Molecular Medicine

Supplemental Data

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

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SUPPLEMENTARY MATERIALS AND METHODS

Drosophila Stocks

For overexpression of the genes, the UAS-GAL4 binary system for targeted gene expression was used (1). Eip55E (*Drosophila* CSE) cDNA was cloned into the pUAST vector and verified by sequencing. Transgenic CSE fly lines were generated by Genetic Services Inc. (Sudbury, USA) in a w^{1118} (y^1w^{1118}) background by random insertion into the genome. Fly stocks bearing the CSE transgene were backcrossed into control lines for six generations. CSE-1A was isogenized with the w^{1118}-*A* line; and CSE-2B and CSE-3B were isogenized with the w^{1118}-*B* line. Transgenes were selectively overexpressed in the eyes by using GMR-GAL4 driver flies (stock #1104), ubiquitously overexpressed using the daughterless driver flies (stock #8641) for the qPCR analysis or overexpressed using the actin driver (stock #4414) for the S-sulfhydration measurements. To test whether a titration effect existed, a UAS-GFP (stock #6658) and two UAS-YFP (stock #6659; #6660) lines were used. The driver stocks and stocks bearing fluorescent proteins under the control of UAS were ordered from the Bloomington Drosophila stock center (Indiana University, USA). All crosses were performed at 25°C according to standard protocols. Male flies were used for all experiments. See table at the top page S2.

Fly Food and Supplementation of PPG and STS

Fly strains were raised and crossed on Nutri-Fly Bloomington food (Brewer's Yeast, Sucrose, Agar Type II, Glucose, Yeast Extract, $MgSO_4$ x $6H_2O$, Peptone, $CaCl_2$ x $2H_2O$; Genesee Scientific). For inhibition of CSE, fly crosses were set up on propargylglycine (PPG)- supplemented food. PPG (Sigma, Zwijndrecht, the Netherlands) was added to freshly prepared fly food to a final concentration of 2 mM as previously described (2). In the same manner, STS (Sigma, Zwijndrecht, the Netherlands) was administered to the fly food reaching final concentrations of 20 mM, 80 mM and 120 mM. Every two days, the relevant concentration of STS dissolved in distilled water was added to the vials during development of the flies.

Light and Electron Microscopy

To enable correlative analysis, for each condition, the same fly head was imaged with both light microscopy (LM) and scanning electron microscopy (SEM). One-day-old flies were decapitated, heads were dehydrated through an ethanol series, and after acetone as an intermediate step, air dried with tetramethylsilane (Sigma-Aldrich). Light microscopy images of fly eyes were taken with a Leica M165 FC stereo microscope followed by focus stacking using Adobe Photoshop. For the scanning electron microscopy, the same eyes were gold/paladium-coated (3 nm) and analyzed with a Zeiss Supra 55 SEM at 2KV using a SE2 detector.

Quantitative RT-PCR

Human pontine tissue was homogenized in lysis buffer and total RNA was extracted using the RNeasy Mini Kit (Qiagen). To verify CSE overexpression in transgenic *Drosophila* lines under control of the daughterless driver, total RNA was isolated from 10-15 one-day old flies using the RNeasy Mini Kit (Qiagen). For each genotype/treatment, at least three independent extractions were prepared. cDNA was synthesized using Superscript II with random hexamer primers (Invitrogen, Carlsbad, USA). Gene expression was determined by quantitative real time-PCR (qPCR) using a SYBR green mastermix (iQ SYBR GREEN Supermix; Bio-Rad). Rp49 and TBP were used as normalization reference house-keeping genes for *Drosophila* and human pontine tissue, respectively. The PCR profile consisted of 15 min at 95°C, followed by 40 cycles with heating of 95°C for 15 s and cooling to 60°C for 1 min. To detect mRNA levels of immune response genes,

Genotypes of fly lines used per experiment.

Experiment	Genotype
qRT-PCR analysis of CSE-overexpressing lines (Suppl. Figure S1; Figure 3)	y1, w1118-1; daughterless-GAL4/+ y1, w1118-1; daughterless-GAL4/+; UAS-CSE-1/+ y1, w1118-2; daughterless-GAL4/+ y1, w1118-2; daughterless-GAL4/+; UAS-CSE-2/+ y1, w1118-2; daughterless-GAL4/UAS-CSE-3
Co-overexpression of CSE and SCA3 in *Drosophila* eyes (Figures 2, 3); qRT-PCR analysis of antimicrobial peptide mRNA abundance (Figure 5, Suppl. Figure S3); Oxidative stress assay (Figure 4); Western Blot analysis of SCA3trQ78 aggregation (Figure 4, Suppl. Figure S2); Protein persulfidation analysis (Figure 6, Suppl. Figure S4)	y1, w1118-2 y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/+ y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/+; UAS-CSE-1/+ y1, w1118-2 y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/+ y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/+; UAS-CSE-2/+ y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/UAS-CSE-3
Effect of thiosulfate supplementation on the eye phenotype of SCA3 flies (Figure 7)	y1, w1118-1; GMR-GAL4-UAS-SCA3trQ78/+ y1, w1118-2; GMR-GAL4-UAS-SCA3trQ78/+
Protein persulfidation analysis (Figure 6, Suppl. Figure S4)	y1, w1118-1; actin-GAL4/+ y1, w1118-1; actin-GAL4/+; UAS-CSE-1/+ y1, w1118-2; actin-GAL4/+ y1, w1118-2; actin-GAL4/+; UAS-CSE-2/+ y1, w1118-2; actin-GAL4/UAS-CSE-3

the same procedure was used with the exception that ten-day-old flies co-overexpressing CSE and SCA3 under control of the GMR driver were used. All samples were normalized to their housekeeping gene, the average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the delta Ct. Results were expressed as $2^{-\Delta Ct.}$ The following primers were used: see table at the bottom of this page.

Western Blot Analysis

For the SCA3tr78 insoluble/soluble fraction analysis, one-day-old flies were directly frozen in liquid nitrogen and decapitated. Twenty-five heads per condition were homogenized in Laemmli Sample Buffer (62,5 mM Tris/HCL pH 6,8; 2% SDS; 10% glycerol; bromophenol blue) containing 2% beta-mercaptoethanol. Ten μL of each sample was loaded onto 12.5% SDS-polyacrylamide gels. Proteins were transferred from the gels onto nitrocellulose membranes and immunostained. Aspecific binding of the antibodies was prevented using 5% milk in Tris-buffered saline-0.1% Tween-20 (TBST). Membranes were incubated with the primary antibody solution in 5% milk in TBST overnight at 4°C. As a primary antibody, rat monoclonal high affinity anti-HA-peroxidase (1:500) (clone 3F10; Roche, Indianapolis, USA) was used to detect SCA3tr-78, and mouse anti-Tubulin (Sigma, Zwijndrecht, the Netherlands) was used for Tubulin detection. Afterwards, membranes were incubated with the secondary antibody in TBST. Goat anti-rat IgG horseradish peroxidase (1:5000) (GE Healthcare UK Limited) was used as the secondary antibody for SCA3tr-78 detection. As the secondary antibody for Tubulin detection, sheep anti-mouse IgG horseradish peroxidase (1:4000) (GE Healthcare UK Limited) was used. The blot was scanned with an imaging densitometer, and optical densities

Gene	Primer
Drosophila CSE (Eip55E)	For - TGAACGGTCATACGGATGTG Rev - ACCTGATAGCAGTCGAATGG
Drosophila rp49	For - GCACCAAGCACTTCATCC Rev - CGATCTCGCCGCAGTAAA
Drosophila IM1	For - TGCCCAGTGCACTCAGTATC Rev - GATCACATTTCCTGGATCGG
Drosophila IM2	For - AAATACTGCAATGTGCACGG Rev - ATGGTGCTTTGGATTTGAGG
Drosophila Drosomycin	For - GTACTTGTTCGCCCTCTTCG Rev - GATTTAGCATCCTTCGCACC
Drosophila Diptericin	For - ACCGCAGTACCCACTCAATC Rev - ACTTTCCAGCTCGGTTCTGA
Drosophila Attacin	For - GCTTCGCAAAATAAACTGG Rev - TCCCGTGAGATCCAAGGTAG
Drosophila Cecropin	For - GAACTTCTACAACATCTTCGT Rev - TCCCAGTCCCTGGATTGT
Human CSE	Assay-on-Demand Applied Biosystems ID: Hs00542284_m1
Human TBP	For - GCCCGAAACGCCGAATAT Rev - CCGTGGTTCGTGGCTCTCT

were quantified. To calculate the ratio between the SCA3tr-78 insoluble and soluble fraction, the total intensity of the signal in the stacking gel normalized to Tubulin was divided by the intensity of SCA3tr-78 monomer band normalized to Tubulin. Western blots from five independent experiments with unique sets of samples were used for quantification using ImageJ.

Protein Oxidation Detection

Ten-day-old flies were directly frozen in liquid nitrogen and decapitated. Per condition, 15 heads were homogenized in RIPA buffer containing 2% beta-mercaptoethanol. Protein oxidation was assessed with an OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, USA) according to manufacturer's instructions. In this method, carbonyl groups of the proteins are derivatized using 2,4-dinitrophenylhydrazine (DNPH) reagent, the sample is then processed on the SDS-PAGE in a similar fashion as in the Western blot analysis and, as a result, the total levels of oxidized proteins in the sample are detected (3). As a loading control, blots were immunostained with a rabbit anti-Tubulin (Sigma, Zwijndrecht, the Netherlands) antibody. The relative levels of oxidized proteins were measured by comparing total chemiluminescence of the sample lanes normalized to Tubulin levels using ImageJ.

Collection of Human Pontine Tissue

Brains from 7 genetically confirmed SCA3 patients and 7 controls without medical histories of neuropsychiatric diseases were analyzed (Supplementary Table S1). Immediately after brain autopsy samples (15x15x5mm) of the base of the pons were snap frozen. Informed consent was obtained from all SCA3 patients. Control cases were anonymized and coded according to the National Code for Good Use of Patient Material. All procedures were approved of and in accordance with the Medical Ethical Committee of the University Medical Center Groningen.

Supplementary Table 1. Characteristics of the SCA3 and control patients.

Patient	Age	Gender	Cause of death	CAG repeats
Control 1	79	female	metastasized lung carcinoma	-
Control 2	53	male	metastasized signet-ring carcinoma	-
Control 3	16	female	ALL + sepsis	-
Control 4	17	male	lung transplantation + alveolar hemorrhage	-
Control 5	83	male	contusio cerebri	-
Control 6	27	male	car accident	-
Control 7	58	male	bronchopneumonia + lung emboli	-
SCA3 1	70	female	-	68
SCA3 2	63	male	-	70
SCA3 3	38	male	-	81
SCA3 4	71	male	-	70
SCA3 5	88	female	-	65
SCA3 6	34	female	-	77

Immunohistochemistry for CSE

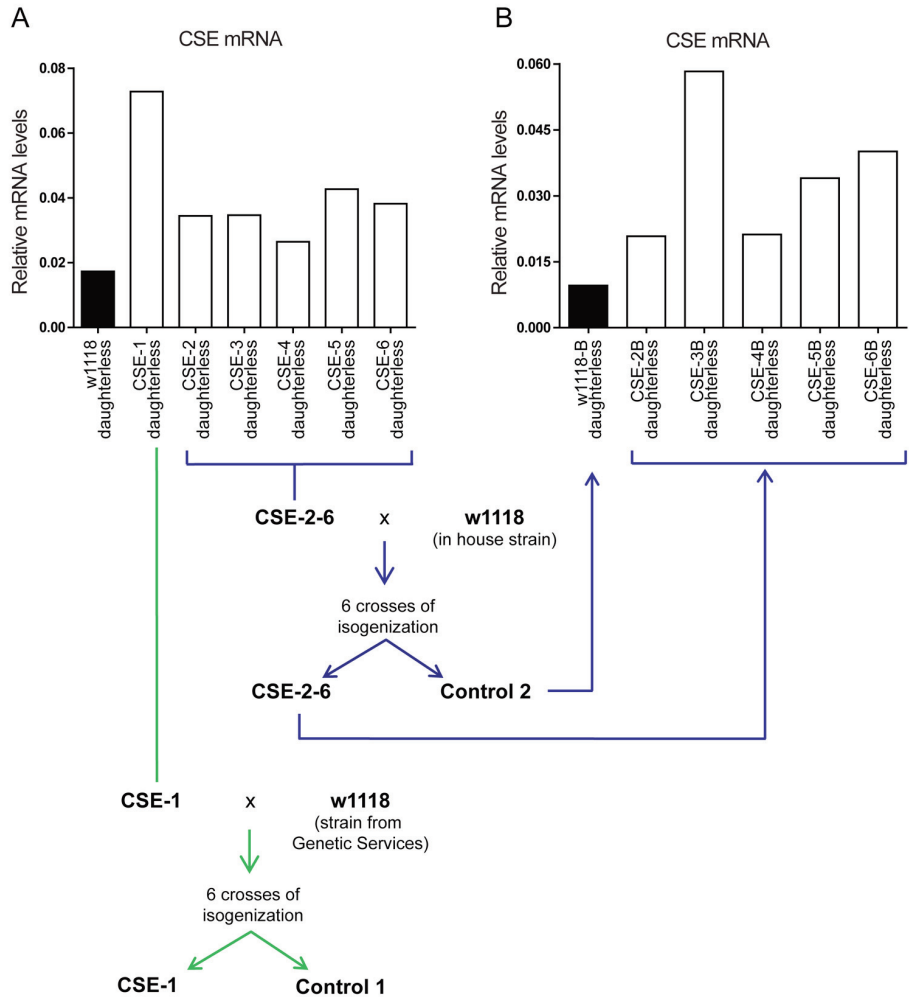
For immunostaining, frozen human pontine sections were dried and fixed in acetone. Subsequently, sections were incubated at room temperature with the primary antibody (Proteintech rabbit polyclonal CSE antibody 11217-1-AP (1:500)) for 60 min. Endogenous peroxidase was blocked with H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 30 min. Binding was detected using sequential incubation with a peroxidase-labeled secondary antibody (Dakopatts, Glostrup, Denmark) for 30 min. All antibodies were diluted with PBS supplemented with 1% BSA. At the secondary antibody dilution, 1% human AB serum was added. Peroxidase activity was developed using filtered 3-amino-9-ethylcarbazole for 15 min containing H_2O_2. Counterstaining was performed using Mayer's hematoxylin. Appropriate isotype and PBS controls were consistently negative.

Statistical Analysis

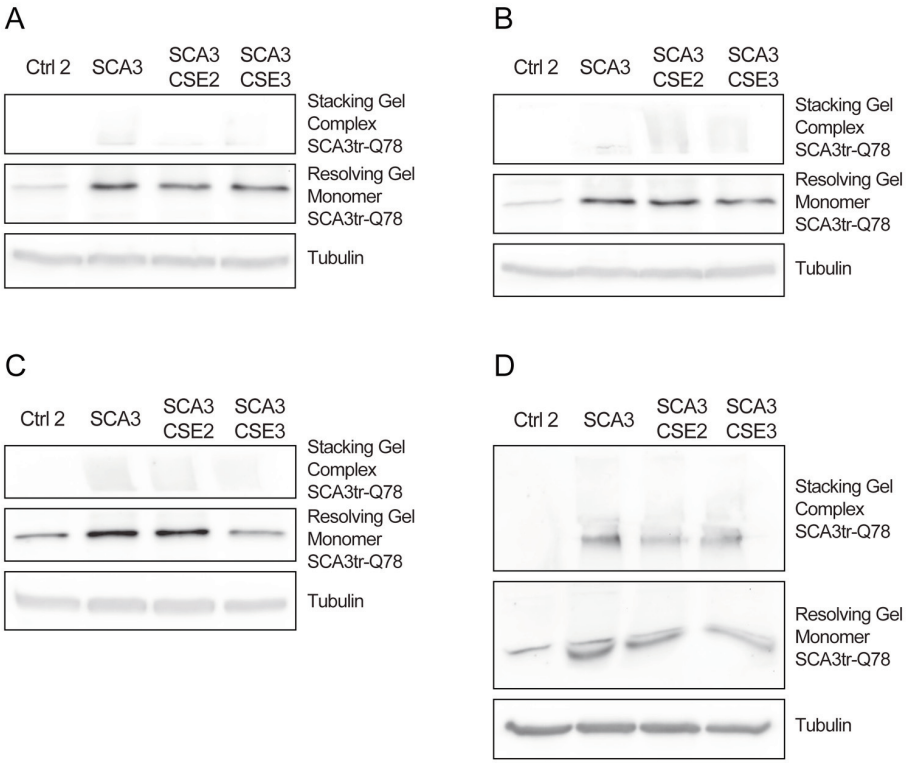
Data were analyzed using GraphPad Prism 5.0 and IBM SPSS 20.0 software. Normality was tested using the Kolmogorov-Smirnov test. The unpaired Student *t*-test was used for comparisons between 2 groups with normal distribution. Non-parametric data were compared using the Mann-Whitney U-test. For comparisons between three groups, an ANOVA with Bonferroni post-test was used for parametric data and a Kruskal Wallis with Dunnett's post-test was used for non-parametric data. For the analysis of the number of degenerated eyes, a logistic regression was used. The number of replications of each experiment was at least three. Statistical significance was accepted at $p<0.05$. All data are expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise.

SUPPLEMENTARY REFERENCES

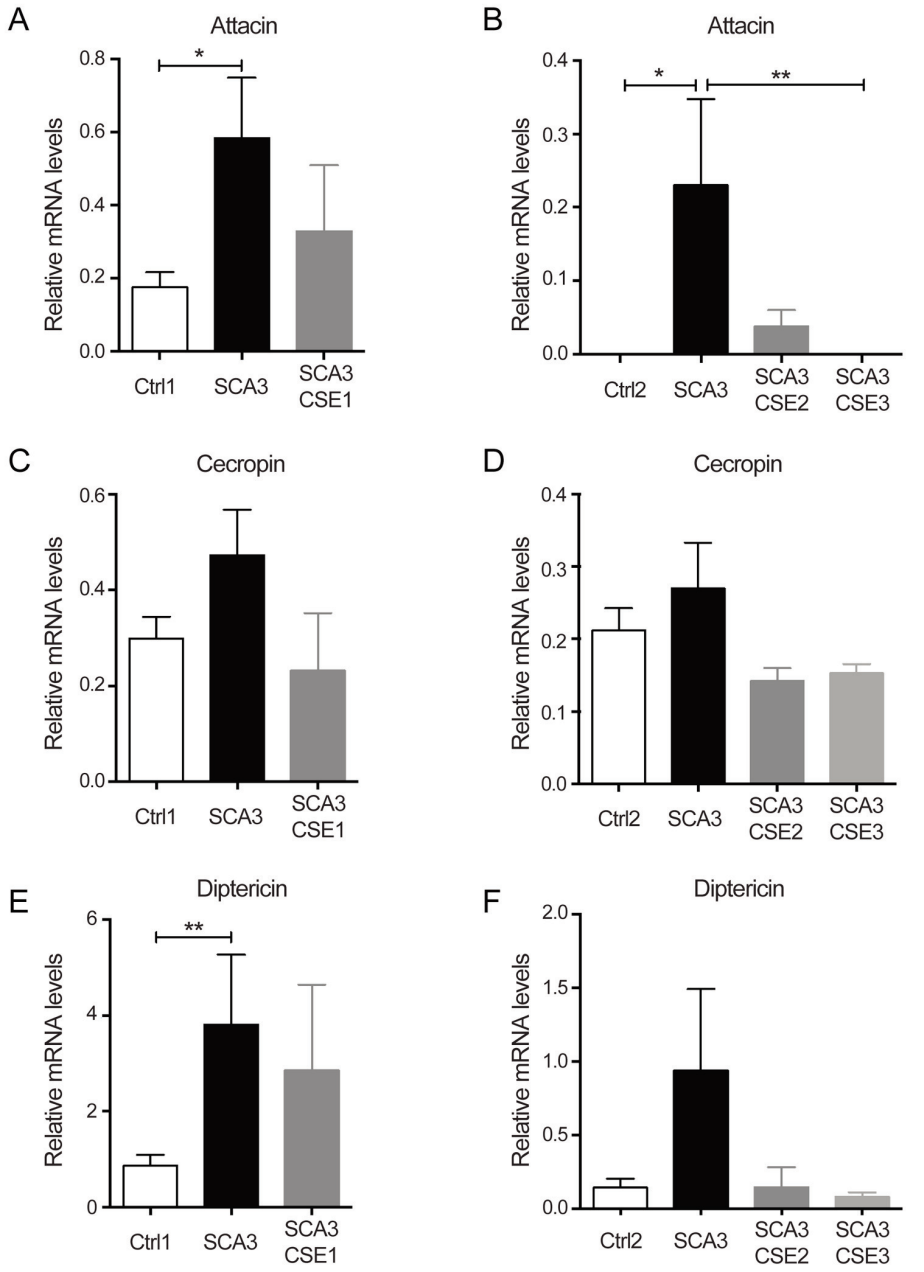
1. Brand AH, Perrimon N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118:401–15.
2. Kabil H, Kabil O, Banerjee R, Harshman LG, Pletcher SD. (2011) Increased transsulfuration mediates longevity and dietary restriction in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* 108:16831–6.
3. Rana A, *et al*. (2010) Pantethine rescues a Drosophila model for pantothenate kinase-associated neurodegeneration. *Proc. Natl. Acad. Sci. U. S. A*. 107:6988–93.



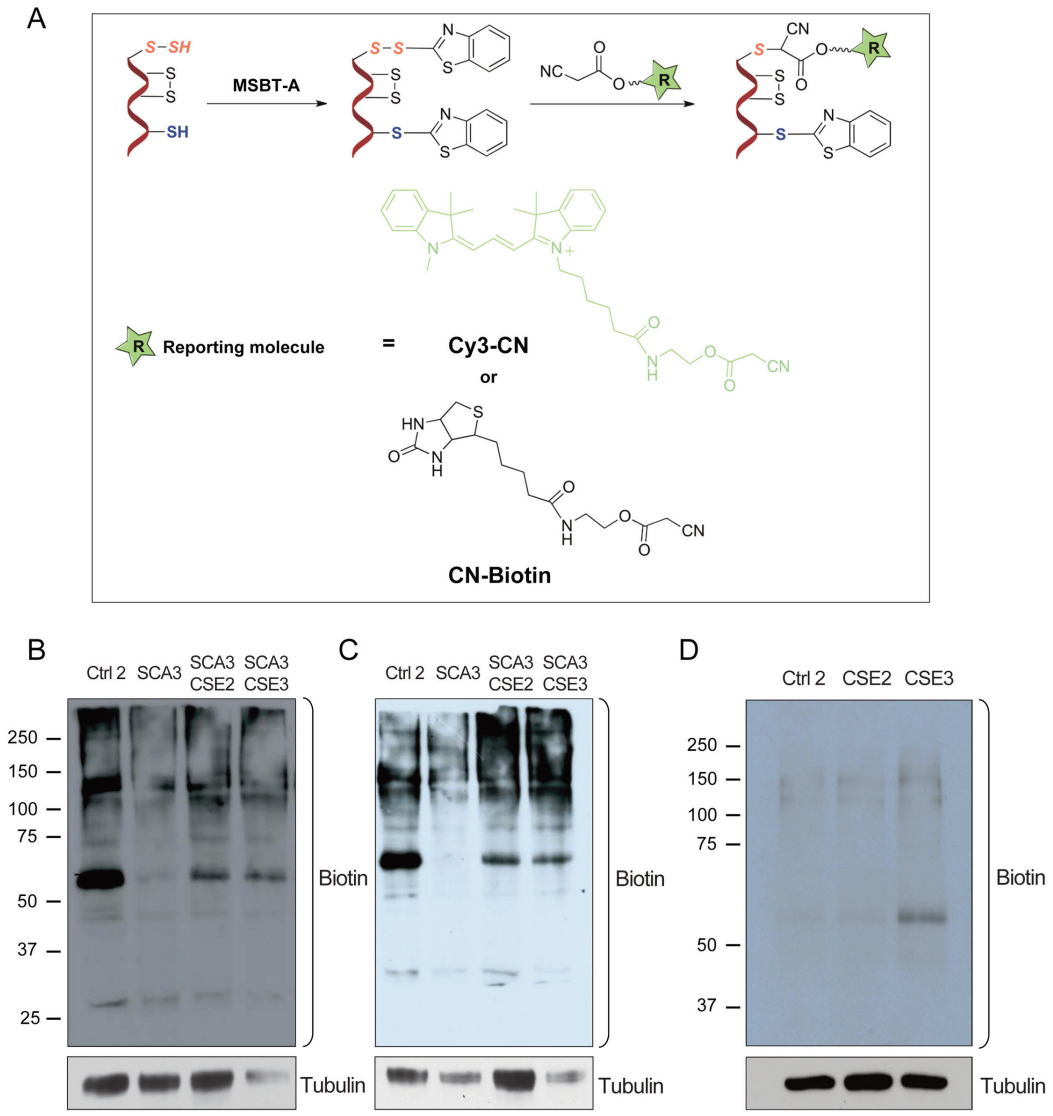
Supplementary Figure S1. CSE mRNA levels of different CSE overexpression lines and schematic representation of backcrossing of relevant CSE lines. (A) Relative CSE mRNA levels in fly lines received from Genetic Services and (B) in lines after backcrossing for 6 generations into the in-house w^{1118} strain expressed under control of the daughterless driver. Below: scheme of crosses to isogenize fly lines with 2 different w^{1118} control strains. Both experiments were used as an indication to select fly lines for further experiments and, therefore, were performed once. Genetic crosses to generate isogenic lines: As an isogenic control, one strain (strain CSE-1, was backcrossed with the w^{1118} control strain that was used to generate the transgenic lines. The backcrossed CSE strain 1–overexpressing line is further referred to as CSE1, and its isogenic non-CSE-expressing control line is further referred to as Control 1. Other lines (CSE-2 to CSE-6) were backcrossed with the in-house w^{1118} strain to generate an isogenic control line. From these, two lines were selected: one line overexpressing CSE to a lower level (further referred to as CSE2) and one line overexpressing CSE to a higher level (further referred to as CSE3). The isogenic control of these lines is further referred to as Control 2.



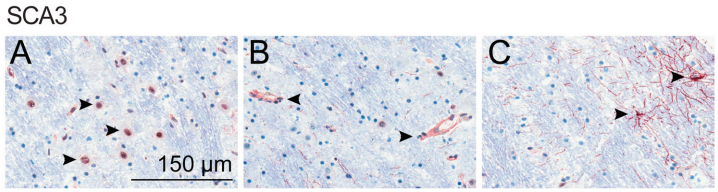
Supplementary Figure S2. Overexpression of CSE in a SCA3 background SCA is not associated with a decrease in insoluble/soluble fraction ratio of SCA3tr-78 protein. (A-D) Set of Western blots used for analysis of fly heads and used for quantification of Figure 4B of the insoluble/soluble fraction ratio. The samples were analyzed for the amount of SCA3tr78 protein and its aggregation using an anti-HA antibody. Alpha-tubulin was used as a loading control. In SCA3 flies, both soluble monomer (in the resolving gel) and aggregated protein (in the stacking gel) fractions of SCA3tr78 protein were detected. Neither the expression levels, nor the insoluble/soluble ratio of the mutant protein were majorly modified by overexpression of CSE.



Supplementary Figure S3. Inflammation genes that are variously affected by CSE overexpression in a SCA3 background. Expression of inflammation genes from the Relish pathways upon the overexpression of SCA3 alone or in combination with CSE. (A and B) Attacin is induced by SCA3 but not differently expressed in the CSE1 line. (B) Attacin mRNA levels are reduced in the CSE3-expressing flies compared to SCA3-expressing flies. (C and D) Cecropin is not significantly influenced by CSE overexpression. (E) Diptericin is significantly induced in the Control 1 background but not in the (F) Control 2 background. (E and F) In all CSE overexpression lines, mRNA levels of Diptericin are not different from the isogenic control lines.



Supplementary Figure S4. Overexpression of CSE increases protein persulfidation in the wild type background and partly rescues decreased protein persulfidation of SCA3 flies. (A, B) Sulfhydration levels in the SCA3 fly heads are decreased compared to control flies. Protein sulfhydration was determined using the biotin-labeling assay. Tubulin staining was shown to demonstrate equal protein loading of the samples. Extracts of control flies and SCA3 flies were loaded. Two blots represent two biological replications. (C) CSE overexpression in wild type flies elevates levels of sulfhydration, which is higher in CSE3 than in CSE2-overexpressing line corresponding with the mRNA levels of CSE overexpression. Protein sulfhydration in (A-C) was determined using the biotin-labeling assay. Tubulin staining was shown to demonstrate equal protein loading of the samples.



Supplementary Figure S5. Localization pattern of CSE is not affected in pontine tissue of SCA3 patients. Immunohistochemistry using an anti-human CSE antibody revealed that in pontine tissue of an additional SCA3 patients (Sample 4, Supplementary Table S1) (representative images are shown in A-C), CSE is localized in (A) neurons of the pontine nuclei, (B) the vasculature and (C) astrocytes. Black arrows indicate the mentioned structures. Scale bar, 150 µm.