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

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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)- β -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- β -globin intron-GFP, were then
119 digested with MluI and ligated to yield a hybrid Pol II/Pol III construct expressing
120 CMV- β -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°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 β -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 14th day and persisted until at least the 26th 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 25th 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 β -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 α -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⁺) 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.

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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
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386

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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 β -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 μ 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 μ 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 β -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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