

Near-infrared light (670 nm) reduces MPTP-induced parkinsonism within a broad therapeutic time window

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Received: 7 December 2015 / Accepted: 28 January 2016
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Abstract We have shown previously that near-infrared light (NIR), when applied at the same time as a parkinsonian insult (e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP), reduces behavioural deficits and offers neuroprotection. Here, we explored whether the timing of NIR intervention—either before, at the same time or after the MPTP insult—was important. Mice received MPTP injections (total of 50 mg/kg) and, at various stages in relation to these injections, extracranial application of NIR. Locomotor activity was tested with an open-field test, and brains were processed for immunohistochemistry. Our results showed that regardless of when NIR was applied in relation to MPTP insult, behavioural impairment was reduced by a similar magnitude. The

beneficial effect of NIR was fast-acting (within minutes) and long-lasting (for several days). There were more dopaminergic cells in the NIR-treated MPTP groups than in the MPTP group; there was no clear indication that a particular combination of NIR treatment and MPTP injection resulted in a higher cell number. In summary, irrespective of whether it was applied before, at the same time as or after MPTP insult, NIR reduced both behavioural and structural measures of damage by a similar magnitude. There was a broad therapeutic time window of NIR application in relation to the stage of toxic insult, and the NIR was fast-acting and long-lasting.

Keywords Parkinson's disease · Photobiomodulation · Substantia nigra · Neuroprotection · Open-field test

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Abbreviations

ATP	Adenosine triphosphate
LED	Light-emitting diode
MG	Medial geniculate nucleus
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NIR	Near-infrared light
PaG	Periaqueductal grey matter
PBS	Phosphate-buffered saline
Red	Red nucleus
SNC	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
6OHDA	6-Hydroxydopamine

Introduction

There is an ever increasing need to develop neuroprotective approaches that help slow, or even stop, the degeneration

of brain cells in a number of conditions, from Parkinson's to Alzheimer's disease and from macular degeneration to motor neurone disease. In Parkinson's disease for example, although there are very good treatments that offer symptomatic relief (e.g. dopamine drug therapy), none arrest the pathology effectively; the key midbrain dopaminergic cells continue to die during the course of treatment (Olanow et al. 2008; Bezard et al. 2013; Jankovic and Poewe 2012; Schapira et al. 2014). Hence, when an intervention is reported to offer neuroprotection to these vulnerable cells, particularly across a number of animal models of the disease, one has a sense of encouragement. Near-infrared light (NIR) therapy is one such intervention ($\lambda = 600\text{--}1070\text{ nm}$; Quirk et al. 2012; Johnstone et al. 2014a, 2016).

Previous studies have shown that NIR treatment—when applied at the same time as the parkinsonian toxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)—neuroprotects many midbrain dopaminergic cells in mice (Shaw et al. 2010; Peoples et al. 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014; El Massri et al. 2015) and monkeys (Darlot et al. 2015). Further, such treatment has been reported to reduce clinical impairment and improve locomotive behaviour in both these species (Whelan et al. 2008; Moro et al. 2013; Reinhart et al. 2014; Darlot et al. 2015). Similar beneficial outcomes have been reported in other animal models of Parkinson's disease, including rats lesioned with 6-hydroxydopamine (6OHDA) (Reinhart et al. 2015) or injected with adeno-associated virus inducing overexpression of α -synuclein (Oueslati et al. 2015), and K369I transgenic mice (Purushothuman et al. 2013).

In this study, we explored the patterns of behaviour and neuroprotection when NIR was applied at different stages in relation to MPTP insult, either before (pre-), at the same time (simultaneous-) or after (post-) treatment. From these experiments, we hoped to determine: (1) the importance of the timing of treatment in relation to the insult, (2) the therapeutic time window for NIR effectiveness and (3) the time course of NIR-induced effects.

Materials and methods

Subjects

Male BALB/c mice ($n = 147$) were housed on a 12-h light/dark cycle with unlimited access to food and water. Animals were 8–10 weeks old. All experiments were approved by the Animal Ethics Committee of COMETH (Grenoble).

Experimental design

Following previous work, we used an acute MPTP mouse model (Schober 2004; Shaw et al. 2010, 2012; Blesa et al.

2012; Bové and Perier 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014; El Massri et al. 2015). Briefly, we made two MPTP (25 mg/kg injections; total of 50 mg/kg per mouse) or saline injections over a 24-h period (see Fig. 1). Mice were exposed to NIR (670 nm) from a light-emitting diode (LED; Quantum Devices WARP 10; Fig. 2a). We have estimated the energy levels reaching the midbrain at 5.3 mW/cm^2 , equating to $\sim 0.5\text{ J/cm}^2$ (Shaw et al. 2010). The different combinations and exposures of NIR for each experimental group are shown in Fig. 1. As a rule, mice had two exposures (90 s each) on any given day, approximately six hours apart (1 J/cm^2 per day). Hence, we had single (four NIR exposures over 2 days; NIR-MPTP, MPTP = NIR and MPTP-NIR groups), double (eight treatments over 4 days; NIR-MPTP = NIR and MPTP = NIR-NIR groups) and triple (twelve treatments over 6 days; NIR-MPTP = NIR-NIR group) sets of treatments. After the last treatment, the majority of mice were allowed to survive until 13d before perfusion (Fig. 1). Some mice were perfused at earlier stages, on 5.5d and 7.5d; these shorter survival periods were used to compare dopaminergic cell number at different stages, in particular in relation to the initial behavioural changes after MPTP injection (see below). The mice with the shorter survival periods had the same combinations of MPTP and NIR treatments as the corresponding groups with the longer 13-d survival (e.g. MPTP, MPTP = NIR and MPTP-NIR), up until the time of perfusion on 5.5d and 7.5d (Fig. 1). For example, the mice in the MPTP = NIR group perfused on 5.5d had only a single MPTP injection and NIR treatment and were perfused six hours later (Fig. 1).

Open-field behavioural testing

From 0d to 12d, we performed a standard open-field test as described previously (Moro et al. 2013; Reinhart et al. 2014). Briefly, we used the Noldus Ethovision (XT10) program (Noldus Information Technology, Wageningen, the Netherlands) to measure activity (i.e. levels of locomotion; velocity and mobility). The changes in activity over the experimental period for each mouse were calculated as a percentage value compared to the baseline, with the baseline being 100 %; in Fig. 3, the values shown reflect the average for each group. Each animal was tested at fourteen time points (for 10 min at each point): twice on 3d to 8d (the individual values of both tests were similar for each animal and hence pooled) and once on 0d (baseline) and 12d. For comparisons between groups in the behavioural analysis, a one-way ANOVA was performed (F and p values; GraphPad Prism program).

Immunohistochemistry and cell analysis

On either 5.5d, 7.5d or 13d, mice were anaesthetised after intraperitoneal injection of chloral hydrate (4 %;

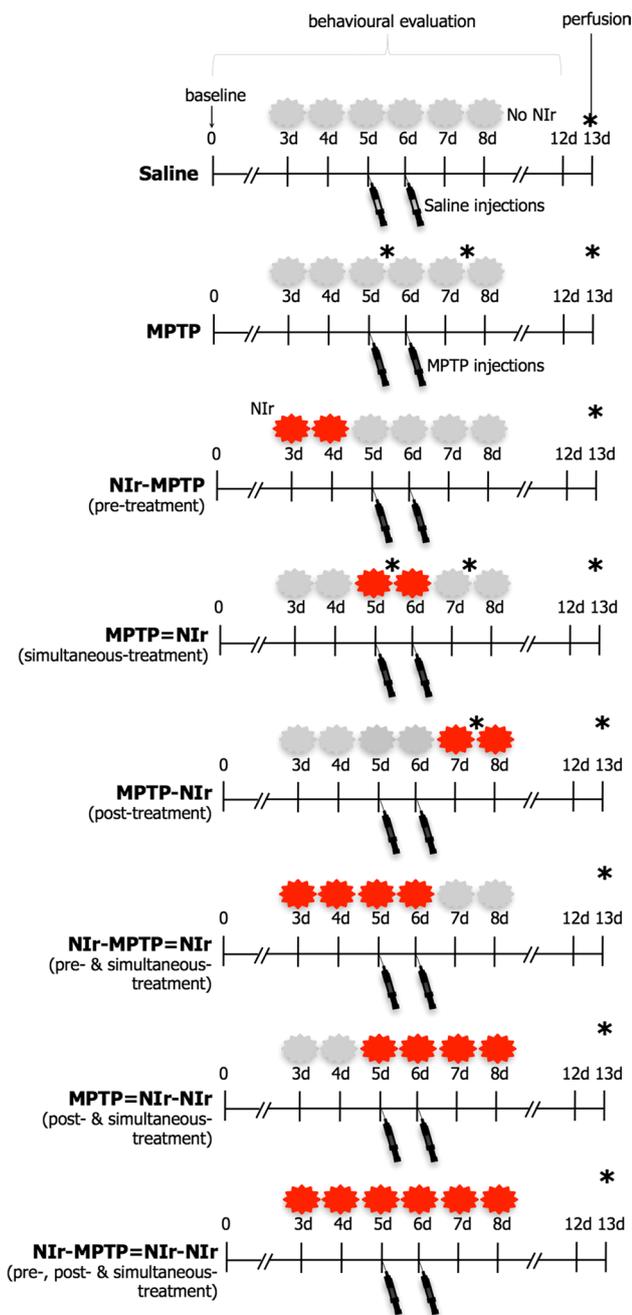


Fig. 1 Outline of the different experimental groups. Mice were administered single MPTP (or saline) injections (25 mg/kg each) on 5d and 6d, and on the particular days that they were Nir-treated, two exposures were given, approximately 6 h apart, each lasting 90 s using a WARP-LED. For each MPTP-Nir groups, there was a corresponding saline-Nir group that had the same patterns of Nir treatments and time lines, except that saline injections were made instead of MPTP injections. For each experimental group, there were at least nine animals used. Note that the majority of groups were perfused on 13d, while some groups (MPTP, MPTP = Nir and MPTP-Nir) had mice that were perfused on either 5.5d or 7.5d as well. These shorter survival periods were used to compare cell number at different stages of the experimental period. The asterisk* designates the days of perfusion in each of the different groups

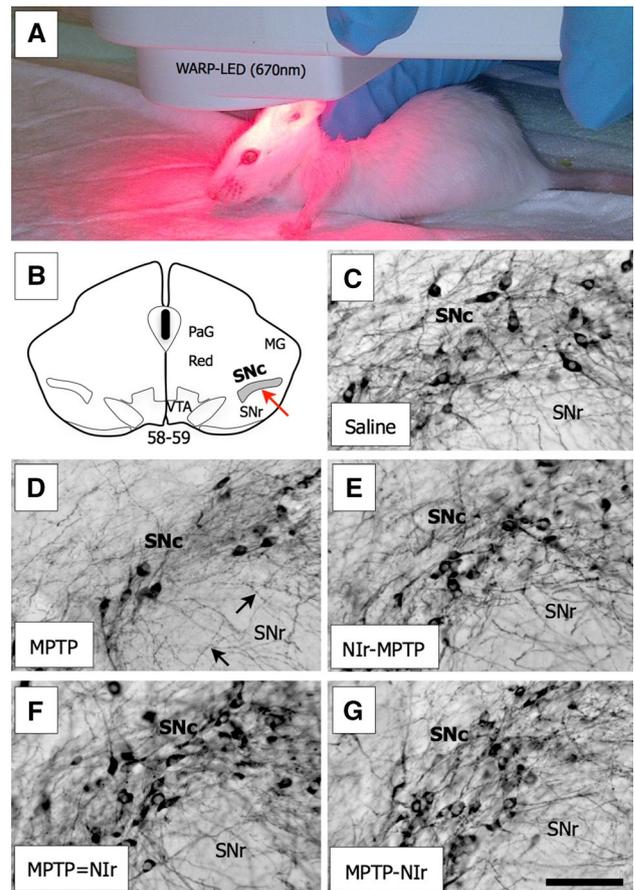
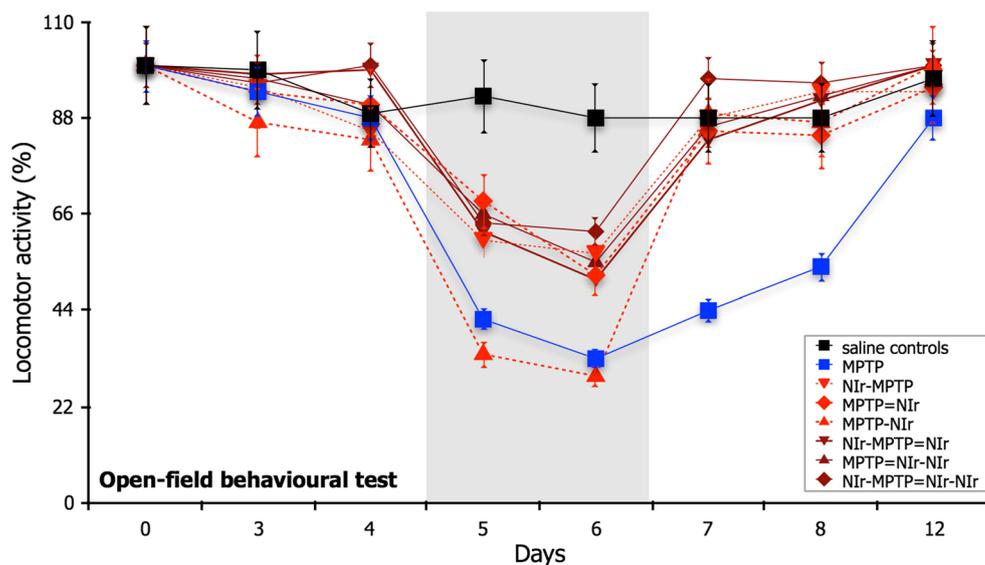


Fig. 2 a Our application of Nir to mice. The WARP-LED was held over the mouse's head for 90 s (for a single exposure). b Schematic diagram of coronal section, corresponding to plates 58–59 of mouse atlas (Paxinos and Franklin 2001), from where the photomicrographs were taken (*arrow*). c–g Photomicrographs of TH⁺ cells in the SNc of the saline (c), MPTP (d), Nir-MPTP (e; pre-treatment), MPTP = Nir (f; simultaneous-treatment) and MPTP-Nir (g; post-treatment) groups (13d survival). The patterns of morphology in the other MPTP-Nir-treated groups were similar to those shown in (e–g), hence not shown. The *arrow* in (d) indicates degenerating axonal profiles. All figures are of coronal sections; dorsal to top and lateral to right. Scale bar 100 μm

1 ml/100 g), and their brains processed for immunohistochemistry as described previously (Shaw et al. 2010; Peoples et al. 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014; El Massri et al. 2015). Briefly, brains were aldehyde-fixed (4 % buffered paraformaldehyde), cryoprotected (buffered 30 % sucrose) and sectioned coronally using a freezing microtome. Sections were incubated in anti-tyrosine hydroxylase (TH; 1:500; T8700 Sigma), followed by the Extravidin rabbit peroxidase staining kit (1:20 EXTRA3-1KT Sigma). They were then reacted in a 3,3'-diaminobenzidine tetrahydrochloride solution (D3939 Sigma) and coverslipped.

Fig. 3 Graph showing the locomotor activity of mice as assessed by an open-field test. The different markers show results for the different experimental groups. The activities of the saline and all NIR-treated saline groups were similar and hence pooled. The changes in locomotor activity are expressed as an average percentage of the baseline value for each group, with the baseline being 100 %. Each animal was tested at 14 time points: twice on 3–8d (the individual values of both tests were similar for each animal and hence pooled) and once on 0d (baseline) and 12d. The grey shading represents the 2 days of MPTP (or saline) injection



The number of TH⁺ cells within the substantia nigra pars compacta (SNc) of the midbrain was estimated using the optical fractionator method (StereoInvestigator, MBF Science), as outlined previously (Shaw et al. 2010; Peoples et al. 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014, 2015; El Massri et al. 2015). For comparisons between groups in the cell analysis, a one-way ANOVA was performed as above.

Correlation coefficients

In order to explore the relationship between behaviour and the extent of SNc lesion in the different experimental groups, we used the GraphPad Prism program to calculate correlation coefficients (Pearson's test) as described previously (Darlot et al. 2015). Locomotor activity was expressed by averaging the percentage activity values (as described above) of individual mice in each group. The extent of SNc lesion for the MPTP and each of the NiR-treated MPTP groups on 13d was expressed as a percentage change of TH⁺ cell number in the SNc from mean values attained from the saline controls.

Results

Behavioural analysis

Figure 3 shows the locomotor activity of mice, expressed as a percentage of the baseline value, in the different experimental groups on each day of testing. The activity of mice in the saline and all the NiR-treated saline groups was similar across the survival period (ANOVA: $F = 0.5$, $p = 0.7$); hence, their values were pooled (saline controls, Fig. 3).

On the days before the first MPTP injection (3d and 4d), the locomotor activity of mice in all groups was similar, being near the baseline values (i.e. 100 %; ANOVA: $F = 1.0$, $p = 0.4$; Fig. 3). During the days of the MPTP injections (5d and 6d), however, clear differences between some of the groups emerged (ANOVA: $F = 9.0$, $p < 0.0001$; Fig. 3a). While the activity of mice in the saline controls remained close to baseline values, the activity of mice in the MPTP group declined dramatically (60–70 %; Fig. 3). All the mice in the NiR-treated MPTP groups that had either pre-treatment or simultaneous-treatments (in any combination), had a reduction in activity during this period also (40–50 %), but it was not as severe as the mice in the MPTP group (Fig. 3). The exception to this pattern was the mice in the MPTP-NiR (post-treatment) group, those that had yet to receive NiR treatment; the activity of these mice mirrored those in the MPTP group (Fig. 3). The day after the last MPTP injection (7d), the activity of mice in all the NiR-treated MPTP groups—in particular, those in the MPTP-NiR (post-treatment) group—returned to baseline values and remained there up until the last day of testing (12d; Fig. 3). It should be noted that in the MPTP-NiR (post-treatment) group, the locomotor activity of mice returned to baseline almost immediately after the first NiR treatment on 7d; in fact, these animals were moving more freely around their box within only 20 min after their NiR treatment. The activity of the mice in the MPTP group by contrast was still much lower than baseline over the 2 days post-MPTP injections (45–55 %), but by the last of testing, had returned close to baseline as with all the other groups (ANOVA: $F = 0.3$, $p = 1.0$; Fig. 3). Finally, there were no major differences in the locomotor activity of MPTP-treated mice that received either single (NiR-MPTP, MPTP = NiR, MPTP-NiR), double (NiR-MPTP = NiR, MPTP = NiR-NiR) or triple (NiR-MPTP = NiR-NiR) sets of NiR treatments; they

all had similar degrees of behavioural impairment and patterns of activity during the experimental period (Fig. 3).

Cellular analysis

Figure 2c–g shows photomicrographs of TH⁺ cells in the SNc of the saline (Fig. 2c), MPTP (Fig. 2d), Nlr-MPTP (Fig. 2e; pre-treatment), MPTP = Nlr (Fig. 2f; simultaneous-treatment) and MPTP-Nlr (Fig. 2g; post-treatment) groups. Although there were fewer TH⁺ somata in the MPTP group (Fig. 2d; see below), those remaining were similar in overall appearance to those seen in the other groups. The bulk of cells had round or oval-shaped somata with one to two labelled dendrites. There were also more degenerating axonal profiles (arrows Fig. 2d) in the MPTP group compared to the other groups.

For the numerical analysis, we interpreted a change in TH⁺ cell number after experimental manipulation as an index of cell survival (Shaw et al. 2010; Peoples et al. 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014, 2015; El Massri et al. 2015). Figure 4 shows the estimated number of TH⁺ cells in the SNc of the different experimental groups with different survival periods: on 13d (black columns), a day after the last behavioural test and, on 7.5d (blue columns) and 5.5d (green columns), 2 days when the mice treated with MPTP had the least locomotor activity recorded during the experimental period (see Fig. 3).

In the saline and all the Nlr-treated saline groups, the number of TH⁺ cells was similar (ANOVA: $F = 0.4$, $p = 0.8$); hence, their data were pooled (saline controls, Fig. 4). On 13d, although the locomotor activity of mice in all groups was similar (Fig. 3), there were clear differences in the number of TH⁺ cells between the saline controls and the MPTP

group, and between the MPTP group and the Nlr-treated MPTP groups (Fig. 4; ANOVA: $F = 3.4$; $p < 0.001$). In fact, the number of TH⁺ cells in the Nlr-treated MPTP groups averaged ~20 % (range 11–27 %) higher than in the MPTP group, indicating Nlr-induced neuroprotection. There was no clear and consistent evidence indicating that the groups that had double (Nlr-MPTP = Nlr, MPTP = Nlr-Nlr) or triple (Nlr-MPTP = Nlr-Nlr) sets of Nlr treatments, resulted in a greater protection of SNc TH⁺ cells than the groups that had a single set of treatments (Nlr-MPTP, MPTP = Nlr, MPTP-Nlr); TH⁺ cell number was similar in all the different Nlr-treated MPTP groups (Fig. 4; ANOVA: $F = 1.6$; $p = 0.2$).

For the shorter survival periods of 5.5d and 7.5d—during stages when mice treated with MPTP showed the least locomotor activity—the number of TH⁺ cells was already lower than the saline controls. On 5.5d in particular, TH⁺ cell number in the MPTP group was very much lower than in controls (33 %); by 7.5d, this number had recovered to levels evident on 13d (Fig. 4). Even at these early stages, TH⁺ cell number averaged ~20 % higher in the Nlr-treated MPTP (MPTP = Nlr and MPTP-Nlr) groups than in the MPTP group on these days (range 10–25 %), similar to the value on 13d (Fig. 4).

Correlation between behaviour and anatomical lesion

In order to explore any correlation between behaviour and anatomical lesion, we compared locomotor activity to the extent of SNc TH⁺ cell loss across the different experimental groups (Fig. 5). There was a clear tendency for the MPTP group to have less locomotor activity and a larger SNc lesion than for other groups (blue squares; Fig. 5). Greater locomotor activity was associated with smaller SNc lesions and seen in the Nlr-treated MPTP groups (red

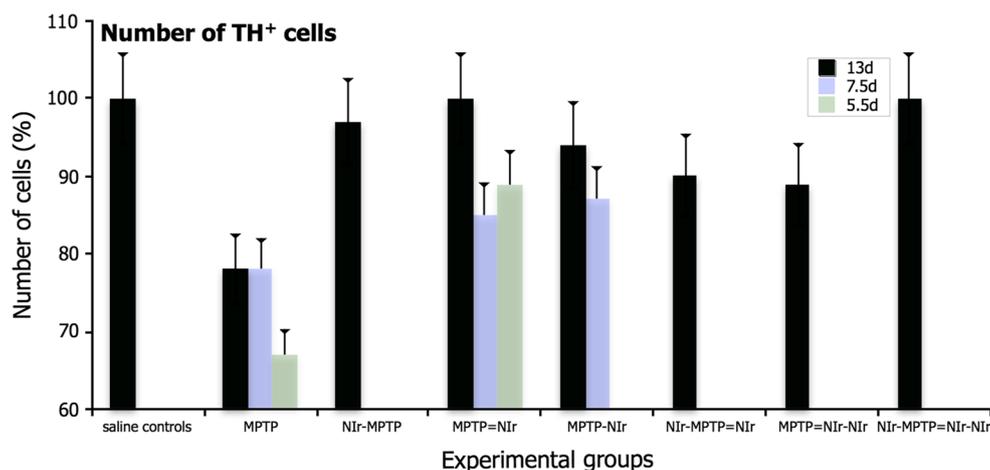


Fig. 4 Graph showing the estimated total number of TH⁺ cells in the SNc in each of the experimental groups on 13d (black columns), 7.5d (blue columns) and 5.5d (green columns). The number of cells in the saline and all Nlr-treated saline groups were similar and hence

pooled. Note that the number of cells in the MPTP group was lower than in all the other groups of the same survival time, in particular all the Nlr-treated MPTP groups; the fewest TH⁺ cells were evident in the MPTP group on 5.5d (colour figure online)

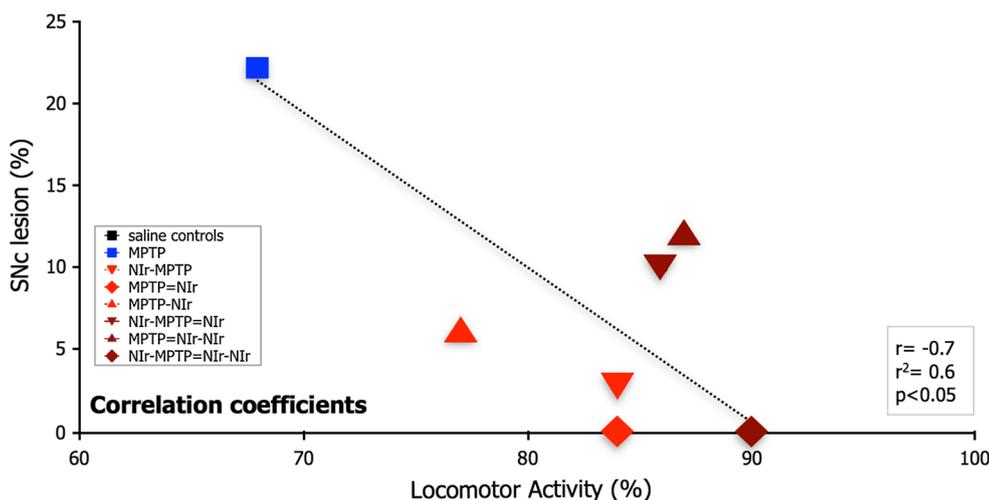


Fig. 5 Correlation between locomotor activity and SNc lesion in the different experimental groups. Locomotor activity was expressed as an average percentage activity from all animals in each group. The extent of SNc lesion for each group (on 13d) was expressed as a percentage change in TH⁺ cell number from the saline controls (0 %

lesion and 100 % activity). In general, the MPTP group (blue square) had less activity and a larger SNc lesion, while the NiR-treated MPTP groups (red triangles and diamonds) had more activity and smaller lesions. The different symbols represent the different groups (see legend) (colour figure online)

triangles and diamonds; Fig. 5). Overall, we found a significant correlation between locomotor activity and the extent of SNc lesion in the different experimental groups ($r = -0.7$, $r^2 = 0.6$; $p < 0.05$).

Discussion

Following on from previous reports (Whelan et al. 2008; Shaw et al. 2010; Peoples et al. 2012; Moro et al. 2013, 2014; Johnstone et al. 2014b; Reinhart et al. 2014; El Massri et al. 2015), the main finding of the present study was that NiR treatment was equally effective in reducing motor impairment and offering neuroprotection whether applied before (pre-treatment), at same time (simultaneous-treatment) or after (post-treatment) MPTP insult. From these different combinations of treatment and insult, we found a broad therapeutic time window for NiR effectiveness and that NiR was fast-acting and long-lasting; behavioural improvements were almost immediate following treatment and lasted for several days thereafter. These issues will form the main points of discussion below.

The behavioural results were striking. Although mice in all groups had similar locomotor activity at the end-point of the experimental period (see below), the central feature of our results was that during earlier stages—when MPTP insult alone generated severe behavioural impairment—NiR treatment reduced this impairment (Fig. 3). Further, single, double and triple sets of NiR treatments—regardless of stage of application—all resulted in similar patterns of behavioural improvements. When mice were NiR-treated either before or

at the same time as the MPTP insult, their behavioural impairment reduced substantially; further, their activity returned to control levels well before those in the MPTP group (Fig. 3). Hence, pre- and simultaneous-treatments of NiR not only offset the most debilitating effects of MPTP insult during the injection period, but continue to provide benefit for several days thereafter. Our behavioural results from the series involving the post-treatment of NiR were even more striking. In these cases, when mice were NiR-treated well after the MPTP insult, their behavioural impairment dissipated almost immediately; within minutes after treatment, activity returned to control levels. Overall, these results indicated that NiR was fast-acting and long-lasting (see further below).

During the earlier stages of the experimental period, when clear behavioural differences were evident between some of the groups, our cellular analysis indicated a substrate for these differences. In particular, the MPTP group—with the greatest behavioural impairment—had the fewest cells, while all the NiR-treated MPTP groups—with overall reduced behavioural impairment—showed evidence of neuroprotection, having more dopaminergic cells than the MPTP group. There was no indication that any particular combination of NiR treatment and MPTP insult resulted in a greater magnitude of neuroprotection, with all NiR-treated MPTP groups, whether single, double and triple, each having similar numbers of surviving cells (Fig. 4).

It is likely that the greater survival of dopaminergic cells underpinned the reductions in behavioural impairment during these early stages. The greater number of surviving cells in mice of the NiR-treated MPTP groups presumably resulted in greater dopaminergic transmission through the

striatum, leading to their reduced behavioural impairment (Moro et al. 2013; Reinhart et al. 2014). We showed that NIR treatment was able to “protect” cells against a toxic insult and limit motor deficits both when applied immediately (within minutes; i.e. simultaneous-treatment) or when applied up to 2 days beforehand (i.e. pre-treatment). Further, NIR was also able to “rescue” cells and restore locomotor activity to control levels when applied up to 2 days after the insult (i.e. post-treatment) (Wallace et al. 2007; Peoples et al. 2012). There appeared a relatively broad therapeutic time window for NIR effectiveness; in our acute MPTP model, this window was evident for up to 2 days on either side of the insult. Building on this finding in rodents, it will be a challenge for future studies to determine how long this window remains open, particularly in humans, thereby providing insights into the stages of therapeutic effectiveness of the treatment in relation to the disease progression.

The precise mechanisms of protection and rescue regulated by NIR are not known, but several previous studies have reported that NIR stimulates mitochondria by increasing ATP (adenosine triphosphate) 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 (Liang et al. 2008; Ying et al. 2008; Hamblin and Demidova 2006; Trimmer et al. 2009; Rojas and Gonzalez-Lima 2011; Chung et al. 2012; Quirk et al. 2012; Begum et al. 2013; Gonzalez-Lima and Barrett 2014; Gkotsi et al. 2014). In this study, such factors were likely to have protected and rescued cells against toxic insult, leading to greater cell survival, dopaminergic striatal transmission and reductions in behavioural impairment.

With regard to the recovery in locomotor activity in the MPTP group towards the end of the experimental period, notwithstanding a lower number of dopaminergic cells compared to the others (Figs. 3, 4), we suggest that there were compensatory neural mechanisms at play. In response to the lower number of dopaminergic cells in the SNc in this group, the remaining cells may have been stimulated—for example by motor cortex or pedunculo-pontine tegmental nucleus (Aravamuthan et al. 2008; Valencia et al. 2014)—to increase dopaminergic transmission through the striatum, thereby leading to the improvement in locomotor activity. A future, more functional analysis may explore this issue further.

In conclusion, within the limitations of an acute MPTP mouse model of Parkinson’s disease, our results provide some key insights into the effectiveness of NIR therapy, laying groundwork for future endeavours on humans. We revealed a relatively broad therapeutic time and dose window for NIR, with comparable benefits—namely, reductions in behavioural impairment and magnitudes of

neuroprotection—being evident when NIR was applied either several days before, at the same time or several days after the MPTP insult. We showed also that NIR was fast-acting and long-lasting, with behavioural improvements being evident almost immediately after application and lasting for several days thereafter.

Acknowledgments We are forever grateful to Michael J. Fox Foundation, Credit Agricole Sud Rhones Alpes, Fondation Philanthropique Edmond J. Safra, France Parkinson and the French National Research Agency (ANR Carnot Institute), Tenix corp and Salteri family for funding this work. D.M.J. is an Early Career Fellow of the NHMRC, Australia. J.S. was supported by the Foote Foundation and Sir Zelman Cowen Universities Fund; he is Director of CSCM Pty Ltd.

Author contributions C.M., D.M.J., J.S., A.L.B. and J.M. are full-time members of staff at their respective institutions, while F.R. and N.E.M. are postgraduate students. All authors contributed to the analysis of the data and of the reading and writing of the manuscript. F.R., C.M., N.E.M., A.L.B. and J.M. contributed to the experimental work.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper, except for the funding provided by the organisations mentioned in the Acknowledgements.

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