Glutaredoxin 1 downregulation in the substantia nigra leads to dopaminergic degeneration in mice

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Abbreviations

AAV	Adeno-associated virus
ANOVA	Analysis of variance
ASK1	Apoptosis signal regulating kinase 1
CDLD	Carbidopa-levodopa
CMV	Cytomegalovirus
DA	Dopaminergic
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EBST	Elevated body swing test
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
Grx1	Glutaredoxin 1
GSH	Glutathione
GSSG	Glutathione disulfide
IHC	Immunohistochemistry
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MKK3/6	Mitogen activated protein kinase kinase 3/6
MKK4/7	Mitogen activated protein kinase kinase 4/7
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PP2A	Protein phosphatase 2 A
PrSH	Protein thiol
PrSSG	Protein-glutathione mixed disulfide
qRT-PCR	quantitative real time polymerase chain reaction
scrRNA	scrambled RNA
shRNA	short hairpin RNA
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
Trx1	Thioredoxin 1
VTA	Ventral tegmental area

Abstract:

Background: Parkinson's disease (PD) is characterized by a severe loss of the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Perturbation of protein thiol redox homeostasis has been shown to play a role in the dysregulation of cell death and cell survival signaling pathways in these neurons. Glutaredoxin 1 (Grx1) is a thiol/disulfide oxidoreductase that catalyzes the deglutathionylation of proteins and is important for regulation of cellular protein thiol redox homeostasis. **Objectives:** We evaluated if downregulation of Grx1 could lead to dopaminergic degeneration and PD-relevant motor deficits in mice. **Methods:** Grx1 was downregulated unilaterally through viral vector-mediated transduction of short hairpin RNA (shRNA) against Grx1 into SNpc. Behavioral assessment was performed through rotarod and elevated body swing test. Stereological analysis of tyrosine hydroxylase (TH) and Nissl positive neurons was carried out to evaluate neurodegeneration. **Results:** Downregulation of Grx1 resulted in contralateral bias of elevated body swing and reduced latency to fall off accelerating rotarod. This was accompanied by a loss of TH positive neurons in SNpc and their DA projections in the striatum. Further, there was a loss Nissl positive neurons in SNpc indicating cell death. This was selective to SNpc neurons since DA neurons in VTA were unaffected akin to that seen in human PD. Further, Grx1 mRNA expression was substantially decreased in SNpc from PD patients. **Conclusions:** Our study indicates that Grx1 is critical for the survival of SNpc DA neurons and that it is downregulated in human PD.

INTRODUCTION

Cardinal histopathological feature of Parkinson's disease (PD) is the severe loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the loss of dopaminergic axonal projections to the striatum. This results in reduced DA signaling in the basal ganglia circuit leading to movement deficits, in particular hypokinesia, bradykinesia and rigidity. Selective vulnerability of SNpc DA neurons has been attributed to several putative mechanisms such as mitochondrial dysfunction [1–4], dopamine metabolism [5], iron accumulation [6,7], dysregulation of the ubiquitin proteasome system [8,9] and increased oxidative stress [10–12]. While different mechanisms have been implicated in the pathogenesis of PD, most of them converge on increased oxidative damage to proteins and other cellular constituents.

Perturbation of protein thiol redox homeostasis as a result of increased oxidative stress mediates cell death signaling that could drive neurodegeneration in PD [13,14]. In its reduced state, thioredoxin (Trx1) binds to apoptosis signal-regulating kinase 1 (ASK1), which inhibits ASK1 activity. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, selective thiol oxidation of thioredoxin (Trx1) releases its inhibitory association with ASK1 leading to its activation [15]. Activated ASK1 further activates p38 and c-Jun N-terminal kinase (JNK) through phosphorylation of mitogen activated protein kinase kinase 3/6 (MKK3/6) and mitogen activated protein kinase kinase 4/7 (MKK4/7), respectively that results in cell death. Thiol oxidation of critical cysteines in protein kinase B (Akt) leading to its increased dephosphorylation through enhanced association with protein phosphatase 2A (PP2A) has also been reported to cause downregulation of Akt mediated cell survival in MPTP mouse model [16].

Glutaredoxin 1 (Grx1), a protein thiol/disulfide oxidoreductase, is important for regulating cellular protein thiol redox homeostasis [17]. Glutathione (GSH) is a cellular antioxidant defense mechanism that protects proteins from irreversible oxidative damage through reversible glutathionylation. Grx1 acts as a deglutathionylating enzyme that revives the active protein thiols and restores protein thiol redox homeostasis [13] by catalyzing the reversible deglutathionylation of protein-glutathione mixed disulfide (PrSSG) in an NADPH dependent manner thereby restoring the steady-state function of the protein as depicted in supplementary figure S1 [14,17,18].

Grx1 downregulation could thus, potentially result in diverse alterations within the cell caused by the resulting disequilibrium in protein thiol homeostasis. In the *Caenorhabditis elegans* model of familial PD wherein human pathogenic LRRK2 mutants were overexpressed, downregulation of Grx1 homologue led to exacerbation of neurodegeneration [19]. We, therefore, set out to determine whether Grx1 downregulation would lead to development of dopaminergic degeneration in mouse. To this end, a viral vector coding for a short hairpin RNA (shRNA) against Grx1 was injected unilaterally into mouse SNpc.

MATERIALS AND METHODS

Animals: All experiments were performed with 3-5 months old C57BL/6 male mice (25-30 grams) or post natal day 0-1 pups (p0-1) from C57BL/6 mice obtained from Indian Institute of Science. Detailed description of animal experiments is provided in the supplementary material.

Adeno-Associated Virus (AAV) 6-sh/scRNA-Grx1 vector production: For knocking down Grx1, the downregulation cassettes containing the mU6 promoter and the Grx1

short hairpin RNA (shRNA) or scrambled RNA (scRNA) sequences were subcloned from earlier constructs [20] into the pAAV-Cytomegalovirus (CMV)-β-globin intron-Green Fluorescent Protein (GFP) construct (Stratagene, La Jolla, CA). The shRNA and scRNA sequences are: 5'-TTT GCG GAT GCA GTG ATC TAA TAA GTT CTC TAT TAG ATC ACT GCA TCC GCT TTT T–3' and 5'-CTA GAA AAA GCG GAT GCA CTG ATC TAA TAG AGA ACT TAT TAG ATC ACT GCA TCC G-3' for shRNA, and for scrambled: 5' -TTT GTT GGT TAC GGG GTA TCG ATT CAA GAG ATC GAT ACC CCG TAA CCA ACT TTT T-3' and 5'-CTA GAA AAA GTT GGT TAC GGG GTA TCG ATC TCT TGA ATC GAT ACC CCG TAA CCA A-3'. Briefly, the entire cassette was PCR amplified, and MluI restriction sites were introduced on either end using the following primer sequences: Forward: 5'-AAG ATC ACGCGT GGC CAA GCT TAT CCG ACG C-3'; Reverse: 5'- AAG ATT ACGCGT CC GCG GCC GCA AGA A-3'. These PCR products, along with the pAAV-CMV- β-globin intron-GFP, were then digested with MluI and ligated to yield a hybrid Pol II/Pol III construct expressing CMV-β-globin intron-GFP and upstream mU6-Grx1 shRNA/mU6-Grx1 scRNA in opposite orientation, named AAV-shRNA-Grx1 shRNA and AAV-scRNA-Grx1 respectively. The constructs were tested for efficacy in HEK293T cells (ATCC, Manassas, VA) showing robust Grx1 downregulation within 48 hr.

The downregulation and scrambled pAAV constructs were then packaged as rAAV serotype 6 (AAV6) particles by co-transfecting them individually with pDF6 into AAV-293 cells (Stratagene, La Jolla, CA) [21] using calcium phosphate [22]. At 48 hours after transfection, vector particles were then extracted by cell lysis using repeated freeze-thawing rounds. The lysates were purified and concentrated using fast protein liquid chromatography through a heparin affinity column as described

earlier [23]. Detailed description of estimation of viral titer has been provided in the supplementary material.

Animals were unilaterally injected with AAV-shRNA-Grx1 or AAV-scRNA-Grx1 at a dilution of ~5×10^{10} TU/mL as control. The original titers of the AAV-shRNA-Grx1 and AAV-scRNA-Grx1 vectors were ~2.54×10^{11} TU/mL and ~1.33×10^{11} TU/mL, respectively. These vectors, when used undiluted resulted in high mortality and focalized cell death, as estimated in pilot experiments. Therefore, the AAV-shRNA-Grx1 vector was diluted five times to give an effective titer of ~5×10^{10} TU/mL. The effect of the two dilutions was tested for 14 days and 28 days post-surgery. The scrambled control (scrRNA) was used at a titer equivalent to that of the five-times diluted shRNA-Grx1 vector.

Human PD autopsy samples: All experiments on human autopsy brain tissue were carried out following approval from Institutional Human Ethics Committee (IHEC) and all guidelines were followed (IHEC approval# 4/2010). One half of the brain was fixed and used for neuropathological examination. The other hemi-brain was dissected and flash frozen using dry ice. Sections from the mesencephalon were cut on a cryostat on glass slides kept frozen on dry ice. The SNpc and the VTA were scraped out from the frozen sections and stored at -80°C until use. The whole dissection procedure was performed using RNAse free material. Tissue was prepared from human autopsy midbrain from PD patients and age-matched controls that were obtained from the ICM Brain Bank and the Neuroceb Pitié-Salpêtrière hospital, Paris. A description of the cases studied is provided in the supplementary table S1.

Quantitative real time PCR: A thorough description of RNA isolation and cDNA synthesis is provided in the supplementary text. The nucleotide sequences for primers

used for human and mouse gene expression analysis and the PCR conditions are provided in supplementary tables S2, S3 and S4, respectively.

Statistical Analyses: All statistical analyses were performed using Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla, CA). For comparison of multiple experimental groups, two-way ANOVA with Tukey's multiple comparison tests was performed. Comparison of two groups was performed with unpaired Student's *t*-test. Median absolute deviation (MAD) [24] was the method used for removal of outliers in the qRT-PCR experiments.

RESULTS

Experimental design and Grx1 downregulation using AAV-shRNA-Grx1

The scr/shRNA to Grx1 was cloned under mU6 promoter alongside β-globin-GFP under CMV promoter in the AAV6 backbone (Fig. 1A). Mouse primary cortical neurons were transduced with shRNA to Grx1 and quantification of mRNA levels using qRT-PCR revealed that there was 79% downregulation (Fig. 1B). Grx1 was downregulated by unilateral stereotaxic transduction of AAV-shRNA-Grx1 into mouse SNpc (Fig. 1C). Contralateral bias of elevated body swing (E) as well as latency to fall off rotarod (R) were evaluated prior to surgery and were subsequently monitored over 28 days after virus injection as described in figure 1D.

Locomotor deficits observed post unilateral Grx1 downregulation

In order to quantify asymmetric, motor deficits following unilateral viral injection of SNpc, elevated body swing test (EBST) was performed [25]. Increased bias to turn to the contralateral side of viral injection as measured through EBST (contralateral bias) emerged by the 14^{th} day and persisted until at least the 26^{th} day post-surgery,

implying decreased striatal dopaminergic tone and/or degeneration of SNpc dopaminergic neurons following Grx1 downregulation. Contralateral bias was not observed in animals injected with AAV-scrRNA-Grx1 (Fig. 1E). Further, motor deficits were evaluated by measuring the performance of the mice on rotarod under increased speed of rotation. Latency for falling off rotarod was measured before surgery to rule out inherent animal biases. The AAV-shRNA-Grx1 injected animals showed a significant drop in latency to stay on the rotarod towards 25th day post injection, which was not observed in animals injected with AAV-scrRNA-Grx1 (Fig. 1F).

Loss of SNpc TH positive neurons post Grx1 downregulation

Stereological analysis was performed at 14 days and 28 days post transduction to assess the loss of TH positive neurons in SNpc and VTA. While significant decrease in total number of TH positive neurons in the ipsilateral SNpc was observed at both 14 days (~74%) and 28 days (~72%) post injection, TH positive neurons on the contralateral side and in both hemispheres in scrambled controls were preserved (Fig. 2A). TH positive neurons were also conserved in the SNpc of mice injected with PBS showing that the procedure of stereotaxic injection *per se* did not lead to neurodegeneration (Fig. 2A).

To verify that the loss of TH in SNpc following Grx1 downregulation was indeed due to cell loss and not due to reduced expression of TH protein, stereological analysis of Nissl positive neurons was performed. Indeed, loss of total number of Nissl positive neurons was observed in the ipsilateral hemisphere injected with AAV-shRNA-Grx1 at both 14 days (~51%) and 28 days (~51%) (Fig. 2B). Mice transduced with AAV-shRNA-Grx1 did not show robust loss of TH positive neurons in ipsilateral VTA (Fig. 2C). The number of TH positive neurons also did not change significantly in both ipsilateral and contralateral hemispheres of the AAV-scrRNA-Grx1 and PBS injected

mice in the VTA (Fig. 2C). Representative micrographs showing dopaminergic degeneration following Grx1 downregulation are shown in figure 2D.

Protein thiol levels following Grx1 downregulation

In order to determine if the protein thiol levels were altered, we estimated the levels of GSH and PrSH after dissecting out SNpc from the ipsilateral and contralateral sides 14 days after unilateral injection of AAV with shRNA to Grx1 or a scrambled sequence. We found no difference in GSH levels between the ipsilateral SNpc and contralateral SNpc, where no virus was injected indicating that the total glutathione levels were unaffected following viral injection of shRNA to Grx1 (Fig. 3A). In further validation of this observation, no difference was found in PrSH levels in SNpc between the animals that received shRNA or the scrambled sequence through AAV injection after 14 days (Fig. 3B).

We also stained brain sections from mice treated with AAV-scr/shRNA-Grx1, 14 days post-surgery, for glial fibrillary acidic protein (GFAP) and Iba1. This was done in order to determine if there was reactive astrogliosis and presence of activated microglia, which could contribute to raise in GSH and protein thiol levels in SNpc. Previous studies have reported increased levels of GSH in astrocytes and microglia as compared to neurons [26,27]. It has also been shown that the levels of GSH further increase in microglia upon activation [28,29]. The presence of reactive astrogliosis is evident from the GFAP staining along with the presence of hypertrophic morphology and shorter and thicker processes of the astrocytes on the ipsilateral SNpc (Fig. 3C). The presence of activated microglia with large cell body and thick processes on the ipsilateral SNpc in contrast to the resting microglia with long thin processes on the contralateral SNpc is also evident after Iba1 immunohistochemistry [30] (Fig. 3D). Reactive astrogliosis and activated microglia were, therefore, observed in the

ipsilateral but not in the contralateral SNpc in mice that were injected with shRNA to Grx1 (Fig. 3C and D, respectively). These observations indicate that the reactive astrogliosis and activated microglia in SNpc could have contributed to the GSH and PrSH levels seen in SNpc (Fig. 3A and B, respectively). Therefore, any loss of GSH or PrSH caused by Grx1 downregulation would have been compensated by the reactive astrogliosis and activated microglia.

Loss of TH positive fibers in striatum post Grx1 downregulation

Analysis of fluorescence intensity following immunohistochemistry for TH on serial sections containing striatum was performed to quantify the loss of TH positive axon terminals in the striatum. A loss of TH positive fibers was observed in ipsilateral striatum in the AAV-shRNA-Grx1 injected mice at both 14 days (~80%) and 28 (~64%) days, while TH fibers in mice injected with AAV-scrRNA-Grx1 were preserved (Fig. 4A and B).

Reduced expression of Grx1 mRNA in human PD autopsy tissue

Evaluation of Grx1 mRNA levels in SNpc from human PD autopsy tissue using qRT-PCR showed reduced Grx1 mRNA expression in PD tissue as compared to age-matched controls (~56%) (Fig. 5A). Detailed information on the cases studied is provided in supplementary table 1. Further, as a confirmation that the decrease in the mRNA levels of Grx1 in PD was not a consequence of cell loss, we showed that the mRNA levels of β-actin and GAPDH were not altered in SNpc from PD tissue as compared to controls (Fig. 5B and C, respectively). Further, to ensure that the loss of Grx1 mRNA expression in human autopsy SNpc was not a result of prolonged treatment with dopamine replacement drugs such as carbidopa and levodopa, we evaluated the mRNA expression of Grx1 after per oral treatment of C57BL/6 mice with a combination of carbidopa-levodopa (30mg-10mg per kg body weight in 10% w/v

sucrose) every day for 14 days. Surprisingly, carbidopa and levodopa treatment upregulated Grx1 (~40%; Fig. 5D).

DISCUSSION

Grx1 is a protein thiol/disulfide oxidoreductase that is critical for maintaining the thiol status in cells. As summarized in supplementary figure S1, in this study we observed that downregulation of Grx1 in SNpc through unilateral stereotaxic injection of AAV-shRNA-Grx1 led to the development of motor deficits. This was accompanied by loss of TH positive neurons in ipsilateral SNpc and their fiber terminals in ipsilateral striatum, while VTA was unaffected. Further, decrease in the number of Nissl positive neurons in SNpc confirmed that there was indeed neuronal degeneration. Finally, we observed significant downregulation of Grx1 mRNA in SNpc from human PD autopsy tissue as compared to controls indicating that Grx1 is potentially important for DA neuron maintenance and survival in SNpc.

Grx1 is expressed in neuronal and glial cells as shown by us and others previously [31]. The functional activity of Grx1 is highly dependent on availability of GSH and NADPH [32]. GSH is present in higher levels in glial cells as compared to neurons [26,27,33] and there is a constant cross talk between neurons and glia [34]. In co-culture of neurons with astrocytes, the latter helped alleviate thiol mediated stress response in a manner similar to that seen with GSH [35]. Furthermore, astrocytes release GSH constantly into the medium, which impacts the neuron through xCT (cysteine glutamate transporter) [36]. Thus, a global knock-down of Grx1 would better represent the situation that might be seen in PD patients rather than knocking it down selectively only in the neurons. Thus, the viral transduction of shRNA-Grx1 would result in Grx1 downregulation in all cell types in SNpc, presumably not at similar levels [37]. The neurodegeneration of TH positive neurons

277 in SNpc could, therefore, have been influenced by the altered redox homeostasis
278 caused by global Grx1 downregulation across cell types including glia.
279 Downregulation of Grx1 is expected to lead to an increase in protein-
280 glutathione mixed disulfide (PrSSG) resulting in the loss of PrSH and GSH.
281 However, we did not find significant difference in the levels of total GSH or protein
282 thiols in the ipsilateral SNpc wherein Grx1 was downregulated as compared to the
283 contralateral SNpc or scrambled control. This is surprising since downregulation of
284 Grx1 has been shown to alter the status of cysteine thiols in a variety of proteins
285 including those involved in glycolysis [38]. However, we did see reactive astrogliosis
286 and activated microglia (Fig. 3C and D, respectively) in the ipsilateral SNpc 14 days
287 after the viral injection. Since the tissue is homogenized for the measurement of
288 GSH and PrSH levels, the levels assayed would represent the contribution from all
289 cell types including the above. The lack of reliable histological methods for
290 measuring GSH or PrSH in specific cell types precludes the estimation of these
291 thiols in individual cells. Therefore, while it is well acknowledged that Grx1
292 downregulation leads to alteration in thiol redox homeostasis, we are unable to
293 demonstrate it specifically in the TH positive neurons. Nevertheless, our
294 observations clearly show the loss of TH neurons and increased reactive astrogliosis
295 and activated microglia in the ipsilateral SNpc when Grx1 is downregulated. It could
296 be argued that the estimation of GSH and PrSH could have been done at an earlier
297 time point after the viral injection. However, any measurement done before TH cell
298 loss can be presumed to be transient. It was for this reason that we chose to
299 measure GSH and PrSH levels 14 days after the viral transduction when we saw
300 loss of ~70% TH positive cells.

In contrast to our study, it has been reported that TH loss (assessed by immunoblotting) was not seen in 10 months old Grx1 knock-out mice [39]. However, it is noteworthy that in this study the quantification of TH was done using whole brain homogenate. Since TH expression in the brain is restricted to monoaminergic neurons, assessment of TH expression using whole brain homogenate would result in averaging of signals and any differences that may exist in selective cell populations, such as SNpc, would not be detected. Therefore, the impact of the knock out of Grx1 in dopaminergic neurons of SNpc needs to be ascertained by careful stereology of SNpc neurons carried out after TH staining. Thus, in the present study we clearly demonstrate that viral mediated knock-down of Grx1 results in loss of TH positive neurons in SNpc specifically since dopaminergic neurons in VTA are unaffected (Fig. 2C).

Change in Grx1 activity is particularly relevant in PD since it affects several cellular processes related to DA neurodegeneration in SNpc. For example, downregulation of Grx1 in Neuro-2A cells leads to loss of mitochondrial membrane potential [40] and restoration of its activity reverses the mitochondrial complex I loss seen after MPTP exposure in mice [41,42]. Further, Grx1 downregulation leads to loss of DJ-1, which is a multifunctional protein whose mutations are linked to familial PD [43,44], facilitating cytosolic translocation of the death associated protein (Daxx) thus triggering apoptosis [20]. Grx1 downregulation also exacerbates neurodegeneration in *C. elegans* model of PD that carries LRRK2 mutations [45]. Thiol anti-oxidants such as α-lipoic acid (ALA) when co-administered with MPTP affords protection against loss of TH neurons in substantia nigra and also reverses the loss of complex I activity seen in animals treated with MPTP for 8 days [46]. Further, using human primary neurons we have shown that over-expression of Grx1

reverses 1-methyl-4-phenylpyridinium (MPP^+) mediated loss of phosphorylated Akt (pAkt). In the same study we have also demonstrated that the loss of pAkt is due to the oxidation of the cysteine sulfhydryl groups in Akt which is also reversed by over-expression of Grx1 [16]. These studies clearly demonstrate that Grx1 over-expression can afford protection and reverse the pathogenic processes that lead to degeneration of SNpc neurons in model systems of PD even under conditions in which Grx1 is not the primary target of the toxic compound used to kill dopaminergic neurons. Grx1, therefore, plays a critical role in maintaining protein thiol homeostasis and prevents irreversible oxidation of protein thiols, which could potentially initiate the death signaling cascade [20,47] as seen in PD mouse models treated with MPTP. Thus, there are multiple consequences of Grx1 downregulation and these effects acting alone or in concert may be responsible for the neurodegeneration seen in the current study.

Interestingly, the extent of cell death did not increase between the 14 days and 28 days post viral injection in our study (Fig. 2A). This suggests that TH neurons infected initially with AAV-shRNA-Grx1 die within 14 days of transduction, and cell death does not progress beyond this time-point. Further, we did not observe significant loss of DA neurons in VTA. This selectivity is reminiscent of human PD and the MPTP-induced neurodegeneration in mouse and non-human primate models [48] and suggests that VTA dopaminergic neurons may be protected from Grx1 downregulation through cell-intrinsic and/or extrinsic mechanisms. Several other factors that make VTA DA neurons relatively less vulnerable have also been reported [48–50]. For example, DA neurons in the VTA are more protected against oxidative stress than nigral neurons because they are in an environment with more astrocytes expressing the anti-oxidant enzyme glutathione peroxidase [51].

An important observation was the downregulation of Grx1 mRNA in SNpc from PD patients (Fig. 5A). In order to determine if the downregulation of Grx1 in human PD could have been caused by the dopaminergic treatment taken by all the subjects, we performed a parallel experiment in mice. We fed mice with carbidopa-levodopa (30 mg-10 mg/ kg body weight) for 14 days orally and measured Grx1 in SNpc using qRT-PCR (Fig. 5D). We observed that there was, in fact, an upregulation of Grx1 in mice treated with carbidopa-levodopa as compared to the vehicle controls. Thus, it is unlikely that the decrease in Grx1 seen in human PD patients as reported in the current study is caused by dopaminergic treatment.

The fundamental conclusion of this paper is that knock-down of Grx1 is adequate to cause degeneration of SNpc DA neurons very selectively while preserving them in VTA.

We postulate that the pro-oxidant environment of SNpc coupled with the low levels of GSH in SNpc (which are further lowered in PD) [52] could result in dysregulation of protein thiol homeostasis. The inability to restore the equilibrium could potentially lead to downregulation of glutaredoxin further exacerbating the degeneration process. In a complex disorder such as Parkinson’s disease, it would not be right to attribute Grx1 as a primary mediator of neurodegeneration seen in SNpc. Nevertheless, our study shows that Grx1 is important for maintenance of dopaminergic neurons in SNpc and any perturbation of Grx1 expression can lead to neurodegeneration of DA neurons in SNpc.

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Authors' Contributions

Research project was conceptualized by VR, organized by AV, AR, EH and VR and executed by AV, AR, DB, LD, RPK and BS. Statistical analysis was designed by AV and AR, executed by AV, and reviewed and critiqued upon by EH and VR. First draft of the manuscript was written by AV and VR and was further reviewed and critiqued upon by AR, DB, BS, LD, RPK, EH and VR.

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FIGURE LEGENDS

Figure 1: Locomotor deficits following unilateral AAV mediated downregulation of Grx1 in mouse SNpc

(A) Schematic representation of the AAV6 backbone containing sh/scrRNA-Grx1 cloned under mU6 promoter along with β-globin-GFP under CMV promoter. (B) Significant downregulation of Grx1 was observed in primary cortical cultures following transduction with AAV-shRNA-Grx1. (C) Stereotaxic injection of AAV-sh/scrRNA-Grx1 into SNpc of C57BL/6 mice following anatomical coordinates for SNpc. (D) Diagrammatic representation of the experimental design showing schedule of behavioral recordings. Letter E represents elevated body swing test and R represents rotarod test. (E) Animals injected with AAV-shRNA-Grx1 showed locomotor deficits as compared to those injected with AAV-scrRNA-Grx1 as estimated by measuring fraction of contralateral bias with respect to the total turns in either direction. (F) Measurements of time to fall off rotarod with increased speed of rotation before and at three time points after unilateral, stereotaxic AAV-sh/scrRNA-Grx1 injections in SNpc. The animals with unilateral SNpc injections with AAV-shRNA-Grx1 presented a decrease in time to fall off the rotarod as compared to those injected with AAV-scrRNA-Grx1. Unpaired Student's *t*-test was performed to compare two groups. Two-way ANOVA with Tukey's test was performed for multiple comparisons of the data. Data are represented as mean ± SEM. n=5-8 animals, * stands for $p<0.05$.

Figure 2: Loss of TH positive neurons in SNpc but not in VTA post Grx1 downregulation

(A) Stereological quantification of the number of TH positive neurons in AAV-sh/scrRNA-Grx1 injected ipsilateral (injected) and contralateral (un-injected) SNpc, 14 and 28 days, and PBS injected, 28 days post-surgery. (B) Stereological quantification of the number of Nissl positive neurons in SNpc of AAV-shRNA-Grx1 injected animals at 14 and 28 days post-surgery. (C) Number of TH positive neurons in the ipsilateral and contralateral VTA of AAV-shRNA-Grx1, AAV-scrRNA-Grx1 and PBS after 28 days of injection. (D) Representative micrographs showing TH positive neurons in SNpc post AAV-sh/scrRNA-Grx1 and PBS injection. GFP under CMV promoter is also expressed along with the sh/scrRNA and hence GFP labelled cells are observed on the ipsilateral side of injection. Each dot in the figure represents an individual animal. Two-way ANOVA with Tukey's test was performed for multiple comparisons on the data. Error bars indicate ±S.E.M. n= 4-8. *$p<0.05$. Scale bar represents 500 μm.

Figure 3: Glutathione and protein thiol levels after Grx1 downregulation

(A) The levels of total GSH assayed in the SNpc from ipsilateral and contralateral hemisphere of C57BL/6 mice after unilateral injection of AAV-scr/shRNA-Grx1, 14 days post-surgery. (B) Total protein thiol levels assayed from ipsilateral and contralateral SNpc 14 days after unilateral injection of an AAV-scr/shRNA-Grx1. (C) Representative images depicting reactive astrocytes as observed through immunohistochemistry for GFAP in SNpc 14 days after unilateral injection of AAV-scr/shRNA-Grx1. (D) Representative images depicting activated microglia as observed through immunohistochemistry for Iba1 in SNpc 14 days after unilateral injection of AAV-scr/shRNA-Grx1. Two-way ANOVA with Tukey's test was performed

for multiple comparisons on the data. Data are represented as mean ± SEM. n=5-6, * stands for $p<0.05$. Scale bar represents 20 μm.

Figure 4: Loss of TH positive fibers in striatum post AAV-shRNA-Grx1 injection in SNpc in mice

(A) Fluorescent micrographs illustrating loss of TH positive fibers in striatum on the ipsilateral hemisphere of AAV-shRNA-Grx1 injection. The contralateral hemisphere as well as the hemispheres injected with AAV-scrRNA-Grx1 retain the fibers staining. (B) Quantification of mean fluorescent intensity for A. The ipsilateral hemisphere injected with AAV-shRNA-Grx1 showed significant loss of TH positive fibers as compared to the contralateral hemisphere. Each dot in the figure represents an individual animal. Two-way ANOVA with Tukey's test was performed for multiple comparisons on the data. Data are represented as mean ± SEM. n=5-8, * stands for $p<0.05$. Scale bar represents 1 mm.

Figure 5: Downregulation of Grx1 mRNA in human PD autopsy tissue

(A) Significant downregulation of Grx1 mRNA was observed in PD as compared to controls using qRT-PCR on human autopsy tissue. (B) and (C) No significant change was observed in the mRNA levels of either β-actin or GAPDH, respectively, in SNpc from PD autopsy tissue samples as compared to SNpc from controls. (D) Upregulation of Grx1 mRNA in C57BL/6 mice upon per oral treatment with carbidopa-levodopa (30 mg-10 mg/ kg body weight) for 14 days. Each dot in the figure represents an individual or mouse. Unpaired, two tailed Student's *t*-test was performed to compare two groups. Data are represented as mean ± SEM. n=5-8, * stands for $p<0.05$.

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