Cell Stress and Chaperones (2021) 26:617–627
https://doi.org/10.1007/s12192-021-01219-z

MINI REVIEW

Heat shock proteins: a history of study in Russia

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Received: 7 June 2021 / Revised: 18 June 2021 / Accepted: 21 June 2021 / Published online: 28 June 2021

Abstract

This review describes a brief history of the discovery and studies in Russia and associated countries of the main stress protein (Hsp70) that plays important roles both in the normal function of the cell and body as well as under various stressful stimuli. Research on this protein at the Institute of Molecular Biology (Moscow) began with the elucidation of its adaptive functions at the cellular level and at the level of the whole organism. These studies examined the function of Hsp70 under normal and extreme conditions using a wide range of model and non-model animal species, from *Leishmania* and *Drosophila* to camels and humans. These analyses made it possible to elucidate the primary regulations in the evolution and function of heat shock (HS) genes in the studied organisms. Next, we studied the structure and characteristic features of heat shock genes and proteins in species with contrasting habitat temperatures. The systems of Hsp70 expression and isolation we developed using various research objects allowed us to proceed to study the protective properties of human recombinant Hsp70 in normal-aging animal models as well as animal models experiencing sepsis, Alzheimer's disease, and stroke. The results obtained open the prospects of using recombinant Hsp70 for the treatment of various neuropathologies in humans. This review describes the logic and history of investigation of Hsp70 performed by one group of scientists from Engelhardt Institute of Molecular Biology, Russian Academy of Sciences. It was not the goal of this paper to give a comprehensive general picture of other similar studies carried out in Russia during this period.

Keywords Heat shock proteins · Hsp70 · Adaptation · Inflammation · Ageing · Sepsis · Alzheimer's disease · Transcriptomics

Discovery of heat shock proteins

It all started with a curious incident in a laboratory in Italy sometimes described as serendipity (Ritossa 1962). The now famous Italian geneticist Ferruccio Ritossa sat at a light microscope observing *Drosophila* larval chromosome squashes. They showed a different puffing pattern than he expected. Ritossa determined that the larvae had been accidentally placed in a 37°C incubator before the chromosomes were prepared for viewing.

The numerous swellings or "puffs" that represent active genes had disappeared, and several new large puffs had appeared in places where they did not exist prior to the heat shock (HS). Importantly, this pattern was repeated after every HS. Ritossa made his discovery in 1962, but at that time, it did not arouse much interest (Ritossa 1962) and was considered just a laboratory artefact. Almost 15 years later, nearly simultaneously and independently in two leading genetic centres in England and the USA, researchers returned to Ritossa's observation at a molecular level and showed that in these puffs that arise in response to HS, RNAs that encode a special group of proteins that were mistakenly called "heat shock proteins" are synthesized (Lewis et al. 1975; Tissiéres et al. 1974). This was a misnomer because the same puffs, and therefore the proteins, arise in the chromosomes of flies after HS and under the influence of dozens of other harmful effects, including anaerobic conditions, exposure to heavy metal salts, arsenic compounds, alcohol, and LSD (Lindquist 1986; Evgen'ev et al. 2014). Therefore, it would have been more correct to call these proteins "stress proteins", but the original name, "heat shock proteins" remained, and it is still customary to call a group of genes induced by HS or other stresses heat shock genes. At the time of their discovery, it was not yet known how conservative these genes and, accordingly, proteins were; in other words, it was unknown how similar their primary structure was among both closely and distantly related organisms. To answer this question, in 1978, we decided to isolate RNA after HS from one fly species, *Drosophila*

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melanogaster, label this RNA with an isotope (^{125}I), and hybridize it (i.e. apply the labelled RNA to the polytene chromosome preparation) using chromosomes from another very distantly related fly species (*D. virilis*). There are approximately 50–60 million years of evolution between these species. To our delight, the RNA from *D. melanogaster* perfectly hybridized with the chromosomes of the distant species, and the radioactive label was localized only in *D. virilis* puffs that occurred after HS (Evgen'ev et al. 1978). Therefore, we established that *Hsp* genes are very conserved and have a similar sequence, even in very distantly related organisms. Subsequently, other researchers showed that the homology of the main *hs* gene, *Hsp70*, which has a molecular weight of 70 kDa, between mice and humans is greater than 90%, and that between *Escherichia coli* and humans is 55% (Evgen'ev et al. 2014; Hunt and Morimoto 1985).

Next, we were interested in understanding how, after an increase in temperature, the entire "battery" of *Hs* genes located on different chromosomes undergoes coordinated and very fast (3–5 min) simultaneous activation. Apparently, there must be some gene or several regulatory genes that quickly turn on all the *Hs* genes in response to stressful stimuli. The question was how to find these genes in the "haystack"—that is, in the *Drosophila* genome. Here, the well-studied genetics of this model organism came to our aid. For many years, geneticists from different countries had studied lethal mutations, such as those that lead to the death of a fly during a particular stage of development (egg, larva, pupa, or imago). A special group comprises so-called temperature-sensitive mutations (ts-lethals), that is, damaged genes that encode a defective protein that can somehow function at normal temperatures but lead to the death of the fly at elevated temperatures. We suggested that, if indeed there was a specialized gene or genes that regulated the battery of *Hs* genes, then mutations in such a "switch" should be lethal and belong to a category of polyphase temperature-sensitive lethal genes. Mutations in such genes lead to the death of a fly in response to an increase in temperature during any stage of development. An analysis of the literature showed that there are very few polyphasic ts-lethals, and we obtained all of them (five strains) from the *Drosophila* collection in the late 1970s. The flies were tested simply by administering HS; after HS, we monitored how much of the radioactive label (^{35}S-methionine) was incorporated in the proteins after HS compared to the control wild-type flies. Our results revealed that the second ts-lethal strain tested in this way after HS had incorporated the label ten times less than the rest of the studied lines, including the control strain (Evgen'ev et al. 1979). However, all HS-induced puffs in this strain were of normal size after the temperature elevation. Therefore, the "switch" worked fine, the *hs* genes were induced, and the observed compromised synthesis of Hsps in this strain did not result from inefficient transcriptional induction of the corresponding genes. Subsequently, it turned out that our logic was generally correct, and later, the American scientist Carl Wu described the desired *Drosophila* heat shock factor (HSF1) that regulates all *hs* genes at once (Wu 1995) using our logic, as he later communicated in a private conversation. Soon after, a similar transcription factor was discovered in humans (Morimoto 1998). Although Carl Wu's findings were more fortuitous, the ts-lethal we selected and investigated in detail was also very interesting. Later, scientists from different countries showed that the gene we were studying, known as *sbr* in *Drosophila*, is responsible for the transport of RNA from the nucleus to the cytoplasm, and its homologue was identified in various organisms from yeast to humans (Tret'iakova et al. 2001).

Heat shock genes and adaptations to extreme environmental conditions

After the discovery of Hsps and the description of the expression of corresponding genes in various organisms, molecular work was performed to investigate this interesting system from different points of view, including the chromatin structure of these loci and the features of their transcription and translation. Indeed, the HS gene system was ideal for studying the regulation of gene expression in response to heat stress in a cell culture or an entire body of a model organism to elucidate the consequences of this challenge.

In the early 1980s, the molecular aspects of the expression of *hs* genes were also studied in the laboratory of Prof. A. D. Mirzabekov at the IMB RAS; in particular, his group was the first to describe the characteristic changes in chromatin that occur in the regulatory regions of *hs* genes before and after temperature elevation (Karpov et al. 1984). A chance conversation with Andrey Mirzabekov determined the direction of my research on the *hs* gene system for many years. Once we had lunch together in the dining room of IMB and discussed *hs* genes in flies, and Andrey suddenly asked: "…and what do you think; how do the hs genes work in organisms that inhabit, say, the deserts of Central Asia or the Sahara and every day they have to warm up and live at elevated, and sometimes extreme, temperatures?". I could not answer this question at that time, but I began to think.... Indeed, I thought, if two closely related species have lived in temperature-contrasting environments for millions of years, they must have evolved mechanisms to survive in thermally distinct environments. It is possible that the *hs* gene system, which plays a crucial role in survival when temperatures rise, has evolved differently in such species. Although the system of *hs* genes was being actively investigated at that time, a massive endeavour was being made primarily on the molecular structure of *hs* genes and the details of their regulation. Biochemists and molecular biologists were conducting research on this system at that time (in the 1980s), and news of the discovery of the *hs* gene

system had not yet reached field zoologists and evolutionary biologists. We had a head start of nearly 10 years in this respect, and we tried to take full advantage of it.

In our laboratory, the cell lines of two silkworm species were identified for different reasons. One line was obtained from the gypsy moth, which lives in the middle belt of Russia and, from time to time, ravages the forests of the Moscow region. Another line was obtained from a silkworm species, the main producer of silk. This species is of southern origin and is found in Central Asia and Southern China as well as in other regions with hot climates.

In 1985–1986, my colleague Vladimir Scheinker and I compared the incorporation of a radioactive label into the proteins of these two cultured cell lines at different temperatures and obtained amazing data. Cells of the northern species (gypsy moth) are able to synthesize Hsps at temperatures in the range of 31–37°C, similar to cultured *Drosophila* cells, while cells of the southern species of silkworm can incorporate a label in Hsps at extreme temperatures, up to 48°C (Figure 1) (Evgen'ev et al. 1987).

In other words, we obtained the exact results we expected. Species that inhabit zones with elevated temperatures were able to synthesize Hsps at temperatures much higher than their northern "relatives". Everything would turn out fine, but I could not help but think that we were dealing with an artificial situation in which a cell culture that had been grown in the laboratory for a long time could undergo changes in its original characteristics during its growth and development. It would be better to compare the living representatives of these contrasting species rather than their cell cultures. But where can one obtain them? Then, one of those "accidents" occurred that often radically change our fate. A stout, mustachioed, and always smiling young man looked into my laboratory and introduced himself: "Khayot Ulmasov from Ashgabat". This encounter determined my research, and my fate in general, for the next several decades. Khayot had somehow learned about our "heat shock games" and suggested that I go to Ashgabat (Turkmenistan), where we would have the opportunity to compare the response to HS in vivo in two species of silkworm using the cell cultures of the species from which we had obtained such remarkable results in Moscow. I soon went to Ashgabat, where we conducted experiments in live silkworm individuals at different stages of development and fully confirmed the characteristic differences in the responses of these species to HS that we had observed earlier when exploring cell cultures of the same species.

In Ashgabat, I met and befriended local zoologists, and this joint work continued for many years until their political leader, Turkmenbashi demolished the Institute of Zoology where Khayot worked to build the Intourist Hotel, and my wonderful friend and student Khayot Ulmasov, who was excommunicated from science, died of a heart attack. Our constant contact with field zoologists, taxonomists, and ecologists allowed us to advance our

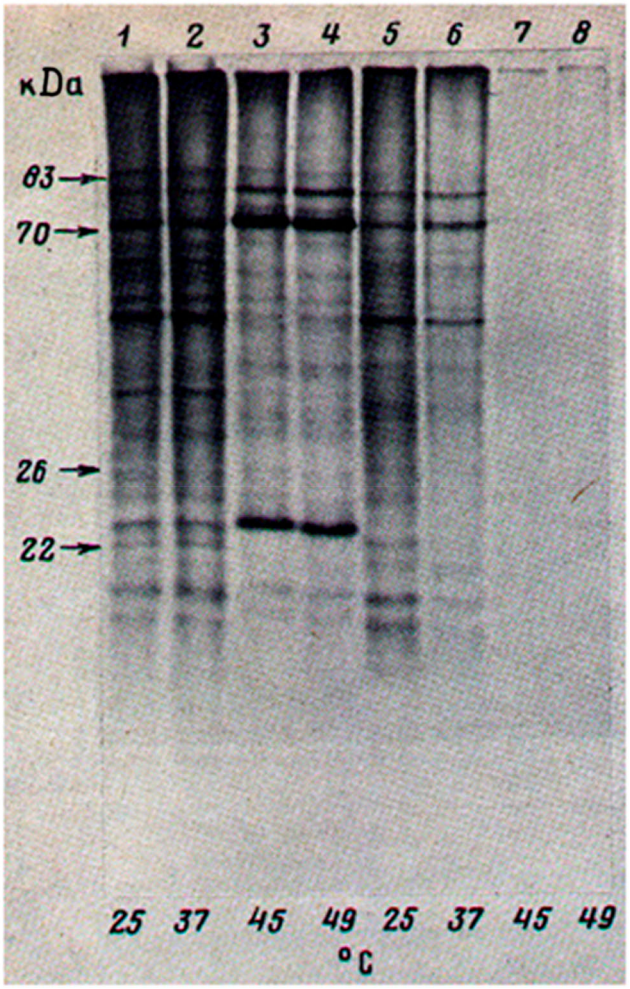


Fig. 1 One-dimensional, "historical" electrophoresis of labelled proteins of cell cultures of two silkworm species. 1–4, silkworm; 5–8, gypsy moth. The silkworm cells were able to incorporate a radioactive label (C^{14} amino acids) at temperatures of 45°C and 48°C, while the gypsy moth cells did not incorporate the label at these temperatures

understanding of the role of Hsps in adapting to extreme conditions and to overtake most other groups in other countries that were studying the role of stress genes from the environmental aspect by at least 10 years.

At that time, in the late 1980s, together with a few other scientists interested in the role of Hsps in adaptations (Lindquist 1986; Norris et al. 1995; White et al. 1994), we began to "plow" an almost uncultivated field connecting the activity and structure of *hs* genes and proteins with the adaptation of species to extreme living conditions. On the advice of local Turkmen zoologists, our next research target for several years was lizards. Local ecologists and herpetologists obtained several species of lizards for us that differed sharply in the temperature of their habitats. This "set" included desert lizards that were active during the hottest time of the day, a species of desert lizard that was active only at night, and, finally, a species from the temperate climate zone, representatives of which we caught for the experiments near Moscow. We used a range

of modern methods, including two-dimensional electrophoresis, as well as analyses of RNA and individual proteins. At that time, it was already known from the work of molecular biologists that proteins and, naturally, heat shock genes are a composite group, with members that differ in size and are located in different loci on the chromosomes (Parsell and Lindquist 1993; Morimoto 1998).

In our research, we focused primarily on the main inducible heat shock protein with a molecular weight of 70 kDa, Hsp70, which is usually encoded in a wide variety of organisms with several identical copies (Evgen'ev et al. 2014). Hsp70 undoubtedly occupies a special place among heat shock proteins and has been intensively studied in particular. Various molecular and biological studies have shown that Hsp70 is able to exit cells and serve as a danger signal, performing its most important functions under both normal and stressful conditions (Asea 2008). Normally, Hsp70 binds to newly synthesized proteins and acts as a "nurse" to deliver them in their native state to various compartments of the cell. Due to these features, inducible and constitutive or cognate Hsc70 are classified as a "chaperone" protein. In both normal conditions and under stress, these proteins are also actively involved in the detection and destruction of denatured or defective proteins (Hartl et al. 2011; Hightower 1991; Mayer 2010).

The results of studies of the thermally contrasting lizard species exceeded all expectations and formed the basis of our ideology for explaining the reaction of an individual and the species as a whole in response to an increase in temperature (HS). In the cells of day-time heat-resistant desert lizards, Hsp70 was always present at significant levels, allowing the lizards to tolerate high temperatures without turning on additional SOS systems. In other words, such desert lizards are pre-prepared for heat shock and can move without harming their health, even in high-temperature conditions on hot desert sand. In contrast, in the northern lizards, the Hsp70 levels are barely detectable under normal natural conditions, but with an increase in temperature, a rapid and sharp "burst" of Hsp70 synthesis occurs; this burst quickly disappears after the high temperature ceases. The nocturnal desert lizards occupied an intermediate position between these two extreme variants in relation to the synthesis of Hsps (Ulmasov et al. 1992). Notably, additional long-term studies on various animal species, from shrimp and Leishmania to camels and humans, as well as studies by other authors, have generally confirmed the fundamental conclusion regarding higher constitutive levels of Hsps in species that are adapted to extreme conditions (Ulmasov et al. 1992; Evgen'ev et al. 2014).

In the 1980s, we managed to publish the results of our "lizard" research in a prestigious American journal (Ulmasov et al. 1992), and at that time, I received several offers for collaboration from different countries. In particular, Dr. Wehner, who, as I later learned, was at that time the director of the Institute of Zoology in Zurich, wrote to me from Switzerland. Wehner had read our article on desert lizards and suggested collaborating on a project similar to our "lizard" work in a desert ant species that he wanted to compare to an ant from a temperate zone. For a number of personal reasons, I refused to work together at that time and forgot about this generally flattering offer. However, exactly 3 years later, I was reminded of this offer when Wehner's work appeared in the same journal (Proceedings of the National Academy of Sciences USA) that published our article on lizards in collaboration with the famous German molecular geneticist Walter Goering (Gehring and Wehner 1995). The authors obtained exactly the same results as we did on lizards and also found high basal levels of Hsp70 in a desert ant in the absence of any HS. Alas, the authors "forgot" to reference our pioneering work, of which they were well aware, as it had been published 3 years earlier in the same journal. I sent the same reproachful letter to both authors, but did not receive any response. Working with the lizards of Turkmenistan, we obtained another important piece of evidence regarding the adaptive role of Hsp70 in the normal functions of animals. We were able to show that levels of Hsp70 in the body of a lizard living in the desert significantly fluctuated during the day and directly depended on the temperatures of both the soil and air (Ulmasov et al. 1999).

During the many years we worked with our Turkmen colleagues, they often suggested that it would be good to study the response of the camel, a desert inhabitant, to heat shock at the molecular level, as well as that in the Turkmens themselves, who have long inhabited the deserts of Central Asia. I had long refused because I thought that warm-blooded animals that maintain their body temperature regardless of the environment were unlikely to serve as a good model for our research on the role of Hsps in adapting to elevated temperatures. However, I was wrong. Although the internal organs of mammals do indeed maintain approximately the same temperature under normal conditions, this is not the case for the skin of mammals. As it turned out, the skin of a camel standing in the sun can reach temperatures up to 41°C and above!

For our research, we obtained a culture of camel skin cells (fibroblasts), compared them to a culture of human cells, and observed characteristic differences in the responses of these cells to an increase in temperature. For this work, the skin cells of a camel were cut off from the eyelid of this large and rather aggressive animal using a razor blade. Fortunately, we did not have to euthanize or kill these beautiful animals; our Turkmen colleagues visited an Ashgabat meat processing plant on the days when camels were killed and their meat was added to expensive varieties of sausage, and the cells necessary for analysis were cut from the eyelid of a newly killed animal. Similarly, we compared the synthesis of Hsps in fibroblast cultures obtained from Russians as well as in cultures obtained from Turkmens, who have long lived in the deserts of Central Asia. In this case, we did not have to go to the meat processing

plant; the cell cultures were taken from volunteers of different nationalities and, as a rule, from the participants of the work.

The cells of both camels and Turkmen were able to synthesize Hsp70 at higher temperatures than other mammals (Ulmasov et al. 1993; Lyashko et al. 1994). That is, in the case of warm-blooded animals, the HS gene system is also obviously involved in the evolution of adaptations to extreme environmental conditions.

Characteristic structure of genes and chromosomal loci encoding Hsp70

A new direction in our research on the *hs* gene system in higher organisms was provided by an expedition to Kunashir Island (Kuril Islands). Here, as is often the case, "chance" again intervened in our favour. In the mid-1980s, with my colleague and friend Vladimir Scheinker, I went to the Far East to collect my favourite fruit flies. One day, two entomologists from the Zoological Institute of the Russian Academy of Sciences, who had just returned from Kunashir Island, where they were collecting dragonflies and grasshoppers, came to the biological station near Vladivostok, where we were staying. At a joint meal, they casually mentioned that on Kunashir, in the sulphur-rich silt near a hot spring, they observed the quite large larvae of some flies. At that time, we had already started our molecular and ecological work on the *hs* gene system, and I, of course, was very excited by this information and, returning to Moscow, began to organize an expedition to the island of Kunashir. It turned out that the Southern Kuril Islands, including Kunashir, were annexed by the USSR from Japan after World War II, and at that time belonged to the border zone; a special permit from the Ministry of Defence and a large amount of other paperwork were required to travel there. In the late 1980s, I was not up to this endeavour, and I left Russia for a long time for the USA, but I did not forget the "Kunashir flies".

Fifteen years later, I learned that my friend Boris Margulis (of the Institute of Cytology of the Russian Academy of Sciences) was organizing an expedition to this island. The expedition included professional entomologist Andrey Przhyboro from the Zoological Institute of the Russian Academy of Sciences and my employee, molecular biologist David Garbuz, who hoped to catch larvae and, with luck, adult flies from the hot sulphur springs and bring them to Moscow for analysis. The expedition was successful, and both live flies and their larvae were collected in the hot, sulphurous springs (Figure 2).

Andrey Przhyboro identified the collected flies, which, as it turned out, belonged to the Stratiomyidae family (Diptera) and live in a variety of climatic zones. In particular, one of the species of this group inhabits the cold lakes of the Pskov and Leningrad regions. As expected, the larvae from the hot springs of Kunashir Island were able to survive and synthesize Hsp70 at much higher temperatures than their "relatives" living in the clean but very cold lakes of the Pskov region. We decided to use these two species of flies, contrasting in the temperatures of their habitats, to obtain genomic libraries that would allow us to clone and "read" large sections of genomic DNA. By obtaining libraries from these two species of flies, we were able to isolate and study the structure of the chromosome regions of the compared species that contained genes encoding the main stress protein (Hsp70). The analysis showed that the genes encoding Hsp70 in the thermoresistant species from the island of Kunashir were compactly located on the chromosome and that all family members in this species have almost identical structures; on the other hand, the *Hsp70* genes of the related species from cold springs were located a greater distance from each other and differed significantly in their sequence (Garbuz et al. 2011b). Interestingly, we observed a similar pattern when comparing two species of *Drosophila* flies that live in ecological niches that contrasted in terms of their habitat temperatures (Evgen'ev et al. 2004). Apparently, the compact structure of the locus containing the *Hsp70* genes allows more efficient induction and functioning of the entire battery of these genes in species living in extreme, fluctuating conditions.

Fig. 2 Appearance of the larvae (in actual size) of a heat-resistant species of Stratiomyidae flies in a sulphur spring (Kunashir Island)

Remarkably, during the selection process, changes in the stress genes, particularly in the genes encoding Hsp70, also occurred at the level of regulatory sequences. We came to this conclusion after we were able to clone all genes encoding Hsp70 in the genome of a camel and in the genome of two fly species inhabiting ecological niches with contrasting

temperatures (Astakhova et al. 2015; Garbuz et al. 2011a). In our experiments, which allowed us to quantify the strengths of regulatory elements ("promoters"), we showed that in human cell cultures under normal physiological conditions, a foreign camel promoter of the Hsp70 gene worked more efficiently than its own human promoter of the same gene.

Moreover, Hsp70 itself, synthesized in heat-resistant species of Stratiomyidae flies, surpassed the corresponding *Drosophila* proteins in its protective properties. We came to this conclusion as a result of the following experiments. In our collection, a *Drosophila* strain was obtained from the Stock Centre, from which all the genes (6 copies) encoding Hsp70 were removed by genetic manipulation (Gong and Golic 2004). From this strain, we obtained two derivatives: in the first strain, we introduced one "native" *Drosophila Hsp70* gene, and in the second, one *Hsp70* gene was isolated from the genome of a heat-resistant species of Stratiomyidae. To our surprise, larvae of the strain with the "foreign" *Hsp70* gene turned out to be more thermoresistant than flies into which the one corresponding "native" *Drosophila* gene has been introduced (Shilova et al. 2018).

In recent years, we cloned the *Hsp70* genes of these two temperature-contrasting Stratiomyidae species in expression vectors and obtained the corresponding proteins in bacteria for analysis. It turned out that in our tests, the Hsp70 encoded by the gene of a thermoresistant fly species surpassed the corresponding protein from a cold-adapted species in its chaperone activity, and judging by experiments conducted with thermal denaturation, also surpassed the cold-adapted species protein in its thermal stability (Garbuz et al. 2019).

Therefore, our large-scale studies have shown that the system of stress genes, particularly those in the *Hsp70* gene family, plays an obvious and important role in the adaptation of an organism to rapidly changing and/or extreme environmental conditions and can undergo the characteristic changes described herein during the adaptive evolution of the species.

A potential practical use for Hsp70 in the treatment of various diseases

During our camel research, when such significant differences were found in the responses of cells to HS among different mammalian species, the idea to isolate Hsp70 from camel tissue and try to use it for therapeutic purposes naturally arose. Hsp70 is found in large quantities under normal physiological conditions without shock in many mammalian tissues, especially in the muscles and hearts of mammals. A method of isolating Hsp70 from muscle tissue has been previously described, and with great difficulty and adventure, we brought a "fresh" camel heart from Ashgabat by plane on ice and began isolating Hsp70. We were able to isolate a significant amount of Hsp70 from this animal, but it was unclear on which model we should test the possible protective properties of the isolated protein. Again, serendipity intervened, as Arkady Murashov (Centre for Drug Testing, Pushchino) had a "running" rat model of sepsis ("blood poisoning") caused by the addition of endotoxins lipopolysaccharide (LPS) or lipoteichoic acid (LTA) from various bacteria. We decided to test our dedicated camel Hsp70 on this model. The resulting protein was administered intravenously once before and once after the administration of toxins. The results exceeded all expectations: the pre-administration of Hsp70 20 min before the addition of toxins reduced the blood pressure and normalized heart rate of the model rats as well as all the main blood parameters that are typically disturbed in sepsis. At the same time we began to use in our experiments human recombinant Hsp70 (hrHsp70) expressed in *E.coli.*

An example of this protection using a recombinant form of HSPA1A, here termed human Hsp70 (hrHsp70), is shown in Figure 3.

Most importantly, the pre-administration of Hsp70 reduced the mortality of animals in response to the dose of toxins used, both in the case of Gram-positive and Gram-negative bacteria, by 50% (Kustanova et al. 2006; Rozhkova et al. 2010). Unfortunately, while therapeutic (after toxin injection) administration of Hsp70 significantly improved most blood parameters in experimental animals (rats), it did not significantly increase the survival rate of rats that were injected with LPS (Kustanova et al. 2006).

After obtaining these results in a sepsis model, we decided to test whether chronic administration of recombinant Hsp70 could affect the ageing process in model animals. There were prerequisites for this approach, since a number of studies have shown that with age in mammals and in humans, the level of a number of stress proteins in the brain in particular decreases, including Hsp70 (Leak 2014). In our experiments, Hp70 was administered intranasally and daily for several months to mice of different ages.

First, it was necessary to ensure that this large protein could cross the blood-brain barrier and enter the brain in an intact form using this method of administration. To do this, the recombinant protein was labelled with a radioactive isotope (^{125}I) before administration and injected into the nose of mice. Next, total proteins were isolated from the brains of the injected mice and separated by electrophoresis (Yurinskaya et al. 2015). These experiments convincingly demonstrated that the labelled rHsp70 quickly enters the brain of animals and does not disintegrate, reaching various areas of the brain in an intact form (Figure 4).

These experiments yielded the expected results. Chronic administration of recombinant human Hsp70 significantly increased the life expectancy of old experimental animals (Figure 5) and "rejuvenated" their brains based on a number of criteria compared to control animals (Bobkova et al. 2015).

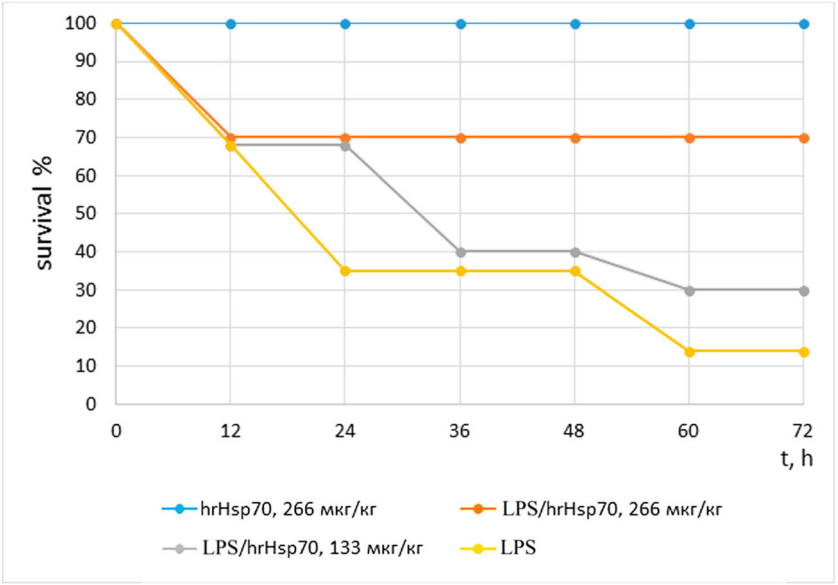


Fig. 3 The survival rate of experimental animals after the introduction of bacterial toxins (LPS) was significantly improved after the preventive intravenous introduction of recombinant human Hsp70 (hrHsp70)

Remarkably, administration of Hsp70 increased levels of the synaptophysin protein involved in nerve impulse transmission and reduced concentrations of the "ageing marker" lipofuscin in the brain (Figure 6).

The next model on which we tested human Hsp70 was the original model of Alzheimer's disease (AD) developed by N. V. Bobkova (Institute of Cellular Biophysics, Pushchino). In her experiments, Bobkova showed that if the olfactory bulbs are surgically removed in mice or rats, the animals develop AD with all the associated symptoms, including impaired memory and learning ability, and ending with an increase in the levels of amyloid, which is toxic to brain cells, and mass death of neurons in various brain regions (Nesterova et al. 2008). It is remarkable that the symptoms of AD in this model

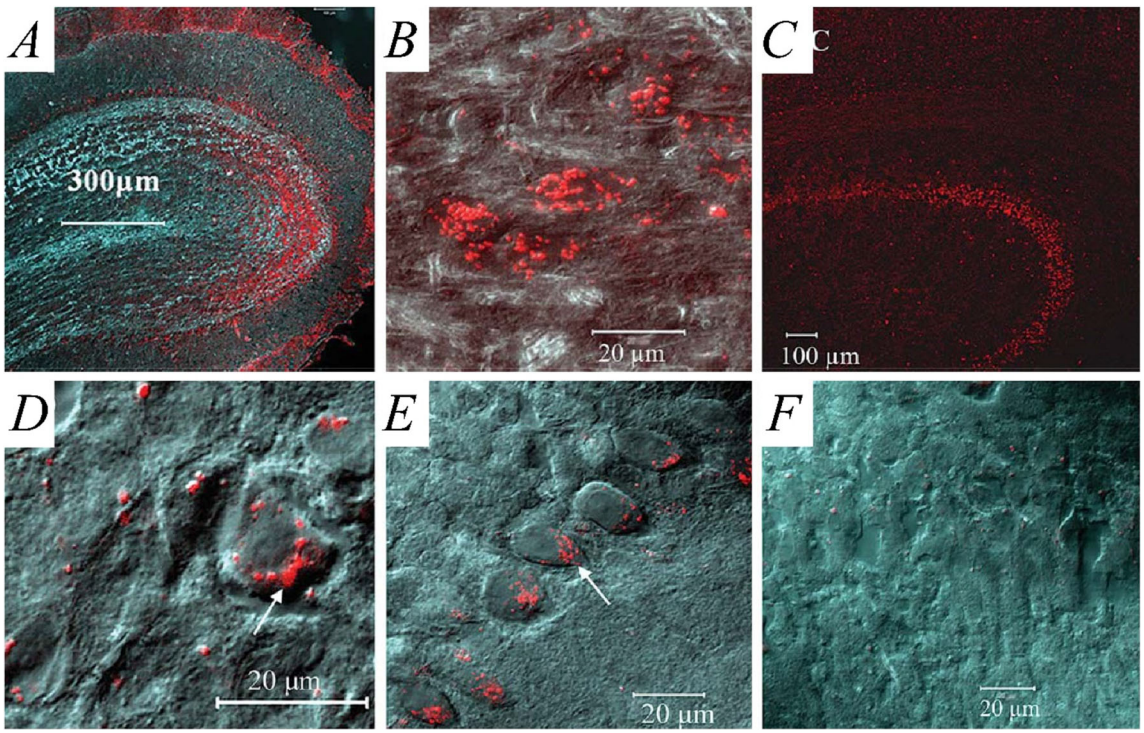


Fig. 4 Fluorescently labelled rHsp70 and the confocal microscopy of brain sections. **A** Olfactory bulbs, **B** dorsal suture nucleus, **C** and **D** hippocampus, and **E** cerebellum. Localization of the label in the perinuclear region is indicated by the white arrows. **F** Absence of fluorescence in control mice (administration of unlabelled Hsp70). Bulbectomized mice and 5XFAD line mice show similar label distributions

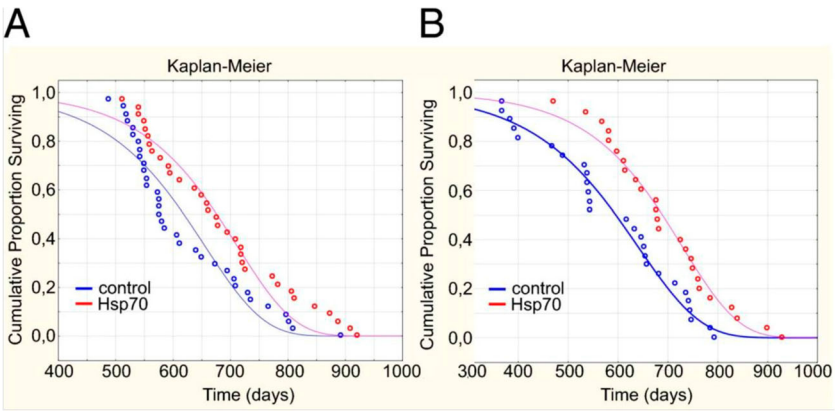


Fig. 5 Chronic administration of Hsp70 increases the life expectancy of aged mice

manifest with strictly regular dynamics. The first peak of the disorder was observed 4 months after removal of the olfactory bulbs; then, within a few months, there was remission. However, by 8–9 months after the operation, the maximum manifestation of AD symptoms developed, and the death of the animals occurred. Studies conducted using antibodies to Hsp70 revealed a curious pattern. In bulbectomized mice (i.e. mice with olfactory bulbs removed), the content of endogenous Hsp70 also changed in a characteristic way, and the peak of Hsp70 synthesis precisely coincides with the period when "remission" was observed in the operated mice with respect to behavioural and other symptoms of AD (Bobkova et al. 2013). This coincidence led to the idea that artificial administration of recombinant Hsp70 may help relieve or remove the symptoms of AD in the model developed by Bobkova. Obviously, in this case, it was unlikely that a positive result could be expected from a single intravenous injection of Hsp70. Therefore, we injected the protein intranasally, as we did when studying the ageing model, a few weeks after removal of the olfactory bulbs. In these experiments, recombinant Hsp70 in saline solution was intranasally administered daily for 3 weeks to the experimental group of mice, and other control groups were injected with either saline or the same protein that was first inactivated by boiling, 3 weeks after the removal of the olfactory bulbs. The results fully confirmed our expectations. The bulbectomized mice that received Hsp70 for several weeks learned faster, had better memory, and had lower levels of beta-amyloid in the brain compared to the controls (Figure 7A, B).

Most importantly, the state of the studied brain regions (cortex and areas of hippocampus) in these mice differed favourably from the state of the brain of the operated animals that were injected with saline or inactivated Hsp70 (Bobkova et al. 2014). Subsequently, similar experiments were independently conducted using another, generally accepted, gene-engineered mouse model of AD (5XFAD), wherein the intranasal administration of Hsp70 also yielded highly significant positive results, leading, in particular, to a decrease in the number of β-amyloid plaques in the cortex and hippocampus (Figure 7C). At the same time, this model of AD also exhibited a decrease in the accumulation of beta-amyloid, attenuated activation of neurogenesis in the hippocampus and prefrontal cortex, and improvements in cognitive function, in particular, with respect to the ability to learn (Evgen'ev et al. 2017; Evgen'ev et al. 2019).

Molecular mechanisms of the protective action of Hsp70

To understand the molecular basis of the protective anti-inflammatory effect of Hsp70 observed in different models, in recent years, we have conducted large-scale studies on various cell cultures, as well as transcriptomic studies using different parts of the mouse brain, where we compared the activity of the genome in the brain of mice, using lines that represent valid models of AD. In a wide variety of human and mouse cell cultures, including cells of neuronal origin (human

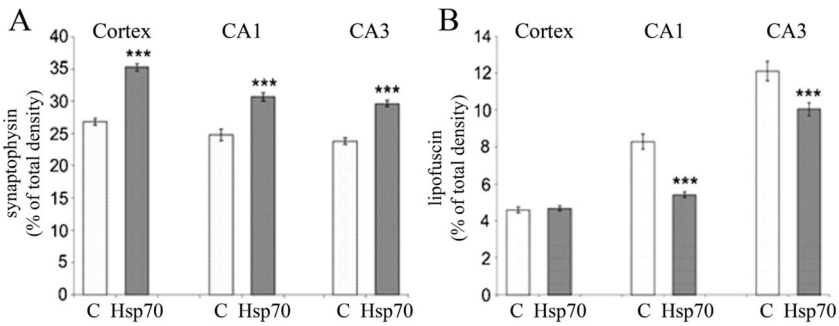
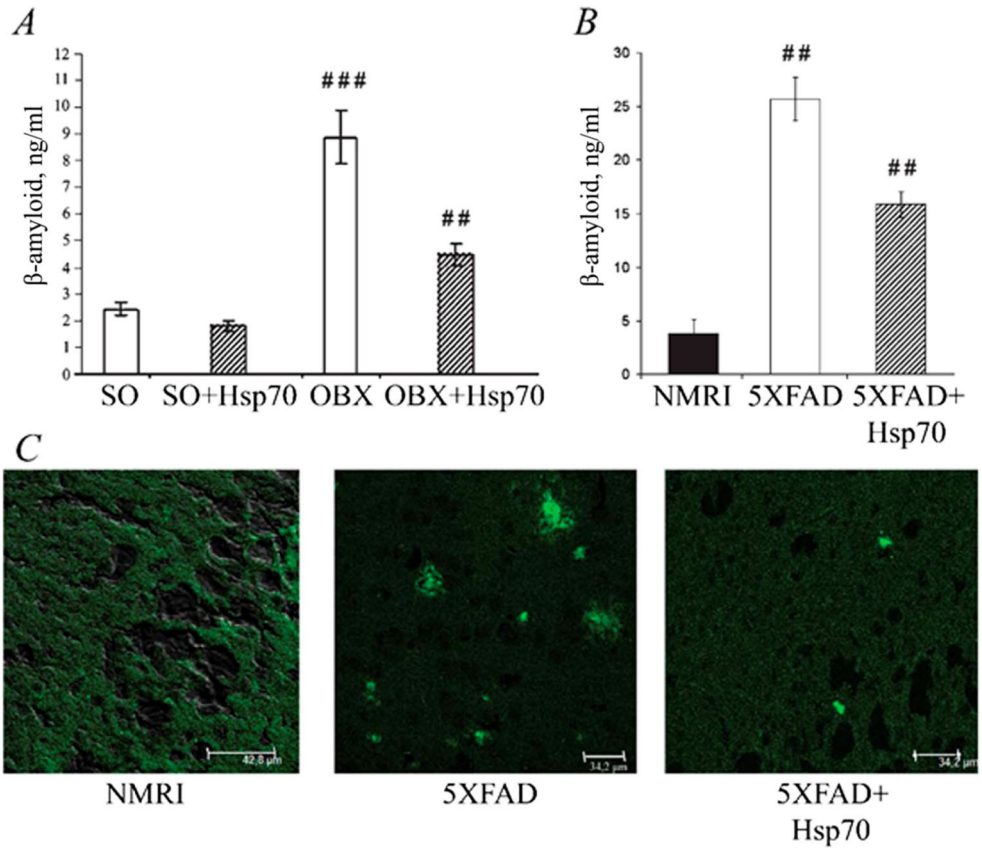


Fig. 6 Effect of exogenous Hsp70 on synaptophysin (**A**) and lipofuscin (**B**) levels in the brains of aged mice. The ratio of the stained area to the total area of the field of view is shown. ***p ≤ 0.01



Fi.e 7 **A**, **B** Levels of beta-amyloid in the brains of mice: sham-operated (SO), bulbectomized (OBX) NMRI lines, and transgenic 5XFAD and control mice subjected to intranasal administration of recombinant Hsp70 (+Hsp70). **C** The densities of the amyloid plaques in the brains of 5XFAD mice are shown without and after the administration of recombinant Hsp70. A micrograph of an NMRI of a mouse brain slice is shown as an example of a negative control (tissue without Aß plaque formation). ##p ≤ 0.01; ###p ≤ 0.001

neuroblastoma), we, together with Maxim Vinokurov's group (IBK RAS, Pushchino), found that the addition of recombinant Hsp70 to the medium effectively reduces the synthesis of the primary proinflammatory mediators, such as reactive oxygen species (ROS), NO, interleukins, and TNF-α, caused by the addition of bacterial toxins (LPS or LTA) to cell cultures (Shilova et al. 2018; Vinokurov et al. 2012; Yurinskaya et al. 2020).

Our transcriptome studies on transgenic mice (AD model) revealed that sub-chronic intranasal introduction of recombinant Hsp70 leads to a decrease in the expression of genes responsible for the development of neuroinflammation in the brain tissues. In addition, this treatment modulates the expression of genes responsible for the presentation of antigens. This suggests that the mechanism of action of exogenous Hsp70 in the AD models is based on its ability to suppress hyperactivation of the innate immune system (Evgen'ev et al. 2017; Evgen'ev et al. 2019). In our transcriptomic studies, we also showed that the introduction of recombinant Hsp70 in AD models in the cortex and hippocampus activates a number of regulatory pathways, which leads to a marked improvement in synaptic signal transmission and the induction of neurogenesis in the affected areas of the brain.

Sub-chronic (within a few weeks) intranasal administration of recombinant human Hsp70 proved to be a successful approach when studying a number of other models associated with inflammation and mass death of brain cells. In particular, our use of this approach revealed a highly significant protective effect in the case of artificially induced stroke, where the introduction of Hsp70 reduced the area of the brain that was affected and reduced the level of apoptosis in neuronal cells (Demyanenko et al. 2021).

Conclusions

Our long-term studies of the system of genes encoding the main stress protein Hsp70 have shed light on the evolution of this universal and ancient SOS system, as well as on the role of Hsp70 in the adaptation of a wide variety of organisms to extreme and/or rapidly changing environmental conditions. We have also developed a variety of methods and experimental approaches for the expression and isolation of Hsp70 from various sources, from bacteria to transgenic mice. The use of isolated recombinant Hsp70 in various models of neurodegeneration and normal ageing has shown promising prospects for the use of this protein as a therapeutic and preventive agent in sepsis, normal ageing, and the treatment of many human pathologies, including Alzheimer's disease and stroke.

Acknowledgements Dozens of people from various institutes and universities in Russia and other countries participated in this long-term work to varying degrees. I would especially like to mention the role of the late Khayet Ulmasov, who conducted the primary field research in Turkmenistan, as well as my long-term collaborators Olga Zatsepina and David Garbuz, who participated in the study of Hsps during the main stages. I would also like to thank Natalia Bobkova, together with whom all the experiments on ageing and the study of AD models were performed, as well as Maxim Vinokurov and Marina Yurinskaya, who performed all the work with cell cultures. At many stages of the work, we discussed our results with Boris Margulis and Irina Guzhova, who are our co-authors on a number of articles. The author expresses his deep gratitude to Dr. Lawrence Hightower and Dr. Robert Tanguay for reading the MS and many useful suggestions and corrections.

Funding This work was supported by Russian Science Foundation Grant 17-74-30030.

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