Does melatonin help save dopaminergic cells in MPTP-treated mice?

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ABSTRACT

This study explores whether melatonin neuroprotects dopaminergic cells of the substantia nigra pars compacta (SNc) from degeneration in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (well-known animal model of Parkinson disease). BALB/c albino mice were divided into four experimental groups. In each, mice received three series (over a 24-h period) of two intraperitoneal injections (1 h apart) in different combinations. The different groups and their combinations of injections were: (1) Saline (saline, saline); (2) Mel (melatonin, saline); (3) MPTP (saline, MPTP); (4) Mel-MPTP (melatonin, MPTP). Six days after the last injection, all mice were perfused transcardially with aldehyde fixative. Brains were processed for routine tyrosine hydroxylase (TH; rate limiting enzyme for dopamine production) immunohistochemistry and Nissl staining. Our results – using unbiased stereology – showed that there were more TH⁺ (50%) and Nissl-stained (30%) cells in the SNc of the Mel-MPTP group compared to the MPTP group, indicating a clear saving or neuroprotection of these cells. In fact, we found no significant difference between the number of TH⁺ and Nissl-stained SNc cells in the Mel-MPTP group compared to the controls, namely Saline and Mel groups. This indicated that melatonin pre-treatment potentially neuroprotected all the SNc cells from MPTP toxicity and death.

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1. Introduction

Parkinson disease is characterised by a loss of dopaminergic cells within the SNc of the midbrain [1]. This cell loss results in a reduction of dopamine levels in the striatum, which in turn, triggers a cascade of abnormal neural circuits that manifest in the distinct signs of Parkinson disease, namely lead-pipe rigidity, akinesia and tremor [2]. The factors that generate the initial loss of SNc dopaminergic cells are not entirely clear, but there is evidence for mitochondrial dysfunction as a result of exposure to an environmental toxin [2].

The first line treatment for patients with Parkinson disease is dopamine-replacement drug therapy. Perhaps the best known example of this therapy is Levodopa, which remains the “gold standard”. However, the use of Levodopa – as well as many other

drugs – has been shown to have its problems. For instance, long-term use leads to major motor side-effects and decreased effectiveness [3]. Further, and perhaps most importantly, such treatment is largely symptomatic and does not stop the pathology [3,4]. For this reason, there is a need to develop new treatments aimed at stopping or slowing down the progression of the disease. Indeed, there have been many studies over the years – mainly in animal models of the disease – reporting that some substances, or treatments (e.g., deep brain stimulation [4]), save or offer neuroprotection to the dopaminergic system in parkinsonian cases. One such substance is the indoleamine, melatonin.

Melatonin is released naturally into the blood stream mainly by the pineal gland, but also by other structures such as the retina, bone marrow and gastrointestinal system. The classical function associated with melatonin is entraining body circadian rhythms, an internal 24-h time-keeping system [5]. In more recent years, melatonin has been shown to also have an anti-oxidant function that protects cells from oxygen free radical damage [6]. In particular, melatonin has been shown to either stimulate radical detoxifying enzymes or scavenge hydroxyl radicals and offer protection against oxidative stress [7].

In this study, we have explored the cell saving or neuroprotective effects of melatonin in MPTP-treated mice. There has been few previous studies that have reported on the impact of melatonin on the morphology and number of dopaminergic cells in

Abbreviations: 6OHDA, 6-hydroxydopamine; CoQ10, co enzyme Q 10; cp, cerebral peduncle; CPu, caudate-putamen complex; GDNF, glial cell line derivative neurotrophic factor; ic, internal capsule; Mel, melatonin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PaG, periaqueductal grey matter; PBS, phosphate buffered saline; Pt, pretectum; SCol, superior colliculus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TH, tyrosine hydroxylase; VTA, ventral tegmental area; ZI, zona incerta.

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the SNc of parkinsonian animals [8,9]; previous studies have focussed mainly on changes in activity or dopamine levels in the striatum [10–17] or in the SNc [18]. In each of these studies, melatonin was administered either just prior to or immediately after parkinsonian insult (either 6-hydroxydopamine (6OHDA) lesion or MPTP treatment). In this study, we have followed a similar paradigm and combination of melatonin administration and parkinsonian insult. This paradigm is somewhat unlike the clinical reality where SNc cell loss occurs prior to therapeutic intervention. However, in our experimental study – as with the above mentioned previous ones – we hoped to explore the maximum effect of melatonin treatment on the number of SNc cells and hence determine more systematically its neuroprotective effects.

2. Materials and methods

2.1. Subjects

Male albino BALB/c mice (~20g; ~8 weeks old; *n* = 53) were used. Animals were housed in a 12 h light/dark cycle with unlimited access to food and water. All experiments were approved by the Animal Ethics Committee of the Australian National University and University of Sydney.

2.2. Experimental design

There were four experimental groups in this study (see Fig. 1). In each, mice received three series (over a 24-h period) of two intraperitoneal injections (1 h apart) in different combinations. The different groups and their combinations of injections were;

- (1) *Saline group* (*n* = 11): injections of saline (5% ethanol) followed by saline.
- (2) *Melatonin (Mel) group* (*n* = 10): injections of melatonin (20 mg/kg, in 5% ethanol/saline) followed by saline.
- (3) *MPTP group* (*n* = 18): injections of saline (5% ethanol) followed by MPTP (20 mg/kg in saline).
- (4) *Mel-MPTP group* (*n* = 14): injections of melatonin (20 mg/kg) followed by MPTP (20 mg/kg).

The quantities of melatonin and MPTP injected were within the range used by previous studies [8–18]. It should be noted that these studies have used different means of administering melatonin, either via drinking water [11,12], by subcutaneous mini-pumps [11] or by intraperitoneal injection [8–10,13–16]. In this study, we chose the more quantifiable route of intraperitoneal injection. For the MPTP doses, we used a milder dose of 60 mg/kg (3 × 20 mg/kg injections) that results in a less severe loss of SNc cells. This allows for more time for therapeutic intervention and greater sign of possible neuroprotection [4].

After the last series of injections, mice were allowed to survive for 6 days; this survival period is sufficient for MPTP to impart toxicity among dopaminergic cells [4]. After each injection, particularly after the MPTP ones, the mice were monitored closely for any changes in behaviour.

2.3. Histology and immunohistochemistry

After the survival period was complete, mice were anaesthetised after an intraperitoneal injection of sodium pentobarbital (60 mg/ml). They were then perfused

transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 4% buffered formaldehyde. The brains were removed and post-fixed overnight in the same solution. Next, brains were placed in PBS with the addition of 30% sucrose until the block sank. The forebrain and midbrain were then sectioned coronally and serially (at 55 µm thickness) using a freezing microtome. All sections were collected in PBS. One series of sections (1:2) was mounted onto gelatinised slides and then stained for Nissl (neutral red) using routine procedures. The other series were stored in PBS and used for immunohistochemistry. For this, sections were immersed in a solution of 0.1% Triton (Sigma) and 10% normal goat serum in PBS at room temperature for 1 h. Sections were incubated in anti-TH (Chemicon; 1:500) for 24 h. Next, sections were incubated in biotinylated anti-mouse IgG (Sigma; 1:200) for 2 h at room temperature. Finally, sections were incubated in Extravidin-peroxidase complex (Sigma; 1:200) at room temperature for 1 h. To visualise the bound antibody, sections were reacted in nickel-Tris-buffered saline (pH 7.4)–3,3'-diaminobenzidine tetrahydrochloride (Sigma) solution. In between each incubation, sections were washed in three changes of PBS. Sections were mounted onto gelatinised slides, air dried overnight, dehydrated in ascending alcohols, cleared in Histoclear and coverslipped using DPX. Some immunostained sections were counterstained lightly with Neutral Red as well. For controls, sections were processed as described above, except that there was no primary antibody used. Control sections were immunonegative.

2.4. Analysis

The total number of cells in the SNc was estimated using routine stereological methods. Counts were made from the SNc (right hand side), the main region of compact cells that has been shown to undergo the most degeneration after parkinsonian insult [1,4]. This region was distinguished from the smaller cells of the ventral tegmental area (VTA) that were located more medial [4,19].

In the SNc, cell number was analysed using TH immunoreactivity and Nissl staining. These two methods were used to explore whether the loss of cells after MPTP treatment was due to loss of antigen expression (TH) or to a loss of the cells themselves (Nissl); in other words, we explored whether there was functional and/or true neuroprotection. TH immunohistochemistry labels “healthy” cells that express the dopamine phenotype, that is, this labelling provides an index of function of dopaminergic cells. On the other hand, Nissl staining labels all cells regardless of phenotypic expression [4].

Following procedures as outlined by previous studies, the outline of the SNc (from TH-immunostained sections that defines the nucleus most distinctly) was drawn and the volume of the region was calculated using Cavalieri’s formula. The estimated total number of cells within the SNc was obtained using the optical fractionator method [4]. Systematic random sampling of locations (with an unbiased counting frame) within the defined SNc boundaries of every 2nd section was undertaken (by two observers, blind to treatment). All cells (nucleated only) that came into focus within an unbiased virtual counting space were counted. As a general rule, the number of cells and volumes (mean of two observer counts) were expressed as the mean ± standard error.

For comparisons between groups (using the StatGraphPad programme), a one-way ANOVA test (*F* test; *p* value) was performed with a Tukey multiple comparison test (*q* test; *p* value). Schematic diagrams and digital images were constructed using Adobe Photoshop and Microsoft PowerPoint programmes.

3. Results

The results that follow are presented in five main parts: topography of dopaminergic cells, MPTP toxicity, morphology of dopaminergic cells, volume of the SNc and number of dopaminergic cells.

Fig. 1. Outline of the different experimental groups used in this study, namely Saline, Mel (melatonin), MPTP, Mel-MPTP. The injections (in italics) were intraperitoneal. There were 3 series of injections – in different combinations for each group – over the first 24 h. After the last injection, mice were allowed to survive for 6 days – to ensure adequate time for toxicity of MPTP – before being perfused and prepared for immunocytochemistry and histology.

3.1. Topography of dopaminergic cells

The SNc was identified as the compact group of cells located immediately superior to the substantia nigra pars reticulata (SNr), and lateral to the smaller cells of the VTA [4,19]. Fig. 2A shows schematic diagrams of the brainstem sections matching closely to those used to count the number of SNc cells. Fig. 2B shows a TH-immunostained section indicating the boundaries of the SNc which was used to measure area and subsequently volume and cell number. In our analysis, we counted only the TH⁺ and Nissl-stained

cells that had a nucleus (arrows Fig. 2C,D); non-nucleated cells were not counted (arrowheads Fig. 2C,D). Such a method ensured consistency in our approach, even though our counts of every second section made it unlikely that we would count the same cell twice [4].

3.2. MPTP toxicity

The success of our MPTP treatment was measured in two ways. First, we examined the patterns of TH immunoreactivity in the midbrain of each of the groups used in this study. Fig. 3 shows

Fig. 2. Schematic diagrams of coronal sections of the rat brainstem. The number in italics for each section correspond to plates in the mouse brain atlas [19]; the higher the number, the more caudal the section. These sections correspond to those in which the number of cells in the SNc (shaded) were counted. (B) TH immunostained section showing the major region analysed in this study; the SNc. The boundaries of the SNc indicated were used to measure the area and subsequently the volume and cell number. Photomicrograph of TH⁺ (C) and Nissl-stained (D) cells of the SNc; arrows indicate "typical" nucleated cells that were counted, while the arrowheads indicate non-nucleated cells that were not counted. For (B)–(D) dorsal to top, lateral to right. Scale bar = 100 μm.

TH-immunostained midbrain sections from the Saline (Fig. 3A), Mel (Fig. 3B), MPTP (Fig. 3C) and Mel-MPTP (Fig. 3D) groups. There were fewer TH⁺ cells in the SNc of the MPTP group compared to all the other groups, namely Saline, Mel and Mel-MPTP. A more quantitative analysis of the differences in TH⁺ (+Nissl-stained) cell number in the different groups is considered later in this manuscript. Second, we examined the patterns of TH⁺ terminal labelling in the CPu. In the MPTP group, we observed fewer TH⁺ terminals in the CPu (Fig. 3E), when compared to the Saline, Mel and Mel-MPTP (Fig. 3F) groups.

We did not observe any major behavioural deficits in the mice after our MPTP injections, although in some instances, the mice would become quiescent immediately after injection. However, these mice soon returned to normal activity – eating and grooming – within the next few hours. Following the saline and melatonin injections, no behavioural deficits were seen in any of the mice.

3.3. Morphology of dopaminergic cells

We examined the morphology of midbrain dopaminergic cells in the four experimental groups using TH immunoreactivity and Nissl staining. In general, there were no substantial differences in the overall morphology of TH⁺ or Nissl-stained cells in each of the groups.

Fig. 4 shows typical examples of labelled cells in the SNc in each of the experimental groups, namely Saline (Fig. 4A,B), Mel (Fig. 4C,D), MPTP (Fig. 4E,F) and Mel-MPTP (Fig. 4G,H). In each case, TH immunoreactivity and Nissl staining was seen in cells with generally rounded somata; TH immunoreactivity also revealed immunoreactive dendrites that often formed a dense lattice work within the area.

3.4. Volume of the SNc

The volume of the SNc in each of the experimental groups was very similar. In the Saline group, SNc volume was $0.14 \pm 0.01 \text{ mm}^3$, while in the Mel group, it was $0.16 \pm 0.010.01 \text{ mm}^3$. In the MPTP group, SNc volume was $0.14 \pm 0.010.01 \text{ mm}^3$, while in the Mel-MPTP group, it was $0.15 \pm 0.010.01 \text{ mm}^3$ (Fig. 5).

When comparing these values between the four experimental groups using an ANOVA test, no significant differences were found ($F = \pm 0.011.4$; $p \pm 0.01 = \pm 0.010.26$). A multiple comparison test also revealed no significant differences between the volumes of these groups (for each group comparison, $q \pm 0.01 < \pm 0.013.8$; $p \pm 0.01 > \pm 0.010.05$). Thus, there was no major impact on the overall volume of the SNc after melatonin or MPTP treatment in our study.

Fig. 3. Photomicrographs of TH immunoreactivity in coronal sections of the midbrain (A–D) and caudate–putamen complex (CPu; E,F). Images are shown from the Saline (A), Mel (B), MPTP (C,E) and Mel-MPTP (D,F) groups. For the CPu, the patterns of immunoreactivity in the Mel-MPTP group (F) were similar to those seen in the Saline and Mel groups (not shown). Sections were immunostained for TH and then counterstained lightly with neutral red. For all images, dorsal to top and lateral to right. Scale bars = 100 μm .

Fig. 4. Photomicrographs of TH⁺ (A,C,E,G) and Nissl-stained (B,D,F,H) cells in the SNc. Photomicrographs from the Saline (A,B), Mel (melatonin; C,D), MPTP (E,F), Mel-MPTP (G,H) groups are shown. Each photomicrograph of each group was taken from a central region of the SNc (from sections corresponding to plate 59 of mouse atlas [19]). All figures are of coronal sections; dorsal to top and lateral to right. Scale bar = 100 μ m.

3.5. Number of dopaminergic cells

The number of TH⁺ and Nissl-stained cells in the SNc of each of the experimental groups in this study will be considered separately below.

3.5.1. TH⁺ cells

The graph in Fig. 6 shows the estimated number of TH⁺ cells in the SNc of the four experimental groups. In the Saline and Mel

groups, the total number of TH⁺ cells was very similar, being $12,150 \pm 0.01800$ and $13,200 \pm 0.01800$ (Fig. 6). These estimates for the total number of TH⁺ cells in the SNc of mice were within the range reported by previous studies [20].

In the MPTP group, the number of TH⁺ cells was 6250 ± 0.01550 , indicating a 50% reduction in SNc cell number from the Saline and Mel groups. For the Mel-MPTP group, the number of TH⁺ cells was $12,350 \pm 0.01850$, which was similar to

Fig. 5. Graphs showing the volume of the SNc in the four experimental groups used in this study. Columns show the mean $\pm$ 0.01 standard error of the total volume of the SNc (of right hand side of TH⁺ sections) in each group. There were no significant differences ($p > 0.05$) in SNc volume between the different groups. There was little variability in the SNc volume in different individual cases of the same group, as indicated by the small standard error bars.

the number of cells found in the Saline and Mel groups (see above; Fig. 6). Hence, compared to the MPTP group, the Mel-MPTP group had 50% more TH⁺ cells in the SNc. These differences were significant using an ANOVA test ($F = 21.7$; $p < 0.0001$). Further, using the multiple comparison test, significant differences (if $q > 3.8$) were found between the MPTP group and each of the other groups, namely the Saline ($q = 7.9$; $p < 0.001$), Mel ($q = 9.6$; $p < 0.001$) and, in particular, the Mel-MPTP ($q = 9.0$; $p < 0.001$; see Fig. 6).

In summary, there were 50% fewer TH⁺ cells in the MPTP group than in the two controls, namely the Saline and Mel groups. The Mel-MPTP group had values similar to the controls and 50% higher than in the MPTP group. These results indicated that melatonin pre-treatment neuroprotects TH expression among SNc cells from the MPTP insult.

3.5.2. Nissl-stained cells

The graph in Fig. 7 shows the estimated number of Nissl-stained cells in the SNc of the four experimental groups. In the Saline and Mel groups, the total number of Nissl-stained cells were very similar, being $14,850 \pm 0.011600$ and $15,450 \pm 0.011150$ (Fig. 7).

Fig. 6. Graphs showing the number of TH⁺ cells in the SNc in the four experimental groups used in this study. Columns show the mean $\pm$ 0.01 standard error of the total number of SNc cells (of right hand side) in each group. † represents significant difference ($p < 0.001$) in cell number between different groups. There was a 50% increase in TH⁺ cell number in the Mel-MPTP group compared to the MPTP group. There was little variability in the number of cells in different individual cases of the same group, as indicated by the small standard error bars.

In the MPTP group, the number of Nissl-stained cells was 9900 ± 0.01600 , indicating ~30% reduction in SNc cell number from the Saline and Mel groups. For the Mel-MPTP group, the number of Nissl-stained cells was $14,100 \pm 0.011000$, which was similar to the number of cells in the Saline and Mel groups (see above; Fig. 7). Compared to the MPTP group, the Mel-MPTP group had ~30% more Nissl-stained cells in the SNc. These differences were significant using an ANOVA test ($F = 6.7$; $p < 0.0001$). Further, using the multiple comparison test, significant differences (if $q > 3.8$) were found between the MPTP group and each of the other groups, namely the Saline ($q = 4.7$; $p < 0.01$), Mel ($q = 5.4$; $p < 0.01$) and the Mel-MPTP ($q = 4.3$; $p < 0.05$; see Fig. 7).

In summary, there were ~30% fewer Nissl-stained cells in the MPTP group than in the two controls, namely the Saline and Mel groups. This decrease was within the range, although slightly smaller, than the decrease evident in TH⁺ cell number of the MPTP group. The Mel-MPTP group had values similar to the controls and ~30% higher than in the MPTP group. Again, this increase was within the range, although slightly smaller, than the increase seen in TH⁺ cell number of the Mel-MPTP group. These results indicated that melatonin pre-treatment not only largely neuroprotected TH expression among SNc cells, but also helped neuroprotect the cells themselves from MPTP insult.

4. Discussion

The most striking finding of this study was that there were more SNc cells in the Mel-MPTP compared to the MPTP group. This indicated that melatonin pre-treatment in MPTP-treated mice offered neuroprotection for these cells. We suggest that this was due to the anti-oxidant properties of melatonin reducing MPTP toxicity. This issue forms the focus of the discussion below. First, a comparison with previous studies is considered.

4.1. Comparison with previous experimental studies

To the best of our knowledge, there have only been two other studies exploring the impact of melatonin administration on the

Fig. 7. Graphs showing the number of Nissl-stained cells in the SNc in the four experimental groups used in this study. Columns show the mean $\pm$ 0.01 standard error of the total number of SNc cells (of right hand side) in each group. † represents significant difference at $p < 0.01$ in cell number between different groups. * represents significant difference at $p < 0.05$ in cell number between different groups. Overall, there was ~30% increase in Nissl-stained cell number in the Mel-MPTP group compared to the MPTP group. There was little variability in the number of cells in different individual cases of the same group, as indicated by the small standard error bars.

number of SNc cells after parkinsonian insult [8,9]. These studies used either 6OHDA-lesioned rats [8] or MPTP-treated mice [9] and found similar patterns of cell saving shown in our study (although not from unbiased stereological methods as in present study). It should be noted that there have been many other previous studies examining the effect of melatonin in parkinsonian cases, but these have focussed mainly on changes in dopamine [17], TH [12,13,17], oxidative [15,16] and particular molecular [3,17] activities in the striatum or TH activity in the SNc [18]. There have also been reports on behavioural changes after administration of apomorphine in melatonin-treated 6OHDA-lesioned rats [8,17]. In each case, melatonin increased – most notably – striatal dopamine levels and TH activity and also reduced apomorphine-induced rotational behaviour generated by parkinsonian insult [13,17]. In contrast to all the above-mentioned reports, there are two experimental studies that did not find a neuroprotective effect – at least for various dopaminergic markers in the striatum – for melatonin in MPTP-treated mice [10,11]. The reasons for these differences are not clear since the markers analysed and regimes of melatonin and MPTP used by these studies were not too dissimilar to those used by the many positive studies. For example, one of these negative studies had a total melatonin dose of 30 mg/kg [10], which is well within the range used by the many positive studies (6–60 mg/kg; see below).

Other substances, in addition to melatonin, have been shown to offer neuroprotection to SNc cells in various animal models of Parkinson disease. For example, caffeine [21], tomato powder [22], glial cell line derived neurotrophic factor [23] and various dopamine agonists [24], all have been shown to increase dopaminergic cell survival in the SNc or activity in the striatum after parkinsonian insult. Further, neuroprotection in the SNc has also been shown after lesion of deep brain stimulation of various centres that have a glutamatergic inputs to the SNc, for example the subthalamus [4,25]. These studies have reported anywhere between 15 and 50% saving of SNc cells. Despite differences in experimental design, the overall magnitude of neuroprotection found in our present study on melatonin (30–50%) is comparable – and indeed at the upper end of the scale – to that found in these previous studies. In fact, our results indicated that melatonin potentially neuroprotected all the SNc cells from MPTP insult (see below).

4.2. Experimental design and limitations of study

A limitation of this study was that our experimental design did not follow the clinical reality of the parkinsonian condition, where individuals first suffer insult (and SNc cell death) and then have therapeutic intervention. However, our experiments were designed to explore the maximum effect of melatonin on SNc cell number and hence quantify more precisely its neuroprotective effects. This may have been more difficult to achieve if there was a degree of cell death before melatonin administration. Our model was also different to that seen in humans in that it resulted in only 30–50% death of SNc cells, not as extensive as is normally seen in Parkinson disease when first diagnosed (70–80% [26]). However, it has been shown that with greater SNc cell death, then there is a smaller magnitude of neuroprotection [4]. Hence, our model was ideal in the sense that it did not generate a massive death of SNc cells, thereby allowing time for therapeutic intervention and a greater scope of possible neuroprotection offered by melatonin [4].

Previous studies have shown that a range of melatonin doses, from 6 to 60 mg/kg, generate neuroprotection of dopaminergic activity in both 6OHDA-lesioned rats and MPTP-treated mice [8,9,12–18] (except for the two studies mentioned above [10,11]). In fact, authors have reported no major differences in the magnitude of neuroprotection – in terms of striatal activity – with different dose regimes [13,15,16]. In our study, we chose to use a higher dose

of melatonin (~60 mg/kg), in order to explore its maximum neuroprotective effect. Future studies may explore further whether lower doses of melatonin have a lesser neuroprotective effect in MPTP-treated animals. In 6OHDA-lesioned rats at least, only very small differences have been noted in behaviour and in SNc cell number with different doses of melatonin (6–30 mg/kg [8]).

4.3. Neuroprotection of SNc cells by melatonin

There were 30–50% more SNc cells in the Mel-MPTP compared to the MPTP group, indicating neuroprotection. In fact, we found no significant difference between the number of SNc cells in the controls, namely the Saline and Mel groups, and the Mel-MPTP group, indicating that melatonin treatment neuroprotected nearly all these cells from MPTP toxicity.

Our results did not address directly the mechanism that provided this neuroprotection, but it was likely to be due to the anti-oxidant properties of melatonin. MPTP is thought to impart its toxicity of dopaminergic cells through complex mechanisms. Briefly, once in the brain, MPTP is converted into the active toxin, MPP⁺, that then concentrates within the mitochondria. There, it inhibits the activity of complex 1 of the electron transport chain leading ultimately to ATP depletion and oxygen free radical production; this process causes much damage to cell proteins, including TH [27]. Melatonin, with its known potent free radical scavenger properties and ability to increase both mitochondrial respiration and ATP synthesis, presumably helped the dopaminergic cells to become more resistant to the above-mentioned damaging effects of MPTP [16]. Hence, and in relation to our findings in this study, the overall numbers of SNc cells in the Mel-MPTP group were much higher than in the MPTP group.

4.4. Functional and true neuroprotection

The present finding that there were both more TH⁺ and Nissl-stained cells in the Mel-MPTP compared to the MPTP group, indicated that there was both a functional and true neuroprotection by melatonin. In other words, there was an actual increase in SNc cell survival and not just a rescue of the dopaminergic phenotype. This result confirms the findings of some studies [4], but not of another [28]. One may argue that it is difficult to compare the findings of these previous studies and ours, mainly because of the different experimental designs. Each study, for example, had a different means of cell labelling (e.g., tracer-labelled vs Nissl-stained) and used different animal models of the disease (e.g., 6OHDA-lesioned rats vs MPTP-treated mice or monkeys). Perhaps a more insightful comparison on the issue of functional vs true neuroprotection may come from a study that incorporates a common experimental design.

Our results indicated also that in each group, there were always more Nissl-stained than TH⁺ cells in the SNc. This finding may have reflected several – not necessarily mutually exclusive – features. First, a proportion of SNc cells were “sick”, due to MPTP toxicity, and not expressed TH. These cells, although functionally impaired, were still Nissl-stained. It has been shown that toxins such as MPTP have initial impact on transmitter phenotype (i.e., TH) and then, after a longer period, on the overall cell itself, manifesting in death [29]. Second, there were different patterns of SNc cell labelling by the two techniques used, namely immunohistochemistry and Nissl staining. For instance, immunohistochemistry perhaps did not label all the cells in the section (i.e., those in the middle) because of limited antibody penetration. Nissl staining has been shown to label all the cells across the entire thickness of the section [30]. Third, there may have been differences in defining the SNc border using the two methods; the borders with Nissl staining were not as clear and some more cells (outside the SNc) may have been included in these

counts. Finally, TH immunohistochemistry did not reveal a distinct population of non-TH⁺ cells in the SNc; these cells would have been revealed by Nissl staining [31]. Taken together, these features were likely to have contributed to the finding that there were fewer TH⁺ than Nissl-stained cells in all the experimental groups of this study.

5. Conclusion and clinical relevance

Our results indicated that melatonin administration in MPTP-treated mice potentially saved all SNc cells from death. We suggest that the mechanism underlying this saving is the anti-oxidant properties of melatonin. Future studies may explore whether this suggestion is, in fact, the case.

There are several conflicting reports on the validity of using melatonin therapy in a clinical setting. On the one hand, melatonin treatment has been reported to have little symptomatic relief for patients [32] and in some cases, may even exacerbate the symptoms by inhibiting dopamine production by cells in the SNc [33]. Further, some studies have indicated that melatonin levels are not necessarily low in parkinsonian patients [34]. Finally, it has been reported that melatonin administration may have many side-effects on patients [35]. On the other hand, however, previous work has in fact reported symptomatic relief in parkinsonian cases with melatonin therapy [36]; further, that dysfunction of the pineal gland – the major site of melatonin production – plays an important role in the generation of parkinsonism [37]. In this context, our results – albeit on an animal model of Parkinson disease – provide some encouragement that perhaps at the right dose, melatonin may be used to help save or prevent the death of SNc cells in Parkinson disease. Future studies may explore this issue further.

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