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Differential Regulation of Small Heat Shock Proteins in Transgenic Mouse Models of Neurodegenerative Diseases

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Abstract

Previously, several studies have demonstrated changes in the levels of small heat shock proteins (sHSP) in the transgenic mouse models of familial amyotrophic lateral sclerosis (fALS) linked to mutations in Cu/Zn superoxide dismutase. Here, we compared the expression of sHSPs in transgenic mouse models of fALS, Parkinson's disease (PD), dentato-rubral pallido-luysian atrophy (DRPLA) and Huntington's disease (HD); where the expression of mutant cDNA genes was under the transcriptional regulation of the mouse prion protein promoter. These models express G37R mutant Cu/Zn superoxide dismutase (SOD1G37R; fALS), A53T mutant α-synuclein (α-SynA53T; PD), full-length mutant atrophin-1-65Q, and htt-N171-82Q (huntingtin N-terminal fragment; HD). We found that the levels and solubilities of two sHSPs, Hsp25 and αB-crystallin, were differentially regulated in these mice. Levels of both Hsp25 and αB-crystallin were markedly increased in subgroups of glias at the affected regions of symptomatic SODG37R and α-SynA53T transgenic mice; abnormal deposits or cells intensely positive for αB-crystallin were observed in SODG37R mice. By contrast, neither sHSP was induced in spinal cords of htt-N171-82Q or atrophin-1-65Q mice, which do not develop astrocytosis or major motor neuron abnormalities. Interestingly, the levels of insoluble αB-crystallin in spinal cords gradually increased as a function of age in nontransgenic animals. In vitro, αB-crystallin was capable of suppressing the aggregation of α-SynA53T, as previously described for a truncated mutant SOD1. The transgenes in these mice are expressed highly in astrocytes and thus our results suggest a role for small heat shock proteins in protecting activated glial cells such as astrocytes in neurodegenerative diseases.

Keywords

neurodegeneration; aging; Hsp25; αB-crystallin

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1. Introduction

Heat shock proteins (HSPs) contribute to a repertoire of molecular chaperones that function to regulate the folding of cellular proteins. In certain physiologic settings, and under conditions of stress, the HSPs, which include HSP110, HSP90, HSP70, HSP60, HSP40, and the small HSP family, engage in a variety of functions to regulate protein folding. Small heat shock proteins (sHSPs) are a family of proteins with molecular mass ranging from 12 to 43 kDa, with the defining feature of the α-crystallin domain, which is highly conserved from prokaryotes to mammals [9]. Although implicated in diverse pathways, the physiological functions of sHSPs remain unclear. Unlike other catalytic chaperons, sHSPs lack ATPase domains but several members can stabilize unfolded proteins. Most sHSPs form multi-subunit oligomers in physiological conditions; the α-crystallin domain, a conserved β-sheet sandwich structure, can engage in an intersubunit composite β-sheet to form a dimer, a potential building block for higher-order structures [15].

Mammalian sHSPs comprise at least ten members; only Hsp25 (Hsp27 in human), αB-crystallin, and the recently identified HSPB8 are stress-inducible [38]. Hsp25 is induced during development and stressful conditions [7;31]; and has been reported to exhibit anti-apoptotic function through possible mechanisms including interfering with the caspase pathway [12; 34], modulating oxidative stress [29], or regulating cytoskeleton [23]. αB-crystallin is a major structural protein in the vertebrate lens, but is also expressed in many other tissues, with suggested chaperon-like in vitro activities [16].

While the role of the sHSPs in the nervous tissue is not established, a variety of HSPs, including sHSPs, have been implicated in neurological disorders. Missense mutations in Hsp27 are linked to the sensory and motor neuropathies in Charcot-Marie-Tooth disease and distal hereditary motor neuropathies [10]. A mutation in αB-Crystallin is associated with desmin-related myopathy [40]. Induction of αB-Crystallin has been reported in Alexander's disease [18], Creutzfeldt-Jacob disease [33], Alzheimer's disease [26], and other neurological conditions [19]. Hsp25 and αB-crystallin have been reported to be induced in mouse models of SOD1-linked amyotrophic lateral sclerosis [41;43].

Transgenic mice expressing mutant SOD1, either in a human gene cassette with ubiquitous expression [6;14;43;47] or under the control of a more restrictive promoter, the mouse prion promoter (PrP) [45], recapitulate many features of motor neuron degeneration in human Amyotrophic Lateral Sclerosis (ALS). Transgenic mice expressing human α-synuclein (α-Syn) harboring the A53T mutation, under the control of the same prion promoter, recapitulate features of Parkinson's Disease (PD) and other α-synucleinopathies [13;24]. Both the mutant SOD1 and α-Syn models develop motor deficits leading to paralysis, which are associated with motor neuron loss and ubiquitinated inclusions in the brain stem and the spinal cord. We have recently described up-regulation of two small heat shock proteins, Hsp25 and αB-crystallin, in the disease tissue of transgenic mice carrying the human genomic SOD1 gene (Gn.SOD1) [43], or the PrP.SOD1 cassette [45]. To explore the relationship between chaperone proteins and neurodegenerative diseases associated with abnormal protein aggregates, we have systematically investigated the regulation of a panel of chaperones in four transgenic mouse models of neurodegenerative disease that involve an accumulation of aggregated protein. The four models we study here, SOD1G37R [45], α-SynA53T [24], Atrophin-1-65Q [36] and htt-N171-82Q [35], express mutant genes via a vector derived from the mouse prion protein. Our study demonstrates that induction of Hsp25 and αB-Crystallin in spinal cords of PrP.SOD1-G37R and PrP.α-SynA53T mice correlates to the presence of astroglial responses and that both proteins are highly induced in glias rather than neurons. Neither of these sHSPs is induced in the spinal cords of either of the polyglutamine mouse models, which also lack evidence of astrocytic responses or degenerative changes in spinal cord. As previously demonstrated for

mutant SOD1L126Z [44], αB-crystallin was found to inhibit the in vitro aggregation of α-SynA53T, suggesting that αB-crystallin may slow aggregation of mutant protein in glias. Robust increases in insoluble αB-crystallin were also noted as a function of age in non-transgenic animals, suggesting the aging process produces signals that modulate αB-crystallin. These findings provide evidence that the astroglial response and normal aging, in mice, is associated with the induction of inhibitors of protein aggregation.

2. Materials and Methods

2.1. Transgenic mice

Mice harboring fragments of the human genomic SOD1 gene encoding the G37R mutation, Gn.SOD1G37R (line 29) or the wild-type sequence (line 76) have been previously reported [47]. The SOD1 cDNA G37R transgenic mouse model (line 110) was recently reported [45], using the previously described PrP promoter vector [4]. PrP.α-synuclein (line G2-3 and O2), PrP.htt-N171-82Q (expressing a mutant N-terminal fragment of huntingtin; line 81) and PrP.AT65Q (expressing a full-length atrophin-1; line 150) mice were previously reported [24;35;36]. All the transgenic mice were generated by injecting DNA into mouse embryos [C3H/HeJ X C57BL/6J F2]. All lines were maintained by crossing transgenic males to non-transgenic [C57BL/6J X C3/HeJ F1] females, except for PrP.α-synuclein mice which have been successively backcrossed into the C57BL6/*J* strain. Non-transgenic mice [C57BL/6J X C3/HeJ F1] were purchased from Jackson Laboratories (Bar Harbor, ME). The procedures involving animals were approved by the Animal care & Use Committee of The Johns Hopkins Medical Institutions.

2.2. Detergent extraction and gel electrophoresis

The methods used in detergent extraction of tissue homogenates have been previously described [43]. Briefly, tissue homogenates were extracted by sonicating in buffer A (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, and 100mM NaCl; 1% Nonidet P40; proteinase inhibitor cocktail 1:100 dilution <P 8340, Sigma, St. Louis, MO>), and then centrifuged at >100,000 g for 10 minutes to separate supernatant S1 and pellet P1. The P1 pellet was extracted once more with buffer A, and centrifuged to produce the pellet P2, which was either suspended in buffer B (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, and 100mM NaCl; 0.5% Nonidet P40; 0.5% deoxycholic acid; 2% SDS) for denaturing SDS-PAGE or in buffer C (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 9M urea, 2% Nonidet P40, 40mM DTT) for 2D gel electrophoresis. Samples were mixed with 4X Laemmli Buffer and boiled before denaturing SDS-PAGE and immunoblotting. Equal protein loading and transfer was routinely monitored by ponceau-S staining of the nitrocellulose membranes.

2.3. Immunoblotting and Immunohistochemistry

Antibodies against Hsp25 (SPA-801), αB-crystallin (SPA-222), Hsp40 (SPA-450), Hsp60 (SPA-806), Hsp70 (SPA-812), and Hsp90 (SPA-830) were purchased from Stressgen (Victoria, BC, Canada). A monoclonal antibody for α-Syn was purchased from BD transduction laboratories (San Diego, CA). The antibody against glial fibrillary acidic protein (GFAP) was purchased from Dako Corp (Carpenteria, CA). Luxol fast blue was purchased from Sigma (St. Louis, MO). Immunoblotting and Immunocytochemical analysis was performed as previously described [24;43;45]. Luxol fast blue staining for myelin was done on paraffin sections (12 μm). The sections were treated with xylene and 95% alcohol and stained with 0.1% Luxol Fast Blue in 95% ethanol, 0.5% acetic acid and destained using 0.05% $LiCO_3$.

2.4. Cell-free aggregation assay

Brains from young PrP.α-SynA53T transgenic mice were homogenized with a Dounce homogenizer in phosphate buffered saline (PBS, pH 7.4) with proteinase inhibitors. The homogenate was centrifuged at 150,000 g and 4 °C for 50 minutes, and the supernatant was adjusted to 2 mg/ml of proteins with PBS. To induce aggregation, 50 μl of samples were added to a 200 μl tube, and shaken on a titer plate shaker (750 rpm) at 37 °C for 12 hrs. To study the affects of αB-crystallin on α-synuclein aggregation, purified α-crystallin (predominantly αB-crystallin, SPP-225, Stressgen, Victoria, BC, Canada) was added at 0.4 ug/ul. In some reactions, both α-crystallin (0.4 ug/ul) and purified mouse monoclonal anti-αB-crystallin IgG (0.16 ug/ul) were present in the reaction. For the control reactions, equal amount of solvent alone were added. The resulting samples were centrifuged in a Beckman airfuge (>100,000 g) for 10 minutes, and the pellets were washed twice with PBS. The resulting pellets were considered aggregated proteins, and resuspended in PBS with 2% SDS for further analyses.

3. Results

3.1 Up-regulation of Hsp25 and αB-crystallin in murine models of ALS and an α-SynA53T model of PD, but not in models of polyglutamine diseases

The SOD1G37R and α-SynA53T models have similar cellular pathology and behavioral phenotypes; both develop motor deficits leading to paralysis with associated cellular pathology in the brain stem and the spinal cord. To investigate the responses of molecular chaperones in these two models, levels of major chaperone proteins, in both their soluble and insoluble forms, were investigated. For Hsp90, Hsp70, Hsp60, and Hsp40, no significant changes in expression levels were associated with the transgene expression or the presence of symptoms in either SOD1 or α-Syn mice (Fig. 1). We also examined how the levels of these chaperones are regulated by age. As with the study of adult mice, there was no evidence of changes in the levels of Hsp90, Hsp70, Hsp60, and Hsp40, as function of age (not shown).

To examine the sHSPs, several strains of SOD1 and α-Syn transgenic mice were used. The mutated human genomic SOD1 (Gn.SOD1G37R) gene expresses the protein ubiquitously in the mouse and these animals develop ALS-like motor neuron disease, whereas Gn.SOD1WT (line 76) expresses wild-type human SOD1 at comparable levels but does not cause disease [47]. The PrP promoter drives expression of transgenes in neurons and astrocytes of the CNS, and in other tissues including muscle [45]. A line of mice expressing SOD1G37R under the control of the PrP promoter (line 110) is free of disease as hemizygous transgenic mice, but develops motor neuron disease as early as 7 months of age when the transgene dose is increased by breeding to homozygosity [45]. The same PrP promoter vector was used to establish a PD mouse model by expressing the α-SynA53T; mice from line G2-3 exhibit brain stem and spinal cord pathology and motor deficit phenotypes. By contrast mice expressing α-SynA30P at a comparable level (line O2), via the same vector, do not develop disease symptoms or pathology [24;28]. Since both SOD1 and α-Syn models have spinal cord pathology, this tissue was harvested to study and compare the expression of sHSPs (Fig. 2).

In both mouse models, Hsp25 was significantly up-regulated, in both the supernatant and pellet fractions of non-ionic detergent extracts in spinal cords from all symptomatic SOD1 and α-Syn mice (Fig. 2A–B, left). The induction of Hsp25 was selectively associated with neurodegeneration since the Hsp25 levels were not elevated in transgenic mice expressing high levels of non-pathogenic SOD1 (SOD1WT) or α-Syn (A30P) protein (Fig. 2A, left). In symptomatic mice from both models, αB-crystallin was also induced but the solubility profiles of αB-crystallin and Hsp25 differ. In both models, the levels of Hsp25 are significantly increased in both the soluble and insoluble fractions, whereas the bulk of the induced αB-crystallin is associated with the detergent insoluble fraction (Fig. 2A–B). Further, while the

levels of αB-crystallin in the insoluble fraction (P2), from both Gn.SOD1G37R and PrP.SOD1G37R, were significantly higher than controls in virtually all of the symptomatic SOD1 mice (>10 fold), the levels of insoluble αB-crystallin were highly variable in the mutant α-Syn transgenic mice. For example, in some symptomatic α-SynA53T mice, significant increases in the levels of detergent soluble αB-Crystallin were not accompanied by a corresponding increase in the insoluble fraction (Fig. 2A, right panels, last right lane). Thus, our results indicate that accumulation of insoluble αB-crystallin is a variable feature of the α-SynA53T disease model. In the course of our analysis, we also observed that normal aging has a noticeable effect on induction of αB-crystallin (see below), which may explain the low level in the 1.2 month old PrP.SOD1G37R mouse (Fig. 2A, right panels, third lane). The increases in the Hsp25 and αB-crystallin levels were clearly restricted to the pathologically affected regions (brain stem and spinal cord). Expression levels of both sHSPs in unaffected regions (e.g. cortex) were very low and not different from non-transgenic animals (not shown).

Because Hsp25 and αB-crystallin are known to be phosphorylated at several sites [3;30], we used 2D-PAGE to examine whether the induction of these sHSPs is associated with posttranslational modifications. For both proteins, immunoblot analysis of isoforms resolved by 2D-PAGE revealed several isoelectric variants in the soluble and insoluble fractions; these isoforms correspond to those reported to be generated by phosphorylation (Fig. 2C) [11;17]. However, because of very low level of Hsp25 level in control samples, we were not able to accurately detect the more acidic Hsp25 isoforms. Thus, we could not determine if the relative abundance of Hsp25 isoforms change with the disease. Overall, it appears that the the major isoforms are similarly represented in the both the soluble and insoluble fractions from the affected tissues.

Polyglutamine diseases such as Huntington's Disease and Dentato-Rubral Pallido-Luysian Atrophy, have been modeled in mice by expressing a mutant N-terminal fragment of hungtingtin (htt-N171-82Q; line 81) or a full-length atrophin-1 (AT65Q; line 150), respectively, via the same PrP vector. Both polyglutamine proteins undergo protein aggregation, a process implicated in pathogenesis of both diseases. In both the htt-N171-82Q and AT65Q mice, all major regions of the brain and brain stem exhibit nuclear inclusion pathology [35;36]. Motor neurons of the spinal cord also exhibit nuclear inclusions, particularly in the large motor neurons (Jiou Wang, Gabriele Schilling, and David R Borchelt, unpublished observations). However, these models of polyglutamine toxicity are not associated with significant astrogliosis in any part of the CNS [35;36]. In contrast to the strong induction of sHSPs in the mutant SOD1 and α-Syn mice, there is little or no change in the levels of Hsp25 or αB-Crystallin in the spinal cords of either polyglutamine model (Fig. 2D). Thus the expression of mutant SOD1 and α-Syn is associated with a specific induction of sHSPs in the degenerating spinal cord.

During the course of the present study, we noticed that multiple samples from older mice exhibited higher sHSP levels than younger mice, suggesting that aging may be a factor affecting the levels or solubility of sHSPs in CNS. To test this idea, mice of the same gender with the same genetic background (C57BL/6J X C3H/HeJ) were analyzed at multiple ages. Significant increases in the levels of soluble and insoluble αB-Crystallin were noted (Fig. 3). Less obvious, but significant, changes in Hsp25 were noted (Fig. 3). Gender did not have a detectable effect on the levels of either protein (not shown). It is notable that neurodegeneration has a much more remarkable effect on the up-regulation of both proteins, as demonstrated by a parallel sample from a symptomatic α-SynA53T mouse (Fig. 3A). We have not observed any obvious change in the distribution of either sHSP by immunohistochemistry in normal aging mice (not shown).

3.2. Up-regulation of Hsp25 and αB-crystallin occur primarily in reactive astroglia and oligodendrocytes, respectively

The distribution of induced Hsp25 and αB-Crystallin in PrP.SOD1G37R and PrP.α-SynA53T transgenic mice was examined by immunohistochemical analyses of tissues from transgenic and non-transgenic mice. In normal adult mice, Hsp25 is expressed at very low levels in the forebrain, which is confirmed by Western analyses (not shown). In the cerebellum, Hsp25 distinctly marks a subset of Purkinje cells (Fig. 4B). In the brain stem and spinal cord, the Hsp25 antibody stains a subset of large well-defined neurons (not shown), largely in agreement with previous reports [2;44].

In the symptomatic mutant PrP.SOD1G37R transgenic mice, the most obvious increase in Hsp25 immunoreactivity was associated with reactive astrocytes (Fig. 4). In addition to the occasional Hsp25 positively stained cells in the forebrain, clusters of heavily stained glial cells were present in the inferior colliculus, cerebellar white matter (Fig. 4A), and the neuropil throughout the brain stem and spinal cord (Fig. 4C, D). Overall, there was no obvious increase in neuronal Hsp25 staining, and most, if not all, of the cells that stain intensely for Hsp25 have cellular and nuclear morphology that is consistent with astrocytes (Fig. 4C,D). In some cells, intense Hsp25 staining suggests inclusion-like deposits (Fig. 4D). While further studies are needed to confirm the cellular identity of cells with Hsp25 “aggregates”, the cellular morphology (presence of processes) suggest that they are also astrocytes. Up-regulation of Hsp25 in astrocytes of PrP.SOD1G37R transgenic mice is consistent with previous studies showing similar induction of Hsp25 expression in the astrocytes of Gn.SOD1-G93A and Gn.SOD1-L126Z transgenic mice [41;44]. In symptomatic PrP.α-SynA53T mice, similar but less profound Hsp25 pathology is found (Fig. 5A–D). As in the mutant SOD1 transgenic mice, increased Hsp25 expression is largely localized to cells with astroglial morphology and is restricted to regions associated with robust α-synucleinopathy (Fig. 5A–D). Unlike the mutant SOD1 transgenic mice, heavily Hsp25-postive astroglial deposits were not observed in the disease-affected α-Syn transgenic mice.

In normal adult mice, the αB-crystallin positively stained cells are restricted in white matter of the brain, while evenly distributed in both the gray matter and the white matter of the spinal cord (Fig. 4F, H, I). Based on the nuclear morphology and scant cytoplasm of the immunoreactive cells, it is likely that the αB-crystallin expression is localized to the oligodendrocytes in spinal cords of normal mice (Fig. 4H, I) [44]. In the disease-affected PrP.SOD1G37R transgenic mice, αB-crystallin localization was highly abnormal as indicated by the presence of heavily stained “clumps” in the neuropil, with either irregular or round shapes, that are likely cellular inclusions or cell remnants (Fig. 4E, G). The cellular origin of these intensely stained clumps is unclear. While the induction of αB-Crystallin seems to occur in astroglial cells in the transgenic model expressing the truncated SOD1 mutant, Gn.SOD1-L126Z [44], the abnormal αB-crystallin “aggregates” here are not clearly associated with any cells of specific morphology. Given that the expression αB-Crystallin is limited to oligodendrocytes in nontransgenic and in young PrP.SOD1G37R mice, it is likely that the αB-Crystallin “aggregates” may be associated with oliogodendrocytes. Overall, the distribution of these αB-crystallin deposits in the CNS closely parallels the distribution of Hsp25-stained astrocytes and neuropathology (i.e., most abundant in the inferior colliculus, cerebellar white matter, brain stem, and spinal cord).

Consistent with variable levels of αB-crystallin expression in the PrP.α-SynA53T transgenic mice, there were no obvious abnormalities in αB-crystallin localization in the spinal cords of symptomatic α-Syn transgenic mice. However, overall αB-crystallin staining was increased throughout the neuropil (not shown). The lack of abnormal αB-crystallin localization in α-Syn transgenic mice is not because of the absence of astrogliosis (Fig. 5E) or myelinated axon degeneration in brain stem and spinal cord. Luxol fast blue staining of the brain stem and spinal

cord from the disease-affected mutant α-Syn transgenic mice shows significant disintegration of myelinated fiber tracts (Fig. 5G).

Collectively, these results suggest that Hsp25 is induced in astrocytes as a general component of reactive astrocytic response in the mutant SOD1 and mutant α-Syn transgenic mouse models. However, despite the degeneration of myelinated axons and disintegration of CNS myelin in the α-SynA53T mice, accumulation of αB-crystallin into abnormal morphologies such as “clumps” appear to be specific to the mutant SOD1 mice.

3.3 Suppression of α-SynA53T aggregation by αB-crystallin

In a previous study, we found that αB-crystallin can inhibit aggregation of the truncation mutant SOD1-L126Z in a cell-free aggregation assay [44]. To ask whether αB-Crystallin can exert such chaperone activity on α-SynA53T, we examined the affects of αB-crystallin on the aggregation of this protein from mouse tissues using the same cell-free assay. Incubation of high-speed supernatants from brain at 37°C leads to formation of insoluble α-SynA53T aggregates within few hours. But in the presence of αB-crystallin the formation of insoluble α-SynA53T aggregates is significantly reduced (Fig. 6). The activity of αB-crystallin was partially neutralized by an inhibitory monoclonal anti-αB-crystallin antibody, confirming that αB-crystallin is an inhibitor of α-SynA53T aggregation.

4. Discussion

The present study documents differences in the regulation of heat shock protein responses in mouse models of familial ALS, PD, HD, and DRPLA. The transgene expression in the models studied here is regulated by the mouse prion protein vector. Thus, while these mouse models express mutant genes that are specific for different diseases, they are completely comparable in terms of the expression patterns of genes. Remarkable increases in the levels of two sHSPs, Hsp25 and αB-crystallin, were associated with disease in mutant SOD1 and mutant α-Syn transgenic mouse models. In contrast, the expression of two distinct polyglutamine proteins, an N-terminal fragment of hungtingtin and a full-length atrophin-1, did not induce significant alterations in the levels of either of these sHSPs. We also show that changes in the levels of these sHSPs in both SOD1 and α-Syn models are associated with the presence of reactive astroglia. Most of the induced Hsp25 is found in cells with typical astroglia morphology, whereas the cellular distribution of αB-crystallin appears to be primarily oligodendrocytes with clumps of reactivity in neuropil. In a prior study of a model with a different mutant SOD1 L126Z, induction of αB-crystallin in astrocytes was demonstrated [44]. Thus, it is possible that part of the neuropil staining seen in the PrP.SOD1G37R mice originates from astroglial cells. Other studies have also shown induction of sHSP in glial cells with disease in mutant SOD1 transgenic mice [27;41]. The increase in glial Hsp25 expression contrasts with a decrease in Hsp25 expression in motor neurons of presymptomatic Gn.SOD1G93A mice [27] and in surviving motor neurons of mutant SOD1 L126Z transgenic mice [44]. While we have not focused on the neuronal expression of sHSPs in the current study, the surviving motor neurons in the SOD1 transgenic mice did show reduced Hsp25 staining (data not shown) and the ventral horn motor neurons in α-SynA53T transgenic mice seem to stain less intensely for Hsp25 than in control mice (see Fig. 5 C, D).

Between the SOD1 and α-Syn models, partitioning of sHSPs into detergent insoluble complexes was exhibited at a higher degree in the mutant SOD1 model. Furthermore, we noted that normal aging of mouse CNS is also associated with increased partitioning of αB-crystallin into the detergent insoluble phase. As was previously reported for mutant SOD1L126Z, αB-Crystallin was capable of inhibiting the aggregation of α-SynA53T. Collectively, our results indicate that a component of the glial response is to induce sHSPs, including αB-crystallin which is capable of preventing the aggregation of mutant SOD1 [44] and α-SynA53T in vitro.