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Original Contribution

Protective effects of the thioredoxin and glutaredoxin systems in dopamine-induced cell death

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ABSTRACT

Although the etiology of sporadic Parkinson disease (PD) is unknown, it is well established that oxidative stress plays an important role in the pathogenic mechanism. The thioredoxin (Trx) and glutaredoxin (Grx) systems are two central systems upholding the sulfhydryl homeostasis by reducing disulfides and mixed disulfides within the cell and thereby protecting against oxidative stress. By examining the expression of redox proteins in human postmortem PD brains, we found the levels of Trx1 and thioredoxin reductase 1 (TrxR1) to be significantly decreased. The human neuroblastoma cell line SH-SY5Y and the nematode *Caenorhabditis elegans* were used as model systems to explore the potential protective effects of the redox proteins against 6-hydroxydopamine (6-OHDA)-induced cytotoxicity. 6-OHDA is highly prone to oxidation, resulting in the formation of the quinone of 6-OHDA, a highly reactive species and powerful neurotoxin. Treatment of human cells with 6-OHDA resulted in an increased expression of Trx1, TrxR1, Grx1, and Grx2, and small interfering RNA for these genes significantly increased the cytotoxic effects exerted by the 6-OHDA neurotoxin. Evaluation of the dopaminergic neurons in *C. elegans* revealed that nematodes lacking *trxr-1* were significantly more sensitive to 6-OHDA, with significantly increased neuronal degradation. Importantly, both the Trx and the Grx systems were also found to directly mediate reduction of the 6-OHDA-quinone in vitro and thus render its cytotoxic effects. In conclusion, our results suggest that the two redox systems are important for neuronal survival in dopamine-induced cell death.

Parkinson disease (PD) is the second most common neurodegenerative disease, with a prevalence of 1–2% in the population over 50. It is clinically characterized by motor problems such as tremor, poor balance, slow voluntary movements, and rigidity. PD is a progressive disease caused by the loss of dopamine-producing neurons and their axons mainly in the substantia nigra of the human brain. It is characterized morphologically by the presence of Lewy bodies, which are aggregates of misfolded α-synuclein in the cytoplasm of dopaminergic neurons ([1,2] and references therein). The pathogenesis of PD is still unknown, but among other causes mitochondrial dysfunction with malfunction of complex I has been implicated, leading to an increased leakage of electrons from the mitochondria, generating increased reactive oxygen species (ROS) and, consequentially, oxidative stress within the cell. In PD, the dopaminergic neurons of the substantia nigra are degenerating to a greater extent than other neurons, most likely owing to dopamine itself. Dopamine is normally stored at millimolar concentrations in vesicles; however, this storage is disrupted in the presence of α-synuclein and oxidative stress, leading to an increased concentration of dopamine in the cytoplasm [3]. In the presence of oxygen, dopamine will oxidize and form 6-hydroxydopamine (6-OHDA) [4], which in turn will efficiently autoxidate and generate the highly neurotoxic compound 6-OHDA-quinone (Fig. 1). This quinone is electron deficient, and if not eliminated by cellular antioxidants such as glutathione (GSH), it reacts readily with cellular nucleophiles such as sulfhydryl groups on proteins, thereby affecting several biological systems in the cell [5]. In addition, during the formation of 6-OHDA-quinone ROS are generated in the form of H_2O_2 [6], and in turn this reactive species causes oxidative stress and damage within the cell that eventually can lead to neuronal death [4].

To sustain the thiol redox homeostasis cells have evolved two separate pathways, the thioredoxin (Trx) and the glutaredoxin (Grx) systems. The main function of both these systems is to reduce disulfide bonds and thereby rescue cells against injuries

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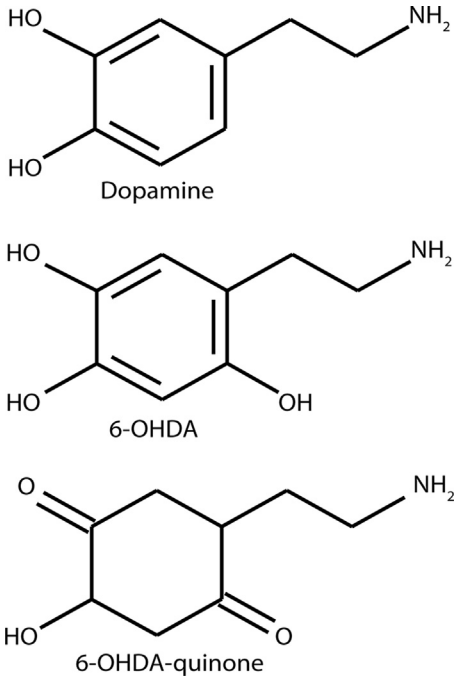

Fig. 1. Chemical structure of dopamine, 6-OHDA, and 6-OHDA-quinone.

sustained in an oxidative environment [7]. The thioredoxin system comprises Trx together with the selenoenzyme thioredoxin reductase (TrxR). It has both cytosolic (Trx1 and TrxR1) and mitochondrial (Trx2 and TrxR2) isoforms [8]. The glutaredoxin system consists of glutathione reductase (GR) and GSH, which is required for the reduction of Grx. There are four human Grx's: cytosolic Grx1, Grx2c, Grx3, and Grx5; mitochondrial Grx2a; and nuclear Grx2b [9]. Both systems are dependent on NADPH as electron donor. The active site of Trx and the dithiol Grx's (Grx1 and Grx2) is composed of two cysteines, separated by two amino acids (CXXC), whereas the monothiol Grx's (Grx3 and Grx5) are lacking the C-terminal cysteine and instead use GSH for their catalytic activity [9]. These systems are known to regulate many biological systems, including the synthesis of deoxyribonucleotides, nitric oxide signaling, and protein folding, as well as acting as key redox regulators of kinases and transcription factors such as ASK-1 and p53 [8,10].

In this study we aimed to investigate the role of redox proteins in the defense against 6-hydroxydopamine-induced cell death. We postulate that Trx and Grx are involved in dopamine-induced neurodegeneration and that increased levels of these proteins would protect the cell against the reactive dopamine metabolite 6-OHDA-quinone.

Materials and methods

Clinical material

Paraffin-embedded tissue samples from substantia nigra from patients with PD ($n = 11$) and age-matched controls ($n = 10$) were randomly selected from the archives of the Laboratory for Clinical Pathology, Karolinska University Hospital, Sweden, after approval of the Regional Ethics Review Board in Stockholm (Dnr: 488/02).

Immunohistochemical staining for redox proteins in substantia nigra

The immunohistochemical (IHC) staining was performed using the following antibodies and corresponding dilutions against the respective human proteins: Trx1 (IMCOcorp, 1:36,000), Trx2 (Proteintech Europe, TXN 13089-1-AP, 1:25), TrxR1 (Upstate, 07-613, 1:50), Grx1 (IMCOcorp, 1:1000), and Grx2 (Agrisera (CYLKKSKRKEFQ), 1:50). IHC was performed by the accredited laboratory facility at the Division of Pathology, Karolinska University Hospital, Sweden. TrxR2 was also examined with several different antibodies, but all were proven to be nonspecific for IHC in the tissue examined and could not be used for further studies. Microphotographs of the various stainings were acquired using a microscope with an inbuilt camera (Nikon Eclipse E1000) at 4 × magnification. To evaluate the IHC, the total stained area was measured, using Nikon's image acquisition tool, NIS-Elements 3.0. The expression of redox proteins was calculated as the stained area of neuronal cells in each section divided by the total number of cell nuclei in the same image.

Cell culturing

The neuroblastoma cell line SH-SY5Y was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), under normal conditions, 5% CO_2 at 37 °C. To induce differentiation, cells were treated with 10 μM all-*trans*-retinoic acid (Sigma–Aldrich) in DMEM, supplemented with 3% FBS, for 48 h. To investigate the involvement of antioxidants, the cells were pretreated with sodium selenite (Se; 0.5 μM; Sigma–Aldrich) for 24 h, followed by treatment with 6-OHDA (40 μM; Sigma–Aldrich). Viability was measured using the XTT kit (Cell Proliferation Kit II, Roche). The medium was changed before addition of the substrate, and wells with only medium were used as blanks. Values were normalized to untreated control.

Transfection

When seeded into appropriate culturing plates SH-SY5Y cells were transfected with small interfering RNA (siRNA) for Trx1 (Silencer siRNA TXN ID 117158; Ambion), TrxR1 (Silencer siRNA TXNRD ID 111302; Ambion), Grx1 (Silencer siRNA GLRX ID 117030; Ambion), Grx2 (GLRX2HSS147234; Invitrogen), and negative control siRNA (ID AM4611; Ambion) using 33 nM Lipofectamine 2000 (Invitrogen). All treatments were performed 24 h after seeding/transfection of cells.

Quantitative PCR

Cells were lysed using the QiaShredder (Qiagen), and RNA was extracted using the RNeasy Plus Mini Kit (Qiagen), according to the protocol supplied by the manufacturer. The concentration of the mRNA was determined using the NanoDrop Spectrophotometer ND-1000. The Omniscript reverse transcription kit (Qiagen) was used for the cDNA synthesis using 2 μg of RNA and oligo(dT) (Qiagen) as primer (final concentration 40 ng/μl). Real-time quantitative PCR was performed on a C1000 thermal cycler (Bio-Rad) with 30 ng cDNA per reaction in triplicates on 96-well plates using iQ SYBR Green Supermix (Bio-Rad). The final volume for each reaction was 10 μl. Primer sequences and concentrations are listed in Supplementary Table 1. The qPCR was programmed according to the following steps: initiation, 50 °C, 2 min, and 95 °C, 2 min; denaturation (40 ×), 95 °C, 15 s; elongation, 60 °C, 30 s; and melt curve (80 ×) start temperature 55 °C, with an increase of 0.5 °C per 10-s cycle. The housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as endogenous control, and results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Samples were separated by SDS–PAGE (12% Mini-Protean TGX gel; Bio-Rad) at 140 V followed by semidry electroblotting onto a

nitrocellulose membrane for 45 min at 40 mA. Membranes were probed with anti-TrxR1 (1:500), anti-Grx2 (1:3000), and anti-actin (Sigma; 1:3000) and incubated at 4 °C overnight. The membranes were blocked with 5% dried milk for 2 h at room temperature, followed by incubation with secondary antibodies for 2 h at room temperature. For Grx2 horseradish peroxidase-conjugated secondary antibody was used (Sigma–Aldrich, 1:3000), and for TrxR1 and actin infrared-labeled antibodies IRDye 800CW and IRDye 700RD, respectively, were used (Licore; 1:10,000). Bound antibodies were detected with chemiluminescence or in the infrared range using the Odyssey Fc infrared imaging system (Li-Cor) according to the manufacturer's instructions.

ELISA

ELISA was performed as previously described [11,12] for the quantification of Trx1, Trx2, and Grx1. In brief, 96-well plates were coated with primary antibody and incubated at 4 °C overnight (2.5 μg/ml anti-Trx1 (Agrisera), 5 μg/ml anti-Trx2 (Agrisera), 2 μg/ml anti-Grx1 (IMCOcorp)). The plates were blocked with 150 μl blocking buffer, and standard and samples were incubated on the plates overnight at 4 °C. The plates were incubated with secondary antibody 2 h at room temperature (5 μg/ml biotinylated Trx1 (Agrisera), 5 μg/ml biotinylated Trx2 (Agrisera), 1 μg/ml biotinylated Grx1 (IMCOcorp)). Alkaline phosphatase-conjugated streptavidin (1:1000) was incubated on the plates for 1 h before addition of phosphatase substrate. The plates were read at 405 nm in a PowerWave HT (BioTek) spectrometer.

Caenorhabditis elegans strains

C. elegans strains were maintained at 20 °C on NGM agar plates seeded with a lawn of *Escherichia coli* OP50 as food source. The worm strains used in this study were OH7193 [13], *otIs181[Pdat-1::mCherry; Pttx-3::mCherry] III; him-8(e1489) IV;* VB1363 [14], *trxr-1 (sv43) IV;* and RB1637, *trx-5 (ok2014) V*. Crossing of the *Pdat-1::mCherry* reporter strain with the deletion mutants generated the following new strains: OE4550, *otIs181[Pdat-1::mCherry; Pttx-3::mCherry] III; trxr-1(sv43) IV,* and OE4551, *otIs181[Pdat-1::mCherry; Pttx-3::mCherry] III; trx-5(ok2014) V]*. PCR conditions and primer sequences to follow up the respective deletion mutants will be provided upon request.

6-OHDA treatment of C. elegans

Worms were treated with 6-OHDA as previously described [15]. In brief, worms were synchronized by placing 50 gravid hermaphrodites on seeded agar plates for 4 h, and their progeny were allowed to grow until the L3–L4 stage at 20 °C. These worms were then washed off the plates in distilled water and washed additionally three times. A stock solution of 100 mM 6-OHDA was prepared fresh in 20 mM ascorbic acid before treatment. The stock solution was diluted to the desired concentrations in water (20 or 40 mM 6-OHDA). Worms were treated in 1 ml for 1 h, at room temperature with gentle shaking. After 1 h, the treatment was removed and the worms were washed once in water and three times in M9 buffer. Worms were plated and allowed to recover for 24 h, followed by evaluation of neuronal degeneration by microscopy on a Zeiss Axioplan II. Evaluations were performed with a Cy3/DsRed filter at 200 ms exposure time, with 20–40 × magnification.

Evaluation of neuronal degeneration in C. elegans

Approximately 40 worms were anesthetized in 3 mM levamisole on agarose pads. Thirty worms per treatment were scored for neuronal degeneration. Neuronal degeneration was evaluated according to the following criteria: unaffected, with no effect on the fluorescent neurons; blebbing, in which changes in the dendrite or axon and slightly decreased fluorescence of the cell body were observed; snap, defined by loss of dendrite or axon, low florescence, and rounding of the cell body; and complete cell loss, in which all but the nondopaminergic AIY neurons were missing (Supplementary Figs. 1A–1E), as previously described by Tucci et al. [15].

Determination of thioredoxin and thioredoxin reductase activity

The activity measurements were performed as previously described [16] with some modifications. A mixture of 50 mM Tris, pH 8, 1 mM EDTA, pH 8.0, and 0.15 mg/ml NADPH was prepared. 6-OHDA was dissolved in MilliQ H_2O and added to a 96-well plate (25, 50, and 100 μM final concentration). Mammalian TrxR1 (30 nM; IMCOcorp) and Trx1 (2 μM; Promega), and catalase (20 U; Sigma–Aldrich) were added to the mixture, and the consumption of NADPH was monitored at A_{340} for 20 min.

Determination of glutaredoxin activity

The assay was performed as previously described, with minor modifications [17]. A mixture of 0.1 M Tris, pH 8, 2 mM EDTA, pH 8, 0.1 mg/ml bovine serum albumin, 50 μM GSH, pH 5.0, 2 mg/ml NADPH, and 0.008 OD/ml yeast GR was prepared (all reagents were purchased from Sigma–Aldrich). 6-OHDA was dissolved in MilliQ H_2O and added to a 96-well plate (with a final concentration of 25, 50, or 100 μM). Reactions were performed with 1 μM hGrx1 (IMCO Corp.) and 20 U catalase (Sigma–Aldrich). A 100-μl mixture was added, and the final volume was adjusted to 110 μl per well. Consumption of NADPH was monitored at A_{340} for 1 h.

Statistical analysis

The Kruskal–Wallis statistical method was used to calculate the significance level in the quantification of the IHC, and *t* test for independent samples was used for calculating the significance from the experiments conducted in SH-SY5Y cells and *C. elegans*. All statistical calculations were performed using the Statistica Program (Statsoft).

Results

The thioredoxin system but not the glutaredoxin system is downregulated in PD brains

To investigate the expression pattern of the redox proteins in PD brains, paraffin-embedded tissue from substantia nigra from Parkinson patients ($n = 11$) and controls ($n = 10$) were sectioned and stained for Trx1, Trx2, TrxR1, Grx1, and Grx2 (Fig. 2). The tissue was also stained for TrxR2, but all antibodies tested were proven to be nonspecific for IHC in the tissue examined and therefore TrxR2 was not evaluated. To evaluate the immunohistochemical staining, the stained area was measured and divided by the number of nuclei in the section. A significant downregulation of Trx1 and TrxR1 in substantia nigra of PD patients compared to controls was observed, which was not detected for the other proteins examined (Fig. 2).

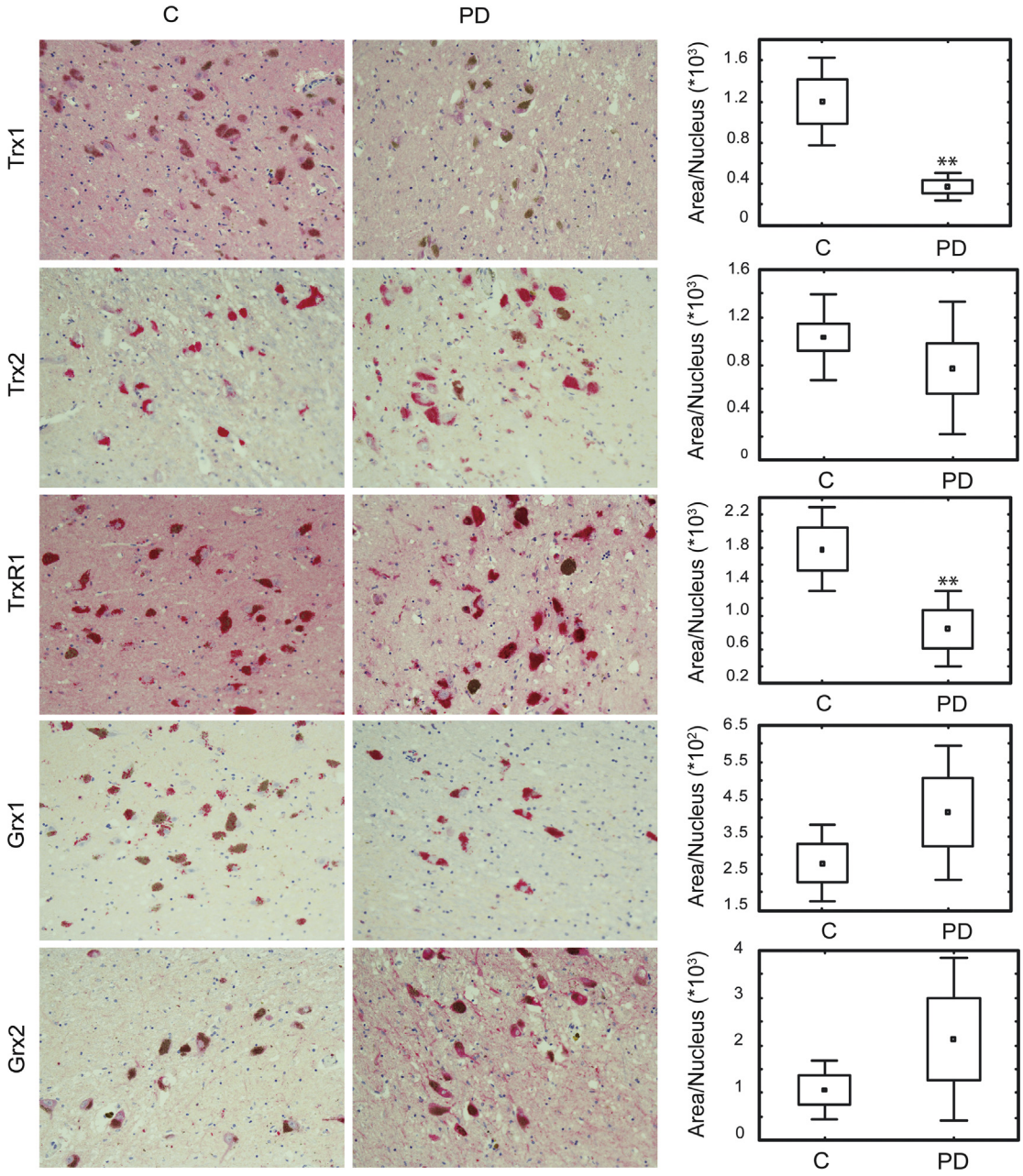

Fig. 2. Immunohistological staining of redox proteins in substantia nigra of PD patients ($n_C = 10$, $n_{PD} = 11$). Representative figures of staining with Trx1, Trx2, TrxR1, Grx1, and Grx2 and quantification of immunohistological stainings from substantia nigra (stained area/nuclei). Pictures were taken at 20 × magnification. The dot represents the mean value, the box indicates the mean ± standard error, and the brackets represents mean ± standard deviation. The Kruskal–Wallis statistical method was used to calculate the significance level ($^{**}p < 0.01$).

Treatment with 6-OHDA in the human neuroblastoma cell line SH-SY5Y altered the expression of redox proteins

To further explore the involvement of redox proteins in relation to dopamine-induced cell death, the human neuroblastoma cell line (SH-SY5Y) was differentiated with retinoic acid and the viability was evaluated after treatment with increasing concentrations of 6-OHDA. As no difference in toxicity was observed in differentiated cells compared to undifferentiated cells (data not shown), all subsequent experiments were conducted in undifferentiated cells. The IC_{50} value of 6-OHDA in undifferentiated cells was determined to 40 μM, as calculated from the dose–response curve (data not shown).

In addition to 6-OHDA, cells were also pretreated with selenite (0.5 μM) for 24 h. Selenium compounds have known antioxidant properties at low to moderate doses, possibly through their incorporation into selenoproteins (including TrxR1 and TrxR2) [18]. Our results demonstrate a strong protective effect of selenite pretreatment, with a 50% higher viability compared to when cells were treated only with 6-OHDA (Fig. 3A). Next, the mRNA levels of the redox proteins were determined in SH-SY5Y cells treated with 6-OHDA. Increased mRNA levels of Trx1, TrxR1, TrxR2, Grx1, and Grx2 (total), as well as the Grx2 splice forms Grx2a and Grx2c, were observed (Fig. 3B and C). However, there was no difference in terms of mRNA expression of redox proteins between the samples pretreated with selenite and those without, suggesting that the increase is caused by the addition of 6-OHDA and not by the selenite pretreatment. Treatment with 6-OHDA was also shown to increase the total enzymatic activity of TrxR1 and TrxR2 (Fig. 3F), as well as increasing the protein levels of Trx2 (Fig. 3E) and Grx1

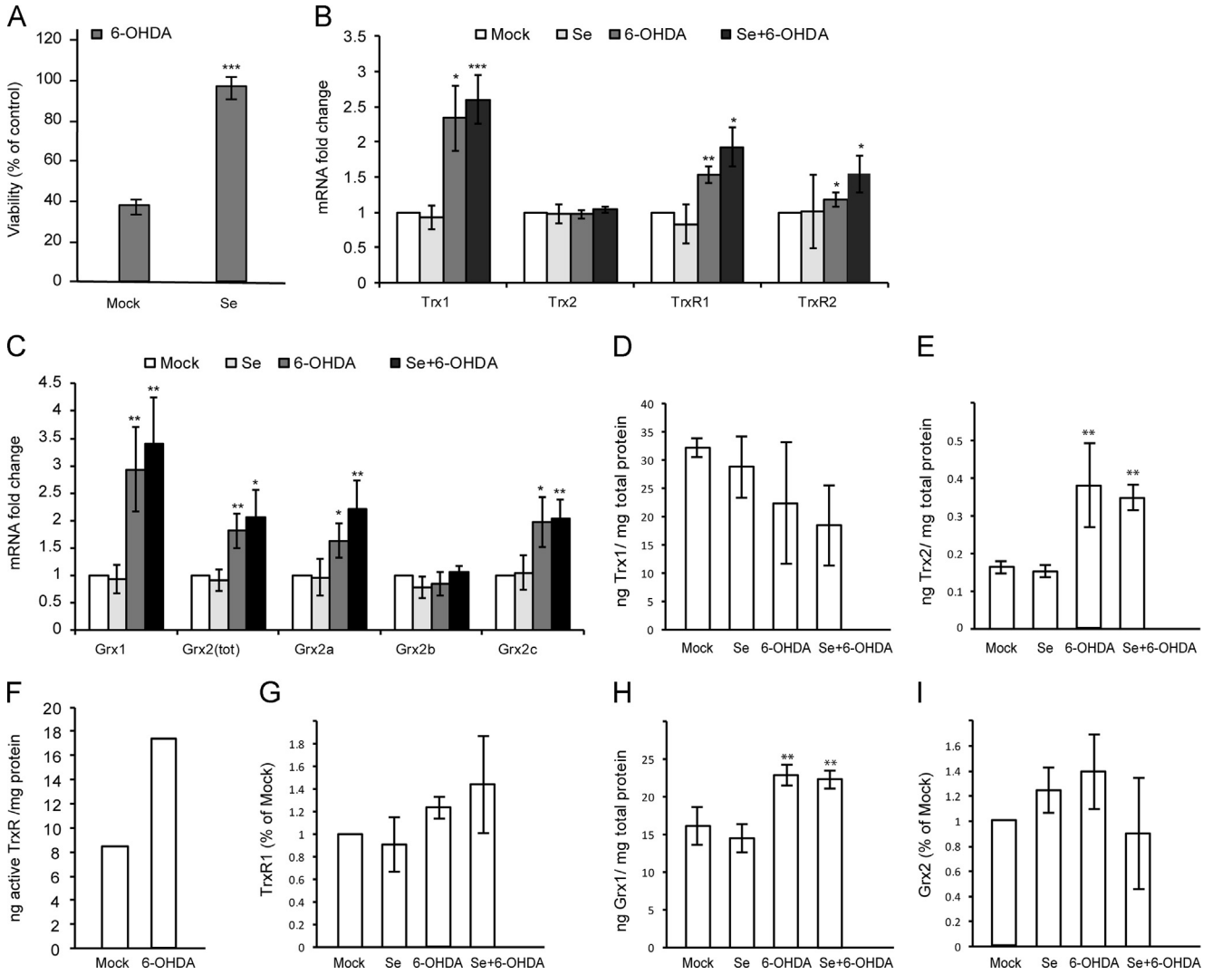

Fig. 3. Changes in redox response in SH-SY5Y cells upon pretreatment with selenite and treatment with 6-OHDA (Se 0.5 μM, 6-OHDA 40 μM). (A) Viability in cells after pretreatment with selenite (0.5 μM) and treatment 6-OHDA (40 μM) measured by XTT. Values were normalized to mock-treated cells. (B and C) Relative mRNA levels after treatment with Se and 6-OHDA. HPRT was used as reference gene and all values were normalized to untreated control. (D) Protein levels of Trx1. (E) Protein levels of Trx2. (F) Total TrxR, enzymatic activity measured in cell lysate after treatment with 6-OHDA for 24 h. (G) Protein level of TrxR1. (H) Protein level of Grx1. (I) Protein level of Grx2. Protein levels were measured after treatment with Se and/or 6-OHDA and measured either with ELISA (Trx1, Trx2, and Grx1) or with Western blot (Grx2 and TrxR1). For Western blot actin was used as loading control and values were normalized to mock-treated cells. Statistical analysis was performed with *t* test for independent samples (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

(Fig. 3H). In contrast, the protein levels of Trx1, TrxR1, and Grx2 remained unaffected (Fig. 3D, G, and I).

Knockdown by siRNA of redox proteins increases the toxicity induced by 6-OHDA in neuroblastoma cells

With the observed changes in the mRNA levels of Trx1, TrxR1, Grx1, and Grx2 upon treatment with 6-OHDA, the potential impact of proteins belonging to the thioredoxin and glutaredoxin systems on the cytotoxicity of 6-OHDA was examined by knockdown of Trx1, TrxR1, Grx1, and Grx2 using siRNA. Increased cytotoxicity of 6-OHDA was observed in cells after knockdown of TrxR1, Grx1, and Grx2 but not in Trx1 siRNA (Fig. 4A). Control experiments with siRNA for Trx1, TrxR1, Grx1, and Grx2 are illustrated in Fig. 4B.

C. elegans trxr-1 mutants are more sensitive to 6-OHDA toxicity

As the brains of PD patients exhibited low levels of Trx1 and TrxR1, the effects of dopaminergic cell degeneration in connection to the thioredoxin system were studied in *C. elegans*, an amenable animal model system with a simple, well-described dopaminergic system consisting of eight dopaminergic neurons in the hermaphrodite (two ADE and four CEP neurons in the head plus two PDE neurons in the posterior part of the body) [19]. The *C. elegans* thioredoxin system is composed of five thioredoxins (*trx-1* to *trx-5*) and two thioredoxin reductases *trxr-1* and *trxr-2*. *C. elegans trx-1* to *trx-4* have known localizations outside the dopaminergic neurons [20–22] (and A. Miranda-Vizuete, unpublished data) and were therefore not chosen for this study. In addition, both *trxr-2* and *trx-2* are known for their mitochondrial localization, where dopamine is expected to have less effect, and based on this were also excluded. Therefore, we focused our approach with *C. elegans* on the *trxr-1* and *trx-5* genes. Animals harboring the *trxr-1(sv43)* [14] and *trx-5(ok2014)* null alleles were used and treated with 20 and 40 mM 6-OHDA. The neuronal integrity was evaluated by scoring the degeneration of the four dopaminergic CEP (marked with dat-1::mCherry) neurons and the two nondopaminergic AIY (marked with ttx-3::mCherry) neurons (Supplementary Fig. 1).

The stages of neuronal degeneration were defined as unaffected, neurite blebbing, dendritic snap, and complete cell loss (the classification is thoroughly explained under Materials and methods) [15]. The internal control cells (AIY) showed no degeneration in any of the treatments. Increased neuronal degeneration in the nematodes was observed in the *trxr-1* mutant when treated with 40 mM 6-OHDA, as illustrated in Fig. 5 and Supplementary Table 2. The degeneration in the *trxr-1* mutant had significant differences comparing the unaffected neurons ($p_{trxr\text{-}1} = 0.004$ and $p_{trx\text{-}5} = 0.08$) and complete cell loss ($p_{trxr\text{-}1} = 0.03$ and $p_{trx\text{-}5} = 0.13$).

6-OHDA-quinone as a substrate for the glutaredoxin and thioredoxin systems

To assess whether the protective effects of the redoxins were indirect via protection against oxidative stress or direct through an interaction and reduction of the 6-OHDA-quinone, enzymatic activity measurements of the thioredoxin and glutaredoxin systems using 6-OHDA as substrate were conducted and the consumption of NADPH was monitored over time. To exclude that the reaction observed was not due to the known peroxidase activity of the Trx or Grx system [23,24] acting on the spontaneously formed hydrogen peroxide when 6-OHDA converted to 6-OHDA-quinone in the presence of oxygen, catalase was included in excess in the reaction mixture. Our data demonstrate that TrxR1 has the capacity to reduce the 6-OHDA-quinone and that this reaction was enhanced in the presence of Trx1 (Fig. 6A, Supplementary Fig. 2A). When examining the Grx system, we observed a modest increased consumption of NADPH with increasing concentration of 6-OHDA in the presence of GSH alone (Supplementary Fig. 2B), as previously reported by others [25]. However, this reaction rate was significantly increased with the addition of Grx1 to the reaction mixture compared to the reaction catalyzed by GSH alone (Fig. 6B). Both systems have well-documented peroxidase activities, and consequently part of the total activity measured is probably due to the peroxidase activity exhibited by these redox systems. However, oxidation of NADPH remained even after the addition of catalase to the reaction mixture (Fig. 6), thus confirming the ability of these proteins to reduce the 6-OHDA-quinone. The reduction of the 6-OHDA-quinone was further shown to occur in a concentration-dependent manner (Supplementary Figs. 2A and 2B). To explore the catalytic mechanism, we used a human Grx1 mutant variant lacking the C-terminal cysteine in the active site and compared it to the wild-type protein. As illustrated in Supplementary Fig. 2C, no NADPH consumption was obtained with the mutated glutaredoxin, confirming that the dithiol mechanism is required for the reduction of the 6-OHDA-quinone. These redoxins thus exert their protective effect against PD-induced neuronal cell death by being able to reduce the neurotoxin 6-OHDA-quinone directly.

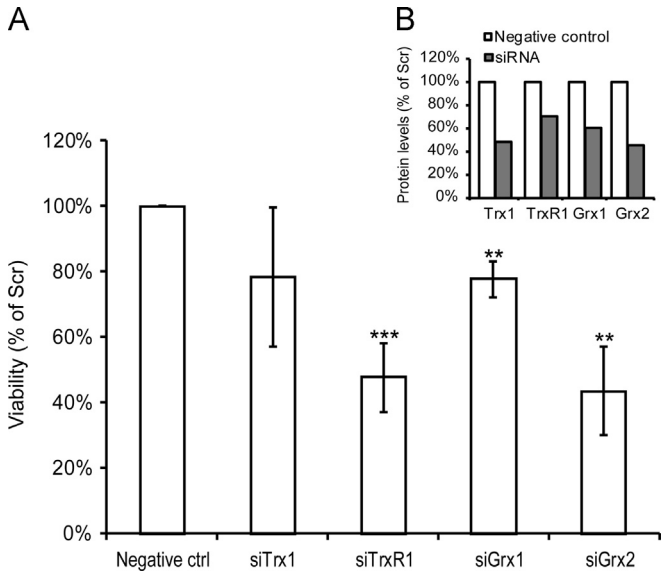

Fig. 4. Knockdown of redox proteins with siRNA. (A) Viability of SH-SY5Y cells after knockdown of redox proteins followed by 6-OHDA treatment. Levels of significance were calculated using *t* test for independent samples (**$p < 0.01$ and ***$p < 0.001$). (B) A representative graph of protein levels of Trx1, TrxR1, Grx1, and Grx2 after 24 h siRNA treatment, measured by ELISA (Trx1, Trx2, and Grx1) or Western blot (TrxR1 and Grx2).

Discussion

Although PD is the second most common neurodegenerative disorder, affecting a growing number of people owing to increased age, the etiology of PD is largely unknown. A deeper understanding of the underlying mechanisms behind dopamine-induced cell death would be essential for the development of efficient treatment strategies. This study demonstrates that the thioredoxin and the glutaredoxin systems not only play an important role against dopamine-induced cell death, but also

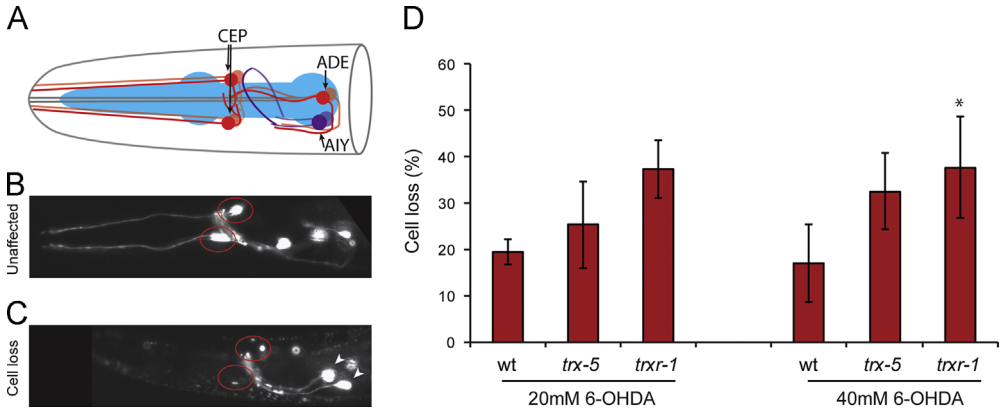

Fig. 5. Role of redox proteins in neuronal toxicity in *C. elegans*. (A) Schematic overview of neurons marked with mCherry in the worm model. Dopaminergic (four CEP and two ADE) are in red and nondopaminergic (AIY) in purple. (B) Unaffected neuron. (C) Complete loss of the CEP dopaminergic neurons, AIY unaffected. (D) Loss of dopaminergic CEP in *C. elegans* with deletion of *trx-5* or *trxr-1*. AIY was used as a survival control, and only animals with intact AIY were calculated ($n = 30$ for each experiment). Levels of significance were calculated using *t* test for independent samples (*$p < 0.05$). A full representation of all measured parameters can be found in Supplementary Fig. 1.

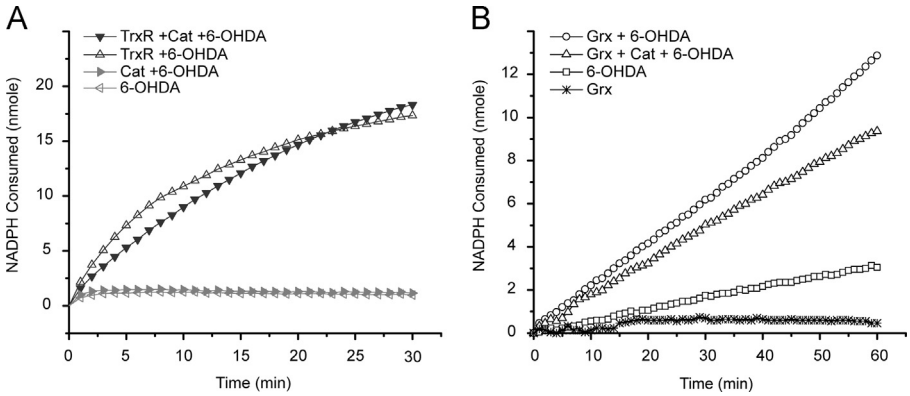

Fig. 6. Activity of TrxR1 and Grx1 in the presence of 6-OHDA. (A) TrxR1 activity assay with 100 μM 6-OHDA in the presence or absence of 20 U catalase. (B) Activity assay for Grx1 with 100 μM 6-OHDA in the presence or absence of 20 U catalase.

exert critical protective effects by direct enzymatic reduction of the neurotoxic dopamine metabolite 6-OHDA-quinone.

Familial PD has been linked to mutations in the α-synuclein gene, and it has been suggested that a loss of the normal function as well as a toxic effect of the mutated form of this protein promotes the accumulation of dopamine in the cytoplasm [6]. Free in the cytosol, dopamine will autoxidize to form 6-OHDA and 6-OHDA-quinone with H_2O_2 as a by-product that will further contribute to the increased oxidative stress in the nigrostriatal regions, where most of the neurotransmitter is synthesized and stored. Low levels of the cytosolic thioredoxin system in substantia nigra, as was found in the human tissue examined from PD patients, would therefore lead to a decreased defense against the oxidative stress, and this is further supported by the increased level of protein oxidation in this region [26].

TrxR1 and TrxR2 are two of the 25 selenoproteins known in humans [27]. Selenium-containing proteins have vital functions in the body, making selenium an essential trace element. It has been shown that the highest retention of selenium occurs in the brain in comparison with other tissues [28], placing the brain at the top of a tissue hierarchy. Feeding rats with a selenium-deficient diet resulted in a high priority of selenium distribution to the brain. The Se-deficient rats showed dramatically decreased levels of selenium in liver and kidneys, whereas the levels in brain were far less affected [29]. The preferential supply of selenium in the brain during prolonged periods of selenium deficiency has also been reported by others [30] and gives a strong indication of an important function of this essential trace element in the brain. In three large trials carried out among elderly persons, low selenium levels were associated with faster decline in cognitive functions and poor performance in tests assessing coordination and motor speed [31]. Low levels of selenium have further been observed in patients suffering from PD [32]. Selenium-deficient diet has also resulted in a large decrease in activity of the selenoproteins TrxR and GPx in rat liver [33]. With immunohistochemical evaluations of human postmortem substantia nigra we showed a decreased level of the selenium-containing protein TrxR1, which might possibly be explained by the low selenium levels reported by others in PD.

In previous studies with 6-OHDA, pretreatment with sodium selenite resulted in an upregulation in antioxidant status and lowered dopamine loss in a rat model [34]. Sulfhydryl antioxidants protected against neuronal degradation in the striatum, particularly in the case of cysteine, and this was attributed to its capacity to remove the H_2O_2 produced in the autoxidation of 6-OHDA [35]. Furthermore, SH-SY5Y cells exposed to 50 μM 6-OHDA have been reported to increase the GSH concentrations 12-fold, but with no change in GSH:GSSG ratio, suggesting an induction of oxidative stress, with an adaptive increase in intracellular GSH [36]. Based on the indicative role of selenium in PD, selenium pretreatment was performed before the addition of 6-OHDA. Addition of selenium to the neuroblastoma cells protected the cells against 6-OHDA. The protective effect seen by selenite could, however, not be explained by an increased expression of the redoxins examined in this setup, as the expression did not differ significantly from that of the 6-OHDA treatment alone. The protective effect observed by the selenite pretreatment might instead be explained by the involvement of other selenoproteins. One very likely candidate is the selenium-containing glutathione peroxidase, which exhibits well-established peroxidase activity [37]. These peroxidases would be able to protect against the spontaneously formed hydrogen peroxide when 6-OHDA is autoxidized to the 6-OHDA-quinone.

The involvement of TrxR's in protecting against oxidative stress, mitochondrial dysfunction, and cell death in dopaminergic cells has been previously reported by inhibition of total TrxR with the specific gold compound auranofin and through knockdown of TrxR2 with shRNA. Knockdown of TrxR2 potentiated H_2O_2 release and cell death, resulting from subtoxic concentrations of paraquat in two dopaminergic cell lines [38]. Additionally, inhibition of TrxR's in N27 dopaminergic cells before treatment with 6-OHDA increased the levels of H_2O_2 and subsequent cell death [38]. In agreement with these results, we show increased levels of Trx1 and both the cytosolic TrxR1 and the mitochondrial TrxR2 mRNA upon treatment with 6-OHDA in the neuroblastoma cell line SH-SY5Y. Increased enzymatic activity of total TrxR upon treatment with 6-OHDA was also seen, further strengthening the suggested role of the Trx system in the protection against dopamine-induced cell death. The mRNA levels of Grx1, Grx2(tot), Grx2a, and Grx2c were also increased after 6-OHDA treatment. Toxicity induced by 6-OHDA has previously been shown to be inhibited by Grx1 overexpression [39], and *E. coli* Grx2 has been shown to protect cerebellar granule neurons from dopamine-induced apoptosis, by activating the NF-κB signaling pathway through Ref-1 [40]. The protection was further shown to be attributed to the activation of the Ras/PI3K/Akt and JNK/AP-1 pathways, culminating in NF-κB activation [41].

Knocking down TrxR1, Grx1, and Grx2 with siRNA in our setup increased the vulnerability to 6-OHDA. Trx1 displayed a similar trend but with no significant changes. However, 6-OHDA treatment did result in a clear increase in Trx1, and previous reports have shown a protective effect of Trx1 in PD, after treatment with MPTP in a mouse cell line, by suppressing endoplasmic reticulum stress [42]. One can therefore not rule out the beneficial role Trx1

might have, but rather speculate on the possibility of a compensatory mechanism during siRNA inhibition for Trx1 in our human neuroblastoma cell line. Moreover, Trx1 is known to interact and regulate signaling molecules such as NF-κB and ASK-1 [43], all suggested to be involved in 6-OHDA-induced cell death [44,45]. Increased dopaminergic cell death was also observed upon treatment with 6-OHDA in a *C. elegans trxr-1*-null mutant strain. No significant difference could be seen after deletion of *trx-5,* even though a strongly similar trend was observed.

Through direct enzymatic interaction, both the thioredoxin and the glutaredoxin systems were able to reduce the neurotoxic 6-OHDA-quinone. The thioredoxin system presented the highest activity in vitro for this neurotoxin, and with significantly lowered levels in substantia nigra of PD patients, this might imply a diminished protective effect of the neuronal cells in substantia nigra against this highly cytotoxic metabolite. It has previously been shown that GSH can reduce the 6-OHDA-quinone to dopamine or covalently bind to dopamine-quinone [5]. We can demonstrate that the glutaredoxins are much more efficient at reducing 6-OHDA-quinone than GSH alone. The direct reduction of the 6-OHDA-quinone by these systems strongly suggests that the thioredoxins and the glutaredoxins are able to render the cytotoxic effects generated by the neurotoxin, and this is supported by the increased sensitivity of the SH-SY5Y cells to 6-OHDA during downregulation of the redoxins. Apart from the reduction of the 6-OHDA-quinone, the redoxins may potentially also protect the cells against the spontaneous ROS formation when 6-OHDA autoxidates or through their well-documented regulation of several signaling molecules [43]. Taken together, this work reveals the importance of the Grx and Trx systems in the defense against dopamine-induced cell death.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.05.011.

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Supplementary Table 1.

Primer sequences and concentrations for qPCR reaction.

Primer	Sequence	Concentration (nM)
HPRT	Fwd 3'-GCAGACTTTGCTTTCCTTGG-5'	300
	Rev 3'-TATCCAACACTTCGTGGGGT	900
Grx1	[46]	
Grx2 (tot)	[46]	
Grx2a	[46]	
Grx2b	[46]	
Grx2c	Fwd 3'-TAAGCAAGATGGAGAGCAA-5'	900
	Rev 3'-GTTCCACCACTTTATAGTTA-5'	900
Grx3	Fwd 3'-GTGAAGTTGGAAGCTGAAGGTGT-5'	900
	Rev 3'-CACTAGATGCATGTCGCTGAAC-5'	900
Grx5	Fwd 3'-GCTCCGACAAGGCATTAAAGAC-5'	300
	Rev 3'-TTCAGTTCTTCCACCAAGTCCC-5'	300
Trx1	Fwd 3'-GATCCATTTCCATCGGTCCTTACA-5'	900
	Rev 3'-AGAGAGGGAATGAAAGAAAGGCTT-5'	900
Trx2	Fwd 3'-GTCCTCATCTTGGTCCCTTCC-5'	900
	Rev 3'-ACAAAACAGCAGCTGGAAAGAG-5'	900
TrxR1	[47]	
TrxR2	Fwd 3'-TCAGAAGATCCTGGTGGACTCC-5'	300
	Rev 3'-TCGTGGGAACATTGTCGTAGTC-5'	300

Supplementary Table 2.

Levels of degeneration of dopaminergic CEP neurons in C. elegans after 20 or 40 mM 6-OHDA treatment. Levels represent the percentage (0.00-1.00) of total number of cells from each treatment. Significance was calculated by t-test for independent samples, trx-5 or trxr-1 compared to wt.

Genotype	6-OHDA (mM)	Unaffected	p-value	Neurite blebbing	p-value	Dendritic snap	p-value	Cell loss	p-value
wt	20	0.42		0.26		0.12		0.19	
trx-5	20	0.28	0.25	0.31	0.62	0.15	0.42	0.25	0.35
trxr-1	20	0.22	0.10	0.32	0.31	0.09	0.44	0.37	0.07
wt	40	0.42		0.30		0.11		0.17	
trx-5	40	0.25	0.08	0.33	0.84	0.10	0.94	0.33	0.13
trxr1	40	0.18	**0.004**	0.31	0.93	0.13	0.58	0.38	**0.03**

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