Exogenous Hsp70 delays senescence and improves cognitive function in aging mice

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Molecular chaperone Heat Shock Protein 70 (Hsp70) plays an important protective role in various neurodegenerative disorders often associated with aging, but its activity and availability in neuronal tissue decrease with age. Here we explored the effects of intranasal administration of exogenous recombinant human Hsp70 (eHsp70) on lifespan and neurological parameters in middle-aged and old mice. Long-term administration of eHsp70 significantly enhanced the lifespan of animals of different age groups. Behavioral assessment after 5 and 9 mo of chronic eHsp70 administration demonstrated improved learning and memory in old mice. Likewise, the investigation of locomotor and exploratory activities after eHsp70 treatment demonstrated a significant therapeutic effect of this chaperone. Measurements of synaptophysin show that eHsp70 treatment in old mice resulted in larger synaptophysin-immunopositive areas and higher neuron density compared with control animals. Furthermore, eHsp70 treatment decreased accumulation of lipofuscin, an aging-related marker, in the brain and enhanced proteasome activity. The potential of eHsp70 intranasal treatment to protect synaptic machinery in old animals offers a unique pharmacological approach for various neurodegenerative disorders associated with human aging.

aging | Hsp70 | therapy | memory | proteasome

Heat shock proteins (HSPs) serve to maintain intracellular protein homeostasis and have been shown to prevent protein damage during aging in different animal models (1). HSPs are required for longevity (2, 3), and a number of studies suggest that longer-lived species have higher constitutive expression of HSPs (4–7). Consistent with this finding, overexpression of HSP genes increased longevity in *Drosophila*, *Caenorhabditis elegans*, and vertebrates (1, 8, 9). Hsp70 is the major cytoprotective molecular chaperone with many different functions in the cell (10–12). Observations suggest that genetic variants of the Hsp70 family contribute to longevity in a wide range of organisms (9, 13, 14). Its defensive role in multiple neurodegenerative disorders (15, 16) can be explained by the multifaceted action of this protein. Indeed, the induction of Hsp70 has been shown to diminish oxidative stress damage (17, 18), suppress apoptosis (19), support proteasomal and lysosomal functioning (20), suppress toxic protein aggregation such as Aβ (21), inhibit proinflammatory signaling (22), and increase survival of endogenous neural progenitor cells (21). Notwithstanding Hsp70's importance, its chaperone activity, as well as the rate of its synthesis and induction in response to stimuli, decreases in neurons with age (3, 6, 21, 22), suggesting that a pharmacological approach aiming to recover this chaperone in the aging brain may counter neurodegeneration.

To our knowledge, the effect of exogenous HSPs on longevity has not yet been investigated. We previously showed that intranasally injected Hsp70 rapidly entered the brain of wild-type mice and was transported within neurons (23, 24). Furthermore, chronic Hsp70 treatment ameliorated multiple behavioral and molecular disturbances in two models of Alzheimer's disease (AD)-type neurodegeneration (23). In this study, we explored the geroprotection potential of recombinant exogenous Hsp70 (eHsp70) in healthy mice. For all of the described experiments, we used highly pure LPS-free human eHsp70 (25), which rules out a possibility of confounding inflammatory responses associated with contaminated Hsp70. Our results demonstrate that long-term intranasal administration of human eHsp70 improves longevity and ameliorates aging-related behavioral deficits and molecular alterations to synaptic structure in the brains of aging mice.

Results

eHsp70 Treatment Prolonged Lifespan. Chronic eHsp70 treatment significantly prolonged the lifespan of old animals when eHsp70 administration started at 17 mo of age (Fig. 1*A*, Table 1, and Table S1). Similarly, a pronounced effect in survival was seen when administration was started at 12 mo of age, indicating that middle-aged animals also responded (Fig. 1, Table 1, and Table S1). It is noteworthy that, although eHsp70 treatment only moderately increased the maximum lifespan (Table 1), the overall mortality rate in eHsp70-treated animals was much lower compared with the control groups (Table 1). After 22 mo, in the untreated middle age group, survival was just over 22%, whereas in the experimental group almost 55.5% were still alive. After 22 mo, in the untreated old age group, survival was just over 35.2%, whereas in the experimental group almost 54.5% were still alive (Fig. 1*B* and Table 1).

Significance

The compromised ability of neurons to express Heat Shock Protein 70 (Hsp70) correlates with aging-related neurodegeneration. In this study, middle-aged and old mice were treated chronically until death with human Hsp70 delivered intranasally and were investigated after 5 or 9 mo of Hsp70 treatment for their cognitive ability and synaptic density. Hsp70 treatment extended mean and maximum lifespan, improved learning and memory in old animals, increased curiosity, decreased anxiety, and helped maintain synaptic structures that degrade with age. These results provide evidence that intranasal administration of Hsp70 could have significant therapeutic potential in preserving brain tissue and memory for middle-age and old individuals and could be applied either as unique self-contained treatment or in combination with other pharmacological therapies.

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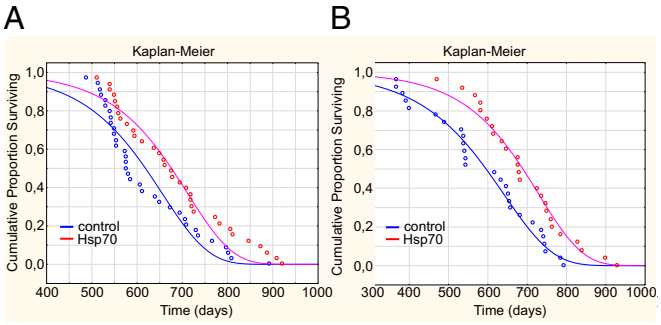

Fig. 1. Effect of chronic eHsp70 treatment on longevity. (*A*) Longevity of old animals (experimental and control mice; $n = 33$ and 34, respectively) when eHsp70 treatment was started at 17 mo of age and lasted until animals' death. (*B*) Longevity of middle-aged animals (experimental + control mice $n = 25 + 27$) when eHsp70 treatment was started at 12 mo of age and lasted until animals' death. Experimental results were statistically processed with the use of the Statistica.10 software (Statsoft). The choice of the most appropriate model was carried out with the use of the weighted least-squares method. Parameters of the Gompertz model were estimated by using the maximum-likelihood ratio. Kaplan and Meier product-limit method was used to compare untreated (control) and eHsp70-treated samples of mice. Experimental data are designated with color circles. Cox's *F* test: $F(66, 68) = 1.661273$; $P = 0.01959$ for old mice; Cox's F-Test: $F(50, 54) = 1.927759$; $P = 0.00949$ for middle-aged mice. Confidence intervals for the parameters were obtained by using the bootstrap method. Treatment with eHsp70 improved longevity from 29 to 31 mo in both age groups, but more importantly, eHsp70 improved survival rates; at 22 mo, 55.5% of animals in the experimental middle-aged group were alive, compared with 22.2% in the control group. After 22 mo, in the untreated old-age group, survival was just over 35.2%, whereas in the experimental group, 54.5% were still alive.

eHsp70 Improved Learning and Ameliorated Memory Impairment Observed in Old Animals. We investigated the effect of chronic administration of eHsp70 on learning and spatial memory of the animals using the Morris Water Maze test. Preliminary experiments demonstrated that all animals could swim and were free from visual impairments; control and treated mice of old and middle age were able to find the visible platform with similar latency: for old mice (16 ± 2.5 and 13 ± 2.7 s) and (20 ± 3.4 and 18 ± 2.7 s) after 5 and 9 mo of eHsp70 treatment; for middle-aged mice (12 ± 2.1 and 10 ± 1.7 s) and (17 ± 2.8 and 15 ± 2.2 s) after 5 and 9 mo, respectively. At every testing period, training trials were carried out over 5 d, and spatial memory was assessed in a probe trial on day 6. The rate of learning was determined based on the time the animal spent looking for the hidden platform (latency period). In both age groups, treatment with eHsp70 for 5 mo (Fig. 2 *A* and *B*) or 9 mo (Fig. 2 *C* and *D*) significantly reduced the latency to find the platform. Statistical analysis revealed a significant main effect of the trial day on latency in all animals groups ($P < 0.001$). There was also a significant effect of duration (5 or 9 m) of eHsp70 treatment in middle age and old mice ($P < 0.05$). One-way ANOVA showed a significant difference between age groups for escape latency ($P < 0.05$). In general, old and middle-age control mice learned more slowly than animals treated with eHsp70 (Fig. 2).

Spatial memory was tested in a probe trial 24 h after the last training session. Analysis of spatial memory used two independent measures: the number of entries into each sector and the time spent in each sector. Control middle-aged mice made multiple entries into, and spent more time in, the sector where the platform had been located during the training sessions. In general, control old mice have worse spatial memory compared with control middle-aged mice and could not recognize the target sector in both time periods of testing (Fig. 3 *A* and *C*). This finding confirms the results of many studies demonstrating cognitive decline in the elderly (26, 27). Middle-aged animals easily found the sector of the water maze, where the platform had been located during training trials, regardless of treatment (Fig. 3 *B* and *D*). In contrast, old animals in the control group encountered difficulties in finding the target. Treatment with eHsp70 significantly improved spatial memory, but only after 9 mo of eHsp70 administration (Fig. 3 *A* and *C*). Importantly, these results show cognitive protection after chronic eHsp70 treatment, even when eHSP administration began in old age, but in this case longer treatment (9 mo) is required. We hypothesized that eHsp70 administration protects normal function of neurons during aging. Similarly, overexpression of HSPs in aging cells in culture or in the intact brain has a protective effect (28, 29).

eHsp70 Improved Exploratory Behavior and Lessened Anxiety in Old Animals. We investigated locomotor activity after 5 and 9 mo of chronic eHsp70 intranasal administration using the open field test (OFT). We failed to observe any significant differences in the number of crossed sectors between eHsp70-treated and control

Table 1. Lifespan parameters of NMRI mice treated and untreated with Hsp70

Parameters	Control	Hsp70	Ratio
Old mice			
No.	34	33	
Mean lifespan (LS), d	632.0 ± 18.29	686.1 ± 20.73	8.6%
Median	584	677	15.9%
Mean LS of last 10% survivors	834.7 ± 28.76	906.0 ± 9.64	8.5%
Maximum LS, days	892	921	3.3%
Initial mortality rate (β), d^{-5}	9.32 (9.14; 12.87)	9.54 (8.99; 12.13)	1.02 times
Aging rate (α), d^{-3}	5.98 (4.92; 6.25)	5.46 (4.61; 5.70)	−0.91* times
MRDT, d	115.83 (110.81; 140.85)	115.83 (110.81; 140.85)	1.1 times
Middle-aged mice			
No.	27	25	
Mean LS, d	589.8 ± 25.93	693.3 ± 22.63	17.55%
Median	617	679	10%
Mean LS of last 10% survivors	775.7 ± 13.98	889.7 ± 26.77	14.7%
Maximum LS, d	793	930	17.3%
Initial mortality rate (β), d^{-5}	12.70 (7.27; 33.55)	7.00 (4.54; 12.34)	−1.8** times
Aging rate (α), d^{-3}	6.43 (4.86; 7.42)	6.37 (5.37; 7.54)	0.99 times
MRDT, d	107.87 (93.42; 142.62)	108.85 (91.94; 129.04)	1.009 times

MRDT, mortality rate doubling time. **$P < 0.01$; *$P < 0.05$.

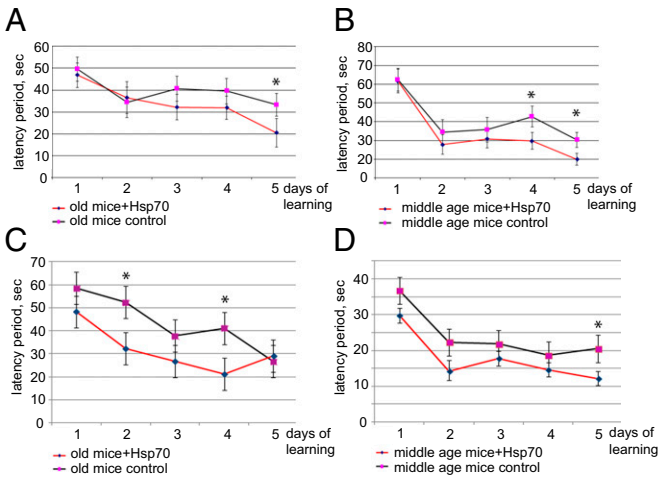

Fig. 2. Effect of eHsp70 treatment on learning in old and middle-aged mice. Learning was assessed in old ($n = 9$; *A*) and middle-aged ($n = 9$; *B*) animals after 5 mo of eHsp70 treatment and again in old ($n = 8$; *C*) and middle-aged ($n = 9$; *D*) animals after 9 mo of eHsp70 treatment. Treatment with eHsp70 decreased latency to finding the platform in both old and middle-aged mice at both time points investigated. *$P < 0.05$.

groups in middle-aged animals (Fig. 4 *B* and *D*). In old animals treated with eHsp70 for 5 mo, this parameter was significantly higher in comparison with the control group in the first 3 min of observation (Fig. 4*A*). After 9 mo of eHsp70 treatment, this difference was not so pronounced, but we observed a slight tendency toward increased horizontal activity (Fig. 4*C*). Middle-aged animals treated with eHsp70 and control animals exhibited a normal pattern of horizontal activity in the OFT (Fig. 4 *B* and *D*).

Our results indicate that changes in the behavioral profile begin from 22 mo of age. A decline in locomotor activity was seen in control old animals (Fig. 4 *A* and *C*). The middle-aged animals demonstrated a higher level of horizontal activity, which might mask any positive effect of eHsp70 treatment in this group (Fig. 4 *B* and *D*).

A similar effect of eHsp70 treatment was observed with rearing, a measure of exploratory behavior. A significant increase in vertical activity was seen in eHsp70-treated old animals in comparison with the control group (Fig. S1*A*); this increase was not seen in the middle-aged groups (Fig. S1*B*). Interestingly, old animals treated with eHsp70 exhibited exploratory behavior similar to middle-aged control mice. Similarly, eHsp70-treated old animals exhibited lower anxiety, based on an increased crossing of the central zone of the open field (Fig. S1*C*). Middle-aged eHsp70-treated animals did not differ from the controls in these behavioral parameters (Fig. S1*D*). Furthermore, in the middle-aged control group, all of the parameters at the time of monitoring were at a relatively high level. Therefore, any effect of eHsp70 treatment might not be evident until the age when behavioral parameters were already decreased,—i.e., after 22 mo of age.

Analysis of nest investigations, indicating curiosity, showed that middle-aged animals treated with eHsp70 did not differ from controls (3.75 ± 0.35 and 3.66 ± 0.28, respectively). However, old mice treated with eHsp70 exhibited more curiosity than the controls (3.52 ± 0.29 vs. 1.2 ± 0.26, respectively) and did not differ from the middle-aged controls.

These significant positive effects of eHsp70 treatment on spatial memory and behavior suggested a molecular basis. Therefore, we examined levels of synaptophysin and other age markers in the brains of eHsp70-treated and control animals.

Levels of Synaptophysin, Lipofuscin, and Neuronal Density in the Brains of Aged Mice Depend on eHsp70 Treatment. A drastic decrease in neuron density in various brain regions is an important morphological feature of the aging process. We measured neuron density and found a significant protective effect of eHsp70 treatment in the cortex and in the CA1–CA2 region of the hippocampus (Fig. S2).

Synaptophysin is a 38-kDa Ca^{2+}-binding glycoprotein widely found in presynaptic membranes and neurotransmitter vesicles. It is a major component of synaptic vesicles and plays an important role in vesicle trafficking (30–32). The level of synaptophysin directly reflects the health of synapses and is decreased in aged primates (33). Therefore, it was of great interest to determine whether eHsp70 treatment altered synaptophysin concentration. Areas of synaptophysin immunostaining in slices of the temporal cortex, CA1 and CA3 hippocampal zones were measured. In old eHsp70-treated mice, synaptophysin-immunopositive areas were significantly larger in all studied brain regions than in the control animals of the same age and were similar to those seen in middle-aged animals (Fig. 5*A*).

Lipofuscin is another marker of neuronal age (34). In contrast to synaptophysin, lipofuscin concentration increases with aging. In human brain, lipofuscin is generated and accumulated in neurons as part of the aging process (35). It is hypothesized that prevention of lipofuscin accumulation in the brain will slow aging processes and inhibit the development of neurodegenerative diseases (33, 35, 36). In previous studies, accumulation of lipofuscin was higher in the hippocampus in comparison with the cortex in aged animals (37). In our old eHsp70-treated mice, lipofuscin autofluorescence was significantly lower compared with controls in the CA1 and CA3 regions of the hippocampus ($P < 0.01$; Fig. 5*B* and Fig. S3); there was no significant difference in the cortex due to eHsp70 treatment. Age-related lipofuscin accumulation may result from impairment of the lysosomal and proteasomal degradation

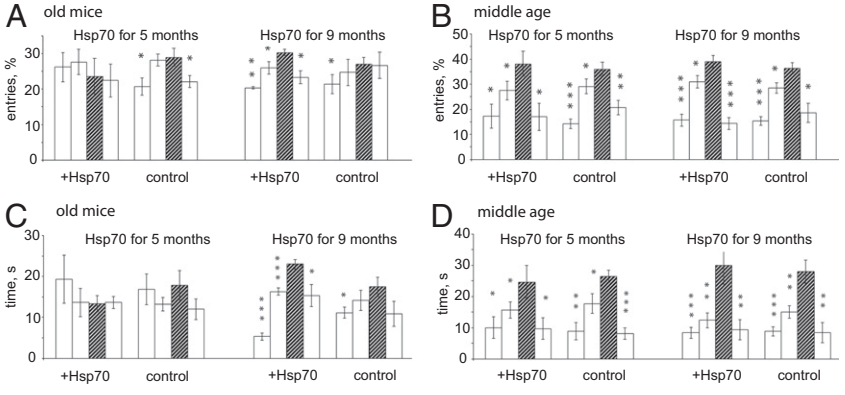

Fig. 3. The effects of chronic intranasal administration of eHsp70 for 5 or 9 mo on spatial memory in old and middle-aged animals. Animals were subjected to probe trial after 5 d of training trials in the Morris water maze. (*A* and *B*) The number of entries into each the four sectors of the Morris water maze was assessed in old ($n = 13$; *A*) and middle-aged ($n = 14$; *B*) animals. (*C* and *D*) Time spent in each sector of the Morris water maze was also analyzed for old ($n = 13$; *C*) and middle-aged ($n = 14$; *D*) animals. The target sector is shaded. In middle-aged animals, treatment with eHsp70 for 5 or 9 mo did not induce changing in recognition of target sector in comparison with age-matched control mice. In contrast, old animals only improved their spatial memory after 9 mo of treatment with eHsp70. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with age-matched control groups.

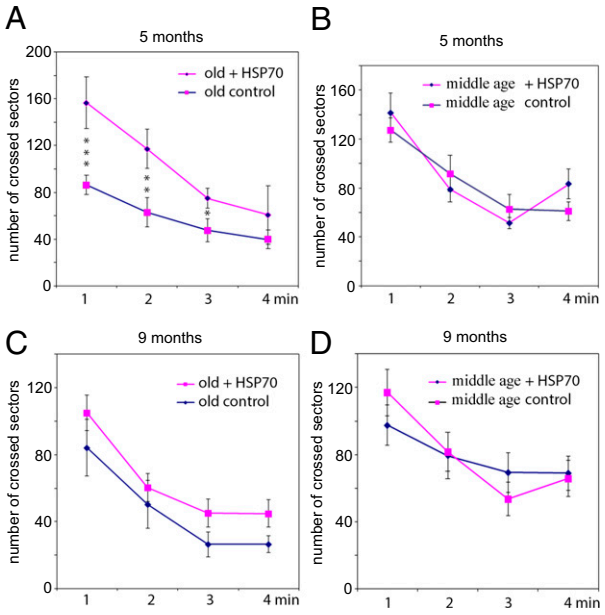

Fig. 4. Effect of chronic intranasal administration of eHsp70 for 5 or 9 mo on locomotor activity in an OFT. We assessed locomotor activity in middle-aged ($n = 26$; *B* and *D*) and old ($n = 27$; *A* and *C*) mice during each minute of the test. In old animals treated with eHsp70 for 5 mo, locomotor activity increased compared with age-matched control ($n = 7$; *A*). This effect was not apparent after 9 mo of treatment. Furthermore, eHsp70 treatment had no effect in middle-aged animals. $^{**}P < 0.01$; $^{***}P < 0.001$.

pathways (37), suggesting that eHsp70 may augment proteasome activity in the hippocampus of old mice.

eHsp70 Promoted Proteasome Activity in the Cortex of Old Mice. We hypothesized that eHsp70 exerts its protective effects on behavior by decreasing the production and/or accumulation of damaged proteins in the brain. The observed decrease in neuronal lipofuscin reactivity (Fig. 5*B*) in response to eHsp70 supports this notion. A majority of intracellular proteins are degraded by proteasomes; thus, the catalytic activity of proteasomes is critical to the homeostatic process. Because proteasome activity decreases with age in brain (38), we investigated whether eHsp70 treatment altered proteasome activity in the brains of treated mice.

Homogenates of brain tissue were obtained from eight animals treated with eHsp70 and five control NMRI mice (2-y-old mice were used in all experiments). To characterize proteasome function in these tissues, caspase- and chymotrypsin-like activities and expression of proteasomal subunits were determined. Caspase-like activity in the cortices of control animals was comparable to that detected in brains of eHsp70-treated mice. In contrast, chymotrypsin-like activity in mice of the eHsp70 group was significantly increased (17%; Fig. 5*C*). We analyzed the subunit composition of proteasomes in the lysates by Western blot (Fig. S4). In homogenates from eHsp70-treated animals, there was a significant elevation in expression of the Rpt6 subunit (20%). Expression of the β6 subunit was elevated by 66%, and the β5i subunit was increased by 139% (Fig. 5*D*). These findings suggest that the elevated chymotrypsin-like activity in the lysates from eHsp70-treated animals is likely due to the increased amount of proteasomes containing the β5i subunit.

Discussion

Cumulative inflammatory and oxidative processes contribute to aging, and minimizing these processes may help extend the active lifetime of an individual. HSPs, specifically Hsp70, provide resistance to stress, and their induction can increase tolerance to stress and extend longevity (9, 13). In previous studies, we explored two mouse models of AD-like neurodegeneration and found that intranasal administration of eHsp70 normalized neuron density in the neocortical and hippocampal areas. This finding correlated with diminished β-amyloid accumulation. eHsp70 treatment also protected spatial memory in both models (23).

Based on these results, we investigated the effects of chronic eHsp70 administration on behavioral and molecular parameters of aging. We found that eHsp70 treatment substantially increased median lifespan in both age groups examined (Table 1 and Fig. 1). In addition, treatment improved many behavioral parameters in old mice, including exploratory behavior and spatial memory. Importantly, the longer period of treatment resulted in more protection in almost all of the parameters studied.

The potential for eHsp70 treatment to protect synaptic machinery in old animals is of great relevance for neurodegenerative diseases, such as AD and Parkinson's disease. One mechanism underlying our results may be the neuroprotective effect of Hsp70 on synaptic machinery. An investigation of recombinant Hsp70 localization after intracerebroventricular injection found the protein in neurons and colocalized with synaptophysin (39). In the present study, total neuron density was significantly higher in the cortex and CA1–CA2 areas of the hippocampus in eHsp70 treated mouse brain. This finding confirms our previous data demonstrating significantly higher neuronal density in brain regions of eHsp70-treated 5XFAD mice compared with control mice (23).

The specific signaling pathways activated by intranasal administration of eHsp70 are unknown. Maintenance of normal protein synthesis, folding and degradation, which can be disturbed during aging, depends on hundreds of genes (1, 3, 40). It is possible that prolonged intranasal administration of eHsp70 may ameliorate chronic inflammation characteristically seen in aging neuronal tissues,

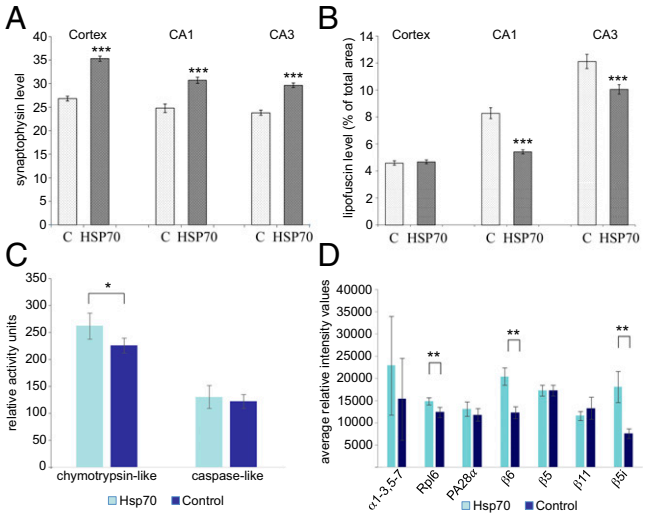

Fig. 5. Effect of eHsp70 administration for 5 mo on molecular markers of aging. (*A* and *B*) Levels of synaptophysin (*A*) or lipofuscin (*B*) were measured in the brains of eHsp70-treated old mice ($n = 9$) and age-matched controls ($n = 8$). Error bars represent mean SEM. Treatment with eHsp70 increased synaptophysin immunostaining in the cortex and hippocampus and decreased lipofuscin autofluorescence in the hippocampus. (*C*) Proteolytic activities of proteasomes in lysates from the cerebral cortex of experimental old mice. Average relative activity levels in 1 μL of tissue lysate of eHsp70-treated ($n = 8$) and control ($n = 5$) groups are shown. Treatment with eHsp70 increased chymotrypsin-like activity, but had no effect on caspase-like activity. (*D*) Expression of proteasome subunits in cortex lysates. Treatment with eHsp70 increased expression of the Rpt6, β6, and β5i subunits. Error bars represent SD of the mean. Significance was tested by using the Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, calculated by two-tailed Student's *t* test.

potentially by decreasing reactive oxygen species production and proinflammatory cytokines; an anti-inflammatory effect of eHsp70 has been demonstrated (22, 23, 40–43).

To characterize the intracellular molecular changes induced by eHsp70, we examined the activity and subunit composition of proteasomes in eHsp70-treated old mice. Aging is associated with increased cellular protein load due to the accumulation of damaged and nonfunctional proteins (1, 44). This process is in part due to a decline in the proteolytic capacity of proteasomes (38, 45). Our data indicated that eHsp70 treatment increased chymotrypsin-like catalytic activity and significantly elevated levels of the immune subunit β5i in old mice. Immune subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) replace constitutive β1, β2, and β5 subunits of the proteasome in response to inflammation, tissue damage, or oxidative stress, forming the immunoproteasome (45, 46). Interestingly, we did not find up-regulation of β1i in brain lysates, suggesting an increase in proteasomes containing both constitutive subunits and β5i. Immune subunits and constitutive proteolytic subunits do incorporate into proteasomes together; in healthy organs, from one-third to half of all proteasomes are actually "mixed" proteasomes (for instance, containing catalytic subunits β1, β2, and β5i or β1i, β2, and β5i) (41). The existence of mixed proteasome subtypes is important for the generation of unique peptides for presentation by class I MHC and influences immune recognition (47, 48). Moreover, β5i chaperone and proteolytic activity enhanced proteasome maturation and ensured optimal class I MHC expression (25), which is important for synaptic plasticity and refinement in the CNS (49–51). Finally, correlation between increased proteasomal activity and longevity was demonstrated: Elevated levels of proteasomes with immune subunit β5i were revealed in primary cells from longer-lived primates in comparison with ones from shorter-lived species. Importantly, enhanced proteasome activity and β5i expression was observed in livers of mice treated with drugs that extend lifespan (52). It is thus possible that elevation of β5i levels in response to eHsp70 treatment facilitated proteasome maturation, enhancing degradation of damaged proteins, and abated age-related synaptic dysfunction. Therefore, it could not be excluded that differences observed in behavioral tests (Fig. 4) between old and middle-aged animals are in part due to the elevated proteasomal activity in brains of old mice treated with eHsp70. In fact, proteasomal activity usually declines with age (38, 45), and it is reasonable that in old animals, it is lower than in middle-aged ones, and thus the effect of proteasome activity up-regulation in old animals should be more pronounced and have clearer manifestations.

Evidence from *C. elegans* and *Drosophila* points to a connection between neuronal proteostasis and somatic aging (44, 53–55). Stress response and protein homeostasis maintenance in those organisms are orchestrated at least in part via cell nonautonomous neuronal control. The present study suggests that neuronal cell nonautonomous control of aging operates in mammals as well. This finding raises exciting questions concerning neuronal protein homeostasis and its role in enhancing lifespan and diminishing aging-associated pathology in peripheral tissues. Our data suggest that eHsp70 can be used as a powerful molecular tool in revealing candidate signal pathways and identify therapeutic targets for aging and neurodegenerative diseases.

Materials and Methods

eHsp70. Human recombinant Hsp70 expressed in army worm (*Spodoptera frugiperda*) cells was purified as described (42, 56). The resulting eHsp70 was free of LPS, as confirmed by the Limulus Amebocyte Lysate assay (25), which negates the possibility of confounding inflammatory responses associated with Hsp70 contamination by LPS. Fig. S5 illustrates the high purity of eHSP70 used in the study.

Animals. A total of 119 male NMRI mice were used to investigate lifespan, as well as behavior. Molecular changes associated with aging were studied in additional groups of 2-y-old mice. All animals were given free access to food and water under constant temperature (23 ± 1 °C) and humidity (55 ± 5%) and were adapted to a standard 12-h light/dark cycle (lights on from 9:00 to 21:00). No significant weight differences were observed between control and experimental groups (Table S2). The procedures used in this study were in accordance with the *Guide for the Care and Use of Laboratory Animals* (56) published by the National Institutes of Health. All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry.

To investigate the effect of chronic eHsp70 administration on behavior, cognitive parameters, and synaptic structure, we investigated eight groups of NMRI mice. The first four groups included "old" animals 17 mo of age (n = 67), whereas the second four groups comprised middle-aged mice 10 mo of age (n = 52). The first (n = 33) and the fifth (n = 25) groups of mice were treated intranasally with eHsp70. The other groups served as negative controls and were treated with saline, BSA, or heat-denatured Hsp70. Because we failed to detect any differences in the lifespan of these control groups, they were all grouped as a control for middle-aged group (n = 27) and old animals (n = 34).

Notably, in our previous work (23), we also failed to observe any protective effect of boiled eHsp70. Experimental middle-aged animals of the treated group received eHsp70 from 12 mo of age, whereas experimental eHsp70-treated old animals were treated with eHsp70 starting from 17 mo of age (dose 2 μg per mouse per day). A total of 1× and 2× of the current eHSP70 dose did not show any difference in neuroprotection and memory improvements in the mouse models of AD-like neurodegeneration (23), suggesting that we have been working within an optimal therapeutic window with respect to intranasal eHsp70.

Investigation of Exploratory and Locomotor Activity in Mice. The OFT measures locomotor activity, exploratory behavior, curiosity, and anxiety in rodents. The OFT consisted of a 70-cm-diameter arena divided into 60 sectors. Each sector had a 0.8-cm-diameter hole in the center, imitating a nest. The walls of the arena were 70 cm high, and it was illuminated by 60-V lamp for the first 3 min of the testing period. Then the light was switched off for the fourth and final minute of testing. All behavioral experiments were carried out between 09:00 and 11:00 in a soundproof chamber with red diffuse light.

To assess locomotor activity, exploratory behavior, curiosity, and anxiety, each mouse was placed in the central zone. Latency to leave the central zone and the number of sectors crossed per minute were measured to quantify locomotor activity. The number of rearings was used to quantify exploratory behavior, and the frequency of investigation into the central zone was used as an indicator of curiosity. Changes in the number of crossings of the central zone, which indicates the level of fear and anxiety, were also monitored. The floor of the apparatus was cleaned with a wet sponge and dry paper between testing periods.

Measurement of Neuron Density. After dissection, one cortical hemisphere was fixed in 4% (vol/vol) fresh paraformaldehyde in 0.1 M phosphate buffer for 48 h. The brain was rinsed in 0.1 M phosphate buffer (pH 7.4) for 12 h and was then washed in deionized water for 12 h. Serial coronal sections 20-μm thick were cut on a rotating freezing microtome (Reichert). Sections were stained for Nissl substance by using Cresyl Violet and were viewed at a magnification of 20× or 40×. Digitized images were captured by using a DXM1200 camera mounted on a Nikon EM1000 light microscope. Only neurons with well-defined cellular contour, nucleus, and nucleoli were analyzed. Cell density was determined in 1 mm^2 of the temporal cortex (superior and inferior regions), and the CA1–CA2 stratum radiatum of dorsal hippocampus. To measure neuron density in different brain structures, an eyepiece with a special built-in grid was used, with square sides estimated by standard object-micrometer. The cell number was determined in 10 squares of the grid, or ∼0.036 mm^2, when the 40× objective was used. Density measurements were performed in 10 microscopic views. Neuron density (D_n) was estimated by using the formula $D_n = k \times x$, where x equals the average cell number in one microscopic view and k is the constant coefficient (27.7) estimated by taking into account the square of microscopic view.

Proteasome Activity Assay. Chymotrypsin- and caspase-like activities were measured in cortical lysates by using the fluorogenic substrates Suc-LLVY-AMC and Z-LLE-AMC (Enzo), respectively. Aliquots of lysate were mixed with lysis buffer and reaction buffer containing 40 mM Tris·HCl (pH 7.5), 1 mM DTT, 30 μM Suc-LLVY-AMC or Z-LLE-AMC, 5 mM $MgCl_2$, and 1 mM ATP. Additional control reactions with 10 μM proteasome inhibitor MG132 (Tocris) were performed to ensure lack of unspecific substrate degradation. Samples were incubated for 20 min at 37 °C, and reactions were stopped by using 2% (wt/vol) SDS. Fluorescence was measured with a VersaFluor Fluorometer (Bio-Rad). Relative activity was calculated by subtracting

the activity level with MG132 from values detected in samples containing tissue lysates. For each sample, average activity in 1 μL of the homogenate was calculated. Values were further normalized by protein concentration. Finally, average activity for the control and eHsp70 groups was calculated.

See *SI Materials and Methods* for additional information.

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