NEAT1_1 LONG NON-CODING RNA REDUCES THE SURVIVAL OF PRIMARY NEURONAL CELLS UNDER ER-STRESS

Pukaeva NE[1,2], Zalevskaya VN[2], Deykin AV[3], Taubinskaya MI[2], Kukharskaya OA[2], Ovchinnikov RK[1,2], Antohin AI[1], Kukharsky MS[1,2] ✉

[1] Pirogov Russian National Research Medical University, Moscow, Russia
[2] Institute of Physiologically Active Compounds at Federal Research Center of Problems of Chemical Physics and Medicinal Chemistry, Russian Academy of Sciences, Chernogolovka, Russia
[3] Belgorod State National Research University, Belgorod, Russia

NEAT1 long non-coding RNAs play an important role in the central nervous system (CNS) and are associated with a number of pathological conditions. Increased levels of NEAT1 in the brain have been observed in neurodegenerative and psychiatric diseases — the significance of such an increase is still poorly understood. Functionally, NEAT1 is associated with cellular stress pathways in the nervous system. The aim of the current study was to evaluate the effect of increased levels of the short isoform NEAT1_1 on survival of mice primary hippocampal cultures under ER-stress induced by MG132 proteasome inhibitor. Primary cultures were obtained from transgenic animals expressing human NEAT1_1. Cellular composition and apoptosis were assessed using immunocytochemical staining. The expression of apoptosis signaling pathway genes was analyzed by quantitative PCR with reverse transcription. No differences in cellular composition and morphological characteristics of neurons were observed in primary neuronal cultures obtained from transgenic animals as compared to wild type cultures. Induction of ER-stress resulted in a more significant increase in apoptotic death of cells including neurons in NEAT1_1 expressing cultures in comparison with the wild type cultures. ER-stress signaling pathway genes *Atf4* and *Ddit3* were less expressed in transgenic cultures under stress. Expression of *Bcl2l2* and *Mcl1* anti-apoptotic genes was reduced as well. Thus, high levels of NEAT1_1 in primary neuronal cultures increased apoptotic cell death under ER-stress.

Keywords: long non-coding RNAs, NEAT1_1, ER-stress, primary neuronal cultures, apoptosis

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✉ **Correspondence should be addressed:** Michail S. Kukharsky
Ostrovitianova, 1/9, Moscow, 117997, Russia; kukharskym@rsmu.ru

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ДЛИННАЯ НЕКОДИРУЮЩАЯ РНК NEAT1_1 СНИЖАЕТ ВЫЖИВАЕМОСТЬ ПЕРВИЧНЫХ НЕЙРОННЫХ КЛЕТОК ПРИ ЭПР-СТРЕССЕ

Н. Е. Пукаева[1,2], В. Н. Залевская[2], А. В. Дейкин[3], М. И. Таубинская[2], О. А. Кухарская[2], Р. К. Овчинников[1,2], А. И. Антохин[1], М. С. Кухарский[1,2] ✉

[1] Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия
[2] Институт физиологически активных веществ Федерального исследовательского центра проблем химической физики и медицинской химии Российской академии наук, Черноголовка, Россия
[3] Белгородский государственный национальный исследовательский университет, Белгород, Россия

Длинная некодирующая РНК NEAT1 играет важную роль в работе центральной нервной системы (ЦНС) и участвует в развитии ряда патологических состояний. Повышение уровня NEAT1 в мозге происходит при нейродегенеративных и психических заболеваниях. Какую роль играет такое повышение остается неясным. Функционально NEAT1 связывают с реализацией ответа на клеточный стресс в нервной системе. Целью работы было оценить влияние повышенного уровня короткой изоформы NEAT1_1 на выживаемость первичных гиппокампальных культур мышей, при ЭПР-стрессе, индуцированном с помощью ингибитора протеасом MG132. Первичные культуры были получены от трансгенных животных, экспрессирующих NEAT1_1 человека. Характеристику клеточного состава и оценку апоптоза проводили с помощью иммуноцитохимического окрашивания. Анализ экспрессии генов сигнальных путей апоптоза осуществляли методом количественной ПЦР с обратной транскрипцией. Первичные нейронные культуры, полученные от трансгенных животных, не отличались от культур дикого типа по клеточному составу и морфологическим характеристикам нейронов. При индукции ЭПР-стресса происходило усиление апоптотической гибели клеток, в том числе нейронов, в культурах, экспрессирующих NEAT1_1 в сравнении с культурами дикого типа. Активация генов сигнальных путей ЭПР-стресса *Atf4* и *Ddit3* была менее выражена в трансгенных культурах при стрессе. Кроме того, был снижен уровень экспрессии антиапоптотических генов *Bcl2l2* и *Mcl1*. Таким образом, повышенный уровень NEAT1_1 в первичных нейронных культурах усиливает апоптотическую гибель клеток при ЭПР-стрессе.

Ключевые слова: длинные некодирующие РНК, NEAT1_1, ЭПР-стресс, первичные нейронные культуры, апоптоз

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✉ **Для корреспонденции:** Михаил Сергеевич Кухарский
ул. Островитянова, д. 1, стр. 9, г. Москва, 117997, Россия; kukharskym@rsmu.ru

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Long noncoding RNAs (lncRNAs) are molecules longer than 200 nucleotides without open reading frames [1]. lncRNAs are involved in various physiological and pathological processes such as cell cycle regulation, cell differentiation, apoptosis, and inflammation [2]. The role of lncRNAs in evolution of mammalian and human brain seems to be of particular importance. This is supported by the fact that about 40% of all identified human lncRNAs are specifically expressed in the brain [3]. Many studies have demonstrated the involvement of lncRNAs in the development of the nervous system, neuronal plasticity as well as in the pathogenesis of neurological diseases [4]. NEAT1 (nuclear enriched abundant transcript 1) is one of the lncRNAs that has been convincingly shown to be associated with the development of pathological conditions of the nervous system. Changes in NEAT1 levels in the brain are observed in a number of neurodegenerative diseases and psychiatric disorders such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Huntington's disease (HD), Alzheimer's disease (AD), and schizophrenia [5]. In most cases, increased NEAT1 levels in the brain of patients with these diseases are [6–8]. Whether the role of such increase is protective or pathogenetic is still unclear. Experimental data suggest that NEAT1 is a stress-activated gene and its expression is increased in response to pathological effects of stress at the cellular level [9, 10]. NEAT1 gene is transcribed into two RNA isoforms, NEAT1_1 (3.7 kb) and NEAT1_2 (23 kb) overlapping at their 5'-ends [5]. Accumulation of the long NEAT1_2 isoform in human nervous system cells has been confirmed for developing ALS conditions only [11, 12]. For other diseases, NEAT1 dysfunctions are apparently associated with changes in the short isoform levels (NEAT1_1) [13]. In mice, the long isoform was not normally detected in the nervous tissue while the short isoform is expressed in all parts of the CNS [14].

Under physiological conditions, endoplasmic reticulum (ER) is the main subcellular compartment involved in protein quality control, where proteins are properly folded, matured, and degraded [15]. When these processes are disrupted, unfolded and misfolded proteins are accumulated in cells resulting in ER stress, a specific type of cellular stress [16]. This most common pathologic event at the cellular level occurs in neurons located in the lesion zone during neurodegeneration [17]. In case of impossibility to restore protein homeostasis, cell adaptive programs shift towards induction of apoptotic signaling pathways leading to death of the irreversibly damaged neurons [18].

NEAT1 is involved in forming specific ribonucleoprotein (RNP) complexes in cells [6, 11, 13]. Pathological aggregation of RNP proteins TDP-43 and FUS leads to ER stress and neuronal death. TDP-43 and FUS aggregation in the nervous system was found in ALS, FTD, AD and other neurodegenerative diseases. The direct interaction of NEAT1 with such proteins raises the question about the possible influence of this RNA on the development of pathological aggregation and cell death [12, 13].

In the current study, we investigated the effect of the increased levels of NEAT1_1 lncRNA on cell survival in primary hippocampal cultures obtained from transgenic mice under ER stress induced by the proteasome inhibitor MG132.

METHODS

Primary hippocampal cultures

The study was performed on primary neuronal cultures obtained from NEAT1_1Tg transgenic mice expressing the short isoform of human NEAT1_1 in the nervous system. The mice were obtained by transgenesis as described previously [19] and harbor a transgene encoding human NEAT1_1 in the genome under the control of the pan-neuronal Thy1 promoter on a C57BL6 genetic background (in press). The transgene presence was confirmed by PCR. Animals were maintained at a 12h light/12h dark cycle, with food and water supplied *ad libitum*.

Primary neuronal cultures were obtained from the hippocampus of NEAT1_1Tg transgenic mice and wild-type (WT) control mice on the third day after birth (P3) as described previously [20]. Hippocampi isolated from at least three animals of the same genotype were used for culture; each experiment was independently repeated at least twice. After hippocampal dissection, the samples were incubated in a solution of 0.1% trypsin in Hanks' balanced salt solution (HBSS) with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 1 mM sodium pyruvate for 40 min. This was followed by mechanical dissociation in a solution of Neurobasal medium (PanEco; Russia) containing 50 units/mL penicillin/streptomycin, 0.2% beta-mercaptoethanol, 500 μM L-glutamine, 0.36% glucose, and 10% horse serum. Samples were centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in freshly prepared medium, and cell counting was performed using a Goryaev chamber (hemocytometer) and trypan blue staining. B27 supplement (Thermo Fisher Scientific; USA) or NeuroMax supplement (PanEco; Russia) was added to the medium to increase the survival of primary neurons in culture. Cells were seeded on 12-mm-diameter coverslips coated with poly-L-lysine, with approximately 3×10^4 cells per glass. On the next day, medium was changed to fresh medium without serum. A subsequent medium change was performed every 3 days replacing only half of the medium. Cultures were incubated at 37 °C, 5% carbon dioxide, and 95% humidity. The cultures were analyzed after 7 days from seeding.

To induce endoplasmic reticulum stress (ER stress), cells were treated with a solution of MG132 proteasome inhibitor (Sigma-Aldrich; USA) in DMSO (PanEco; Russia) with a final concentration of 200 μM or 10 μM.

Immunocytochemical staining using microtubule-associated protein Tau antibody (SAB4300377; Sigma-Aldrich, USA) was performed to visualize neuronal bodies and outgrowth processes, for subsequent Sholl analysis; to confirm the neuronal phenotype, cells were stained with NeuN marker (antibody MAB377; Millipore, USA). Images were obtained using a Carl Zeiss Axio Observer 3 microscope (Germany) equipped with an Axiocam 712 mono camera (Carl Zeiss; Germany). Semi-automatic Sholl analysis was performed using the ImageJ plugin as described earlier [10]. For each genotype, 30 neurons were analyzed.

Immunocytochemical staining

To characterize the cellular composition of primary neuronal cultures, we performed the immunocytochemical staining using markers of the main types of neuronal tissue cells: NeuN, a marker of differentiated neurons (antibody MAB377; Millipore, USA), GFAP, a marker of astrocytes (antibody G9269; Sigma-Aldrich, USA), Olig2, a marker of oligodendrocytes (antibody ab109186; Abcam, UK), and Iba1, a marker of microgliocytes (antibody ab178846; Abcam, UK). For each marker, six glass coverslips from two independent cultures for each genotype were analyzed. Apoptotic cell death was assessed by staining with antibodies to the activated form of caspase 3, CC3 (antibody AB3623; Sigma-Aldrich, USA). Cells were washed from the medium with 1× PBS (phosphate-buffered saline),

Table. Sequences of primers used for RT-qPCR

Name of primer	Sequence of forward primer	Sequence of reverse primer
B2m	CATGGCTCGCTCGGTGAC	CAGTTCAGTATGTTCGGCTTCC
Atf4	GGGTTCTGTCTTCCACTCCA	AAGCAGCAGAGTCAGGCTTTC
Ddit3	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
Hspa5	TCGACTTGGGGACCACCTAT	AGTGAAGGCCACATACGACG
Emc4	ATACCAGCGTGCAAGAGACC	GGAATCTGCTTGAGGGGACC
Bcl2l1	CGGATTGCAAGTTGGATGGC	TGCTGCATTGTTCCCGTAGA
Bcl2l2	ATTGGATGGTGGCCTACCTG	CCCGTATAGAGCTGTGAACTCC
Mcl1	AACGGGACTGGCTTGTCAAA	CTGATGCCGCCTTCTAGGTC
Casp3	CGGGGAGCTTGGAACGGTA	CCACTGACTTGCTCCCATGT

fixed with 4% paraformaldehyde for 15 min and permeabilized with ice-cold methanol for five minutes. Non-specific binding was blocked using 5% goat serum blocking solution in PBS with Tween 20 for 1 hour at room temperature. The coverslips were then incubated with primary antibodies at 1:1000 dilution in a blocking solution for 1 hour at room temperature, washed out with 1× PBS, and incubated with secondary fluorescently labeled antibodies Goat anti-Rabbit IgG Alexa Fluor™ 568 (A-11011; Thermo Fisher Scientific, USA) and Goat anti-Mouse IgG Alexa Fluor™ 488 (A-11029; Thermo Fisher Scientific, USA) at 1:1000 dilution in PBS-Tween 20 solution for 90 minutes at room temperature. Cell nuclei were stained with DAPI solution (Sigma-Aldrich; USA). The coverslips were mounted on slides, in a drop of Immu-Mount medium (Thermo Fisher Scientific; USA). For analyzing cell types and apoptosis, coverslips were imaged on a Cytation 3 Reader (BioTek; USA) with Gen5 3.08 Software (BioTek; USA). At least 3000 μm × 3000 μm area was scanned in a multichannel fluorescence mode. The scanned area was stitched into a single panoramic image, and a number of positively stained cells for each specific marker was analyzed. For each marker, the results were normalized to the total number of cells estimated by DAPI-stained nuclei counting.

Gene Expression Analysis

Expression of genes involved in ER stress major signaling pathways and apoptosis were assessed by quantitative real-time reverse transcription PCR (RT-PCR) on a CFX96 instrument (Bio-Rad; USA). Cells were grown on a six-well plate for 7 days. Total RNA was isolated using ExtractRNA reagent (Evrogen; Russia) according to the manufacturer's protocol. RNA samples were further treated with DNase (Sigma-Aldrich; USA) according to the manufacturer's protocol. The concentration of purified RNA was measured using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific; USA). Synthesis of complementary cDNA strand was performed using the Magnus Reverse Transcriptase kit (Evrogen; Russia) according to the manufacturer's protocol. Quantitative real-time PCR was performed on a CFX96 instrument (Bio-Rad; USA) using qPCRmix-HS SYBR kit (Evrogen; Russia) according to the manufacturer's protocol. Beta-2-microglobulin gene (B2m) was used as an endogenous control. The list of primers we used is presented in Table.

The program for qPCR included initial denaturation for 5 min at 95 °C, followed by 40 cycles: at 95 °C for 20 s, at 60 °C for 30 s, at 68 °C for 30 s. The results were analyzed using Bio-Rad CFX Manager software (Bio-Rad; USA).

Statistical Analysis of Data

Statistical analysis was performed using Statistica 12.0 (StatSoft, Inc.; USA) and GraphPad Prism 8 (GraphPad Software; USA) software. In all cases, results are presented as arithmetic mean and standard error of the mean (x ± m) with individual values where appropriate. Details of the statistical analysis for each group of data are presented in the figure description. Differences in the obtained results were considered statistically significant at a significance level of $p < 0.05$.

RESULTS

Cellular composition and morphology of neurons in primary neuronal cultures expressing NEAT1_1 do not differ from control ones

Primary hippocampal cell cultures obtained from NEAT1_1Tg transgenic mice and from wild-type (WT) animals on the third day of postnatal development (P3) were used in the experiment. The isolated cells were cultured in vitro for 7 days. Culturing conditions (use of serum, nutrient supplement B27, etc.) were selected to obtain a mixed culture containing all major cell types of nervous tissue. Staining for markers of cell types showed that the primary neuronal cultures contained approximately 9 ± 1.3% neurons, 61 ± 5.6% astrocytes, 28 ± 2.5% oligodendrocytes, and 2 ± 0.5% microgliocytes (Fig. 1A, B). No differences were found between cultures of different genotypes — NEAT1_1Tg and WT - in terms of cellular composition.

To characterize neuron morphology and assess the effect of increased NEAT1_1 expression on morphofunctional characteristics related to cell activity and plasticity, we performed Sholl analysis evaluating the neurite outgrowth. No significant differences in the branching complexity in neurons of two different genotypes (NEAT1_1Tg and WT) were detected although a tendency of decreasing number of neurites in neuron proximal regions was observed for transgenic NEAT1_1Tg cultures (Fig. 2A, B). Thus, NEAT1_1 expression was shown to have no effect on neuronal plasticity.

In transgenic NEAT1_1Tg cultures, apoptotic cell death was more often observed under ER stress

Analysis of apoptotic cell death by immunocytochemical staining for activated caspase-3 (CC3) showed that under normal conditions, a small number (2–4%) of CC3-positive ($CC3^+$) cells were detected in cell cultures while the signal was mostly not colocalized with NeuN marker indicating the glial nature of dying cells (Fig. 3 A–B).

Acute and moderate ER stress was induced by treatment with MG132 proteasome inhibitor (200 μM and 10 μM MG132 concentrations, respectively). On the seventh day of cultivation, MG132 proteasome inhibitor was added to the medium for 4 h, after that the medium was changed. Apoptosis levels were evaluated after 4 h, 12 h and 24 h. The total cell death

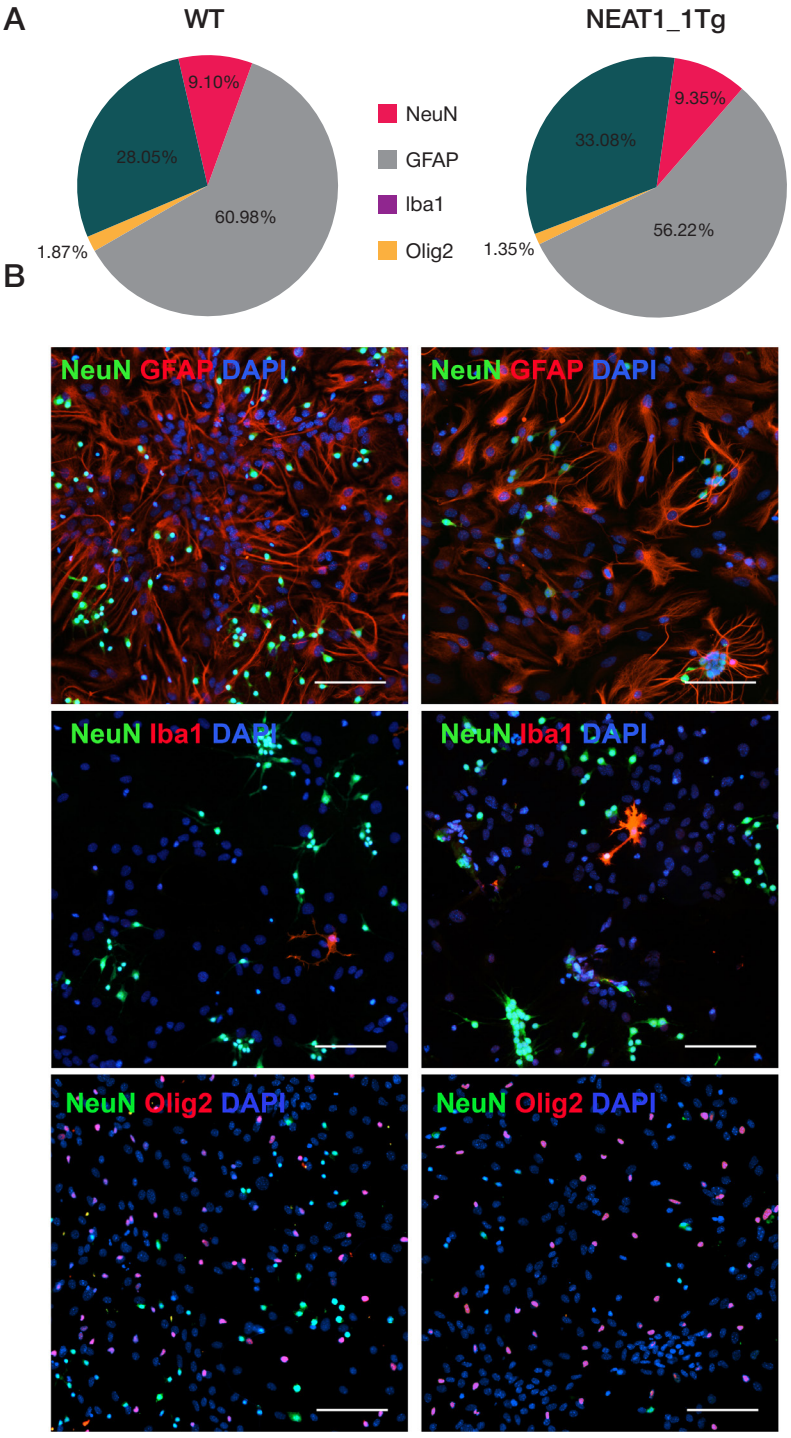

Fig. 1. Cellular composition of hippocampal primary cultures derived from transgenic (NEAT1_1Tg) and wild-type (WT) mice **A**. Percentages of neurons (NeuN), astrocytes (GFAP), microgliocytes (Iba1), and oligodendrocytes (Olig2) in primary cultures. **B**. Representative photographs of primary cultures stained for markers of different cell types. Cell nuclei are stained with DAPI. The scale bar is 100 μm

was analyzed, i.e. all $CC3^+$ cells (both neurons and glia) were counted.

Under acute ER stress induced by high concentration of MG132 (200 μM) in WT cultures, cell death was detected in 4 h after treatment. Number of cells dying as a result of apoptosis increased in 12 h and remained high in 24 h. At the same time, in cultures obtained from transgenic animals, a significantly higher number of apoptotic cells was detected 4 h after treatment, whereas at subsequent time points this index was comparable to WT cultures treated with MG132 (Fig. 3A).

A low concentration of MG132 (10 μM) was used to model a moderate transient ER stress. In this case, total apoptotic death also increased in 4 hours however, in 12 hours the number of $CC3^+$ cells decreased, and in 24 hours it matched the values for control cultures without MG132 treatment indicating recovery of cell cultures after stress. Compared with WT after stress, in NEAT1_1Tg cultures the number of apoptotic cells was higher 4 hours after treatment, and the same trend persisted for 12 hours, whereas the difference leveled off at 24 hours (Fig. 3B).

To assess how ER stress affects neuronal survival, we performed co-staining for CC3 and the differentiated neuron marker NeuN, and then counted cells expressing both markers. In cultures with a high concentration of MG132, neuronal death peaked at 4 h (Fig. 4A), whereas at a low MG132 concentration

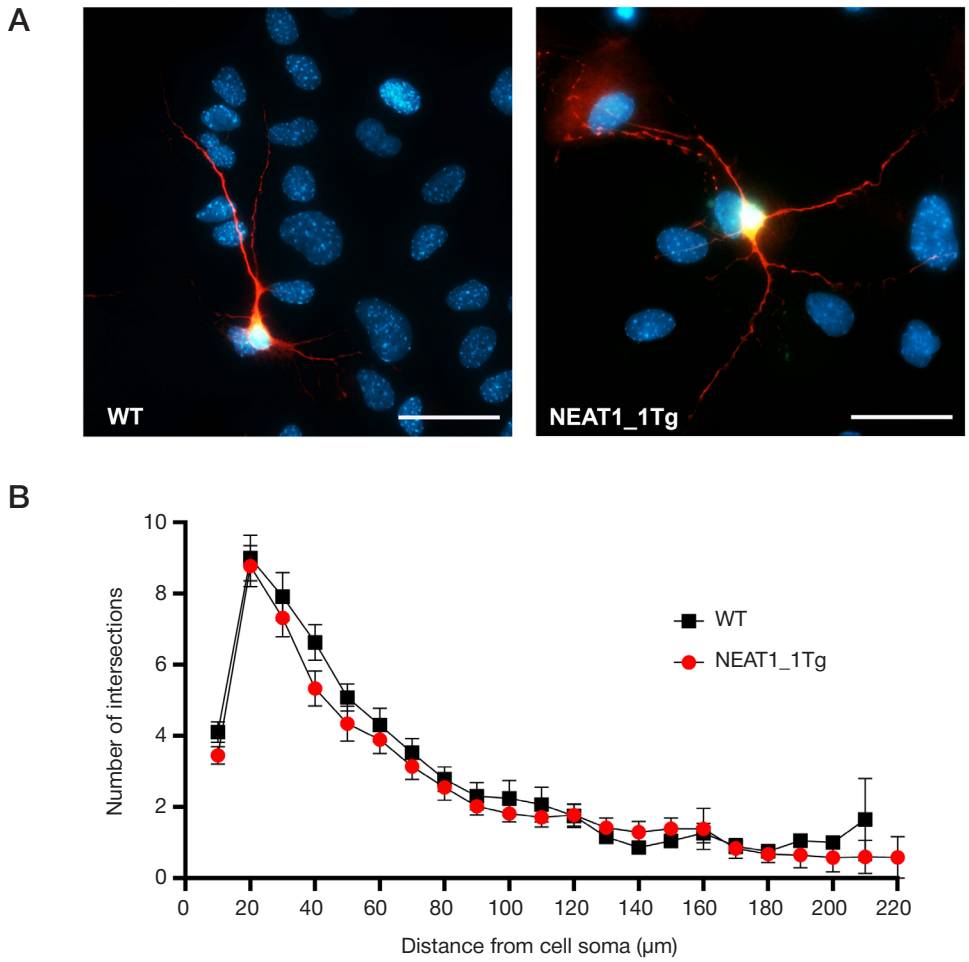

Fig. 2. Morphofunctional characterization of primary neurons. **A**. Representative photographs of wild-type (WT) and transgenic neurons (NEAT1_1Tg). Immunocytochemical staining for microtubule-associated protein Tau (shown in red), neuronal marker NeuN (shown in green), and cell nuclei stained with DAPI. The scale bar is 50 μm. **B**. Analysis of branching of primary neuron processes (Sholl-analysis). The graph shows the average number of intersections of circles of a given radius with processes. Multiple t-test with Holm-Sidak correction

it peaked at 12 h (Fig. 4B). In each case, neuronal death was significantly higher in cultures expressing the NEAT1_1Tg transgene.

Response to ER-stress is altered in transgenic NEAT1_1Tg cultures

To determine the possible mechanism of increased death of cells, particularly neurons, in transgenic neuronal cultures expressing human NEAT1_1, we analyzed the expression of genes involved in signaling pathways of cellular response to ER stress as well as apoptosis genes. It was shown that 4 h after treatment with 10 μM MG132, there was an increase in the expression of the following ER stress marker genes: transcription factor *Atf4*, proapoptotic gene *Chop* (*Ddit3*), chaperone Hspa5 (*Grp78* or *BiP*) (Fig. 5A–B). Similar changes in the expression of these genes occurred in transgenic cultures after MG132 treatment. At the same time, the expression of *Atf4* and *Ddit3* did not reach the levels observed in WT cultures after ER stress induction. The expression level of the gene encoding ER protein *Emc4* having protective properties under stress [21] was significantly reduced in NEAT1_1Tg cultures both under normal conditions and under ER stress (Fig. 5G).

Caspase-3 (*Casp3*) mRNA levels did not differ between transgenic and control cultures and did not change in response to stress (Fig. 5D). Analysis of expression of apoptosis inhibitor genes (*Bcl2l1*, *Bcl2l2*, *Mcl1*) (Fig. 5E–Z) showed that induction of moderate ER stress in WT cultures resulted in increased mRNA levels for *Bcl2l2* and *Mcl1* genes, whereas *Bcl2l1* expression remained unchanged. In contrast, in transgenic NEAT1_1Tg cultures, no significant activation of *Bcl2l2* and *Mcl1* was observed under stress, with some upward trend for *Mcl1* though.

DISCUSSION

NEAT1 has been convincingly shown to be involved in various pathological processes associated with neurodegeneration, including neuroinflammation and apoptosis [5]. *NEAT1* gene promoter has a binding site with p53 protein which is capable of increasing NEAT1 levels [22]. An extended experimental data indicates a protective role of NEAT1. In mouse experiments using an adenoviral vector for RNA delivery, Neat1_1 was shown to have an anti-apoptotic effect in a brain injury model as well as in cell culture of hippocampal neurons from HT-22 mice under hypoxia and glucose deficiency [23]. NEAT1 knockdown in human neuroblastoma SH-SY5Y cells increased apoptotic death in the simulation of viral infection using double-stranded RNA treatment [12]. At the same time, NEAT1 overexpression can stimulate ferroptosis in HepG2 hepatocarcinoma cells [24], and inhibition of NEAT1 in toxic (MPP^+) cell and animal models of Parkinson's disease led to a decrease in cell death and proapoptotic markers [25, 26]. Thus there are conflicting data on the effect of NEAT1 on cell death. Specific pro- or anti-apoptotic effect of NEAT1 may possibly depend on a type of damaging effect. Understanding the mechanism of NEAT1

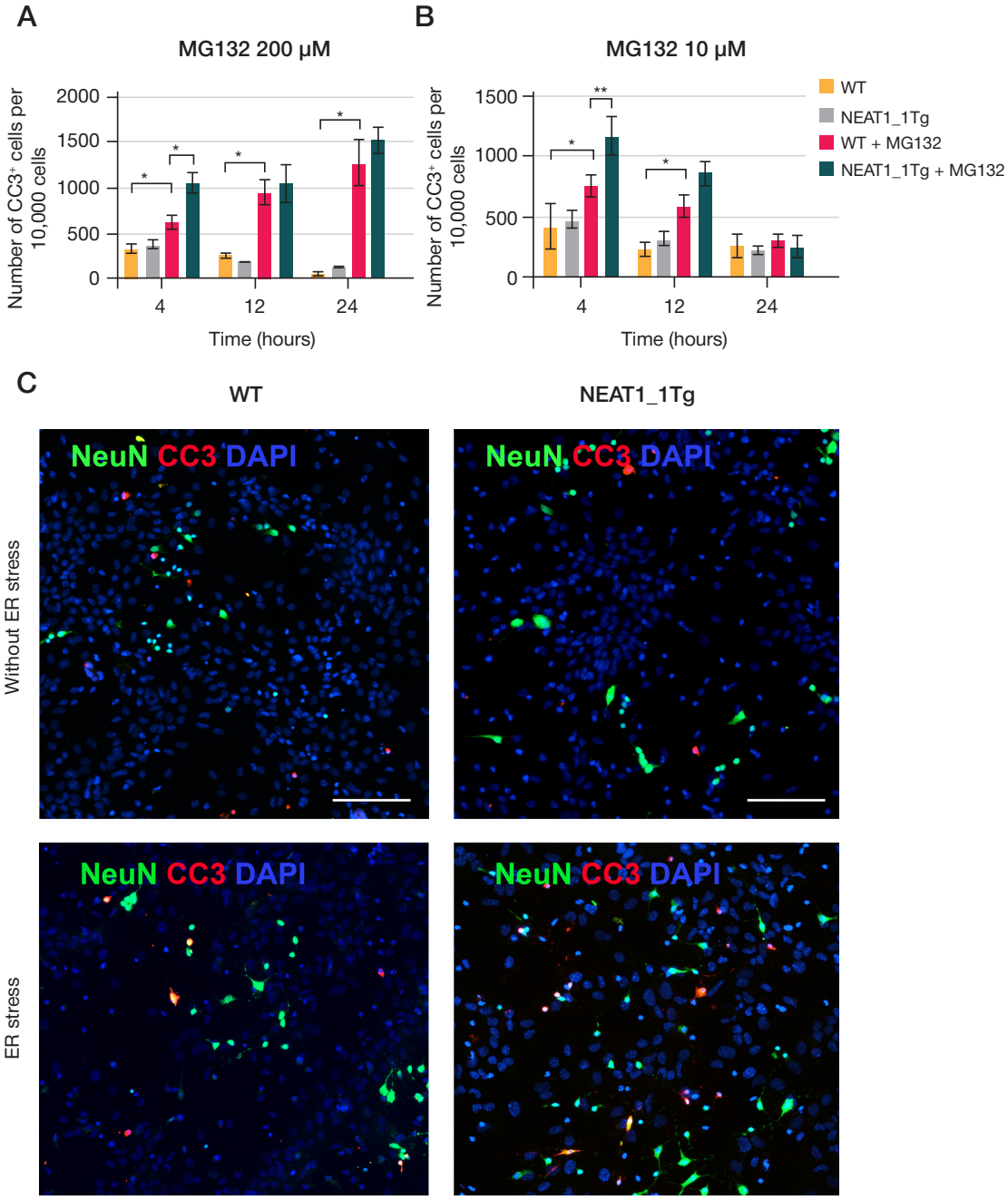

Fig. 3. Apoptotic death in transgenic (NEAT1_1Tg) primary cultures and wild-type (WT) cultures. **A**. Apoptosis in cultures when treated with 200 μM MG132. **B**. Apoptosis in cultures when treated with 10 μM MG132. Two-factor analysis of variance, Fisher's test. * — $p < 0.05$; ** — $p < 0.01$. **B**. Representative photographs of cell cultures upon treatment with 10 μM MG132 after 12 h. Neurons stained for the differentiated neuron marker NeuN (shown in *green*), the apoptotic cell death marker activated caspase 3, CC3 (shown in *red*), and nuclei stained with DAPI (shown in *blue*). Scale section is 100 μm

action is further complicated by the fact that unlike *in vivo* nervous tissue and primary neurons, in most cell cultures the long isoform (NEAT1_2), a basis for paraspecles assembly, is expressed as well [5, 12]. Formation of paraspecles, in turn, can be stimulated by cellular stress of various types including proteasome inhibition [27]. Therefore, it is difficult to identify independent roles of short and long NEAT1 isoforms in the cellular response to stress.

We have shown that ectopic expression of the transgene encoding the short isoform of human NEAT1_1 in the mouse nervous system had no effect on cellular composition (Fig. 1) and morphology of primary neuronal cultures (Fig. 2) derived from hippocampi. Induction of ER stress with the proteasome inhibitor MG132 resulted in apoptotic cell death of primary culture cells (Fig. 3), and apoptosis intensity depended on stress level (MG132 concentration). At the same time, the number of apoptotic cells was higher in cultures expressing the NEAT1_1 transgene compared with WT cells in stress conditions. As it was shown earlier for mouse embryonic Neat1 knockout fibroblasts, the absence of Neat1 increases death of these cells in response to MG132 treatment [27]. Stable Neat1 levels might be important for cell survival under stress or its effect on cell death depends on type of cells. The primary culture used in this study was mixed and contained both neurons and glial cells. This made it possible to assess survival of neurons in the experiment under the mutual influence of different cell populations of neuronal origin. To determine the specific effect of increasing NEAT1_1 levels on neuronal survival, we estimated the number of cells expressing both the mature neuronal marker NeuN and activated caspase-3 (CC3). Stress resulted in a more

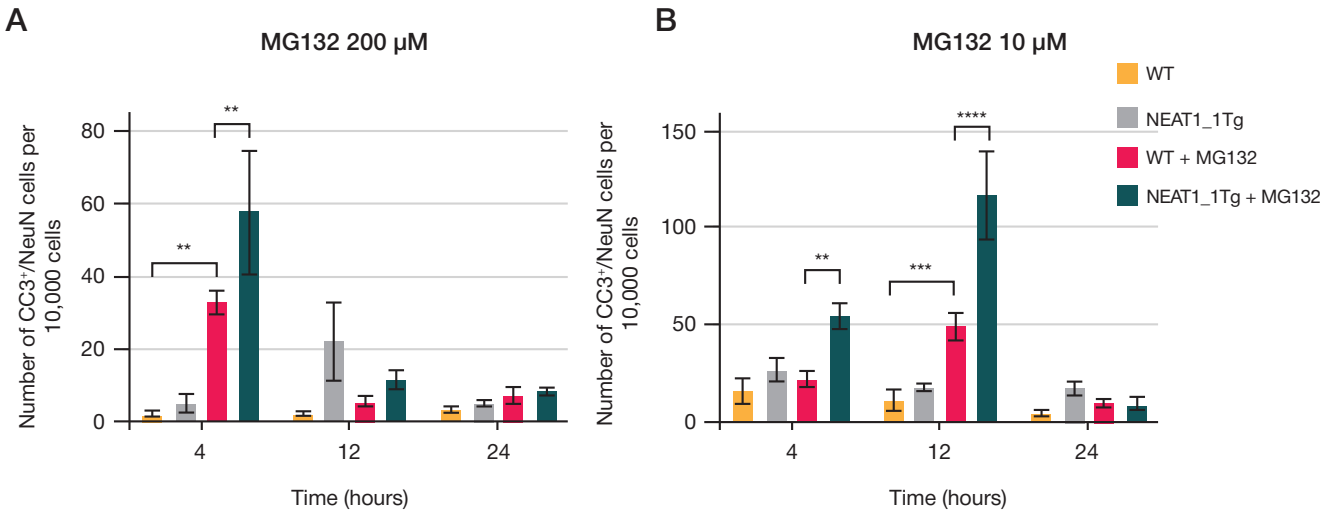

Fig. 4. Apoptotic neuronal death in transgenic (NEAT1_1Tg) primary and wild-type (WT) cultures. **A**. Apoptosis in cultures treated with 200 μM MG132. **B**. Apoptosis in cultures treated with 10 μM MG132. Two-way analysis of variance, Fisher's test. ** — $p < 0.01$; *** — $p < 0.001$; **** — $p < 0.0001$

intensive neuronal death in transgenic cultures (NEAT1_1Tg) compared with wild-type cultures (WT) (Fig. 4). Thus, NEAT1_1 expression enhanced apoptosis of primary neurons under ER stress. An increased number of apoptotic neurons in transgenic cultures without treatment compared with control cultures was observed for all time points. Although these differences are not statistically significant, it can be assumed that there is some predisposition of transgenic neurons to trigger apoptosis under normal conditions, and this should be investigated further.

Cellular ER stress response includes three different signaling pathways based on kinases acting as stress sensors: PERK, ATF6, and IRE1 [16]. When they are activated, an overall protein synthesis is inhibited and protective mechanisms are triggered, primarily aimed at adapting to stress and restoring protein homeostasis. However, under severe stress or its prolonged persistence, the balance shifts towards activation of proapoptotic signaling pathways leading to cell death [28]. We have shown that ER stress causes activation of the PERK pathway evidenced by an increase in *Atf4* mRNA levels however, this activation was less in NEAT1_1Tg cultures (Fig. 5). Accordingly, activation of proapoptotic *Ddit3* (*Chop*) gene which belongs to the same PERK/ATF4/CHOP signaling pathway and acting as activator of cell transition to apoptosis under ER stress, was also reduced in NEAT1_1Tg cultures [29]. In both

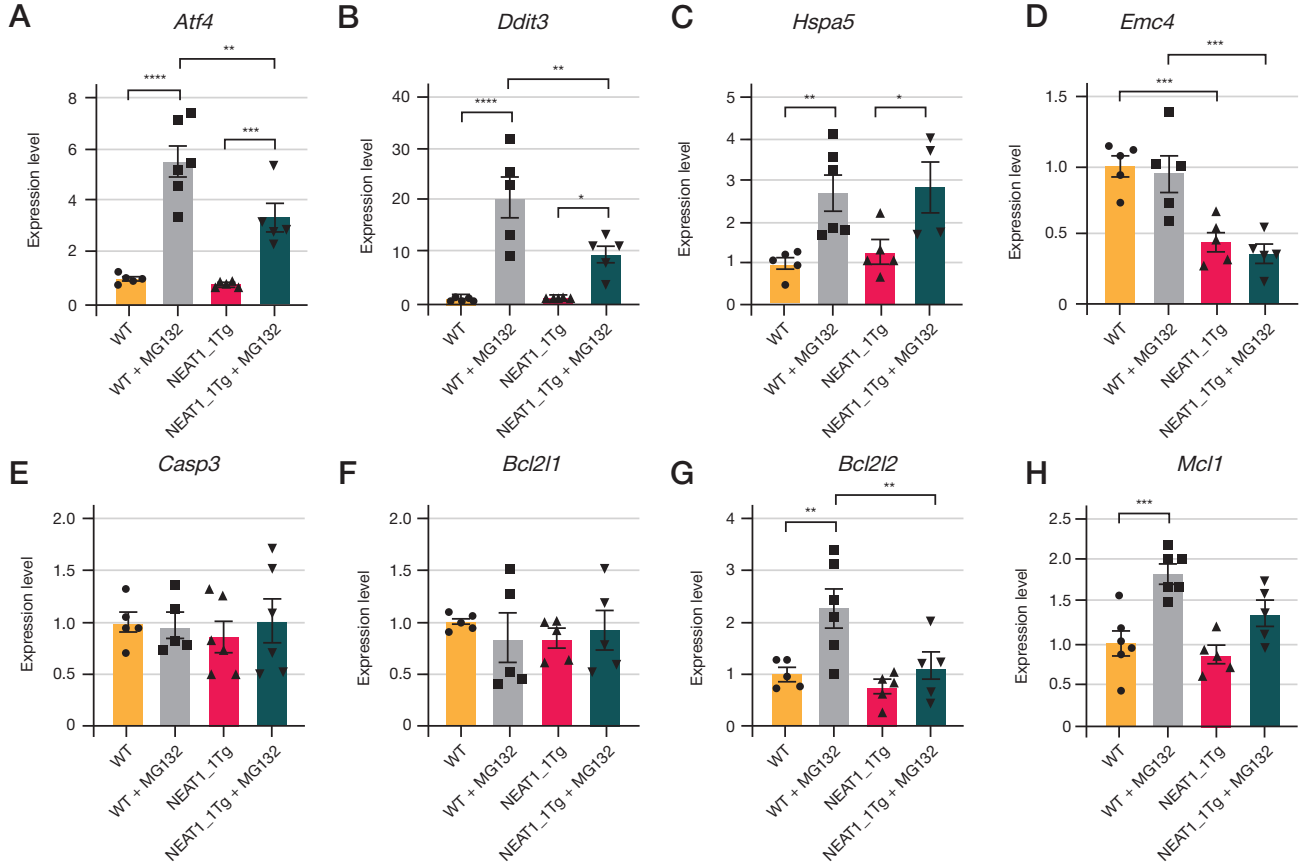

Fig. 5. Gene expression changes in transgenic (NEAT1_1Tg) primary and wild-type (WT) cultures treated with 10 μM MG132. **A–G**. Expression of genes associated with ER stress. **D–Z**. Expression of apoptosis-related genes. One-way analysis of variance, Fisher's test. * — $p < 0.05$; ** - $p < 0.01$, *** — $p < 0.001$; **** — $p < 0.0001$

cultures, expression of Hspa5 chaperone gene (BiP) increased under ER stress. Rapid response of NEAT1_1Tg cells to stress and triggering of apoptosis through caspase-3 activation could be explained by initial differences in the amounts of caspase-3 inactive form in cells. Analysis of caspase-3 (*Casp3*) expression showed that at least at the initial stage of stress development, 4 h after MG132 treatment of cells, its levels were similar in all four groups. Since the number of cells stained with antibodies against caspase-3 activated form was increased in 4 hours after the stress induction, the difference between NEAT1_1Tg- and WT-cell death levels is likely to be due to some other regulatory factors, in particular the cleavage of procaspase-3 with formation of its activated form. We analyzed the expression of the following apoptosis inhibitor genes: *Bcl2l1*, *Bcl2l2*, and *Mcl1* [30]. *Bcl2l1* expression did not change in response to ER stress. For *Bcl2l2* and *Mcl1* genes, an increase in their levels was observed in WT cultures while in NEAT1_1Tg cultures such activation was practically absent for *Bcl2l2* and was significantly less for *Mcl1*. In NEAT1_1Tg cultures, there was a decrease in mRNA level of *Emc4* gene related to conserved transmembrane proteins required for protein stacking in ER. The lack of Emc proteins can lead to ER stress [31]. Thus, an increased level of NEAT1_1 in neuronal cultures during ER stress results in a decrease in regulatory anti-apoptotic signals which enhances cell death including neuronal death.

CONCLUSIONS

Additional expression of transgenic human NEAT1_1 long non-coding RNA under ER stress promotes apoptotic neuronal death in primary hippocampal cultures. Increasing levels of this RNA in the nervous system of patients suffering neurodegenerative diseases (ALS, FTD, HD) with protein aggregation and ER stress can be considered as a pathogenetic factor.

References

1. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs. Cell. 2018; 172 (3): 393–407.
2. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009; 10 (3): 155–9.
3. Zimmer-Bensch G. Emerging Roles of Long Non-Coding RNAs as Drivers of Brain Evolution. Cells. 2019; 8 (11).
4. Wan P, Su W, Zhuo Y. The Role of Long Noncoding RNAs in Neurodegenerative Diseases. Mol Neurobiol. 2017; 54 (3): 2012–21.
5. An H, Williams NG, Shelkovnikova TA. NEAT1 and paraspeckles in neurodegenerative diseases: A missing lnc found? Noncoding RNA Res. 2018; 3 (4): 243–52.
6. Tollervey JR, Curk T, Rogelj B, Briese M, Cereda M, Kayikci M, et al. Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. Nat Neurosci. 2011; 14 (4): 452–8.
7. Sunwoo JS, Lee S, Kim JH, Lim JA, Kim TJ, Byun JI, et al. Altered functional connectivity in idiopathic rapid eye movement sleep behavior disorder: a resting-state EEG study. Sleep. 2017; 40 (6).
8. Cheng C, Spengler RM, Keiser MS, Monteys AM, Rieders JM, Ramachandran S, et al. The long non-coding RNA NEAT1 is elevated in polyglutamine repeat expansion diseases and protects from disease gene-dependent toxicities. Hum Mol Genet. 2018; 27 (24): 4303–14.
9. An H, Tan JT, Shelkovnikova TA. Stress granules regulate stress-induced paraspeckle assembly. J Cell Biol. 2019; 218 (12): 4127–40.
10. Kukharsky MS, Ninkina NN, An H, Telezhkin V, Wei W, Meritens CR, et al. Long non-coding RNA Neat1 regulates adaptive behavioural response to stress in mice. Transl Psychiatry. 2020; 10 (1): 171.
11. Nishimoto Y, Nakagawa S, Hirose T, Okano HJ, Takao M, Shibata S, et al. The long non-coding RNA nuclear-enriched abundant transcript 1_2 induces paraspeckle formation in the motor neuron during the early phase of amyotrophic lateral sclerosis. Mol Brain. 2013; 6: 31.
12. Shelkovnikova TA, Kukharsky MS, An H, Dimasi P, Alexeeva S, Shabir O, et al. Protective paraspeckle hyper-assembly downstream of TDP-43 loss of function in amyotrophic lateral sclerosis. Mol Neurodegener. 2018; 13 (1): 30.
13. Matsukawa K, Kukharsky MS, Park SK, Park S, Watanabe N, Iwatsubo T, et al. Long non-coding RNA NEAT1_1 ameliorates TDP-43 toxicity in in vivo models of TDP-43 proteinopathy. RNA Biol. 2021; 18 (11): 1546–54.
14. Nakagawa S, Naganuma T, Shioi G, Hirose T. Paraspeckles are subpopulation-specific nuclear bodies that are not essential in mice. J Cell Biol. 2011; 193 (1): 31–9.
15. Wiseman RL, Mesgarzadeh JS, Hendershot LM. Reshaping endoplasmic reticulum quality control through the unfolded protein response. Mol Cell. 2022; 82 (8): 1477–91.
16. Kukharsky MS, Everett MW, Lytkina OA, Raspopova MA, Kovrazhkina EA, Ovchinnikov RK, et al. Protein homeostasis dysregulation in pathogenesis of neurodegenerative diseases. Mol Biol (Mosk). 2022; 56 (6): 1044–56.
17. Merighi A, Lossi L. Endoplasmic reticulum stress signaling and neuronal cell death. Int J Mol Sci. 2022; 23 (23).
18. Galehdar Z, Swan P, Fuerth B, Callaghan SM, Park DS, Cregan SP. Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. J Neurosci. 2010; 30 (50): 16938–48.
19. Deikin AV, Kovrazhkina EA, Ovchinnikov RK, Bronovitskii EV, Razinskaia OD, Smirnov AP, et al. A mice model of amyotrophic lateral sclerosis expressing mutant human FUS protein. Zh Nevrol Psikhiatr Im S S Korsakova. 2014; 114 (8): 62–9.
20. Kukharsky MS, Quintiero A, Matsumoto T, Matsukawa K, An H, Hashimoto T, et al. Calcium-responsive transactivator (CREST) protein shares a set of structural and functional traits with other proteins associated with amyotrophic lateral sclerosis. Mol Neurodegener. 2015; 10: 20.
21. Bai L, You Q, Feng X, Kovach A, Li H. Structure of the ER membrane complex, a transmembrane-domain insertase. Nature. 2020; 584 (7821): 475–8.
22. Mello SS, Sinow C, Raj N, Mazur PK, Bieging-Rolett K, Broz DK, et al. Neat1 is a p53-inducible lincRNA essential for transformation suppression. Genes Dev. 2017; 31 (11): 1095–108.
23. Zhong J, Jiang L, Huang Z, Zhang H, Cheng C, Liu H, et al. The long non-coding RNA Neat1 is an important mediator of the therapeutic effect of bexarotene on traumatic brain injury in mice. Brain Behav Immun. 2017; 65: 183–94.
24. Zhang Y, Luo M, Cui X, O'Connell D, Yang Y. Long noncoding RNA NEAT1 promotes ferroptosis by modulating the miR-362-3p/MIOX axis as a ceRNA. Cell Death Differ. 2022; 29 (9): 1850–63.
25. Dong LI, Zheng Y, Gao L, Luo X. lncRNA NEAT1 prompts autophagy and apoptosis in MPTP-induced Parkinson's disease by impairing miR-374c-5p. Acta Biochim Biophys Sin (Shanghai). 2021; 53 (7): 870–82.
26. Liu Y, Lu Z. Long non-coding RNA NEAT1 mediates the toxic of Parkinson's disease induced by MPTP/MPP+ via regulation of gene expression. Clin Exp Pharmacol Physiol. 2018; 45 (8): 841–8.
27. Hirose T, Virnicchi G, Tanigawa A, Naganuma T, Li R, Kimura H, et al. NEAT1 long noncoding RNA regulates transcription via protein sequestration within subnuclear bodies. Mol Biol Cell. 2014; 25 (1): 169–83.
28. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, et al. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol. 2006; 4 (11): e374.
29. Hu H, Tian M, Ding C, Yu S. The C/EBP Homologous Protein (CHOP) Transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. Front Immunol.

2018; 9: 3083.
30. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. Mol Cell. 2010; 37 (3): 299–310.
31. Tian S, Wu Q, Zhou B, Choi MY, Ding B, Yang W, et al. Proteomic analysis identifies membrane proteins dependent on the ER membrane protein complex. Cell Rep. 2019; 28 (10): 2517–26 e5.

Литература

1. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs. Cell. 2018; 172 (3): 393–407.
2. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009; 10 (3): 155–9.
3. Zimmer-Bensch G. Emerging Roles of Long Non-Coding RNAs as Drivers of Brain Evolution. Cells. 2019; 8 (11).
4. Wan P, Su W, Zhuo Y. The Role of Long Noncoding RNAs in Neurodegenerative Diseases. Mol Neurobiol. 2017; 54 (3): 2012–21.
5. An H, Williams NG, Shelkovnikova TA. NEAT1 and paraspeckles in neurodegenerative diseases: A missing lnc found? Noncoding RNA Res. 2018; 3 (4): 243–52.
6. Tollervey JR, Curk T, Rogelj B, Briese M, Cereda M, Kayikci M, et al. Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. Nat Neurosci. 2011; 14 (4): 452–8.
7. Sunwoo JS, Lee S, Kim JH, Lim JA, Kim TJ, Byun JI, et al. Altered functional connectivity in idiopathic rapid eye movement sleep behavior disorder: a resting-state EEG study. Sleep. 2017; 40 (6).
8. Cheng C, Spengler RM, Keiser MS, Monteys AM, Rieders JM, Ramachandran S, et al. The long non-coding RNA NEAT1 is elevated in polyglutamine repeat expansion diseases and protects from disease gene-dependent toxicities. Hum Mol Genet. 2018; 27 (24): 4303–14.
9. An H, Tan JT, Shelkovnikova TA. Stress granules regulate stress-induced paraspeckle assembly. J Cell Biol. 2019; 218 (12): 4127–40.
10. Kukharsky MS, Ninkina NN, An H, Telezhkin V, Wei W, Meritens CR, et al. Long non-coding RNA Neat1 regulates adaptive behavioural response to stress in mice. Transl Psychiatry. 2020; 10 (1): 171.
11. Nishimoto Y, Nakagawa S, Hirose T, Okano HJ, Takao M, Shibata S, et al. The long non-coding RNA nuclear-enriched abundant transcript 1_2 induces paraspeckle formation in the motor neuron during the early phase of amyotrophic lateral sclerosis. Mol Brain. 2013; 6: 31.
12. Shelkovnikova TA, Kukharsky MS, An H, Dimasi P, Alexeeva S, Shabir O, et al. Protective paraspeckle hyper-assembly downstream of TDP-43 loss of function in amyotrophic lateral sclerosis. Mol Neurodegener. 2018; 13 (1): 30.
13. Matsukawa K, Kukharsky MS, Park SK, Park S, Watanabe N, Iwatsubo T, et al. Long non-coding RNA NEAT1_1 ameliorates TDP-43 toxicity in in vivo models of TDP-43 proteinopathy. RNA Biol. 2021; 18 (11): 1546–54.
14. Nakagawa S, Naganuma T, Shioi G, Hirose T. Paraspeckles are subpopulation-specific nuclear bodies that are not essential in mice. J Cell Biol. 2011; 193 (1): 31–9.
15. Wiseman RL, Mesgarzadeh JS, Hendershot LM. Reshaping endoplasmic reticulum quality control through the unfolded protein response. Mol Cell. 2022; 82 (8): 1477–91.
16. Kukharsky MS, Everett MW, Lytkina OA, Raspopova MA, Kovrazhkina EA, Ovchinnikov RK, et al. Protein homeostasis dysregulation in pathogenesis of neurodegenerative diseases. Mol Biol (Mosk). 2022; 56 (6): 1044–56.
17. Merighi A, Lossi L. Endoplasmic reticulum stress signaling and neuronal cell death. Int J Mol Sci. 2022; 23 (23).
18. Galehdar Z, Swan P, Fuerth B, Callaghan SM, Park DS, Cregan SP. Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. J Neurosci. 2010; 30 (50): 16938–48.
19. Deikin AV, Kovrazhkina EA, Ovchinnikov RK, Bronovitskii EV, Razinskaia OD, Smirnov AP, et al. A mice model of amyotrophic lateral sclerosis expressing mutant human FUS protein. Zh Nevrol Psikhiatr Im S S Korsakova. 2014; 114 (8): 62–9.
20. Kukharsky MS, Quintiero A, Matsumoto T, Matsukawa K, An H, Hashimoto T, et al. Calcium-responsive transactivator (CREST) protein shares a set of structural and functional traits with other proteins associated with amyotrophic lateral sclerosis. Mol Neurodegener. 2015; 10: 20.
21. Bai L, You Q, Feng X, Kovach A, Li H. Structure of the ER membrane complex, a transmembrane-domain insertase. Nature. 2020; 584 (7821): 475–8.
22. Mello SS, Sinow C, Raj N, Mazur PK, Bieging-Rolett K, Broz DK, et al. Neat1 is a p53-inducible lincRNA essential for transformation suppression. Genes Dev. 2017; 31 (11): 1095–108.
23. Zhong J, Jiang L, Huang Z, Zhang H, Cheng C, Liu H, et al. The long non-coding RNA Neat1 is an important mediator of the therapeutic effect of bexarotene on traumatic brain injury in mice. Brain Behav Immun. 2017; 65: 183–94.
24. Zhang Y, Luo M, Cui X, O'Connell D, Yang Y. Long noncoding RNA NEAT1 promotes ferroptosis by modulating the miR-362-3p/MIOX axis as a ceRNA. Cell Death Differ. 2022; 29 (9): 1850–63.
25. Dong LI, Zheng Y, Gao L, Luo X. lncRNA NEAT1 prompts autophagy and apoptosis in MPTP-induced Parkinson's disease by impairing miR-374c-5p. Acta Biochim Biophys Sin (Shanghai). 2021; 53 (7): 870–82.
26. Liu Y, Lu Z. Long non-coding RNA NEAT1 mediates the toxic of Parkinson's disease induced by MPTP/MPP+ via regulation of gene expression. Clin Exp Pharmacol Physiol. 2018; 45 (8): 841–8.
27. Hirose T, Virnicchi G, Tanigawa A, Naganuma T, Li R, Kimura H, et al. NEAT1 long noncoding RNA regulates transcription via protein sequestration within subnuclear bodies. Mol Biol Cell. 2014; 25 (1): 169–83.
28. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, et al. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol. 2006; 4 (11): e374.
29. Hu H, Tian M, Ding C, Yu S. The C/EBP Homologous Protein (CHOP) Transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. Front Immunol. 2018; 9: 3083.
30. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. Mol Cell. 2010; 37 (3): 299–310.
31. Tian S, Wu Q, Zhou B, Choi MY, Ding B, Yang W, et al. Proteomic analysis identifies membrane proteins dependent on the ER membrane protein complex. Cell Rep. 2019; 28 (10): 2517–26 e5.