New striatal dopamine neurons in MPTP-treated macaques result from a phenotypic shift and not neurogenesis

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We investigated whether there is neurogenesis in the striatum of aged monkeys, and whether dopamine (DA) depletion induces the genesis of new DA neurons in this structure. Six aged macaques received repeated intraperitoneal injections of bromodeoxyuridine (BrdU) over a 3 week period to label dividing cells. Three macaques were injected in parallel with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to decrease dopaminergic innervation of the striatum. The brains were analysed 3 weeks after the last BrdU injection. In MPTP-treated aged macaques, the number of tyrosine hydroxylase (TH) immunoreactive (ir) striatal neurons increased 2.3-fold compared with controls. These TH-ir striatal cells did not express dopamine beta hydroxylase (DBH) but the dopamine transporter (DAT), suggesting that they are functional DA neurons. They were also negative for calbindin (CB), neuropeptide Y (NPY) and parvalbumin (PV), and a small proportion expressed calretinin (CR). This suggests that these cells stained for TH are interneurons. All these cells also co-expressed glutamic acid decarboxylase (GAD). They thus resemble the small, aspiny, GABAergic interneurons. None of the BrdU-labelled cells in the striatum expressed the neuronal markers neuronal nuclei (NeuN), or GAD or TH, and none of TH-ir cells incorporated BrdU. These data indicate that neurogenesis did not occur in the striatum of aged macaques. The new striatal TH-ir neurons observed after DA depletion was therefore derived from pre-existing GABAergic interneurons. Understanding of the molecular signals mediating this phenotypic shift might help in developing novel and elegant strategies for a cell-based therapy for Parkinson’s disease that would avoid many of the drawbacks of cell transplantation.

Keywords: primates; Parkinson’s disease; neurogenesis; advancing age

Abbreviations: BrdU = bromodeoxyuridine; CB = calbindin; DA = dopamine; DAPI = 4’,6’-diamidino-2-phenylindole-dihydrochloride; DAT = dopamine transporter; DBH = dopamine beta hydroxylase; GAD = glutamic acid decarboxylase; ir = immunoreactive; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN = neuronal nuclei; NPY = neuropeptide Y; PBS = phosphate-buffered saline; PV = parvalbumin; TH = tyrosine hydroxylase


Introduction

A decrease in the dopamine (DA) innervation of the striatum due to loss of DA neurons in the substantia nigra is responsible for the core motor symptoms of Parkinson’s disease. Anti-parkinsonian therapies are therefore aimed at restoring DA transmission in this brain structure. Transplantation of DA precursor cells into the striatum has been shown to ameliorate motor deficits in animal models of Parkinson’s disease (Redmond et al., 1986), but less in patients, due to insufficient graft survival and incomplete and inhomogeneous re-innervation (Lindvall et al., 2004). A small number of DA neurons have been found in the striatum itself, in both rats and primates (Dubach et al., 1987; Tashiro et al., 1989; Betarbet et al., 1997; Porritt et al., 2000). Furthermore, these neurons increase in number following degeneration of the
nigrostriatal pathway (Betarbet et al., 1997; Porritt et al., 2000; Palgi et al., 2002). It is tempting to speculate that the new neurons result from neurogenesis as the injured brain attempts to repair itself. This hypothesis is particularly attractive since the striatum is adjacent to the subependymal zone, a germinal area in adult brain containing stem cells with the potential to produce neurons (Sanai et al., 2004), including DA neurons (Hack et al., 2005). It has already been shown that the subependymal zone reacts to a DA deficiency with an overall reduction in cell proliferation, but a selective increase in the production of DA neurons (Hoglinger et al., 2004; Yamada et al., 2004; Winner et al., 2006). Furthermore, the striatal DA neurons lack lipofuscin, a pigment associated with ageing (Betarbet et al., 1997; Porritt et al., 2000), suggesting that these neurons might have been generated in adulthood. The recent observation that 30% of DA neurons in the human striatum co-express Tuji, a marker of early committed neurons, supports this hypothesis (Cossette et al., 2005).

However, an attempt to demonstrate the adult genesis of DA neurons in the striatum of mice in which striatal levels of DA were decreased by treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was unsuccessful (Kay and Blum, 2000; Mao et al., 2001). This might be due to the capacity of the nigrostriatal DA projections in mice to recover rapidly and completely from MPTP-induced damage (Mao et al., 2001; Petroske et al., 2001; Hoglinger et al., 2004), obviating the need for neurogenesis in this species. We have, therefore, addressed this question in aged macaques in which MPTP induces a profound and stable striatal DA depletion, a model that more closely reflects the situation in human Parkinson’s disease.

Material and methods

Animal treatments

All experiments were performed in accordance with the European Communities Council Directive of 1986 (86/609/EEC). Six male macaques (Macaca mulatta) weighing 8–15 kg that had never been used for experimentation or undergone surgery were used. They were 20–25 years old.

To label mitotic cells, thymidine analogue bromodeoxyuridine (BrDU) (Sigma, St Louis, MO, USA) was administered under anaesthesia (ketamine, 10 mg/kg, i.m.) in 10 intraperitoneal injections (40 mg/kg in 0.9% NaCl with 0.007 M NaOH), once every 2–3 days for 3 weeks. The cumulative dose was 400 mg/kg body weight. The brains were analysed after a survival time of three further weeks. Thus, newborn precursor cells had a maximum of 8 weeks, from first BrDU injection to sacrifice, to differentiate into their mature phenotype. Three of the six monkeys were also injected under anaesthesia (ketamine, 10 mg/kg) with MPTP (0.4 mg/kg, i.m. in NaCl 0.9%) 4 days before and every 2–3 days during the BrDU treatment until their parkinsonism stabilized (4–6 injections depending on the severity of signs in the animals). The cumulative dose per animal ranged from 1.2 to 2 mg/kg. The other three monkeys were used as controls.

The three MPTP-treated monkeys were examined and recorded on video every 2 days. Clinical disability was scored from 1 to 25, as described (Luquin et al., 1993). All animals were euthanized 3 weeks after the last BrDU injection (4–5 weeks after the last MPTP injection).

Tissue processing

After a lethal overdose of anaesthetic, the monkeys were perfused intracardially with 0.9% NaCl. The brain was removed and the hemispheres were separated. The right hemisphere of each brain was divided into blocks that were post-fixed for 3 days [4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS)] and then cut on a freezing microtome in serial, free-floating 40 μm-thick transverse sections.

To reveal incorporated BrdU, the sections were treated with 2 N HCl (37°C, 30 min) to denature the DNA and then rinsed for 10 min in 0.1 M boric acid. Non-specific binding was blocked with PBS 0.2% Triton X100 4.5% normal goat serum for 30 min. The sections were then incubated with the primary antibodies, at optimal dilutions, in the same solution (Table 1) for 2 days at 4°C, washed several times in PBS and then incubated with secondary antibodies from the appropriate species: fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG, Cy 3-conjugated goat or donkey anti-mouse or Cy 3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) (1:200 in PBS and 4.5% normal goat serum, 1 h 30 min at room temperature). Neuronal nuclei (NeuN) immunoreactivity was visualized using Cy3-streptavidin. Sections were then rinsed and mounted in anti-fade medium (Moviol medium; Calbiochem, San Diego, CA, USA). Sections incubated without the primary antibodies remained unstained and served as controls. Cross-reactivity of secondary antibodies was systematically checked by varying the order of staining in the different experiments. Sections were counterstained with 4',6'-diamidino-2-phenylindole-dihydrochloride (DAPI). After immunocytochemistry, the sections were treated with a solution of Sudan Black B (Allied Chemical, New York, NY, USA) in 70% methanol for 5 min in order to reduce lipofuscin autofluorescence (Schnell et al., 1999).

For peroxidase immunohistochemistry, immunoreactivity was visualized using a Vector avidin–biotin complex (ABC) kit (Vector Laboratories, Burlington, CA, USA) with diaminobenzidine (DAB; Sigma) as a chromogen. Sections were counterstained with cresyl violet.

Data analysis

Light microscopic analysis was performed using an epifluorescence microscope (Axioplan 2, Zeiss, Germany) equipped with a video-camera (Retiga, Qimaging, BC) and a computer-based image analysis system (Fluo-up, ExploraNova, La Rochelle, France). Cells that appeared to be double-labelled were then analysed in three dimensions by confocal laser-scanning microscopy (Leica DM IRBE, Germany). Tyrosine hydroxylase (TH)-immunoreactive (ir) neurons and BrDU-ir nuclei in the striatum were quantified using an unbiased sampling technique (Debeir et al., 1999), as follows.

The number of TH immunoperoxidase stained cells, defined as a cell body with two or more processes, were counted in the whole striatum (caudate, putamen, nucleus accumens) on four sections covering the anteroposterior extent of the structure with a
computer-based system (Mercator, ExploraNova, La Rochelle, France). The sections were matched anatomically in each of the animals, verifying that the cross-sections of the striatum were similar in controls and MPTP-treated monkeys. The numbers of cells were averaged for each animal and the density was assessed by dividing the number of TH-ir cells by the area of the striatum surveyed. The density of cells was then expressed as the mean (± SD) number of TH-ir cells per 100 mm².

BrdU-ir cells were quantified on two sections at the level of the anterior striatum (where the nucleus accumbens is greatest) and two sections at the posterior level (where the most anterior part of the internal pallidum is evident) in five areas. These areas selected for quantification covered a large part of the caudate nucleus, putamen and nucleus accumbens (445 000 m²). The numbers of cells in each selected area were averaged for each animal, and the mean (± SD) number of BrdU-ir nuclei per square millimetre was calculated for the controls and MPTP-treated animals. DA depletion was evaluated by measuring the optical density of TH immunoreactivity in the striatum with a computer-based system (Mercator). Statistical analysis was performed using a one-factor or two-factor ANOVA (clinical state factor: control or MPTP-treated; striatal region factor: caudate nucleus, putamen or accumbens) followed by Tukey’s post hoc analysis. A P value of < 0.05 was considered statistically significant.

## Results

### MPTP-induced parkinsonism in aged monkeys

MPTP induced a stable parkinsonian syndrome characterized by persistent akinesia, rigidity and postural instability and intermittent episodes of action and resting tremor in all three monkeys. When sacrificed, two had a moderate parkinsonian syndrome (clinical scores, 10–14), and one had severe parkinsonism (clinical score, 20).

Histological analysis and quantification confirmed that there was a dramatic loss of TH-ir neurons in the substantia nigra pars compacta in MPTP-treated monkeys compared with controls (not shown) and a significant decrease in the optical density of TH immunoreactivity in the dorsal striatum (caudate nucleus and putamen) (4.71 ± 3.73 versus 19.14 ± 6.28, P < 0.05) (Fig. 1A–C) but not in the nucleus accumbens (14.22 ± 1.56 versus 16.15 ± 4.58).

### The number of striatal TH-ir cells increases in MPTP-treated aged monkeys

In control primates, TH-ir cells, defined as a cell body with two or more processes, were distributed preferentially at the periphery of the striatum throughout the entire rostrocaudal extent, as reported by others (Dubach et al., 1987; Palfi et al., 2002). Their cell bodies measured 10–18 μm and had two to five primary aspiny dendrites (Fig. 1D).

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**Table 1** List of primary antibodies and details of immunohistochemical procedure used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Animal source</th>
<th>Dilution</th>
<th>Source</th>
<th>References</th>
</tr>
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<tr>
<td>BrdU</td>
<td>Rat</td>
<td>1 : 200</td>
<td>Immunologicals Direct, Kidlington, UK</td>
<td>Hoglinger et al., 2004</td>
</tr>
<tr>
<td>Calbindin (CB)</td>
<td>Mouse</td>
<td>1 : 2000</td>
<td>Sigma, St Louis, MO</td>
<td>Betarbet et al., 1997</td>
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<td>Calretinin (CR)</td>
<td>Rabbit</td>
<td>1 : 5000</td>
<td>Swant, Switzerland</td>
<td>Parent et al., 1995</td>
</tr>
<tr>
<td>Dopamine beta hydroxylase (DBH)</td>
<td>Rabbit</td>
<td>1 : 3000</td>
<td>Chemicon, Temecula, CA</td>
<td>Cossette et al., 2005</td>
</tr>
<tr>
<td>Dopamine transporter (DAT)</td>
<td>Rat</td>
<td>1 : 7500</td>
<td>Chemicon</td>
<td>Cossette et al., 2005</td>
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<tr>
<td>Glial fibrillary acid protein (GFAP)</td>
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<td>1 : 250</td>
<td>Sigma</td>
<td>Tonchev et al., 2003</td>
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<tr>
<td>Glutamate decarboxylase (GAD-67)</td>
<td>Rabbit</td>
<td>1 : 500</td>
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<td>Betarbet et al., 1997</td>
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<td>Neuronal nuclei (NeuN)</td>
<td>Mouse</td>
<td>1 : 200</td>
<td>Chemicon</td>
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<tr>
<td>Neuropeptide Y (NPY)</td>
<td>Rabbit</td>
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<tr>
<td>Parvalbumin (PV)</td>
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<td>1 : 5000</td>
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<tr>
<td>S100β</td>
<td>Mouse</td>
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<td>Tyrosine hydroxylase (TH)</td>
<td>Rabbit</td>
<td>1 : 250</td>
<td>Pel-Freez, Rogers, AR</td>
<td>Betarbet et al., 1997</td>
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**Fig. 1** TH immunostaining in control and MPTP-treated monkeys. TH-ir axons in the striatum of a control macaque (A) and an MPTP-treated macaque (B). (C) A significant decrease in the optical density of TH immunoreactivity was found in the MPTP-treated group. *P < 0.05. (D) Examples of bipolar and multipolar TH-ir cells in the striatum after MPTP treatment. (E) Distribution of TH-ir cells in the striatum of an MPTP-treated macaque. (F) A significant increase in the number of TH-ir cells was found in the MPTP-treated group. *P < 0.05. Acc: nucleus accumbens; Cd: caudate nucleus; Pu: putamen; V: ventricle. Scale bar, 2 mm in A, B, and 25 μm in D.
The morphology of the striatal TH-ir cells was similar in the MPTP-treated monkeys, but they were distributed homogeneously throughout the striatum (Fig. 1E). They were also 2.3-fold more numerous in the MPTP-treated monkeys in the DA-depleted striatum (quantification on four sections covering the anteroposterior extent of the structure), including the nucleus accumbens where the dopaminergic innervation was less affected by the treatment (76.00 ± 23.65 versus 32.33 ± 12.77, P < 0.05) (Fig. 1F).

**Striatal TH-ir cells are interneurons**

We then characterized the phenotype of the striatal TH-ir cells in aged normal and MPTP-treated animals. First we verified that the TH-ir structures had DAPI-positive nuclei confirming that they were indeed cells (Fig. 2A and B) because degenerating, swollen dopaminergic axons might be mistaken for bipolar cell bodies (Fig. 2C). More than one-third of the TH-ir striatal cell bodies displayed a few lipofuscin granules, as visualized with TH immunohistochemistry where Sudan Black B was omitted (Fig. 2D). None of the TH-ir cells observed co-expressed the markers glial fibrillary acid protein (GFAP) or S100 beta demonstrating that they were not astroglia (Fig. 2E). All of the TH-ir cells in the striatum of MPTP-treated monkeys expressed dopamine transporter (DAT) (Fig. 2F), but none expressed dopamine beta hydroxylase (DBH) (Fig. 2G), indicating that they were dopaminergic and not noradrenergic neurons. Virtually all the TH-ir neurons were negative for calbindin (CB) (Fig. 2H), suggesting that they were interneurons rather than projection neurons. The vast majority of them had a small cell body and were therefore not cholinergic interneurons, which have large cell bodies. They did not express neuropeptide Y (NPY) (Fig. 2I) or parvalbumin (PV) (Fig. 2J). A small proportion (4 cells out of 90) expressed calretinin (Fig. 2K), as previously reported (Cicchetti et al., 1998). Virtually all cells that stained for TH also intensely co-expressed glutamic acid decarboxylase (GAD)-67 (Fig. 2L), demonstrating that they were phenotypically similar to small, aspiny, GABAergic interneurons. Although most of the striatal GAD-67-ir neurons contain the ageing pigment lipofuscin, a very few appeared devoid of granules in MPTP-treated monkeys and in controls, as visualized with GAD immunohistochemistry where Sudan Black B was omitted.
The number of newborn cells increases in the striatum of MPTP-treated aged monkeys

Many newborn BrdU-ir cells could be seen in the striatum of control monkeys. As described previously (Bedard et al., 2002a), they were homogeneously distributed throughout the striatum. The absence of a gradient with respect to the subependymal zone argues against a massive migration of newborn cells into the striatum. Furthermore, many of the BrdU-ir cells appeared in pairs (Fig. 3A, insert), suggesting that they had divided in situ. After DA depletion, the number of BrdU-ir cells increased 3-fold (15.26 ± 3.18 and 45.84 ± 5.50 cells/mm²; P < 0.05) in the whole striatum (caudate nucleus, putamen and nucleus accumbens) (Fig. 3A–C).

The striatal TH-ir cells induced by MPTP treatment are not newborn neurons

To determine whether the striatal TH-ir cells in normal or MPTP-treated aged monkeys were produced by adult neurogenesis, we examined whether the BrdU-ir neurons in striatum expressed neuronal markers. None of the more than 1000 BrdU-ir nuclei analysed in the striatum of control or MPTP-treated animals expressed the neuronal markers NeuN (Fig. 4A), GAD-67 (Fig. 4B) or TH (Fig. 4C). We then examined 500 TH-ir cells in the striatum after MPTP treatment for BrdU incorporation. None of these cells were BrdU-positive, demonstrating that they were not newborn. This negative result cannot be attributed to inadequate histological processing, since BrdU–NeuN-labelled cell bodies were detected in the hippocampal dentate gyrus (Fig. 4D).

Discussion

Our study provides evidence that the TH-ir neurons that appear after DA depletion in the striatum of aged macaques do not result from neurogenesis but rather from a phenotypic shift of pre-existing neurons. We worked with aged macaques (20–25 years of age) because the prevalence of Parkinson’s disease increases with age. We induced an experimental DA depletion using repeated MPTP injections according to an established protocol (Elsworth et al., 1987), except that the injections were separated by longer time intervals owing to the increased susceptibility of aged animals to MPTP (Ovadia et al., 1995). The increase in the number of striatal DA-ir neurons induced by experimental DA depletion in the aged primates (+150%) was smaller than that described previously in younger macaques (+250 to +300%) (Betarbet et al., 1997; Falci et al., 2002), but greater than the increase observed in the post-mortem striatum of older Parkinson’s disease patients (+84%) (Porritt et al., 2000). These observations suggest that the appearance of new DA neurons decreases with increasing age.

In agreement with previous studies (Betarbet et al., 1997; Cossette et al., 2005), virtually all of the striatal TH-ir cells co-expressed the DAT, suggesting that they can function as DA neurons. None of TH-ir cells clearly expressed CB suggesting that they are interneurons (Gerfen et al., 1985). However, the possibility that a small proportion of TH-ir cells belong to the class of spiny neurons, as reported in the literature (Betarbet et al., 1997; Cossette et al., 2005), cannot be excluded. The absence of PV and NPY, and the presence of the GABAergic marker GAD-67, suggest that they may correspond to a subtype of striatal interneurons, as previously reported in humans (Cossette et al., 2005). Owing to the small size of their cell bodies and to the fact that they have two to five dendritic stems, they may be the microneurons we described previously in macaques using Golgi-impregnated material (Yelnik et al., 1991).

Although we detected newborn neurons in the hippocampal nucleus, putamen and nucleus accumbens) (Fig. 3A–C). (20–25 years of age) because the prevalence of Parkinson’s shift of pre-existing neurons. We worked with aged macaques do not result from neurogenesis but rather from a phenotypic appear after DA depletion in the striatum of aged macaques...
were produced by adult neurogenesis. This is in contradiction with the results of Bedard et al. (2002a), who found, with a similar cumulative dose of BrdU and an identical survival time after the last BrdU injection, the neuronal marker NeuN in 5 to 10% of the BrdU-ir nuclei in the striatum of young adult (4 to 6 years) squirrel monkeys. This discrepancy may be due to differences in the strains of monkeys used (Kornack and Rakic, 2001; Bedard et al., 2002b), the enriched environment of the monkeys in the study of Bedard et al. (2002b), a condition known to induce an increase in neurogenesis (Kempermann et al., 1997), or differences in age, since ageing has been shown to affect neurogenesis in hippocampus and subventricular zone (Gould et al., 1999; Jin et al., 2003; Bondolfi et al., 2004). It was still surprising that not a single new striatal neuron was detected in our animals, given the existence of quiescent stem cells with neurogenic potential within the striatal parenchyma itself (Reynolds and Weiss, 1992), as well as in the proximal subventricular zone (Yang et al., 2004). It is possible that the 3–6 weeks survival time was not long enough, in aged macaques, for proliferating cells to differentiate into mature neurons. The time needed for a newborn BrdU-ir neuroblast to develop a mature neuronal phenotype was estimated to be 2 weeks in the dentate gyrus of the hippocampus (Gould et al., 1999), but 97 days in the olfactory bulb (Kornack and Rakic, 2001). Thus, we cannot completely rule out the existence of spontaneous or MPTP-induced neurogenesis in the striatum of aged primates. However, we can conclude with certainty that the DA neurons that appeared in the striatum after MPTP treatment were not newborn cells but rather pre-existing cells that had changed their phenotype. The presence of a few lipofuscin granules, at least in more than one-third of the TH-ir micro-neurons in aged monkeys, seems to favour the notion that some TH-ir cells may be mature. It is possible that the reduced quantity of lipofuscin is a characteristic of striatal micro-neuron population compared to striatal projection neurons, as already reported for the monkey subthalamic interneurons compared to projection neurons (Rafols and Fox, 1976). The existence of some GABAergic striatal cells apparently devoid of lipofuscin in control macaques strengthens the view that TH-ir cells may be a result of a transdifferentiation of pre-existing GABAergic neurons.

In summary, we have shown that there was no neurogenesis in the striatum of aged control macaques. However, the striatum of aged primates is still able to react to DA depletion with the formation of new intrinsic DA neurons. They do not result from adult neurogenesis, but rather from the phenotypic transdifferentiation of pre-existing neurons, which appear to be a specific subtype of GABAergic microneurons. Since injections, infusions or gene delivery of growth factors such as the glial cell line-derived neurotrophic factor and the brain-derived neurotrophic factor have potent trophic effect on DA neurons in normal and MPTP-treated monkeys (Palfi et al., 2002; Gash et al., 2005), it is possible that these trophic molecules may be directly implicated in the DA transdifferentiation postulated from our results. A detailed understanding of the molecular signals mediating the phenotypic shift might help in developing strategies to amplify these spontaneous attempts of the damaged brain to repair itself. If the TH-ir cells induced indeed provide DA to the normal targets of the nigrostriatal pathway, this might constitute a novel and elegant cell-based therapy for Parkinson’s disease that would avoid many of the drawbacks of cell transplantation.

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