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Aditi Verma, Ajit Ray, Deepti Bapat, Latha Diwakar, Reddy Peera Kommaddi, Bernard L Schneider, Etienne C Hirsch, Vijayalakshmi Ravindranath

### ► To cite this version:

Aditi Verma, Ajit Ray, Deepti Bapat, Latha Diwakar, Reddy Peera Kommaddi, et al.. Glutaredoxin 1 downregulation in the substantia nigra leads to dopaminergic degeneration in mice. *Movement Disorders*, 2020, 35 (10), pp.1843-1853. 10.1002/mds.28190 . inserm-02994639

**HAL Id: inserm-02994639**

**<https://inserm.hal.science/inserm-02994639>**

Submitted on 8 Nov 2020

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

3 Aditi Verma, PhD<sup>1</sup>; Ajit Ray, PhD<sup>1</sup>; Deepti Bapat, MSc<sup>1</sup>; Latha Diwakar, PhD<sup>1,2</sup>; Reddy  
4 Peera Kommaddi, PhD<sup>1,2</sup>; Bernard L Schneider, PhD<sup>3</sup>; Etienne C. Hirsch, PhD<sup>4</sup> and  
5 Vijayalakshmi Ravindranath, PhD<sup>1,2</sup> \*

- 6 1. Centre for Neuroscience, Indian Institute of Science, Bangalore-560012, India  
7 2. Centre for Brain Research, Indian Institute of Science, Bangalore-560012, India  
8 3. Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, CH-1015  
9 Lausanne, Switzerland  
10 4. Institut du Cerveau – ICM Inserm U 1127, CNRS UMR 7225, Sorbonne  
11 Université, F-75013, Paris, France

12  
13 Address for correspondence

14

15 Vijayalakshmi Ravindranath,  
16 Centre for Brain Research,  
17 Indian Institute of Science,  
18 C.V. Raman Avenue,  
19 Bangalore - 560012, India,  
20 Phone: +91-80-22933433,  
21 Fax: +91-80-23603323,  
22 email: [viji@iisc.ac.in](mailto:viji@iisc.ac.in)

23

24 Keywords: Parkinson's disease; dopaminergic neurons; glutaredoxin 1; shRNA;  
25 tyrosine hydroxylase

26 Word count (excluding references, abstract and figure legends): 3688

27 Number of references (main text): 52

28 Number of grayscale illustrations: 1 (Figure 5)

29 Number of color illustrations: 4

30

31 Running Title: Glutaredoxin 1 loss leads to DA neuron death

32

## 33 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

35 **Abstract:**

36 **Background:** Parkinson's disease (PD) is characterized by a severe loss of the  
37 dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc).

38 Perturbation of protein thiol redox homeostasis has been shown to play a role in the  
39 dysregulation of cell death and cell survival signaling pathways in these neurons.

40 Glutaredoxin 1 (Grx1) is a thiol/disulfide oxidoreductase that catalyzes the  
41 deglutathionylation of proteins and is important for regulation of cellular protein thiol  
42 redox homeostasis. **Objectives:** We evaluated if downregulation of Grx1 could lead

43 to dopaminergic degeneration and PD-relevant motor deficits in mice. **Methods:**

44 Grx1 was downregulated unilaterally through viral vector-mediated transduction of  
45 short hairpin RNA (shRNA) against Grx1 into SNpc. Behavioral assessment was  
46 performed through rotarod and elevated body swing test. Stereological analysis of

47 tyrosine hydroxylase (TH) and Nissl positive neurons was carried out to evaluate

48 neurodegeneration. **Results:** Downregulation of Grx1 resulted in contralateral bias of

49 elevated body swing and reduced latency to fall off accelerating rotarod. This was

50 accompanied by a loss of TH positive neurons in SNpc and their DA projections in

51 the striatum. Further, there was a loss Nissl positive neurons in SNpc indicating cell

52 death. This was selective to SNpc neurons since DA neurons in VTA were

53 unaffected akin to that seen in human PD. Further, Grx1 mRNA expression was

54 substantially decreased in SNpc from PD patients. **Conclusions:** Our study

55 indicates that Grx1 is critical for the survival of SNpc DA neurons and that it is

56 downregulated in human PD.

## 57 INTRODUCTION

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

68 Perturbation of protein thiol redox homeostasis as a result of increased  
69 oxidative stress mediates cell death signaling that could drive neurodegeneration in  
70 PD [13,14]. In its reduced state, thioredoxin (Trx1) binds to apoptosis signal-  
71 regulating kinase 1 (ASK1), which inhibits ASK1 activity. In the 1-methyl-4-phenyl-  
72 1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, selective thiol oxidation of  
73 thioredoxin (Trx1) releases its inhibitory association with ASK1 leading to its  
74 activation [15]. Activated ASK1 further activates p38 and c-Jun N-terminal kinase  
75 (JNK) through phosphorylation of mitogen activated protein kinase kinase 3/6  
76 (MKK3/6) and mitogen activated protein kinase kinase 4/7 (MKK4/7), respectively  
77 that results in cell death. Thiol oxidation of critical cysteines in protein kinase B (Akt)  
78 leading to its increased dephosphorylation through enhanced association with  
79 protein phosphatase 2A (PP2A) has also been reported to cause downregulation of  
80 Akt mediated cell survival in MPTP mouse model [16].

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

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

98

## 99 **MATERIALS AND METHODS**

100 Animals: All experiments were performed with 3-5 months old C57BL/6 male mice  
101 (25-30 grams) or post natal day 0-1 pups (p0-1) from C57BL/6 mice obtained from  
102 Indian Institute of Science. Detailed description of animal experiments is provided in  
103 the supplementary material.

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

106 short hairpin RNA (shRNA) or scrambled RNA (scRNA) sequences were subcloned  
107 from earlier constructs [20] into the pAAV-Cytomegalovirus (CMV)- $\beta$ -globin intron-  
108 Green Fluorescent Protein (GFP) construct (Stratagene, La Jolla, CA). The shRNA  
109 and scRNA sequences are: 5'-TTT GCG GAT GCA GTG ATC TAA TAA GTT CTC  
110 TAT TAG ATC ACT GCA TCC GCT TTT T-3' and 5'-CTA GAA AAA GCG GAT GCA  
111 CTG ATC TAA TAG AGA ACT TAT TAG ATC ACT GCA TCC G-3' for shRNA, and  
112 for scrambled: 5' -TTT GTT GGT TAC GGG GTA TCG ATT CAA GAG ATC GAT  
113 ACC CCG TAA CCA ACT TTT T-3' and 5'-CTA GAA AAA GTT GGT TAC GGG GTA  
114 TCG ATC TCT TGA ATC GAT ACC CCG TAA CCA A-3'. Briefly, the entire cassette  
115 was PCR amplified, and MluI restriction sites were introduced on either end using  
116 the following primer sequences: Forward: 5'-AAG ATC ACGCGT GGC CAA GCT  
117 TAT CCG ACG C-3'; Reverse: 5'- AAG ATT ACGCGT CC GCG GCC GCA AGA A-  
118 3'. These PCR products, along with the pAAV-CMV-  $\beta$ -globin intron-GFP, were then  
119 digested with MluI and ligated to yield a hybrid Pol II/Pol III construct expressing  
120 CMV- $\beta$ -globin intron-GFP and upstream mU6-Grx1 shRNA/mU6-Grx1 scRNA in  
121 opposite orientation, named AAV-shRNA-Grx1 shRNA and AAV-scRNA-Grx1  
122 respectively. The constructs were tested for efficacy in HEK293T cells (ATCC,  
123 Manassas, VA) showing robust Grx1 downregulation within 48 hr.

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



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

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

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

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

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

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

## 162 **RESULTS**

### 163 Experimental design and Grx1 downregulation using AAV-shRNA-Grx1

164 The scr/shRNA to Grx1 was cloned under mU6 promoter alongside  $\beta$ -globin-  
165 GFP under CMV promoter in the AAV6 backbone (Fig. 1A). Mouse primary cortical  
166 neurons were transduced with shRNA to Grx1 and quantification of mRNA levels using  
167 qRT-PCR revealed that there was 79% downregulation (Fig. 1B). Grx1 was  
168 downregulated by unilateral stereotaxic transduction of AAV-shRNA-Grx1 into mouse  
169 SNpc (Fig. 1C). Contralateral bias of elevated body swing (E) as well as latency to fall  
170 off rotarod (R) were evaluated prior to surgery and were subsequently monitored over  
171 28 days after virus injection as described in figure 1D.

### 172 Locomotor deficits observed post unilateral Grx1 downregulation

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

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

#### 185 Loss of SNpc TH positive neurons post Grx1 downregulation

186 Stereological analysis was performed at 14 days and 28 days post transduction  
187 to assess the loss of TH positive neurons in SNpc and VTA. While significant decrease  
188 in total number of TH positive neurons in the ipsilateral SNpc was observed at both 14  
189 days (~74%) and 28 days (~72%) post injection, TH positive neurons on the  
190 contralateral side and in both hemispheres in scrambled controls were preserved (Fig.  
191 2A). TH positive neurons were also conserved in the SNpc of mice injected with PBS  
192 showing that the procedure of stereotaxic injection *per se* did not lead to  
193 neurodegeneration (Fig. 2A).

194 To verify that the loss of TH in SNpc following Grx1 downregulation was indeed  
195 due to cell loss and not due to reduced expression of TH protein, stereological analysis  
196 of Nissl positive neurons was performed. Indeed, loss of total number of Nissl positive  
197 neurons was observed in the ipsilateral hemisphere injected with AAV-shRNA-Grx1 at  
198 both 14 days (~51%) and 28 days (~51%) (Fig. 2B). Mice transduced with AAV-  
199 shRNA-Grx1 did not show robust loss of TH positive neurons in ipsilateral VTA (Fig.  
200 2C). The number of TH positive neurons also did not change significantly in both  
201 ipsilateral and contralateral hemispheres of the AAV-scrRNA-Grx1 and PBS injected

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

204 Protein thiol levels following Grx1 downregulation

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

214 We also stained brain sections from mice treated with AAV-scr/shRNA-Grx1,  
215 14 days post-surgery, for glial fibrillary acidic protein (GFAP) and Iba1. This was done  
216 in order to determine if there was reactive astrogliosis and presence of activated  
217 microglia, which could contribute to raise in GSH and protein thiol levels in SNpc.  
218 Previous studies have reported increased levels of GSH in astrocytes and microglia  
219 as compared to neurons [26,27]. It has also been shown that the levels of GSH further  
220 increase in microglia upon activation [28,29]. The presence of reactive astrogliosis is  
221 evident from the GFAP staining along with the presence of hypertrophic morphology  
222 and shorter and thicker processes of the astrocytes on the ipsilateral SNpc (Fig. 3C).  
223 The presence of activated microglia with large cell body and thick processes on the  
224 ipsilateral SNpc in contrast to the resting microglia with long thin processes on the  
225 contralateral SNpc is also evident after Iba1 immunohistochemistry [30] (Fig. 3D).  
226 Reactive astrogliosis and activated microglia were, therefore, observed in the

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

### 233 Loss of TH positive fibers in striatum post Grx1 downregulation

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

### 240 Reduced expression of Grx1 mRNA in human PD autopsy tissue

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

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

## 254 **DISCUSSION**

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

265 Grx1 is expressed in neuronal and glial cells as shown by us and others  
266 previously [31]. The functional activity of Grx1 is highly dependent on availability of  
267 GSH and NADPH [32]. GSH is present in higher levels in glial cells as compared to  
268 neurons [26,27,33] and there is a constant cross talk between neurons and glia [34].  
269 In co-culture of neurons with astrocytes, the latter helped alleviate thiol mediated  
270 stress response in a manner similar to that seen with GSH [35]. Furthermore,  
271 astrocytes release GSH constantly into the medium, which impacts the neuron  
272 through xCT (cysteine glutamate transporter) [36]. Thus, a global knock-down of  
273 Grx1 would better represent the situation that might be seen in PD patients rather  
274 than knocking it down selectively only in the neurons. Thus, the viral transduction of  
275 shRNA-Grx1 would result in Grx1 downregulation in all cell types in SNpc,  
276 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.

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

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



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

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

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

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

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

372 **Acknowledgements**

373 The authors are grateful to Prof. Patrick Aebischer (Ecole Polytechnique Fédérale de  
374 Lausanne, Switzerland) for providing lab resources and Indo-Swiss Bilateral Research  
375 Initiative for providing funding support to AR for carrying out AAV production and  
376 titration. We thank Ms. Rehab Hussain for assistance with immunohistochemistry. We  
377 would also like to thank Pr Charles Duyckaerts for the neuropathological examination  
378 and Annick Prigent and Sabrina Leclere-Turbant for preparing the human post-mortem  
379 samples.

380 **Authors' Contributions**

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

386

387 **Financial Disclosure Statement:**

388 The study was funded by TATA Trusts, India (VR). AV received research fellowship  
389 from Council of Scientific and Industrial Research (CSIR), Government of India. EH  
390 acknowledges the financial support from Neuroceb, CNRS, INSERM, ICM, Sorbonne  
391 Université and from the program "Investissements d'avenir" ANR-10-IAIHU-06. The  
392 authors declare no other financial disclosures.

393 **FIGURE LEGENDS**

394

395 Figure 1: Locomotor deficits following unilateral AAV mediated downregulation of

396 Grx1 in mouse SNpc

397 (A) Schematic representation of the AAV6 backbone containing sh/scrRNA-Grx1

398 cloned under mU6 promoter along with  $\beta$ -globin-GFP under CMV promoter. (B)

399 Significant downregulation of Grx1 was observed in primary cortical cultures

400 following transduction with AAV-shRNA-Grx1. (C) Stereotaxic injection of AAV-

401 sh/scrRNA-Grx1 into SNpc of C57BL/6 mice following anatomical coordinates for

402 SNpc. (D) Diagrammatic representation of the experimental design showing

403 schedule of behavioral recordings. Letter E represents elevated body swing test and

404 R represents rotarod test. (E) Animals injected with AAV-shRNA-Grx1 showed

405 locomotor deficits as compared to those injected with AAV-scrRNA-Grx1 as

406 estimated by measuring fraction of contralateral bias with respect to the total turns in

407 either direction. (F) Measurements of time to fall off rotarod with increased speed of

408 rotation before and at three time points after unilateral, stereotaxic AAV-sh/scrRNA-

409 Grx1 injections in SNpc. The animals with unilateral SNpc injections with AAV-

410 shRNA-Grx1 presented a decrease in time to fall off the rotarod as compared to

411 those injected with AAV-scrRNA-Grx1. Unpaired Student's *t*-test was performed to

412 compare two groups. Two-way ANOVA with Tukey's test was performed for multiple

413 comparisons of the data. Data are represented as mean  $\pm$  SEM. n=5-8 animals, \*

414 stands for  $p < 0.05$ .

415

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

417 downregulation

418 (A) Stereological quantification of the number of TH positive neurons in AAV-  
419 sh/scrRNA-Grx1 injected ipsilateral (injected) and contralateral (un-injected) SNpc,  
420 14 and 28 days, and PBS injected, 28 days post-surgery. (B) Stereological  
421 quantification of the number of Nissl positive neurons in SNpc of AAV-shRNA-Grx1  
422 injected animals at 14 and 28 days post-surgery. (C) Number of TH positive neurons  
423 in the ipsilateral and contralateral VTA of AAV-shRNA-Grx1, AAV-scrRNA-Grx1 and  
424 PBS after 28 days of injection. (D) Representative micrographs showing TH positive  
425 neurons in SNpc post AAV-sh/scrRNA-Grx1 and PBS injection. GFP under CMV  
426 promoter is also expressed along with the sh/scrRNA and hence GFP labelled cells  
427 are observed on the ipsilateral side of injection. Each dot in the figure represents an  
428 individual animal. Two-way ANOVA with Tukey's test was performed for multiple  
429 comparisons on the data. Error bars indicate  $\pm$ S.E.M. n= 4-8. \*p<0.05. Scale bar  
430 represents 500  $\mu$ m.

431

432 Figure 3: Glutathione and protein thiol levels after Grx1 downregulation

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

442 for multiple comparisons on the data. Data are represented as mean  $\pm$  SEM. n=5-6,  
443 \* stands for p<0.05. Scale bar represents 20  $\mu$ m.

444

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

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

456

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

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

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