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

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Running title: Redox proteins in dopamine induced cell death

Highlights

- The levels of Trx1 and TrxR1 are decreased in substantia nigra of human PD brains.
- The cytotoxic effect of 6-OHDA is increased after downregulation of redoxins.
- Increased dopaminergic degeneration by 6-OHDA in *C. elegans trxr-1* null mutants.
- Both the Trx and Grx systems directly reduce the 6-OHDA-quinone.
- The Trx and Grx systems may protect cells against the neurotoxin 6-OHDA-quinone.

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, the levels of Trx1 and thioredoxin reductase 1 (TrxR1) were found to be significantly decreased. The human neuroblastoma cell line SH-SY5Y and the nematode *Caenorhabditis elegans* (*C. elegans*) were used as model systems to explore the potential protective effect 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 siRNA 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 significant 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.

Key words: 6-hydroxydopamine, thioredoxin, thioredoxin reductase, glutaredoxin, neuronal degeneration

Introduction

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 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 substantia nigra are degenerating to a greater extent than other neurons, most likely due to dopamine itself. Dopamine is normally stored at mM 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 autooxidate and generate the highly neurotoxic compound 6-OHDA-quinone (Figure 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 sulphydryl 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].

In order 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 sustained in an oxidative environment [7]. The thioredoxin system is comprised of 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 glutathione (GSH) which is required for the reduction of Grx. There are four human Grxs; 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 Grxs (Grx1 and Grx2) is composed of two cysteins, separated by two amino acids (CXXC), whereas the monothiol Grxs (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 like 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 Ethical Review Board in Stockholm (Dnr: 488/02).

Immunohistolochemical 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, 1:36000), 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), 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 unspecific for IHC in the tissue examined and could not be used for further studies. Microphotographs of the different stainings were acquired using a microscope with an inbuilt camera (Nikon Eclipse E1000) at 4x 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 DMEM (Gibco) supplemented with 10% FBS (Gibco), under normal conditions, 5% CO_2 at 37 ºC. To induce differentiation, cells were

treated with 10 μM all-trans-retnoic acid (Sigma-Aldrich) in DMEM media, supplemented with 3% FBS for 48 hours. To investigate the involvement of antioxidants, the cells were pre-treated 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 media was changed before addition of the substrate, and wells with only media were used as a blank. Values were normalized to untreated control.

Transfection

When seeded into appropriate culturing plates SH-SY5Y cells were transfected with 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^{TM}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 RNeasy Plus Mini Kit (Qiagen), according to protocol supplied by the manufacturer. The concentration of the mRNA was determined using the NanoDrop® Spectrophotometer ND-1000. 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 (40x): 95 °C, 15 sec, and elongation: 60 °C, 30 sec, Melt curve (80 x): start temperature 55 °C with an increase of 0.5 °C per 10 second 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 on a SDS-PAGE (12% Mini-PROTEAN TGX gel, BioRad) at 140 V followed by semi-dry electro blotting 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 over night. The membranes were blocked with 5% dried milk for 2 h in room temperature, followed by incubation with secondary antibodies for 2 h at room temperature. For Grx2 HRP 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, 1:10000). Bound antibodies were detected with chemiluminescence or in the infrared range using the Odyssey Fc infrared imaging system (LI-COR) according to 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 over night [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 over night 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), 1µg/ml biotinylated Grx1 (IMCOcorp)]. ALP conjugated streptavidin (1:1000)

was incubated on the plates for 1h prior to addition of phosphatate substrate. The plates were read at 405 nm in a PowerWave HT (BioTek) spectrometer.

C. elegans strains

C. elegans strains were maintained at 20 °C on NGM agar plates seeded with a lawn of *E. 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,* RB1637 (obtained from the publicly funded Caenorhabditis Genetics Center/CGC), *trx-5 (ok2014) V*. Crossing of the P*dat1-::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 can be provided upon request.

6-OHDA treatment of *C. elegans*

Worms were treated with 6-OHDA as previously described [15]. In brief, worms were synchronised by placing 50 gravid hermaphrodites on seeded agar plates for four hours, and their progeny was 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 were prepared freshly in 20 mM ascorbic acid prior to treatment. The stock solution was diluted to the desired concentrations in water (20 mM or 40 mM 6-OHDA). Worms were treated in 1 ml for one hour, at room temperature with gentle shaking. After one hour, 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 hours, followed by evaluation of neuronal degeneration by

microscopy on a Zeiss Axioplan II. Evaluations were performed with Cy3/DsRed filter at 200ms exposure time, with 20-40x magnification.

Evaluation of neuronal degeneration in *C. elegans*

Approximately 40 worms were anaesthetised in 3 mM levamisole on agarose pads. 30 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, where changes of the dendrite or axon and slightly decreased fluorescence of the cell body was observed, Snap, defined by loss of dendrite or axon, low florescence and rounding of the cell body, and complete cell loss where all but the non-dopaminergic AIY neurons were missing (Supplementary figure 1 A-E), 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 (20U, Sigma-Aldrich) was added to the mixture, and the consumption of NADPH was monitored at A_{340} for 20 minutes.

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 BSA, 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 Corporation) and 20U 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 one hour.

Statistical analysis

The Kruskall-Wallis statistical method was used to calculate the significance level in the quantification of the IHC, and t-test for independent samples were 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 (Figure 2). The tissue was also stained for TrxR2, but all antibodies tested were proven to be unspecific for IHC in the tissue examined and was therefore 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 (Figure 2).

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, suggestively through the 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 only treated with 6-OHDA (Figure 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, Grx2(total), as well as the Grx2 splice forms Grx2a and Grx2c were observed (Figure 3B and C). However, there was no difference in terms of mRNA expression of redox proteins between the samples pretreated with selenite and the ones without, suggesting that the increase is caused by the addition of 6-OHDA and not by the selenite pre-treatment. Treatment with 6-OHDA was also shown to increase the total enzymatic activity of TrxR1 and TrxR2 (Figure 3F), as well as increasing the protein levels of Trx2 (Figure 3E) and Grx1 (Figure 3H). In contrast, the protein levels of Trx1, TrxR1and Grx2 remained unaffected (Figure 3D, 3G and 3I)

Knock-down 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 knock-down of Trx1, TrxR1, Grx1 and Grx2 using siRNA. Increased cytotoxicity of 6-OHDA was observed in cells after knock down of TrxR1, Grx1 and Grx2 but not in Trx1 siRNA (Figure 4A). Control experiments with siRNA for Trx1, TrxR1, Grx1 and Grx2 are illustrated in figure 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 was 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 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 non-dopaminergic AIY (marked with ttx-3::mCherry) neurons (Supplementary Figure 1). The stages of the neuronal degeneration were defined as unaffected, neurite blebbing, dendritic snap, and complete cell loss (the classification is thoroughly explained in material 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 Figure 5 and Supplementary Table 2. The degeneration in the *trxr-1* mutant had significant differences when comparing the unaffected neurons (p_{trxr-1} = 0.004 and p_{trx-5}= 0.08) and complete cell loss (p_{trxr-1} = 0.03 and p_{trx-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 systems [23, 24] acting on the spontaneously formed hydrogen peroxide when 6-OHDA converts to 6-OHDA-quinone in the presence of oxygen, catalase was included in excess to 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 (Figure 6A, Supplementary Figure 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 Figure 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 (Figure 6B). Both systems have well documented peroxidase activities, and consequently part of the total activity measured is likely 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 (Figure 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 figure 2 A-B). 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 figure 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 directly being able to reduce the neurotoxin 6-OHDA-quinone.

Discussion

Although PD is the second most common neurodegenerative disorder, affecting a growing number of people due 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 they also 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 auto-oxidize to form 6-OHDA and 6-OHDA-quinone with H_2O_2 as a byproduct that will further contribute to the increased oxidative stress in the nigrostratial 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.

TrxR1 and TrxR2 are two of the 25 selenoproteins known in human [26]. 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 [27], 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 [28]. The preferential supply of selenium in the brain during prolonged periods of selenium deficiency has also been reported by others [29], 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 [30]. Low levels of selenium have further been observed in patients suffering from PD [31]. Selenium-deficient diet has also resulted in a large decrease in activity of the selenoprotein containing TrxR and GPx in rat liver [32]. With immunohistochemical evaluations of human post mortem 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, pre-treatment with sodium selenite resulted in an upregulation in antioxidant status and lowered dopamine loss in a rat model [33]. Sulfohydryl antioxidants protected against neuronal degradation in the stratium, particularly in the case of cysteine and was attributed to its capacity to remove the H_2O_2 produced in the auto-oxidation of 6-OHDA [34]. 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 [35]. Based on the indicative role of selenium in PD, selenium pretreatment was performed prior to 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 set up, as the expression did not differ significantly from 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 that exhibits well-established peroxidase activity [36]. These peroxidases would be able to protect against the spontaneously formed hydrogen peroxide when 6-OHDA is autooxidised to the 6-OHDA-quinone.

The involvement of TrxRs 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 [37]. Additionally, inhibition of TrxRs in N27 dopaminergic cells prior to treatment with 6-OHDA increased the levels of H_2O_2 and subsequent cell death [37]. 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 [38], and *Escherichia coli* Grx2 has been shown to protect cerebellar granule neurons from dopamine induced apoptosis, by activating NF-κB signaling pathway through Ref-1 [39]. 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 [40].

Knocking down TrxR1, Grx1, and Grx2 with siRNA in our setup increased the vulnerability to 6-OHDA. 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 implicate a diminished protective effect of the neuronal cells in substantia nigra to 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 suggest that the thioredoxins and the glutaredoxins are able to render the cytotoxic effects generated by the neurotoxin, and is supported by the increased sensitivity of the SH-SY5Y cells to 6-OHDA during down regulation of the redoxins. Taken together, the present work reveals the importance of Grx and Trx systems in the defense against dopamine induced apoptosis.

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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	[41]	
Grx2 (tot)	[41]	
Grx2a	[41]	
Grx2b	[41]	
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	[42]	
TrxR2	Fwd 3'-TCAGAAGATCCTGGTGGACTCC-5'	300
	Rev 3'-TCGTGGGAACATTGTCGTAGTC-5'	300

Supplementary Table 2.

Levels of degeneration on 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**

Figure legends

Figure 1. Chemical structure of DA, 6-OHDA and 6-OHDA-quinone.

Figure 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 with 20x magnification. The dot represents the mean value, the box indicates the mean +/- standard error, and the brackets represents mean +/- standard deviation. The Kruskall-Wallis statistical method was used to calculate the significance level (*p<0.05, **p<0.01).

Figure 3. Changes in redox response in SH-SY5Y cells upon pre-treatment with selenite and treatment with 6-OHDA (Se 0.5 µM, 6-OHDA 40 µM). A. Viability in cells after pretreatment with selenite (Se, 0.5µM) and treatment 6-OHDA (40 µM) measured by XTT. Values are normalized to mock treated cells. **B-C.** Relative mRNA levels after treatment with Se and 6-OHDA. HPRT was used as reference gene and all values are 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 24h **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).

Figure 4. Knock-down 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<0.05, **p<0.01 and ***p<0.001). **B.** A representative figure of protein levels of Trx1, TrxR1, Grx1 and Grx2 after 24h siRNA treatment, measured by ELISA (Trx1, Trx2, and Grx1) or Western Blot (TrxR1 and Grx2).

Figure 5. Role of redox proteins in neuronal toxicity in *C. elegans*. A. Schematic overview of neurons marked with mCherry in the worm model. Dopaminergic (4 CEP and 2 ADE) in red and

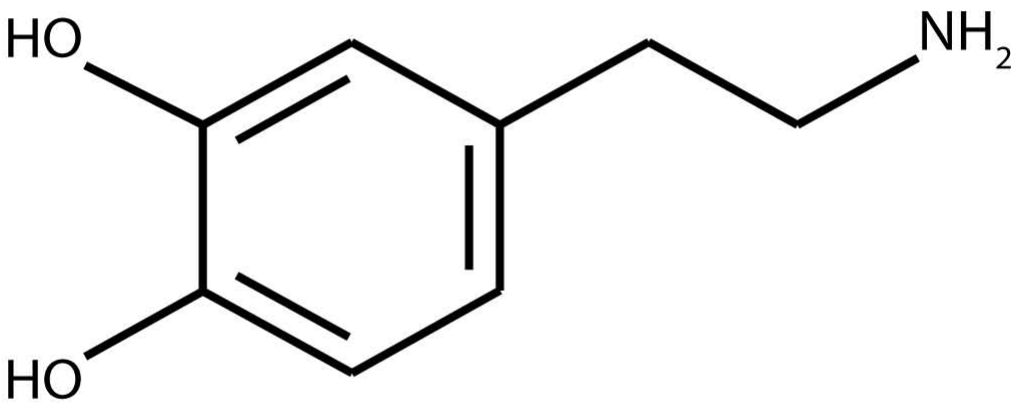
non-dopaminergic (AIY) in purple. **B.** Unaffected neuron. **C.** Complete loss of the CEP dopaminergic neurons, AIY unaffected. **D.** Loss of dopaminergic CEP in *C. elegans* with deletion of *trx-5* or *trxr-1*. AIY was used as survival control cells, and only animals with intact AIY were calculated (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 figure 1.

Figure 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.

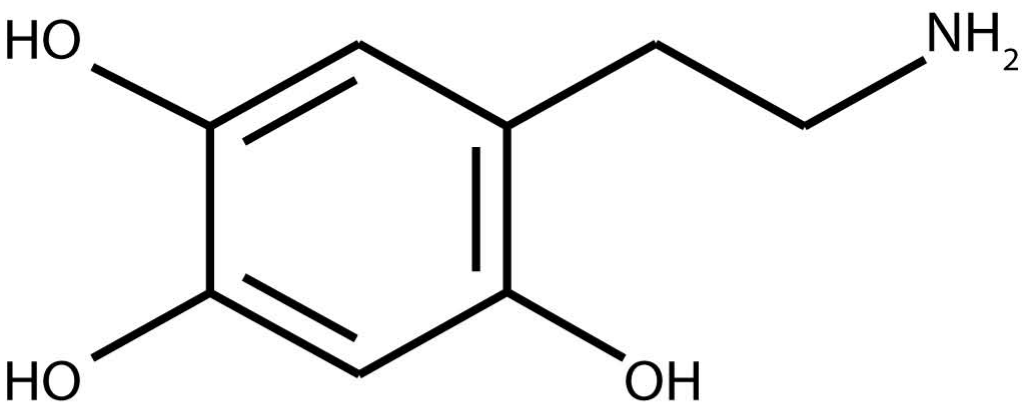
Supplementary Figure 1. Neuronal degeneration in *C. elegans*; Dopaminergic CEP neurons visualized by red rings, and unaffected non-dopaminergic AIY neurons are indicated with purple arrows. **A.** Schematic overview of the neurons studied in this paper. The dopaminergic neurons are shown in red and the non-dopaminergic AIY neurons in purple. **B**. Unaffected; no degeneration of either the CEP neurons (red circle) or the AIY neurons (purple arrow). **C**. Neurite blebbing; blebbing of the dendrite visualized by the white arrows, and slightly lower fluorescence of the cell body (red ring). **D**. Dendritic snap; the dendrite is completely broken as indicated with the white arrows, and the cell body is rounded and the fluorescence is low. **E**. Cell loss; the entire CEP neuron is missing, only low fluorescence seen as apoptotic bodies remain. **F**. Degeneration of the dopaminergic CEP neurons in *C. elegans* with deletion of *trx-5* or *trxr-1*. AIY was used as survival control cells, and only animals with intact AIY were calculated (n = 30 for each experiment). Animals were scored according to above mentioned criteria. Levels of significance were calculated using t-test for independent samples (*p<0.05, **p<0,01).

Supplementary Figure 2. 6-OHDA-quinone as substrate for the thioredoxin and glutaredoxin systems. A. Assay for the thioredoxin system with different concentrations of 6-OHDA. **B.** Grx1 activity assay with different concentrations of 6-OHDA. **C.** Reaction with 100 μM 6-OHDA and wild type Grx1 or the mutant $Grx1_{C14S}$.

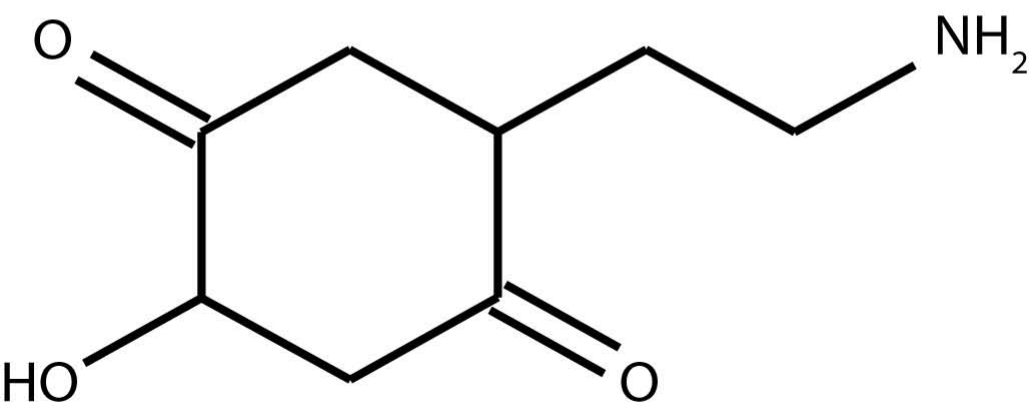
Figure 1



Dopamine



6-OHDA



6-OHDA-quinone

Figure 2

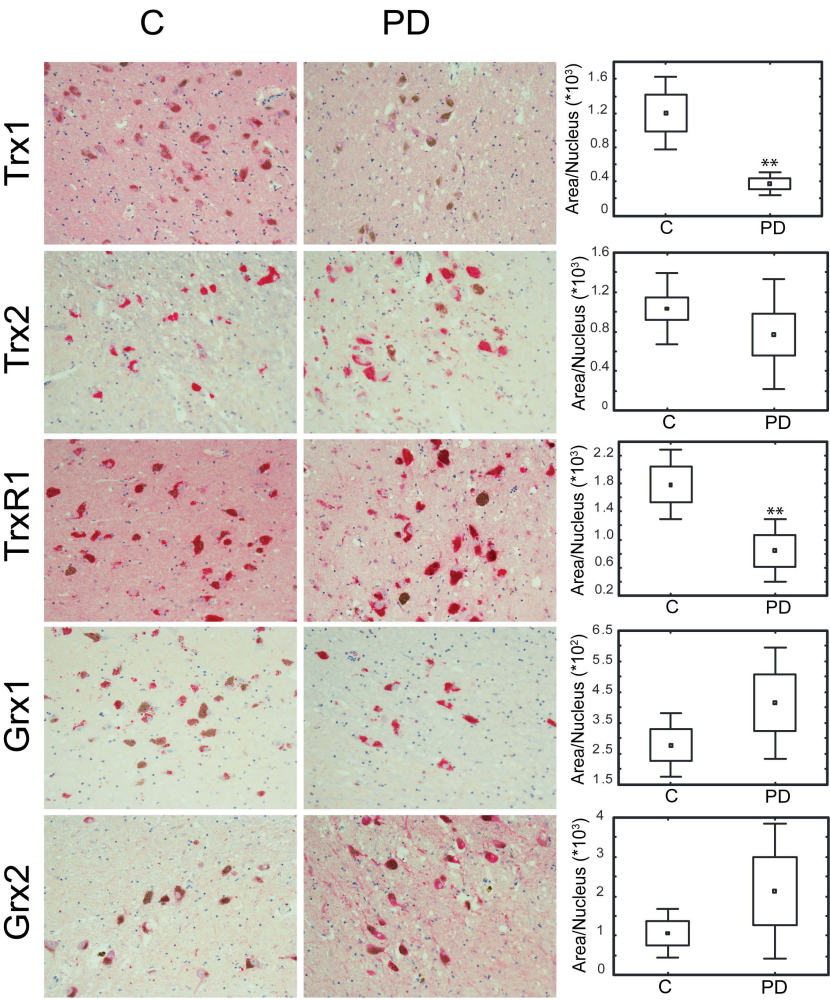

C
PD
Trx1
Trx2
TrxR1
Grx1
Grx2
Area/Nucleus (*10³)
Area/Nucleus (*10²)
**
C
PD

Figure 3

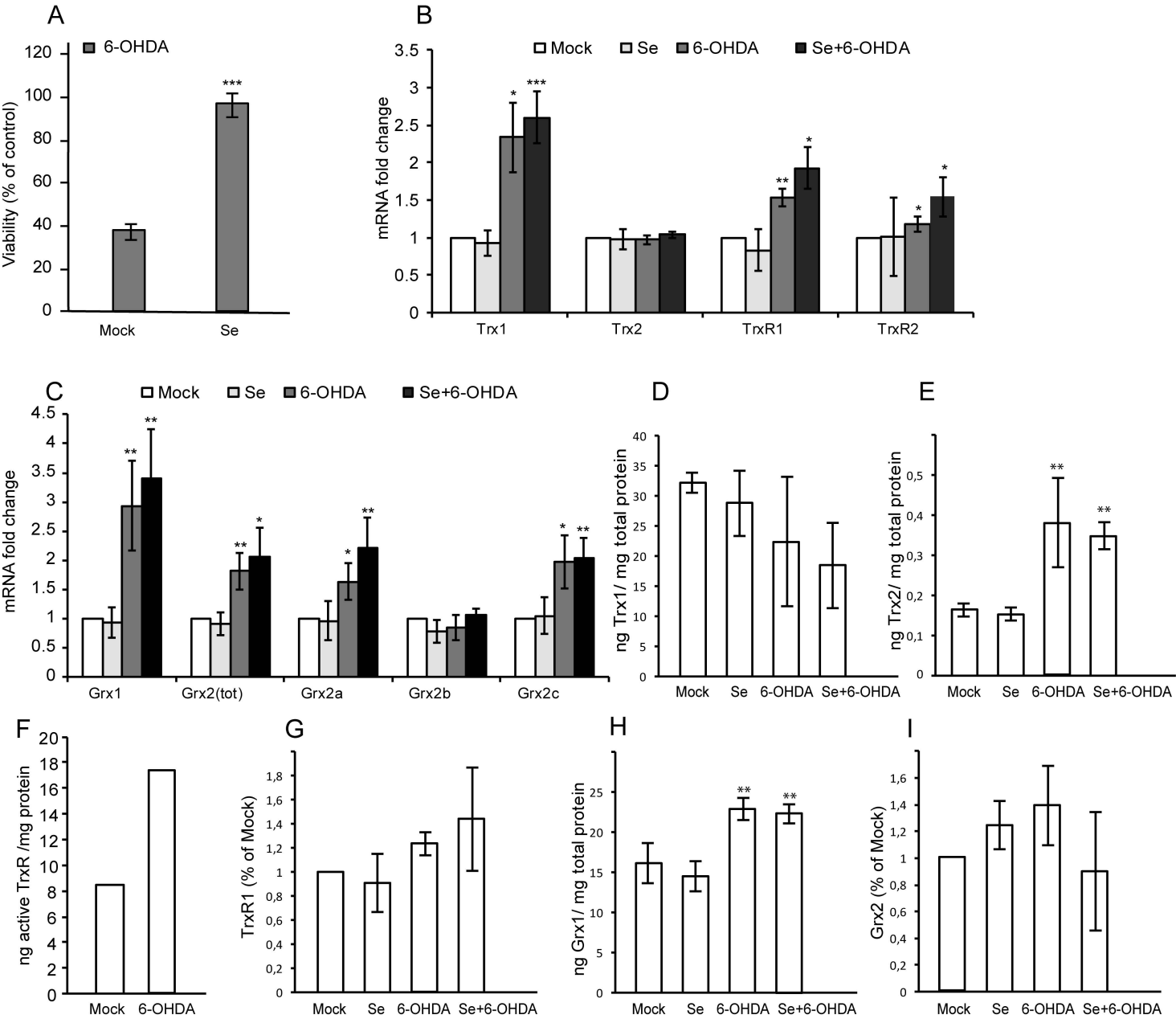

A
6-OHDA
Viability (% of control)
Mock
Se
B
Mock
Se
6-OHDA
Se+6-OHDA
mRNA fold change
Trx1
Trx2
TrxR1
TrxR2
C
Mock
Se
6-OHDA
Se+6-OHDA
mRNA fold change
Grx1
Grx2(tot)
Grx2a
Grx2b
Grx2c
D
ng Trx1/ mg total protein
Mock
Se
6-OHDA
Se+6-OHDA
E
ng Trx2/ mg total protein
Mock
Se
6-OHDA
Se+6-OHDA
F
ng active TrxR /mg protein
Mock
6-OHDA
G
TrxR1 (% of Mock)
Mock
Se
6-OHDA
Se+6-OHDA
H
ng Grx1/ mg total protein
Mock
Se
6-OHDA
Se+6-OHDA
I
Grx2 (% of Mock)
Mock
Se
6-OHDA
Se+6-OHDA

Figure 4

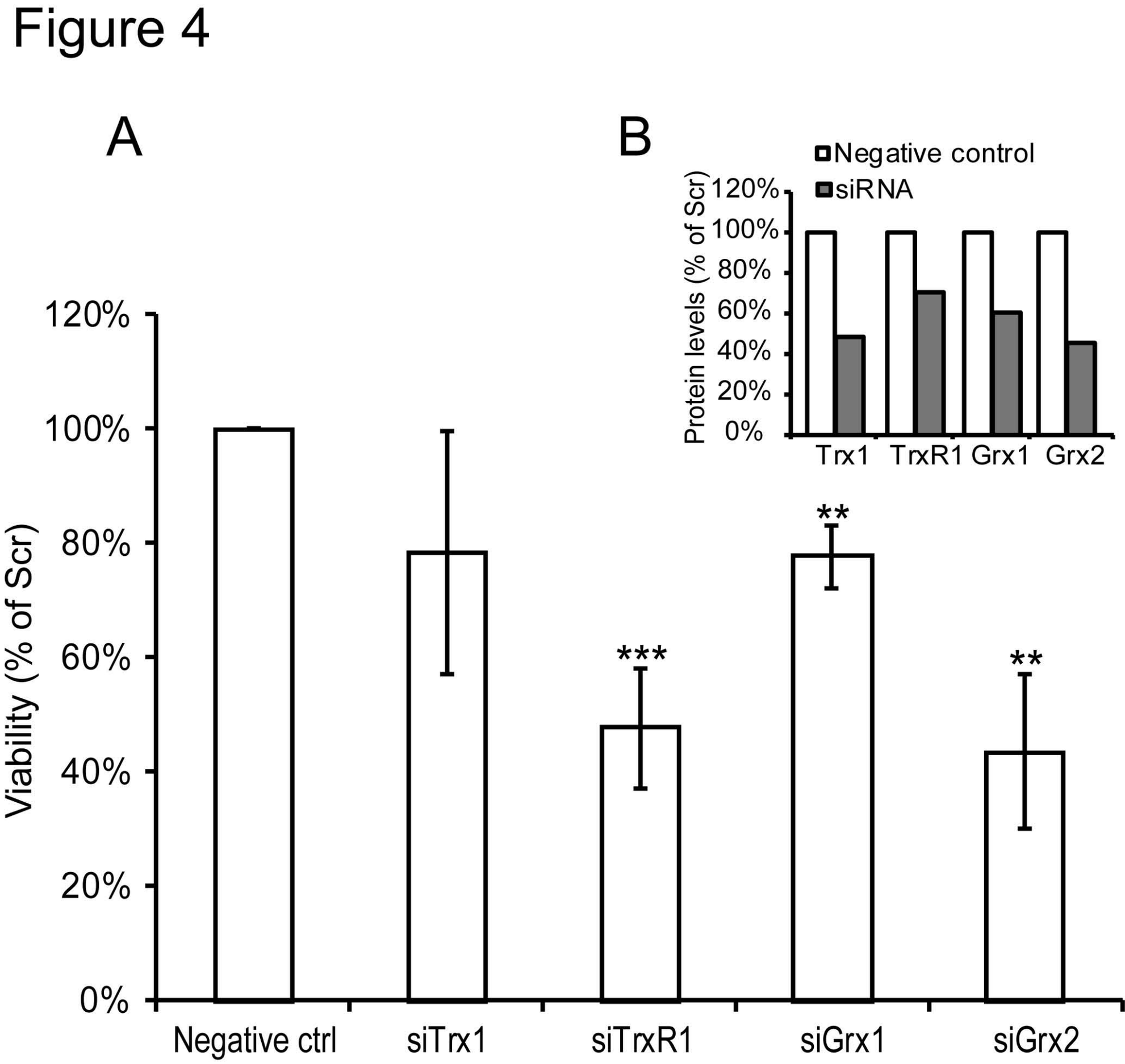
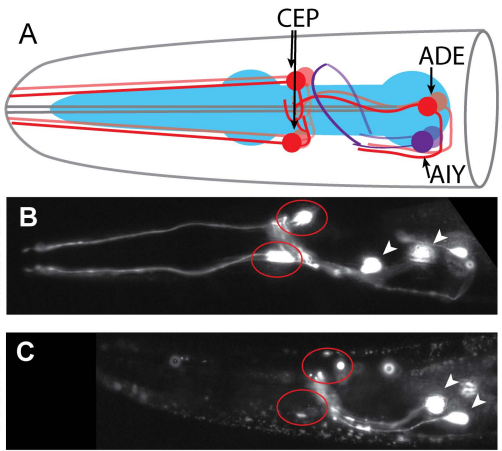

A
B
Negative control
siRNA
Protein levels (% of Scr)
120%
100%
80%
60%
40%
20%
0%
Trx1
TrxR1
Grx1
Grx2
Viability (% of Scr)
***
**
**
Negative ctrl
siTrx1
siTrxR1
siGrx1
siGrx2

Figure 5


A
CEP
ADE
AIY
B
Unaffected
C
Cell loss

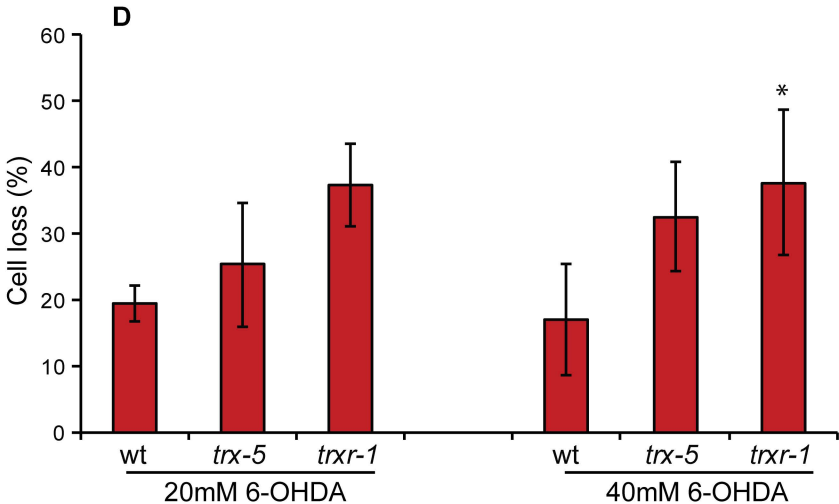
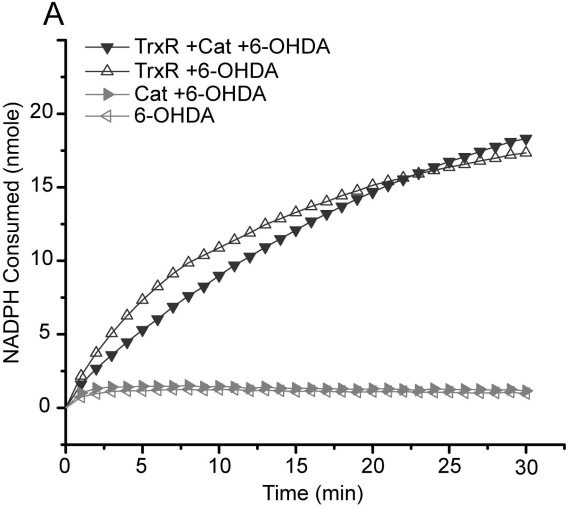
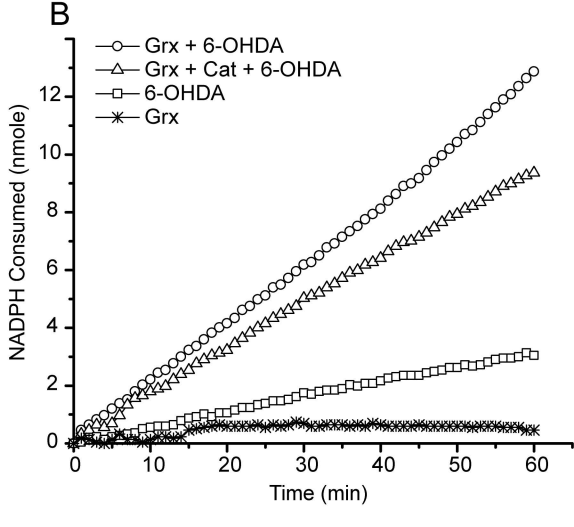
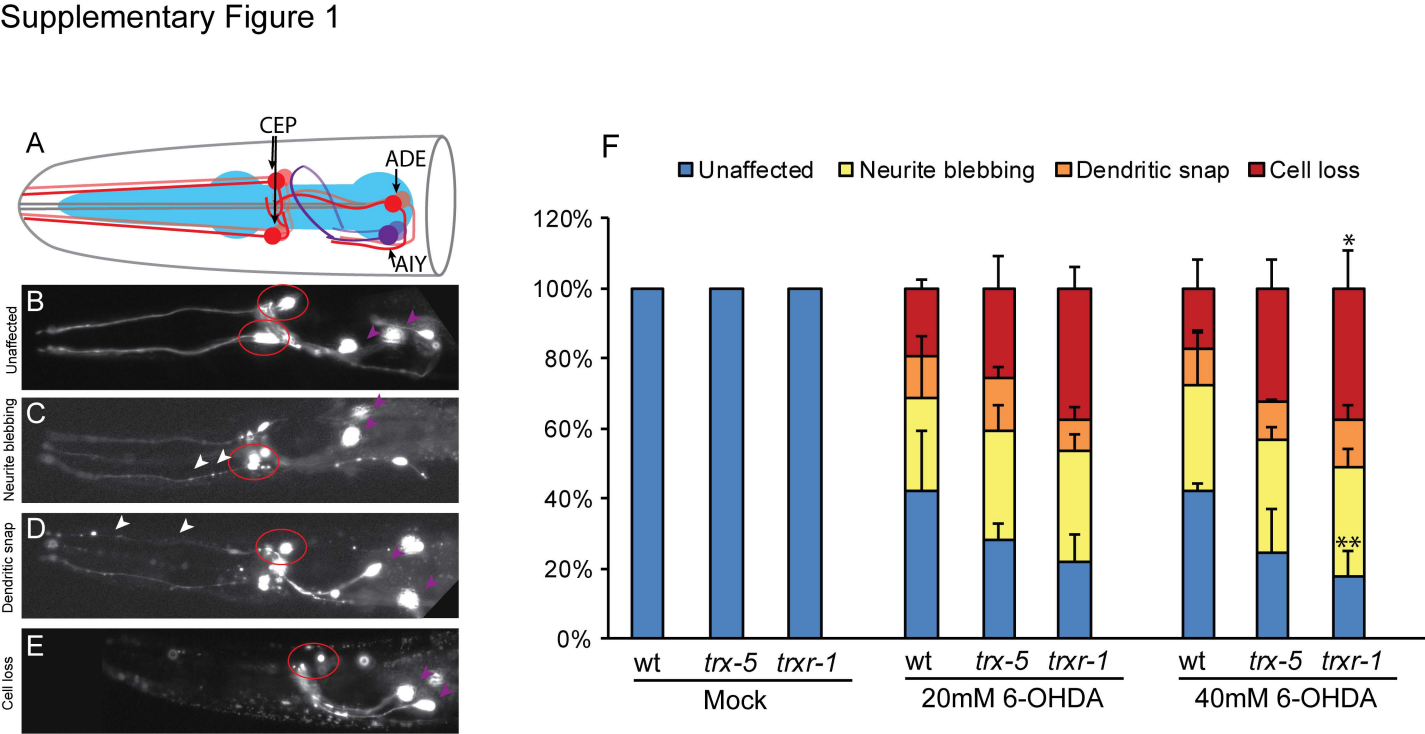

D
Cell loss (%)
60
50
40
30
20
10
0
*
wt
trx-5
trxr-1
20mM 6-OHDA
wt
trx-5
trxr-1
40mM 6-OHDA

Figure 6


A
TrxR +Cat +6-OHDA
TrxR +6-OHDA
Cat +6-OHDA
6-OHDA
NADPH Consumed (nmole)
Time (min)
0
5
10
15
20
25
30


B
Grx + 6-OHDA
Grx + Cat + 6-OHDA
6-OHDA
Grx
NADPH Consumed (nmole)
Time (min)
0
2
4
6
8
10
12
0
10
20
30
40
50
60

Supplementary Figure 1



Supplementary figure 2