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Overexpression of Cystathionine γ-Lyase Suppresses Detrimental Effects of Spinocerebellar Ataxia Type 3

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Spinocerebellar ataxia type 3 (SCA3) is a polyglutamine (polyQ) disorder caused by a CAG repeat expansion in the ataxin-3 (*ATXN3*) gene resulting in toxic protein aggregation. Inflammation and oxidative stress are considered secondary factors contributing to the progression of this neurodegenerative disease. There is no cure that halts or reverses the progressive neurodegeneration of SCA3. Here we show that overexpression of cystathionine γ-lyase, a central enzyme in cysteine metabolism, is protective in a *Drosophila* model for SCA3. SCA3 flies show eye degeneration, increased oxidative stress, insoluble protein aggregates, reduced levels of protein persulfidation and increased activation of the innate immune response. Overexpression of *Drosophila* cystathionine γ-lyase restores protein persulfidation, decreases oxidative stress, dampens the immune response and improves SCA3-associated tissue degeneration. Levels of insoluble protein aggregates are not altered; therefore, the data implicate a modifying role of cystathionine γ-lyase in ameliorating the downstream consequence of protein aggregation leading to protection against SCA3-induced tissue degeneration. The cystathionine γ-lyase expression is decreased in affected brain tissue of SCA3 patients, suggesting that enhancers of cystathionine γ-lyase expression or activity are attractive candidates for future therapies.
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INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, is a rare progressive neurodegenerative disease and the most common dominantly inherited ataxia worldwide. SCA3 is a polyglutamine (polyQ) disorder caused by a CAG-trinucleotide repeat expansion encoding glutamine within the sequence of the ataxin-3 (*ATXN3*) gene. The length of the repeat expansion is directly related to the aggregation propensity of the ataxin3 protein and is inversely related to the age of onset of the disease. Protein aggregates are considered to be the cause for neuronal dysfunction and death, which is supported by several lines of evidence showing that aggregate prevention or increased (autophagic) clearance delays neuronal death and degeneration in multiple model systems (1–4).

The pathophysiological sequel of neurodegeneration in SCA3 is not fully understood, although proteotoxic stress, transcriptional dysregulation, mitochondrial dysfunction, oxidative stress and inflammation have been implicated (5–7). To date, there are no disease-modifying treatments for polyQ diseases such as SCA3.

Cystathionine γ-lyase (CSE) is one of the central enzymes in cysteine and hydrogen sulfide metabolism (H_2S). Homocysteine is a substrate for CSE leading to the production of H_2S, α-ketobutyrate, ammonia, homolanthionine and cystathionine, with the latter serving as a CSE substrate to produce cysteine (8,9). Cysteine is also a substrate for CSE leading to the production of H_2S, cystathionine and pyruvate (8,9). H_2S and CSE are linked to aging and age-related pathologies (10–13). H_2S can act as an endoge-

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nous modulator of oxidative stress either by direct scavenging of reactive oxygen species (ROS) and nitrogen species (14) or through increasing the intracellular glutathione (GSH) pool (15,16). H_2S also confers cytoprotection via suppression of inflammation (17) and by protecting mitochondrial function and integrity (17,18). Decreased levels of H_2S in brain tissue are associated with neurodegenerative age-related diseases such as Parkinson's (19), and administration of H_2S has been shown protective in experimental models for this disease (20–22). Decreased levels of CSE have recently been observed in human Huntington disease tissues and in a mouse Huntington model (22). After addition of sodium hydrogen sulfide and L-cysteine, levels of protein persulfidation (also called protein S-sulfhydration) increased in a CSE-dependent manner *in vitro* (23), suggesting an influential effect of this type of posttranslational protein modification. Indeed, protein persulfidation has been demonstrated to mediate the activity of parkin, to serve as an antioxidant and to protect against cellular senescence (24–26). A possible link between SCA3, CSE and protein persulfidation remains to be determined as well as the potential neuroprotective effects of overexpressing the CSE enzyme directly in a neurodegenerative background.

Here, we investigated the possible protective role of CSE in a *Drosophila* model for SCA3. *Drosophila* was chosen because *CSE* is highly conserved between humans and flies (http://flybase.org/blast), and an established *Drosophila* model for SCA3 is available (27,28). In the fly model for SCA3, a truncated version of the pathogenic human *ATXN3* gene containing a multiple CAG repeat is expressed, and key features of SCA3 disease are present (27–29). This model is suitable because the CAG repeat, and not the mutated protein, is considered to be the disease-causing entity in SCA3 and in several other polyQ diseases as well (29,30). We used the *Drosophila* SCA3 model (also called SCA3 flies) to investigate the effects of *CSE* overexpression. We identified additional phenotypes in the SCA3 model, such as loss of tissue integrity in degenerative patches in the fly eye, increased activation of the innate immune response and decreased protein persulfidation. We found that transgene-mediated increased expression of *CSE* rescues these novel and also previously reported phenotypes of the fly SCA3 model. Rescue was also observed after addition of sodium thiosulfate, a drug approved by the U.S. Food and Drug Administration and a component of the transsulfuration pathway in which CSE plays a central role (9,31). The CSE-mediated rescue occurs independently of protein aggregate formation, indicating that rescue effects occur downstream of the formation of these toxic entities. We also found endogenous expression of CSE in brain areas that are affected in SCA3 patients. In addition a lower expression was observed in patient samples compared with controls. We present and discuss a possible role for CSE in polyQ disease pathology and treatment.

MATERIALS AND METHODS

Below we provide a brief overview of the methods used for experiments presented in this article. For further details, please see the Supplementary Materials and Methods.

Drosophila Stocks

As wild-type control, the y^1w^{1118} *Drosophila* line was used. *Eip55E* (*Drosophila CSE*)-overexpressing lines were generated in the laboratory. The *GMR-GAL4 UAS-SCA3trQ78* fly stock was a gift from Nancy M Bonini (28,32). The detailed description of the *Drosophila* lines and fly food, backcrossing and supplementation of chemical compound information can be found in the Supplementary Materials and Methods.

Eye Degeneration Assay

To evaluate relative degeneration percentage, we used an eye scoring method that was previously described (33,34). Irregularly structured depigmented eyes without dark patches were defined as rough. The presence of one or more black patches along with the irregular structure and depigmentation was considered a degenerated rough eye. Each eye of 1-d-old flies was scored as a singular entity. We scored the total amount of degenerated eyes as opposed to the total amount of eyes (rough + degenerated). Total count of eyes scored per condition was between 100 and 1,000, depending on the number of progeny of a particular genotype.

Protein Persulfidation Assay

To detect protein persulfidation, a tag-switch assay was used as previously described (35,36). Briefly, protein extracts were prepared from either whole flies (three flies per sample, 100 µL extraction buffer) for persulfidation analysis of *CSE* overexpression effect under control of actin driver in a nondisease background or fly heads (25 heads per sample, 65 µL of extraction buffer) for persulfidation analysis of *CSE* overexpression effect under control of a glass multiple reporter (*GMR*) driver in the SCA3 background. Extracts were prepared using HEN buffer (50 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], 2 mmol/L ethylenediaminetetraacetic acid [EDTA], 1 % NP-40) additionally supplemented with 2% sodium dodecyl sulfate (SDS), 1% protease inhibitors and 20 mmol/L 2-benzo[d]thiazol-2-ylsulfonyl)acetic acid (MSBTA), a water-soluble methylsulfonyl benzothiazole derivate (37). The extracts were incubated for 30 min on ice and 30 min at 37°C. Proteins were then purified by using water/methanol/chloroform precipitation (4/4/1, v/v/v). Samples were redissolved in phosphate-buffered saline containing 2% SDS and treated with CN-biotin (Supplementary Figure S4A, D) overnight. Protein concentrations were adjusted to the same value before the SDS electrophoresis was performed. Biotinylation of the samples was detected by Western blot, using anti-biotin antibodies (Sigma-Aldrich). To improve the assay, we used a new probe, CN-Cy3, which served as an alternative for CN-biotin and al-

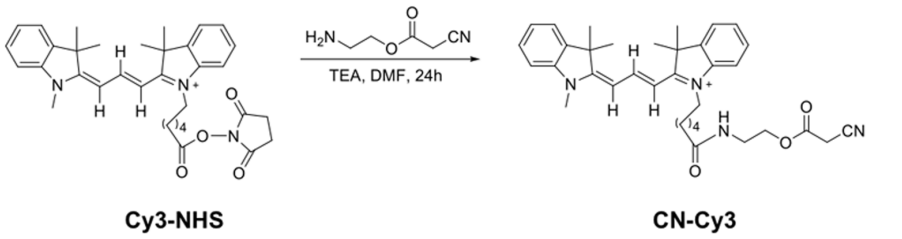

Figure 1. Synthesis of Cy3-CN from a commercial Cy3-NHS ester. TEA, triethylamine; DMF, dimethylformamide.

lowed direct in-gel fluorescence measurements. CN-Cy3 is a cyanoacetate derivative of a cyanine-based Cy3 fluorescence dye. (See Figure 1).

Molecular Biology Techniques

For a detailed description of quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), Western blot and protein oxidation analyses, and immunohistochemistry used in the current study, please see the Supplementary Materials and Methods.

All supplementary materials are available online at www.molmed.org.

RESULTS

SCA3 Flies Show Increased Tissue Degeneration

A *Drosophila* model was used to further investigate a possible protective role of overexpression of *CSE* in SCA3. First, we performed an extended phenotypic analysis of this model. Previously, it was shown that flies bearing *UAS-SCA3trQ78*—an inducible truncated version of the human *ATXN3* gene containing 78 CAG repeats—under the control of the GMR driver (also called *GMR-GAL4-UAS-SCA3trQ78* or SCA3 flies) develop progressive cellular eye degeneration. These flies develop a fully penetrant "rough eye" phenotype, and a certain percentage of the rough-eyed flies possess a variable amount of patches with increased degeneration in these rough eyes (27,28). Rough eyes containing these degenerative patches (further called "degenerated rough eyes") are considered to be more affected compared with the rough eyes without these patches; therefore, this is a useful tool to identify enhancers or suppressors of the SCA3-induced toxicity (32). To visualize these phenotypic differences at a higher magnification and to evaluate the severity of the rough versus degenerated rough eye phenotypes in more detail, we performed correlative light microscopy and scanning electron microscopy on the eyes of wild-type flies (Figure 2A–A″) and flies overexpressing human *ATXN3* (Figures 2B, C). It appeared that the rough eyes consist of irregular-shaped ommatidia with irregularly arranged bristles (Figures 2B–B″). In contrast, the degenerative patches contained an undefined structure, and bristles were absent (Figures 2C–C″). These results confirmed that degenerative patches can indeed be classified as more severely affected tissue parts compared with the rest of the rough eye structures. Therefore, an intervention that causes a decreased amount of rough eyes with degenerative areas within the SCA3 background can be classified as protective.

Generation and Characterization of Various *CSE* Transgenic Lines

To further investigate a possible modulating role of *CSE* in SCA3 pathogenesis, we investigated the effect of *CSE* overexpression in the SCA3 fly model. Six different fly lines overexpressing *Eip55E*, a highly conserved *Drosophila* ortholog of the human *CSE* gene (http://flybase.org/blast) (38) under a GAL4-inducible promoter, were created. Because of the absence of an antibody against *Drosophila* CSE, the characterization of the lines was performed by using qRT-PCR analysis. CSE expression levels of each inducible line were determined in the presence of a daughterless driver, resulting in ubiquitous expression of the construct. All six lines showed increased mRNA expression of CSE compared with the in-house control w^{1118} strain (Supplementary Figure S1A). Because the genetic background of *Drosophila* plays an important role in the severity of specific phenotypes (39), all transgenic overexpressing lines were backcrossed for at least six generations to create isogenic controls. This step resulted in the generation of *CSE* overexpressing strain 1 (called CSE1) and its specific isogenic control (called control 1) and to the generation of *CSE*-expressing strain 2 and 3 (called CSE2 and CSE3) and their isogenic control (called control 2). This approach allowed us to compare the effect of *CSE* overexpression in two genetic backgrounds, to investigate the effect of variations in overexpression levels and to compare this to isogenic controls. By using qRT-PCR, we demonstrated that CSE1 showed a 2.1-fold induction of CSE compared with its isogenic control and that CSE2 and CSE3 showed a 2.2- and 5.3-fold increased expression of CSE compared with their isogenic control, respectively (Figures 3A, B). Extensive description of all used *Drosophila* genotypes is presented in the Supplementary Materials and Methods and Supplementary Figures S1A, B).

Overexpression of *CSE* Partially Rescues the Phenotype of SCA3 in *Drosophila*

To investigate a possible effect of *CSE* overexpression on the eye phenotype, we scored the percentage of degenerated eyes (i.e., when degenerated patches visualized in Figures 2C–C″ are present) 1 d after eclosion by using light microscopy. In the SCA3 background, *CSE*-overexpressing flies showed a significant decrease in the percentage of degenerative rough eyes (Figures 3C, D). Suppression of the SCA3-degenerative rough eye

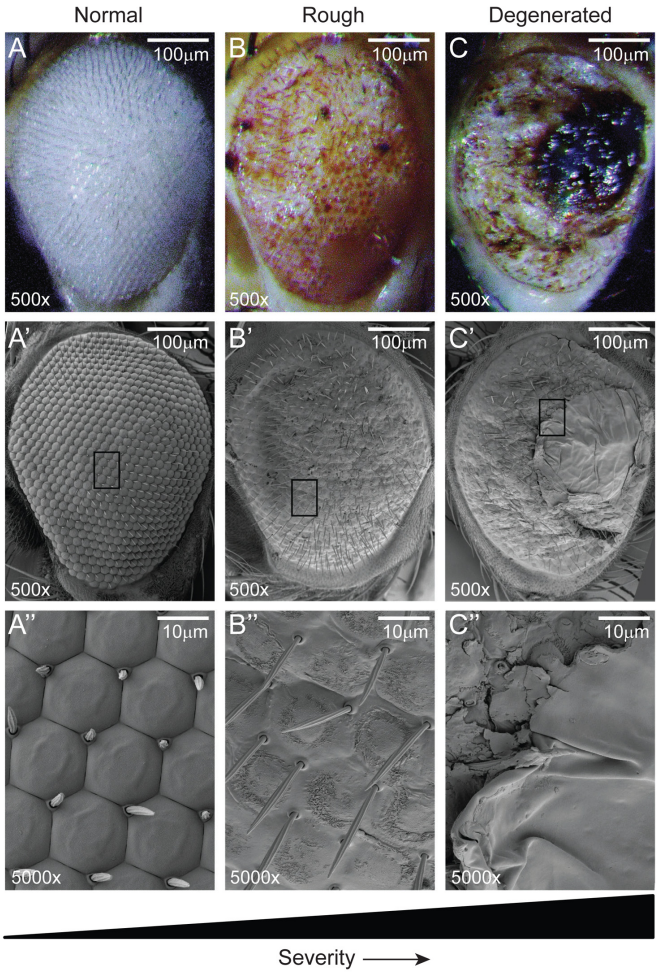

Figure 2. Increased tissue degeneration is present in neurodegenerative patches within the rough eye background of SCA3 flies. Eyes of SCA3 and control flies were visualized in detail using light end electron microscopy. Representative light microscopy pictures (A, B, C) with correlative scanning electron microscopy pictures (A′, A,″ B′, B,″ C′, C″) of eye phenotypes are shown. (A, A′, A″) Normal control eye phenotype (B, B′, B″) SCA3-overexpressing eye with a phenotype classified as rough. (C, C′, C″) SCA3-overexpressing eye with a phenotype classified as degenerated; black patches show less preservation of tissue integrity.

phenotype was observed in all *CSE*-overexpressing lines compared with their *SCA3*-expressing isogenic control lines. Similar results were obtained in both genetic backgrounds. The CSE3 line with the highest level of *CSE* overexpression reduced the number of degenerative eyes to a greater extent than the CSE2 line (Figure 3D). To further strengthen the rescue potential of CSE, we pharmacologically inhibited CSE with propargylglycine (PPG) as previously described (10). Supplementation of PPG to the fly food reversed the protective effect of *CSE* overexpression in the SCA3 background, as evidenced by an increased percentage of degenerated rough eyes (Figures 3C, D). Addition of PPG neither enhanced nor suppressed the percentage of degenerative eyes in the SCA3 background, strongly suggesting that the observed effect in the *CSE*-overexpressing background is due to inhibition of CSE and not due to other effects of PPG. Together, these results indicate that the rescuing potential is mediated by overexpression of *CSE* and is not influenced by the genetic background.

Overexpression of *CSE* Does Not Induce a Change in Levels of Insoluble Proteins

Polyglutamine diseases are thought to be driven by protein aggregation that subsequently triggers a myriad of downstream consequences ultimately leading to neurodegeneration. We thus first tested whether the rescue of SCA3-induced tissue degeneration by *CSE* overexpression was associated with reduced aggregation of the truncated human ATXN3 protein. Hereto, we determined ratios of insoluble versus soluble fractions of ATXN3 proteins in SCA3 flies in the absence and presence of *CSE* overexpression by using Western blot analysis as described previously (29). An increased insoluble/soluble ratio indicates an increase in protein aggregation. Overexpression of *CSE* did not significantly alter the insoluble/soluble ratio in SCA3-expressing flies (Figures 4A, B; Supplementary Figure S2), indicating that the protective effects of *CSE* overexpression are not mediated by decreased toxic protein aggregates and most likely work protectively against damaging effects downstream of the formation of aggregates.

Overexpression of *CSE* Reduces Levels of Oxidative Damage of Proteins in SCA3 Flies

Oxidative stress is associated with the pathogenesis of SCA3 disease (40,41). Previously, it was demonstrated that CSE deficiency is linked to increased levels of oxidative stress (42). As a readout for oxidative stress, we used OxyBlot analysis as previously described (43). SCA3 flies showed increased levels of oxidized proteins (characteristically visible as multiple bands) compared with their isogenic non-SCA3 control lines (Figures 4C, D). The level of oxidized proteins was reduced in all three *CSE* overexpression lines in the SCA3 background compared with the isogenic controls (Figures 4C, D).

Overexpression of *CSE* Prevents SCA3-Associated Immune Induction

Several findings suggest that inflammation contributes to the multifaceted

pathogenesis of SCA3 disease (44). In *Drosophila*, the Toll and immune deficiency (IMD) immune signaling pathways mediate activation of nuclear factor (NF)-κB transcription factors Dif-Dorsal and Relish, respectively. Targets of these transcription factors include antimicrobial peptides (AMPs). These immune pathways are equivalent to NF-κB signaling in mammals and are a key factor in the induction of the innate immune response (45). Immune induction by expression of SCA3trQ78 in *Drosophila* has been previously reported as well (46). To investigate whether CSE-mediated protection against SCA3 is associated with a reduction in the immune response, we performed a qRT-PCR analysis for AMPs, which are targets of either the IMD or the Toll pathway. As targets of the Toll pathway, *immune-induced molecule 1* (*IM1*), *immune-induced molecule 2* (*IM2*) and *drosomycin* were analyzed (47,48), and for the IMD pathway, *attacin*, *cecropin A1* and *diptericin* (45). Expression of SCA3 activated the immune response of both pathways. *CSE* overexpression in the SCA3 background significantly attenuated all investigated players of the Toll pathway (Figure 5). A comparable effect was seen for a subset of AMPs of the IMD pathway (Supplementary Figure S3). This result shows that the suppressive effect of *CSE* overexpression on eye degeneration in SCA3 flies is associated with a dampening of the Toll pathway and a partial dampening of the IMD innate immune response pathway. These data suggest that CSE may exert its beneficial effects on the SCA3 phenotype by attenuating the immune response.

SCA3 Flies Show Reduced Levels of Protein Persulfidation

Decreased levels of CSE are associated with impaired neurological function (22). Here we demonstrated that increased levels of CSE are protective. Because *CSE* overexpression is associated with increased protein persulfidation (25,49), we hypothesized that, in a SCA3 background, protein persulfidation may be decreased and *CSE* overexpression may

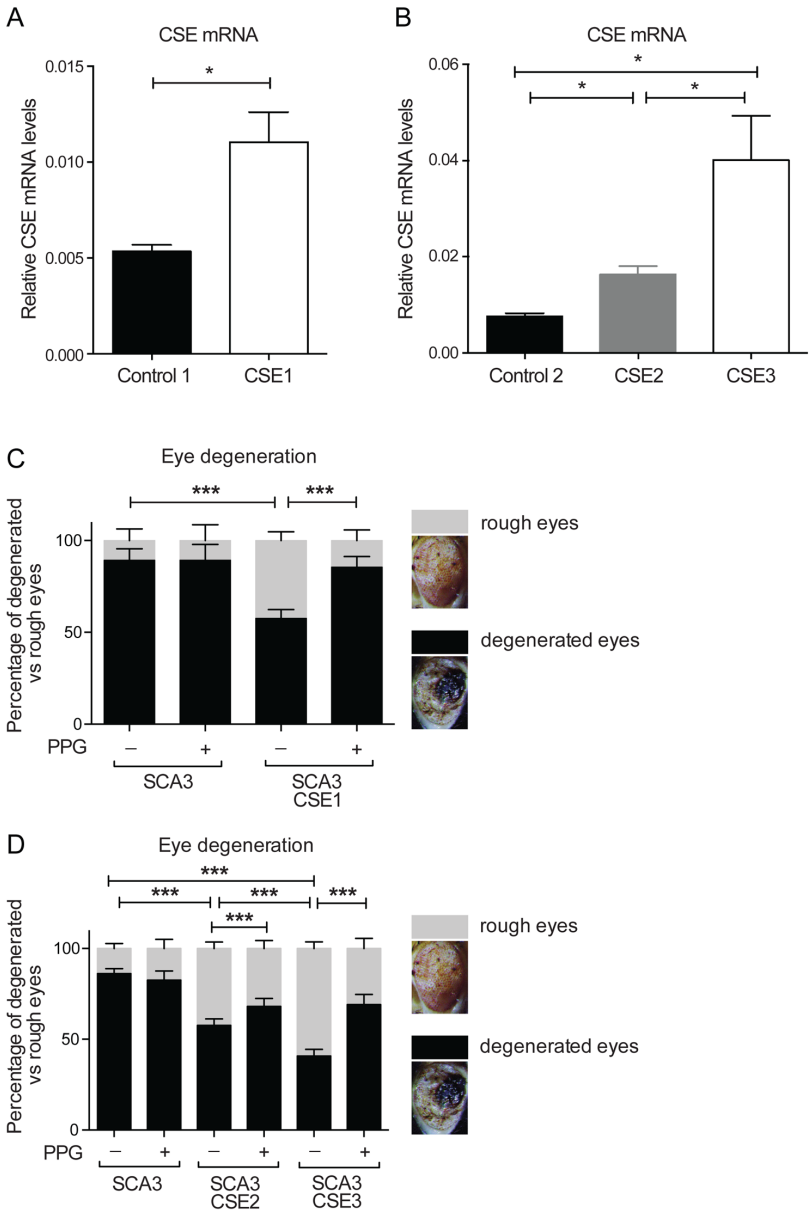

Figure 3. *CSE* overexpression suppresses the SCA3 phenotype. (A, B) *CSE* overexpression was determined by using qRT-PCR in *CSE* (CSE1; CSE2; CSE3) overexpressing transgenic fly lines compared with their isogenic controls (control 1 and control 2). *CSE* was expressed ubiquitously by using an actin-GAL4 driver. In both genetic backgrounds, CSE mRNA levels were increased in the *CSE*-overexpressing lines compared with their isogenic controls. $^{*}p < 0.05$, error bars indicate standard error of the mean (SEM). (C, D) SCA3 flies with and without overexpression of *CSE* (three independent lines) were analyzed. In all three transgenic lines in the SCA3 background, *CSE* overexpression resulted in a decrease of the degree of eye degeneration compared with isogenic SCA3-expressing lines. Inhibition of *CSE* by 2 mmol/L PPG diminished this effect. The presence of degenerative patches (black area) was determined by using light microscopy. For quantification, the number of rough and degenerated eyes in at least three independent experiments (n = 100–300 per experiment) was counted. $^{***}p < 0.001$, error bars indicate SEM. Black area represents the percentage of rough eyes containing neurodegenerative patches. Grey area represents percentage of rough eyes without neurodegenerative patches.

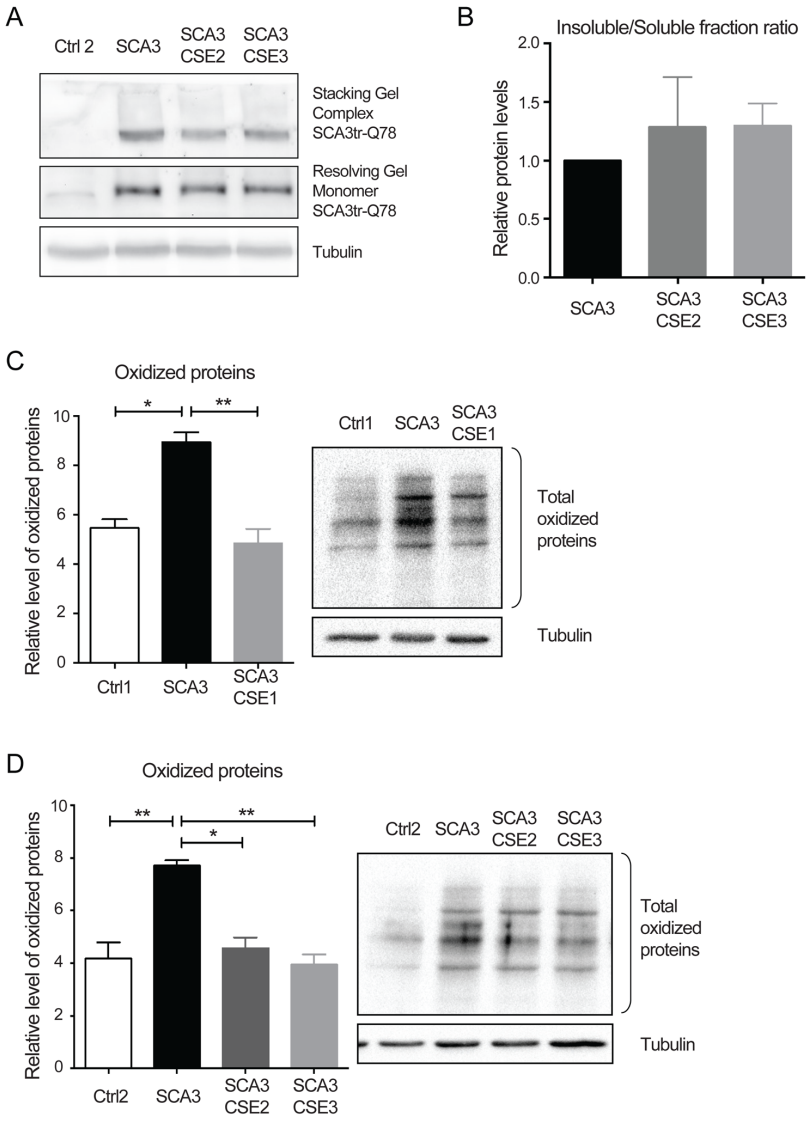

Figure 4. SCA3tr-Q78 protein expression and aggregation is not altered, and oxidative stress is reduced in the presence of CSE overexpression. (A) Western blot analysis of extracts of heads of SCA3-overexpressing flies was used to determine levels of protein aggregation. The samples were analyzed for the amount of soluble SCA3tr-78 protein (present in resolving gel) and levels of SCA3tr-78 protein aggregation (present in stacking gel) by using an anti-HA antibody. α-Tubulin was used as a loading control. In the SCA3 flies, both soluble monomer (in the resolving gel) and aggregated protein (in the stacking gel) fractions were detected. CSE overexpression did not significantly alter the solubility of the SCA3 protein. (B) Quantification of the ratio between the relative intensity of the protein band in the stacking gel and SCA3tr-78 monomer band in the resolving gel. There was no significant change in the protein solubility upon the overexpression of CSE in a SCA3 background (n = 5). (C, D) OxyBlot analysis of extracts derived from fly heads was used to examine levels of oxidized proteins. SCA3 flies had higher total levels of oxidized proteins compared with wild-type flies. CSE overexpression in the SCA3 background showed a reduction of oxidized proteins. Three independent CSE-overexpressing lines were used and compared with their isogenic controls. For quantification, optical density of oxidized proteins was normalized to tubulin; levels of oxidized proteins in CSE-overexpressing SCA3 fly samples were comparable to levels in control fly heads. *$p < 0.05$, **$p < 0.01$, error bars indicate SEM.

lead to restoration of this posttranslational modification.

To selectively detect protein persulfidation, a recently reported tag-switch assay was further optimized and used. The original method labels the persulfidation of proteins with biotin as a reporting tag (Supplementary Figures S4A–C). To further enhance the signal intensity, the tag-switch assay was improved by using a reagent, which directly introduced a cyanine-based Cy3 fluorescence tag, Cy3-CN (Supplementary Figure S4A), to persulfidated proteins. An artificial color scheme was used to better illustrate the persulfidation levels (the color scale is given in the figure). A signal in the yellow-white range indicates high levels of persulfidation, and a signal in the black-blue range indicates a low level (see also the Supplemental Data). Persulfidation of proteins in heads of SCA3 flies was found to be decreased compared with wild-type controls (Figure 6A). Next, we investigated whether the protective effect of *CSE* overexpression was associated with restoration of protein persulfidation. Consistently with our hypothesis, we found that *CSE*-overexpressing flies demonstrated elevated protein persulfidation compared with the controls (Figure 6B, Supplementary Figure S4D). The observed rescue of SCA3 degeneration in *CSE*-overexpressing fly lines was associated with normalization of protein persulfidation levels (Figure 6C, Supplementary Figures S4B, C). These data showed that degenerative defects of SCA3 are associated with decreased levels of protein persulfidation, and this can be reversed by overexpression of *CSE*, an intervention that also protects against tissue loss in the *Drosophila* SCA3 model.

Treatment with Sodium Thiosulfate Reduces Eye Degeneration in SCA3 Flies

To try to pharmacologically rescue SCA3-induced degeneration, we used sodium thiosulfate (STS). STS is a relatively stable nontoxic compound used in

clinical settings to treat caliciphylaxis, extravasations during chemotherapy or cyanide poisoning (50), and is also a component of the transsulfuration pathway. Used as a substrate for rhodanase-like enzymes, thiosulfate could also be a source of targeted persulfidation, as recently proposed by Mishanina *et al.* (31). Therefore, we tested the effects of thiosulfate on SCA3 flies.

Increasing concentrations of STS were fed to SCA3-expressing flies to investigate possible toxicity of STS. Concentrations of ≥120 mmol/L STS induced lethality (data not shown). Upon supplementation of lower concentrations of STS, a dose-dependent decrease in the percentage of degenerated rough eyes was observed (Figure 7A). A total of 80 mmol/L STS reduced the percentage of degenerated rough eyes in *SCA3*-overexpressing conditions in two genetic backgrounds (Figure 7B). These data show that the suppression of SCA3-associated degeneration is achieved not only using a genetic approach by overexpression of *CSE* but can also be achieved pharmacologically using STS.

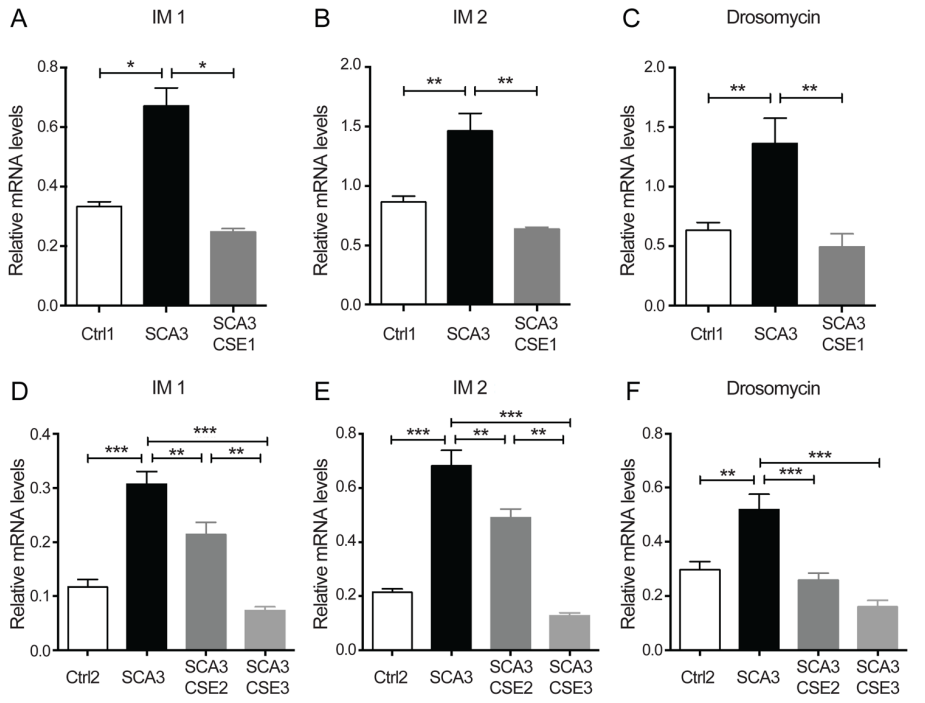

Figure 5. Overexpression of *CSE* prevents SCA3-related immune induction. mRNA levels of various immune response genes (*IM1, IM2, Drosomycin*) were determined by qRT-PCR in control flies, in SCA3 flies (2 genetic backgrounds) and in SCA3 flies overexpressing *CSE* in the same isogenetic background. In *SCA3*-expressing flies, all investigated immune players were upregulated compared with control flies of the same genetic background. In *CSE*-expressing flies in various SCA3 backgrounds, immune response gene expression was lower compared with the isogenetic SCA3 backgrounds. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, error bars indicate SEM. IM 1, immune-induced molecule 1; IM 2, immune-induced molecule 2.

Endogenous CSE Is Present in Affected Brain Tissue of SCA3 Patients

To investigate a possible role of CSE in human SCA3 pathogenesis, we investigated the expression levels and localization of CSE in healthy tissue and in SCA3 disease tissue. To determine the presence and localization of CSE, we performed immunohistochemistry for CSE on postmortem pontine tissue of control individuals and SCA3 patients. From the sparse tissue available for this rare disease, pontine tissue was chosen to analyze the features of this disorder, because in this tissue several types of toxic protein aggregates are present with enough neurons preserved to allow immunohistochemical analysis (7) in contrast to other brain areas that are almost completely degenerated or that hardly show degeneration or protein aggregation (51,52). As control samples, postmortem tissue of individuals without a neurodegenerative or neuropsychiatric disease were used (Supplementary Table S1; n = 7). CSE protein expression was observed in vascular endothelium, neurons and astrocytes (Figures 7C–F). This localization pattern was not affected in pontine tissue of SCA3 patients (Figures 7G–J; Supplementary Figure S5). To investigate expression levels of CSE, we performed qRT-PCR analysis for CSE transcripts on pontine samples of SCA3 patients and control samples. qRT-PCR data revealed the presence mRNA levels of CSE in pontine tissue of control tissue (n = 7) and SCA3 (n = 6) patients; although, in the latter, levels were reduced (Figure 7K). Western blot analysis using an anti-CSE antibody (53) further confirmed the presence and decreased levels of endogenous CSE in pontine tissue of SCA3 patients compared with controls (Figures 7L, L'). Together, these data demonstrated that CSE is endogenously present but decreased expressed in affected brain areas of SCA3 patients.

DISCUSSION

We present evidence that *CSE* overexpression works protectively in a *Drosophila* model for the neurodegenerative disease SCA3. To our knowledge, protective effects by *CSE* overexpression in neurodegenerative animal models have not been described before. However, neuroprotective effects of H_2S have been reported previously, not only in experimental models for Parkinson's disease, (21), vascular dementia (54) and homocysteine-induced neurotoxicity, but also in *in vitro* models for oxidative stress in neurons (55) and Alzheimer's disease (56). In an experimental mouse model for Parkinson's disease, inhalation of H_2S prevents the devel-

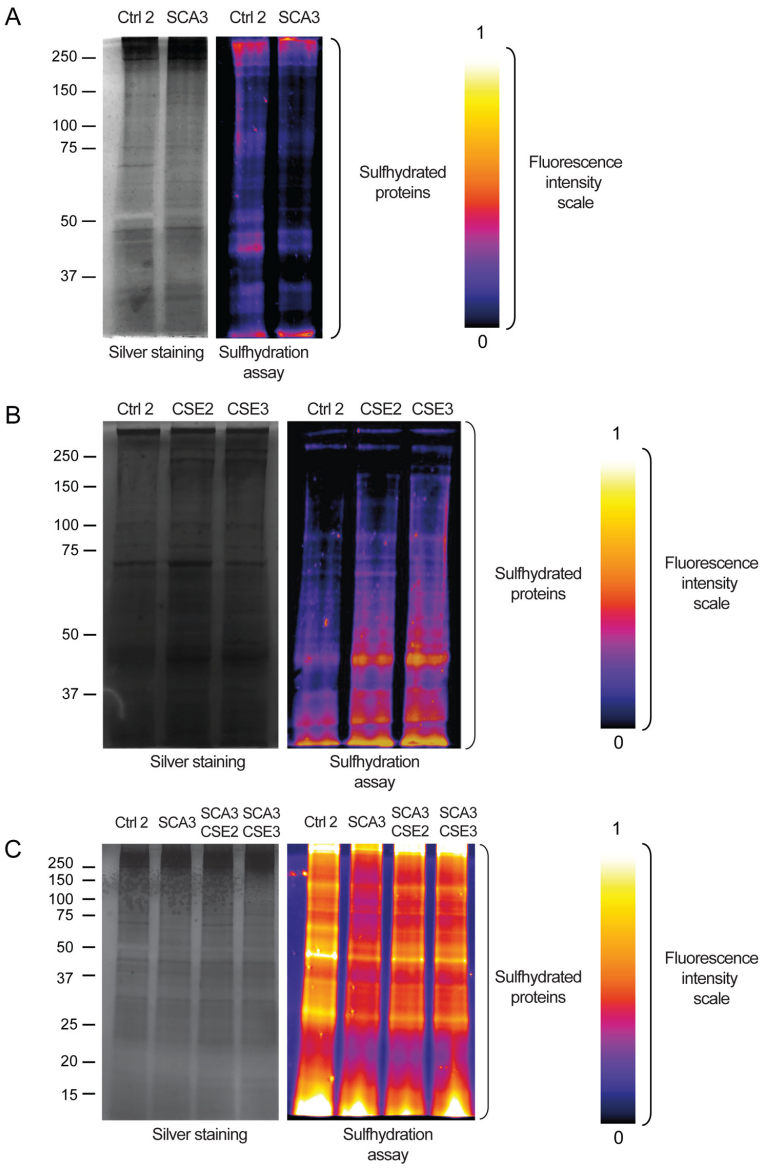

Figure 6. Protein persulfidation is decreased in SCA3 flies and restored by overexpression of *CSE*. (A) Persulfidation levels in the SCA3 fly heads are decreased compared with control flies. Protein persulfidation was determined using the tag-switch assay with direct fluorescence labeling and in-gel fluorescence detection. The gels were artificially colorized in ImageJ for better visualization of the changes in the signal intensity. Fluorescence intensity scale is provided at the right. Silver-stained gels were shown to demonstrate equal protein loading of the samples. Note that because of the sensitivity of the methods, not all bands visualized by silver staining will be detected by the fluorescence detection of protein persulfidation and *vice versa*. Fluorescence intensity scale is provided; signal in the white-yellow range of colors indicates relatively high levels of protein persulfidation; signal in the black-blue range indicates relatively low levels of protein persulfidation. Extracts of control flies and SCA3 flies were loaded. (B) *CSE* overexpression in wild-type flies elevates levels of persulfidation. Protein persulfidation was determined using the tag-switch assay with direct fluorescence labeling and in-gel fluorescence detection. Extracts of flies overexpressing *CSE* showed an increase of protein persulfidation compared with the control flies. (C) Protein persulfidation levels in the SCA3 fly heads are decreased compared with control flies, and *CSE* overexpression in the SCA3 background resulted in the partial restoration of persulfidation levels.

opment of neurodegeneration and movements disorders (20).

The findings in *Drosophila* may be of clinical relevance because we observed that, in SCA3 patients, CSE expression is decreased in affected brain areas compared with controls. Recently, decreased levels of CSE were also demonstrated in striatal brain samples from patients with Huntington disease (22). $CSE^{-/-}$ mice showed impaired locomotor functions (22); therefore, it is possible that low levels of CSE negatively influence the progression of neurodegenerative phenotypes in Huntington disease and SCA3. This result is consistent with our findings showing that, in contrast to decreasing CSE levels, boosting CSE expression in a neurodegenerative background is beneficial. In contrast to our results, CSE protein levels were found unaltered in the cerebellum and cerebral cortex of one spinocerebellar ataxia patient (SCA subtype unknown) (22), suggesting that alteration of CSE levels in SCA patients may be confined to pontine tissue or may depend on the SCA subtype.

CSE is an essential enzyme in the transsulfuration pathway and plays a role in the endogenous production of cysteine (57) and H_2S (58–60). Therefore, the beneficial effects of CSE can be mediated via cysteine, H_2S or both. It is also possible that the increased expression of CSE catalyzes the formation of cysteine persulfides that can trans-persulfidate the proteins without any H_2S being produced (61). The explanation of the beneficial effects by protein persulfidation is in agreement with our observations that this posttranslational modification is increased in *CSE*-overexpressing flies and restored in *CSE*-overexpressing flies in a SCA3 background. Moreover, it also explains the rescue effect of STS, assuming that the proposed effect of STS on protein persulfidation is correct (31). A protective effect of protein persulfidation has been demonstrated in other studies as well. For example, the activity of neuroprotective ubiquitin ligase parkin is regulated by protein persulfidation. Parkin persulfidation is markedly depleted in the brains

of patients with Parkinson's disease (24). Another study demonstrates that the capacity of H_2S to protect against oxidative stress is executed via persulfidation (25,26). These findings together with our results suggest that boosting the transsulfuration pathway may contribute to neuroprotection via increased persulfidation of proteins.

Our results show that overexpression of *CSE* is associated with a dampening of the immune response and decreased levels of protein oxidation. This finding is consistent with previous findings because inflammation has been implicated as a critical mechanism responsible for the progressive nature of neurodegeneration (44,62), and there is an inverse link between an activated transsulfuration pathway and the immune response. In experimental models, H_2S exerts anti-neuroinflammatory effects via inhibition of p38/Jun nuclear kinase and NF-κB signaling pathways (63), and the inhibition of CSE by PPG leads to increased inflammation (64). Furthermore, CSE has been shown to be a modulator of oxidative stress in mice (42). SCA3 is associated with oxidative stress because mutant *ATXN3* is associated with a significantly reduced capability to counteract oxidative stress that contributes to neuronal cell death in SCA3 (65).

On the basis of the discussed results of others and our observations, we propose the following hypothetical model: PolyQ diseases lead to accumulation of toxic protein aggregates, and this somehow reduces levels of CSE and/or protein persulfidation. This result, in turn, contributes to increased oxidative stress and an augmented immune response leading to accelerated neurodegeneration. It is possible that overexpression of CSE induces protein persulfidation (via or independent from induced H_2S and/or cysteine biosynthesis). Increased levels of protein persulfidation reduces the levels of oxidative stress and dampens the immune response, and, by this, the damaging effects of toxic protein aggregates are reduced and tissue integrity is better preserved.

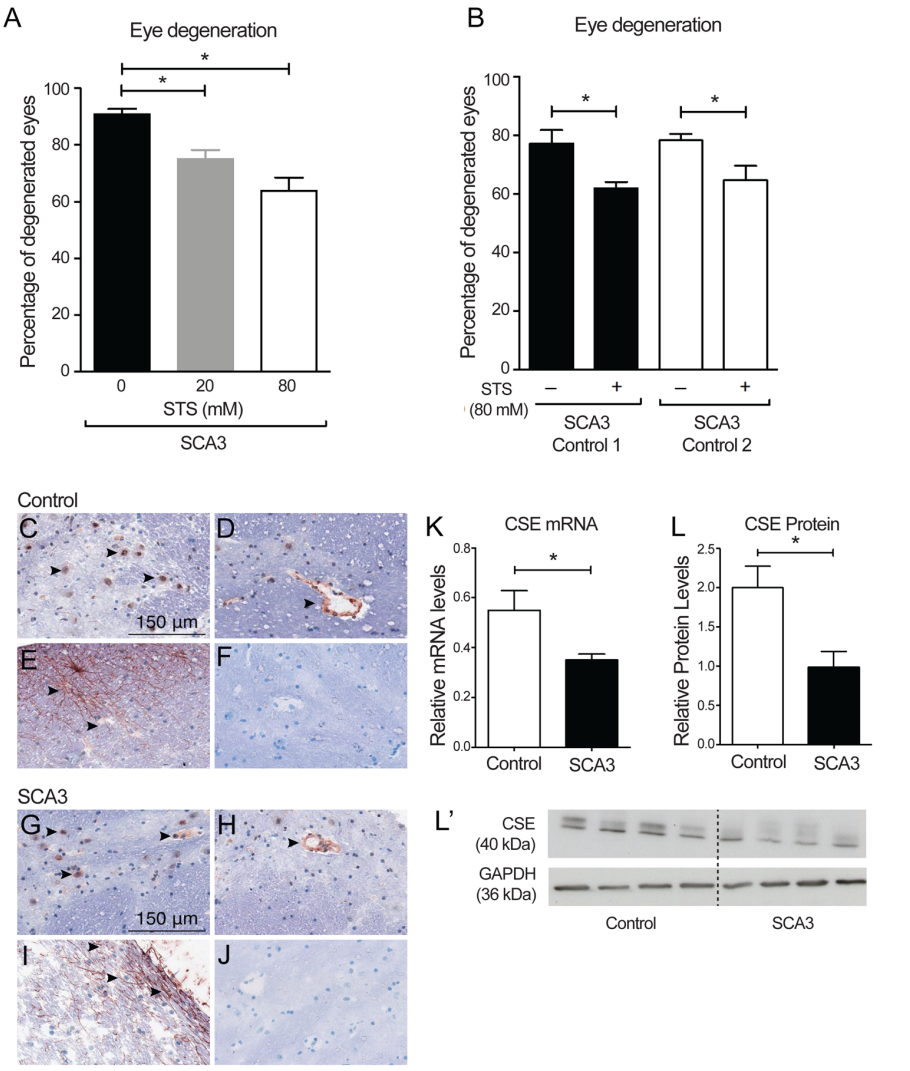

Figure 7. Treatment with STS suppresses SCA3-associated degeneration in *Drosophila*, and CSE levels are decreased in brains of SCA3 patients. (A, B) Effect of the H_2S donor sodium thiosulfate was determined on the degenerative eye phenotype by using light microscopy. The number of rough and degenerated eyes was counted in three independent experiments (n = 100–300 per experiment). (A) Increasing concentrations of STS resulted in a reduced percentage of degenerated rough eyes of *SCA3*-expressing flies in the control1 background. (B) Addition of 80 mmol/L STS to the food of *SCA3*-expressing lines in two genetic backgrounds partly rescued the SCA3-induced eye degenerative phenotype. *$p < 0.05$, error bars indicate SEM. (C–J) Immunohistochemistry by using an anti-human CSE antibody revealed that in control human pontine tissue (C–F) and in pontine tissue of SCA3 patients (sample 5, Supplementary Table S1) (representative images are shown in G–J), CSE is localized in neurons of the pontine nuclei (C, G), the vasculature (D, H) and astrocytes (E, I). Black arrows indicate the mentioned structures. No differences in staining pattern were observed between control and SCA3 brain tissue. Omission of the primary antibody resulted in absence of staining; representative images are shown (F, J). Scale bar indicates 150 µm in all images. (K) CSE mRNA levels were determined by using qRT-PCR (control, n = 7; SCA3, n = 6). (L,L') CSE protein levels were determined using Western blot analysis. Control samples correspond with control: 2, 3, 4, 6, respectively (Supplementary Table S1); SCA3 samples correspond with SCA3: 2, 3, 4, 5, respectively (Supplementary Table S1). *$p < 0.05$, error bars indicate SEM.

Our data show that CSE levels are decreased in tissue of SCA3 patients. However, in our opinion, it is more important that CSE is still expressed in affected tissue and apparently in identical cell types, because this result allows a strategy to increase CSE expression or activity by pharmacological inventions to protect against tissue degeneration in SCA3. Little is known about the regulation of CSE, but there are substances available that are able to influence CSE activity or transcription. There is evidence that myeloid zinc finger 1 and specificity protein 1 transcription factor affect the transcription of *CSE* (66). Furthermore, studies suggest that CSE can be upregulated by bacterial endotoxin (66,67) and by nitric oxide (68). S-adenosylmethionine and pyridoxal-5′-phosphate stimulate CSE activity to increase H_2S production (69,70). Alternatively to increasing *CSE* expression as a therapeutic option, rescue may be provoked by STS because our data show a protective effect of this compound as well, and it is tolerated by humans in high concentrations (71,72).

CONCLUSION

Our data indicate a modifying role of the transsulfuration pathway in SCA3 and suggest that this is mediated via protein persulfidation. The presence of CSE in SCA3-relevant brain regions, together with the protective effects of *CSE* overexpression in *Drosophila*, indicates the relevance for future research on developing clinically applicable activators of CSE or other members of the transsulfuration pathway. As the protective effects occur downstream of the formation of protein aggregates, it may be possible that activation of the transsulfuration pathway is protective for other polyglutamine expansion–induced diseases as well.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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