Phosphorylation Dynamics Regulate Hsp27-Mediated Rescue of Neuronal Plasticity Deficits in Tau Transgenic Mice

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Molecular chaperones regulate the aggregation of a number of proteins that pathologically misfold and accumulate in neurodegenerative diseases. Identifying ways to manipulate these proteins in disease models is an area of intense investigation; however, the translation of these results to the mammalian brain has progressed more slowly. In this study, we investigated the ability of one of these chaperones, heat shock protein 27 (Hsp27), to modulate tau dynamics. Recombinant wild-type Hsp27 and a genetically altered version of Hsp27 that is perpetually pseudo-phosphorylated (3×S/D) were generated. Both Hsp27 variants interacted with tau, and atomic force microscopy and dynamic light scattering showed that both variants also prevented tau filament formation. However, extrinsic genetic delivery of these two Hsp27 variants to tau transgenic mice using adeno-associated viral particles showed that wild-type Hsp27 reduced neuronal tau levels, whereas 3×S/D Hsp27 was associated with increased tau levels. Moreover, rapid decay in hippocampal long-term potentiation (LTP) intrinsic to this tau transgenic model was rescued by wild-type Hsp27 overexpression but not by 3×S/D Hsp27. Because the 3×S/D Hsp27 mutant cannot cycle between phosphorylated and dephosphorylated states, we can conclude that Hsp27 must be functionally dynamic to facilitate tau clearance from the brain and rescue LTP; however, when this property is compromised, Hsp27 may actually facilitate accumulation of soluble tau intermediates.

Introduction

Aberrant protein production is a common feature of many diseases of aging. The majority of these disease-associated proteins are also clients of the chaperone network. These abnormal clients are prone to aggregation, forming pathologic inclusion bodies in neurodegenerative diseases. One family of chaperones that may help offset the toxicity of misfolded substrates is the small heat shock protein (Hsp) family. The primary function of small Hsps is to protect unfolded proteins in the cytosol from entering an aggregation pathway. This function has been defined primarily by investigating one particular small Hsp: Hsp27 (Renkawek et al., 1999; Wyttenbach et al., 2002; Shimura et al., 2004; Sanbe et al., 2007). Hsp27 regulates many disease-related proteins that are

prone to aggregation; however, validation of these findings in mammalian systems has been slow to follow, in large part because of several unique properties that distinguish Hsp27 from more classical chaperones such as Hsp70 and Hsp90 (Perrin et al., 2007).

Hsp27 facilitates degradation and prevents aggregation of aberrant substrates independent of ATP or ubiquitination (Jakob et al., 1993; Shimura et al., 2004). Thus, measuring its activity is difficult because it essentially has no measurable enzymatic function. The most well characterized modification of Hsp27 is its capacity to be phosphorylated by stress-activated kinases at serine residues 15, 78, and 82 (Stokoe et al., 1992). The known consequence of this phosphorylation is disassembly of large (200–800 kDa) dormant Hsp27 multimers into smaller oligomeric and monomeric species (Huot et al., 1991; Landry et al., 1992). These structures are not static, and deciphering the role that cycling between phosphorylated and dephosphorylated states has on chaperoning activity will be a critical step forward in understanding the mechanisms used by Hsp27 to process substrates (Haley et al., 2000; Lelj-Garolla and Mauk, 2005, 2006).

Our previous work has shown that regulation of the microtubule associated protein tau, which accumulates in ~70% of all neurodegenerative diseases, is tightly coupled to the chaperone machinery. Although a role for Hsp27 in tau regulation has been proposed (Shimura et al., 2004), its involvement with tau *in vivo*

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is unknown. Neurons normally have low levels of Hsp27 expression, but this can be induced in response to proteotoxic stress. In fact, increases in Hsp27 correlate with pathological accumulation of aberrant proteins in neurodegenerative diseases, both in neurons and in glia (Renkawek et al., 1994; Koren et al., 2009).

We hypothesized that dynamic regulation of Hsp27 was a requirement for its ability to remove aberrant tau from the brain. Here, we have investigated whether functionally dynamic and static Hsp27 species alter tau aggregation and clearance in the brain. These studies show that, although these Hsp27 variants act similarly at the molecular level, they act quite differently at the organismal level. Moreover, our studies suggest that tau aggregation can be decoupled from neuronal dysfunction.

Materials and Methods

Antibodies. Anti-Hsp27 and human Tau-150 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-human tau was purchased from Dako. Rabbit polyclonal anti-pS199/S202 tau was purchased from Anaspec. Neuronal nuclei (NeuN) antibody was purchased from Millipore. Mouse monoclonal anti-His antibody was purchased from Invitrogen. Secondary antibodies conjugated to HRP and anti-goat secondary antibody conjugated to a fluorophore (594 nm) were purchased from Southern Biotechnology. Alexa Fluor (Invitrogen) were optimized and diluted accordingly as described below.

Virus. Adeno-associated virus serotype 9 (AAV9)-expressing wild-type (wt) Hsp27 was a gift from T.E.G. AAV9-enhanced green fluorescent protein (GFP) was also a gift from Dr. Kevin Nash (University of South Florida Health Byrd Alzheimer's Institute, Tampa, FL). AAV9-3×S/D Hsp27 was a gift from Dr. Grant Mauk (University of British Columbia, Vancouver, British Columbia, Canada).

Recombinant protein. Production and purification of recombinant proteins was based on protocols described previously (Berrow et al., 2006). pET28 vectors carrying His-tagged gene sequences of tau, wtHsp27, and 3×S/D Hsp27 (a genetically altered version of Hsp27 that is perpetually pseudo-phosphorylated) were transformed into the *Escherichia coli* strain BL21 (DE3) Codon Plus. The latter sequence, 3×S/D Hsp27, consists of the wtHsp27 sequence with amino acid substitutions: S15D, S78D, and S82D. Luria broth medium containing kanamycin was inoculated with a respective stationary overnight culture. Cultures were grown at 30°C to an OD₆₀₀ of 0.5. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation at 4000 × g for 15 min at 4°C. Pelleted cells were lysed by sonication. All proteins were purified via a succession of Ni-NTA-Sepharose chromatography (equilibrated in 50 mM NaH₂PO₄, 500 mM NaCl, 30 mM imidazole, pH 7.5, elution by a step gradient to 300 mM imidazole). The purity of all proteins was verified on a Coomassie Brilliant Blue-stained SDS polyacrylamide gel.

Sucrose cushion. Sucrose–PBS solutions of increasing densities (20–50%) were layered in ultracentrifuge tubes. Recombinant proteins at a concentration of 1.2 μg were incubated in 20 mM HEPES or sterile PBS for 30 min at room temperature (RT). Samples were then added to the top of the sucrose layers and centrifuged at 100,000 × g.

Western blot. Western blot (WB) experiments were performed as described previously (Dickey et al., 2009). Samples were mixed with either a 6× WB sample buffer [50 mM Tris, 10% SDS for sucrose cushion (SC) samples] or a 2× Laemmli's sample buffer [for immunoprecipitation (IP) samples; Bio-Rad], boiled for 10 min, and loaded onto 10% Tris-glycine gels (Invitrogen) or 18-well, 10% criterion gels (Bio-Rad). Gel proteins were transferred onto nitrocellulose membranes, which were blocked for 60 min at RT in 7% milk. Then, membranes were incubated with primary antibodies at a dilution recommended by the manufacturer.

Immunoprecipitation. IP experiments were performed as described previously (Dickey et al., 2009). Purified recombinant tau proteins were incubated and agitated with BSA (as a control), wtHsp27, or 3×S/D Hsp27 in protein base buffer (PBB) (57.5 mM Na₂HPO₄, 334.42 mM NaCl, pH 8.0) for 30 min at RT and co-immunoprecipitated with anti-Hsp27 antibody for 1 h at RT. Protein A slurry (50 μl) was added, and the

mixture was incubated at RT for 2 h. Samples were centrifuged, and Protein A beads were precipitated. Pellets were washed four times with PBB. The precipitates were subjected to Western blot detection of Hsp27 and tau using His antibody or tau antibody (Dako).

Animals. All procedures involving experimentation on animal subjects were done in accord with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of South Florida. The rTg4510 and parental mice were maintained and genotyped as described previously (Santacruz et al., 2005). For immunohistochemistry experiments, mice (ten females and six males) were injected with AAV9-expressing vectors at 4 months, and their brains were harvested 2 months after injection. At least three sections were analyzed for each group of mice. Number of mice analyzed: $n_{wtHsp27} = 6$, $n_{3×S/D} = 5$, $n_{GFP} = 4$. Statistical significance was determined with Student's *t* tests. Alternatively, mice (11 females and 16 males) were injected with AAV9-expressing vectors at 1.5 months, and their brains were harvested 2.5 months after injection for electrophysiology experiments. The surgical procedure was performed as described previously (Carty et al., 2008) with some modifications. Twenty-five percent by mass Mannitol (Thermo Fisher Scientific) was delivered to mice by intraperitoneal injections 15 min before surgery. Using isoflurane, mice were anesthetized, and the cranium was exposed with a small incision along the skin covering the medial sagittal plane. Holes were drilled through the cranium over the coordinates to reach the injection sites measured with a stereotaxic apparatus from bregma: −1.5 mm anteroposterior, 2/−2 mm bilateral, and 3.0 mm vertical for frontal cortex; 2.2 mm anteroposterior, 2.2/−2.2 mm bilateral, and 2.3 vertical for cornu ammonis 1 (CA1); and 1.0 mm anteroposterior, 1.0/−1.0 mm bilateral, and 2.5 mm vertical for the lateral ventricles. Burr holes were drilled using a dental drill bit (SSW HP-3; SSWWhiteBurs) or a 21 gauge needle (BD Biosciences). A 2 μl total volume of each of the viral vectors in sterile PBS at a concentration of >10¹¹ vg/ml were dispensed into the each injection site at a rate of 5 μl/min using convection-enhanced delivery (CED) (Bobo et al., 1994). The needle was removed after 2 min of the injection. The incision was then cleaned and closed with Vetbond solution (3M). The animals recovered within 10 min and were housed singly until the time their brains were harvested. CED of AAV9 was performed with a modified 26 gauge blunt needle (Hamilton Co.) attached to a 10 μl syringe (Hamilton Co.). Needles were modified by attaching standard polyimide-coated, flexible-silica capillary tubing (100 ± 04 μm; TSP100170; Polymicro Technologies) into the needle shaft with Super Glue (Loctite), allowing 1 mm to be exposed outside the needle and 3 mm to remain inside.

Immunohistochemistry. Mouse brains were harvested for immunohistochemical analysis as described previously (Dickey et al., 2009). Briefly, mice were overdosed with pentobarbital and perfused transcardially with 0.9% normal saline solution. Brains were removed and immersed in 4% paraformaldehyde. Fixed brains were cryoprotected in successive 24 h increments of 10, 20, and 30% sucrose gradients as described previously (Gordon et al., 2002). Brains were frozen on a temperature-controlled freezing stage, coronally sectioned (25–50 μm) on a sliding microtome, and stored in a solution of PBS containing 0.02% NaN₃ at 4°C. Immunostaining was performed following the floating section procedure described previously (Gordon et al., 1997) with minor modifications. Immunostaining procedure began by immersing brain sections in a 3% solution of H₂O₂ for 15 min at RT to block endogenous peroxidases. Sections were washed in PBS and permeabilized in blocking buffer (4% donkey serum, 1.83% lysine, and 2% Triton X-100) for 30 min at RT and then incubated overnight at 4°C with anti-Hsp27 antibody at a 1:2000 dilution. After washing with PBS, sections were incubated with biotinylated anti-goat secondary antibody for 2 h and then with streptavidin–peroxidase. The peroxidase reactions consisted of 1.4 mM diaminobenzidine with 0.03% hydrogen peroxide in PBS for 5 min. Brain sections for immunofluorescence were blocked as described above and incubated overnight at 4°C with biotinylated NeuN (1:100), anti-pS199/S202 tau (1:1000), and anti-Hsp27 (1:1000) primary antibodies. Slides were washed and then incubated with Alexa Fluor secondary antibodies for 2 h at RT; dilutions were determined according to the manufacturer. Finally, stained sections were mounted on glass slides and imaged. Nonspecific reaction product formation was negli-

ble as assessed by omitting the primary antibody. NeuN staining was selected as a neuronal marker. Because of the potential loss of NeuN recognition in damaged neurons (McPhail et al., 2004; Unal-Cevik et al., 2004; Collombet et al., 2006; Kienzler et al., 2009), neuronal selection may have excluded damaged cells.

Microscopy. Immunohistochemically stained sections were bright-field imaged using the Carl Zeiss AxioImager.Z1 and AxioVision software with the 5×/0.16 dry ECPlanApo objective. Images covering the entire brain sections were captured using an AxioCam MRc5 camera and stitched together as a single image using the Carl Zeiss Panorama program. Immunofluorescently stained brain sections were imaged using the Leica TCS SP2 laser-scanning confocal microscope, which is inverted and has a motorized z-stage (z-Galvo and z-Wide). The fields examined were selected based on the following criteria: regions in the hippocampus that were Hsp27 or GFP positive. Z-stacked images of the selected fields were captured using a 40×/1.25–0.75 PLAN APO Oil objective and a Leica photomultiplier tube. Lasers used were 405 diode (for NeuN-positive signal represented in blue), argon (for Hsp27/GFP-positive signal in green), and helium–neon (for tau-positive signal in red).

Image analysis. Tau signal quantification was performed using NIH ImageJ (Bolte and Cordelières, 2006; Rasband, 2009). The signals from each channel from z-stacked images for the region of interest (ROI) were summed into a final image that was imported from the Leica software into NIH ImageJ (supplemental Fig. 1a, available at www.jneurosci.org as supplemental material). The three channels were split into independent images for NeuN, Hsp27 or GFP, and tau (supplemental Fig. 2B–D, available at www.jneurosci.org as supplemental material). To obtain a mask that revealed only NeuN-positive regions, the blue channel was processed by first removing outliers, resulting in an image highlighting the NeuN-positive regions as shown in supplemental Figure 1e (available at www.jneurosci.org as supplemental material) (process > noise > remove outliers > radius 10 pixels, threshold of 5, and remove bright). The threshold for this image was set to a grayscale range of 70–255, the outline of which is presented in supplemental Figure 1f (available at www.jneurosci.org as supplemental material). We selected the area and the integrated density (integrated density – analyze > set measurements) and redirected the calculations to the red and green channels. The following adjustments were then made in analyze > analyze particles: we further specified our NeuN-specific ROI to areas greater than 100 pixels² and circularity of 0.00–1.00, which increased the selectivity of our measurements to neurons. We also excluded areas along the edges of the field. Because the neuron somas were sometimes dissected by the z-stack, we included the area within the blue delineations, which correspond to areas in which neuron bodies are present. The integrated density values for each NeuN region defined in the outline were divided by the area of the same regions, which resulted in the pixel density per area for tau and Hsp27/GFP. We defined the signal above background as those values that were 2 SDs above the mean. We then determined the mean of the tau signals above background in NeuN-positive regions for each condition: GFP- or Hsp27-expressing brains.

Electrophysiology experiments. Mice were decapitated, and the brains were rapidly removed and briefly submerged in ice-cold cutting saline (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH₂PO₄, 28 NaHCO₃, 0.5 CaCl₂, 5 D-glucose, and 0.6 ascorbate). All solutions were saturated with 95% O₂ and 5% CO₂. Whole brains were then dissected on cutting solution-soaked filter paper and mounted on a glass platform resting on ice. Hippocampal slices (400 μm) were prepared on a vibratome and allowed to equilibrate in a 50% cutting saline and 50% artificial CSF (ACSF) (in mM: 125 NaCl, 2.5 KCl, 1.24 NaH₂PO₄, 25 NaHCO₃, 10 D-glucose, 2 CaCl₂, and 1 MgCl₂) at room temperature for a minimum of 10 min. Slices were transferred to an interface chamber supported by a nylon mesh and allowed to recover for a minimum of 1.5 h before recording. Slices were perfused in ACSF at 1 ml/min. Field EPSPs (fEPSPs) were obtained from

Figure 1. wt and 3×S/D Hsp27 bind tau despite differences in their oligomerization states. **A**, SC–WB for Hsp27. Recombinant wt and 3×S/D Hsp27 were independently subjected to SC ultracentrifugation. Hsp27 levels were analyzed in equal volumes of the recovered SC fractions. **B**, CoIP–WB of His-tagged recombinant tau (rTau) with rabbit anti-tau antibody showed that rTau coprecipitated with both wt and 3×S/D Hsp27 but not BSA. Unlabeled rabbit IgG was used as a negative co-IP control.

area CA1 stratum radiatum. Stimulation was supplied with a bipolar Teflon-coated platinum electrode, and a recording was obtained with the use of a glass microelectrode filled with ACSF (resistance of 1–4 MΩ). fEPSPs were generated using a 0.1 ms biphasic pulse delivered every 20 s. After a consistent response to a voltage stimulus was established for a 5–10 min period, threshold voltage for evoking a fEPSP was determined, and the voltage was then increased incrementally every 0.5 mV until the maximum amplitude of the fEPSP was reached. These data were used to create the input/output (I/O) curve. An fEPSP baseline response, defined as 50% of the stimulus voltage used to produce the maximum fEPSP amplitude as determined by the I/O curve, was then recorded for 20 min. The tetanus used to evoke CA1 long-term potentiation (LTP) consisted of a theta-burst stimulation (TBS) protocol. The TBS consisted of five trains of four pulse bursts at 200 Hz separated by 200 ms, repeated six times with an intertrain interval of 10 s. After TBS, fEPSPs evoked by baseline stimulus were recorded for 60 min. Potentiation was measured as the normalized increase of the mean fEPSP descending slope after TBS normalized to the mean fEPSP descending slope for the duration of the baseline recording. Experimental results were obtained from those slices that exhibited stable baseline synaptic transmission for a minimum of 20 min before the delivery of the LTP-inducing stimulus. Student's *t* tests were performed during the first and last 5 min of the recordings.

Dynamic light scattering. To analyze the effects of Hsp27 on *in vitro* fibril formation by tau, we used correlated dynamic light scattering (DLS) (Hill et al., 2009). Recombinant tau (250 mM) was suspended in buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.4), with Hsp27 at a stoichiometric ratio of 50 parts tau to 1 part Hsp27. All proteins were filtered through 0.2 μm syringe filters (Anotop) and passed through 100 kDa molecular cutoff filters (Nanosep). Heparin, at one-quarter of the molar concentration of tau, was added to initiate aggregation (Barghorn et al., 2004). Samples were placed in glass cuvettes, and aggregation kinetics at 37°C were monitored in 3 min intervals using DLS (Malvern Zetasizer Nano S). The low molar ratios of Hsp27 to tau (typically 1:50) made Hsp27 contributions to the DLS signal negligible.

Atomic force microscopy. Samples were diluted in 10× buffer, and 75 μl of the solution were deposited onto freshly cleaved mica for 15 min, rinsed with deionized water, and dried with dry nitrogen. Samples were imaged in air with an MFP-3D atomic-force microscope (Asylum Research) using PPP-FMR (Nanosensor) silicon tips with nominal tip radii of 7 nm. The cantilever was driven at 60–70 kHz in alternating current mode and a scan rate of 0.5 Hz, acquiring images at a 1024 × 1024 pixel resolution.

Stereology. All slides used to analyze changes of tau levels in neurons expressing AAV9-expressing proteins (see Fig. 4) were used for stereology analysis. First, coverslips were removed by placing all slides in a Coplin jar with Tris-buffered saline and nutating overnight at room temperature. Slides were then stained by the Nissl method. Neurons that were stained with cresyl violet were counted in the CA1 and the dentate

Figure 2. Heparin-induced tau fibril formation is abrogated by wtHsp27 and 3xS/D Hsp27. **A**, Tau (circles) fibril growth rate in the absence or presence of either wtHsp27 (squares) or 3xS/D Hsp27 (triangles). **B**, **C**, Particle size monitoring of tau alone (circles) or in combination with wt (squares) or 3xS/D (triangles) Hsp27 at day 0 (**B**) and day 5 (**C**) by DLS. **D**, AFM images of tau alone or preincubated with either wtHsp27 or 3xS/D Hsp27. Buffer without protein was used as a negative control. Heparin was used to induce tau aggregation for 5 d (**A–C**), and 15 d (**D**). Preincubation consisted of 35 mol of recombinant tau per mole of recombinant Hsp27s.

gyrus (DG) using the optical fractionator method of stereological counting (West et al., 1991) with commercially available stereological software (StereoInvestigator; MBF Bioscience). A systematic random sampling of sections were coded to ensure blinding. The ROIs were defined using specific landmarks within the brain to maintain consistency. A grid was placed randomly over the region of interest slated for counting. At regularly predetermined positions of the grid, cells were counted within three-dimensional optical disectors. Within each disector, a 1 μ m guard distance from the top and bottom of the section surface was excluded. Section thickness was measured regularly on all collected sections to estimate the mean section thickness for each animal after tissue processing and averaged $35.24 \pm 0.46 \mu$ m for all sections analyzed. The total number of neurons was calculated using the following equation: $N = Q^- \times 1/ssf \times 1/asf \times 1/hsf$, where N is total neuron number, Q^- is the number of neurons counted, ssf is section sampling fraction, asf is the area sampling fraction, and hsf is the height sampling fraction.

Statistical analyses. Statistical analyses were performed by Student's t tests as indicated in the figure legends.

Results

Wild-type and mock-phosphorylated Hsp27 bind to and abrogate the aggregation of tau

We generated two recombinant variants of Hsp27: wild-type Hsp27 (wtHsp27) and a mutant form of Hsp27 where serines 15, 78, and 82 are substituted by aspartates (3xS/D Hsp27). We analyzed the oligomerization properties of these Hsp27 variants with 20–50% sucrose cushioning. Sucrose fractions were collected after ultracentrifugation, and Hsp27 was detected by Western blot (Fig. 1A). Wild-type Hsp27 was predominantly found in the 50% fraction, whereas 3xS/D Hsp27 was distributed throughout all sucrose fractions. This confirmed that phosphorylation of Hsp27 abrogates multimer formation, whereas wild-type Hsp27 is more prone to oligomerization (Lelj-Garolla and Mauk, 2005). Both Hsp27 variants were able to bind tau, as demonstrated by co-immunoprecipitation (Fig. 1B).

We then tested whether tau aggregation could be altered by both Hsp27 variants using atomic force microscopy (AFM) and DLS (Reynolds et al., 2005). DLS showed reductions in the growth rate of tau filament formation over a 5 d period (Fig. 2A). No rapid or spontaneous aggregation of tau was observed at day 0 (Fig. 2B). However, after incubation for 5 d, both wtHsp27 and 3xS/D Hsp27 reduced tau aggregation at substoichiometric ratios (35:1) compared with tau incubated with buffer alone (Fig. 2C). However, wild-type Hsp27 ($r \approx 10$ nm; day 5) was more effective than 3xS/D Hsp27 ($r \approx 20$ nm; day 5) at abrogating tau aggregation (Fig. 2A). These samples were then incubated an additional 10 d, and AFM was used to image tau structures with or without each Hsp27 variant (Fig. 2D). Tau incubated with heparin alone formed robust filamentous aggregates. Conversely, tau incubated with wild-type Hsp27 or 3xS/D Hsp27 failed to form filaments, but intermediate aggregates of tau were observed in the presence of both variants. Consistent with our DLS findings, wild-type Hsp27 more potently reduced tau aggregation compared with 3xS/D Hsp27 (Fig. 2D).

Convection enhanced delivery of AAV9 facilitates robust distribution of gene product in the hippocampus

Because both Hsp27 variants were capable of modifying tau biology *in vitro*, we sought to determine their impact in the brain. We implemented an AAV expression system with the goal of delivering both Hsp27 variants to the rTg4510 transgenic mouse model of tauopathy (Santacruz et al., 2005). To test distribution and efficacy of this approach, AAV1 and AAV9 particles expressing wtHsp27 and 3xS/D Hsp27 were generated and delivered to the brain using two different approaches based on previous studies: AAV1 was somatically delivered into the ventricular space of nontransgenic (NTG) P0 pups as described previously (Levites et al., 2006); AAV9 was delivered into the CA1 region of the hippocampus and frontal cortices of adult, nontransgenic mice using

Figure 3. Hsp27 upregulation by intrahippocampal AAV9 injections has robust distribution throughout the hippocampus, but it does not transduce all neurons. **A**, Immunohistochemistry detection of Hsp27 in brain sections of mice injected with AAV1, AAV9, or non-injected mice. Brains were harvested 8 weeks after viral injections. Dark staining, which is not present in the non-injected brain section, corresponds to Hsp27. **B**, Immunofluorescence of the hippocampus neuronal layer using antibodies against Hsp27 and the neuronal marker NeuN shows that not all neurons (blue) expressed Hsp27 (green).

CED combined with intraperitoneal injections of mannitol before surgery (Fu et al., 2003; Hadaczek et al., 2006; Cearley et al., 2008). Eight weeks after injections, mice were killed, and distribution of Hsp27 was assessed by immunohistochemistry. Hippocampal injection of AAV9 caused robust distribution throughout the region, but the cortical injection was much less consistent across animals (Fig. 3A). Although the AAV1 delivery did result in greater overall spread of the viral particles, the intensity of staining in any one region was unremarkable. Moreover, hippocampal staining was minimal (Fig. 3A). Although distribution with AAV9 in the hippocampus was robust, and primarily neuronal, immunofluorescent staining showed that all neurons (NeuN; blue) were not transduced by virus (Hsp27; green) (Fig. 3B). These data suggested that AAV9 administration to the hippocampus using CED and mannitol would provide the most consistent and robust signal in the hippocampus but would require analyses of individual cells to assess changes in tau levels.

Tau clearance by Hsp27 is dependent on phosphorylation dynamics

We used the rTg4510 transgenic mouse model to test the effects of both Hsp27 variants on tau pathology in neurons by bilaterally

injecting their hippocampi with AAV9-expressing wtHsp27, 3×S/D Hsp27, or GFP (Fig. 3). Because these mice begin to develop tangle pathology as early as 3 months of age (Dickey et al., 2009) and we already demonstrated that all neurons were not transduced by AAV, we initiated these studies in 4-month-old mice and harvested tissues 2 months later to ensure that most hippocampal neurons would have tau accumulation (Dickey et al., 2009). This design allowed us to assess the tau burden in neurons that were successfully transduced with viral particles encoding each Hsp27 variant or GFP by triple labeling tissue sections with antibodies specific for NeuN (blue), Hsp27 (green), and tau (red) (Fig. 4A,B). Interestingly, we found very few wtHsp27-expressing neurons that also contained tau aggregates, suggesting that wtHsp27 facilitated tau clearance (Fig. 4B). This was in stark contrast to neurons positively stained for Hsp27 from mice receiving the 3×S/D Hsp27 variant. In these mice, tau levels were actually higher relative to mice injected with GFP-AAV. Quantification of tau (red signal) in neurons (blue areas) sharing positive green signal [Hsp27 or GFP (for additional details, see supplemental Fig. 4, available at www.jneurosci.org as supplemental material)] showed that, compared with GFP-expressing neurons ($n = 264$), tau signal was reduced by $66 \pm 8\%$ ($n = 296$) and increased by $50 \pm 11\%$ in neurons expressing wtHsp27 and 3×S/D Hsp27 ($n = 302$), respectively ($p < 0.001$) (Fig. 4C).

wtHsp27 was able to reduce tau levels in neurons; however, it was unclear whether it was preventing tau aggregation or actively disrupting preformed tau structures. DLS was used to specifically address this question. Recombinant tau was incubated with heparin for 22 d before the addition of recombinant wtHsp27 at a molar ratio equivalent to that used in Figure 2. Wild-type Hsp27 was unable to disrupt preformed aggregates (Fig. 5A), suggesting that, for Hsp27 to impact neuronal function in the rTg4510 model, it should be administered at a time before robust tau aggregation, which would be much earlier than 4 months of age. This result indicates that, for Hsp27 to be effective at modulating tau aggregation, it must interact with tau before filament formation, at a point when tau is in an intermediate state (Fig. 5B). Thus, Hsp27 can block the tendency of tau to aggregate, facilitating its entry into degradation or refolding pathways. However, Hsp27 cannot disaggregate preformed tau fibrils.

Thus, a bias for healthy neurons was indeed introduced by using NeuN in our imaging studies, because damaged neurons may lose antigenicity for NeuN (McPhail et al., 2004; Unal-Cevik et al., 2004; Collombet et al., 2006; Kienzler et al., 2009). However, because damaged neurons are likely a result of tau aggregation, we would speculate that Hsp27 would not impact damaged neurons because it cannot disaggregate preformed tau filaments (Fig. 5A), making such a bias unlikely to affect the interpretation of our results.

We performed hippocampal stereology in the CA1 (injection site) and DG of these same 6-month-old rTg4510 mice using Nissl stain to evaluate whether wtHsp27 could confer neuroprotection compared with 3×S/D Hsp27 and GFP-AAV9. Although there was a trend that wtHsp27-injected and 3×S/D Hsp27-injected mice appeared to have higher and lower numbers of CA1 neurons, respectively; however, these differences were not statistically significant (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This same trend was not observed in the dentate gyrus.

Hippocampal plasticity deficits in the rTg4510 mice are rescued by AAV9–Hsp27 injections

The next goal of these studies was to determine the physiological outcome of Hsp27 overexpression in the brain. Because Hsp27

Figure 4. Neuronal overexpression of Hsp27–AAV9 variants differentially modulates neuronal tau levels. **A**, Confocal images of immunofluorescently labeled neurons (blue), tau (red), and Hsp27 or GFP (green) in CA1 from rTg4510 mice injected with AAV9-expressing wtHsp27, 3×S/D Hsp27, or GFP (scale bar, 125 μ m). **B**, Higher zoom images of fields from CA1 regions of immunofluorescently labeled sections from AAV9-injected mice (scale bar, 50 μ m). **C**, Quantification of red signal (tau) in blue areas (neurons) sharing positive green signal (Hsp27 or GFP). Statistical significance was determined with Student's *t* tests. ****p* = 0.001.

Figure 5. wtHsp27 is unable to disrupt preformed tau aggregates. **A**, DLS scatter plot measuring the particle size of stably formed recombinant tau fibrils before and after addition of wtHsp27. Heparin was used to induce aggregation over the course of 22 d. wtHsp27 was then incubated, and particle size was measured over time. **B**, Tau can enter 3 protein folding pathways once it loses form and function: aggregation (K_{agg}), degradation (degrade), or refolding (K_{refold}). Aggregation and refolding of tau is based on equilibrium, as indicated by the bidirectional arrows. Hsp27 prevents tau aggregation and facilitates the entry of tau into degradation or refolding pathways. Because refolding, or recycling, of tau is in equilibrium with unfolded intermediates, it is possible that Hsp27 may increase the prevalence of these intermediates. Hsp27 is not able to disaggregate tau once fibrillization has already occurred.

was unable to disaggregate preformed tau filaments, we speculated that any effects Hsp27 might have on the tau-induced phenotype of the rTg4510 mice would be most pronounced if administration of Hsp27 was initiated before any tangle formation. Previous work showed that tangle formation in the rTg4510 hippocampus begins at ~2–3 months, and neuronal loss caused by this aberrant tau aggregation is only evident after 5 months. Thus, the CA1 subfields of hippocampi of 2-month-old rTg4510 mice and wild-type littermates (NTG) were injected with saline or AAV9 encoding wtHsp27, 3×S/D Hsp27, or GFP, with the goal being to test the effects of Hsp27 overexpression on LTP in the rTg4510 hippocampus before tangle formation. Mice were then killed at 4 months of age before neuronal loss manifests to assess whether Hsp27 was able to ameliorate the neuronal dysfunction brought on by accumulation of aberrant tau intermediates over the 2 month period.

Hippocampal slices from each treatment group were stimulated by a high-frequency TBS to assess LTP over a 60 min period. Whether injected with saline or GFP–AAV9, there was no difference in the pattern of LTP in mice of the same genotype (Fig. 6A). The rTg4510 mice had significant reductions in LTP relative to NTG controls in either treatment (**p* < 0.001), showing LTP dysfunction in the rTg4510 model for the first time. Interestingly, LTP of hippocampal slices taken from rTg4510 mice transduced with wtHsp27–AAV9 was identical to NTG mice injected with GFP–AAV9. Western blot confirmed reductions in tau levels (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Thus, wtHsp27 was able to functionally rescue hippocampal LTP deficits. Conversely, LTP deficits were equivalent in rTg4510 mice receiving 3×S/D Hsp27–AAV9 and GFP–AAV9 (Fig. 6B). Input/output analysis was done by plotting the fEPSP slopes to presynaptic fiber volley amplitude and showed no significant differences between rTg4510 mice receiving either Hsp27 variant compared with those receiving GFP (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). This analysis indicates that the functional rescue of LTP by wtHsp27 overexpression in rTg4510 mice was not attributable to changes in baseline synaptic transmission nor was viral induction generally altering presynaptic activity. These studies demonstrated that, for Hsp27 to facilitate clearance of abnormal tau intermediates, it must be functionally intact with regard to

phosphorylation dynamics. Moreover, because the LTP analyses were performed on 4-month-old rTg4510 hippocampal tissues, we can conclude that tau accumulation is sufficient to disrupt hippocampal function without killing neurons, because these mice do not have neuronal loss until after 5.5 months of age.

Discussion

The mechanisms used by Hsp27 to facilitate abnormal protein clearance have been difficult to define. Here, using a combination of biophysical and physiological approaches, we have discovered an important aspect of Hsp27 biology that, to this point, was unknown. Although both mock-phosphorylated Hsp27 and wild-type Hsp27 could bind and prevent tau aggregation *in vitro*, only wild-type Hsp27 was able to clear tau from the brain and rescue functional deficits. Because the mock-phosphorylated form of Hsp27 actually stabilized tau in the brain, it suggests that Hsp27 must be able to cycle between phosphorylated and non-phosphorylated states for it to succeed at clearing abnormal clients such as tau. Otherwise, Hsp27 might actually facilitate accumulation of soluble tau intermediates (Fig. 7).

Both phosphorylated and nonphosphorylated Hsp27 can bind to tau in Alzheimer's brain (Shimura et al., 2004). However, although both variants can bind and abrogate tau aggregation, preventing Hsp27 dephosphorylation might thwart its capacity to clear bound clients, preserving them as aberrant intermediates. Thus, Hsp27 may be a double-edged sword depending on the context of the neuron: Hsp27 may be highly effective at preventing aggregation of unfolded proteins resulting from acute stress events in neurons. The bound client would be preserved for recycling and functional restoration, the most energetically favorable outcome for proteostasis in otherwise healthy neurons. However, chronic stress would likely also cause chronic protein instability in neurons. In this scenario, functional recycling of unfolded or aberrant proteins may not be feasible in neurons that are already operating at suboptimal levels. Simply sequestering these clients into ordered aggregates would become the best option for survival, albeit at a reduced capacity (Hensley et al., 1999; Santacruz et al., 2005; Ben-Zvi et al., 2009; Miller, 2009; Rocher et al., 2009; Cohen et al., 2010; Frost and Diamond, 2010). Based on this model, Hsp27 phosphorylation would counteract the aggregation pathway, perhaps accelerating neuronal dysfunction. As novel mechanisms linking Hsp27 to both the degradation and aggregation pathways in neurons continue to be defined, therapeutics can be specifically tailored to target these mechanisms based on the pathogenic context.

Our findings also show that the oligomerization state of Hsp27 is not critically linked to its ability to prevent tau aggregation; only its ability to promote tau clearance *in vivo*. Perhaps discrete multimeric Hsp27 species can prevent tau aggregation via distinct physical interactions, but the ultimate outcome in the cell is the same. Thus, these unique Hsp27 multimers may represent different structural entities despite their primary composition. Each of these entities could have their own function and targets. This property could eventually lead to specific therapeutics targeting individual small Hsp multimers. Because there are ~11 small Hsps in the human proteome that can

Figure 6. LTP deficits in rTg4510 mice are rescued by wtHsp27 but remain unaffected with 3×S/D Hsp27. After recording baselines for 20 min, LTP was induced with TBS (5 bursts of 200 Hz separated by 200 ms, repeated 6 times with 10 s between the 6 trains), and LTP was recorded for 60 min. Changes in fEPSP slope are expressed as a percentage of baseline. **A**, Representative fEPSP traces for rTg4510 and age-matched NTG mice injected with either saline or GFP-AAV9. Changes in the slopes of NTG-GFP ($n = 6$) and NTG-saline ($n = 9$) were significantly different ($*p < 0.01$) from that of TG-GFP ($n = 5$) and TG-saline ($n = 9$). LTP was not significantly different between mice of the same genotype despite treatments ($p > 0.05$). **B**, Representative fEPSP traces for rTg4510 injected with wtHsp27-AAV9 ($n = 7$), 3×S/D-AAV9 Hsp27 ($n = 9$), or GFP-AAV9 ($n = 7$) and age-matched NTG mice injected with GFP-AAV9 ($n = 6$). Changes in fEPSP slopes between NTG-GFP versus TG-wtHsp27 and TG-3×S/D versus TG-GFP were not significantly different ($p > 0.05$). NTG-GFP and TG-wtHsp27 fEPSP slopes were significantly different from both TG-3×S/D and TG-GFP ($p < 0.001$). p values were determined by Student's t tests using data from the first and last 10 min of the LTP curves.

Figure 7. Proposed mechanism of wtHsp27 and 3×S/D Hsp27 modulation of tau aggregation. During stress-induced client denaturation or loss of function, both wtHsp27 and phospho-Hsp27 (S/DHsp27) bind and prevent client aggregation (*in vitro*). Client-bound wtHsp27 is then able to facilitate client clearance. Conversely, retention of Hsp27 in a mock-phosphorylated state subverts client clearance, leading to accumulation of soluble intermediates (*in vivo*).

cross-interact with each other, the number of potential superstructures that these proteins may form could number in the tens of thousands, similar to the diversity we achieve with just 20 amino acids. This concept of a “quaternary proteome” could represent a new landscape for understanding protein biology.

This study is also the first description that a cellular correlate of hippocampus-dependent learning and memory could be improved by extrinsic delivery of a tau-modifying protein to tau transgenic mice. This is in part attributable to the unique nature of the rTg4510 model. The forebrain accumulation of tau pathology in this model allows studies to be designed that can assess the functional consequences of altering tau pathogenesis in regions affected by Alzheimer’s disease (Ramsden et al., 2005; Santacruz et al., 2005; Dickey et al., 2009). This study also demonstrates the associative and cooperative properties of hippocampal neuronal signaling (LTP) as a correlate for learning and memory in the rTg4510 model: despite the fact that Hsp27 did not affect every neuron, it was still able to rescue the electrical potential of the hippocampal field in general. As efforts continue to be made toward understanding how mechanisms can be linked to physiological outcomes, we anticipate that other chaperone proteins will emerge as important therapeutic targets for tauopathies.

In summary, therapeutic approaches to modify Hsp27 levels and function may be effective strategies for treating distinct neurological disorders. However, the contextual environment of the neuron must be considered to select the most appropriate strategy for each disease. In some circumstances, increasing or decreasing Hsp27 levels may be beneficial, but in others it might be more appropriate to regulate the phosphorylation state and thus the oligomerization properties of Hsp27. By defining these important mechanisms, we can move toward more individualized and effective therapeutic strategies targeting the chaperone network.

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