Therapeutic Effects of Cystamine in a Murine Model of Huntington's Disease

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The precise cause of neuronal death in Huntington's disease (HD) is unknown. Proteolytic products of the huntingtin protein can contribute to toxic cellular aggregates that may be formed in part by tissue transglutaminase (Tgase). Tgase activity is increased in HD brain. Treatment in R6/2 transgenic HD mice, using the transglutaminase inhibitor cystamine, significantly extended survival, improved body weight and motor performance, and delayed the neuropathological sequela. Tgase activity and N^{Σ}-(γ -L-glutamyl)-L-lysine (GGEL) levels were significantly altered in HD mice. Free GGEL, a specific biochemical marker of Tgase activity, was markedly elevated in the neocortex and caudate nucleus in HD patients. Both Tgase and GGEL immunoreactivities colocalized to huntingtin aggregates. Cystamine treatment normalized transglutaminase and GGEL levels in R6/2 mice. These findings are consistent with the hypothesis that transglutaminase activity may play a role in the pathogenesis of HD, and they identify cystamine as a potential therapeutic strategy for treating HD patients.

Key words: Huntington's disease; cystamine; transglutaminase; glutamyl lysine; neuroprotection; transgenic R6/2 mice

The disease phenotype in Huntington's disease (HD) is caused by an expansion of a polyglutamine tract in the protein, huntingtin (htt) (Huntington's Disease Collaborative Research Group, 1993), leading to conformational change, abnormal protein–protein interactions, and eventual neuronal death. Mutant htt undergoes proteolytic processing, in part by the pro-apoptotic enzyme caspase-3, releasing an N-terminal fragment containing the polyglutamine tract (Goldberg et al., 1996). This fragment forms macromolecular aggregates with itself and other proteins that become ubiquitinated and large enough to be visible in the processes, cytoplasm, and nuclei of neurons (Davies et al., 1997; DiFiglia et al., 1997; Scherzinger et al., 1997; Kuemmerle et al., 1999). Aggregation of the N-terminal fragments of huntingtin is CAG-length dependent, occurring once the polyglutamine tract is >36 amino acids long and increases with greater lengths (Li and Li, 1998; Martindale et al., 1998). Thus, it has been hypothesized that aggregation may be the trigger for a toxic gain of function, leading to neurodegeneration.

At least three transglutaminase (Tgase) isoenzymes are found in the brain (Tgase1, 2, and 3) of which Tgase 2 (tissue Tgase) is the most abundant (Kim et al., 1999). It has been suggested that Tgase may be involved in the etiology of HD by catalyzing the formation of γ-glutamyl isopeptide bonds between polyglutamine tracts and a lysine protein substrate, rendering the resulting cross-linked protein complexes insoluble (Folk, 1983; Green, 1993). N^{Σ}-(γ -L-glutamyl)-L-lysine (GGEL) is, therefore, a specific biomarker of Tgase activity. It is of interest that GGEL levels have been reported to be significantly elevated in the CSF of HD patients (Jeitner et al., 2001).

Tgase activity is upregulated in several other neuronal injury models and neurodegenerative diseases and may be a generalized response during neurodegeneration (Gilad et al., 1985; Holmes and Haynes, 1996; Fujita et al., 1998; Kim et al., 1999; Singer et al., 2002).

It has been shown *in vitro* that polyglutamine repeat domains and mutant htt are substrates for Tgase (Kahlem et al., 1996; Cariello et al., 1996; Cooper et al., 1997a,b; Kahlem et al., 1998). The substrate activity increases with increasing size of the polyglutamine domain (Karpuj et al., 1999; Gentile et al., 1998; de Cristofaro et al., 1999). Tgase activity is increased in postmortem HD brain in a grade-dependent manner (Karpuj et al., 1999; Lesort et al., 1999). The formation and maintenance of htt inclusions may therefore be the result, in part, of Tgase activity. Whereas Tgase 2 is predominantly a cytoplasmic protein, with

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increasing intracellular calcium levels, active Tgase 2 translocates to the nucleus and is placed in a position to contribute to the formation of nuclear inclusions (Karpuj et al., 1999; Lesort et al., 1999). Quantitative differences in brain Tgase activity and Tgase isoenzymes may possibly explain selective neuronal vulnerability in HD (Cooper et al., 2002).

We examined whether the administration of cystamine reduces Tgase activity and GGEL levels, lessens the behavioral and neuropathological severity, and extends survival in R6/2 transgenic HD mice.

These findings have been reported in preliminary form (Ferrante et al., 2001).

MATERIALS AND METHODS

Human tissue specimens. Postmortem tissue specimens of striatum and frontal cortex from 14 adult-onset HD patients (five grade 3, and nine grade 4 cases; mean age, 66.3 years; range, 53–74 years), and six age-matched patients without any known neurological sequela (mean age, 68.1 years; range, 62–79) were dissected fresh and either placed in cold (4°C) 2% paraformaldehyde-lysine-periodate solution for 24–36 hr or flash frozen using liquid nitrogen vapors. Brain tissue specimens were received from the Bedford Veterans Affairs Medical Center Brain Tissue Archive, St. Louis Medical Center, and Emory University. The postmortem intervals did not exceed 18 hr (mean time, 8.1 hr; range, 4–18 hr). The range of CAG repeats in the HD patients was 41–52. Each HD patient had been clinically diagnosed based on known family history and phenotypic symptoms of HD. The diagnosis of HD was confirmed by neuropathological examination and graded by our severity scale (Vonsattel et al., 1985). Tissue blocks were processed for histology, as previously described (Kuemmerle et al., 1999).

Animals. Male transgenic HD mice of the R6/2 strain were obtained from The Jackson Laboratory (Bar Harbor, ME). The male R6/2 mice were bred with females from their background strain (B6CBAFI/J). The offspring were genotyped using a PCR assay on tail DNA. The mice were housed four per cage under standard conditions with *ad libitum* access to water and food. To ensure homogeneity of the cohorts used in these experiments, we have standardized our criteria for placement of mice into testing groups. Mice were randomized from 38 litters all within 2 d of the same age from the same "f" generation. Any mice that had altered base-paired banding identified from PCR analysis were excluded from the study. All mice were weighed before placement and equally distributed according to weight within each cohort. Enrichment conditions were not applied to any cages because of its effect on improving phenotype in R6/2 mice. All mice were handled under the same conditions by one investigator. Equal numbers of mice from both genders were included in the experimental paradigm. We have not observed gender differences in survival in the R6/2 transgenic HD mouse model. All animal experiments were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by both the Veterans Administration and Boston University Animal Care Committees.

Intraperitoneal dosing. Based on the study of Boyko et al. (1998), we completed a dose–response study, treating wild-type (Wt) and R6/2 mice with 112, 225, and 400 mg/kg daily intraperitoneal injection of cystamine dihydrochloride (Sigma, St. Louis, MO) dissolved in PBS. Approximately 100 mice were used in the dosing study. At 21 d of age, groups of 20 R6/2 and littermate wild-type control mice were treated with 112 mg/kg and 225 mg/kg cystamine, PBS, or untreated. In all, behavioral and survival data were obtained from ~180 R6/2 and littermate wild-type mice. During the temporal progress of the disease, the intraperitoneal injection was 100 μl/20 gm/mouse until endstage (17 weeks).

Oral dosing. Prenatal oral dosing at 225 mg/kg cystamine was initiated in breeding wild-type females and continued postnatally in the drinking water. Based on water consumption of 5 ml/d per 20 gm mouse, a cystamine concentration of 900 mg/l tap water was used. Eight pregnant dams were used in the study, four cystamine-treated and four untreated under standard conditions with *ad libitum* access to water and food. Pups were genotyped at 15 d, and mixed R6/2-positive and wild-type litters were kept together after weaning (21 d) in groups of four mice throughout the course of the survival experiment. Body weight and survival data were recorded for 30 cystamine-treated mice (16 R6/2-positive; 14 wild-type littermate mice) and 26 untreated mice (10 R6/2-positive; 16 wild-type littermate mice).

Motor performance and weight assessment. Motor performance was assessed weekly from 21–63 d of age and twice weekly from 63 d of age in the R6/2 mice. The mice were given two training sessions to acclimate them to the rotarod apparatus (Columbus Instruments, Columbus, OH). Mice were placed on a rotating rod at 16 rpm. The length of time remaining on the rod was taken as the measure of competency. The maximum score was 60 sec, and each mouse performed three separate trials. The three results were averaged and recorded. Body weights were recorded twice weekly.

Survival. R6/2 mice were observed twice daily, mid-morning and late afternoon. Their motor performance and ability to feed was closely monitored and was the basis for determining when to euthanize the mice. The criteria for killing was the point in time in which the HD mice were unable to right themselves after being placed on their back and initiate movement after being gently prodded for 30 sec. HD mice have lost ~40–50% of their body weight at this time point. Two independent observers confirmed the criteria for killing (R.J.F. and A.D.).

Transglutaminase and GGEL assays. At 21 d of age, groups of 10 R6/2 and littermate wild-type control mice were treated with daily 112 mg/kg cystamine or PBS intraperitoneal injections. The mice were killed at 63 d of age, and the brains were rapidly frozen and stored at −80°C. Tgase activity was determined by a previously described method that measures tritiated putrescine in a protein substrate (Lesort et al., 1999). Putrescine incorporation was determined by liquid scintillation counts and calculated as picomoles per hour per milligram of tissue protein. Free GGEL levels were determined by liquid chromatography with electrochemical detection (LCEC). Brain samples were placed in cold (4°C) 50% methanol (100 mg/400 ml methanol), sonicated for 3 × 10 sec cycles, protein levels were determined via Coomassie Protein Assay (Pierce, Rockford, IL), samples were centrifuged (4°C, 40,000 rpm for 1 hr), and supernatant was extracted for GGEL assay. GGEL was analyzed using LCEC after *O*-phthaldialdehyde (OPA)/β-mercaptoethanol derivatization, using a recently reported method (Jeitner et al., 2001) with the exception that an XTerra MS 5 μm, 4.6 mm × 25 cm C18 column (Waters, Milford, MA) was used for the separations. GGEL levels are reported as picomoles per microgram of tissue protein. Each of the sample measurements was performed twice, and identification was blinded to the investigators performing assays (J.K.K., S.M., M.B., and T.M.J.).

Histologic evaluation. At 21 d, R6/2 transgenic mice and wild-type littermate control mice were treated with daily 112 mg/kg cystamine or PBS intraperitoneal injections. Groups of 10 animals from each treatment paradigm were deeply anesthetized and then transcardially perfused with 4% buffered paraformaldehyde at 90 d of age. Approximately 40 mice were used for data collection in the neuropathological analysis and processed for histopathology, as previously described (Ferrante et al., 2002). Serially cut tissue mouse and human tissue sections were stained for Nissl substance and immunostained for htt, using a polyclonal rabbit antibody (EM48; dilution, 1:1000; S. M. Hersch), two Tgase 2 antibodies (mouse monoclonal antibody, dilution, 1:200, NeoMarker Inc., Fremont, CA; goat polyclonal antibody, dilution, 1:400, Upstate Biotechnology, Lake Placid, NY), and an antibody to GGEL (anti-N epsilon gamma glutamyl lysine mouse monoclonal antibody, dilution, 1:500, Abcam Limited, Cambridge, UK), using a previously reported conjugated second antibody method in human and murine brain tissue samples (Ferrante et al., 2002). Specificity for the antisera used in this study was examined in each immunochemical experiment to assist with interpretation of the results. Preabsorption with excess target proteins, omission of the primary antibodies, and omission of secondary antibodies was performed to determine the amount of background generated from the detection assay.

Double immunofluorescence was performed using a previously described method (Ferrante et al., 1997) by incubating R6/2 mouse tissue sections in polyclonal rabbit htt antisera (EM48, dilution, 1:1000) and in either a monoclonal mouse Tgase antisera (NeoMarkers; dilution, 1:250) or in a mouse monoclonal GGEL antisera in Tris HCl buffer containing 0.3% Triton X-100 for 24–72 hr at 4°C. htt antisera resulted in the presence of green fluorescence, whereas Tgase and GGEL antisera resulted in the presence of red fluorescence. Identical microscopic fields were photographed with a Nikon fluorescent microscope, delineating the location of htt and Tgase or GGEL immunoreactivities within the same brain tissue section and merged.

Stereology/quantitation. Serial cut coronal tissue-sections from the rostral segment of the neostriatum and neocortex at the level of the anterior commissure (interaural 5.34 mm/bregma 1.54 mm to interaural 3.7 mm/bregma −0.10 mm), were used for htt aggregate analysis. Unbiased

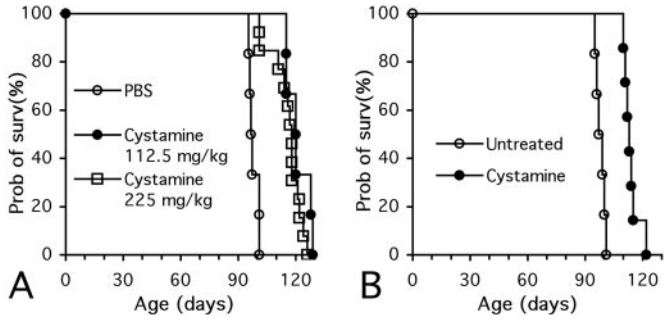

Figure 1. Survival in cystamine-treated R6/2 mice. Kaplan–Meier probability of survival analysis for cystamine treatment using intraperitoneal injection of 112 and 225 mg/kg in R6/2 mice and untreated R6/2 mice showing cumulative survival (*A*). Survival analysis of oral treatment using 225 mg/kg (*B*). Both intraperitoneal and oral cystamine treatment significantly extended survival in R6/2 transgenic mice ($p < 0.001$).

stereologic counts of htt-positive aggregates (≥1.0 μm) were obtained from the neostriatum in 10 mice each from cystamine-treated and PBS-treated R6/2 mice at 90 d using Neurolucida Stereo Investigator software (Microbrightfield, Colchester, VT). The total areas of the neostriatum and neocortex were defined in serial sections in which counting frames were randomly sampled. The dissector counting method was used in which htt-positive aggregates were counted in an unbiased selection of serial sections in a defined volume of the neostriatum and neocortex. Striatal neuron areas were analyzed by microscopic videocapture using a Windows-based image analysis system for area measurement (Optimas; Bioscan Incorporated, Edmonds, WA). The software automatically identifies and measures profiles. All computer-identified cell profiles were manually verified as neurons and exported to Microsoft Excel. Cross-sectional areas were analyzed using Statview.

Statistics. The data are expressed as the mean ± SEM. Statistical comparisons of rotarod, weight data, and histology data were compared by ANOVA or repeated measures ANOVA. Survival data were analyzed by the Kaplan–Meier survival curves.

RESULTS

The effects of intraperitoneal injection and oral administration of cystamine on survival in HD R6/2 transgenic mice are shown in Figure 1. Intraperitoneal administration of cystamine significantly extended survival in R6/2 mice at both the 112 mg/kg and 225 mg/kg doses (PBS-treated R6/2 mice: 101.1 ± 3.6 d; 112 mg/kg cystamine-treated R6/2 mice: 120.8 ± 5.8 d, $p < 0.001$; and 225 mg/kg cystamine-treated R6/2 mice: 118.3 ± 4.3 d, $p < 0.001$) (Fig. 1). All mice at a 400 mg/kg dosing regimen died within 2–5 d after treatment was initiated at 21 d. At ~83 d survival, during each of the two 225 mg/kg cystamine experiments, the treated mice became moribund, and treatment was stopped. Cystamine toxicity was suspected. Cystamine treatment was started again after a 7 d drug holiday at 90 d. Although greater survival was observed using the 112 mg/kg dose, it was not significantly different from the 225 mg/kg dose. The percentage increase in survival for 112 mg/kg and 225 mg/kg cystamine dosing paradigms were 19.5 and 17.0%, respectively.

The effects of prenatal oral administration of cystamine in drinking water administered to pregnant dams significantly increased survival in R6/2 littered-mice, as compared with unsupplemented dams and R6/2 littered-mice (Fig. 1). Oral cystamine treatment was continued in the drinking water from those litters born to cystamine-treated dams. Oral cystamine-treatment (225 mg/kg) significantly extended survival in R6/2 mice by 16.8% (unsupplemented R6/2 mice: 98.2 ± 2.3 d; cystamine-treated R6/2 mice: 114.1 ± 5.5 d, $p < 0.0$ 1). No significant differences in survival were observed between the oral postnatal

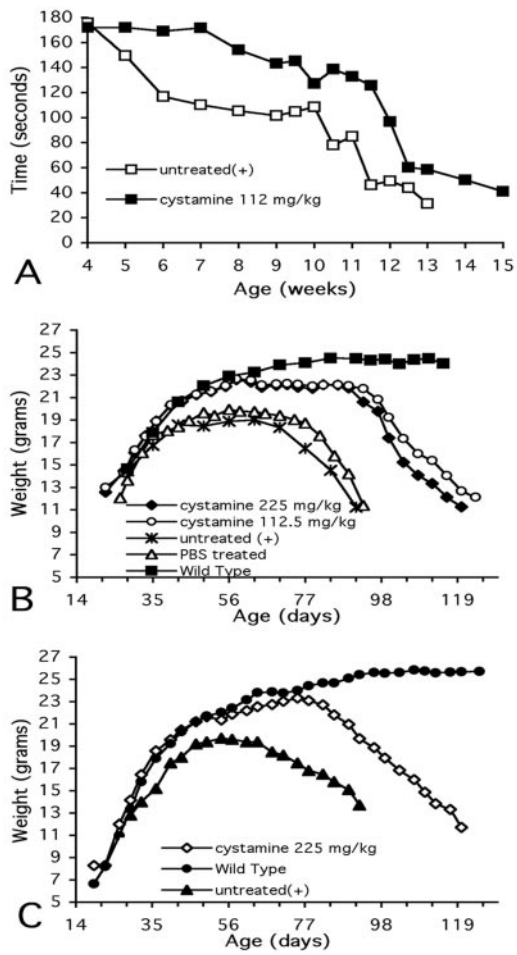

Figure 2. Motor performance and body weight analysis in cystamine-treated R6/2 mice. Effects of intraperitoneal cystamine treatment (112 mg/kg) on rotarod performance (*A*) significantly improved motor performance in R6/2 HD transgenic mice throughout the temporal sequence of the experiment (4–16 weeks). Effects of intraperitoneal (112 and 225 mg/kg) (*B*) and oral (225 mg/kg) (*C*) cystamine treatment on body weight in R6/2 HD transgenic mice. Greater body weight improvement was observed in both the intraperitoneal and oral paradigms.

treatment and the intraperitoneal postweaning treatment using cystamine at 225 mg/kg in both experiments.

Intraperitoneal cystamine treatment (112 mg/kg) significantly improved rotarod performance throughout the entire measured (4–15 weeks) life span of the R6/2 mice in contrast to PBS-treated R6/2 mice (PBS-treated R6/2 mice: 79 ± 26 sec; 112 mg/kg cystamine treated R6/2 mice: 137 ± 17 sec, $p < 0.01$). The data represent combined means and SDs from 5 to 12.5 weeks (Fig. 2*A*). The improvement in rotarod performance was 27%.

The effects of 112 mg/kg and 225 mg/kg intraperitoneal cystamine treatment and prenatal oral treatment (225 mg/kg) on body weight in HD R6/2 transgenic mice are shown in Figure 2*B*. Each cystamine regimen resulted in significant improvement of body weight in comparison with unsupplemented R6/2 mice. Intraperitoneal cystamine treatment, 112 and 225 mg/kg, resulted in significantly greater body weight gains in R6/2 mice ($p < 0.01$) in comparison with untreated R6/2 mice and untreated mice (Fig. 2*B*). Significantly greater body weight measurements were present throughout the temporal sequence of measurements (4–17 weeks) in both 112 and 225 mg/kg cystamine-treated R6/2

mice. At 9 and 12 week time points, the average differences between PBS-treated and both cystamine treatment paradigms were 15.2 and 48.9%, respectively. The total gain in body weight from 4 to 17 weeks for both 112 and 225 mg/kg cystamine-treated R6/2 mice was 15.4 and 13.6% greater, respectively, in comparison with untreated R6/2 mice. Oral cystamine treatment (225 mg/kg) also resulted in significant body weight improvement across the life span of R6/2 mice ($p < 0.01$) (Fig. 2*C*). The total gain in body weight from 4 to 17 weeks for orally cystamine-treated R6/2 mice (225 mg/kg) was 12.7% greater, in comparison with unsupplemented R6/2 mice.

At 90 d, there was a 21.1% reduction in brain weight in unsupplemented R6/2 mice, in contrast to littermate controls. In comparison, there was only a 5.7% brain weight loss in the 112 mg/kg cystamine–treated R6/2 mice (Wt littermate mice: 461 ± 12 mg/kg; PBS-treated R6/2 mice: 364 ± 17; 112 mg/kg cystamine-treated R6/2 mice: 435 ± 10, $p < 0.01$). Concomitant with brain weight loss, marked gross atrophy with bilateral ventricular enlargement and flattening of the medial aspect of the striatum was present in the untreated R6/2 brains at 90 d (Fig. 3), as previously shown (Ferrante et al., 2000, 2002). Consistent with the brain weight findings, cystamine treatment reduced the gross brain atrophy in R6/2 mice in comparison with untreated mice (Fig. 3). In addition to the decrease in gross brain weight and brain atrophy, there was significant atrophy of striatal neurons at 90 d in R6/2 mice. Although neuronal size was smaller in the 112 mg/kg cystamine-treated mice than in littermate control mice, the cytoprotective effect of 112 mg/kg cystamine treatment significantly delayed striatal neuron atrophy in comparison with unsupplemented R6/2 mice (Wt littermate control: 134 ± 11 μm^2; unsupplemented R6/2: 57 ± 15 μm^2; cystamine: 92 ± 14 μm^2, $p < 0.02$) (Fig. 3).

In the neostriatum and neocortex of R6/2 mice, there is an early and progressive accumulation of htt-positive aggregates, as well as an increase in aggregate size, from 21 to 90 d of age (Ferrante et al., 2000). Aggregates are much more prominent within the cortex in comparison with the neostriatum. Cystamine treatment of R6/2 mice resulted in a significant reduction in striatal and cortical aggregate number at 90 d of age ($p < 0.01$) (Fig. 4), more so than in any other reported treatment paradigm to date. At 90 d, the decreases in aggregate number in cystamine-treated R6/2 mice were 68 and 47% in the neostriatum and neocortex, respectively, as compared with untreated R6/2 mice (untreated R6/2 mice neocortex: 815 × 10^3; untreated R6/2 mice neostriatum: 520 × 10^3; cystamine-treated R6/2 mice neocortex: 424 × 10^3; cystamine-treated striatum: 166 × 10^3).

Brain Tgase activity was significantly elevated in R6/2 mice in comparison with wild-type littermate controls (Wt littermate mice: 0.65 ± 0.10 pmol · hr^{-1} · mg^{-1} protein; R6/2 mice: 0.87 ± 0.11 pmol · hr^{-1} · mg^{-1} protein, $p < 0.01$) (Fig. 5*A*). In addition, daily intraperitoneal cystamine treatment in R6/2 mice significantly reduced levels of Tgase activity to the normal range found in littermate control mice (cystamine-treated R6/2 mice: 0.57 ± 0.15 pmol · hr^{-1} · mg^{-1} protein, $p < 0.01$). There was no significant difference between cystamine-treated R6/2 mice and littermate control mice.

Whereas Tgase 2 immunoreactivity was present within both R6/2 and littermate control mouse brains (Fig. 5*B,C*), it was markedly greater in R6/2 tissue specimens at 90 d. Tgase immunoreactivity was present in both the nucleus and cytoplasm of immunostained neurons, as well as in vascular elements. These findings are similar to those we observed in HD patients (Lesort et al., 1999). Intensely immunostained Tgase 2-positive structures morphologically similar to mutant htt aggregates were found in R6/2 tissue specimens within neurons and the neuropil (Fig. 5*C*). Further characterization of these structures, using combined immunofluorescence for htt (FITC) and Tgase (TRITC) immunoreactivities, showed colocalization of htt aggregates and Tgase immunoreactivity (Fig. 5*D–F*). Approximately 10% of the mutant htt aggregates colocalized with Tgase-positive aggregates.

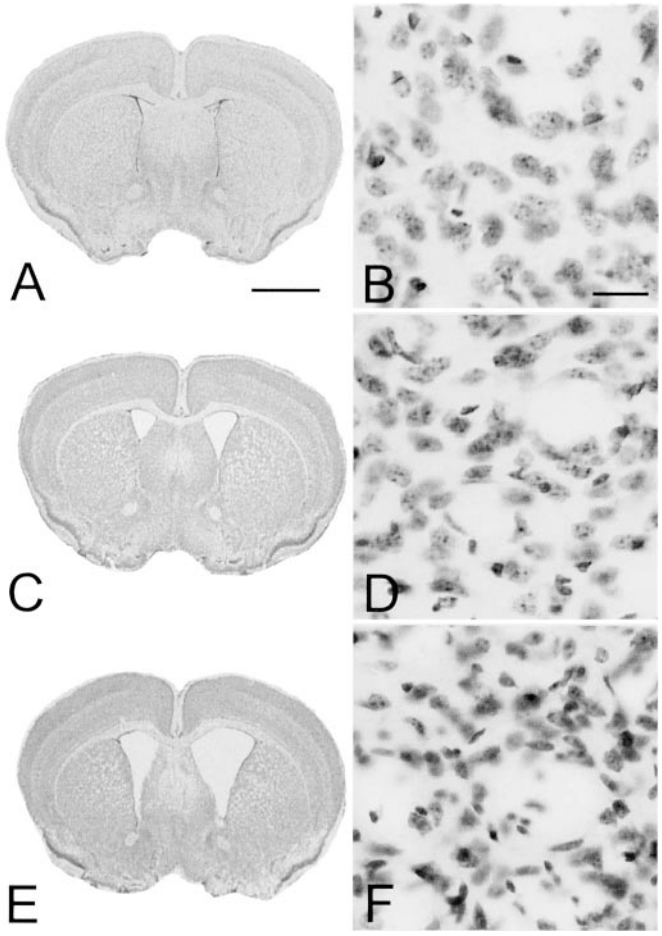

Figure 3. Gross brain and histopathological neuroprotection with cystamine treatment. Photomicrographs of coronal sections through the rostral neostriatum at the level of the anterior commissure in a wild-type littermate mouse (*A*), cystamine-treated (*C*), and untreated (*E*) R6/2 HD transgenic mice at 90 d. Note the generalized gross atrophy of the brain in the untreated R6/2 mouse along with enlargement of the lateral ventricles (*E*). In contrast, the cystamine-treated R6/2 mouse at 90 d (*C*) shows significantly less atrophy and ventricular enlargement than the unsupplemented mouse. Corresponding Nissl-stained tissue sections from the dorsomedial aspect of the neostriatum (*B, D, F*) with *A, C*, and *E*, respectively. Note the reduced neuronal size in the unsupplemented R6/2 mouse, with delayed neuronal atrophy in the cystamine-treated R6/2 mouse, in comparison with the control (*A*). Scale bars: (in *A*) *A, C, E*, 2 mm; (in *B*) *B, D, F*, 50 μm.

Free GGEL levels were significantly elevated in both the neocortex and caudate nucleus of severe to very severe grades in HD patients in comparison with non-neurologic age-matched control brain samples (HD neocortex: 525 ± 104 pmol/mg protein; control neocortex: 69 ± 13 pmol/mg protein, $p < 0.001$) (HD caudate nucleus: 653 ± 129 pmol/mg protein; control caudate nucleus: 62 ± 12 pmol/mg protein, $p < 0.001$) (Fig. 6*A*). In contrast, free GGEL levels in unsupplemented R6/2 mice were

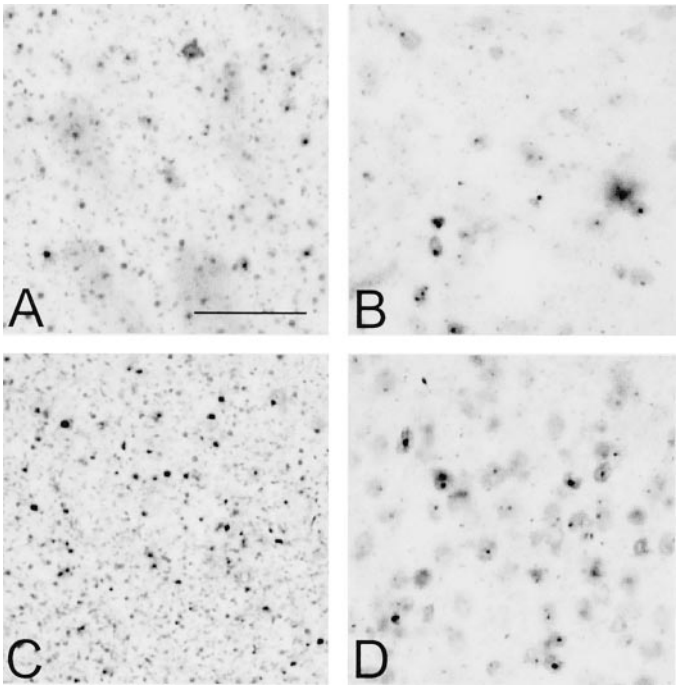

Figure 4. Huntingtin immunoreactivity in cystamine-treated R6/2 mice. htt-immunostained tissue sections from the neostriatum and layer six of the neocortex in untreated (*A, C*, respectively) and cystamine-treated (*B, D,* respectively) R6/2 HD transgenic mice at 90 d. Although there is diffuse immunoreactivity within nuclei in the cystamine-treated mice, the number and size of htt aggregates is significantly greater in the untreated R6/2 mice, in comparison with the cystamine-treated R6/2 mice. Diffuse nuclear immunostaining is present in the cystamine-treated mice. Scale bar, 100 μm.

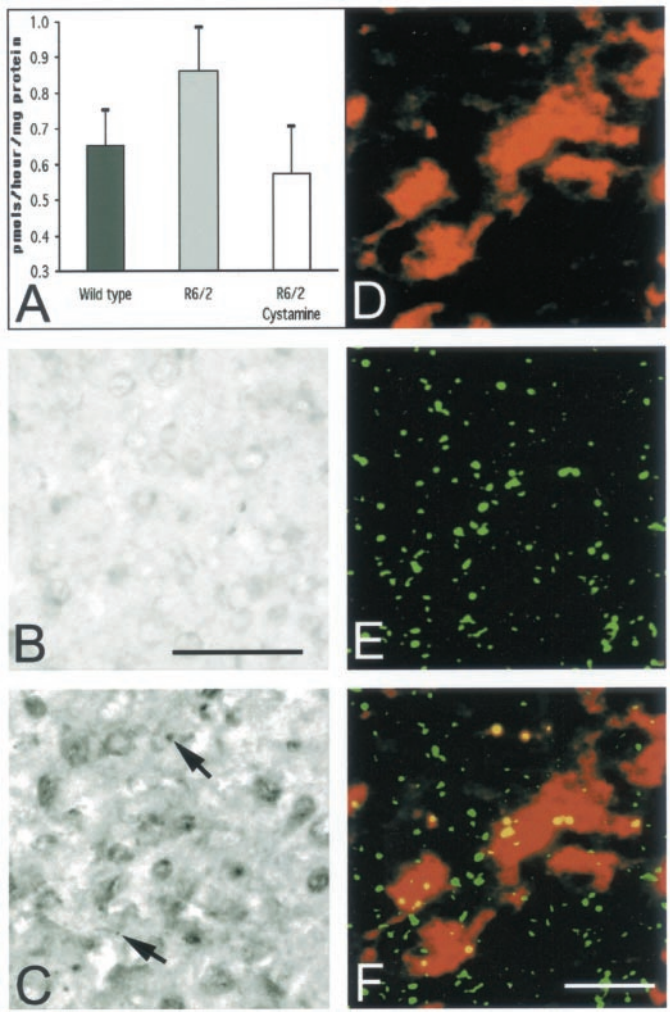

Figure 5. Transglutaminase activities in cystamine-treated R6/2 mice. Tgase activity is significantly increased in unsupplemented R6/2 mice in comparison with wild-type littermate control mice (*A*). Cystamine treatment reduces Tgase activity to control levels (*A*). Tgase immunoreactivity in a wild-type littermate mouse (*B*) and an R6/2 mouse (*C*) at 90 d. There is light immunostaining in the wild-type control with increased immunoreactivity in the R6/2 mouse. Intense aggregate-like figures (*arrows*) are present in neurons and the neuropil of R6/2 mice (*C*). Combined immunofluorescence within the same tissue section of an R6/2 mouse for Tgase (*red*) (*D*) and huntingtin (*green*) (*E*) immunoreactivities show that there is partial colocalization between htt-positive aggregates and Tgase-positive aggregate figures (*yellow*) within the merged figures (*F*). Scale bars: (in *B*) *B*, *C*, 100 μm; (in *F*) *D*, *E*, *F*, 20 μm.

significantly less in comparison with wild-type littermate controls (Wt littermate mice: 232 ± 25 pmol/mg protein; R6/2 mice: 151 ± 30 pmol/mg protein, $p < 0.01$) (Fig. 6*B*). Intraperitoneal cystamine-treatment in R6/2 mice significantly increased GGEL levels, in comparison with untreated R6/2 mice (cystamine-treated R6/2 mice: 263 ± 45 pmol/mg protein, $p < 0.01$), and is consistent with a beneficial therapeutic effect. There was no significant difference, however, in GGEL levels between cystamine-treated R6/2 mice and littermate controls. This finding in the mice may be related to the degree of sequestered GGEL in the insoluble mutant htt aggregate.

The GGEL immunocytochemical findings showed a marked increase in GGEL immunointensity in brain sections from both HD patients and R6/2 mice (Fig. 7). GGEL immunoreactivity was prominent in neurons and the vasculature. As with Tgase, there were GGEL-positive aggregates in R6/2 mice and HD patients (Fig. 7*B,E*). Both GGEL aggregates and immunoreactive intensity were reduced in the cystamine-treated R6/2 mice (Fig. 7*C*). Combined immunofluorescence for htt (FITC) and GGEL (TRITC) immunoreactivities showed colocalization of htt aggregates with GGEL immunoreactivity (Fig. 8*A–C*).

DISCUSSION

Although there have been enormous strides in the understanding of HD and the mutant gene, treatment to slow or prevent disease progression remains elusive. There has been, however, great excitement surrounding drug treatment in HD mice. The study of therapeutics in the transgenic mouse models has helped to develop a number of potential treatment strategies. Several pilot clinical trials in HD patients have recently been initiated based on findings observed in mouse trials (remacemide, coenzyme Q10, minocycline, creatine) (Chen et al., 2000; Ferrante et al., 2000, 2002; Andreassen et al., 2001).

Green first hypothesized the involvement of Tgase in HD (Green, 1993). There are several strong lines of evidence in support of this hypothesis. Tgases are a family of calcium-activated enzymes, which catalyze the formation of γ-glutamyl isopeptide bonds between substrate proteins, often rendering the resulting cross-linked protein complexes insoluble. Expanded polyglutamine repeats are excellent glutamyl-donor substrates of tissue Tgase. Studies have shown that htt, and other polyglutamine-containing constructs, are *in vitro* substrates of Tgase and that Tgase may be involved in htt aggregation (Kahlem et al.,

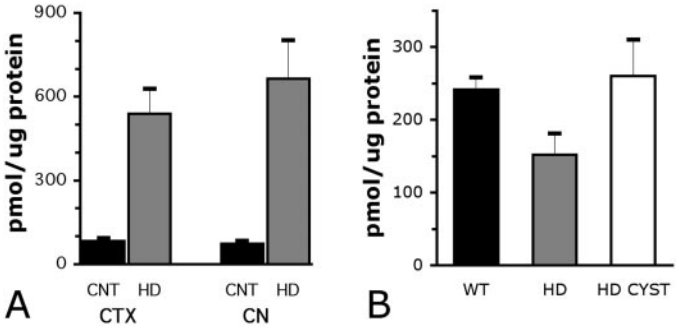

Figure 6. Brain GGEL levels in Huntington's disease patients and R6/2 mice. Free GGEL levels in both the neocortex and caudate nucleus in severe grades of HD were markedly elevated in HD patients as compared with non-neurologic control patients (*A*). Free GGEL levels in unsupplemented R6/2 mice, however, were significantly reduced in comparison with WT littermate control mice, with improved GGEL levels in cystamine-treated R6/2 mice. htt aggregate formation is markedly greater in R6/2 mice than in HD patients. GGEL is colocalized with and sequestered in insoluble htt aggregates. This may result in artificially lowered free GGEL levels observed in R6/2 mice.

1996; Cooper et al., 1997, 2000, 2002; Karpuj et al., 2002b). We and others have reported that levels of Tgase are elevated in HD in a grade-of-severity-dependent manner (Karpuj et al., 1999; Lesort et al., 1999). It has been demonstrated in cell model systems that Tgase inhibitors suppress aggregate formation and reduce cell death (Igarashi et al., 1998; de Cristofaro et al., 1999; Oliverio et al., 1999). Tgase mediates htt aggregation *in vitro* and has a direct correlation to polyQ domain size (Karpuj et al., 2002b). In addition, it has been reported that induction of Tgase 2 gene expression, as a consequence of retinoic acid treatment, results in *in vitro* cell death (Oliverio et al., 1999). Together, these findings suggest that Tgase may play a role in aggregate formation and possibly neuronal cell death in polyglutamine repeat diseases.

Cystamine is the disulfide form of the free thiol, cysteamine. Both cystamine and cysteamine have been reported to inhibit Tgase (Lorand and Conrad, 1984; Uhl and Schindler, 1987; Cooper et al., 2002). Cystamine and monodansyl cadaverine, another Tgase inhibitor, can inhibit the formation of cellular aggregates produced by truncated dentatorubral-pallidoluysian atrophy proteins containing expanded polyglutamine stretches and partially suppress apoptotic cell death (Igarashi et al., 1998; Kahlem et al., 1998). The ratio of cellular glutathione to glutathione disulfide ensures that cystamine is significantly reduced to cysteamine (Cooper and Krystal, 1997). Cooper et al. (2002) suggest that cysteamine is the likely inhibitor of Tgase. The extent of the roles both cystamine and cysteamine play in the observed effects are under further investigation.

In the present experiments, we show that both oral and intraperitoneal cystamine treatment significantly extends survival in the R6/2 model of HD by 16.8 and 19.5%, respectively. In addition, cystamine treatment significantly improved motor performance; delayed loss of body weight, gross brain weight and atrophy, and striatal neuron atrophy; and greatly attenuated the development of mutant-htt aggregates. Levels of Tgase activity and Tgase 2 immunoreactivity were greater in R6/2 mice than in littermate control mice and were reduced by cystamine treatment. Tgase immunoreactivity colocalized to mutant htt aggregates. Cystamine treatment significantly increased free GGEL in the R6/2 mice, consistent with a therapeutic effect. Protein-bound GGEL immunoreactivity determined histologically was markedly

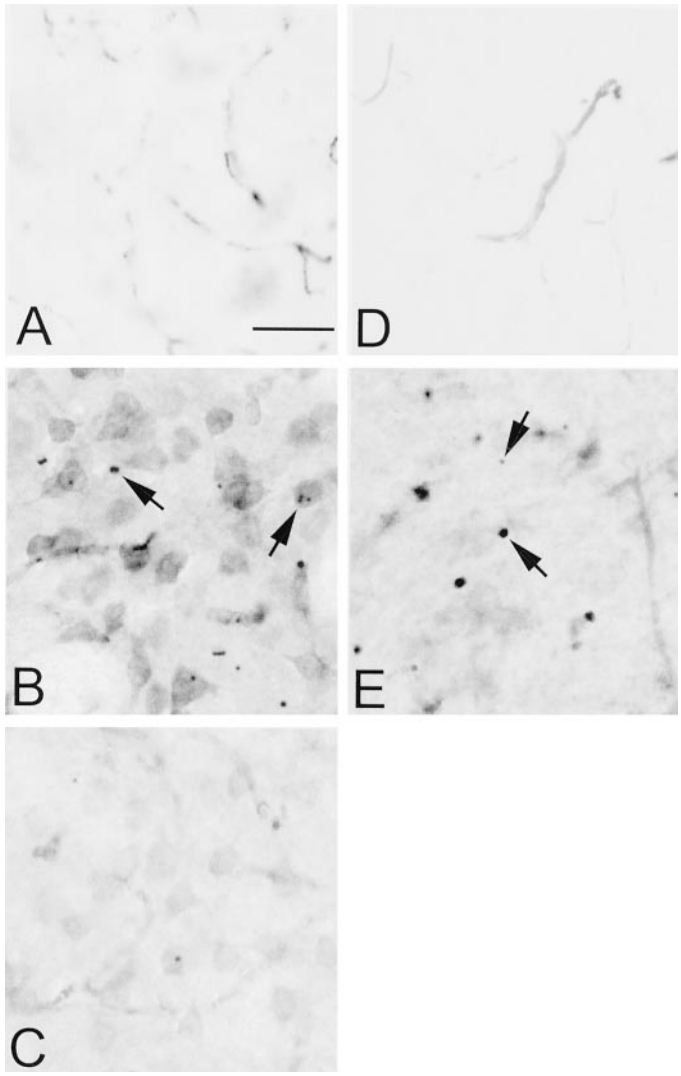

Figure 7. Protein-bound GGEL immunoreactivity in R6/2 mice and HD patients. GGEL immunocytochemical findings in R6/2 mice (*A–C*) show a marked increase in GGEL immunointensity in brain sections from R6/2 mice at 90 d (*B*), in comparison with wild-type littermate control mouse (*A*). GGEL immunoreactivity was found in neurons and the vasculature in R6/2 mice, with intensely immunostained aggregate-like structures in both neurons and the neuropil (*arrows*). In contrast, cystamine-treated R6/2 mice show reduced GGEL immunoreactivity and fewer aggregates (*C*), consistent with reduced htt aggregates in treated R6/2 mice seen in Figure 4. The neocortex (lamina 6) from a grade 3 HD patient shows a similar increase in GGEL immunoreactivity (*E*), in comparison with an age-matched control (*D*). GGEL-positive aggregates are present in the HD neocortex (*arrows*). Scale bar, 50 μm.

increased in both HD patients and R6/2 mice and colocalized with mutant htt aggregates. These findings demonstrate that cystamine has significant efficacy in improving the neurological and neuropathological phenotype observed in the R6/2 transgenic model of HD and strongly suggests that Tgase plays a role in HD.

Tgase catalyzes the formation of covalent linkages between a glutamine protein residue and lysine protein residue, forming a GGEL linkage. GGEL, therefore, is a specific biochemical marker of Tgase activity. We show that free GGEL levels were significantly elevated in both cortex and caudate nucleus of HD patients. In support of our findings, it has recently been shown that free GGEL is significantly increased in the CSF of HD

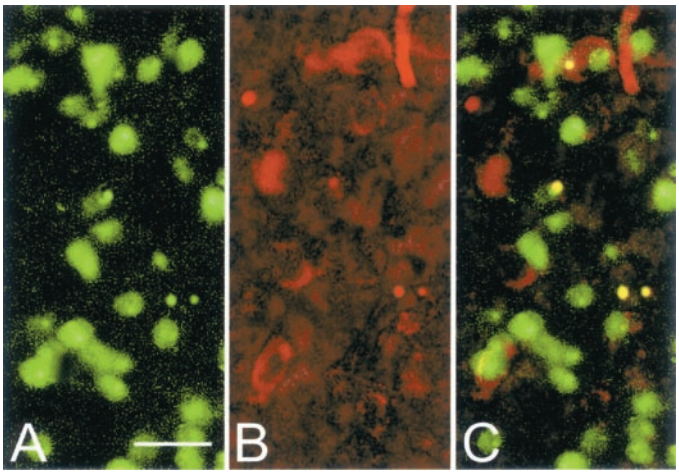

Figure 8. Combined GGEL and mutant htt immunofluorescence in R6/2 mice and HD patients. Combined immunofluorescence for htt (*green*) (*A*) and GGEL (*red*) (*B*) immunoreactivities within the same tissue specimens from the neostriatum of a 90-d-old R6/2 mouse show colocalization of htt aggregates and GGEL immunostaining in the merged figure (*yellow*) (*C*). Scale bar, 100 μm.

patients (Jeitner et al.,2001). Although protein-bound GGEL histologic immunoreactivity was markedly increased in R6/2 mice, free GGEL levels measured biochemically were reduced in unsupplemented R6/2 mice, in comparison with both wild-type and cystamine-treated R6/2 mice. This finding in the mice may be related to the degree of sequestered GGEL in the insoluble mutant htt aggregate. There are markedly greater numbers of htt aggregates within the R6/2 mouse model of HD than in patients with HD. It is possible that the difference may be the result of a decrease in free GGEL formation (from the proteolysis of cross-linked proteins) caused by sequestration in insoluble deposits and/or to increased degradation of free GGEL by γ-glutamylamine cyclotransferase and membrane-bound γ-glutamylamine transpeptidase (Danson et al., 2002).

Karpuj et al. (2002a) have recently reported that cystamine treatment initiated at 7 weeks, after clinical signs have appeared, prolonged survival by ~12% in R6/2 HD mice. This was much less than the present findings and may reflect delayed treatment after symptoms were present or dosing differences. In addition, although there was a delay in both weight loss and limb clasping, neuropathological analysis did not show any amelioration of htt aggregates between cystamine-treated and PBS-untreated R6/2 mice. In contrast, we found a marked reduction in htt aggregates in R6/2 mice in which cystamine treatment was initiated at an earlier time point. It is possible that Tgase may be involved with the cross-linking of htt in smaller fragments (microaggregates), resulting in the nidus for disease. This is consistent with other findings that macroscopic aggregates do not correlate with cell death (Saudou et al., 1998; Klement et al., 1998; Kuemmerle et al., 1999). Collectively, our findings suggest that cystamine can inhibit aggregate formation and may be most beneficial as a treatment given before the onset of clinical phenotype.

Three theories, which are not mutually exclusive, have been proposed concerning the potential mechanism of htt aggregation in HD. Perutz et al. (1994) has suggested that expanded CAG repeats interact to form a polar zipper. Polymerization of htt and aggregate formation occurs *in vitro* only when the polyglutamine repeat is above 36 (Scherzinger et al., 1997). The second hypothesis, proposed by Green (1993) and Kahlem et al. (1996) suggests that Tgases may cross-link polyglutamine tracts into htt aggregates in HD. Aggregates can occur in the absence of Tgase, as shown *in vitro* in cell-free systems (Scherzinger et al., 1997). However, it is possible that initial polymerization could occur by a polar zipper mechanism followed by covalent cross-linking by Tgase. Finally, a toxic-channel hypothesis has been suggested in which long-chain polyglutamines form relatively stable microhelical channels that remain in an open state (Monoi et al., 2000). These channels are permeable to monovalent cations and dissipate electrochemical proton and voltage gradients across membranes, reducing ATP production.

The exact mechanism or mechanisms by which cystamine treatment is beneficial to R6/2 mice is unclear and may be multifold. Cystamine has a therapeutic role in a number of clinical conditions (McDonnell et al., 1997; Boyko et al., 1998; Iwata et al., 1998; Misik et al., 1999; Qiu et al., 2000). In addition to modulating Tgase activity and subsequent protein aggregation, cystamine may act as an antioxidant. Oxidative stress may play a role in both HD mice and patients (Browne et al., 1999; Bogdanov et al., 2001). We found that application of cystamine in an *in vitro* model of oxidative stress is cytoprotective and increases glutathione levels (R. R. Ratan, unpublished data). Glutathione is a principle substrate for the detoxification of reactive oxygen species. Maintenance of high glutathione levels may be an important mechanism by which cystamine treatment improves the behavioral and neuropathological phenotype in the R6/2 transgenic mouse model of HD. Cystamine has also been suggested to ameliorate apoptosis (Igarashi et al., 1998; Oliverio et al., 1999). The potential for cystamine to play a neuroprotective role via glutathione replenishment and/or caspase inhibition, therefore, needs further investigation.

Although the cause of neuronal death in HD remains unknown, specific early molecular events may lead to a progressive cascade of generic pathogenic processes. It has been widely postulated that the mutant htt protein may cause toxic effects in neurons, leading to a cascade of pathogenic mechanisms, including oxidative stress, mitochondrial dysfunction, energy metabolism defects, apoptosis, and excitotoxicity. An important event in this cascade may be transcriptional dysregulation through direct binding of the mutant htt protein to transcription factors, disrupting the normal pattern of gene transcription and altering gene expression in those pathogenic mechanisms associated with HD (Cha et al., 2000; Lin et al., 2000; Steffan et al., 2000). It is possible that cystamine may act by increasing transcription of neuroprotective factors in HD (Karpuj et al., 2002a). Alternatively, cystamine attenuates the development of huntingtin inclusions and, therefore, may act in part by reducing transcription factor sequestration, which is known to occur within insoluble htt aggregates (Cha, 2000; Steffan et al., 2000).

The prospects for neuroprotective treatment in HD patients are rapidly brightening. Our findings underscore the importance of the power of transgenic mouse models of HD for the screening of novel therapeutics. The positive effects of cystamine in R6/2 transgenic mice provide further evidence that Tgase may contribute to HD pathogenesis, although it is unclear what role other mechanisms of action of cystamine may play in improving both the behavioral and neuropathological phenotype. Regardless, these studies have identified a novel therapeutic strategy that may be successfully translated to human clinical trials and the subsequent treatment of HD patients.

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