Human Molecular Genetics, 2001, Vol. 10, No. 14 **1511–1518**

Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in *SCA1* mice

Christopher J. Cummings[1], Yaling Sun[1], Puneet Opal[2], Barbara Antalffy[3], Ruben Mestril[6], Harry T. Orr[7], Wolfgang H. Dillmann[8] and Huda Y. Zoghbi[1,2,4,5,*]

[1]Department of Pediatrics, [2]Department of Neurology, [3]Department of Pathology, [4]Department of Molecular and Human Genetics, [5]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA, [6]Department of Physiology, Loyola University, Maywood, IL 60153, USA, [7]Departments of Laboratory Medicine and Pathology and Institute of Human Genetics, University of Minnesota, Minneapolis, MN 55455, USA and [8]Department of Medicine, Division of Endocrinology and Metabolism, University of California at San Diego, San Diego, CA 92103, USA

Received April 18, 2001; Revised and Accepted May 14, 2001

Many neurodegenerative diseases are caused by gain-of-function mechanisms in which the disease-causing protein is altered, becomes toxic to the cell, and aggregates. Among these 'proteinopathies' are Alzheimer's and Parkinson's disease, prion disorders and polyglutamine diseases. Members of this latter group, also known as triplet repeat diseases, are caused by the expansion of unstable CAG repeats coding for glutamine within the respective proteins. Spinocerebellar ataxia type 1 (SCA1) is one such disease, characterized by loss of motor coordination due to the degeneration of cerebellar Purkinje cells and brain stem neurons. In SCA1 and several other polyglutamine diseases, the expanded protein aggregates into nuclear inclusions (NIs). Because these NIs accumulate molecular chaperones, ubiquitin and proteasomal subunits—all components of the cellular protein re-folding and degradation machinery—we hypothesized that protein misfolding and impaired protein clearance might underlie the pathogenesis of polyglutamine diseases. Over-expressing specific chaperones reduces protein aggregation in transfected cells and suppresses neurodegeneration in invertebrate animal models of polyglutamine disorders. To determine whether enhancing chaperone activity could mitigate the phenotype in a mammalian model, we crossbred SCA1 mice with mice over-expressing a molecular chaperone (inducible HSP70 or iHSP70). We found that high levels of HSP70 did indeed afford protection against neurodegeneration.

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disorder characterized by progressive ataxia and degeneration of cerebellar Purkinje cells, inferior olivary neurons, and neurons within the brain stem. Symptons typically strike in midlife and worsen over the next 10–15 years; there is no established therapy to delay the onset or slow the progression of the disease (1). SCA1 is caused by the abnormal expansion of polyglutamine within the SCA1 gene product ataxin-1; normal alleles have between six and 44 glutamines, whereas disease alleles may bear as many as 82 units (2). At least seven other human neurodegenerative diseases are caused by a polyglutamine repeat expansion, including Huntington's disease, dentatorubro-pallidoluysian atrophy, spinocerebellar ataxia types 2, 3, 6, 7 and 12, and spinobulbar muscular atrophy (3). The mechanism by which expanded proteins lead to long-term neurodegeneration remains elusive, but it is clear from numerous studies that the expanded polyglutamine tract confers a toxic property or 'gain of function' on the otherwise unrelated disease proteins.

One hallmark of these diseases—and many other neurodegenerative disorders, including Alzheimer' disease, Parkinson' disease, amyotrophic lateral sclerosis and the prion disorders—is the formation of insoluble protein aggregates or inclusion bodies. These aggregates are immunoreactive for ubiquitin, and most have been reported to contain molecular chaperones and components of the proteasome (4). In previous work we put forward the hypothesis that the expanded polyglutamine tract alters the conformation of the ataxin-1, and that the misfolded protein is targeted for re-folding and proteolysis by the ubiquitin-proteasome pathway. The earliest suggestion that this might be the case came from a study showing that nuclear inclusions (NIs) in both SCA1 patient tissue and transgenic mice stain positively for the molecular chaperones Hsp70 and Hsp40, ubiquitin and proteasomal subunits (5). In

*To whom correspondence should be addressed at: Department of Pediatrics, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030, USA; Tel: +1 713 798 6558; Fax: +1 713 798 8728; Email: hzoghbi@bcm.tmc.edu
Present address: Christopher J. Cummings, AGY Therapeutics, 290 Utah Avenue, South San Francisco, CA 94080, USA

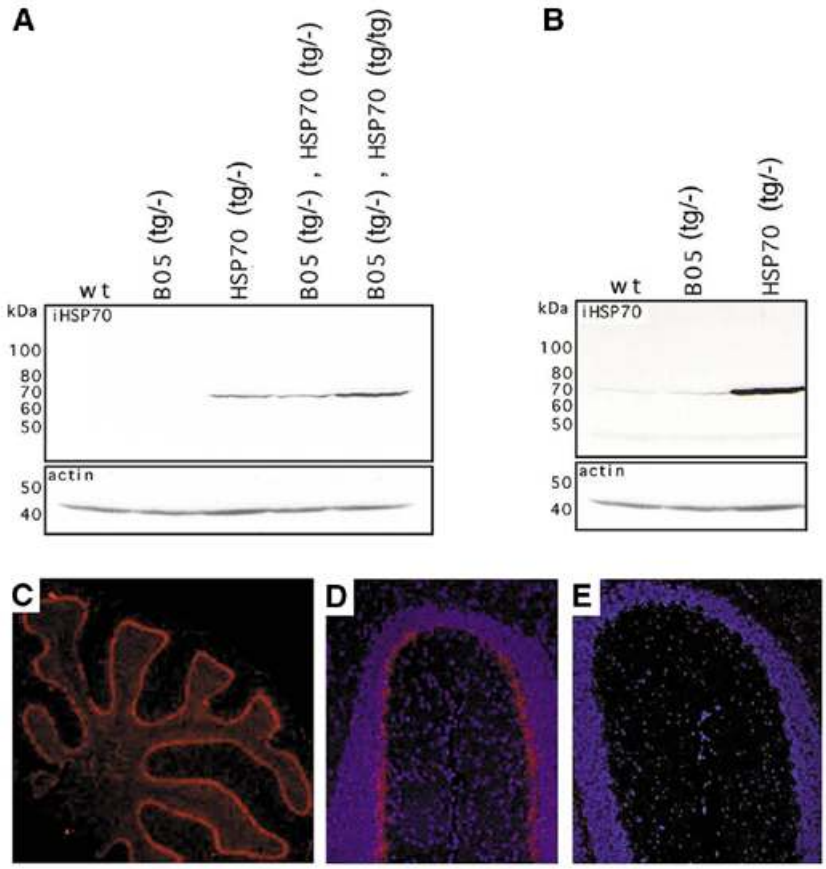

Figure 1. Transgenic mice ($HSP70^{tg/-}$; $B05^{tg/-}$, $HSP70^{tg/-}$; and $B05^{tg/-}$, $HSP70^{tg/tg}$) express high levels of rat iHSP70 in cerebellar Purkinje cells. Note that the SCA1 transgene in the double transgenic does not alter HSP70 levels. (**A**) Western blot analysis of total cerebellar protein lysate with antibodies specific for the inducible form of HSP70 shows a single band migrating at ~70 kDa in HSP70 transgenic mice but not in wild-type or B05 mice. (**B**) Maximum exposure of a similar western blot to reveal the low levels of endogenous iHSP70 demonstrates that rat iHSP70 expression is ~10-fold greater than endogenous iHSP70. (**C**–**E**) *In situ* RNA analysis demonstrates that the transgene is predominantly expressed in the Purkinje cell layer. Higher magnification in (E) reveals that the iHSP70 transcript in wild-type control animals is only slightly above the background. Therefore, unstressed transgene-positive mice express high levels of iHSP70 in cerebellar Purkinje cells. This high level of expression appears to occur without any apparent detrimental effect on gross cerebellar morphology.

support of a role for chaperones, we found that over-expression of an HSP40 chaperone, HDJ-2, reduces aggregation in tissue culture (5). To investigate the role of the ubiquitin-proteasome pathway in SCA1 pathogenesis, we crossed SCA1 transgenic mice (the B05 line) with mice lacking the ubiquitin E3 ligase, Ube3a. Neuronal degeneration was accelerated in these double mutants, and these results provided the first *in vivo* (albeit indirect) support for our hypothesis (6,7).

A genetic screen in an SCA1 fly model provided the most recent evidence that protein clearance pathways are involved in SCA1 pathology. Significantly, some of the more prominent exacerbations of the SCA1 phenotype occurred in the context of deficiency of chaperone proteins, including HSP70 (8). Other *Drosophila* models have also been used to demonstrate that overproduction of the Hsp70 and Hsp40 molecular chaperones suppresses polyglutamine-induced neurotoxicity (8–11). Chan *et al.* (11) further demonstrated that over-expression of the HSP40 chaperone dHdj-1 can suppress polyglutamine toxicity only in the presence of functional HSP70. Since HSP40 chaperones present substrates to HSP70 and stimulate its activity, it is not surprising that dHdj-1 would require functional HSP70 in order to modify the phenotype in flies.

RESULTS

Crossbreeding and iHSP70 expression analysis of HSP70/SCA1 mice

To determine whether over-expression of HSP70 could ameliorate the disease phenotype in an animal model which closely approximates the human disease, we crossed the well characterized B05 line (12) with mice that over-express the inducible form of rat HSP70, under the control of the human cytomegalovirus enhancer and chicken β-actin promoter (13). To evaluate their suitability for studies of the role of HSP70 on SCA1-induced neurodegeneration, we first sought to determine whether they express high levels of the transgene in cerebellar Purkinje cells. We used western blot analysis to compare the expression of endogenous HSP70 with that of iHSP70 in whole cerebellar extracts from Hsp70 transgenic and wild-type mice, B05 and B05/HSP70 double transgenics. Levels of endogenous iHSP70 in non-stressed wild-type mice are extremely low; HSP70 transgene expression is ~10-fold higher in $HSP70^{(tg/-)}$ mice (Fig. 1A and B). It is interesting that there is no difference between wild-type and B05 animals in HSP70 expression, which suggests that mutant ataxin-1

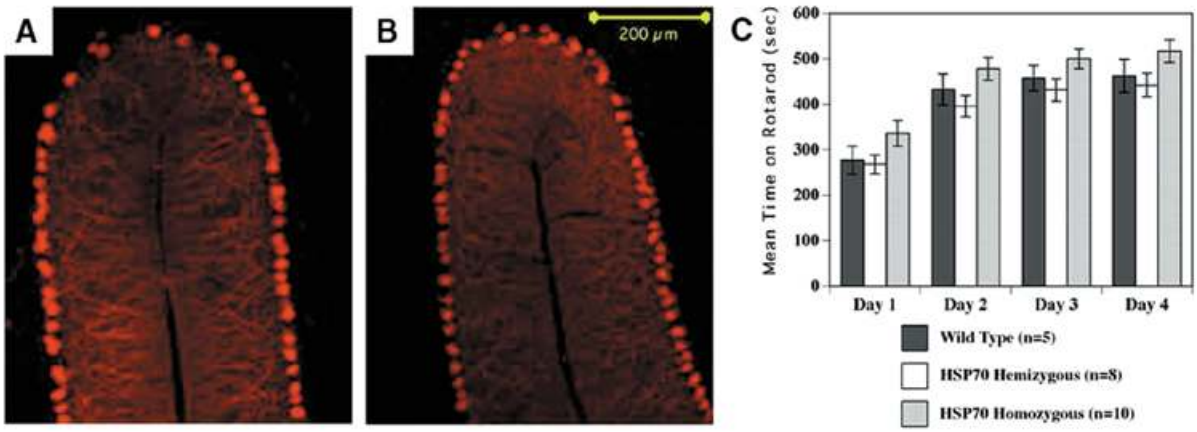

Figure 2. Cerebellar morphology and function in transgenic mice expressing rat iHSP70. Calbindin staining of cerebellar sections from 12.5-week-old wild-type (**A**) and HSP70$^{(tg/-)}$ mice (**B**) show similar morphology, indicating that HSP70 over-expression has no detectable effect on cerebellar and Purkinje cell morphology. (**C**) Expression of HSP70 alone has no significant effect on Rotarod performance.

expression alone does not elicit a notable HSP70 stress response. HSP70$^{(tg/-)}$ and B05$^{(tg/-)}$/HSP70$^{(tg/-)}$ mice demonstrate equal HSP70 levels (Fig. 1B). As homozygotes, HSP70$^{(tg/tg)}$ (data not shown) or B05$^{(tg/-)}$/HSP70$^{(tg/tg)}$ mice exhibit twice the HSP70 levels of HSP70 hemizygotes. Since it is conceivable that the abundant expression might be contributed by non-Purkinje cells—either neuronal or glial populations—we performed RNA *in situ* analysis to examine HSP70 expression within the cerebellum. We found that the strongest *in situ* signal localizes to the Purkinje cell layer (Fig. 1C and D). Immunohistochemical studies of the same HSP70 transgenic line confirmed that Purkinje cells contain high levels of the transgene product (14) and that the protein is predominantly cytoplasmic.

Chaperone over-expression alone is not deleterious to morphology or function

Because molecular chaperones have such a wide variety of functions, we first sought to determine whether over-expression of HSP70 might have deleterious effects on neuronal architecture or function. Because our subsequent analysis would be restricted to the cerebellum, we focused on cerebellar cyto-architecture and Purkinje cell dendritic morphology using the Purkinje cell-specific marker, calbindin. The fissura prima, which defines the anterior boundary of the central lobe, shows a similar degree of neuronal degeneration from one B05 animal to another, so we examined it at high magnification for comparison. Examination of wild-type and HSP70$^{(tg/-)}$ sections at 12.5 weeks (Fig. 2A and B, respectively) reveals that over-expression of rat iHSP70 alone does not have any detectable effect on normal cerebellar development or Purkinje cell morphology. Cerebellar function in HSP70 mice also seems unaffected; accelerating Rotarod analysis of 9.5-week-old mice revealed no difference between non-transgenic and transgenic mice over-expressing HSP70 at either hemizygous or homozygous levels (Fig. 2C; statistical analysis of variance revealed no differences in performance). These studies indicate that over-expression of the iHSP70 chaperone alone does not impair Purkinje cell development, survival or function.

High levels of chaperone expression mitigate the SCA1 behavioral phenotype

Having established that HSP70 over-expression does not itself cause a phenotype that would confound the results of our proposed study, we performed accelerating Rotarod tests at 9.5 and 12.5 weeks with B05 and B05/HSP70$^{(tg/-)}$ animals (Fig. 3A and B). B05 transgenic mice become ataxic by home cage behavior at 12 weeks of age and show motor incoordination by Rotarod testing as early as 5 weeks after birth (6,12). At 9.5 weeks of age, the B05 mice were significantly more impaired on the Rotarod than B05 littermates over-expressing HSP70$^{(tg/-)}$ (for the latter 3 of 4 days of trial) (Fig. 3A). Although both B05 and B05/HSP70$^{(tg/-)}$ mice performed worse at 12.5 weeks than at 9.5 weeks, the B05 littermates expressing HSP70$^{(tg/-)}$ (Fig. 3B) were notably better than the B05 mice. The 12.5-week-old B05/HSP70$^{(tg/-)}$ mice often lasted on the Rotarod until it reached maximum speed. In fact, two of the six B05/HSP70$^{(tg/-)}$ mice performed until the trial was complete (600 s). This level of coordination is very rare in B05 mice at 12.5 weeks (6,12).

To ensure that the improved motor coordination was due to the activity of HSP70 rather than genetic background effects, we next extended the Rotarod analysis to include B05/HSP70$^{(tg/tg)}$ mice at 9.5 weeks of age. The doubled HSP70 levels provided even greater improvement over the B05 animals (Fig. 3C). During all four days of the trial, the B05/HSP70$^{(tg/tg)}$ mice showed a statistically significant increase in mean performance time, suggesting that higher HSP70 levels offer protection against the polyglutamine-induced behavioral phenotype.

High HSP70 levels reduce SCA1 pathological changes

As reported previously, anti-calbindin immunofluorescence studies of B05 mice at 9.5 weeks revealed thinning of Purkinje cell dendritic arborization and slightly misaligned Purkinje cell layers with heterotopic Purkinje cells (Fig. 4A) (12). B05/HSP70$^{(tg/-)}$ mice at 9.5 weeks manifest a similar thinning of the Purkinje cell dendritic arborization but less spatial alteration in the Purkinje cell layer (Fig. 4B). In light of the dose-dependent protection seen by Rotarod analysis, we expected to find better

Purkinje cell morphology in B05 mice doubly transgenic for HSP70$^{(tg/tg)}$. Indeed, a 9.5-week-old B05/HSP70$^{(tg/tg)}$ cerebellum shows numerous Purkinje cells having thicker and more arborized dendritic branches than B05 neurons (Fig. 4C). The protective effect of HSP70 was still apparent at 12.5 weeks, as can be seen by comparing the B05 neuropathology with that of B05/HSP70$^{(tg/tg)}$ mice. The B05 cerebella contain numerous heterotopic Purkinje cells (arrowheads) with dramatically reduced dendritic arborization (Fig. 4D). Cerebella from B05/HSP70$^{(tg/tg)}$ mice, on the other hand, show markedly fewer heterotopic Purkinje cells and more robust dendritic arborization (Fig. 4E). Over-expression of iHPS70 thus suppresses the polyglutamine-induced neuropathology of SCA1 transgenic animals.

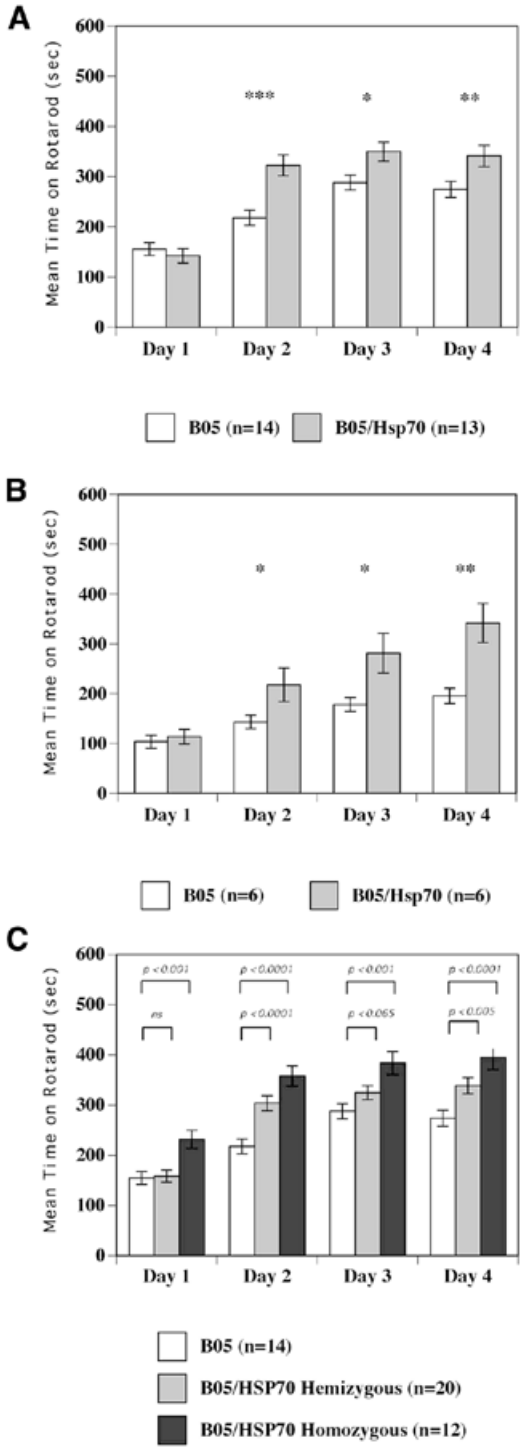

Figure 3. B05 transgenic mice expressing rat iHSP70 perform better on Rotarod than B05 littermates. For 3 of the 4 days of trial at both 9.5 (**A**) and 12.5 (**B**) weeks, the B05 transgenic mice expressing rat iHSP70$^{(tg/-)}$ last longer on the Rotarod than B05 littermates. Although both groups of animals show progressive loss of neurological function, the B05 animals expressing higher chaperone levels are able to maintain their balance on the rotating rod when it reaches the maximum speed (240 rotations/s). At 12.5 weeks, two of six double transgenic animals stayed on the Rotarod until the trial was complete at 600 s. This is rare with B05 animals at 12.5 weeks (none of six in this study). The performance levels with statistical difference between B05/HSP70$^{(tg/-)}$ and B05 littermates are noted with asterisks; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$. (**C**) At 9.5 weeks of age, B05 mice expressing homozygous levels of HSP70$^{(tg/tg)}$ perform better on the Rotarod than B05/HSP70$^{(tg/-)}$ and B05 animals. In a larger set of animals the analysis was expanded to include B05 mice expressing homozygous levels of Hsp70$^{tg/tg}$. B05/HSP70$^{tg/tg}$ performed better than B05/HSP70$^{tg/-}$ animals, which in turn performed better than B05 mice.

Chaperone over-expression does not alter NI formation

In Purkinje cells from B05 mice, ataxin-1 is localized primarily to a single nuclear structure, with limited distribution throughout the nucleus (Fig. 5A) (5,7,15). Purkinje cells from age-matched (9.5 weeks) littermate B05 animals over-expressing inducible HSP70$^{(tg/tg)}$ had identical staining patterns (Fig. 5B). To quantify the occurrence of NI in the different lines, we divided the number of Purkinje cells with aggregates by the total number of Purkinje cells counted in 5 µm sections of the entire midsagittal cerebellar hemisphere. The percentage of B05 Purkinje cells bearing NI increases with age from ~27.5% at 6.5 weeks to ~55% at 9.5 weeks (Fig. 5C) (7,15). The double transgenic mice manifested similar rates of NI formation: ~25% at 6.5 weeks and ~50% at 9.5 weeks. Like the B05/HSP70$^{(tg/-)}$ at 9.5 weeks, B05/HSP70$^{(tg/tg)}$ mice had similar percentages of Purkinje cells with NI as the B05 line (~49%). These results are reminiscent of two studies in *Drosophila* which found that over-expression of HSP70 with or without dHdj-1 suppressed neuronal degeneration but did not noticeably affect NI formation (9,11).

DISCUSSION

This report is the first to demonstrate that high expression levels of iHSP70 afford protection against polyglutamine-induced neurodegeneration in a mammalian model of SCA1. By what mechanisms might the chaperones be conveying their ameliorative effect? One possibility is that they moderate the folding or ubiquitin-proteasomal clearance of mutant ataxin-1 (and other polyglutamine proteins). We previously showed that the NIs were smaller in the presence of higher-than-normal levels of DNAJ/HDJ-2, and Fernandez-Funez *et al.* (8) found that over-expression of dHdj-1 changed the pattern of NI formation in flies over-expressing mutant ataxin-1 (5). Others also found that hsp40 and hsp70 were able to inhibit polyglutamine fibril formation *in vitro* (16). Although Hsp70 and dHdj1 have not appreciably reduced the size of NI in a *Drosophila* model of SCA3, more of the mutant protein is extractable in these flies, since a greater proportion of the

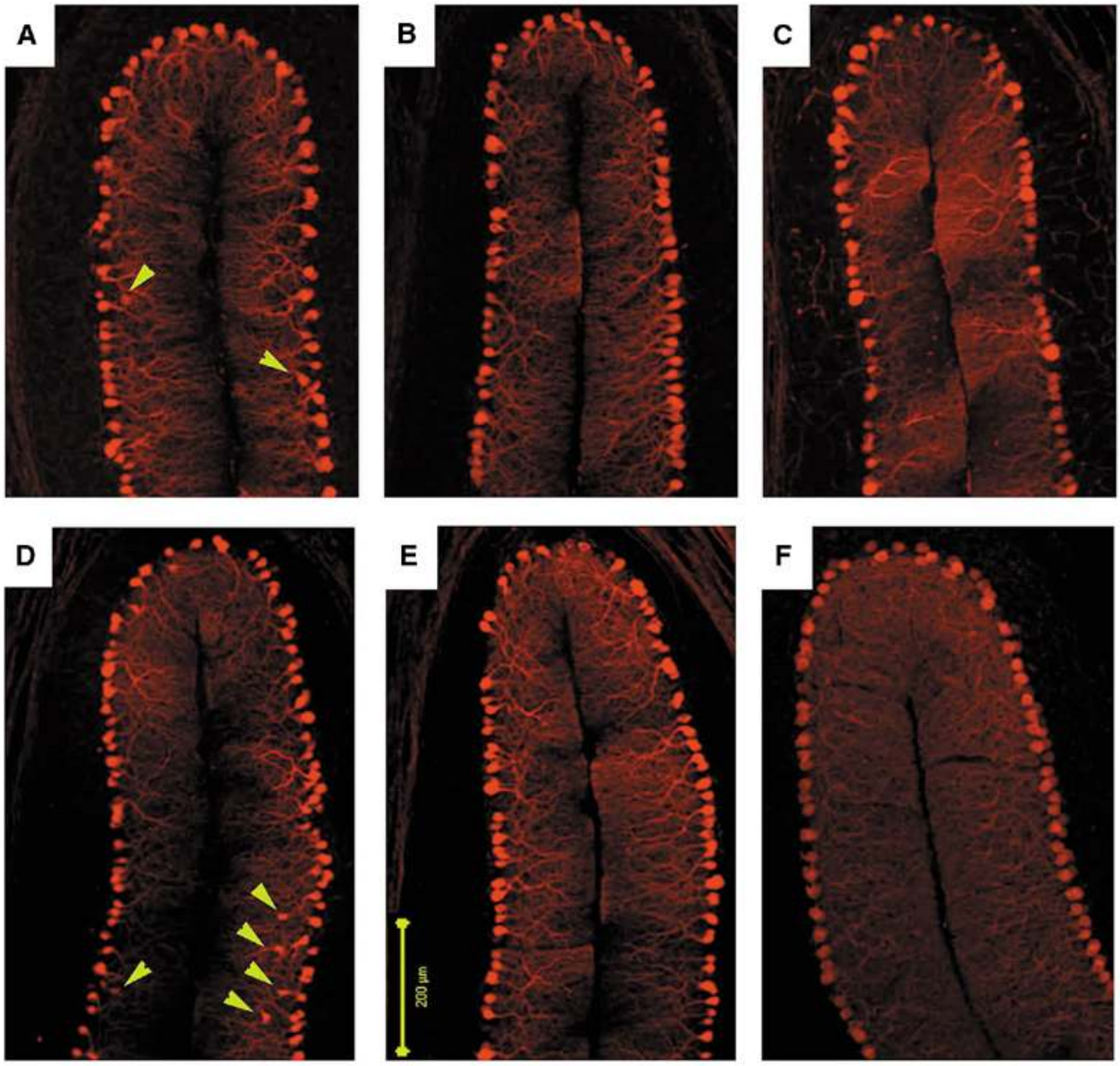

Figure 4. Cerebellar morphology in *SCA1* B05 transgenic mice expressing rat iHSP70. Calbindin staining of cerebellar sections at the level of the fissura prima between the anterodorsal and central lobes (A–F). Cerebellar section from (**A**) a 9.5-week-old B05 mouse demonstrating early alterations in Purkinje cells morphology, including thinning of the dendritic arborization and disruption of the Purkinje cell layer (arrowhead). (**B**) Sections from B05/HSP70$^{(tg/-)}$ mice show similar Purkinje cell alterations. Transgenic mice hemizygous for B05 and homozygous for iHSP70 (**C**), however, have slightly thicker Purkinje cell dendritic arborization at 9.5 weeks than B05 and B05/HSP70$^{(tg/-)}$ littermates. Progressive Purkinje cell pathology is seen in B05 mice at 12 weeks in (**D**), including loss of dendritic arborization and heterotopically localized Purkinje cells (arrowheads). (**E**) B05/HSP70$^{(tg/tg)}$ mice at 12 weeks have better dendritic arborization than B05 littermates, with marked reduction in Purkinje cell heterotopia [compare (D) and (E)]. (**F**) Wild-type at 12.5 weeks.

protein is visualized as a monomer on SDS gels when either or both of these chaperones are expressed (11).

Mutant ataxin-1 has proven to be difficult to extract or solubilize from cerebella of transgenic SCA1 mice (6). We did attempt to ascertain whether increased levels of Hsp70 in Purkinje cells could render expanded ataxin-1 more extractable or soluble. High salt and SDS extraction treatment of cerebella from B05 and B05/HSP70 mice, however, showed no appreciable effect; indeed, virtually none of the protein was soluble in SDS in either instance (data not shown). This lack of effect on protein solubilization might lie in the properties of complexed ataxin-1 in Purkinje cells (as opposed to complexes of other polyglutamine proteins such as ataxin-3 in *Drosophila*). Also, other factors such as the context of the polyglutamine tract, the stoichiometry of the chaperones, and cell-specific factors might contribute to differences in extractibility (17). It might be possible to solubilize ataxin-1 with more stringent extraction protocols; nonetheless, the disease phenotype is clearly mitigated with little apparent difference in the properties of the aggregating polyglutamine protein.

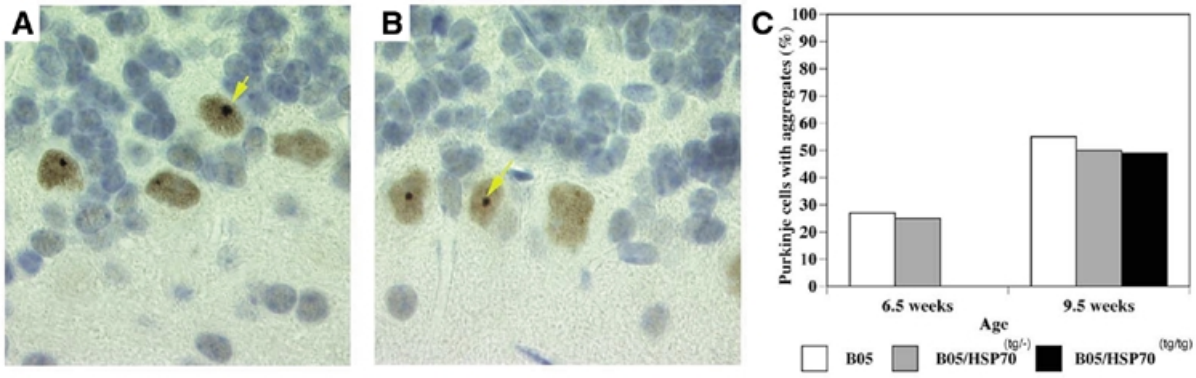

Figure 5. *SCA1* B05 transgenic mice expressing rat iHSP70 have NIs. Immunohistochemistry of cerebellar sections from 6.5-week-old B05$^{(tg/-)}$ (**A**) and B05/HSP70$^{(tg/tg)}$ littermate (**B**) stained with anti-ataxin-1 antibody. Both animals have Purkinje cells containing ataxin-1-positive nuclear aggregates. (**C**) B05 mice expressing HSP70 have approximately the same numbers of Purkinje cells with nuclear aggregates as age-matched B05 mice. The bars represent the percentage of Purkinje cells with nuclear aggregates at 6.5 and 9.5 weeks. The data were generated from two B05/HSP70$^{(tg/-)}$ animals at each age, one B05 at 6.5 weeks, and one B05/HSP70$^{(tg/tg)}$ at 9.5 weeks. The total number of Purkinje cells used to calculate the frequency of aggregates is: n = 2046 and 2027 for B05/HSP70$^{(tg/-)}$ at 6.5 and 9.5 weeks, respectively; n = 1292 for B05 6.5 weeks; and n = 930 for B05/HSP70$^{(tg/tg)}$.

There are other avenues by which chaperones may act to improve polyglutamine-induced phenotypes. HSP70 over-expression could act competitively to protect against toxic interactions between mutant ataxin-1 and its normal or acquired interacting partners. Alternatively, HSP70 could suppress apoptosis by acting downstream of cytochrome c release and upstream of caspase-3 activation (18,19). HSP70 is able to suppress the stress kinase c-Jun W-terminal kinase (JNK) (20); this activity is independent of HSP70's protein refolding function, as it can be mediated by a deletion mutant which lacks the ATP-binding domain (21). JNK activation has been observed in a hippocampal neuronal cell line which expresses expanded huntingtin (22,23), raising the possibility that activation of stress-signaling kinase and JNK might be one of the pathways involved in polyglutamine-induced neurodegeneration.

Notwithstanding our present uncertainty as to mechanism, there are three important outcomes of this study. First, chaperone over-expression in Purkinje cells does not appear to have untoward effects on the morphology of these neurons or their role in motor coordination. Second, because the same molecular changes (altered subcellular distribution of chaperones and components of the ubiquitin proteasome pathway, and downregulation of Purkinje cell-specific genes) (5,24) are seen in the SCA1 B05 mice and in human patients, the success of the double mutant mice gives hope that upregulating chaperone activity offers a possible therapeutic strategy to suppress polyglutamine-induced neurotoxicity in a mammalian central nervous system. This beneficial effect is assayable and responsive to chaperone dosage. Of course, the studies in mice do not parallel the human condition completely; the B05 line expresses ataxin-1 82Q at 50–100× endogenous levels. Either substantial over-expression or an extremely long polyglutamine tract is necessary to produce a disease phenotype in the mouse, presumably because the life-span of the mouse is too short for its neurons to accumulate damage from glutamine toxicity (a process which takes decades in humans). On the other hand, this may explain why the phenotype in the double mutant mice was still apparent, if diminished; the iHSP70 mice over-express the chaperone by only 10–20× endogenous levels. It is tempting to speculate that further benefit might be achieved by enhancing expression of the iHSP70 transgene in Purkinje cells or increasing the ratio of chaperone to mutant protein expression in favor of the former.

The third important outcome of this study is proof of the principle that both *Drosophila* and cell culture provide reliable models for high-throughput screening assays, and that pathways discovered in these models to affect polyglutamine-induced neurodegeneration are likely to carry over into mammalian systems. Such screens might uncover a plethora of candidate targets whose biological significance is yet to be deciphered, but from a therapeutic standpoint this issue might be moot if beneficial effects can be brought to the bedside.

MATERIALS AND METHODS

Generation and maintenance of transgenic mice

Transgenic mice expressing rat iHSP70 were generated and characterized as described previously (13,14). Three homozygous HSP70$^{(tg/tg)}$ male mice, strain CB6, were mated with 1–2 female B05 heterozygous mice (6), producing mixed CB6 and FVB genetic backgrounds. First or second generation B05$^{(tg/-)}$/HSP70$^{(tg/-)}$ mice were mated with either HSP70$^{(tg/-)}$ or HSP70$^{(tg/tg)}$ mice to produce all combinations and numbers for pathology and Rotarod analysis.

Immunohistochemistry, immunofluorescence and immunoblotting

Immunohistochemical and immunofluorescence staining were performed as described previously (7), using monoclonal anti-iHSP70 (StressGen, SPA-810) and monoclonal anti-calbindin (Sigma, CL300). We performed western blot analysis as described previously (7) with 100 µg/lane dounce-homogenized total cerebellar lysates [2% SDS, 100 mM Tris pH 6.8, 25 mM DTT, protease inhibitors (Boehringer Manheim)]. Nitrocellulose blots were probed with anti-iHSP70 (1:1000) and anti-actin (1:500) (Sigma, AC-40).

To determine whether chaperone over-expression affects solubility or extractability of ataxin-1, the cerebella of 6-week-old mice of each genotype were cut into two sagittal sections along the midline so as to allow two distinct extraction procedures; half the cerebellum was minced by a razor blade, then sonicated in 700 µl of 5× Laemmli sample buffer containing 3% 2-mercaptoethanol, and 8 M urea with protease inhibitors; the other half was homogenized in 0.25 M Tris pH 7.5, containing 0.2 mM sodium vanadate and 50 mM sodium fluoride drawn through an 18 gauge needle to ensure complete homogenization, and briefly spun at 2500 r.p.m. on the microcentrifuge (600 *g*). Protein determinations of the supernatant of these samples were performed using the Bradford reagent to confirm equivalent levels of each protein, and 100 µg of protein from each sample was dissolved in 5× Laemmli buffer to be loaded on a single lane of a 15-well BioRad minigel apparatus using standard conditions for running and staining.

In situ hybridization

Following a previously described method (25), 12 µm sections were cut from fixed and wax-embedded wild-type and $HSP70^{(tg/-)}$ cerebellum. Sections were rehydrated, treated with proteinaseK, acetylated and dehydrated. S^{35}-labeled probes were generated with the T7/SP6 *in vitro* transcription kit (Boehringer-Mannheim). Sections were incubated with probe overnight at 55°C, washed at 62°C in 1× SSC and formamide, and RNAse A treated. The slides were dehydrated, dipped in emulsion and developed, then viewed by indirect microscopy.

Rotating rod analysis

Rotarod analysis was performed on naïve animals at 9.5 and 12.5 weeks. Test and control animals matched for age, sex and weight were tested on the Jones and Roberts accelerating Rotarod apparatus (Stoelting, IL, Ugo Bassili). The instrument accelerates from 4 to 40 r.p.m. over a period of 4 min and 30 s. Time was recorded when an animal fell off or made two consecutive revolutions, holding onto the rod. A trial was allowed to proceed for 10 min (600 s). The procedure was repeated, with four trials a day, for four consecutive days. Weights were recorded on the last day of the trial. Statistical analysis of variance and student's *t*-test were performed with Microsoft Excel (98) or InStat (2.03) and the histograms were generated in DeltaGraph (4.0).

ACKNOWLEDGEMENTS

The authors thank Dr N. Bermingham for performing the *in situ* work and Dr J. Botas, Dr R. Siefers, Dr T. Melcher and V. Brandt for constructive criticism. This work is supported by grants from the NINDS (NS27699) to H.Y.Z. and by the Neuropathology Core of the Baylor Mental Retardation Research Center. H.Y.Z. is a Howard Hughes Medical Institute Investigator.

REFERENCES

1. Zoghbi, H.Y., Pollack, M.S., Lyons, L.A., Ferell, R.E., Daiger, S.P. and Beaudet, A.L. (1988) Spinocerebellar ataxia: variable age of onset and linkage to human leukocyte antigen in a large kindred. *Ann. Neurol.*, **23**, 580–584.
2. Orr, H., Chung, M.-y., Banfi, S., Kwiatkowski, T.J.,Jr, Servadio, A., Beaudet, A.L., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zoghbi, H.Y. (1993) Expansion of an unstable trinucleotide (CAG) repeat in spinocerebellar ataxia type 1. *Nat. Genet.*, **4**, 221–226.
3. Cummings, C.J. and Zoghbi, H.Y. (2000) Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.*, **9**, 909–916.
4. Kaytor, M.D. and Warren, S.T. (1999) Aberrant protein deposition and neurological disease. *J. Biol. Chem.*, **274**, 37507–37510.
5. Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T. and Zoghbi, H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.*, **19**, 148–154.
6. Burright, E.N., Clark, H.B., Servadio, A., Matilla, T., Feddersen, R.M., Yunis, W.S., Duvick, L.A., Zoghbi, H.Y. and Orr, H.T. (1995) SCA1 transgenic mice: a model for neurodegeneration caused by an expanded CAG trinucleotide repeat. *Cell*, **82**, 937–948.
7. Cummings, C.J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y.-h., Ciechanover, A., Orr, H.T., Beaudet, A.L. and Zoghbi, H.Y. (1999) Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron*, **24**, 879–892.
8. Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J. *et al.* (2000) Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature*, **408**, 101–106.
9. Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L. and Bonini, N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.*, **23**, 425–428.
10. Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*, **287**, 1837–1840.
11. Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L. and Bonini, N.M. (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in *Drosophila*. *Hum. Mol. Genet.*, **9**, 2811–2820.
12. Clark, H.B., Burright, E.N., Yunis, W.S., Larson, S., Wilcox, C., Hartman, B., Matilla, A., Zoghbi, H.Y. and Orr, H.T. (1997) Purkinje cell expression of a mutant allele of *SCA1* in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. *J. Neurosci.*, **17**, 7385–7395.
13. Marber, M.S., Mestril, R., Chi, S.H., Sayen, M.R., Yellon, D.M. and Dillmann, W.H. (1995) Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J. Clin. Invest.*, **95**, 1446–1456.
14. Rajdev, S., Hara, K., Kokubo, Y., Mestril, R., Dillmann, W., Weinstein, P.R. and Sharp, F.R. (2000) Mice overexpressing rat heat shock protein 70 are protected against cerebral infarction. *Ann. Neurol.*, **47**, 782–791.
15. Skinner, P.J., Koshy, B., Cummings, C., Klement, I.A., Helin, K., Servadio, A., Zoghbi, H.Y. and Orr, H.T. (1997) Ataxin-1 with extra glutamines induces alterations in nuclear matrix-associated structures. *Nature*, **389**, 971–974.
16. Muchowski, P.J., Schaffar, G., Sittler, A., Wanker, E.E., Hayer-Hartl, M.K. and Hartl, F.U. (2000) Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl Acad. Sci. USA*, **97**, 7841–7846.
17. Krobitsch, S. and Lindquist, S. (2000) Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. *Proc. Natl Acad. Sci. USA*, **97**, 1589–1594.
18. Li, C.Y., Lee, J.S., Ko, Y.G., Kim, J.I. and Seo, J.S. (2000) Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J. Biol. Chem.*, **275**, 25665–25671.
19. Jana, N.R., Zemskov, E.A., Wang, G. and Nukina, N. (2001) Altered proteasomal function due to the expression of polyglutamine- expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum. Mol. Genet.*, **10**, 1049–1059.
20. Gabai, V.L., Meriin, A.B., Yaglom, J.A., Volloch, V.Z. and Sherman, M.Y. (1998) Role of Hsp70 in regulation of stress-kinase JNK: implications in apoptosis and aging. *FEBS Lett.*, **438**, 1–4.
21. Mosser, D.D., Caron, A.W., Bourget, L., Meriin, A.B., Sherman, M.Y., Morimoto, R.I. and Massie, B. (2000) The chaperone function of hsp70 is

required for protection against stress-induced apoptosis. *Mol. Cell. Biol.*, **20**, 7146–7159.
22. Liu, Y.F., Dorow, D. and Marshall, J. (2000) Activation of MLK2-mediated signaling cascades by polyglutamine-expanded huntingtin. *J. Biol. Chem.*, **275**, 19035–19040.
23. Liu, Y.F. (1998) Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. *J. Biol. Chem.*, **273**, 28873–28877.
24. Lin, X., Antalffy, B., Kang, D., Orr, T. and Zoghbi, H.Y. (2000) Polyglutamine expansion in ataxin-1 downregulates specific neuronal genes before pathogenic changes in spinocerebellar ataxia type 1. *Nat. Neurosci.*, **3**, 157–163.
25. Albrecht, U., Eichele, G., Helms, J. and Lu, H.-C. (1997) Visualization of gene expression patterns by *in situ* hybridization. In Daston, G.P. (ed.), *Molecular and Cellular Methods in Developmental Toxicology*. CRC Press, Boca Raton, FL, 49–78.