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Title: Induced Cognitive Impairments Reversed by Grafts of Neural Precursors: a Longitudinal Study in a Macaque Model of Parkinson's Disease

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One Sentence Summary: Empirical evidence that cell therapy efficiently reverts cognitive and clinical motor symptoms in the non-human primate model of Parkinson's disease.

Abstract:

Parkinson's disease (PD) evolves over an extended and variable period in humans; several years prior to the onset of classical motor symptoms, cognitive deficits as well as sleep and biological rhythm disorders develop and worsen with disease progression, significantly impacting the quality of life of patients. The gold standard MPTP macaque model of PD recapitulates the progression of motor and non-motor symptoms over contracted periods of time.

Here, this multidisciplinary and multiparametric study follows, in five animals, the steady progression of motor and non-motor symptoms and describes their reversal following bilateral grafts of neural precursors in diverse functional domains of the basal ganglia.

Results show unprecedented recovery from cognitive symptoms in addition to a strong clinical motor recuperation. Both motor and cognitive recovery and partial circadian rhythm recovery correlate with the degree of graft integration into the host environment as well as with in-vivo levels of striatal dopaminergic innervation and function.

Given inter-individuality of disease progression and recovery the present study underlines the importance of longitudinal multidisciplinary assessments in view of clinical translation and provides empirical evidence that integration of neural precursors following transplantation efficiently restores function at multiple levels in parkinsonian non-human primates.

40 **Main Text:**

41 **INTRODUCTION**

42 Parkinson's disease (PD) is a neurodegenerative condition affecting up to 10M of the worldwide
43 population with incidences increasing with age, making PD the fastest growing neurological disorder in
44 a globally aging population [1]. The clinical manifestation is primarily motor with a typical parkinsonian
45 syndrome including bradykinesia, rigidity and resting tremor and is confirmed by clinical diagnostic
46 tools including *in-vivo* imaging of the denervation of the nigrostriatal dopaminergic (DA) axis [2].
47 Unfortunately, 60-80% of DA cells are lost prior to the onset of clinically diagnosed motor symptoms
48 [3]. During this so-called premotor period preceding the clinical threshold, the DA-lesion is progressive
49 and accompanied by the manifestation and further deterioration of non-motor symptoms including
50 cognitive disorders as well as perturbation of circadian rhythm and sleep disorders [4, 5]. Although
51 considered secondary, non-motor symptoms nonetheless have a significant impact on quality of life; the
52 cognitive abilities of the vast majority of PD patients deteriorate leading to psychiatric disturbances [6]
53 with circadian perturbations affecting biological rhythms [7].

54 DA lesion is the hallmark of PD and in addition to circadian regulation affects multiple frontal lobe
55 functions including performance monitoring, motivational aspects of behavior and motricity. However,
56 there is currently no cure for PD and palliative therapies such as levodopa and Deep-Brain Stimulation
57 (DBS), mainly correct motor symptoms and often give rise to behavioral perturbation [8]. For example,
58 DBS shows no long-lasting effect on axial symptoms e.g. freezing of gait and negatively impacts
59 cognitive and neuropsychiatric symptoms [9, 10]. Importantly palliative therapies do not solve the issue
60 of neuronal loss and the long-term prognosis. Consequently, cell replacement therapy for PD has been
61 recently re-evaluated as a potential cure for PD [11], leading to recent trials [12] aimed at improved
62 grafting procedures, and to pave the way for stem-cell based transplantation in humans. In this respect
63 there is a recognized need for more detailed perspectives from pre-clinical investigations in animal
64 models [13].

65 With the aim of optimizing the therapeutic use of cell replacement in PD we have examined the global
66 consequence of cell grafts at different stages of the disease in a macaque model of PD. While numerous
67 efforts have been deployed to use non-motor symptoms as premotor markers for the prognosis of PD
68 [14], virtually no studies have addressed the impact of cell replacement on non-motor symptoms [15]
69 leading to insufficient appraisal of their multi-parametric impact at either pre-clinical or clinical stages
70 [8, 16]. This is problematic because advanced PD with an extensive nigrostriatal DA lesion constitutes a
71 highly deleterious environment for the survival of grafted cells [17, 18]. Hence in the present study we
72 use an experimental design that allows us to address the capacity of cell grafts to reverse the disease at
73 disease onset in the motor phase and assess the impact of DA lesion extent on graft efficiency. This
74 design also allowed us to characterize the dynamics of graft induced recovery by comparing it to
75 spontaneous recovery in the premotor stage. Because non-motor symptoms have a gradual onset prior to
76 clinical manifestations of DA denervation, evaluation of the effects of NP grafts in parkinsonian non-
77 human primates (NHP) requires a longitudinal multi-parametric investigation, which we have
78 implemented in the present study. We grafted neural precursors (NPs) because they hold the promise of
79 enhanced efficiency compared to DA neurons [19-21], as in addition to their neuro-restorative potential
80 NPs have the capacity to differentiate into neuroprotective astroglial cells [22-24].

81 Repeated systemic injections of low-doses of MPTP (0.2 mg/kg every 3-4 days) induced a parkinsonian
82 syndrome with a slow and typically dorso-ventral progression of the nigrostriatal denervation, together
83 with a premotor expression of cognitive and circadian *i.e.* non-motor symptoms [25-27]. Here, we
84 implemented a multidisciplinary / multiparametric approach to assess the therapeutic potential of
85 bilateral grafts of NPs in multiple functional domains of the basal ganglia of NHP (*macaca fascicularis*).
86 For ethical reasons we reduced the number of animals used by monitoring individual macaques so that

87 each case constitutes its own control. This required continuous assessment of clinical motor and non-
88 motor symptoms as well as *in-vivo* monitoring of the nigrostriatal lesion with Positron Emission
89 Topography (PET) imaging of DA transporters (DAT). Monitoring was prior to and following the
90 induction of stable motor symptoms [27], and subsequent to transplantation of NPs. In addition, Fluoro-
91 DOPA (¹⁸F-DOPA) imaging allowed assessment of the impact of the NP grafts on DA function [28].
92 Finally, *post-mortem* immunohistological examination of NP grafts was performed in order to evaluate
93 the impact of the graft on host tissue as well the survival, integration and differentiation fate of the grafted
94 cells into the host brain.

95 RESULTS

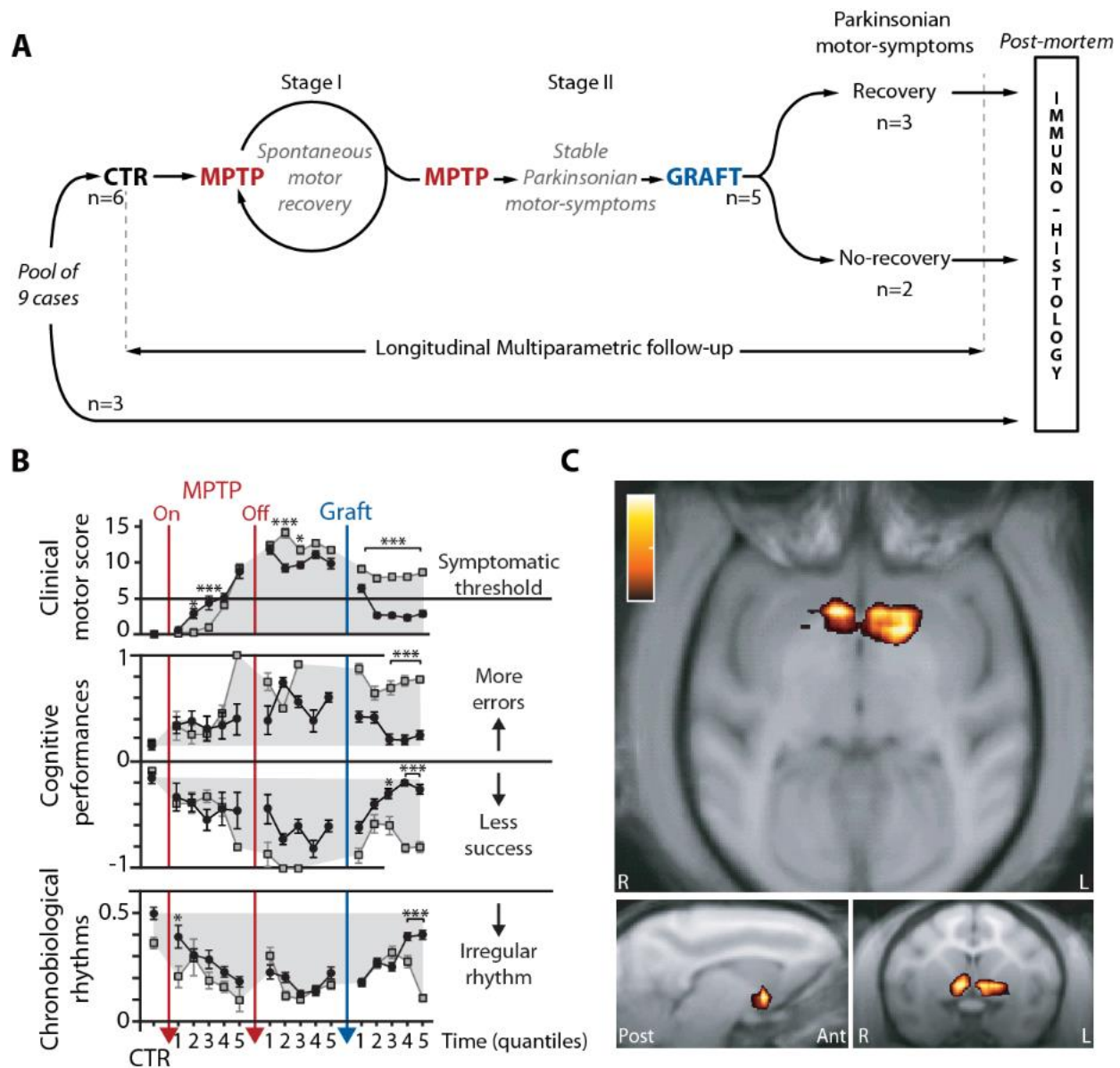
96 The experimental design (**Fig.1A, Table S1**) allowed full characterization of MPTP intoxication. In
97 Stage I, all animals show non-motor symptoms [25, 26] and a nigrostriatal DA denervation superior to
98 70% [27] but, at this stage, spontaneous recovery from motor symptoms occurs and this was investigated
99 in 3 out of the 6 individual cases [25, 27, 29]. Comparison of cognitive, circadian and DA function
100 markers enabled characterization of spontaneous and graft-induced recovery. Subsequent to Stage I,
101 resuming MPTP injections until a clinical score of 10 is reached on two consecutive days [27] leads to a
102 Stage II, where motor symptoms become permanent. In order to implement this experimental strategy it
103 is necessary to adapt cumulated MPTP-doses to individual clinical motor scores (**Table S2**) thereby
104 taking into account the known variability of response to MPTP and adjustment of the intoxication
105 protocol (**Fig.1A, Table S2, see Materials and Methods**). Clinical scores and behavioral measures were
106 divided into quantiles (Q1-5, as described in [25], see **Materials and Methods**) in order to compare
107 cases on the basis of the presumed DA-lesion rather than on the time spent in the premotor (MPTP),
108 motor (post-MPTP/pre-graft) and post-graft periods. We compared groups (*recovered* vs. *non-recovered*)
109 across control, premotor and motor quantiles (pre-/post-MPTP) to evaluate differences in behavioral
110 markers between groups due to MPTP intoxication and, across pre- and post-grafts quantiles (pre-/post-
111 graft) in order to evaluate differences between groups due to NP grafts (one-way anova: F-test
112 (groups,df), p-value, effect-size (η_p^2), see **Materials and Methods**).

113 Behavioral and functional markers of chronic MPTP-induced Parkinsonism

114 During Stage II, all cases displayed a stable parkinsonian syndrome with clinical motor scores ranging
115 from mild to severely symptomatic (**Figs.1B, S1A**). In two out of the five grafted cases, we failed to
116 observe recovery from motor symptoms whereas the three other cases exhibited robust recovery from
117 motor symptoms with a clinical motor score returning below the symptomatic threshold (pre-/post-graft:
118 $F(2,10) = 126, p < 2E-16, \eta_p^2 = 0.50$ while effect-size was clearly reduced over pre-/post-MPTP
119 quantiles: $F(2,11) = 11, p < 2E-16, \eta_p^2 = 0.14$, **Figs.1B, S1A, Table S8**). We accordingly assigned
120 cases to the *non-recovered* or *recovered* group based on clinical motor outcome following NP grafts
121 (**Figs.1A-B, S1A**).

122 During the pre-Graft period, non-motor symptoms emerged in all animals with progressive deterioration
123 during the premotor period *i.e.* before motor scores reached clinical thresholds (**Figs.1B, S1**). Alterations
124 of circadian rhythms appeared progressively during the premotor phase (**Figs.1B, S1C**) to reach peak
125 levels during the symptomatic period as previously described [26]. This pattern of decreased (increased)
126 activity during the Light (Dark) phase resembles circadian alterations of the sleep-wake cycle observed
127 in PD patients which manifests as increased daytime sleepiness and fragmented sleep structure [30]. The
128 premotor and motor alteration of rest-wake locomotor rhythms in all cases exhibited reduced light/dark
129 (LD) ratios (minimal effect-size of group difference across pre-/post-MPTP quantiles: $F(2,11) =$
130 $4.3, p = 3.2E-16, \eta_p^2 = 0.07$, **Fig.1B, Table S8**) and relative amplitude, as well as increased rhythm
131 variability and fragmentation (**Fig.S1C**). In all cases cognitive performance deteriorated during MPTP
132 intoxication and after offset (pre-/post-MPTP quantiles, no group difference in Success: $F(2,11) =$

133 1.8, $p > 0.05$, $\eta_p^2 = 0.11$, and similarly minor effect-size of group for Errors: $F(2,11) = 2.1$, $p =$
 134 0.03, $\eta_p^2 = 0.12$, **Figs.1B, S1B**).

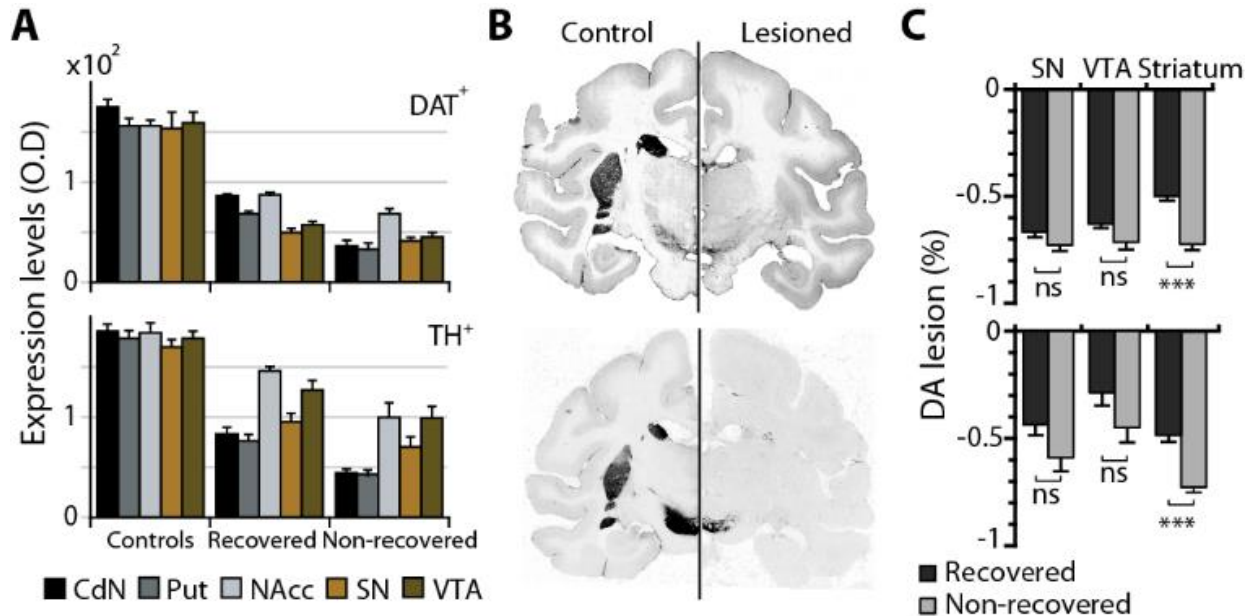


135

136 **Fig. 1. Motor, non-Motor and Functional Markers of Graft-induced Recovery.** (A) Study design. At early stages
 137 of MPTP treatment (Stage I) there is spontaneous recovery from motor symptoms but not from cognitive and circadian
 138 symptoms. Continued MPTP intoxication leads to Stage II where motor symptoms now become permanent. (B) Cases are
 139 grouped in *recovered* (black) vs. *non-recovered* (grey) according to the level of parkinsonian motor-symptoms following cell
 140 therapy. Case2 excluded because of per-operative brain hemorrhage following graft. Motor symptoms clinically scored with
 141 the Parkinsonian Monkey Rating Scale (PMRS top panel), Cognitive performances (range normalized to control performance,
 142 -1 for success, middle panel) and, Chronobiological rhythms reflected by the ratio of spontaneous locomotor activity during
 143 the light and dark periods (normalized contrast with Light/Dark ratio in control conditions, bottom panel). Means \pm SE, anova
 144 between groups, adjusted p-values for multiple comparisons * < 0.05 , *** < 0.001 , see **Table S8**. (C) Increase of nigrostriatal
 145 innervation. Parametric maps of DAT binding potential with axial (top), sagittal (bottom-left panel) and coronal (bottom-right
 146 panel) views of significant positive difference for the contrast Pre- vs. Post-Graft (color scale from 0.2-1, case1, recovered
 147 group). See **Fig.S1** for individual behavioral markers and **Fig.S9** for statistical thresholding in (C) and negative difference.

148 Nigrostriatal DA innervation evaluated by striatal DAT binding, decreased by more than 80% relative to
 149 control measures of striatal DAT levels with a relatively spared binding in the ventral striatum [27]. This

150 dorsoventral pattern of the nigrostriatal lesion was later confirmed by semi-quantitative evaluation of
 151 *post-mortem* immunostaining for DAT and TH (Fig.2) with a significantly more advanced lesion in *non-*
 152 *recovered* cases (Fig.2C).



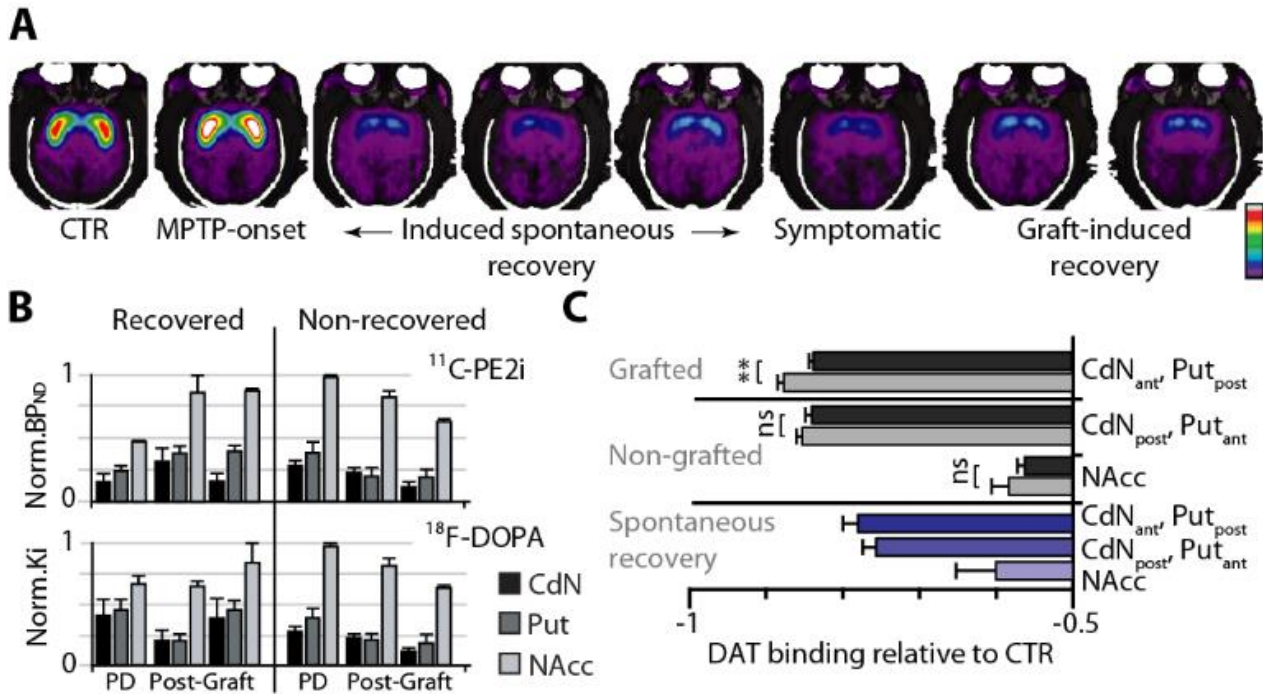
153

154 **Fig. 2. Dopaminergic nigrostriatal lesion.** Immunostaining for DAT (upper row) and for TH (lower row). (A) Optical
 155 density (O.D.) measures of DA lesion (arbitrary units) is made on *post-mortem* tissue of controls, *recovered* MPTP, *non-*
 156 *recovered* MPTP. (B) Illustrative histological sections showing immunostaining. (C) Percent change in expression levels
 157 between MPTP and Controls provides DA lesion estimates by comparing MPTP sub-groups (two-sided Wilcoxon rank sum
 158 test, Bonferroni correction for multiple comparisons *** $p < 0.001$, $Z \geq 5.75$), *Recovered* (black) vs. *Non-recovered* (gray).
 159 Mean±SE. *Recovered* group: cases 1, 4 and 5; *Non-recovered* group: cases 3 and 6; Controls group: n=3.

160 Behavioral and functional markers following NP grafts

161 NPs were derived from a rhesus embryonic stem cell line (LYON-ES1) stably expressing the fluorescent
 162 marker tau-GFP [31, 32], which allows precise tracking of the grafted NPs and their integration into the
 163 host circuits. We chose to graft NPs given their inherent neuroprotective potential resulting from
 164 interaction with host-cells [33] and their observed higher survival rate compared to mature DA cell grafts
 165 [22, 34, 35]. Because *in-vitro* NPs show both astroglial and neuronal -including DA- fates (Fig.S2), it is
 166 possible to assess the influence of the host-environment on the differentiation fate of transplanted cells.
 167 NP grafting was delayed by 3 months on average following the last MPTP injection (13 ± 6 weeks, see
 168 **Materials and Methods**). Bilateral transplantations in multiple functional domains of the basal ganglia
 169 potentiated graft efficiency on both motor and non-motor symptoms. Grafts were targeted in striatal
 170 regions involved in cognitive (*i.e.* anterior caudate nucleus – ant-CdN) and motor functions (*i.e.* posterior
 171 putamen – post-Put.), as well as in the substantia nigra (SN), the latter providing the source of
 172 nigrostriatal DA innervation *i.e.* *pars compacta*, and part of the frontal DA innervation.
 173 Following NP grafts, the reduction of the clinical motor score was associated with an additional cognitive
 174 and circadian recovery in the *recovered* group (Figs.1B, S1). In the *recovered* group, clinical scores
 175 progressively and significantly diverged at the 2nd quantile from those of the *non-recovered* group,
 176 whereas group differences were significant after the 3rd quantile for non-motor symptoms (Fig.1B).
 177 Clinical scores dropped below symptomatic thresholds on average 6 weeks following NP grafts, to
 178 ultimately achieve maximal motor recovery 5 weeks later (Figs.1B, S1A). In the *non-recovered* group,
 179 the initial cognitive and LD ratio recovery shortly after the graft was followed by a relapse on the last
 180 three to two quantiles (Fig.1B). However, the effect of the group difference of non-motor markers in pre-
 181 /post-graft was 3-5 times higher than in pre-/post-MPTP (with large effect-size for Success: $F(2,10) =$

182 10, $p = 3.1E-13$, $\eta_p^2 = 0.27$ and Errors: $F(2,10) = 11$, $p = 1.6E-14$, $\eta_p^2 = 0.29$, and moderate for LD
 183 ratio: $F(2,10) = 12$, $p < 2E-16$, $\eta_p^2 = 0.094$, **Fig.1B, Table S8**). Non-parametric circadian rhythm
 184 analyses (see **Material and Methods**) revealed high variability across cases, preventing post-graft group
 185 comparison on those markers (**Fig.S1C**). In the *recovered* group, markers of striatal DA function and
 186 DAT increased following NP grafts, in correlation with the observed behavioral recovery (**Figs.1B-C,**
 187 **3B-C**). Contrasting with the beneficial effects of NP grafts in the *recovered* group, both clinical motor
 188 scores and non-motor symptoms persisted following NP graft in the *non-recovered* group (**Figs.1B, S1**),
 189 in agreement with the absence of change in markers of striatal DA function and DAT (**Fig.3B**).



190

191 **Fig. 3. Functional markers of striatal DA function and DA terminals.** (A) Images of striatal DAT binding potential
 192 (BP_{ND}, color scale 0-7, case1 – see **Fig.S3A** for all cases). (B) Functional markers of nigrostriatal innervation (normalized
 193 BP_{ND} of ¹¹C-PE2i) and DA function (normalized Ki, uptake of ¹⁸F-DOPA) in a *recovered* and a *non-recovered* case) during
 194 expression of stable symptoms (PD) and following transplantation (Post-Graft, minimum of 10weeks between measures).
 195 Min-max normalization per case. (C) Percent change in Striatal DAT binding in Grafted and Non-grafted regions for
 196 *recovered* (case1, black) and *non-recovered* (cases 3 and 6, grey). Purple, Striatal DAT binding following induced
 197 spontaneous recovery (case1). Two-sided Wilcoxon rank sum test, Bonferroni correction for multiple comparisons ** $p <$
 198 0.01, $Z = 2.92$.

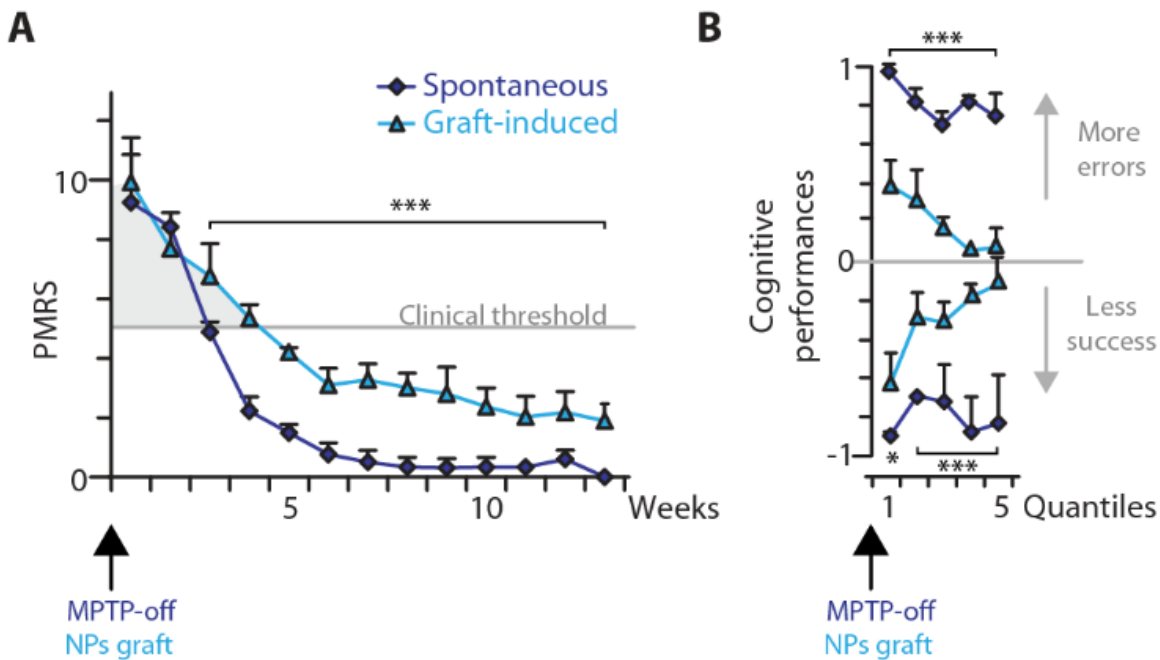
199 In the *recovered* group; we observed that PET-scan estimation of nigrostriatal innervation as revealed by
 200 DAT binding significantly increased bilaterally in the ventral striatum as early as 15 weeks post-graft
 201 (**Fig.1C**). This suggests a primarily protective influence of grafts on the remaining pool of nigrostriatal
 202 projection neurons. Post-graft F-DOPA scans confirmed a selective effect of NPs on DA synthesis
 203 (**Fig.3B**) that was associated with maximal motor recovery. Significant increases in DA function and
 204 DAT binding were also observed in grafted parts of the striatum when compared to the same regions in
 205 *non-recovered* cases (**Fig.3C**).

206 We performed sham transplantation (see **Materials and Methods**), in two out of the four cases following
 207 PET-scan imaging of striatal DAT innervation. No recovery from motor nor from non-motor symptoms
 208 was observed following sham grafts (**Fig.S3B**). Further, functional markers displayed no difference
 209 between *recovered* vs. *non-recovered* cases in striatal DAT binding and no specific increase in sham-
 210 grafted cases (**Fig.S3C**).

211

212 Graft-induced recovery differs from spontaneous recovery

213 We compared the time course of behavioral symptoms and functional markers following graft-induced
214 recovery to those following spontaneous recovery. In half of the cases (n=3), spontaneous recovery was
215 initially induced by halting MPTP intoxication as described previously (Stage I, **Fig.1A**) [25, 27].
216 Spontaneous recovery of clinical motor symptoms displayed faster dynamics and was more complete
217 compared to graft-induced recovery (large effect-size: $F(2,13) = 24, p < 2.2E-16, \eta_p^2 = 0.40$, **Fig.4A**).
218 Importantly, spontaneous recovery was not accompanied by changes in cognitive performance which
219 contrasted with graft induced recovery that was invariably accompanied by progressive cognitive
220 improvements (**Fig.4B**). These findings are intriguing because spontaneous motor recovery occurs
221 despite an average $71.5 \pm 9.7\%$ reduction in striatal DAT relative to control levels and that stable
222 symptoms were observed when the lesion reached $82.3 \pm 7.9\%$ of control [27]. The present findings show
223 that graft-induced recovery occurs despite an average $78.04 \pm 12\%$ reduction in striatal DAT binding
224 (**Fig.3C**). These results confirm the specificity of graft-induced recovery compared to spontaneous
225 recovery, particularly with respect to cognitive deficits (large effect-size for both Success: $F(2,5) =$
226 $39, p < 2.2E-16, \eta_p^2 = 0.40$ and Errors $F(2,5) = 35, p < 2.2E-16, \eta_p^2 = 0.38$, **Fig.4B**).
227



228

229 **Fig. 4. Dynamics of spontaneous vs. graft-induced behavioral recovery.** (A) Clinical motor score over weeks and
230 (B) cognitive performances (over quantiles) comparison between spontaneous recovery (cases 1, 3 and 4) vs. graft-induced
231 recovery (cases 1, 4, 5). Mean \pm SE, anova between groups, adjusted p-values for multiple comparison * < 0.05 , *** < 0.001 .

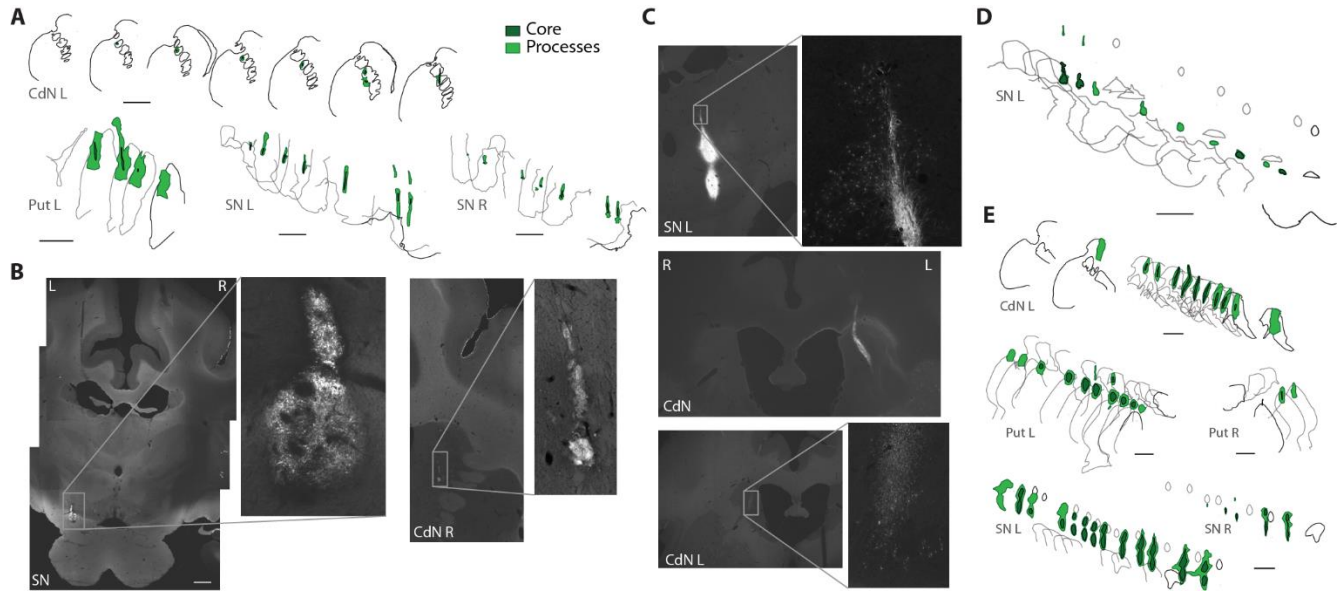
232 Behavioral and functional recovery correlate with graft survival

233 We grafted NPs bilaterally in multiple sites including the ant-CdN, post-Put and SN. Each site received
234 1.5×10^5 - 1×10^6 NP cells (see **Materials and Methods**). Following an average post-graft period of
235 30 ± 8 weeks, we performed a *post-mortem* immuno-histological assessment of tau-GFP⁺ cells survival
236 and integration into the host milieu (**Fig.1A**).

237 Noteworthy, the *non-recovered* group is characterized by a complete absence of tau-GFP⁺ cells (**Fig.S4**).
238 This could be due to the fact that the nigrostriatal DA lesion was most severe in these cases (**Fig.2C**).
239 However, immunological rejection of the grafted NPs cannot be excluded as no immunosuppressive
240 treatment was employed. The fact that non-motor symptoms initially improved in the *non-recovered*

241 group prior to an abrupt deterioration after the 2nd Quantile (**Fig.1B**) is coherent with a chronic graft
242 rejection in the *non-recovered* group (**Fig.S4**).

243 In sharp contrast, we found surviving grafted cells in all cases of the *recovered* group, at locations
244 including the targeted functional domains of the basal ganglia (**Figs.5, S5-8**). The average number of
245 surviving cells was: $1.23 \pm 0.9 \times 10^4$ cells in the ant-CdN, $1.95 \pm 1.1 \times 10^4$ cells in the post-Put and
246 $5.93 \pm 3.0 \times 10^4$ cells in the SN (**Table S3**) which corresponds to an average survival rate of: $6.9 \pm 6.2\%$ in
247 the ant-CdN, $13.0 \pm 7.3\%$ in the post-Put and $15.5 \pm 9.4\%$ in the SN (overall survival rate of $11.6 \pm 4.4\%$,
248 Mean \pm SE).



249

250 **Fig. 5. Post-mortem localization of grafted NPs.** (A) Digital reconstructions of post mortem grafts in case1 (case4
251 shown in **D** and case5 in **E** - scale bars 200 μ m). (B-C) Photomicrographs of brain slices with zoom on tau-GFP⁺ grafts (scale
252 bar 2mm, case4 shown in **B** and case5 in **C**).

253 We observed unilateral survival of NPs in some of the grafted striatal regions (**Table S3**). We could not
254 link asymmetrical survival in the post-Put to a systematic difference in left vs. right motor recovery
255 (**Fig.S9A**) suggesting the induction of known cross-hemispheric compensatory processes [36].
256 Nevertheless, small differences were observed in cognitive performance linked to unilateral survival in
257 the ant-CdN (**Fig.S9B**); differences that were coupled with a minor unilateral DAT binding decrease in
258 the corresponding striatal region (**Fig.S9C**, right).

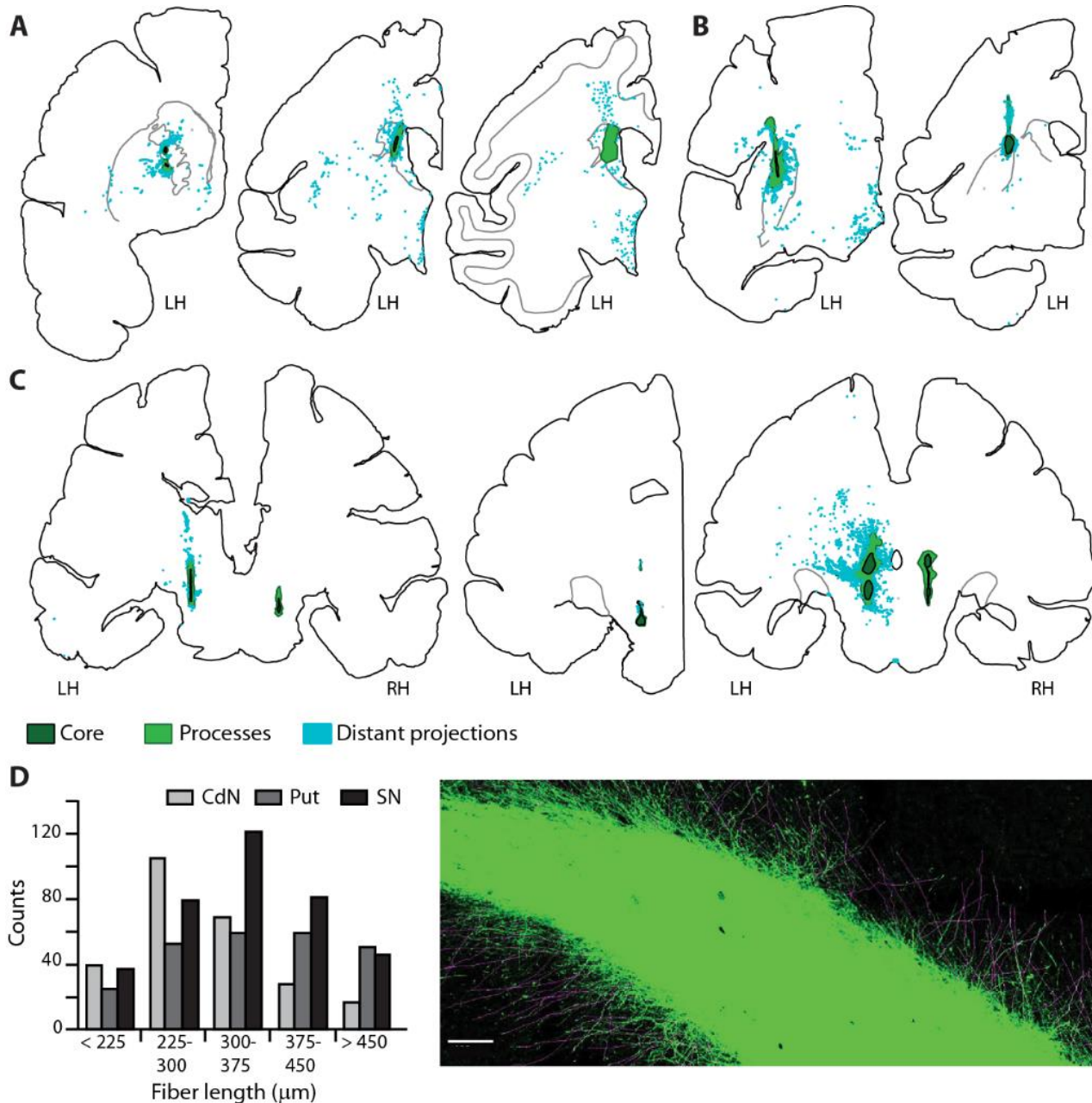
259 Surviving grafts displayed extensive tau-GFP positive projection fibers (**Fig.6A-C**) which extend several
260 millimeters from the graft core, considerably further than the local processes which are less than 1
261 millimeter (**Fig.6D**). These findings indicate that the *recovered* group is characterized by integration of
262 NPs into the host tissue (**Fig.6**).

263 Differentiation fates indicate protective and restorative effects of NP grafts

264 Detailed immuno-histological examination of graft locations reveals that grafted NPs differentiated
265 principally into astrocytes and mature neurons (**Fig.7A-C, D; Fig.S5**). Among surviving NPs, astrocytic
266 fate accounted for $42 \pm 5\%$ (GFAP⁺/tau-GFP⁺ co-expression ratio, **Fig.7A, Table S4**) and neuronal fate
267 for $16 \pm 4\%$ (MAP2⁺/tau-GFP⁺ co-expression ratio, **Fig.7A, Table S4**). Furthermore, we demonstrate that
268 NPs spontaneously differentiate into aminergic (TH⁺/DAT⁺) cells thereby potentially restoring a
269 significant proportion of the lost DA pool (**Fig.7C-D**).

270 On average, the TH⁺/tau-GFP⁺ co-expression ratio implies that $6 \pm 2\%$ of tau-GFP⁺ cells differentiated
271 into aminergic cells (**Fig.7B, Table S4**). Up to 13% of tau-GFP⁺ in SN graft co-localize with TH⁺
272 expression (case4), which corresponds to an estimate of 1.4×10^4 TH⁺/tau-GFP⁺ cells and hence to a

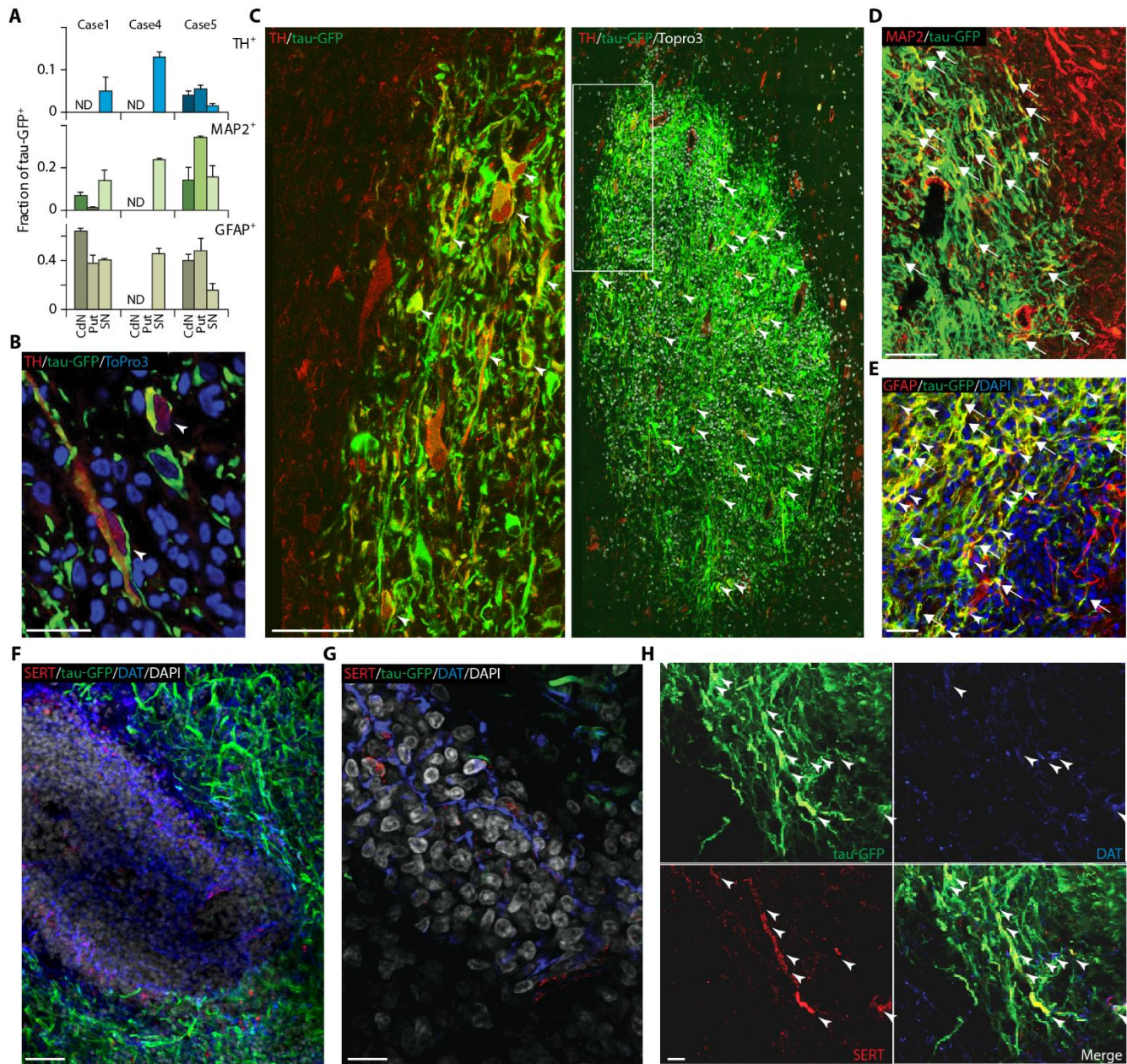
273 restoration of nearly 18% of the original pool of DA-containing midbrain neurons in the same species
 274 [37]. We qualitatively confirmed NP differentiation into both the serotonergic and the dopaminergic
 275 phenotypes (**Fig.7H**). Together with the protective effect of the graft on the survival or upregulation of
 276 DA cells (**Figs.1C, 7F-G**), these results strongly support a role of the graft in the recovery from motor
 277 and cognitive symptoms (**Figs.1, S1** and **Tables S4-S5**).



278

279 **Fig.6. Grafted NPs project over long distances.** Digital post-mortem reconstructions of NP grafts at the level of (A)
 280 anterior CdN in case1 (left) and case5 (middle and right), (B) posterior Put for cases 1 (left) and 5 (right) and, (C)
 281 SN for cases 1, 4 and 5 (from left to right). Tau-GFP+ projection fibers (clear blue) are found at relatively distant locations from the
 282 graft core (dark green), much farther than local graft processes (clear green) emanating from the core. (D) Typical processes
 283 emanating from the core have an average length superior to 340μm. Left, count histogram of processes' length for case5.
 284 Right, typical tau-GFP+ photomicrograph showing graft-core and processes at the level of ant-CdN (case5, faint purple lines
 285 from measurement overlay, scale bar 100μm).

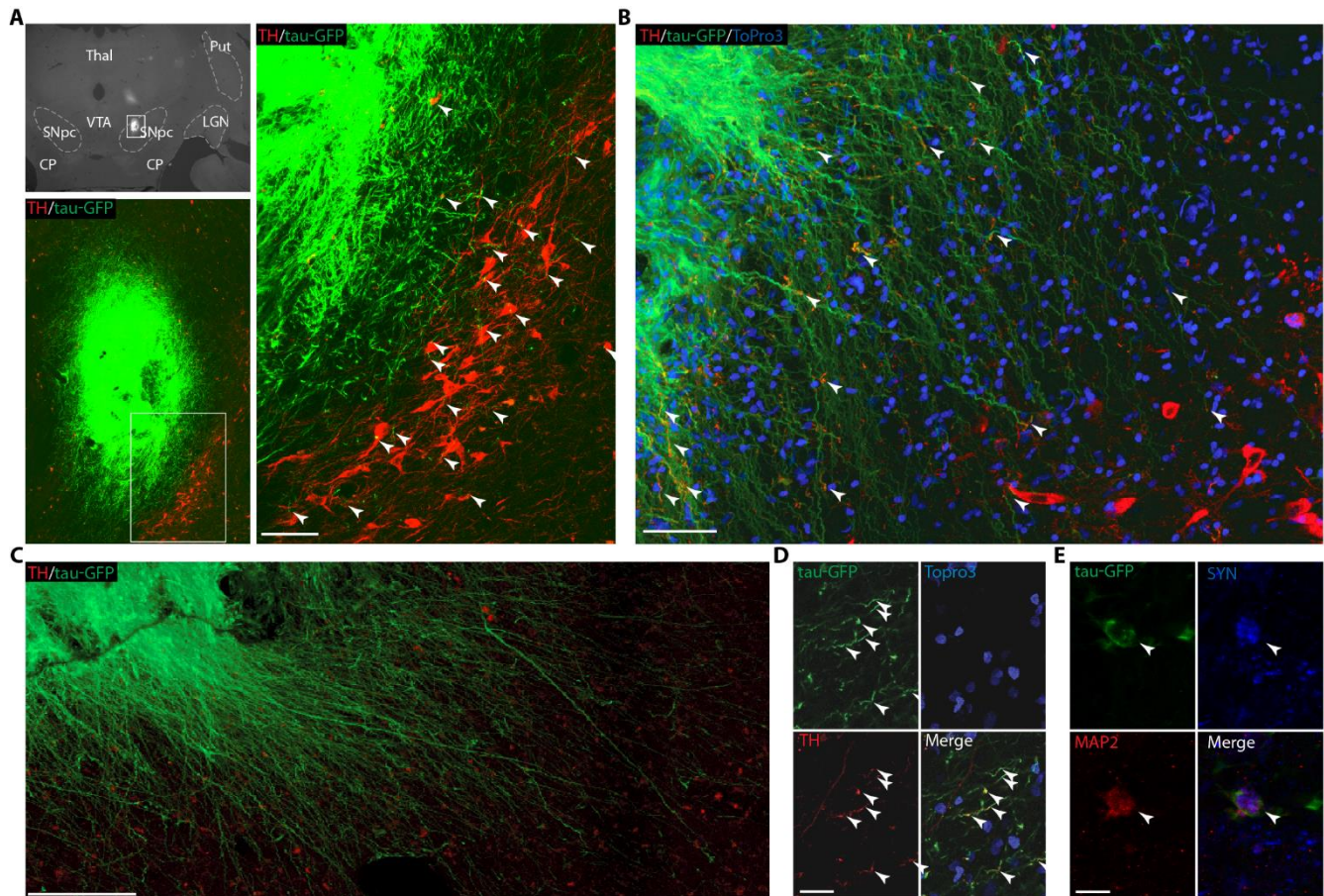
286 At some graft sites, aggregates of host cells (*i.e.* tau-GFP negative, **Figs.S7E, G and S8D, H**) were
 287 observed in the graft-core of nigral transplants, exhibiting aminergic TH⁺, dopaminergic DAT⁺ and
 288 sparse serotonergic SERT⁺ processes (**Figs.7F-G, S7G-J**). Importantly, no post-graft overgrowth was
 289 observed (**Fig.S10A-C**) and few CD68⁺ macrophages were detected in the vicinity of transplanted sites,
 290 despite the absence of immunosuppressive treatment (**Fig.S10D-F**).



291
 292 **Fig. 7. Differentiation Fate of grafted NPs.** (A) Fraction of tau-GFP⁺ co-localized with TH⁺, MAP2⁺ and GFAP⁺.
 293 Illustrative example of NPs differentiated into: (B) aminergic cells, SN, case4 (arrowheads); (C) aminergic cells, SN, case5
 294 (arrowheads, left is enlarged view of region framed on the right); (D) neuronal cells (arrowheads) and processes (arrows),
 295 post-Put, case5; (E) astroglial cells (arrowheads) and processes (arrows), SN, case4. (F-G) Host tissue within the graft core
 296 presenting numerous dopaminergic (DAT⁺, blue) and sparse serotonergic (SERT⁺, red) processes. (H) NPs also display
 297 serotonergic and/or dopaminergic phenotype (arrowheads). Scale bars, B 20µm, C left 50 µm a, D-E 100µm, F 25µm, G
 298 5µm, H 15µm.

299 Finally, we explored the extent to which the grafted NPs were structurally integrated, via contacts with
 300 host neurons. For this purpose, we examined synaptophysin (SYN) immunohistology. SYN is localized

301 in the membrane of pre-synaptic vesicles and provides a specific and sensitive marker for synapses. We
302 explored regions at the periphery of the grafts, where the numerous local processes emanating from the
303 core appear preferentially directed toward host DA cells (**Fig.8A, C**). Confocal microscopy shows that
304 SYN co-localized at the junction between TH⁺ and MAP2⁺ host-cells and tau-GFP⁺ processes from
305 grafted-cells (**Figs.8E, S8E, G, I-K**), indicating that local processes stemming from the graft core
306 establish synaptic contacts with the remaining host DA cells in the SN (**Fig.8**). Together with the presence
307 of long-distance projections emanating from the grafted sites (**Fig.6**), this suggests functional integration
308 of NPs into the host brain circuitry and supports the effect of NP grafts in the observed functional
309 recovery (**Figs.1B-C, 3**).



310

311 **Fig.8. Histological evidence of NP integration and interaction with host cells.** (A) Photomicrographs (up-left
312 panel) shows position of graft in SN, framed region zoomed in bottom-left panel. Right-panel show zoom of framed region
313 in the bottom-left panel. In (A) and (B), Grafted NPs (tau-GFP⁺, green) extend processes (arrowheads) toward surviving host
314 DA cells (SN grafts). (C) NPs display numerous extensive processes projecting outside the graft-core. (D) NPs differentiate
315 into aminergic phenotype displaying TH⁺ processes (arrowhead). (E) Synaptophysin expression (SYN, blue) co-localized
316 with grafted cell (tau-GFP⁺) differentiated into mature neuron (MAP2⁺) suggesting synaptic contact. Scale bars: A-C
317 100μm, D-E 5μm.

318 DISCUSSION

319 We transplanted macaque NPs into multiple functional domains of the basal ganglia, in both hemispheres
320 of middle-aged parkinsonian macaques. Our study demonstrates that the successful integration of the
321 grafts promotes a robust clinical and unprecedented cognitive recovery (**Figs.1B, S1**), as well as partial
322 circadian recovery. Recovery is accompanied by quantitative effects on functional markers of
323 nigrostriatal DA innervation and striatal DA function (**Figs.1C, 3B-C**). This study thus provides major
324 empirical evidences in favor of a promising stem-cell derived cell therapy for PD patients. First, we show

325 for the first time that transplantation and successful engraftment of NPs efficiently promotes recovery
326 from motor and non-motor symptoms. Second, we demonstrate the restorative and the neuroprotective
327 potential of grafted NPs. Our results show that NPs have the capacity to restore a significant pool of
328 mature neurons (**Fig.7A**), including dopaminergic and serotonergic cells. In addition, NPs largely
329 differentiate into astroglial cells that the present study confirms provide a neuroprotective environment
330 for the surviving pool of DA neurons [22-24]. Third, our results also suggest that nigral transplantation
331 of NPs provide neurotrophic effects on the host cells (**Figs.7F-G, S7E, G-J and S8D, H**), orienting
332 endogenous cells towards a DA fate [38, 39]. Finally, we provide cellular evidences of structural and
333 functional integration of grafted NPs (**Figs.6, 8 and S8E, G, I-K**) into the host brain network.

334 The consistent cognitive recovery following successful engraftment of NPs in ant-CdN and SN
335 establishes the potential of cell therapy to alleviate non-motor cognitive symptoms in addition to motor
336 symptoms in PD patients. The specificity of the graft-induced cognitive recovery (**Fig.1B**) was further
337 confirmed by the absence of cognitive improvements following sham-grafts (**Fig.S3B**) and spontaneous
338 motor recovery (**Fig.4B**). These results anticipate the outcome of cell therapy for the majority of PD
339 patients experiencing mild cognitive impairment (MCI) that significantly affects their quality of life [40].
340 Cognitive functions in human and NHP depend on the integrity of the prefrontal cortex (PFC), the DA
341 system and the DA innervation of PFC via direct projections from SN and ventral tegmental area [41-
342 46]. In addition, fronto-striatal circuits are also extensively involved in cognitive function and more
343 particularly the cognitive sectors of the striatum i.e. ant-CdN [47]. Many cognitive symptoms expressed
344 in PD patients not only arise from DA degeneration but also from other monoamine neurotransmitter
345 systems closely connected to the DA system i.e. serotonin and norepinephrine [48, 49]. These systems
346 are also affected in the MPTP-monkey model [50]. With the cognitive task that was used here, increased
347 errors and reduced success rates observed in Stages I and II can be directly related to the reduced control
348 over motor planning and parallel executive dysfunctions observed in PD patients [4, 51]. Hence, the
349 observed reduction of cognitive performances (**Figs.1B, S1B**) can be directly imputed to the nigrostriatal
350 DA lesion, the altered DA innervation of frontal cortex [41] and the wider MPTP-induced alteration of
351 the aminergic system including noradrenalin and serotonin [29, 52-55]. We relate the consistent cognitive
352 recovery following NP grafts (**Fig.1B**) to the efficiency of the transplanted NPs in both SN and ant-CdN
353 of which an average of, respectively $5.93 \pm 3 \times 10^4$ and $1.23 \pm 0.9 \times 10^4$ cells survived, differentiated (**Fig.7**)
354 and integrated the host network through local and long-distance projections (**Figs.6, 8**). Importantly, we
355 show that grafted NPs can differentiate into a substantial proportion of TH⁺ expressing cells (**Fig.7A**),
356 well within the range of previous studies reporting clinical improvement (**Table S4**), but also in the
357 serotonergic phenotype (**Fig.7H**) thereby supporting their role not only in motor but also in cognitive
358 recovery.

359 In addition to these restorative effects, our results suggest that bilateral multi-site NP grafts exert a
360 neuroprotective influence on the pool of surviving DA cells and of their ventro-striatal target. We show
361 a significant and specific increase in striatal DAT binding following graft (**Fig.1C**) and not following
362 sham-surgery (**Fig.S3C**). This increase is located in the ventral striatum that corresponds to the surviving
363 pool of nigrostriatal innervation following induced spontaneous recovery [27]. The large proportion of
364 astroglial differentiation (**Fig.7A**) might support this protective effect of NP grafts, in accordance with
365 previous reports on human NP grafts in MPTP-monkeys [56]. Although we report some degree of
366 recovery of DAT binding in the grafted caudate nucleus and putamen (**Fig.3B-C**), these effects were
367 minimal compared to what was found in the ventral striatum. Longer post-graft survival could lead to
368 greater increases in DAT binding. Indeed, transplanted PD patients display fast clinical improvements in
369 motor function i.e. within 2-3 months after transplantation, whereas it can take 1-2 years to detect via
370 functional imaging significant increase in DA function at the grafted locations [57-62].

371 The impact of cell therapy on chronobiological rhythms as far as we know has not been previously
372 studied in the MPTP-monkey model. The initially observed circadian recovery following NP grafts

373 concerned both groups but was followed very quickly by a deterioration after the third quantile in *non-*
374 *recovered* cases. However, in the *recovered* group the LD ratio recovery was significant but only partial
375 (**Fig.1B**). Previously we showed that following MPTP-induced nigrostriatal DA lesion, environmental
376 cues mask the failure of the intact SCN to drive striatal clock genes and DA functions that control rest-
377 wake locomotor rhythms [26]. Here, the partial recovery of circadian rhythms in the presence of
378 environmental timing cues (**Fig.1B**), could reflect incomplete restoration of DA function. However,
379 following NP grafts, under continuous light conditions (n=4, cases 1, 3, 4 and 5) there was an absence of
380 endogenous circadian rhythm as observed in the MPTP monkey [26]. This again points to NP grafts
381 failing to fully restore the DA network involved in the SCN control of rest-wake rhythms. DA projection
382 neurons from SN and VTA regulate sleep-wake rhythms through interaction with several structures from
383 the circadian network including the locus coeruleus, the lateral hypothalamus and the pedunculopontine
384 nucleus [63]. Those structures, which were not grafted in the present study, are impacted in PD and
385 following MPTP intoxication and thus constitute future candidates for cell replacement protocols [64-
386 66].

387 There are limitations in the use of cell-therapy for PD e.g. presence of Lewy-body formations in the
388 grafted cells [67, 68]. However, the balance benefit-risk of cell-therapy, even in cases of Lewy-body
389 development in the grafted cells, is clearly in favor of the transplanted patients [11]. Three decades after
390 the first clinical trials of transplanting cells from fetal ventral mesencephalon in PD patients [69], cell
391 replacement therapy in PD is still not a proposed treatment. However, this therapeutic approach has
392 recently been shown to be efficient at different levels [61, 62, 70], leading to more recent trials [71]. In
393 parallel, many strategies have been developed in recent years in order to improve the safety and efficiency
394 of using ESCs or iPSCs as a source for the production of the to-be-transplanted cells [21]. Virtually no
395 NHP studies have addressed the impact of cell replacement on non-motor circadian symptoms and only
396 one recent reported beneficial effects on depressive symptoms (**Table S5**). Here we report for the first-
397 time efficient recovery from cognitive and clinical motor symptoms, as well as partial circadian recovery
398 following NP grafts in the NHP model of PD. The present study confirms the neuroprotective and
399 restorative capacities of NP grafts and, underlines the crucial importance of longitudinal and multifaceted
400 approaches for a better translation to the clinic and appraisal of early-phase clinical trials.

401 While there are doubtless limitations in the use of the MPTP-monkey model to study human-specific
402 aspect of PD; this model remains nevertheless the gold standard for modeling PD and for the pre-clinical
403 evaluation of new therapeutic approaches. Previous studies have shown that advanced nigrostriatal lesion
404 is detrimental to the survival of grafted cells [17, 18], which is coherent with our observation of poor
405 survival of grafted NPs in the *non-recovered* group. Further, in the *non-recovered* group immune
406 rejection is likely to have occurred as previously observed following allogenic transplantation in non-
407 immunosuppressed monkeys [72, 73]. These observations suggest that future therapeutic cell
408 replacement procedures should be carried out preferably at early stages of PD.

409

410 MATERIALS AND METHODS

411 In agreement with the 3Rs [74], the rationale for the current study is that each case represents its own
412 control through detailed follow-up of all the periods of the protocol i.e. CTR, MPTP, post-MPTP and
413 Pre- vs. Post-Graft. We published precise and detailed reports for each clinical, behavioral and functional
414 parameter followed as well as for the characterization of NPs grafted in the present study. These precise
415 and detailed control procedures are described in **Table S1**.

416 Study Approval

417 All procedures were carried out according to the 1986 European Community Council Directives
418 (86/609/EEC) which was the official directive at the time of experiments, the French Commission for
419 animal experimentation, the Department of Veterinary Services (DDSV Lyon, France). Authorization
420 for the present study was delivered by the “Préfet de la Région Rhône Alpes” and the “Directeur
421 départemental de la protection des populations” under Permit Number: #A690290402. All procedures
422 were designed with reference to the recommendations of the Weatherall report, “The use of non-human
423 primates in research”.

424 Study Design

425 The objective of the research was to investigate the efficiency of NP grafts to restore motor and non-
426 motor symptoms in parkinsonian macaque monkeys. All behavioral and functional measures were
427 acquired by experimenters blinded to the outcome of the grafts integration as this was evaluated post-
428 mortem. Post-mortem quantifications of graft survival, differentiation rate and length of graft’s processes
429 were done by investigators blinded to the precise evolution of parkinsonian symptoms of the animals
430 following graft. The experimental findings include replication of the procedure in all individual cases
431 separately and conclusions on the findings arise from difference in outcome based on post-mortem
432 evaluation of grafts integration. Cases followed by PET imaging were chosen randomly before
433 experiment starts as well as cases chosen for sham-grafts. Data collection was stopped at least 7 months
434 following transplantation and after last possible PET-scan acquisition. Data-points of the locomotor
435 activity acquired on the day of anesthesia for MRI, PET-scan or surgery were excluded from circadian
436 analyses. Presented results include all other data-points, no outliers were excluded.

437 Nine macaque monkeys (*Macaca fascicularis*, n=9) were used in total for this study. The study was
438 designed such that one third (n=3) were randomly selected as histological controls i.e. never received
439 MPTP injections and, the other two third (n=6) entered the MPTP protocol. Whilst we selected control
440 animals for histological comparison, we designed the study such that each animal would be its own
441 control for clinical, behavioral and functional measures. **Figure 1A** displays a schematic representation
442 of the study design and protocol. Two third of the MPTP group (n=4) were randomly selected to be
443 followed by PET imaging of striatal DA transporters and DA function throughout the full study and, half
444 of them (n=2) were randomly selected to evaluate sham-grafts effects on clinical, behavioral and
445 functional measures. In addition, half of the animals entering the MPTP protocol were randomly selected
446 (n=3) for a two-step MPTP procedure in which MPTP injections were first halted based on clinical score,
447 in order to induce spontaneous recovery [25] for further comparison of this *spontaneous* recovery to the
448 prospective *graft-induced* recovery. MPTP-intoxication was then resumed in order to induce stable motor
449 symptoms [27] over the period preceding cell therapy. Study design is further detailed below.

450 Six late middle-aged – 11-13 (13-17) years old at protocol onset (end) female macaque monkeys (*Macaca*
451 *fascicularis*, 4-5kg) were intoxicated with low-dose 6-methyl-1-Methyl-4-phenyl-1,2,3,6-
452 tetrahydropyridin injections (MPTP, 0.2mg/kg, i.m). Animals were housed in a room dedicated to MPTP
453 experiments, with free access to water and received food twice a day. The neurotoxin was delivered at
454 low-doses chronically each 3-4 days (slowly progressive lesion) during prolonged periods, followed by
455 acute low-doses intoxication (daily injections) in order to ensure stable persistent motor symptoms, as

456 described previously [27, 29]. Cases 1, 3 and 4, were selected to critically compare the evolution of
457 clinical, behavioral and functional markers following spontaneous clinical motor recovery [25, 27] with
458 those obtained during potential recovery induced by cell therapy. In order to do so, we suspended the
459 slowly progressive lesion induced by chronic low-dose MPTP injections as soon as the clinical score
460 reached symptomatic threshold (**Fig.S1** and **Table S2**). In all cases, acute (daily) low-dose MPTP
461 injections induced persistent motor-symptoms. We cautiously stopped daily MPTP injections when the
462 PMRS-motor score was above ten for two consecutive days following one MPTP injection. For results
463 presentation, animals were grouped according to their clinical motor state at the end of the protocol
464 following cell therapy (see **Fig.1**) i.e. 1st group – recovered (n=3, cases 1, 4, 5) and, 2nd group – non-
465 recovered (n=2, cases 3 and 6). Case2 had per operative brain hemorrhage following NPs transplantation
466 and was thus excluded from group comparison. Data are presented according to the following periods of
467 the protocol: 1- CTR (measures acquired before MPTP-onset); 2- MPTP (during MPTP intoxication
468 period); 3- post-MPTP (following arrest of last MPTP injections, clinical motor score remained stably
469 above 5 during this period i.e. motor-symptomatic); 4- post-Grafts (following transplantation, described
470 in **Supplementary Materials and Methods**). Delays between last MPTP injection and cell therapy were
471 4-23 weeks (n=6, all cases) and, delays between last MPTP injection and sham-grafts were 6-16 weeks
472 (n=2, cases 1 and 6).

473 Complete details concerning Parkinsonian Motor Rating Scale – PMRS, Surgical Procedures,
474 Transplanted cells, Cognitive behavior – detour task, Circadian rhythm follow-up, Dopamine function
475 imaging – [¹¹C]-PE2I and [¹⁸F]-FDOPA, Immuno-histological and Quantification procedures, Statistics
476 can be found in the **Supplementary Materials and Methods**.

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504 **Supplementary Materials**

505 Supplementary Material and Methods.

506 Fig.S1. Individual follow-up of behavioral and clinical markers.

507 Fig.S2. Characterization of grafted cells.

508 Fig.S3. Functional follow-up of striatal DAT binding and Sham-grafts consequences on behavioral and
509 functional markers.

510 Fig.S4. Grafted NPs did not survive in the *non-recovered* group.

511 Fig.S5. Post-mortem localization and characterization of transplanted NPs.

512 Fig.S6. Grafted NPs in case1.

513 Fig.S7. Grafted NPs in case4.

514 Fig.S8. Grafted NPs in case5.

515 Fig.S9. Hemispheric differences in graft-induced recovery.

516 Fig.S10. NPs show no overgrowth after transplantation and induce minor immune reaction.

517 Table S1. Longitudinal protocol design, repeated measures and case controls.

518 Table S2. Total cumulated MPTP doses per case.

519 Table S3. Cell survival following transplantation of NPs.

520 Table S4. Grafted cells number, survival rates, differentiation fates and comparison to literature.

521 Table S5. Clinical motor, cognitive and functional impact of grafts described in Table S4.

522 Table S6. Antibodies list.

523 Table S7. Primers for semi-quantitative RT-PCR.

524 Table S8. Group-level stats of Fig.1B.

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