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**BRAIN
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Research Report
**Intraocular microinjections repair experimental
Parkinson's disease**
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ABSTRACT

Circadian involvement in Parkinson's disease (PD) and more specifically in nigro-striatal dopamine (NSD) function is of increasing interest to the neurosciences.

Given that bright light therapy is of therapeutic value in PD, possible mechanisms underlying retinal involvement in this phenomenon was explored further by administering anti-Parkinsonian chemotherapies into the vitreous humour directly adjacent to the retina. 2 μ l of a 100 mM solution of L-Dopa significantly improved motor function in the later stages of degeneration and during the day while the injection of 2 μ l of a 10 mM solution of the melatonin receptor antagonist ML-23 improved motor function in the early stages of PD and during the dark phase of the light/dark cycle. The results suggest that the function of nigral cells is regulated by a more global system embracing circadian physiology that extends from the retina to the pineal. Furthermore, the induction of PD is characterised by an imbalance between melatonin and dopamine (DA) whereby this ratio is elevated at least 6 to 1 in favour of melatonin. The commonly observed treatment failures and side effects of DA replacement therapy probably result from increasing endogenous DA without taking parallel melatonin dysfunction into account. The proposed integrated function of the NSD and circadian systems may permit therapeutic targeting at a level which is safer, more effective and without the side effects of systemically administered regimens of DA replacement.

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1. Introduction

The role of the nigro-striatal dopamine (NSD) system in motor function and in the development of Parkinson's disease (PD) has been determined on the basis of a point to point anatomical approach that ascribes a single cause to the expression of a broad spectrum of symptoms, in a single neuronal subset. This has been universally defended as the key element in understanding NSD system function, even though the brain is conceived and demonstrated to be a functionally inter-dependent circuitous system. For the past forty years, while treatment strategies for PD have progressed through a series

of advances, these have been limited to finding more efficacious ways of replacing deficient dopamine (DA), with little credence given to the involvement of other neurological systems.

The circadian system, in particular, is likely to hold the key for understanding the degenerative process underlying PD (Abbott, 2006). The circuitry underlying this system leaves the retina, traversing and communicating with various structures in the hypothalamus and midbrain as it courses its way to the pineal (Moore, 1996) and thereby mediates control over a number of functions that are compromised in PD. The high incidence of depression (Deniker et al., 1975; Dowling, 1995; Girotti et al., 1986;

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Okun and Watts, 2002) and insomnia (Arnulf et al., 2002; Demet et al., 1999) as well as the favourable therapeutic response of PD patients to phototherapy (Artemenko and Levin, 1996; Paus et al., 2006; Willis and Turner, 2007) all intimate compromised circadian function in this disorder (Guardiola-Le Maitre, 1997; Willis, *in press*). Furthermore, the NSD system and retino-hypothalamic tract (RHT) share the lateral hypothalamus (LH) as a point of anatomical convergence (Moore, 1996; Moore and Bloom, 1978; Moore et al., 1967) and the occurrence of abundant neuropathological changes occurring therein in animals and in man (Kremer, 1992; Langston and Forno, 1978; Willis and Armstrong, 1998; Willis and Smith, 1985), provide anatomical justification for such a hypothesis. Direct evidence showing a relationship between the severity of PD and altered pineal function (Ghaemi et al., 2001), as well as altered therapeutic response to DA replacement after pinealectomy (Willis, 2005b), or altered neuroendocrine function in response to L-Dopa in amaurosis (Bellastella et al., 1990) further support this contention.

In a recent study examining the role of the visual system in experimental PD, enucleation significantly enhanced the expression of motor impairment (Willis et al., *in press*). As RHT system fibres leave the retina they project caudally and contralaterally with the most pronounced enhancement of hemi-Parkinsonian symptoms achieved by enucleation ipsilateral to the hemisphere where the lesion was placed. This, in essence, would produce the equivalent of a bilateral lesion of the NSD and would concomitantly place the organism in a physiological state equivalent to bilateral enucleation or exposure to chronic darkness. In contrast, enucleation contralateral to the placement of NSD lesions facilitated recovery as hemi-Parkinsonian animals showed a behavioural response closely resembling post synaptic denervation supersensitivity whereby preceding damage predisposed

the animal to enhanced recovery (Willis et al., *in press*). When these findings are viewed within a broader context, the role of the circadian system in PD extends far beyond current theories that focus on the therapeutic value of melatonin solely as an antioxidant (Shrinivasan et al., 2005). In fact, contrary to antioxidant based theories, the therapeutic value of reducing the bioavailability of melatonin or antagonising melatonin receptors has tremendous potential in this regard (Willis and Armstrong, 1999; Willis and Robertson, 2004, 2005).

To explore this further, systematic intervention in DA and melatonin function in the retina was undertaken. In consideration of the reciprocating relationship between DA and melatonin in this location (Djamgoz et al., 1997; Dubocovich, 1983; Dubocovich, 1989), and that the retina is the first port of call for the RHT system linking the environment to the brain, the effects of microlitre quantities of drugs that alter melatonin and DA function on the expression of Parkinsonian symptoms were examined after intravitreal (IVIT) injection. In the first study L-Dopa was employed on the basis that it is the mainstay of DA replacement therapy in PD and that it opposes the function of melatonin within the RHT system. In a second study the melatonin antagonists ML-23 was used because melatonin is elevated in PD (Blazejova et al., 2000; Bordet et al., 2003; Catala et al., 1997; Willis, *in press*) and this drug has been shown to exacerbate recovery from experimental PD in several models (Willis, 2005a; Willis and Robertson, 2004, 2005). This approach may help to resolve the issue regarding the involvement of the RHT, and the retina as a component of that system, in the expression of symptoms in PD. This study emerges from the basic assumption that if the retina is involved in the aetiology and progression of PD (Willis et al., *in press*) then it may also be a route for therapeutic intervention.

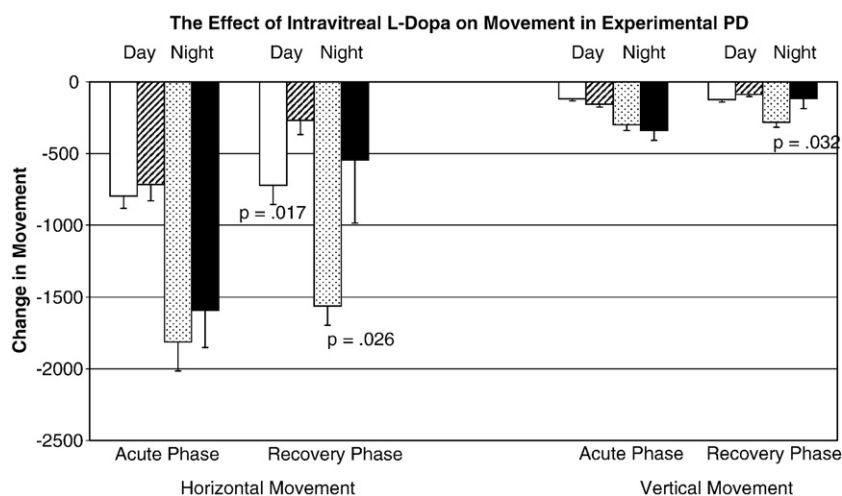


Fig. 1 – The effects of IVIT L-Dopa (2 μ l of a 100 mM solution) on movement in the dark and the light during the acute and recovery phases of experimental PD. A significant improvement in horizontal movement (left trace) was observed during the day (diagonal bar=PD+L-Dopa versus open bar=PD+vehicle) and during the night (PD+L-Dopa=filled bar vs PD+vehicle=PD mottled bar) during the recovery phase. Vertical movement (right trace) also improved during the night of the recovery phase and this was significantly better than vertical movement in rats treated with vehicle. T-bars represent the standard error of the mean.

2. Results

2.1. Study 1: IVIT L-Dopa

The effects of IVIT L-Dopa on movement are expressed in the left traces in Fig. 1. During the day or night, in the acute phase of experimental PD, there was no difference in horizontal movement between animals injected with IVIT L-Dopa and those receiving vehicle (Day-Vehicle; mean = -797 ± 86 vs L-Dopa; mean = -716 ± 113 ; Night-Vehicle; mean = -1813 ± 202 vs L-Dopa; mean = -1592 ± 260). However, during the recovery phase of experimental PD, the L-Dopa injected group crossed significantly more squares in comparison to their control performance (mean = -269 ± 99) and also compared to the vehicle injected group (mean = -722 ± 134) and this was significant ($\chi^2 = 4.318$, $n = 11$, $df = 1$, $p = 0.017$).

In the night, during the recovery phase those rats receiving IVIT L-Dopa were less impaired (mean = -545 ± 441) than those receiving IVIT vehicle (mean = -1562 ± 134) and this was also statistically significant ($\chi^2 = 3.892$, $n = 11$, $df = 1$, $p = 0.026$).

The effect of IVIT L-Dopa on vertical movement is depicted in the right traces in Fig. 1. IVIT L-Dopa had no significant effect on vertical movement in the day or night during the acute phase of experimental PD. Similarly, there was no effect of IVIT injection during the day time measurement in the recovery phase. However, in the dark during the recovery phase the animals injected with L-Dopa showed vertical movement that more closely approximated their control performance (mean = -117 ± 70) than did rats injected with IVIT vehicle (mean = -282 ± 35) and the difference was significant ($\chi^2 = 3.544$, $n = 11$, $df = 1$, $p = 0.032$).

The only time that a difference in latency to retract a limb was observed between the IVIT L-Dopa versus the IVIT vehicle group was during the day of the recovery phase (Fig. 2, left traces). The L-Dopa injected group was almost five times faster (mean = 1.5 ± 0.89) than those injected with vehicle (4.1 ± 1.4) and the difference was highly significant ($\chi^2 = 6.866$, $n = 22$, $df = 1$, $p = 0.003$). The latency to step up or down from a raised platform was not altered by IVIT injection of L-Dopa at any time during the course of experimental PD.

As was observed with the parameter of latency to retract, the only time at which IVIT L-Dopa caused a significant improvement in the latency to ambulate was during the day of the recovery phase (Fig. 2, right traces). The L-Dopa injected group were more than 4 times faster (mean = 2.4 ± 0.6) compared to those injected with vehicle (10.2 ± 2.9) and the difference was highly significant ($\chi^2 = 7.405$, $n = 11$, $df = 1$, $p = 0.002$).

Assessment of residual effects undertaken at least 48 h after completion of the injection regime and in the absence of interference with anaesthetic revealed that all but one of the parameters were unaffected by acute L-Dopa administration into the vitreus. Horizontal movement (Supplementary Data: Fig. 1, left trace), vertical movement (Supplementary Data: Fig. 1, right trace), latency to step and to ambulate (Supplementary Data: Fig. 2, middle and right traces) were not significantly different in L-Dopa versus vehicle injected animals once IVIT injections were withdrawn. However, latency to retract a limb (Supplementary Data: Fig. 2, left trace) improved significantly when tested during the day (L-Dopa, mean = 3.5 ± 1.2 versus vehicle, mean = 9.9 ± 3.1) or the night (L-Dopa, mean = 2.2 ± 0.91 versus vehicle, mean = 8.5 ± 2.6) and these differences were significant ($\chi^2 = 2.959$, $n = 26$, $df = 1$, $p = 0.043$ and $\chi^2 = 4.097$, $n = 26$, $df = 1$, $p = 0.016$, respectively).

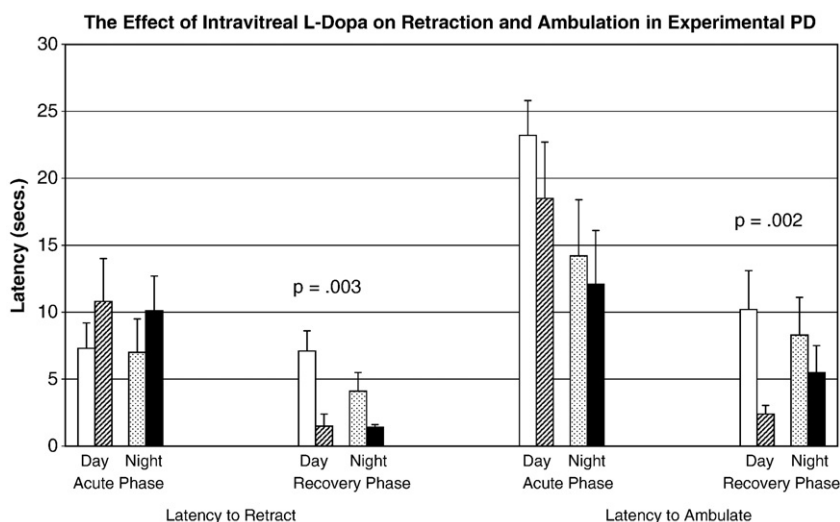


Fig. 2 – The effects of IVIT L-Dopa ($2 \mu\text{l}$ of a 100 mM solution) on latency to retract a limb (left traces) and latency to ambulate (right traces) in experimental PD. While no significant effects were observed on latency to retract during the acute phase, IVIT L-Dopa significantly improved retraction in the light during the recovery phase (open bar) compared to those treated with vehicle (diagonal bar). Similarly, the latency to ambulate was significantly improved in the light during the recovery phase for L-Dopa treated animals (open bar) compared to those treated with vehicle (diagonal bar). No other significant changes were observed in this parameter at any other time. T-bars represent the standard error of the mean.

Fig. 3 depicts the changes in body weight for the duration of the study in L-Dopa or vehicle injected rats. For the 6 day period following 6-OHDA injection just prior to the first spontaneous death, change in body weight was not significantly different between the two groups (ANOVA: $F=0.073$, $df=1,106$, $p=0.65$). When a comparison was made between the two groups for the last six days of measurement after the last spontaneous death, no significant difference was found (ANOVA: $F=3.269$, $df=1, 75$, $p=0.075$). This suggests that the severity of PD induced by intracerebral (I.C.) 6-OHDA was similar and that the effects of L-Dopa administration were attributable to the injection *per se*.

2.2. Study 2: IVIT ML-23

As shown in Fig. 4A the IVIT injection of ML-23 returned horizontal movement to above its control level in the dark, during the night of the acute phase. At that time the 6-OHDA injected rats injected with ML-23 (mean= 31.9 ± 188) were significantly better than those treated with 6-OHDA plus vehicle (mean= -486 ± 215) and the difference was significant ($\chi^2=2.797$, $n=14$, $df=1$, $p=0.046$). Comparison of the 6-OHDA plus ML-23 with the control animals injected with ML-23 (mean= -193 ± 170) revealed that they were not significantly different.

As shown in Fig. 4B, the IVIT injection of ML-23 returned vertical movement to a level similar to that seen in control animals injected with ML-23 in the dark, during the night of the recovery phase. At that time the 6-OHDA injected rats injected with ML-23 (mean= -88 ± 50) were significantly better than those treated with 6-OHDA plus vehicle (mean= -246 ± 32) and the difference was significant ($\chi^2=4.123$, $n=11$, $df=1$, $p=0.024$). Comparison of the 6-OHDA plus ML-23 with the control animals injected with ML-23 (mean= -103 ± 32) revealed that they were not significantly different.

The only time that a difference in latency to retract a limb was observed between the 6-OHDA plus ML-23 group versus the 6-OHDA plus IVIT vehicle group was during the night of the acute phase (Fig. 5, left traces). While the 6-OHDA plus ML-23 group (mean= 3.3 ± 1.3) was similar to the control plus ML-23 group (mean= 2.3 ± 0.96) they were significantly faster on this task than rats injected with 6-OHDA plus IVIT vehicle (9.2 ± 2.1) and the difference was highly significant ($\chi^2=3.318$, $n=28$, $df=1$, $p=0.01$).

Latency to step up or down from a raised platform was significantly improved in 6-OHDA plus ML-23 treated rats on the night of the acute phase (Fig. 5, middle traces). With a mean latency (5.4 ± 2.4) being statistically similar to that of control rats receiving IVIT ML-23 (mean= 5.1 ± 2.6 ; $\chi^2=1.007$, $n=11$, $df=1$, $p=0.267$) the difference between the control group and 6-OHDA treated rats injected with IVIT ML-23 (mean= 11.1 ± 5.1) was significantly different ($\chi^2=1.576$, $n=11$, $df=1$, $p=0.045$). There were no other significant differences in performance on this parameter during other times of testing.

As observed with other parameters, the only time that latency to ambulate (Fig. 5, right traces) was significantly different in the 6-OHDA plus ML-23 group (mean= 6.7 ± 2.5), compared to the 6-OHDA plus vehicle group (mean= 13.9 ± 3.6), was during the night of the acute phase. At that time, this difference was statistically significant ($\chi^2=3.275$, $n=14$, $df=1$, $p=0.014$). The PD plus ML-23 group was not different to control animals injected Intravitreally with ML-23 (mean= 7.7 ± 1.7) at this time nor were there any other differences observed on this parameter at any other night of observation.

Fig. 6 depicts the changes in body weight for the duration of the study in all three groups of animals tested. For the 14 day period following 6-OHDA injection the change in body weight was not significantly different between the 6-OHDA plus IVIT ML-23 or the 6-OHDA plus IVIT vehicle group (ANOVA: $F=15.496$,

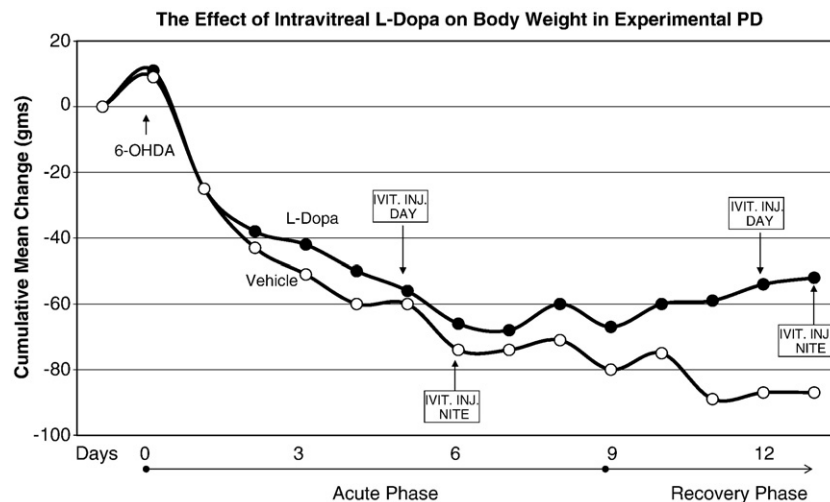


Fig. 3 – The effect of IVIT injections on body weight regulation in experimental Parkinson’s disease after L-Dopa (filled circles; 2 μ l of a 100 mM solution) or vehicle (open circles) on days 5,6,12 and 13. Body weight was monitored for three days prior to and 13 days after the induction of PD using intrahypothalamic injections of 6-OHDA. The cumulative change in body weight for the days of observation is indicated. The acute phase of the disease is represented by the black line extending up to day 9 while the recovery phase is represented by the line extending from day 9 to the end of the study. For the six days following 6-OHDA treatment or the 6 final days of observation no significant difference in the change in body weight was observed between the groups.

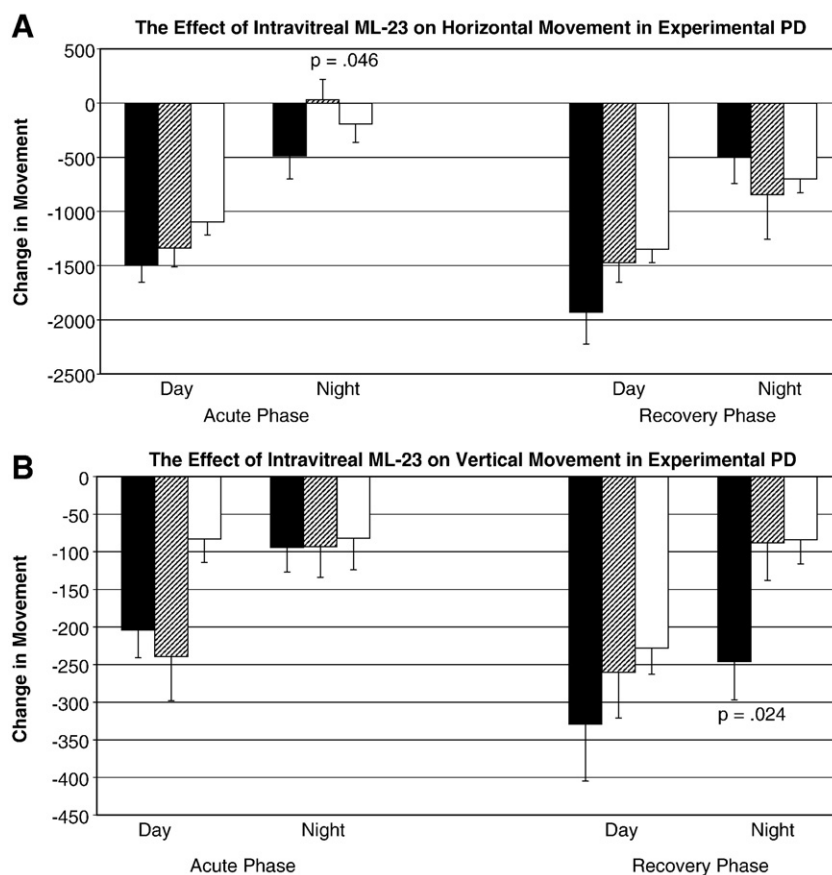


Fig. 4 – A. The effects of IVIT ML-23 (2 μ l of a 10 mM solution) on horizontal movement in experimental PD. While no significant effects were observed on horizontal movement (left trace) during the day of acute phase testing, ML-23 significantly reduced the impairment of horizontal movement during the night at this time (diagonal bar-ML-23 versus closed bar –vehicle). During the recovery phase IVIT injections had no effect on horizontal movement at times of testing during the day or at night. B. The effects of IVIT ML-23 (2 μ l of a 10 mM solution) on vertical movement in experimental PD. ML-23 significantly reduced the impairment of vertical movement during the night of the recovery phase (diagonal bar-ML-23 versus closed bar – vehicle). The open bars represent the ML-23 injected controls. The mean change in movement was determined by comparing test sessions to control sessions and the T-bars represent the standard error of the mean.

df=2,165, $p=0.999$), while both of these groups differed significantly from controls injected with IVIT ML-23 ($p<.001$ for both comparisons).

Post-mortem examination of the brain tissue revealed that the lesions resulting from the injection of 6-OHDA or vehicle into the PLH caused necrotic tissue damage covering a similar volume of tissue and in a similar anatomical placement in animals treated with IVIT L-Dopa (Figs. 7A and B) or ML-23 (Figs. 7C and D). Necrosis depicting the site of injection was located just rostral to the substantia nigra, extending from the LH, through the PLH and into the anterior part of the substantia nigra.

3. Discussion

There are three important findings emanating from the present results. First, the observed involvement of the retina supports the contention that the circadian system plays an important role in the aetiology of PD. For almost 200 years the expression of circadian based symptoms including depression

(Deniker et al., 1975; Girotti et al., 1986; Okun and Watts, 2002), insomnia (Arnulf et al., 2002; Demet et al., 1999; Dowling, 1995; Parkinson, 1817), akathisia (Abbott, 2006; Boeve et al., 2004; Guardiola-Le Maitre, 1997; Schmid, 1993), REM sleep behaviour disorder (RSBD) and nocturnal myoclonus (Abbott, 2006; Willis and Turner, 2007) and associated demetive features (Arnulf et al., 2002) have been observed, but their priority in the aetiology and progression of the disease has remained unexplored. The present work suggests that the retina, as a component of the circadian system, plays an integral role in this regard. Secondly, this work identifies retinal function within a larger anatomical framework that includes the NSD, RHT and other neuroanatomical systems which as been termed the retinal-diencephalic-mesencephalic-pineal (RDMP) axis (Willis, in press). In addition to the attempt to define the mechanism underlying the anti-Parkinsonian effects of bright light therapy, we commenced systematic study of the three main components of this axis which led to the definition of a more comprehensive system controlling motor function. Conceptualization of the pineal as the endocrine endpoint (Moore, 1996), the LH as the functional integratory centre for the

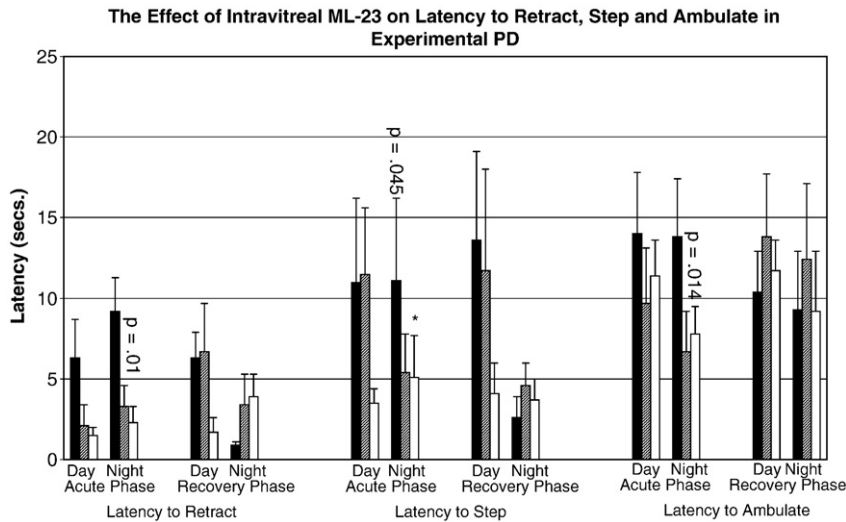


Fig. 5 – The effects of IVIT ML-23 (2 μ l of a 10 mM solution) on latency to retract a limb (left traces), latency to step (middle traces) and latency to ambulate (right traces) in experimental PD. Significant improvement in the latency to perform all three tasks was observed at night during the acute phase of experimental PD and these were significant at the times indicated. The comparison was made between the PD+vehicle group (closed bars) versus the PD+ML-23 groups (diagonal bars) for latency to retract and ambulate while the comparison for latency to step was made between the PD+vehicle group (closed bars) and the ML-23 injected controls (open bars-asterisk). For this parameter no significant difference was observed between the ML-23 plus vehicle group and the controls injected intravitreally with ML-23. No other significant changes were observed in these parameters at any other time. T-bars represent the standard error of the mean.

circadian and NSD systems (Willis, *in press*) and the retina as the site from where the degenerative cycle commences and progresses may serve to explain the therapeutic effects of phototherapy in PD (Artemenko and Levin, 1996; Paus et al., 2006; Willis and Turner, 2007). Thirdly, and most importantly,

the present results demonstrate that as a component of a more global system affected in PD, that is the retina, may be the target for mainstream anti-Parkinsonian medications. That the retina may provide a new site of therapeutic intervention for PD and other neuropsychiatric disorders is the

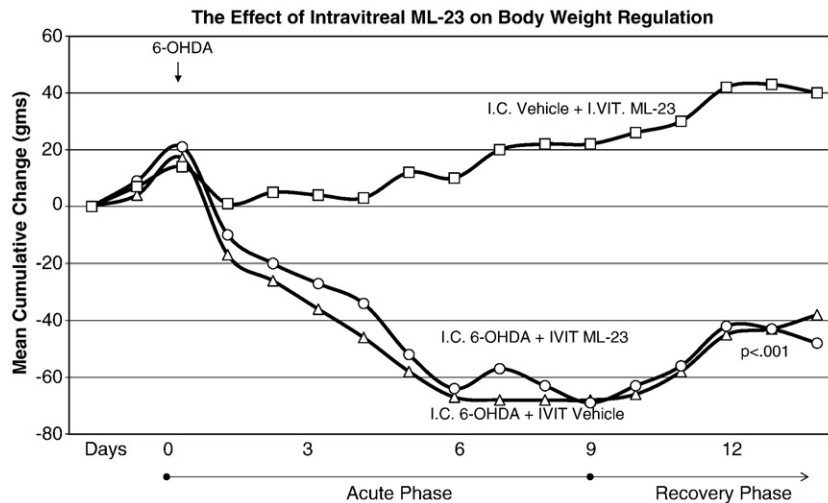


Fig. 6 – The effect of IVIT injections on body weight regulation in experimental PD after ML-23 (circles; 2 μ l of a 10 mM solution) or PD plus vehicle (triangles; 2 μ l of vehicle) or controls plus ML-23 (squares; 2 μ l of a 10 mM solution) on days 2, 3 and 4. Body weight was monitored for three days prior to and 14 days after the induction of PD using intrahypothalamic injections of 6-OHDA. The cumulative change in body weight for the days of observation is indicated. The acute phase of the disease is represented by the black line extending from day zero up to day 9 while the recovery phase is represented by the line extending from day 9 to the end of the study. For the six days following 6-OHDA treatment or the 6 final days of observation no significant difference in the change in body weight was observed between the PD plus vehicle or the PD plus ML-23 groups during the entire course of observation.

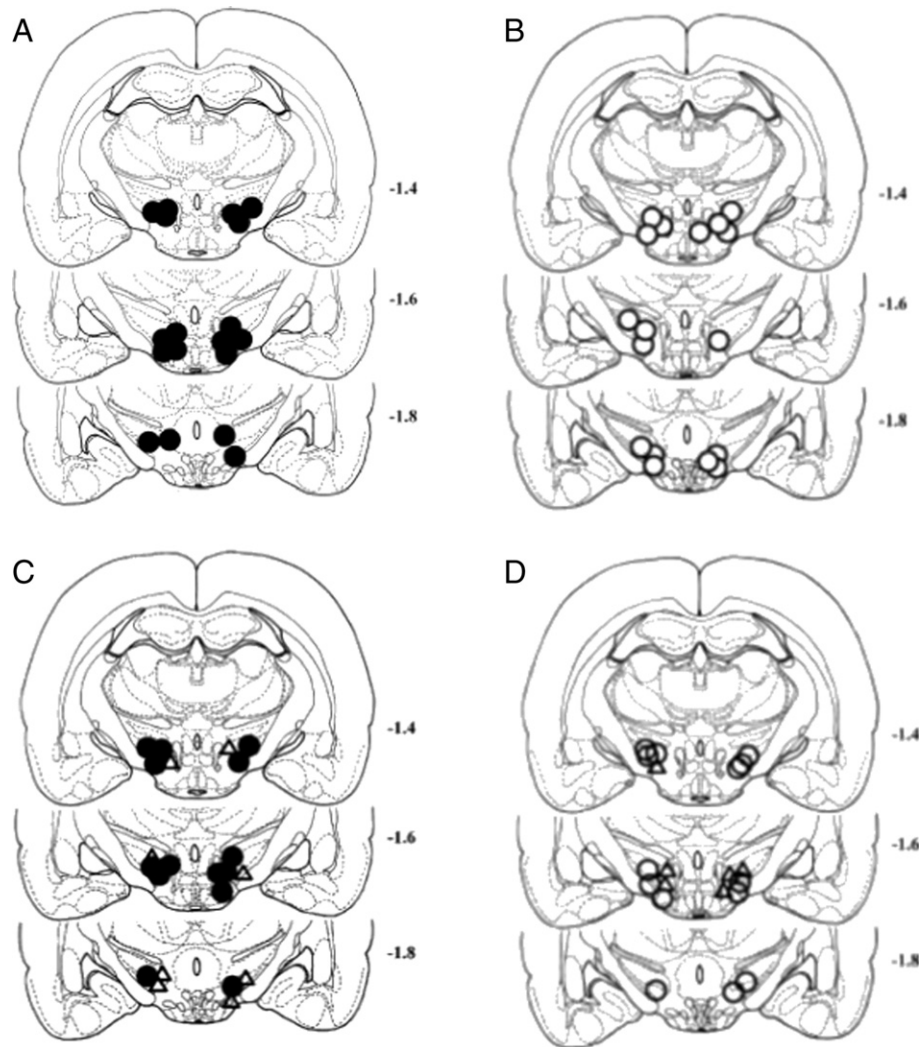


Fig. 7 – Histological plates depicting the sites of injection of 2 μ l of an 8 μ g/ μ l solution of 6-OHDA or of 2 μ l of vehicle in cannulated rats that later received IVIT injections. The method of tissue preparation and histological assessment has been described previously (Willis and Robertson, 2005) and injection sites mapped in regard to anatomical landmarks. In Plates A and B, the sites of 6-OHDA injection in rats receiving IVIT L-Dopa (closed circles) are compared to those injected with IVIT vehicle (open circles). In plates C and D, the sites of 6-OHDA injection in rats receiving IVIT ML-23 (open circles) are similar to those injected with IVIT vehicle (closed circles) while control rats injected I.C. +IVIT ML-23 are depicted by open triangles. The centres of the injection sites were generally located 1.4 to 1.8 mm posterior to bregma (Pellegriano et al., 1979) and were similarly positioned for each group so as to predict a similar pattern of diffusion of 6-OHDA to axons of the adjacent NSD system (Ljungberg and Ungerstedt, 1976) and a homogenous effect of this neurotoxin may therefore be assumed (Willis et al., 1976).

most exciting prospect. The therapeutic effect in this model of PD was achieved with an IVIT dose of L-Dopa that was about 1/1250th of that used systemically (Fink and Smith, 1979; Uretsky and Schoenfeld, 1971) while the dose of ML-23 employed is 1/417th of that found to be effect after systemic administration in rodents and primates (Willis and Robertson, 2004, 2005). Given that the most plaguing problems of Parkinsonian chemotherapy include reduced efficacy with long term treatment, overdose, polypharmacy, side effects and the development of psychosis and involuntary movement (Fahn and the Parkinson Study Group, 2003; Murray et al., 1995; Nutt et al., 1992), IVIT administration might serve to dramatically reduce or eliminate these problems.

The current findings also help to define a more intricate neurochemistry that characterizes PD. That NSD neurones are melanophores developmentally derived from the same part of the neural crest as are retinal and pineal cells intimates that they may possess functional similarities (Borg, 1974; Hadji-constantinou and Neff, 1984). This is particularly true in relation to the function of melatonin and DA within these cells. For example, melatonin and DA sit in functional opposition within the retina and pineal (Djamgoz et al., 1997; Dubocovich, 1983; Dubocovich, 1989). During the day, DA levels are high and melatonin secretion is dramatically suppressed and while such a reciprocating system has not been demonstrated within NSD neurones, circadian based

changes in this system have been reported (O'Neill, 1990). Circadian function expresses a similar relationship as plasma melatonin troughs during the day and peaks at about 0200 h in people exposed to a normal light/dark cycle. In the PD patient, however, this is compromised with the peak occurring at 2100 h (phase advanced) and a general increase in melatonin secretion occurring during the acrophase (Bordet et al., 2003; Catala et al., 1997). The application of light to correct PD symptoms has been undertaken at the onset of the light (Paus et al., 2006) and the dark (Willis and Turner, 2007) cycles, with varying results. While it might be assumed that decreasing melatonin might serve to increase DA this is probably not the case even though pharmacological antagonism of melatonin provides a robust therapeutic effect (Willis and Robertson, 2004). Daily oral administration of ML-23 enhances recovery from experimental PD but does not alter DA levels in the brain (Willis and Robertson, 2005). This suggests that it is not the absolute levels of DA that are crucial in achieving a therapeutic effect but more importantly, the balance between remaining DA and melatonin has to be restored. The present results strongly support this contention in several ways. Firstly, IVIT L-Dopa was more effective during the light phase of the light/dark cycle than it was during the dark phase. Not only were more parameters seen to improve at this time but the therapeutic effect was more pronounced. In fact, this occurred to a point where most animals returned to control performance, as seen in horizontal movement, limb retraction and ambulation. Conversely, the IVIT administration of ML-23 appears to have its most robust effects during the dark phase of the LD cycle with significant effects observed at night for all parameters including horizontal and vertical movement, as well as latency to retract a limb, to step and to ambulate. The existence of such a reciprocating response strongly suggests that the nigra is functionally attuned to retinal function, as is the pineal (Ebadi and Govitrapong, 1986; Willis, 2005b) and it is by such a mechanism that nigral function is intimately modulated.

The time of injection, relative to the stage of the disease experienced, is an important factor to also take into account. For example, IVIT injections of L-Dopa were effective during the recovery phase (after day 9 post 6-OHDA) defined in the present model as the time after which the fulminating form of the disease (Acute Phase, up to day 9 post 6-OHDA) has resolved. During the recovery phase other compensatory processes such as hypersensitivity of residual systems or alternate pathways develop and a gradual recovery ensues (Willis and Smith, 1985). This sequence is, in fact, the opposite of that which routinely occurs in the clinical syndrome whereby the severity of the disease is insidious and progressive. Nevertheless, the finding that peripheral administration of DA replacement during the early degenerative events of bilateral experimental PD is with little or no therapeutic effect (Ljungberg and Ungerstedt, 1976) while increased movement is observed when administered in the recovery phase, confirms the potential of this route of administration as a good method for screening anti-Parkinsonian drugs.

The opposite effect was generally observed with the IVIT injection of ML-23. During the acute phase the pharmacological blocking of melatonin receptors was seen for horizontal, latency to retract, to step and to ambulate after three con-

secutive IVIT injections. In consideration of the possible mechanisms of toxicity that melatonin can express (Willis, *in press*) it is not surprising that melatonin antagonism, by pharmacological (Willis and Robertson, 2004, 2005) or photic intervention (Artemenko and Levin, 1996; Paus et al., 2006; Willis and Turner, 2007), can induce recovery in this disease. Whether the effect pertains to restoration of axoplasmic transport (Huerto-Delgado et al., 1994), dispersion of melatonin (Schorderet and Nowak, 1990), pro-oxidative action of melatonin (Willis, *in press*) or retinal toxicity (Wiechmann and O'Steen, 1992; Yilmaz et al., 2004) these mechanisms may all be engaged in the short term and may well explain the anti-Parkinsonian effects of ML-23 in the acute stages of the disease (Willis and Robertson, 2004).

It is important to note that while the ML-23 group improved significantly on these parameters during the acute phase the therapeutic effects were not long lived and the reduced mortality observed when higher doses of ML-23 are administered systemically (Willis and Robertson, 2005) did not occur. These findings taken together in respect to other reports (Willis, 2005a) show a clear dose dependant effect of ML-23 that is symptom specific. While larger peripheral doses (7×3 mg/kg I.P.) recover motor and vegetative function and reduce mortality, smaller doses administered directly to the retina ($40 \mu\text{g}$ in $2 \mu\text{l}$) only temporarily improve motor function and the usual rate of mortality ensues. In addition, in pilot studies for recent work (Willis and Robertson, 2004) intermediate doses of ML-23 (2×3 mg/kg I.P.) recovered motor function and slightly reduce mortality suggesting that the mechanism of action for affecting vegetative versus motoric function are separate and we suggest further that they may be hypothalamic (Willis and Armstrong, 1998) and retinal (Djamgoz et al., 1997; Manev and Uz, 2006; Willis et al., *in press*), respectively. Which ever the case, the present results demonstrate that retinal administration of ML-23 for motoric symptoms is anatomically specific and dose dependant and, in this regard, confirms the retina as the anatomical site of action of this drug after systemic administration.

The reciprocating effect observed with L-Dopa vs ML-23 in the present study closely approximates the well established relationship between DA and melatonin in melanoophores. This suggests that the same process that governs retinal and/or pineal function (Akhisaroglu et al., 2004; Dubocovich, 1989; Ebadi and Govitrapong, 1986; Manev and Uz, 2006) also operates in the nigra and that it can be modified at the level of the retina. This supports the contention that melatonin and DA function is tightly intertwined along the entire RDMP axis (Willis, *in press*). In PD this relationship is grossly compromised when patients first present as the levels of DA in the NSD system are reduced to 20% of normal (Willis and Armstrong, 1998). With melatonin slightly elevated at this time (Bordet et al., 2003) the ratio of melatonin to DA is elevated at least 6 to 1 in favour of melatonin over DA at the early stages of the disease. As the disease advances the function of the system is compromised further with this ratio increased yet again as excessive doses of DA send the entire system into chaos (Djamgoz et al., 1997). The level of complexity in regard to treatment becomes overwhelming as the DA to melatonin relationship may be compromised at any point along the RDMP axis: that is, at the retina, the mesencephalon or the

pineal (Willis and Armstrong, 1998). On this basis the “more is better...” approach, which has dominated our thinking and practice of DA replacement therapy, may account for the treatment failures, adverse reactions and side effects that make the treatment worse than the disease itself. Only when both melatonin and DA are brought back into normal functional proximity can we expect to see the optimal therapeutic effect. We do, however, exert some caution in this interpretation since neither DA levels in the NSD system nor plasma melatonin levels were measured in the studies reported here. While the methods employed have been shown to have a robust effect on the NSD system (Fink and Smith, 1979; Ljungberg and Ungerstedt, 1976; Willis et al., 1976; Zigmond and Stricker, 1972) and produced a severe form of experimental PD (Balagura et al., 1969; Fink and Smith, 1979; Ljungberg and Ungerstedt, 1976; Willis and Armstrong, 1999; Willis and Robertson, 2004) verification of the interrelated processes operating in the proposed axis will have to be undertaken. This may be best achieved using analytical techniques that simultaneously examine the DA/melatonin relationship in the retina, the mesencephalon and the pineal. Unravelling this level of complexity will prove to be a challenge for future research.

In further consideration of the functional link between melatonin and DA in melanophores it is important to consider the possibility that reducing melatonin might be an endogenous means for increasing DA (Djamgoz et al., 1997; Dubocovich, 1989). However, this is unlikely for several reasons. Firstly, melatonin administration, even in super physiological doses, does not precipitate therapeutic improvement in PD. This failure-to-find phenomena has been replicated and re-replicated on several occasions over the past thirty years (Medeiros et al., 1996; Papavasiliou et al., 1972; Shaw, 1977; Shaw et al., 1973, 1975). Secondly, melatonin antagonism is effective in models of PD including the marmoset (Willis and Robertson, 2005). However, the therapeutic effect seen was independent of DA deficiency and behavioural recovery ensued in spite of the fact that DA deficiency remained unchanged. Thirdly, an indirect method for increasing DA levels (i.e. antagonizing melatonin) would not be expected to facilitate recovery when it can be more readily achieved by injecting DA precursors or synthetic forms of this transmitter at physiological doses. Fourthly, in many PD patients with dyskinesia, light therapy with lower doses of DA replacement has been employed to produce symptomatic control without dyskinesia (Willis and Turner, 2007). Therefore, if light or other methods of antagonizing melatonin are acting by reducing the inhibitory effect of melatonin on DA then a redevelopment of dyskinesia would be expected, but this does not occur. In short, the absence of a dose response where lower doses are therapeutic while higher doses correct dyskinesia would be expected. However, while the use of melatonin to control dyskinesia is accomplished with super pharmacological doses (Abilio et al., 2002; Shamir et al., 2001) the absence of therapeutic effects with lower doses is routinely observed in PD (Medeiros et al., 1996; Papavasiliou et al., 1972; Shaw, 1977; Shaw et al., 1973, 1975). This contradicts the possibility that melatonin antagonism is simply increasing DA by virtue of its reciprocal relationship.

In summary, these results suggest that the retina is a part of a more global system exerting control over motor

function. More importantly this work now presents the possibility that therapeutic intervention is now achievable at the level of the retina in doses which are only a minute fraction of those administered systemically. From this, a better understanding of the systems involved in the aetiology and treatment of PD may be revealed and this may well lead to less invasive methods of therapeutic intervention that do not enhance the morbidity of this debilitating disease.

4. Experimental procedures

31 out bred male, Sprague Dawley rats were obtained from the Bronowski Institute colony or from Monash University Animal Services. Rats were housed individually in wire mesh cages with standard food pellets (Clarke King®/Barastock®) made available *ad lib* from a feeding grid. Tap water was made available *ad lib* from bottles attached to the front of each cage. Animals ranged in weight from 250 to 350 g at the commencement of surgery. Room temperature was maintained at 22 °C ± 2° with a 12:12 h light–dark (LD) cycle with lights on at 0700 h. The room was illuminated with two fluorescent tubes with the intensity of light within each cage averaging 250 lux during the lights on phase of the LD cycle. All experiments were performed under the auspices of the Animal Experimentation Ethics Committee of the Bronowski Institute of Behavioural Neuroscience implementing protocols conforming to the Australian National Guidelines for the Care and Use of Animals for Scientific Purposes.

4.1. Surgery

After habituation into the colony for at least 7 days rats were pre-medicated with atropine sulphate (0.06 mg/kg-S.C.) and then anaesthetized with a Ketamine® (55 mg/kg)/Xylazine® (20 mg/kg) mixture (I.M.). Each rat was then placed in a stereotaxic instrument. The site of cannulation for eventual I.C. injection for achieving experimental PD was the posterior lateral hypothalamus (PLH; Willis and Armstrong, 1998) just rostral to the midbrain diencephalon border in the bundle of NSD system fibres. Two 23 gauge stainless steel cannulae were implanted bilaterally, just dorsal to the intended site of injection at the coordinates AP = –1.8 mm; L = ±1.8 mm; D = –6.1 mm. The injection needle extended 2 mm beyond the cannulae tip in a ventral direction to minimize damage to the injection site (Willis et al., 1976). All coordinates were relative to bregma and in the plane of Pellegrino et al., 1979. While this position has been found to be effective in producing severe Parkinsonian-like effects in animals as it interferes with the axons of the NSD system (Fink and Smith, 1979; Willis and Armstrong, 1998; Willis and Armstrong, 1999; Willis and Robertson, 2004; Willis and Smith, 1985). At the completion of I.C. surgery rats were injected with 12 ml/kg Reversine® (S.C.) which was used as a reversal agent for the Xylazine®. All rats were injected with the analgesic Meloxicam® (10 mg/kg, I.M.) at the completion of surgery. Rats were kept warm after surgery and then allowed at least 10 days of recovery before commencing the formal part of the study.

4.2. Solutions and injections

4.2.1. I.C. 6-OHDA injections

6-hydroxydopamine hydrobromide (Sigma St. Louis MO, USA) was mixed in a concentration of 8 $\mu\text{g}/\mu\text{l}$ and injected in a volume of 2 μl per site. Injections were made at a rate of 1 μl per min and the needle was left *in situ* for at least 30 s after each injection was complete to insure that the drug diffused from the end of the needle. 6-OHDA was dissolved in ascorbic acid solution to prevent rapid oxidation of the drug (Willis and Armstrong, 1998; Willis et al., 1976) and all solutions were brought to isotonicity by adding NaCl. Vehicle injections were made with an isotonic saline/ascorbic acid solution. New solutions of drug were prepared immediately prior to injection with stock solutions kept refrigerated or on ice until used. All solutions were kept shielded from light and then discarded immediately at the end of each injection session.

4.2.2. IVIT injections

Intraocular injections into the vitreous humour were made with the aid of a 10 μl syringe fitted with a 28 g needle that was 75 mm in length. The needle was fitted with a coloured plastic sleeve exposing 3 mm of the tip to allow the experimenter to gauge the depth of needle insertion into the centre of the vitreal mass. L-Dopa (HCl) was obtained from a commercial source (Sigma-Aldrich-U.S.A.) and was dissolved in isotonic saline to achieve a 100 mM solution. Control injections for the L-Dopa study were made with isotonic saline. ML-23 was synthesized by AMRAD Pharmaceuticals and the dissolved in a 70% DMSO solution to achieve a 10 mM concentration. Control injections for this study were made with a 70% DMSO solution. The dose of L-Dopa (2 μl of a 100 mM solution) or ML-23 (2 μl of a 10 mM solution) used for IVIT injection were determined on the basis of previous work using melatonin antagonists and L-Dopa via the systemic or IVIT route (Dubocovich, 1983, 1989; Fink and Smith, 1979; Uretsky and Schoenfeld, 1971; Willis and Robertson, 2004, 2005).

4.3. Behavioural Measures

Dependent variables were measured during the light and the dark phase of the LD cycle between 1000–1500 h and again at 2000–0100 h, respectively, with at least 18 h allowed between consecutive measurements. Assessment after the induction of experimental PD was first undertaken during the “acute phase” which occurs up to 9 days post I.C. injection. This corresponds to a critical time when animals with bilateral lesions of the NSD become severely affected by this acute form of PD whereby death occurs or spontaneous recovery commences (That is, recovery without the aid of artificial feeding). While animals with unilateral lesions do not experience this life threatening form of the disease, this is the time when the most severe deficits are exhibited. The second time of measurement was between days 12–15 after the induction of PD and is termed the “recovery phase” of testing. This period is defined as the time during which recovery from the acute effects of the neurotoxic insult and homeostatic control has returned whereby animals are capable of regulating their nutritive intake on their own and brain lesions are no longer

life threatening. These two phases of testing have been defined previously (Willis and Armstrong, 1999; Willis and Smith, 1985).

Locomotion and rearing were measured with the aid of a 900 mm (length) \times 500 mm (width) \times 300 mm (height) PVC box fitted with machine vision with motion detection capabilities. The total number of movements within the horizontal plane and the number of rearing associated movements in the vertical plane during each 10 min test session were measured and recorded with the aid of specialized software. A series of three motor reflex tests were performed immediately at the conclusion of the open field test (Willis and Armstrong, 1999). These tests included the latency to retract the left and right front limbs when they were elevated 25 mm from the table surface, the latency to step up or down from a raised platform when the rear torso was elevated 25 mm and the latency to ambulate outside of a 90 \times 170 mm rectangle. These tests are derivations of those described previously (Balagura et al., 1969). The test chamber and all surfaces and apparatus were thoroughly washed between the testing of each animal to avoid contamination which may cause distraction during testing.

Body weight was measured intermittently commencing at about 1000 h for at least 3 days prior to and up to 15 days after 6-OHDA injection in both experiments.

4.4. Procedure: Experiment I

After surgical recovery was complete 20 rats were tested on all parameters during the light and then during the dark phase of the LD cycle and these served as control measures. After this time all of the rats received bilateral I.C. injections of 6-OHDA to render them Parkinsonian. These animals were closely monitored for the first day following I.C. injection and the severity of behavioural impairment was judged on the basis of body weight loss over that period of time. The composition of each of the two groups of 10 rats treated with 6-OHDA was matched so that the severity of Parkinsonism exhibited was similar before commencing IVIT injections.

L-Dopa and testing commenced on the 5th Day following I.C. injection. IVIT injections were performed after animals were placed in an induction chamber for 60–90 s in the presence of Fluorothane® anesthesia. Each animal in one of the 6-OHDA injected groups received a 2 μl IVIT injection of L-Dopa, bilaterally 3 h prior to testing which commenced at 1000 h, 3 h into the light phase. The remaining 10 animals in the second 6-OHDA injected group received IVIT injections consisting of 2 μl of isotonic saline (vehicle), bilaterally, 3 h before testing. This procedure was repeated on day 6 with the IVIT injection commencing at 1900 h, at the onset of the dark phase of the light/dark cycle, with testing commencing at 2200 h. This procedure for testing during the day and night was repeated during the recovery phase on days 12 and 13, respectively. Note that every animal in each group was injected and then tested consecutively during the time of behavioural measurement. The regimen for L-Dopa administration and behavioural testing was selected on the basis of previous work demonstrating that the therapeutic effects of L-Dopa occur within a few hours after drug administration via systemic routes (Fink and Smith, 1979; Uretsky and Schoenfeld, 1971).

4.5. Procedure: Experiment II

After surgical recovery was complete 21 rats were tested on all parameters during the light and then during the dark phase of the LD cycle and these served as control measures. After this time 14 of the rats received bilateral I.C. injections of 6-OHDA to render them Parkinsonian. These animals were closely monitored for the first day following I.C. injection and the severity of behavioural impairment was judged on the basis of body weight loss over that period of time which is a standard index depicting the severity of experimental PD in this model (Ljungberg and Ungerstedt, 1976; Lorefalt et al., 2004; Willis and Smith, 1985; Zigmond and Stricker, 1972). The composition of each of the two groups of seven rats treated was matched so that the two groups of 6-OHDA treated rats were similar in severity before commencing IVIT injections. The remaining group of animals served as controls and were injected I.C. with 2 μ l of isotonic saline–ascorbic acid solution.

Commencing on the first day after I.C. injection all 7 animals in one of the 6-OHDA injected groups and all 7 animals in the vehicle-injected control group received a 2 μ l IVIT injection of ML-23, bilaterally. IVIT injections were performed after animals were placed in an induction chamber for 60–90 s in the presence of Fluorothane® anesthesia. The remaining 7 animals in the second 6-OHDA injected group were injected by the IVIT route with 2 μ l of DMSO vehicle, bilaterally. This procedure was repeated on days 2 and 3 post I.C. injection. The IVIT injections commenced at 1000 h during the day. Testing commenced at 1000 h on day 5 post-6-OHDA and 6 for the light and dark phase testing, respectively, as described earlier. For recovery phase testing light and dark phase testing were undertaken on days 14 and 15, respectively. This regime was adopted on the basis of previous work (Willis and Robertson, 2004, 2005) showing that the therapeutic effect of ML-23 is a gradual process when it is administered in a similar regimen by the oral or I.P. route.

4.6. Histological assessment

At the end of both studies all remaining animals were sacrificed with pentobarbitone sodium (325 mg/ml). Each animal was injected with 0.5 ml of the stock solution. The entire brain was removed and placed in 10% formalin. After fixing each brain they were sectioned and the site of injection was examined to permit the identification of the site of I.C. injection. The extent of damage and anatomical position for each injection site was defined in relation to anatomical landmarks and then transcribed onto mapped coronal sections as defined by Pellegrino et al, 1979.

4.7. Statistical analysis

The statistical analyses employed were either one-way ANOVA with Tukey's HSD for post hoc multiple comparisons or Chi-Square test (linear by linear association; SPSS 14.0 for Windows), Levene's test for homogeneity of variance was performed and if significant, or if the data were badly skewed or if *n* was dramatically reduced as a result of natural attrition due to the acute effects of the experimental form of the disease non-parametric analyses were employed. In some instances

data transformation was employed when the distribution for a given set of scores was not normal. In this instance analysis was performed on ratio scores obtained by dividing the test score by the control score. As the hypothesis permitted prediction of the direction of the expected outcome, a one sided test was employed with exact significance. Alpha was set a priori at $p < .05$ and alpha values ranging from .06 to .09 indicated significant trends.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2008.03.083.

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