Intracranial application of near-infrared light in a hemi-parkinsonian rat model: the impact on behavior and cell survival

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OBJECTIVE The authors of this study used a newly developed intracranial optical fiber device to deliver near-infrared light (Nlr) to the midbrain of 6-hydroxydopamine (6-OHDA)–lesioned rats, a model of Parkinson's disease. The authors explored whether Nlr had any impact on apomorphine-induced turning behavior and whether it was neuroprotective.

METHODS Two Nlr powers (333 nW and 0.16 mW), modes of delivery (pulse and continuous), and total doses (634 mJ and 304 J) were tested, together with the feasibility of a midbrain implant site, one considered for later use in primates. Following a striatal 6-OHDA injection, the Nlr optical fiber device was implanted surgically into the midline midbrain area of Wistar rats. Animals were tested for apomorphine-induced rotations, and then, 23 days later, their brains were aldehyde fixed for routine immunohistochemical analysis.

RESULTS The results showed that there was no evidence of tissue toxicity by Nlr in the midbrain. After 6-OHDA lesion, regardless of mode of delivery or total dose, Nlr reduced apomorphine-induced rotations at the stronger, but not at the weaker, power. The authors found that neuroprotection, as assessed by tyrosine hydroxylase expression in midbrain dopaminergic cells, could account for some, but not all, of the observed behavioral improvements; the groups that were associated with fewer rotations did not all necessarily have a greater number of surviving cells. There may have been other “symptomatic” elements contributing to behavioral improvements in these rats.

CONCLUSIONS In summary, when delivered at the appropriate power, delivery mode, and dosage, Nlr treatment provided both improved behavior and neuroprotection in 6-OHDA–lesioned rats.

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KEY WORDS functional neurosurgery; neuroprotection; Parkinson's disease; photobiomodulation; substantia nigra; 6-hydroxydopamine

PARKINSON'S disease is a progressive disorder with distinct cardinal signs of resting tremor, lead-pipe rigidity, akinesia, bradykinesia, or all of these.^{2,24} The current treatment options of drug therapy and surgery treat the motor signs of the disease very well, at least initially, but they do not stop the progression of the disease. The dopa-

minergic cells in the substantia nigra pars compacta (SNc) continue to die throughout the treatment period.^{3,24,35,49} In essence, these treatments do not offer neuroprotection.

Many recent studies, from the culture dish to various animal models, provide promising evidence that near-infrared light (Nlr) treatment is an effective neuroprotective

ABBREVIATIONS ATP = adenosine-5'-triphosphate; CPu = caudate-putamen complex; GFAP = glial fibrillary acidic protein; IBA1 = ionized calcium binding adaptor molecule 1; LED = light-emitting diode; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Nlr = near-infrared light; NlrC = continuous Nlr at weaker power; NlrC+ = continuous Nlr at stronger power; NlrP = pulse Nlr; PBS = phosphate-buffered saline; RHS = right-hand side; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; TH = tyrosine hydroxylase; VTA = ventral tegmental area; 6-OHDA = 6-hydroxydopamine.

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agent.^{8,9,27,39} NlR treatment has been shown to increase cell survival among dopaminergic cells after various types of parkinsonian insult, for example rotenone,^{29,55} 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),^{12,28,32,33,40,45,52} and the K3691 transgenic mutation.⁴² The precise mechanism involved is not clear, but NlR is believed to repair mitochondrial damage and increase adenosine-5'-triphosphate (ATP) production, as well as decrease oxidative stress and activate various transcription factors.^{7,15,16,47} In addition to this structural evidence of neuroprotection, NlR treatment has been reported to improve locomotor activity in MPTP-treated mice.^{33,45,54}

The neuroprotective effects of NlR have been reported in cases where there have been no (e.g., culture dish) or few tissue barriers (e.g., retina or small mouse brain). In fact, the farthest distance that NlR has been traced from the source is approximately 30 mm, a distance well short of the 80–100 mm required to reach the SNc from the surface of the brain in humans.²⁷ This makes NlR directed transcranially unlikely to be useful for treating midbrain lesions in humans. To overcome this, we have been developing an optical fiber device that can be implanted accurately within the brain, to deliver NlR with minimal attenuation to regions near the SNc. Our recent results in MPTP-treated mice show that intracranially applied NlR does not cause toxic damage to surrounding brain tissue when applied in the region of the lateral ventricle, and that when applied in this fashion and location, it is neuroprotective to dopaminergic SNc cells.³²

In this study, we used the intracranial optical fiber device to deliver NlR to the SNc in another model of Parkinson's disease, the 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat. Because there is no ideal animal model of Parkinson's disease, exploring the neuroprotective and behavioral effects of NlR in a number of models strengthens the case for translation to humans. We have explored 2 main issues in this study. First, we analyzed whether the midbrain is a feasible implant site. Our previous study in mice, for species-specific technical reasons, used the lateral ventricle as a target site,³² but for our planned monkey and human work, we will target a region closer to the SNc, namely the midline midbrain at a point midway between the left and right SNc. Second, we analyzed whether NlR had any impact on apomorphine-induced rotational behavior or was neuroprotective, or both, in 6-OHDA-lesioned rats. We tested the impact of different NlR applications, using 2 powers (333 nW and 0.16 mW), modes of delivery (pulse and continuous), and total doses (634 mJ and 304 J). We have evidence regarding some of these issues in the MPTP-treated mouse model using the intracranial device,³² but no evidence is available in the 6-OHDA-lesioned rat model or on behavioral outcomes. Overall, our findings will indicate whether NlR improves behavior and offers neuroprotection in another model of the disease, as well as whether the midline of the midbrain is a feasible optical fiber implant site.

Methods

Animals

Male Wistar rats (approximately 8 weeks old; $n = 61$)

were used for these experiments. Animals were housed on a 12-hr light/dark cycle with unlimited access to food and water. All experiments were approved by the Animal Ethics Committee COMETH (Grenoble) and by the French Ministry for Research.

Experimental Design

We set up 5 experimental groups (Fig. 1). All groups received intrastriatal injections of either 6-OHDA or saline, combined with light-emitting diode (LED)-optical fiber implants delivering NlR, or not. The different groups were saline (saline injections, no NlR; $n = 8$), 6-OHDA (6-OHDA injections, no NlR; $n = 15$), and 6-OHDA-NlR (6-OHDA injections, with NlR; $n = 38$) groups. There were 3 6-OHDA-NlR groups, each of which received different doses of NlR during the course of the survival period. These were the 6-OHDA-NlRP (NlR delivered in pulses at 0.16 mW power; $n = 16$), 6-OHDA-NlRC+ (NlR delivered continuously at 0.16 mW power; $n = 13$), and 6-OHDA-NlRC (NlR delivered continuously but at 333 nW power; $n = 9$) groups. A saline group exposed to NlR was not included, because we have previously published that NlR has no impact on the survival and function of cells in normal brains.^{12,28,32,33,40,45,52} Given the consistency of these previous results, and the complexity of our surgical protocol, we believed that the extra control group was redundant.

Our experimental paradigm of simultaneous administration of parkinsonian insult and therapeutic application was similar to that of previous studies of animal models of Parkinson's disease.^{12,28,32,33,40,41,45,52,53} This paradigm is unlike the clinical reality in which there is cell loss prior to therapeutic intervention. However, in our experimental study, we hoped to determine the maximum effect of any NlR neuroprotection by the LED-optical fiber implant.

6-OHDA Injections

Following protocols used in many previous studies,^{13,18,31,41} rats were anesthetized after an intraperitoneal injection of ketamine (100 mg/kg; Imalgène 1000; Merial) and xylazine (10 mg/kg; Rompun; Bayer Animal Health), and they had 6-OHDA (7.5 $\mu\text{g}/\mu\text{l}$ saline with addition of 0.2% ascorbic acid; approximately 4 μl injected over 8 minutes) or saline (plus 0.2% ascorbic acid) injected stereotactically into the caudate-putamen complex (CPu; coordinates: anteroposterior +1 mm, lateral +3 mm, dorsal-ventral –6.3 mm to skull)³⁹ of the right-hand side (RHS). We injected the CPu, rather than the medial forebrain bundle, because we wanted a smaller lesion in the SNc (20%–30%); medial forebrain bundle injections are known to generate larger lesions and massive SNc cell death (> 70%), a situation not ideal for neuroprotection studies.^{13,18,31,41}

Optical Fiber NlR Implants

After the 6-OHDA injection, we implanted the LED-optical fiber device, as described previously in mice.³² Briefly, a small 670-nm LED (SMT 670; Epitex) was attached to an optical fiber (FT300EMT; Thorlabs) with a tip diameter of 300 μm (Fig. 2A). The step index optical fiber was plastic coated, as to limit leakage of NlR along the

FIG. 1. Outline of the experimental design of this study. Surgery on rats, namely 6-OHDA or saline injection and implantation of optical fiber, was performed on Day 0. Animals were allowed to survive for 23 days thereafter. In the 6-OHDA-NIrP group, NIR was applied for 90 seconds twice a day, while in the 6-OHDA-NIrC groups, NIR was applied continuously during the survival period; in the 6-OHDA-NIrC+ group, NIR was emitted at a higher power than in the other NIR groups. Rats were tested for apomorphine-induced turning behavior on Day -1 (day before surgery) as well as Day 7, Day 14, and Day 21 after surgery. Figure is available in color online only.

length of the fiber and focus to the tip. The LED was attached with electrical cables that would be connected subsequently to a current regulator circuit and battery source (Fig. 2A). All LED-optical fibers were tested for power output using a calibrated light sensor (Optical power meter, Anritsu ML9002A). We found that the LED emitted no heat and was satisfactory for use over longer periods. The optical fiber was implanted stereotactically into a region near the SNc, incorporating the red nucleus and ventral tegmental area (VTA), toward the midline (Fig. 2B; coordinates to bregma: anteroposterior -5.6 mm, lateral

+2.9 mm, dorsal-ventral -8.5 mm to skull, at an angle of 20°).³⁹ We chose this site because it is close to the SNc of both sides. After implantation, biological cement was used to attach the device to the cranium, and the proximal ends of the battery cables were attached to the device, while the distal ends were attached to a battery source placed on top of the rat cage (Fig. 2C-E).³² Immediately following the surgery, rats in the 6-OHDA-NIrP group had 0.16 mW power (the same power as used previously for neuroprotection in MPTP-treated mice)³² delivered in pulses, twice a day for 90 sec (approximately 6 hours apart)

FIG. 2. **A:** Image of the LED-optical fiber device; *white arrow* indicates the LED, while the *black arrow* indicates the optical fiber (these arrows indicate corresponding structures in B–E). **B:** Schematic diagram of optical fiber located within our target region near the SNc. **C:** Image of rat, soon after surgery, with cable attached to battery source on top of the cage. This setup allowed the rat to move freely around the cage, eating and drinking at will. **D and E:** Images of a rat soon after surgery with the implanted LED-optical fiber device turned off (D) and on (E). This animal was still anesthetized following surgery. Note that the Nlr was visible through the biological cement surrounding the device, the skin, and auricles, and although not obvious in this image, the eyes. The *black arrows* indicate the tip of the optical fiber, while the *white arrows* indicate the LED device. Red = red nucleus. Figure is available in color online only.

over the 23-day survival period, resulting in a total dose of 634 mJ (Table 1). Rats in the 6-OHDA-NlrC+ group also had 0.16 mW power, but this was delivered continuously over the 23-day survival period, resulting in a much higher total dose of 304 J. These 2 groups thus allowed us to compare the effectiveness of 2 Nlr doses delivered at the same power. Finally, the rats in the 6-OHDA-NlrC group had a weaker 333 nW power delivered continuously over the survival period, resulting in the same total dose as the 6-OHDA-NlrP group of 634 mJ. Hence, the 6-OHDA-NlrP and 6-OHDA-NlrC groups allowed us to compare the effectiveness of the same dose, but delivered by 2 different Nlr powers (Table 1).

For several reasons, we are confident that any neuroprotective or behavioral improvement after Nlr treatment would not be related to a direct deactivation or degradation of the toxin by Nlr. First, Nlr offers beneficial effects

to diseased or damaged neurons in a broad range of neurological conditions and animal models of disease. For example, Nlr is neuroprotective after 1) insult by a number of different toxins (e.g., MPTP, rotenone, methanol)^{11,29,55} 2) traumatic brain injury,^{37,44} 3) stroke,³⁶ 4) macular degeneration,²¹ and 5) transgenic mutation in mouse models

TABLE 1. The modes of Nlr delivery, power, and total doses used in the different groups of this study

Group	Mode of Nlr Delivery	Power	Total Dose
Saline	None	None	None
6-OHDA	None	None	None
6-OHDA-NlrP	Pulse, 90 sec twice a day	0.16 mW	634 mJ
6-OHDA-NlrC	Continuous	333 nW	634 mJ
6-OHDA-NlrC+	Continuous	0.16 mW	304 J

of multiple sclerosis,³⁴ Alzheimer's disease,¹⁰ and parkinsonism.⁴² Second, NIR is equally effective when applied either before or after toxic insult; pre- or posttreatment of NIR, either *in vitro*⁵⁵ or *in vivo*,⁴⁰ mitigates MPTP-induced cell damage. Third, our measurements using high-performance liquid chromatography and liquid chromatography/mass spectrometry indicate that NIR does not directly degrade MPTP (unpublished observations). Finally, NIR has been shown to have a direct influence on intrinsic cellular activity,^{7,15,16,47} as well as being directly effective in reducing cell death induced by apoptosis.⁴⁴ Taken together, these findings all strongly support the idea that NIR provides genuine cellular interaction and neuroprotection.

Apomorphine-Induced Rotational Behavior

Rats were given an intraperitoneal injection of apomorphine (Apokinin, dopaminergic agonist; 0.5 mg/kg; Aguettant), and they were monitored for any rotational behavior postsurgery.^{14,51} A measure of the extent of the 6-OHDA lesion is the number of contralateral rotations a rat may make soon after each injection. The number of rotations of each rat was counted for 10 minutes postinjection^{14,51} the day before surgery (Day -1) and Day 7, Day 14, and Day 21 postsurgery.

Immunohistochemical Analysis

At the end of the experimental period, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). They were then perfused transcardially with phosphate-buffered saline (PBS) followed by 4% buffered paraformaldehyde. The brains were removed and postfixed overnight in the same solution. Next, brains were placed in PBS with the addition of 30% sucrose until the block sank. The forebrain was then sectioned coronally and serially (at 50 μ m) using a freezing microtome. All sections were collected in PBS, then immersed in a solution of PBS and 10% normal mouse serum (M5905 Sigma), and then in PBS and 1% Triton (X100 Sigma) each for approximately 1 hour at room temperature. Sections were then incubated in either anti-tyrosine hydroxylase (TH; 1:500; T8700 Sigma), anti-glial fibrillary acidic protein (GFAP; 1:500; Z033429-2 Dako), or anti-ionized calcium binding adaptor molecule 1 (IBA1; 1:500; 019-19741 Wako) for 48 hours (at 4°C), followed by biotinylated anti-rabbit IgG for 4 hours and then Extravidin-peroxidase complex for 2 hours (both at room temperature; 1:20; EXTRA3-1KT Sigma). To visualize the bound antibody, sections were reacted in a 3,3'-diaminobenzidine tetrahydrochloride solution (D3939 Sigma). Sections were mounted onto gelatinized slides, air-dried overnight, dehydrated in ascending alcohols, cleared in Histo-Clear (Sigma), and coverslipped using DPX mounting medium (Sigma). As a rule, forebrain sections (including CPu) were immunostained for TH, while midbrain (including implant site) sections were immunostained for TH, GFAP, or IBA1 or were Nissl-stained with cresyl violet. For controls, sections were processed as described above, except that no primary antibody was used. These control sections were immunonegative.

Statistical Analysis

The number of TH+ cells within the SNc (main body of the nucleus, i.e., ventral tier)^{13,46} was estimated using the optical fractionator method (Stereoinvestigator, MBF Science), as outlined previously.^{12,28,32,33,40,42,45,52,53} Briefly, systematic random sampling of sites—with an unbiased counting frame—within defined boundaries of the SNc was undertaken. All cells that came into focus within the frame were counted and at least 7 sites were sampled per section in the SNc. Cell counts were made on the RHS of the brain, the side injected with either 6-OHDA or saline, because most nigral pathways are ipsilateral.³⁸ For group comparisons of cell number, a 1-way ANOVA test was performed (overall comparison of means between groups; F and p values), in conjunction with a post hoc multiple comparison test (comparison of means between pairs of groups; p value) that was incorporated within the software (GraphPad Prism v6).

Results

In the section that follows, the results relating to the apomorphine-induced rotational behavior, the 6-OHDA injection and optical fiber implant sites, together with the number and morphology of SNc cells, will be considered separately.

Apomorphine-Induced Rotational Behavior

No major adverse effects were observed in the rats after surgery for either the implantation of the optical fiber devices or the 6-OHDA lesion. All rats resumed normal activity (eating and grooming) within a few hours after surgery and were never in any visible discomfort during the entire survival period.^{13,18,31,32} There was no obvious impact of NIR on the normal activity of the rats; the activity of NIR-treated rats was not any different from that of untreated rats. In the NIR-treated rats, the NIR filled the cranial cavity, being evident through the biological cement surrounding the LED device, through the skin and hair over the cranium, the auricles, and through the orbit and eyes (Fig. 2D and E). Hence, as with our previous results in mice,³² the intracranially applied NIR was well tolerated by the rats, with no clear deleterious signs.

Figure 3 shows the apomorphine-induced rotational behavior in rats of the different experimental groups. On the day before surgery (Day -1), all the rats in the different groups displayed no rotational behavior after apomorphine injection. In some cases, there was hyperkinetic movement after apomorphine injection, but it soon settled. In the saline group, there was never any apomorphine-induced rotational behavior throughout the 3-week period. In the 6-OHDA group, by contrast, a steady increase in the number of apomorphine-induced rotations, from Day 7 to Day 21, was evident. This indicated the development of a clear functional lesion. In all 6-OHDA-NIR groups, evidence was present of rotational behavior also, but in the 6-OHDA-NIRP and 6-OHDA-NIRC+ groups, it was considerably less than in the 6-OHDA group. This was clearly the case at Day 21, when the differences between the 6-OHDA group and both the 6-OHDA-NIRP ($p < 0.05$) and, in particular, the 6-OHDA-NIRC+ ($p < 0.001$) groups

were significant. The fewer rotations in these groups suggested that Nlr had reduced the functional lesion generated by 6-OHDA. Similar patterns were not evident for the 6-OHDA-NlrC group, however, that received Nlr at a weaker power (Table 1). In this Nlr-treated group, the development and number of apomorphine-induced rotations from Day 7 to Day 21 largely followed that apparent in the 6-OHDA group (Fig. 3). Overall, the differences between the 6-OHDA and 6-OHDA-Nlr groups did not reach significance at Day 7 (ANOVA: $F = 1.4$, $p = 0.3$) and Day 14 (ANOVA: $F = 2.0$, $p = 1.2$), but did so at Day 21 (ANOVA: $F = 4.1$, $p < 0.01$).

6-OHDA CPu Injection Sites

Figure 4 shows TH-immunostained sections of the CPu on the injected RHS of the saline (Fig. 4A), 6-OHDA (Fig. 4B), and 6-OHDA-Nlr (Fig. 4C; 6-OHDA-NlrP) groups. In the saline group, injection sites were characterized by a central core of gliosis, indicating the pipette tract; in the regions immediately surrounding the tract, there was no clear loss of TH immunoreactivity (Fig. 4A). In the 6-OHDA and 6-OHDA-Nlr groups (all 6-OHDA-Nlr groups showed similar patterns), a comparable arrangement of gliosis indicating pipette tract was also seen, but in these cases, the tract was surrounded by a region devoid of dense TH+ terminations; in this region, there were only a few TH+ axons and terminals seen (Fig. 4B and C). This largely barren, TH- region was due to 6-OHDA toxicity and was similar in the 6-OHDA and 6-OHDA-Nlr groups (Fig. 4B and C).

Optical Fiber Midbrain Implant Sites

Figure 5 shows schematic diagrams (Fig. 5A) and a photomicrograph montage (Fig. 5B) of an optical fiber tract through the midbrain. This particular fiber trace was from a rat in the 6-OHDA-NlrP group, but it is typical of the traces we saw in other groups. The implant site in

each rat was close to the midline in the midbrain, traversing the red nucleus and lying close to or within the VTA. The distinct square shape of the optical fiber tip was often identifiable (Fig. 5B).

Figure 6 shows photomicrographs comparing Nissl-stained (Fig. 6A and B), GFAP-immunostained (Fig. 6C and D), and IBA1-immunostained (Fig. 6E and F) cells in regions immediately adjacent to the implant site in saline-sham (Fig. 6A, C, and E) and Nlr-treated (Fig. 6B, D, and F) rats. In general, no major differences were found in the organization and morphology of neurons and glial cells near the implant sites in the different rats. Each of the optical fiber tracts and implant sites generated localized gliosis and some neuronal degeneration, but these patterns were the same in all of the rats, regardless of whether there was Nlr treatment or not. The only cellular damage evident appeared mechanical, being caused after tissue displacement by the optical fiber itself. In addition, we never saw localized zones of necrosis near the tip of the optical fiber, where the Nlr was most intense (Fig. 5B). Finally, there appeared to be no impact of Nlr on the cytoarchitecture in regions near the implant site, most notably, the SNc (Fig. 5B).

Number and Morphology of SNc Cells

In this study, we used TH immunocytochemistry to assess patterns of neuroprotection in the SNc. As with many previous studies, we interpreted TH+ cell number after experimental manipulation as an index of cell survival.^{12,28,32,33,40,42,45,52,53} If cells lose TH expression, then they are likely to undergo death subsequently.^{4,22,25} Notwithstanding a small number of cells that may have transient loss of TH expression, a key outcome measure for our study was whether Nlr treatment mitigated the loss of TH+ cells caused by the 6-OHDA.^{12,28,32,33,40,42,45,52,53}

Figure 7 shows a graph of the estimated total number of TH+ cells in the SNc of the 5 experimental groups. A

FIG. 3. Graph showing the mean number of apomorphine-induced turns of rats (in 10 mins) in the 6-OHDA, 6-OHDA-Nlr, 6-OHDA-NlrC, and 6-OHDA-NlrC+ groups at 4 time points, 1 day before surgery (Day -1) and Day 7, Day 14, and Day 21 postsurgery. The saline group had no rotational behavior throughout the experimental period (not shown). Symbols adjacent to particular markers represent the level of significant difference from the 6-OHDA group on that day of testing; ^ $p < 0.001$, * $p < 0.05$. All columns show the mean $\pm$ standard error in each group. Figure is available in color online only.

FIG. 4. Photomicrographs of saline and 6-OHDA injection sites on the RHS CPu in the saline (A), 6-OHDA (B), and 6-OHDA-NIrP (C) groups (labeling patterns were similar in the other 6-OHDA-NIr groups, hence not shown). Sections were immunostained for TH, and the saline (A) or 6-OHDA (B and C) injection sites are indicated by arrows. In each 6-OHDA injection site, there was a central core of gliosis indicating the pipette tract, surrounded by a striatal region devoid of rich TH immunostaining (B and C). This non-TH immunostained region was as a result of the 6-OHDA toxicity to the dopaminergic terminals. The injection sites in the 6-OHDA and all 6-OHDA-NIr groups were very similar. In each saline injection site, there was a central core of gliosis and pipette tract, but no surrounding region of non-TH immunostaining (A). All figures are of coronal sections; dorsal to top and lateral to right for RHS images and lateral to left for LHS image. Bar = 1 mm.

clear reduction occurred in the number of TH+ cells in the 6-OHDA group compared with the saline group (approximately 25%; $p < 0.05$). In the 6-OHDA-NIrP group, TH+ cell number was considerably higher than that in the 6-OHDA group (approximately 25%; $p < 0.001$), equivalent to that in the saline group, i.e., pulse delivery of NIr appeared to mitigate the cell loss caused by 6-OHDA toxicity. A comparable mitigation was not, however, evident in the other 6-OHDA-NIr groups; the number of TH+ cells in the 6-OHDA-NIrC and 6-OHDA-NIrC+ groups was similar to that in the 6-OHDA group ($p > 0.05$; Fig. 7). Thus, NIr treatment mitigated TH+ cell loss after 6-OHDA lesion, but only under certain conditions. It was far more effective after pulse delivery (6-OHDA-NIrP group) than after continuous delivery either at the same (6-OHDA-NIrC+ group) or weaker (6-OHDA-NIrC group) NIr power. Overall, when comparing all the groups together, the overall differences in the number of TH+ cells were significant (ANOVA: $F = 3.2$, $p = 0.02$).

Figure 8 shows photomicrographs of TH+ cells in the SNc of the saline (Fig. 8A), 6-OHDA (Fig. 8B; similar patterns were seen in the 6-OHDA-NIrC and 6-OHDA-NIrC+ groups), and 6-OHDA-NIrP (Fig. 8C) groups. Although the morphology of TH+ cells was similar in all the groups, the 6-OHDA (and 6-OHDA-NIrC and 6-OHDA-NIrC+) group had fewer cells and more degenerating axonal profiles (Fig. 8B arrow) than the saline and 6-OHDA-NIrP groups had.

Discussion

We have shown that intracranially delivered NIr mitigated behavioral deterioration and the loss of midbrain dopaminergic cells in 6-OHDA-lesioned rats. Quite strikingly, NIr-induced mitigation of dopaminergic cell loss did not account for all the behavioral improvements; the NIr applications that reduced apomorphine-induced rotations did not all necessarily generate a greater number of surviving dopaminergic cells. This discrepancy will be the major focus of the discussion below. First, we will consider the feasibility of the midbrain as a target for optical fiber implants.

Feasibility of a Midbrain Target Site for Intracranial Delivery of NIr

One of our major aims was to explore the feasibility of the midbrain as an implant site. Our previous study in mice targeted the lateral ventricle,³² but for our planned primate endeavors, we hope to target a midline region of the midbrain. This region was chosen because it is near the SNc, and it provides equal doses of NIr to the nucleus of the left and right side. Our results show that, after 23 days of pulse or continuous NIr treatment of the midbrain, there were no adverse effects on the well-being of the rats that, once they recovered from surgery, were never observed to be in any discomfort. Furthermore, our immunohistochemical and histological results revealed little evidence of tissue necrosis or gliosis caused by the intracranially delivered NIr. In each case, the tissue damage observed appeared mechanical, being caused by the optical fiber itself. Taken together, these data indicated that the midbrain is indeed

FIG. 5. A: Schematic diagrams of coronal sections, across the rostrocaudal axis (from left to right; plates 71–87 of rat atlas),³⁹ from where our stereological analysis was undertaken. The red rectangles indicate the typical extent and location of the majority of our implant sites within the midbrain. This case was from the 6-OHDA–NlrP group; implant sites from the other groups were similar. **B:** Photomicrograph montage of a typical implant site from TH+ immunostained sections. This case was from the 6-OHDA–NlrP group and is the same animal as depicted in (A). The arrows in both A and B indicate the approximate region where the photomicrographs in Fig. 6 were taken. Sections are of coronal sections; in B, lateral to left, dorsal to top. MG = medial geniculate nucleus; LG = lateral geniculate nucleus; PaG = periaqueductal gray matter; SCol = superior colliculus. Bar = 1 mm. Figure is available in color online only.

a feasible implant site, and when applied at the therapeutic doses we used (i.e., 0.16 mW power), the intracranially delivered Nlr generated no adverse effect on brain tissue or well-being of the rats. Previous studies have reported that adverse effects may be detected if power output is in a range well over what is needed for neuroprotection, perhaps 10 times more.^{20,25}

Impact of Nlr Treatment on Behavior and Cell Survival in 6-OHDA–Lesioned Rats

Many previous studies have used apomorphine-induced turning behavior as a means of assessing the extent of unilateral 6-OHDA lesions in either the striatum or medial forebrain bundle; the larger the lesion, the more turning behavior is evident.^{5,6,13,14,18,31,41,50,51} In this context, several studies have used this behavioral test to measure the de-

gree of neuroprotection, whether a given neuroprotective agent reduces the number of apomorphine-induced turns in 6-OHDA–lesioned rats.^{1,19,26,30} In this study, we tested various Nlr applications in 6-OHDA–lesioned rats, using 2 modes of Nlr delivery (pulse and continuous), powers (333 nW and 0.16 mW), and total doses (634 mJ or 304 J). We found that the stronger Nlr power, regardless of delivery mode or total dose, generated a clear reduction in apomorphine-induced rotations in 6-OHDA–lesioned rats (6-OHDA–NlrP and 6-OHDA–NlrC+ groups); by contrast, no reduction was evident with the weaker power (6-OHDA–NlrC group). It is likely that the Nlr signal issued by the weaker power did not penetrate the short distance to the SNc and elicit a functional response, while the stronger power did.

The most reasonable explanation for these behavioral

FIG. 6. Nissl-stained (A and B), GFAP+ (C and D), and IBA+ (E and F) cells in regions adjacent to optical fiber implant sites in the midbrain of rats in the saline (A, C, and E) and Nlr-treated (B, D, and F) cases. The region from which these photomicrographs were taken is indicated by arrows in Fig 5. The white arrows indicate the edge of the implant site. Each optical fiber implant site was associated with gliosis; there were no obvious differences between the saline-sham (non-Nlr) and the Nlr-treated cases. The gliosis around the implant site appeared due to mechanical damage caused by the optical fiber itself, with no clear signs of Nlr toxicity; gliosis was evident along the length of the optical fiber, well away from the fiber tip, where the Nlr was most intense. The Nlr-treated case shown here was from a rat in the 6-OHDA-NlrC+ group, representative of all other 6-OHDA-Nlr groups. All figures are of coronal sections, dorsal to top and medial to left. Bar = 100 μ m.

improvements at the stronger Nlr power would be that they reflected a neuroprotection of dopaminergic cells, manifesting in a preservation of functional activity and dopaminergic transmission through the striatum.²⁷ This neuroprotection would have addressed the asymmetry of striatal transmission after a 6-OHDA lesion and contributed to the reduction in turning behavior.^{1,17,26,30} Following this thinking, neuroprotection would have been expected in the groups that had improvements in behavior, namely the 6-OHDA-NlrP and 6-OHDA-NlrC+ groups, but not in the 6-OHDA-NlrC group, the group that had no behavioral improvement. Somewhat surprisingly, however, this was not entirely the case. Although neuroprotection was evident in the 6-OHDA-NlrP group, and not in the 6-OHDA-NlrC group, it was not evident in the 6-OHDA-NlrC+

group, the group that displayed the clearest improvement in behavior (Fig. 3). Hence, Nlr-induced neuroprotection could not account for all the observed behavioral improvements; there may have been other “symptomatic” elements contributing to the improvements in these cases, not just an increase in cell survival. In essence, these results indicated that patterns of cell survival might not necessarily be the most important element of all behavioral changes. The sources of these symptomatic elements are not known, but there are several possibilities. Nlr treatment may have stimulated the activity of the dopaminergic cells, in an attempt to maintain dopaminergic transmission through the striatum and to mask the cellular deficit. This effect may have been heightened in the remaining cells in the 6-OHDA-NlrC+ group to compensate for the smaller size of the

FIG. 7. Graphs showing the number of TH+ cells in each of the experimental groups. Numbers represent counts from the RHS 6-OHDA-lesioned side. The symbols within the columns of the 6-OHDA group represent levels of significant difference from the saline group, while symbols within the columns of any of the 6-OHDA-NIr groups represent significant differences in the 6-OHDA group. ^ $p < 0.001$, * $p < 0.05$. All columns show the mean $\pm$ standard error in each group.

surviving dopaminergic cell population. Alternatively, NIr treatment may have stimulated the activity of nondopaminergic cell groups in the brain, such as those involved in generating movement (e.g., motor cortex, pedunculopontine tegmental nucleus).³⁸ These structures are certainly close enough to the rat midbrain to be influenced by the NIr signal issued from the optical fiber device; previous studies have reported that the NIr signal can be measured approximately 30 mm from the source (see above), and the rat motor cortex (5–10 mm) and pedunculopontine tegmental nucleus (approximately 1 mm) are well within that distance from the midbrain.³⁹ A clear challenge for future studies would be to reveal the source of these symptomatic elements, whether dopaminergic or nondopaminergic, and if they are sustainable, in the longer-term, without any underlying neuroprotection (i.e., in the 6-OHDA-NIrC+ group).

Previous studies have reported that NIr stimulates mitochondria by increasing ATP content and electron transfer in the respiratory chain through activation of photoacceptors (e.g., cytochrome oxidase), together with modulating reactive oxygen species and the induction of various transcription factors.^{7,11,15,16,19,29,47,55} In this study, NIr may have induced such factors to 1) protect cells against toxic insult, leading to a greater cell survival, or 2) stimulate the functional activity of cells, leading subsequently to improvements in behavior, or both 1 and 2. We showed that the pulse mode was more effective in generating both neuroprotection and improved behavior (6-OHDA-NIrP group), while the continuous mode was effective in improving behavior only (6-OHDA-NIrC+ group). The reason why the continuous mode did not induce neuroprotection is not known, but we suggest that it was related to the high total dose of NIr delivered in this group (304 J); this higher dose may have been within the range for inducing an increase

in functional activity but not for inducing neuroprotection, while the lower dose (634 mJ) may have been within the range to induce both beneficial effects. NIr has been shown to be most effective in intermediate doses, but not at very low or extremely high doses, the so-called “hormetic effect.”^{7,19,47} This effect may help explain some of our results here. There may be different hormetic dose-response curves for neuroprotection and functional activity; the curve-inducing cell repair mechanisms and neuroprotection may drop off more quickly with increasing NIr dosage than the curve for activating functional activity, hence contributing to the differences in beneficial effects.⁴⁸ This issue of different biological effects by the same agent at different doses is worthy of further study.

In summary, if given at sufficient power to penetrate brain tissue and reach the SNc (0.16 mW), a pulse delivery of NIr that did not result in a very high total dose (e.g., 634 mJ), generated both neuroprotection and behavioral (functional) improvements. If the NIr power delivered was very weak (e.g., 333 nW) or if it manifested in too high a dose (e.g., 304 J), then not all the beneficial effects (neuroprotection and behavior) were evident.

Conclusions

We have shown that, as in the MPTP-treated mouse model,³² intracranially delivered NIr was well tolerated by the animals, did not cause tissue toxicity, improved behavior, and was neuroprotective in the 6-OHDA-lesioned rat model. These positive findings in a second animal model of Parkinson’s disease provide further encouragement for a positive outcome in humans. Our results show that protection of dopaminergic cells did not account for all the observed behavioral improvements, and that different applications of NIr generated different beneficial out-

FIG. 8. Photomicrographs of TH+ cells in the saline (A), 6-OHDA (B), and 6-OHDA-NlrP (C) groups on the RHS (6-OHDA- or saline-injected sides). Labeling patterns on the LHS for the 6-OHDA and all 6-OHDA-Nlr groups were similar to those in the saline group, while patterns on the RHS in the 6-OHDA group were similar to those observed in the 6-OHDA-NlrC+ and 6-OHDA-NlrC groups (not shown). Arrow in (B) indicates degenerating axons. All figures are of coronal sections; dorsal to top and lateral to right (B, D, and F) and to left (A, C, and E). Bar = 100 μ m.

comes. A careful titration of Nlr application would hence be essential when considering use in humans, establishing a power, mode of delivery, and total dose that generates improvements both in behavior and neuroprotection.

These early findings provide much encouragement for the future.

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Disclosures

Dr. Stone has ownership of CSCM Pty Ltd.

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