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Therapeutic Effect of Exogenous Hsp70 in Mouse Models of Alzheimer's Disease

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Abstract. Brain deterioration resulting from "protein folding" diseases, such as the Alzheimer's disease (AD), is one of the leading causes of morbidity and mortality in the aging human population. Heat shock proteins (Hsps) constitute the major cellular quality control system for proteins that mitigates the pathological burden of neurotoxic protein fibrils and aggregates. However, the therapeutic effect of Hsps has not been tested in a relevant setting. Here we report the dramatic neuroprotective effect of recombinant human Hsp70 in the bilateral olfactory bulbectomy model (OBX mice) and 5XFAD mouse models of neurodegeneration. We show that intranasally-administered Hsp70 rapidly enters the afflicted brain regions and mitigates multiple AD-like morphological and cognitive abnormalities observed in model animals. In particular, in both cases it normalizes the density of neurons in the hippocampus and cortex which correlates with the diminished accumulation of amyloid-β (Aβ) peptide and, in the case of 5XFAD mice, reduces Aβ plaque formation. Consistently, Hsp70 treatment also protects spatial memory in OBX and 5XFAD mice. These studies demonstrate that exogenous Hsp70 may be a practical therapeutic agent for treatment of neurodegenerative diseases associated with abnormal protein biogenesis and cognitive disturbances, such as AD, for which neuroprotective therapy is urgently needed.

Keywords: 5XFAD mice, Alzheimer's disease, amyloid-β, bulbectomized mice, Hsp70

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative pathology in the growing population of elderly humans and leads eventually to dementia and death. Despite tremendous efforts, no effective treatment for AD is currently available. The severity of cognitive impairment in patients with AD usually correlates with the extent of the observed abnormality of neurons, including the quantity of neurofibrillary tangles, the decrease in synaptic density, the enhanced concentration of soluble amyloid-β (Aβ) oligomers, and the amount of neurons that die by apoptosis [1–3].

Heat shock proteins (Hsps) have emerged as critical regulators of neurodegenerative processes associated with protein misfolding in the brains of AD patients [4–6]. Various data suggest that Hsp70 and other molecular chaperones function as a complex neuroprotective system, which fails in the brains of AD patients [5]. Indeed, the expression and functionality of the inducible form of Hsp70 and other major Hsps becomes compromised in aging brain tissue [6]. Yet

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the neuroprotective role of Hsp70 has been demonstrated in several animal models of neurodegeneration [7–10]. In a cellular model of AD, Hsp70 overexpression effectively protected neurons from intracellular accumulation of Aβ [6, 10]. Although a large body of evidence attests to a neuroprotective role of chaperones, there has been no direct demonstration that any of them could either delay the progression of AD or exert any therapeutic effect.

To investigate the potential therapeutic effect of Hsps, we used two mouse models of AD-like neurodegeneration: olfactory bulbectomized mice (OBX) and 5XFAD transgenic mice [11, 12]. OBX animals exhibit functional, morphological, and biochemical characteristics similar to those described for AD patients [2, 17, 18], including severe memory loss [11, 13–16]. Similar to AD patients, OBX mice display a decreased use of cerebral glucose [19] and an increased level of brain amyloid-β protein precursor (AβPP) and Aβ [16, 20, 21]. In OBX guinea pigs, which have the Aβ amino acid sequence identical to that of the human orthologue, intracellular accumulation of Aβ in neurons of the cortex and the formation of extracellular amyloid plaques were observed [11]. Similar to AD patients, OBX mice display a deficit of serotonin, acetylcholine, and glutamatergic brain systems [22–24]. Most importantly, in contrast to many widely used transgenic mice models of AD [25–27], OBX animals are characterized by a substantial death of neurons in the hippocampus and temporal cortex [16, 28, 29], i.e., the brain regions most affected in AD individuals [30].

5XFAD mice represent a widely used conventional model of AD-like neurodegeneration. In contrast to OBX animals, they are characterized by a high level of amyloid plaques that appear early in their lifespan in different brain regions [12].

Recently, we have presented the results of complex analysis of various pathological consequences of olfactory bulbectomy in mice and the results of endogenous Hsp70 levels measurements in the brains of OBX and sham-operated (SO) mice that served as controls in different post-operation time periods [16]. We observed a highly consistent inverse correlation between the level of inducible Hsp70 and AD-like symptoms at different stages of pathology development [16]. Furthermore, it has been recently demonstrated that in transgenic mice constitutively expressing Hsp70 in various brain regions, AD-related phenotypes were suppressed [31]. Since 2000, many studies using animal models of neurological diseases have shown that intranasal administration of large-sized molecules can produce beneficial effects (see [32] for review).

These findings prompted us to investigate a potential pharmacological role of intranasally-injected exogenous human Hsp70 in mouse models of AD. Our results show that Hsp70 treatment is highly effective in ameliorating all major manifestations of neurodegeneration developed in OBX and 5XFAD animals at histological and cognitive levels.

MATERIALS AND METHODS

Animals

Adult males of NMRI mice and transgenic 5XFAD mice were used in experiments. The animals were maintained in their home cages in a climate-controlled room (21–23°C) with a 12:12 h light-dark cycle and had free access to water and food. 5XFAD mice (TG6799) have been described previously [12]. These mice co-express the Swedish (K670N/M671L), Florida (I716V) and London (V717I) mutations in human AβPP(695), and M146L and L286V mutations in PS1, with expression of both transgenes driven by Thy1 promoter. Transgenic mice were acquired from Jackson Laboratory and maintained on a mixed SJL/C57Bl6. Mice were genotyped by PCR analysis of DNA extracted from ears biopsies. The transgenic cassette was detected using primers 5'-AGGACTGACCACTCGACCAG-3' and 5'-CGGGGGTCTAGTTCTGCAT-3', yielding a 377 bp product.

Olfactory bulbectomy procedure

Four groups of NMRI mice aged to10 weeks ($n = 55$) were employed in experiments: in two groups the olfactory bulbs were removed (OBX mice) and the other two groups underwent a sham operation (SO). Mice were anaesthetized with Nembutal (40 mg/kg, i.p.) and 0.5% Novocaine for local anesthesia of the scalp. A single burr hole (2 mm diameter) was drilled over the olfactory bulbs (2 mm anterior to the bregma, 0 mm laterally from the midline). Both the olfactory bulbs were carefully aspirated through a blunt needle attached to a water pump. The extent of the lesion was assessed both visually and histologically at the end of the experimental study. SO mice were treated similarly, except that the olfactory bulbs were not removed. All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry.

Tissue preparation

Mice were anaesthetized with overdose Nembutal (60 mg/kg, i/p) and perfused transcardially with 20 ml of PBS (pH 7.4). Brains were rapidly removed and divided into hemispheres. One hemisphere was frozen on dry ice and stored at −80°C. The other was fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 48 h before being stored in 30% sucrose. Hemibrains were cut in the coronal plane at 20 μm sections on a cryostat and stored in a glycol-based cryoprotectant at −20°C until histological analysis.

Histology and morphology

General histological methods were done as described [16, 29]. A detailed description of all procedures is provided in the Supplementary Data.

Confocal microscopy

Sections of brains from mice with intranasal injection of human HSP70 labeled by Alexa Fluor 647 were inspected under a scanning confocal microscope "Axioplan 2". Sections were observed using 10x/0.3 NA or 20x/0.5 NA Plan-Neofluar or 63x/1.4 NA Plan-Apochromat objective on microscope Axioplan 2 equipped with a Zeiss LSM-510 META scan head. HeNe laser was used to generate the 633 nm line used for excitation, and pinholes were typically set to 1–1.5 airy units. For Alexa Fluor 647, we collected 3–9 images of non-overlapping fields of cells at low power (total of 56 photomicrographs for analysis). Additionally, we collected three images of nonoverlapping fields of labeled cells at high power (total of 36 photomicrographs for analysis). High-power images were collected from the olfactory bulb, the temporal cortex, CA1, and CA3 areas of the hippocampus, the locus coeruleus, the nucleus raphe dorsalis, and cerebellum. Images were exported and stored as TIFF files.

Treatments

Two weeks following bulbectomy or SO procedures, mice were randomly divided into four groups: two groups denoted as (SO + Hsp70) and (OBX + Hsp70) were treated with Hsp70 (2 μg/mouse in 4 μl intranasal injections) daily for 21 days. Intranasal administration of Hsp70 has been carried out with the help of a micropipette. A drop of solution was placed just outside a nostril and was inhaled by an animal without any stress in the process of normal breathing.

In the remaining two groups, denoted as SO and OBX, animals were administered with 0.9% saline (intranasal injection) during the same period and the same volume. Hsp70 or saline were injected 1 h before training and memory testing. In separate experiments, we explored heat-denatured Hsp70 as a control. 5XFAD Tg^+ and Tg^- mice were divided into four groups as well, at 2 months of age, and were treated for 1 month with the same dose of Hsp70 (2 μg/mouse in 4 μl intranasal injections) daily. Groups were denoted as (Tg^+), (Tg^+ + Hsp70), (Tg^-), and (Tg^- + Hsp70).

Morris water maze tests were performed as described [16]. The details of the procedure and maze description are given in Supplementary Data.

Isolation and labeling of human recombinant Hsp70

In our research we used human recombinant Hsp70 expressed in army worm (*Spodoptera*) cells or in *E. coli*. The original clone containing human Hsp70 cDNA (pBlueScriptSK(+)-hsp70) (a generous gift of Dr. R. Morimoto, Northwestern University, Evanston, USA) was subcloned into the donor plasmid pFastBacHTb-hsp70 under polyhedrin promoter. The plasmid was subsequently used in Bac-to Bac system (Invitrogen) in order to express human Hsp70. Recombinant protein contained six His at its N-end which enables its isolation from the cell extracts using Ni-NTA resin columns according to the manufacturer's instructions (QUIAGEN, Ni-NTA Superflow BioRobot Hand Book).

The purity of Hsp70 preparations from *E.coli* and *Spodoptera* cells was confirmed by PAGE-electrophoresis followed by staining with Coomassie Blue and immunoblotting using monoclonal 3B5 anti-Hsp70 and N69 anti-Hsc70 antibodies. Protein concentration was measured according to Bradford's protocol.

For localization experiments, human recombinant Hsp70 was conjugated with Alexa Fluor 647 dye (Invitrogen, Carlsbad, USA) according to instructions of manufacturer.

RESULTS

Hsp70 rapidly enters specific brain regions after intranasal administration to mice

Although previously, using various mammalian cell cultures, it was shown that labeled exogenous Hsp70

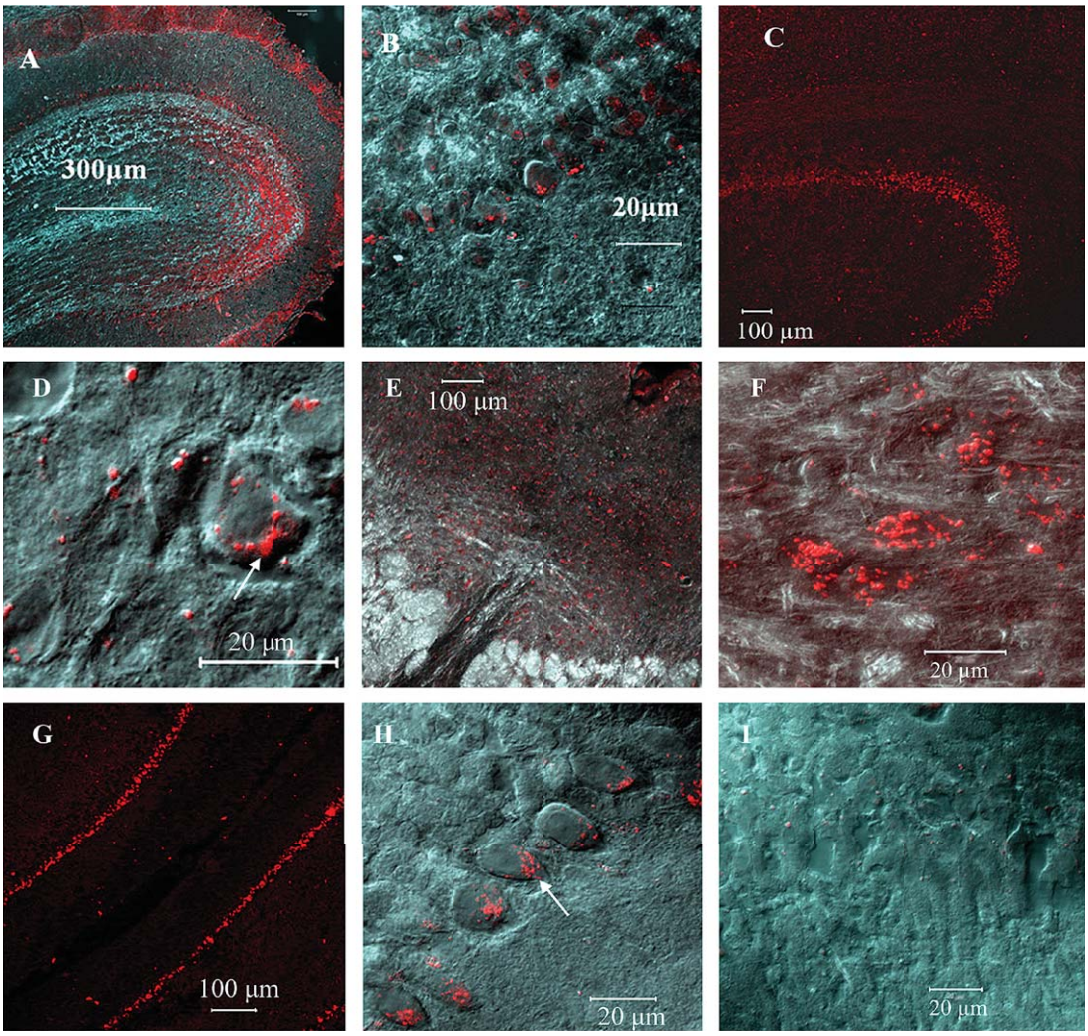

Fig. 1. Localization of exogenous Hsp70 in different brain regions of control mice after intranasal administration. The images show fluorescently labeled with the Alexafluor 647 dye Hsp70 (red) in the brain sections of NMRI mice 3 h after intranasal injection. Hsp70 is distributed non-randomly and concentrated in a few brain structures. Photos show its localization in the olfactory bulbs (A, B), areas of the hippocampus (C, D), in the n. raphe dorsalis (E, F), and in cells of the cerebellum (G, H). In most cases Hsp70 has an intracellular localization (B, D, F, H) and is restricted to the perinuclear zone (indicated by arrow in D and H). Control mice treated with unlabeled Hsp70 do not exhibit any brain fluorescence (I). Scale bars: A = 300 μm; B, D, F, H, I = 20 μm; C, E, G = 100 μm.

is able to penetrate cells [7] and localizes in neurons of the rat brain after intraventricular injection [33], it was necessary to directly prove that after intranasal administration, recombinant Hsp70 was able to enter the brain and find its way to specific brain structures. To trace exogenous Hsp70 and determine whether it could indeed reach the brain to exert its therapeutic effect, we monitored the distribution of Alexa-labeled Hsp70 in specific areas of the brain by confocal microscopy. Three hours after intranasal administration fluorescently-labeled Hsp70 was readily detected in the olfactory bulbs, neocortex, hippocampus, n.raphe dorsalis, locus coeruleus, and cerebellum in intact (non-operated) mice (Fig. 1). In most cases, it showed intracellular localization which was concentrated in the perinuclear zone (e.g., Fig. 1D, H). In similarly treated OBX mice, labeled Hsp70 was observed in essentially the same brain regions, however, the protein formed aggregates more randomly spaced throughout the cytoplasm (Supplementary Figure 1). The fluorescent material was absent in the brain slices of mice following intranasal administration of unlabeled Hsp70 or control naive NMRI mice (Fig. 1I). Therefore, intranasally injected Hsp70 is apparently

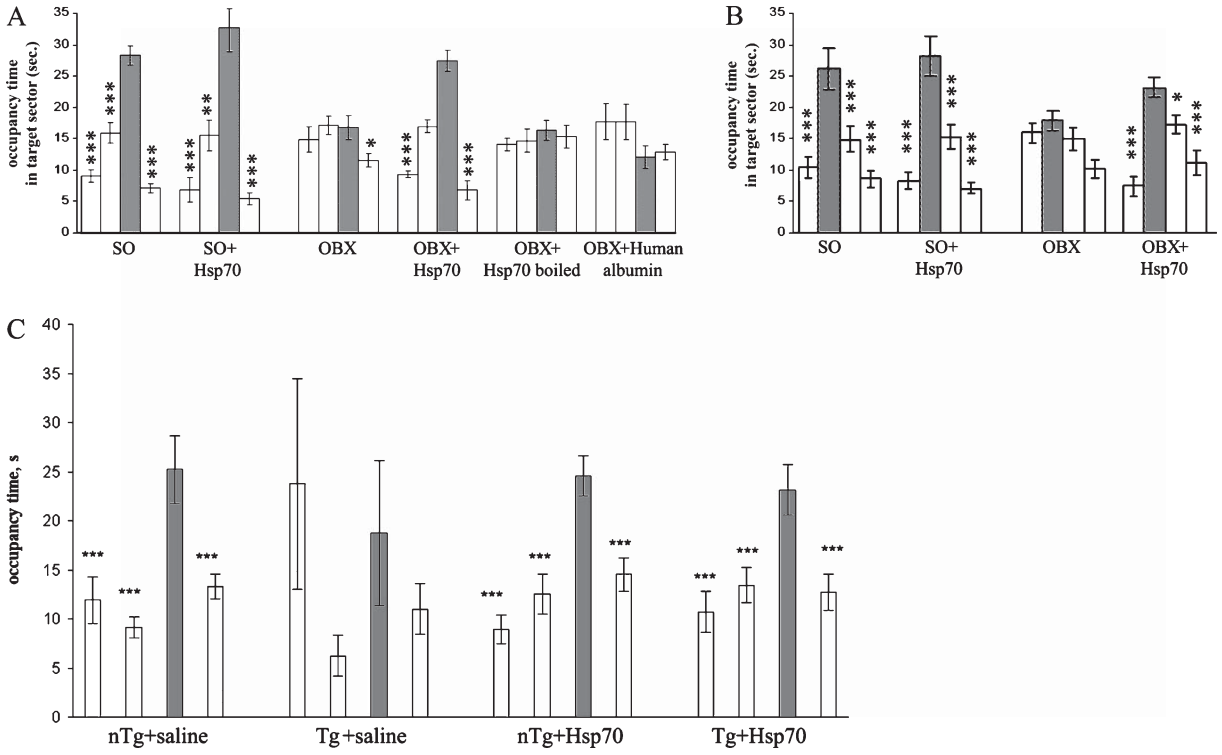

Fig. 2. Hsp70 improves memory in OBX and 5XFAD mice. The protective effects of sub-chronic intranasal administration of exogenous Hsp70 on spatial memory of OBX (A, B) and 5XFAD (C) mice. Results are presented as *post-hoc* data of ANOVA analysis. Hatched bars represent time (in seconds) spent by different groups of mice in the target sectors during probe trial (mean ± SEM); other bars represent time spent in indifferent sectors. Probe trials with independent groups of OBX and SO animals were performed four weeks after bulbectomy immediately after Hsp70 treatment (left panel-A) or eight months after bulbectomy and seven months following Hsp70 treatment (right panel-B). (C) The effect of Hsp70 administration on spatial memory of 5XFAD mice (3 months age). Asterisks indicate significant differences ($*p<0.05$; $**p<0.01$; $***p<0.001$) in comparison with the target quadrant.

able to enter the brain rapidly both in OBX and control mice and be transported within neurons of brain regions, including specific areas where neurodegeneration predominantly develops in AD patients.

Exogenous Hsp70 protects spatial memory in OBX and 5XFAD mice

The results described above provided a rational for the use of intranasally-injected exogenous Hsp70 as a potential protective agent to ameliorate the severe memory impairment observed in OBX and 5XFAD mice. Highly pure human Hsp70 was administered intranasally for a 21-day period starting two weeks after the bulbectomy ($n=13$) or sham operation ($n=13$). In the case of 5XFAD mice, the two month old animals were similarly treated with Hsp70 for one month. The training trials in the Morris water maze were performed either four weeks or eight months after the olfactory bulb ablation. As illustrated by the learning curves (Supplementary Figure 2A-C), all groups improved their performance, i.e., reduced the latent period of finding the invisible platform, with training. OBX mice found the save platform slower than SO mice ($p<0.05$) only on days 1 and 2 of training. However, there was no statistical difference in the learning abilities between any groups over the 5-day training. It is important that the latent periods in experimental groups did not significantly differ on the last day of the training in both testing periods (Supplementary Figure 2).

Figure 2 illustrates the effect of Hsp70 on spatial memory as judged by the time spent in the target sector of Morris water maze in comparison to other indifferent sectors. It is evident that untreated OBX and 5XFAD mice had severely impaired spatial memory. In contrast, OBX+Hsp70 and 5XFAD+Hsp70 mice displayed a significant preference for the target sector as determined by ANOVA and *post-hoc* analysis of their occupancy time (Figs. 2A-C and Supplementary Table 1). While untreated 5XFAD and OBX animals in both periods of testing (one or eight months

Table 1
Effect of Hsp70 intranasal administration on morphology of the temporal cortex and areas of the hippocampus

Brain areas	Groups	Kinds of neuronal pathology observed in %				Normal neurons in %
		Pyknosis	Karyolysis	Cytolysis	Vacuolization	
Temporal cortex	SO	9.9 ± 0.68	3.1 ± 0.23	5.9 ± 0.49	2.7 ± 0.38	75.9 ± 1.1
	SO+Hsp70	11.28 ± 0.43	4.2 ± 0.37	14.6 ± 0.45***	2.9 ± 0.41	67.0 ± 0.91***
	OBX	18.5 ± 0.66***	5.7 ± 0.26***	18.6 ± 0.51***	2.9 ± 0.28	53.6 ± 1.0***
	OBX+Hsp70	9.4 ± 0.68°°°	3.6 ± 0.43°°	19.2 ± 0.82***	2.3 ± 0.28	65.7 ± 1.5*** °°°
CA1	SO	6.3 ± 0.66	2.9 ± 0.26	5.9 ± 0.46	2.2 ± 0.26	82.6 ± 1.08
	SO+Hsp70	7.4 ± 0.55	4.2 ± 0.33***	10.3 ± 0.59	2.4 ± 0.29	75.7 ± 0.95***
	OBX	15.9 ± 0.69***	5.4 ± 0,31***	17.1 ± 0.61***	2.7 ± 0.24	59.0 ± 0.87***
	OBX+Hsp70	6.5 ± 0.33°°°	4.2 ± 0.38** °	19.1 ± 0.89*** °	1.5 ± 0.21°°°	68.9 ± 1.19*** °°°
CA3	SO	4.6 ± 0.43	2.5 ± 0.23	5.9 ± 0.4	1.2 ± 0.16	85.8 ± 0.89
	SO+Hsp70	8.6 ± 0.53**	3.1 ± 0.21***	16.9 ± 0.44***	1.8 ± 0.32*	69.6 ± 0.89***
	OBX	16.7 ± 0.63***	5.2 ± 0.33***	21.1 ± 0.58***	2.6 ± 0.2***	54.0 ± 0.74***
	OBX+Hsp70	6.6 ± 0.4** °°°	5.2 ± 0.37***	18.5 ± 0.65***°°	1.7 ± 0.23°°	67.4 ± 1.02*** °°°

Data are given as mean ± SEM. Comparison between the groups were performed separately for the temporal cortex and both areas of the hippocampus with two-tail Student's t test to compare replicate means with special emphasis on bulbectomy-induced changes (***$p < 0.001$) and effects of Hsp70 treatment in OBX mice (°°°$p < 0.001$).

after olfactory bulb ablation) exhibited impairment of spatial memory and could not recognize the target sector, the Hsp70 treatment of the model mice not only prevented memory deterioration shortly after Hsp70 treatment (e.g., one month following the operation) but also ameliorates memory deficit manifestations in the remote period following the operation. That is OBX mice studied seven months after Hsp70 treatment also demonstrate significant memory restoration probably resulted from Hsp70 pretreatment at the early stage of the pathology development.

A control set of experiments conducted prior to the training trials demonstrated that bulbectomy *per se* as well as Hsp70 administration did not induce motor or visual impairments that could affect the results of the memory test. There were no differences in tests with a visible unhidden platform in the Morris water maze between untreated 5XFAD mice and Hsp70 treated animals. We therefore conclude that subchronic intranasal injection of Hsp70 protected spatial memory in both OBX and 5XFAD animals.

To further characterize the protective role of Hsp70 at the cellular level, we performed detailed analysis of neuronal abnormalities in OBX mice treated by Hsp70 and in untreated control groups.

Hsp70 treatment reduces the accumulation of Aβ in the brains of OBX and 5XFAD mice

An abnormally high accumulation of Aβ peptides in afflicted regions of the brain is a biochemical hallmark of AD patients and also a prominent characteristic of OBX and 5XFAD mice [12, 16]. To examine the effect of Hsp70 treatment on this AD-specific marker, we measured the level of Aβ in an extract of the cortex and hippocampus by using an Invitrogen ELISA mouse beta amyloid (1–40) kit (Invitrogen, Camarillo, CA). We decided to compare the level of "1–40" peptide in both experimental systems because "1–42" peptide for some reason is not detected in OBX mice. The total amount of Aβ (pooled soluble and insoluble fractions) in the untreated groups of OBX and 5XFAD mice was significantly higher than that in the control and groups subjected to Hsp70 treatment ($p < 0.001$) (Fig. 3A, B). Importantly, Hsp70 treatment *per se* did not affect the basal level of Aβ in the control groups. Thus, subchronic Hsp70 treatment effectively protects the brains of mice from the accumulation of potentially toxic Aβ peptide in both animal models of AD.

It is known that 5XFAD mice develop characteristic Aβ plaques in 2-3 months after birth [12]. Intranasal Hsp70 administration for 1 month dramatically diminished the plaque density in the neocortex and CA1 region of the hippocampus in Hsp70-treated 5XFAD mice (Fig. 3C). Examples of typical patterns of plaque distribution in the neocortex in control and experimental animals are depicted in Fig. 3D.

Hsp70 treatment improves neuronal survival and morphology in OBX mice

Despite the pronounced accumulation of Aβ, OBX mice do not develop plaques, but do demonstrate death of neurons in the brain. Therefore, we explored the effect of Hsp70 on state of neurons in the cortex and the hippocampus of OBX animals. To investigate whether the dramatic protective effect of Hsp70 on cognition occurred in parallel with the mitigation of other

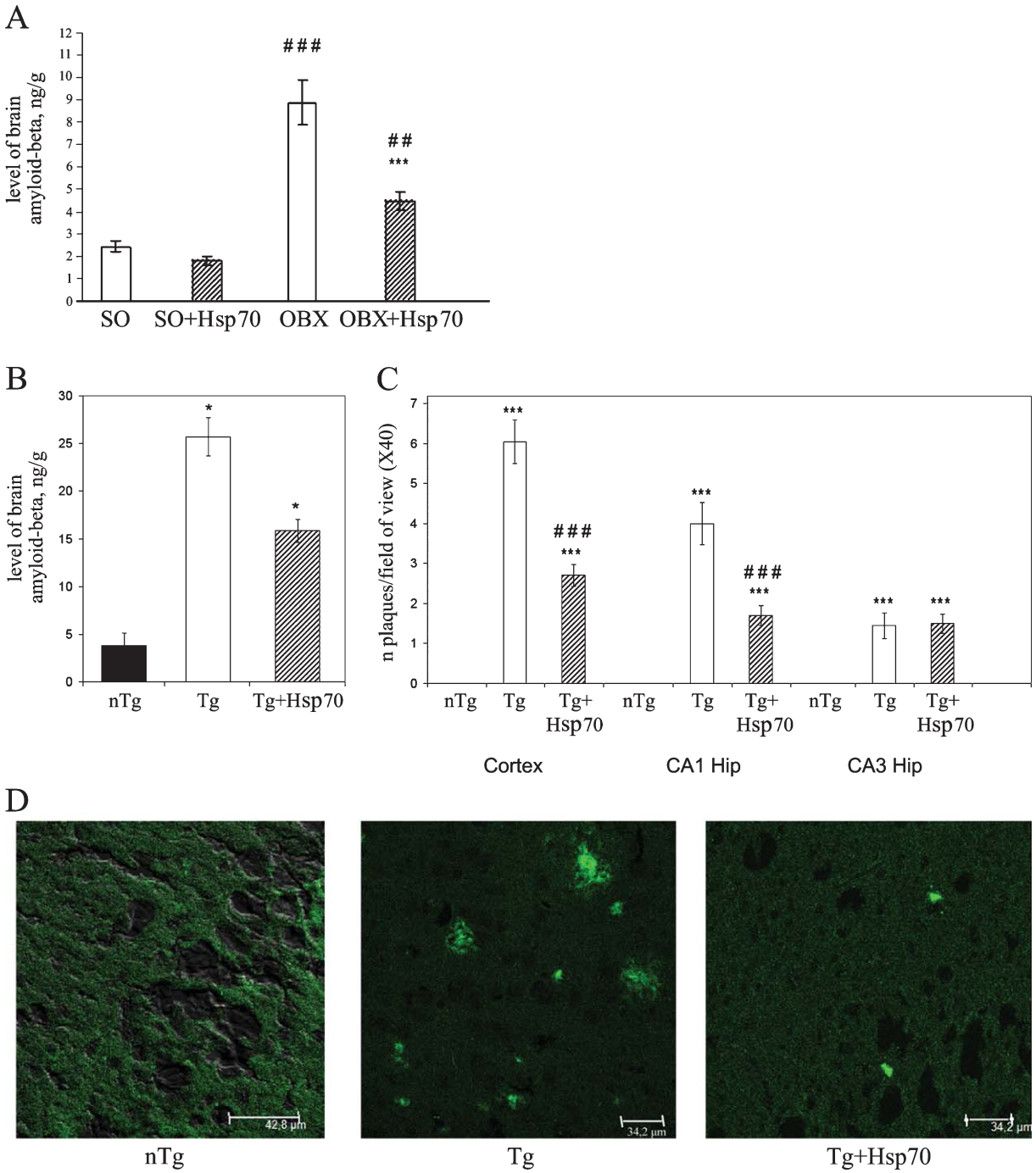

Fig. 3. Exogenous Hsp70 protects the brain of OBX and 5XFAD mice from Aβ accumulation and amyloid plaque formation. A, B) OBX and 5XFAD mice treated with intranasal Hsp70 are characterized by decreased level of Aβ in the brain. Bars indicate the level of Aβ peptides (mean ± SEM) determined by ELISA in the brain tissue (cortex + hippocampus) in the groups of OBX and 5XFAD mice ($n = 6$–11 animals per group). Differences between the levels of Aβ in the groups were determined by using the two-tailed Student's t-test. C) The density of Aβ plaques in Hsp70 treated and untreated 5XFAD mice. D) The representative microphotohraphs of neocortical slices in non transgenic mouse and 5XFAD mice exhibiting Aβ plaques in animals treated with Hsp70 and in control.

AD-like symptoms in OBX mice, we investigated the morphology of neurons and their density in the dorsal hippocampus (CA1 and CA3 areas) and temporal cortex because these regions are central to learning and memory and are the most damaged in AD patients. Importantly, SO mice did not display any significant changes in the form, size, or structural organization of neural cells 5 weeks after the operation (Table 1). Most of the neurons in this group exhibited normal phenotypic characteristics, evenly distributed tigroid and light colored, centrally positioned nuclei with easily distinguishable nucleoli. In contrast, most neurons of the OBX animals displayed fundamental morphological changes in the regions examined. The anomalies included pyknosis, karyolysis, cytolysis, and vacuolization (Table 1, Supplementary Materials and Methods). Characteristically, the proportion of pathologic neurons decreased drastically in OBX+Hsp70 animals. The data demonstrate that the Hsp70 treatment prevents the development of pathology in neurons of the OBX group. Consistent with the protection of cells, Hsp70 significantly improved neuronal survival in OBX mice. Quantitative analysis (Table 1) revealed that the Hsp70-treated OBX mice had significantly more intact neurons in the temporal cortex and hippocampus. The results presented in Table 1 demonstrate that neuronal density in the C1 and C3 regions of the hippocampus in OBX+Hsp70 group is similar to that of control SO mice. Therefore, Hsp70 treatment of OBX mice protects neurons from deterioration and death in brain areas most affected in AD patients.

DISCUSSION

Several observations demonstrate that chaperones belonging to Hsp70 family can ameliorate cellular toxicity of aberrantly folded proteins extant in AD, Parkinson's disease, amyotrophic lateral sclerosis, and polyQ-diseases [4, 34, 35], suggesting that treatment with Hsp70 could potentially be therapeutic for these devastating neurodegenerative diseases. It is of note that an understanding of the role played by stress-related proteins has developed more slowly for AD, mostly due to the lack of animal models that accurately replicate the major characteristics of the human disease. Our results reported herein provide the first direct experimental evidence supporting this hypothesis.

At the first stage of our research, we demonstrated that exogenous Hsp70 promptly enters the mammalian brain and its neuronal cells after intranasal administration, both in OBX and intact control mice. It has been recently shown that direct delivery of various therapeutics including proteins from the nasal cavity into the central nervous system somehow bypasses the blood-brain barrier and provides an attractive alternative to invasive methods of drug administration. Precise mechanisms providing such transport are not known and may involve neuron terminals, bulk flow and diffusion within perineuronal channels, perivascular spaces, or lymphatic channels directly connected to brain tissue [36]. Whatever the mechanism, it is likely to require an as yet unidentified cellular transport machinery specific to Hsp70. It is noteworthy that Hsp70 injected directly into the brain of rats (intracerebroventicularly) is localized in essentially the same brain regions [33]. It has been also shown by this group that fluorescently-labeled Hsp70 is seen in particular neuronal terminals and co-localized in these regions with NMDA receptors, synaptophysin, and the GABA-synthesizing enzyme [33].

Furthermore, in two complementary models of AD, namely in OBX and 5XFAD mice, we showed that the intranasal administration of full-length recombinant human Hsp70 drastically alleviates all symptoms, including memory loss, neuronal death, cellular aberrations, and accumulation of the Aβ peptide. It is noteworthy that major symptoms observed in OBX mice are similar to those of the more conventional transgenic 5XFAD model of AD-like degeneration. In both models, the memory improvement after Hsp70-treatment correlates with the decrease in Aβ levels, and in the case of 5XFAD mice, with the diminished density of amyloid plaques. Interestingly, in contrast to OBX mice, OBX guinea pigs, which naturally express Aβ that is identical to its human counterpart, do generate plaques [11]. The extent of cognitive impairment and neuronal loss, however, is similar between OBX mice and OBX guinea pigs, suggesting that plaque formation *per se* is not a major cytotoxic factor, but rather a phenotypic manifestation of Aβ misfolding, which varies depending on its precise sequence. It is likely that the level of soluble Aβ and its aggregates are mostly responsible for the cytotoxicity [37]. Thus it was demonstrated that intracellular accumulation of Aβ is an early event in neuronal dysfunction [38] and cytosolic Aβ has been shown to be highly cytotoxic to primary neurons [39]. Hsp70 may thus form complexes with soluble Aβ oligomers and suppress their toxicity leading to the observed neuronal protection. Furthermore Hsp70 may deliver the aberrant peptides to the protein degradation machinery such as the proteasome, preventing the increase in its content [40]. Remarkably, the therapeutic effect of Hsp70 in OBX

mice was induced by a single course of treatment with a small dose of protein (see Methods), and in the case of OBX mice lasted for at least eight months (the extent of the experiment). It is clear that such a long-lasting effect of exogenous Hsp70 cannot be readily explained by its direct chaperone activity against misfolded AD proteins, or by indirect effects, such as the suppression of apoptosis and Hsp70-mediated autophagy [41]. In this regard, recent findings that exogenous Hsp70 could stimulate the innate immune response and significantly reduce the level of reactive oxygen species may suggest clues as to the mechanisms responsible for the observed phenomena [42–44].

It is of note that there are other agents besides Hsp70 (e.g., phytic acid) that provide protection against Aβ in cellular and mouse models by attenuating levels of reactive oxygen species production [45].

On the other hand, Hsp70 treatment produces a clear cut adverse effect on the SO mice, i.e., in the CA3 region the level of normal neurons in SO group drops from 87 to 70% (Table 1). Therefore, the treatment apparently results in neuron protection only in the case of neuropathology (5XFAD and OBX mice). Along these lines, it has been previously demonstrated that in Drosophila constitutive expression of Hsp70 in otherwise normal flies has various deleterious effects [46, 47].

It is noteworthy that the use of intranasal administration of recombinant Hsp70 is not the first attempt to treat AD-like symptoms. Thus, intranasal administration of insulin was found effective to modulate verbal memory and plasma Aβ in memory-impaired older adults and in other model systems [48, 49].

Taken together, our findings not only establish exogenous Hsp70 as a potentially practical pharmacological agent for the treatment of various neurodegenerative diseases, but also reveal novel functions of mammalian Hsp70. It is necessary to mention that Hsp70 is able to form complexes with tau and be involved in its degradation process [50], therefore, Hsp70 treatment may be effective not only for AD but other neurodegenerative diseases with tauopathies.

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SUPPLEMENTARY MATERIAL

Supplementary materials are available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-130779.

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