sections, CA1, ob. $\times 100$. Scale bar: 50 μm . Anti-S100 β and DAPI.
- G. Brain sections, CA1, ob. $\times 100$. Scale bar: 50 μm . Anti-NeuN and DAPI (above), Anti-SYP and DAPI (below).
- H. The ratio of GFAP $^{+}$ cells per field of view, %, CA1.
- I. The number of S100 β^{+} cells per field of view, CA1.
- J. NeuN $^{+}$ /DAPI $^{+}$ ratio per field of view, %, CA1.
- K. The ratio of GFAP $^{+}$ cells per field of view, %, DG.
- L. The number of S100 β^{+} cells per field of view, DG.
- M. SYP expression area per field of view, %, CA1.

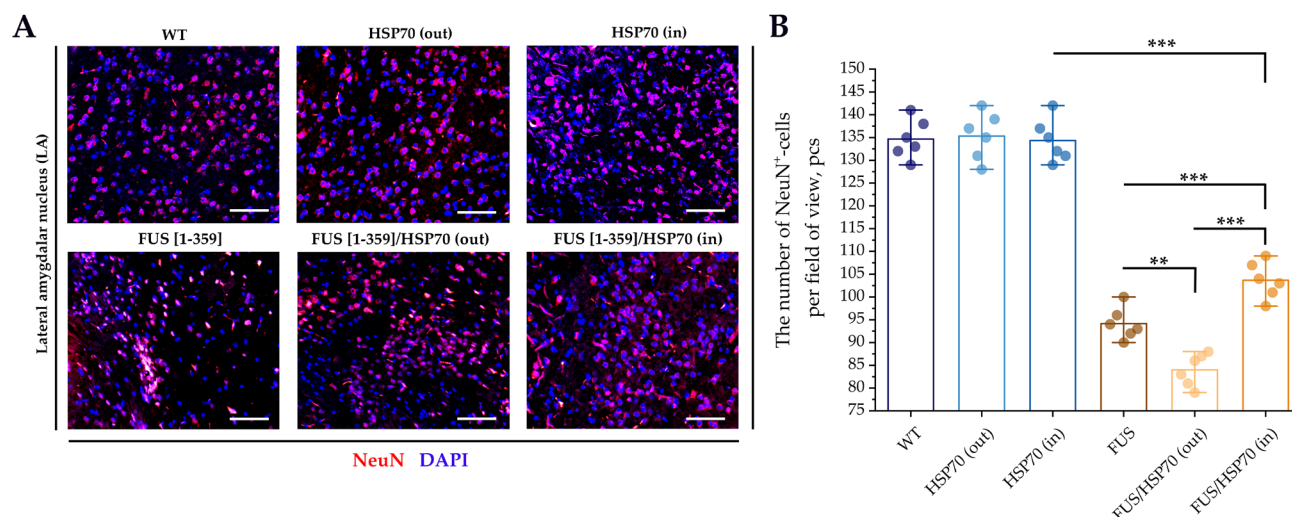


FIG. 7. Immunofluorescent study of amygdala in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: ** $p < 0.01$; *** $p < 0.001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; LA – lateral amygdalar nucleus; NeuN – neuronal nuclear marker.

A. Brain sections, LA, ob. $\times 100$. Scale bar: 50 μm . Anti-NeuN and DAPI.

B. The number of NeuN⁺ cells per field of view, LA.

In the hippocampal regions SUBd, CA1, and DG, this trend was maintained between the groups as described above for the CP and LSc zones. Thus, the number of hyperchromic neurons increased with mutant FUS expression, but in mice with simultaneous intracellular overexpression of HSPA1A (group *FUS[1-359]/HSP70in*) it was lower than in other mice expressing mutant FUS. These changes were accompanied by a decrease in NeuN expression in groups with mutant FUS, least pronounced in *FUS[1-359]/HSP70in* group. In the hippocampus, there was also an increase in the number of GFAP⁺ and S100 β ⁺ cells, and for these parameters, the differences between groups were consistent with the differences in the increase in the number of hyperchromic neurons. Since GFAP and S100 β are markers of activated astrocytes, their increase may reflect the involvement of astroglia into FUS accumulation-driven reactions.

The hippocampus, due to its role in cognitive functions, is of particular interest in ALS. Its sensitivity to changes in FUS activity has been demonstrated in the study by Kino et al. [20]. In mice expressing mutant FUS, cognitive functions and spine formation in the hippocampus were reduced, although there was no cytoplasmic mislocalization of FUS in the hippocampus [21]. It has also been shown that spine density in the hippocampus and cognitive functions stay reduced in transgenic mice expressing mutant FUS and manifesting ALS [22]. However, in our study, no differences were found in the area of hippocampal tissue with a positive reaction to SYP. It is possible that synaptic function is less involved in FUS pathology in ALS than spine density.

An increase in FUS expression, neurodegenerative changes, and subsequent recovery of the number of neurons in a study investigating ischemic effects on the hippocampus were in some cases reported to be accompanied by a transient increase in HSP70 expression [23]. It can be assumed that HSP70 in the hippocampus helps to mitigate the negative effects of FUS accumulation (Fig. 8).

In the amygdala, in most nuclei except the lateral nucleus, the number of neurons decreased in all FUS-expressing groups, but in *FUS[1-359]/HSP70in* group it was minimal. This is consistent with the trend observed in other studied parts of the limbic system. There is little data in the literature about amygdala involvement in ALS, but it has been reported that mutant FUS expression is absent in the amygdala in the FUS variant of ALS [24].

In addition, we have previously examined changes in the primary motor cortex and spinal cord in the same models [25]. In all cases, a reduction in neurodegenerative changes was demonstrated with simultaneous overexpression of the cytosolic form of HSPA1A and mutant FUS protein with cytoplasmic mislocalization.

Limitations of the study

Extrapolation of animal experiments to humans is limited. This study does not cover all areas of the central nervous system. For example, the diversity of cortical areas in humans is inherently greater, as mice lack gyri; mice do not have speech regulation parts in the brain, etc.

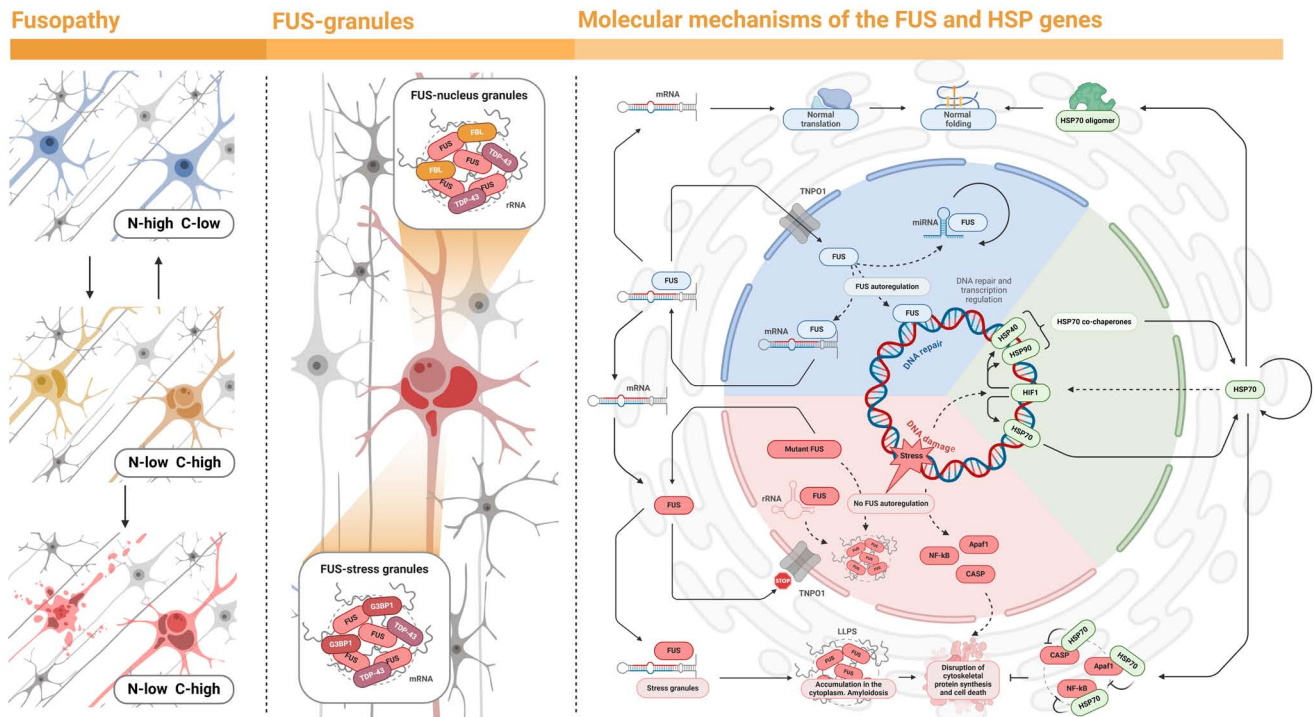


FIG. 8. Mechanisms of FUS-HSP70 interaction in cells.

Left: localization of fused-in-sarcoma (FUS) protein in the nucleus (blue). Loss of the nuclear localization signal leads to the migration of FUS into the cytoplasm (yellow). “N” – nucleus, “C” – cytoplasm.

Center: in the nucleus, FUS, together with fibrillarin (FBL) and the TDP-43 protein, binds rRNA to form nuclear granules. In the cytoplasm, stress granules containing mRNA with the G3BP1 marker are present. FUS in the cytoplasm associates with these granules.

Right: FUS activity (blue sector) supports DNA repair and transcriptional regulation, with autoregulation of FUS activity. FUS located outside the nucleus returns through the nuclear pore with the help of TNPO1 protein, while HSP70 chaperones ensure proper translation and folding. HSP70 activity (green sector) requires interaction with HSP40 and HSP90. Damage induces the synthesis of HSP70, for example through hypoxia-inducible factor 1 (HIF1). Stress-induced DNA damage can lead to mutations in the FUS gene. As a result, mutant FUS loses its nuclear localization signal. The accumulation of FUS in the cytoplasm and its aggregation disrupts liquid-liquid phase separation (LLPS) mechanisms, causing amyloidosis, and together with the activation of apoptosis (CASP; Apaf1; NF-κB) leads to impaired cytoskeleton repair and cell death.

Further research perspectives

We assume that it is a challenging task to establish the molecular mechanisms contributing to the positive impact of HSP70 overexpression on slowing neurodegenerative manifestations in the FUS-related ALS. Recording electrical changes in limbic system structures will allow a more complete description of the double transgenic murine models.

CONCLUSION

In the present study, we explored quantitative parameters of neurodegenerative changes in the limbic system in mice with overexpression of HSP70 in the

form of HSPA1A and co-expression of mutant human *FUS*[1-359] protein related to ALS manifestation. According to data obtained, the co-expression of HSPA1A protein together with the mutant *FUS*[1-359] in the cytoplasm of nervous tissue cells in the limbic system is accompanied by fewer neurodegenerative changes compared to the monoexpression of the mutant FUS. Moreover, pathological changes are most significantly reduced in co-expression of FUS and intracellular HSPA1A overexpressed with HSP70 secretion out of cells. This study indicates HSPA1A overexpression as a promising approach to slow the progression of FUS-related ALS.

AUTHOR CONTRIBUTION

Gennadii A. Piavchenko and Sergey L. Kuznetsov developed the idea and design of the study. Egor A. Kuzmin and Ksenia S. Pokidova carried out the experiments with animals, made histological slides, and provided statistical calculations. Artem A. Venediktov carried out the literature sources and wrote a draft text of the article. Egor A. Kuzmin and Ksenia S. Pokidova designed graphical elements of the work. Gennadii A. Piavchenko and Sergey L. Kuznetsov edited the article. All authors approved the final version of the article.

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ВКЛАД АВТОРОВ


Г.А. Пьявченко и С.Л. Кузнецов сформулировали идею и разработали дизайн исследования. К.С. Покидова и Е.А. Кузьмин провели эксперименты с животными, изготовили гистологические препараты и выполнили статистическую обработку результатов. А.А. Венедиктов изучил литературные источники и написал текст статьи. К.С. Покидова и Е.А. Кузьмин оформили иллюстрации. Г.А. Пьявченко и С.Л. Кузнецов провели научную редактуру статьи. Все авторы статьи утвердили окончательную версию статьи.

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