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Alterations of the circadian timing system in rodent and non human primate models of Parkinson's disease

Karim Fifel

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Docteur de l'Université Claude Bernard Lyon 1

ECOLE DOCTORALE

Neurosciences et Cognition (NSCo)

Par

Karim FIFEL

**Alterations of the Circadian Timing System in Rodent and Non
Human Primate Models of Parkinson's Disease**

Soutenue le 28 Février 2013 devant les Membres de Jury constitué par :

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Abdelhamid BENZAOUZ	Directeur de recherche, INSERM, Bordeaux, France	Rapporteur externe
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Elizabeth S. Maywood	Professeur de l'Université de Cambridge, England	Examineur
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Howard M Cooper	Directeur de recherche, INSERM, Lyon, France.	Directeur de thèse

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ABSTRACT

Since the first description by James Parkinson in his essay on the shaking palsy, Parkinson's disease (PD) was recognized as a motor disease identified by a tetrad of symptoms, namely; akinesia, muscular rigidity, resting tremor and postural instability. These symptoms are known to be related to loss of dopamine (DA) in the striatum following neural degeneration in the substantia nigra (SN). This definition has contributed to the dominance of motor-based animal models of the disease, which, as a consequence, has led to the development of therapeutic strategies aimed to alleviate motor problems. However, it is increasingly recognized that non-motor and perhaps non-dopaminergic related symptoms inevitably emerge and worsen during disease progression. Sleep disruption is one of the major non-motor symptoms and has been suggested as a preclinical marker of the disease.

Models of sleep regulation have emphasized two distinct processes: a sleep-control mechanism, or sleep homeostat, and a circadian oscillator. The circadian oscillator, based in the suprachiasmatic nucleus (SCN), is responsible for the tendency to sleep during certain phases of the 24-hour cycle and the consolidation of sleep and wake into distinct episodes. The sleep homeostat is responsible for monitoring and reacting to the need for sleep, causing the urge to sleep to depend on prior amounts of sleep or wakefulness. While disruptions in the circuitry and the homeostatic processes involved in the regulation of sleep-wake behaviour is well documented in PD, the potential involvement of alterations of the circadian system have not been studied in detail. The aim of my thesis is to investigate alterations in the circadian timing system using two animal models of PD: the mouse and the non-human primate.

We used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a toxin to induce parkinsonism in both monkeys and mice. Circadian rest-activity rhythms of locomotor activity were continuously monitored under different light regimes in both species over several months to years. Clinical state in monkeys was evaluated using Parkinsonian Monkey Rating scale (PMRS), cognitive performance using an Object Retrieval Detour Task (ORDT) and in mice a Radial Water Maze was employed. In addition, hormonal rhythms (cortisol, melatonin) were assessed in monkeys from urinary samples. DA function was followed in monkeys using PET scans (C-PE2I, DAT), post mortem quantification of TH neurons in the brain in both species. In addition, we assessed the status of certain peptidergic systems in the retina and hypothalamus of MPTP-treated monkeys.

In mice MPTP treatment induced 70% degeneration of DA neurons in the SN. However, no alterations in general motor activity, circadian rhythmicity or cognitive performance were observed even nearly one year post treatment. Both untreated controls and treated animals all showed a significant decrease of locomotor activity and of several circadian parameters during aging without any exacerbation in MPTP-treated animals. Monkeys prior to MPTP treatment showed robust daily rest-activity rhythms under a light dark (LD) cycle, with precise onsets and offsets of locomotor activity. Following MPTP treatment the global level of motor activity decreased but daily rhythms were largely conserved, although the amplitude of the rhythm was damped and activity onsets were imprecise. The fragmentation and the stability of the rest-activity rhythm were also affected, sometimes even before the symptomatic stage. In constant light conditions, circadian activity rhythms were severely degraded or completely absent. Cortisol and melatonin rhythms, however, persisted in MPTP treated monkeys. PET scan and TH immunohistochemistry showed no correlation between the extent of degeneration of the dopaminergic system and the circadian behavioral outcome.

Taken together, the studies show that disturbances of circadian functions occur after MPTP treatment in the non-human primate but not in the mouse model of PD. These results emphasize the limitations of the MPTP-treated mouse model of PD for the study of non-motor symptoms, and reinforce previous studies that question the adequacy of this model to replicate cardinal motor features of the disease. In contrast, results in the non-human primate model stress the importance of dopaminergic degeneration in the circadian organisation of behavioral sleep wake cycle in PD.

General Overview

General Overview:

Although early descriptions of Parkinson's disease (PD) exist in several works from different civilizations (Raudino, 2012), the first formal description of the disorder is ascribed to James Parkinson in his original monograph "An Essay on the Shaking Palsy" published in 1817 (Parkinson, 1817). PD is a neurodegenerative disorder of unknown cause with an estimated world-wide prevalence of 1.6-1.8% in persons over 65 years of age (Trenkwalder et al., 1995; Van de Vijver et al., 2001; Von Campenhausen et al., 2005; Muangpaisan et al., 2009). Age is the single most consistent risk factor, and with the increasing age of the general population, the prevalence of Parkinson's disease is expected to rise steadily in the future. The impact of the disease is indicated by the fact that mortality is two to five times as high among affected persons as among age-matched controls (Bennett et al., 1996; Morens et al., 1996; Louis et al., 1997), resulting in a marked reduction in life expectancy (Morens et al., 1996). In fact, neurodegenerative diseases (PD, motor neuron disease and dementia) are projected to surpass cancer as the second most common cause of death among the elderly by the year 2040 (Lilienfeld and Perl 1993). Thus, PD greatly shortens life as well as affecting the quality of life and causing debility during life.

The traditional "textbook" description of PD defines the disease as a progressive disorder characterised by a tetrad of motor symptoms; rigidity, bradykinesia, tremor and postural instability. These features are considered to be due to a selective degeneration of the nigrostriatal pathway and a concomitant reduction in the striatal concentration of Dopamine (Carlsson, 1972). This model has led to the development of efficient existing treatments for alleviating these cardinal motor symptoms of the disease (Wu and Frucht, 2005; Wichmann and DeLong, 2006). However the apparent simplicity of the underlying pathology and optimism of discovering disease modifying, as opposed to symptom-alleviating treatments for the disease has been tempered by the recognition that many non-motor symptoms are inherent to the disease itself and may precede the onset of Parkinsonian features by years and perhaps even decades. Over the course of the disease, these symptoms progressively worsen and may inevitably dominate the clinical picture of PD patients in advanced stages (Chaudhuri et al., 2006; Maetzler et al., 2009).

Although some of these NMS do respond partially to DA-replacement therapy, the majority of them are more refractory and their management requires the introduction of novel treatments (Chaudhuri and Schapira, 2009). The unresponsiveness of NMS to available DA

replacement therapies is explained by the multicentric, complex underlying pathophysiology involving multiple non dopaminergic brain structures (Chaudhuri et al., 2006; Obeso et al., 2010). Indeed, the traditional view that the pathological process in PD begins by degeneration of dopaminergic neurons in the substantia nigra has been challenged by Braak and colleagues (Braak H et al., 2004) who suggested a caudorostral gradient of Lewy body formation progressing from the lower brainstem to the cortex. This model introduces a six-stage pathological process; Braak stage 1 denotes degeneration of the olfactory bulb and the anterior olfactory nucleus, which can clinically manifest as olfactory dysfunction. Braak stage 2 characterises progression of the pathological process to the lower brainstem. These areas, implicated in the preclinical stages 1 and 2 of Parkinson's disease, are also thought to be key areas that mediate non-motor symptoms such as olfaction, sleep homeostasis, and other autonomic characteristics. The typical motor triad of tremor, rigidity, and bradykinesia only emerge at Braak stages 3 and 4 when the topographically stepwise neurodegenerative process has affected the substantia nigra and other deep nuclei of the midbrain and the forebrain. This stage is when Parkinson's disease crosses the threshold from a premotor disorder to a motor disorder and is clinically diagnosed. The final two stages, Braak stages 5 and 6, correlate with the presence of Lewy bodies in limbic structures and mature neocortex (Braak et al., 2005). Patients may have neuropsychiatric symptoms such as depression, cognitive impairment, and visual hallucinations. This model is not, however, without its shortcomings. The staging is based on Lewy-body distribution which differs substantially from the pattern of cell loss (Gibb and Lees, 1988; MacDonald and Halliday, 2002). Furthermore, the validity of the proposed stages correlates poorly with the non-motor clinical manifestation in Parkinson's patients (Kempster et al., 2007; Burke et al., 2008).

Both homeostatic and circadian processes are involved in the regulation of biological rhythms (Borbély, 1982; Dijk and Czeisler, 1994; Borbély and Achermann, 1999; Dijk and Lockley, 2002). Therefore, alteration of one or both of these systems can impact on daily physiological rhythms. The central theme of my thesis is to investigate the proprieties of the circadian system in different animal models of PD and discuss the potential contribution of circadian system impairments to behavioral sleep-wake abnormalities experienced by PD patients in particular and to the wide range of both motor and non-motor symptoms of PD.

Only sparse evidence of circadian abnormalities in PD exists in the literature. In the first chapter of the thesis, I reviewed all available studies pointing to potential changes in the circadian timing system of PD patients. Evidence of such abnormalities has been found in

behavioral, physiological and molecular overt rhythms. I therefore discussed and arranged them accordingly into these categories. The dominating view of the mechanistic control of any overt rhythm was schematized by Eskin (Eskin, 1979, **Figure 1**) and postulates that oscillatory rhythms are generated within a central pacemaker after receiving and integrating information from inputs structures that are thereafter translated to output structures which exteriorate overt rhythmicity. This organization implies that any dysfunctional overt rhythm may arise as a consequence of impairment at the level of the input, the central pacemaker or the output structure. Applied to circadian abnormalities found in PD, I dissected the neural circuitry involved in the regulation of each discussed overt rhythm and defined, when possible, the potential level responsible for abnormalities in the temporal organization of the output oscillation.

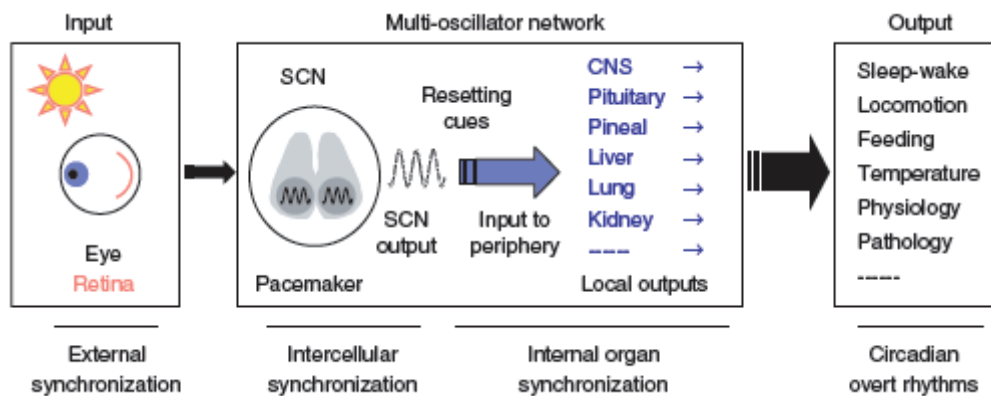


Figure 1: The mammalian circadian timekeeping system. The circadian clock can be conceptualized as three components: the inputs, the ~24-h clock and the outputs. The mammalian circadian clock is organized in a hierarchy of multiple oscillators, in which the SCN is the central pacemaker at the top of the hierarchy. The SCN is synchronized by the external 24-h cycle and in turn coordinates physiological outputs. The multi-oscillator network is synchronized through multiple lines of communication. For the SCN, light represents the primary input and firing rate rhythm an output. Peripheral oscillators are reset by timing cues emanating from the SCN (that is, SCN outputs), which regulate local circadian physiology (local outputs). Intercellular synchronization within the SCN is critically important for the robust operation of the entire body clock, which regulates overt rhythms such as locomotion and body temperature (From Liu et al., 2007b).

To introduce the animal models that we used to tackle our hypotheses, I also discuss briefly in this chapter, the characteristics of the available rodent and non-human primate models of PD. Among the available models, the MPTP-based mouse stands as the most commonly used model in PD-related studies. Yet, one of the well acknowledged limitations of this model is

the absence of Parkinsonism motor symptoms following treatment, let alone non-motor symptoms. The absence of motor and non-motor symptoms in MPTP-treated mice is frequently related to the acute nature of treatment that fails to replicate the slow progressive and widespread neurodegeneration of both DA and non-DA neurons that characterise PD (Jackson-Lewis and Przedborski, 2008; Maetzler et al., 2009). Recent advances in toxin administration methods have shown that chronic delivery of MPTP more reliably reproduces Parkinson-like features in mice, namely behavioral parkinsonism, development of Lewy body like inclusions and degeneration of dopamine and non-dopamine neurons (Fornai et al., 2005). In chapter 2, the potential precipitation of dysfunctional correlates of cognitive and circadian abnormalities experienced by PD patients by chronic MPTP treatment in mice has been experimentally addressed.

Since the discovery of the ability of MPTP to induce Parkinsonism in humans; MPTP has been administered to primates to reproduce a model of PD (Davis et al., 1979; Burns et al., 1983; Langston et al., 1983; 1984; Jenner et al., 1984). As expected, all species of primates so far treated with MPTP develop a Parkinsonian syndrome indistinguishable from the cardinal motor symptoms as they occur in human. Interestingly, and in contrast to its effects in mice, MPTP may trigger the emergence of some non-motor symptoms associated with PD. For example, in common marmosets, constipation, bladder hyper-reflexia and excessive salivation were reported. Sleep disturbances such as excessive daytime sleepiness and fragmentation of sleep-wake behavior do develop after MPTP treatment in both marmosets and cynomolgus macaques (Albanese et al., 1988; Yoshimura et al., 1993; 1998; Barraud et al., 2009). Rhesus monkeys on the other hand, when treated chronically (>3 months) with relatively low doses of MPTP (0.025-0.1 mg/kg) that do not evoke motor deficits, display cognitive deficits similar to those that accompany PD (Taylor et al., 1986; Schneider and Kovelowski, 1990; Vezoli et al., 2011). Given the clinical importance of non motor symptoms and the high degree of construct validity of MPTP-treated primates, it is surprising the lack of studies that have used this model for in-depth investigation of the pathophysiological mechanisms of the wide range of non-motor symptoms of PD. To further our knowledge in this domain, we took advantage of the MPTP-treated NHP to investigate the proprieties of the circadian system following MPTP treatment induced DA loss. In chapter 3, we describe the experimental results of a long-term follow up study of motor, cognitive and rest-activity performances in 4 MPTP treated monkeys.

In light-dark conditions, light (or darkness) exerts strong masking effects on the daily pattern of behavior, fine-tuning the expression of the underlying circadian rhythms controlled by the central SCN clock (Mrosovsky, 1999; Redlin, 2001). Assessment of endogenous circadian rhythmicity requires exposure to constant conditions in which cyclic environmental cues, such as light, temperature and feeding are absent (Pittendrigh and Daan, 1976a; Johnson et al., 2003; Liu et al., 2007b). We therefore assessed the circadian pattern of rest-activity behavior and patterns of hormonal secretion in our MPTP-treated animals. Chapter 4 describes in detail the unexpected manifestation of arrhythmia that MPTP-treated animals show under constant light conditions. To explain our results, we advanced a hypothesis according to which the behavioral deficits could be attributed to an inability of the circadian clock to drive output motor control downstream from the SCN.

Our hypothesis postulates that, in addition to its modulatory role of motor functions, dopamine, as a rhythmic signal would be mandatory for normal circadian expression of DA-related functions. Transposed to Parkinson's patients, this hypothesis implies that during disease progression, the loss of DA will induce a progressive disconnection between SCN output signals and the brain network involved in the expression of locomotor behavior. A validation of this hypothesis would require a model that faithfully replicates both the progressive DA neurodegeneration and behavioral motor symptoms of PD. As it will be discussed in chapter 2, the neurotoxic models (i.e. MPTP) fail to mimic the progressive neural degeneration of DA neurons in the substantia nigra paralleled with motor impairments. Facilitated by an understanding of the genetic basis of PD, multiple genetic mouse models have been developed (Dawson et al., 2010). Although the majority of these transgenic models replicate some form of motor impairment, a major drawback is the lack of brain pathologies characterizing PD (Dawson et al., 2010). The most interesting mouse model developed to date is the so called Mitopark mouse model (Ekstrand et al., 2007). This model experiences a progressive loss of midbrain dopaminergic neurons mirrored with slow motor impairments which are improved markedly by L-DOPA administration; these properties indicate that the Mitopark mouse shares many similarities with human PD. In the fifth chapter, we present the experimental results of the properties of the circadian system in this model showing its potential as a model for a dissection of the physiological and molecular basis of behavioral arrhythmia found in our MPTP treated NHP.

Finally, in the last chapter, I discuss the entire scope of the thesis in relation to (i) inherent circadian and sleep abnormalities of PD. (ii) the relevance of current primate and rodent

models of PD to accurately replicate circadian and sleep dysfunction in PD, and (iii) the implication of our model to light therapy in PD. I conclude the chapter with some short-term perspectives we are currently investigating and would like to expand during my Post-doctoral studies.

Introduction

Introduction:

Idiopathic Parkinson's disease (PD) is the second most common neurodegenerative disease and affects more than 1.6-1.8% worldwide over the age of 65 (Trenkwalder et al., 1995; Van de Vijver et al., 2001; Von Campenhausen et al., 2005; Muangpaisan et al., 2009). Clinically, the disease is mainly characterized by a set of motor abnormalities (bradykinesia, resting tremor, rigidity and postural instability). The tetrad of symptoms results from a progressive and slow (neuro) degeneration of the nigrostriatal dopaminergic system (Carlsson, 1972, Lees et al., 2009) (**Figure 2**). However, a few of decades ago, an increasing awareness of the complex and multifaceted nature of PD has emerged (Chaudhuri et al., 2006; Chaudhuri and Schapira, 2009). Non-motor and non-dopamine related symptoms (NMS) encompass various clinical manifestations, including; neuropsychiatry dysfunctions, behavioral changes, autonomic, gastrointestinal and sensory symptoms. Some of these symptoms are sometimes present during the preclinical phase of PD. In advanced stages, those non-motor symptoms will inevitably dominate the clinical picture (diagnosis) and contribute to an impairment of the quality of life.

Among the NMS of PD, sleep disturbances occur in virtually all patients. Despite this well established association, the cause and effect between sleep disruption and PD related brain dysfunctions are not yet well understood. The regulation of sleep/wake behavior is thought to be governed by two distinct processes (Borbély, 1982; Dijk and Czeisler, 1994; Borbély and Achermann, 1999; Dijk and Lockley, 2002); a sleep dependent mechanism (sleep homeostat) and a circadian sleep regulatory mechanism (or circadian system) (**See section 6**). The timing of sleep can be modulated by independent or concomitant manipulation of those two processes suggesting that any time-dependent sleep dysfunction may be a result of a dysfunction in one or both of those two processes. The relative contributions of homeostatic vs circadian dysfunction regarding the cause of sleep disruption in PD have not been evaluated. Recently, a substantial number of studies have assessed multiple physiological overt circadian rhythms at different stages of the disease. The advances made have allowed a more accurate evaluation of the contribution of the circadian dysfunctions as a cause to sleep abnormalities reported in PD in particular, and to the deterioration of the quality of life in general.

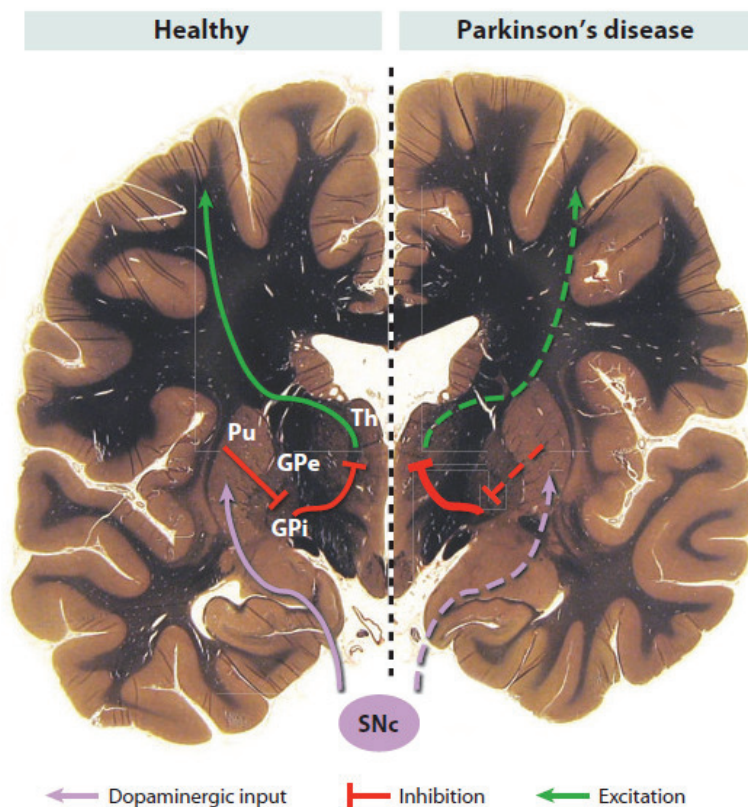


Figure 2: Anatomy and physiology of Parkinson's disease (PD) motor manifestations. A simplified schematic of the neuronal circuits involving the basal ganglia, thalamus, and cortex and their derangement in PD. For simplicity, only the direct pathway is shown. It normally functions to facilitate movements (left), but in PD the output is attenuated (right). The midbrain substantia nigra pars compacta (SNc) provides dopaminergic input to the putamen (Pu), which is excitatory to the direct pathway. The putamen inhibits (red) the globus pallidus interna (GPi), which subsequently inhibits the thalamus (Th). The thalamus projects excitatory input (green) to the motor cortex. In PD, degeneration within the SNc leads to net increased inhibition of the thalamocortical projection. The indirect pathway (not shown), including the globus pallidus externa (GPe) and subthalamic nucleus, is inhibited by SNc dopaminergic input and normally functions to repress movements, but its activity is enhanced in PD (From [Shulman et al., 2011](#)).

The aim of this introduction is to review our current knowledge about the status of the circadian system at different stages of Parkinson's disease and its potential contribution to sleep/wake abnormalities in PD. We aim also to sort out the complexity of the underlying pathophysiology of sleep/wake dysfunction, particularly in advanced forms of the disease which may explain the lack of effective therapeutic strategies. Finally, we bring together findings from recent studies that highlight the possible beneficial effects of light therapy in improving the quality of life of PD patients.

1-The circadian system: general concepts:

Circadian rhythms are daily cycles of physiology and behavior that are driven by an endogenous oscillator with a period of approximately one day (“circa diem”: approximately a day) (Aschoff, 1984; Pittendrigh, 1993). Their rhythmic expression continues even when subjects are temporally isolated. This internalisation of the generation of recurring 24 hrs rhythms constitute a fundamental characteristic of the circadian system that allows an organism to predict daily events, and hence, organize its biology appropriately to cope with environmental constraints. Basically, similar circadian rhythms control a variety of biological processes in living systems, ranging from bacteria to mammals (Lowrey and Takahashi, 2004; Bell-Pedersen et al., 2005; Takahashi et al., 2008). In mammals, lesion and transplantation experiments in the brain have identified a pair of bilateral nuclei of approximately 10.000 neurons each, located in the hypothalamus just above the optic chiasma (hence its denomination as suprachiasmatic nucleus or SCN) as a focal point of circadian rhythms (Moore and Eichler, 1972; Stephan FK and Zucker, 1972; Lehman MN et al., 1987; Ralph et al., 1990; Sujino et al., 2003).

At the molecular level, animal models where single or multiple clock genes have been deleted or mutated have revealed genes that are critically important for circadian rhythms of locomotor behavior, physiology and gene expression. The mammalian circadian clock consists of an autonomous regulatory genetic network involving interconnected negative and positive transcription-translation feedback loops (Ueda et al., 2005) (**Figure 3**). The positive feedback loop consists of the transcriptional activators clock (circadian locomotor output cycles kaput, or NPAS2 in brain tissue) which during the day interacts with BMAL1 to activate and sustain transcription of the Per (Per1, Per2 and Per3) and Cry (Cry1 and Cry2) genes, resulting in high levels of there transcripts. The resulting PER and CRY proteins heterodimerize, the resulting PER-CRY complex which constitute the arm of the negative feedback loop translocate to the nucleus and interact with the CLOCK-BMAL1 complex to inhibit their own transcription. During the night, the PER-CRY complex is degraded, and CLOCK-BMAL1 can then activate a new cycle of transcription. The entire cycle takes approximately 24 hours to complete, hence the term circadian; the complexity and robustness of this network is strengthen and fine tuned by additional interlocking loop involving the nuclear hormone receptor Rev-erba, which is a direct target of CLOCK-BMAL1 and which strongly represses Bmal1 transcription (Preitner et al., 2002; Sato et al., 2004; Dibner et al., 2010). Additionally, a number of other candidate clock components such as (Timeless, Dec1,

Dec2 and E4bp4; reviewed in Lowrey and Takahashi, 2004) as well as a complex post-translational modification of clock proteins have been shown to crucially influence the circadian clock (Lee et al., 2001; Gallego and Virshup, 2007).

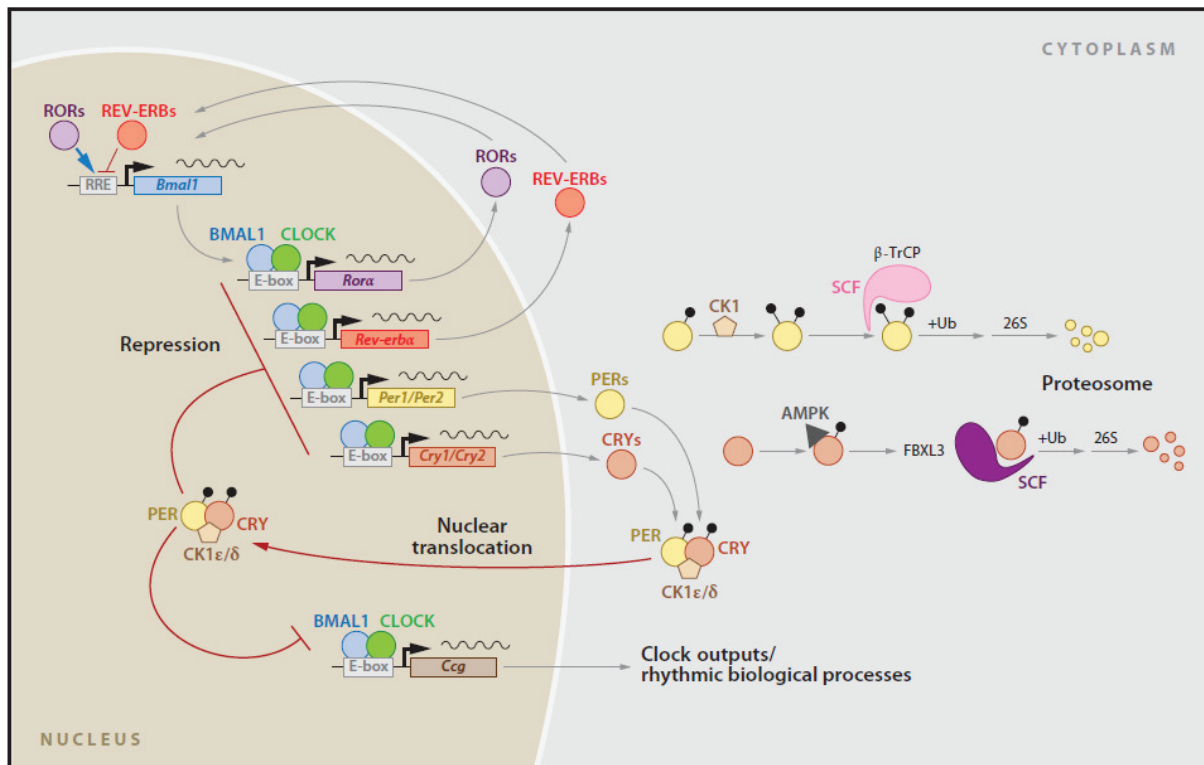


Figure 3: The molecular mechanism of the circadian clock in mammals. Constituting the core circadian clock is an autoregulatory transcriptional feedback loop involving the activators CLOCK and BMAL1 and their target genes *Per1*, *Per2*, *Cry1*, and *Cry2*, whose gene products form a negative-feedback repressor complex. In addition to this core transcriptional feedback loop, other feedback loops are also driven by CLOCK:BMAL1. One feedback loop involving *Rev-erba* and *Rora* that represses *Bmal1* transcription leads to an antiphase oscillation in *Bmal1* gene expression. CLOCK:BMAL1 also regulates many downstream target genes known as clock-controlled genes (*Ccg*). At a post-transcriptional level, the stability of the PER and CRY proteins is regulated by SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complexes involving β -TrCP and FBXL3, respectively. The kinases, casein kinase 1 ϵ/δ (CK1 ϵ/δ) and AMP kinase (AMPK), phosphorylate the PER and CRY proteins, respectively, to promote polyubiquitination by their respective E3 ubiquitin ligase complexes, which in turn tag the PER and CRY proteins for degradation by the 26S proteasome complex (From Mohawk et al., 2012).

Recent advances in genetic, molecular and biochemical approaches revealed that in multicellular organisms such as mammals, physiological circadian oscillations were ubiquitous and that throughout the body. Virtually, there might be as many clocks as there are cells (Yamazaki et al., 2000; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Yoo et

al., 2004). In this perspective, SCN is regarded as a master synchronizer which orchestrates and synchronizes a multitude of peripheral sub-network oscillators into an internally coherent timing system (Yoo et al., 2004; Welsh et al., 2004; Liu AC et al., 2007a; Dibner et al., 2010). To achieve this goal, SCN, after integrating photic informations (which is the primary environmental synchronizer of circadian rhythms in mammals) received through retinohypothalamic tract, uses both neural and a multitude of humoral signals (Kramer et al., 2001; Saper et al., 2005a; Antle and Silver, 2005; Kraves and Weitz, 2006). In addition to these signals, other indirect systemic cues including body temperature, hormonal metabolites and feeding-fasting cycles participate in the synchronization process of the entire circadian system (Dibner et al., 2010). The combination of proper functioning of these direct and indirect factors is responsible for proper timing of the entire circadian system.

2-Sleep and circadian abnormalities in PD patients:

The most obvious circadian abnormalities that affect Parkinson's patients include disruption in both the qualitative and quantitative characteristics of sleep/wake behavior (De Cock et al., 2008). The incidence of sleep disorders in these patients have been reported to be as high as 74-81% (Nausieda et al., 1982; Van Hilten JJ et al., 1993). Those disturbances are experienced mainly as difficulties in falling asleep and maintaining sleep for periods of time (or insomnia), excessive daytime sleepiness and REM behavior disorder (RBD). A detailed description of sleep/wake abnormalities in PD is beyond the scope of this introduction and, interested readers are referred to recent excellent reviews on the topic (De Cock et al., 2008; Arnulf and Leu-Semenescu, 2009; Wulff et al., 2010; Iranzo, 2011; Zhong et al., 2011; Bruin et al., 2012). Besides their high burden on the quality of life mainly in the late stages, strong evidence suggests that some sleep disturbances along side with some other NMS (constipation, olfactory deficit and depression) predate the development of the classical motor signs by more than a decade (Chaudhuri et al., 2006; Chaudhuri and Schapira 2009). This characteristic has prompted the still unconfirmed idea of exploiting these early symptoms as biomarkers in a battery of tests to identify the population at risk of developing PD. The development of such a strategy should open possibilities for the assessment of new therapeutic strategies aimed at preventing or at least delaying the progression of the disease (DeKosky and Marek, 2003; Eller and Williams, 2009; Sherer, 2011; Haas et al., 2012).

The underlying pathophysiological mechanisms of sleep/wake abnormalities are multifactorial. Among the causal factors, neurodegenerative aspects intrinsic to the disease itself have been evoked. These include widespread neurodegeneration within the brainstem, the hypothalamus and the basal forebrain (BF), areas known to be part of the circuitry controlling sleep/wake behavior (**See section 6**). The degenerated systems include multiple wake-promoting neural groups such as noradrenergic neurons in the locus coeruleus (Jellinger, 1991, 2001; Zarow et al., 2003; Fulceri F et al., 2007), cholinergic neurons both in pedunculus pontin nucleus (PPT) and BF (Hirsch et al., 1987), serotonergic neurons in the raphe nucleus (Jellinger, 1991, 2001), dopaminergic neurons in the substantia nigra (SN), ventral tegmental area (VTA) and vPAG (Carlsson, 1972) and the Orexinergic neurons in the lateral hypothalamus (Fronczec et al., 2007, Thannickal et al., 2007). Those abnormalities may be considered as direct pathophysiological correlates of excessive daytime sleepiness and hypersomnia reported in PD (Arnulf and Leu-Semenescu, 2009). In addition, and according to Braak and colleagues (Braak H et al., 2004), the deposition of α -synuclein positive lewy neurite and lewy bodies (a hallmark of pathological features of PD) during the prodromal stage 1 and 2 in lower brainstem nuclei (including PPT and subcoeruleal nucleus) may be the pathological basis of RBD. (**Figure 4**)

Antiparkinsonian therapy constitutes another factor that may induce a disruption of the architecture of sleep/wake cycle. A one year follow-up study have shown for example, that de novo diagnosed parkinson's patients become more sleepy after 1 year treatment with levodopa or a dopamine agonist (Fabbrini et al., 2003). Sleep attacks have also been described in patients using the new DA agonist drugs (pramipexole and ropinirole, Frucht et al., 1999). In patients using DA agonist, a twice higher risk of sleep attacks was found compared to those using L-Dopa alone (Avorn et al., 2005). A suppression of REM sleep and a lengthening of its latency have been attributed also to L-Dopa treatment in both healthy humans (Gillin et al., 1973; Boivin and Montplaisir, 1991) and PD patients (Wetter et al., 2000). The mechanisms of sleep/wake disruption by dopaminergic medication is a complex one, and appear to be dependent on the doses, the time of administration and the dopaminergic receptors targeted. For review see (Monti and Monti, 2007).

Motor and some NMS contribute also as a secondary causes to the disorganization of the circadian pattern of sleep/wake behavior. Re-emergence of resting tremor during microarousals, body movements or sleep state changes (mainly from NREM to REM) may contribute to the fragmentation of sleep (Stern et al., 1968). Restless legs syndrome is another

motor disorder occurring during sleep in up to 30% of PD patients (ref) which was associated with frequent arousal during sleep. In the category of NMSs, nocturia causing bed-wetting and sleep disordered breathing are also important and frequently overlooked causes of sleep/wake behavior morbidity in PD (Chaudhuri et al., 2006).

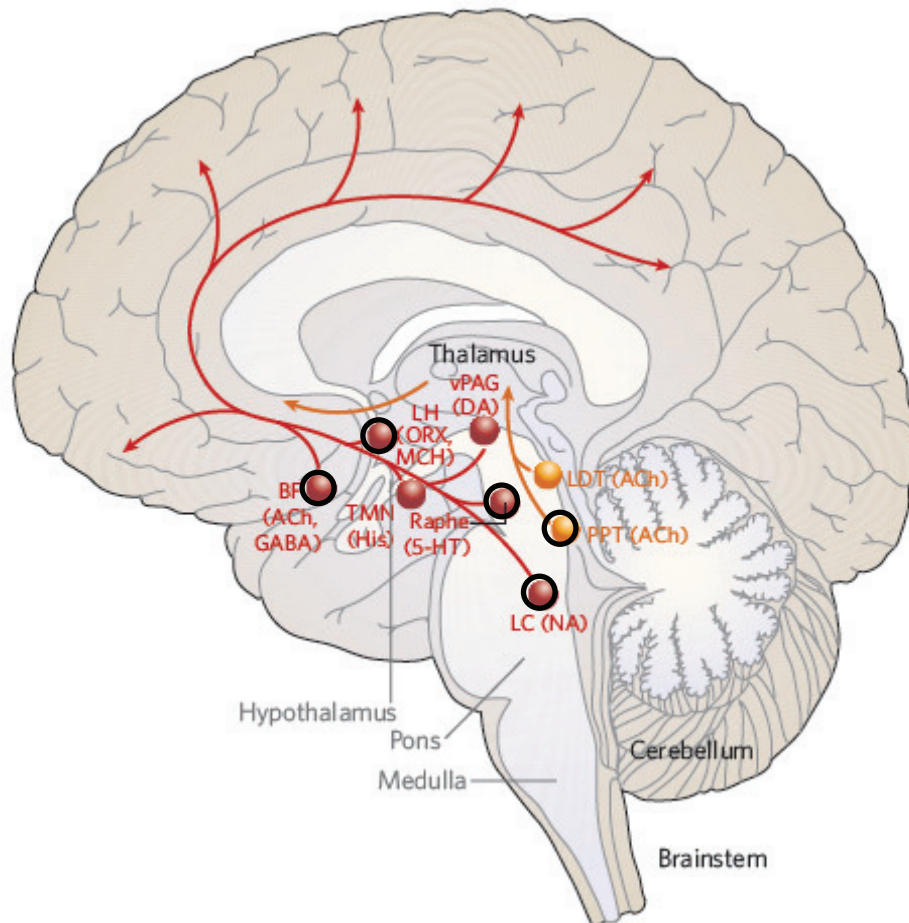


Figure 4: A schematic drawing showing some key components of the ascending arousal system. A major input to the relay and reticular nuclei of the thalamus (yellow pathway) originates from cholinergic (ACh) cell groups in the upper pons, the pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT). These inputs facilitate thalamocortical transmission. A second pathway (red) activates the cerebral cortex to facilitate the processing of inputs from the thalamus. This arises from neurons in the monoaminergic cell groups, including the tuberomammillary nucleus (TMN) containing histamine (His), the A10 cell group containing dopamine (DA), the dorsal and median raphe nuclei containing serotonin (5-HT), and the locus coeruleus (LC) containing noradrenaline (NA). This pathway also receives contributions from peptidergic neurons in the lateral hypothalamus (LHA) containing orexin (ORX) or melanin-concentrating hormone (MCH), and from basal forebrain (BF) neurons that contain aminobutyric acid (GABA) or ACh. Note that all of these ascending pathways traverse the region at the junction of the brainstem and forebrain where von Economo noted that lesions caused profound sleepiness. The circled nuclei constitute sites of neurodegeneration in PD (Modified from Saper et al., 2005a).

As previously mentioned, the dual control of sleep/wake behavior by homeostatic and circadian processes (**See section 6**) implies that any time-dependent sleep/wake dysregulation in PD may arise also as a consequence of a dysfunctional circadian system. Indeed, studies in PD have described daily disturbances at the behavioral, physiological and molecular levels. Behaviorally, by using actigraphy as an estimate to the diurnal motor activity rhythmicity, Van Hilten et al showed an overall flattened diurnal pattern in the most severely affected patients in contrast to the existence of a significant diurnal modulation of activity in healthy controls with a late morning peak (Van Hilten et al., 1993). These results have been substantiated and expanded by three recent studies (Whitehead et al., 2008; Santiago et al., 2010; Niwa et al., 2011). The first (Whitehead et al., 2008) used non parametric circadian rhythm analysis (NPCRA) to assess daily rhythm using rest-activity data derived from actigraphy. This study found an increased intra-daily variability within each 24 hours period in PD patients compared to controls. That is a much more fragmented pattern of activity with frequent transitions from high to low activity bouts. Lower mean amplitude of activity was also reported. The authors interpreted those results as a consequence of an exacerbation of normal ageing processes on circadian system in PD patients. The other two studies confirmed this irregularity in the activity-rest rhythm by showing an increased Day-to-Day variability in initiating bedtimes, waking up and immobility onset and offset times (Santiago et al., 2010) and a decreased nycthemeral rest-activity pattern with disease severity in PD compared to controls (Niwa et al., 2011).

The evidence that the circadian system is affected in PD is often based on alteration of SCN-controlled physiological functions that are also compelling. In addition to basal hypercortisolemia, the 24 hrs cortisol secretory profile was found to be flatter in PD patients compared to Alzheimer's disease patients and control subjects (Hartmann et al., 1997). The nycthemeral pattern of melatonin secretion, another hormonal rhythm under the control of the SCN, was also affected in both its amplitude and its phase of entrainment in relation to LD condition (Bordet et al., 2003). PD patients also suffer from an internal desynchronization of a variety of rhythms in peripheral organs. Daily abnormalities in the cardiovascular control of blood pressure in PD are well documented. Reversal or loss of nycthemeral rhythm of blood pressure (Ejaz et al., 2006), post prandial hypotension (Brevetti et al., 1990), loss of nycthemeral heart rate variability and loss of the sympathetic morning peak were reported in multiple studies using 24 hrs ambulatory blood pressure monitoring that provides a comprehensive information about the rhythmic pattern of blood pressure (Pursiainen et al.,

2002; Schmidt et al., 2009). Disturbed rhythms in renal fluid handling are also among the commonest NMS in PD. The daily rhythm of urine excretion in healthy individuals is characterized by a maximal urine flow in the early afternoon and a minimal around midnight (Hineno et al., 1994). In contrast, Parkinsonian patients excreted a smaller volume of their urine (43%) during the daytime than during the night time (57%). Finally, daily disturbances and fluctuations of contrast sensitivity have been reported in PD compared to unchanged sensitivity through the day in age-matched controls (Struck et al., 1990).

At the molecular level, sparse evidence is available regarding a deregulation of the molecular clock. Recently, Cai et al. (Cai et al., 2010) reported a selective dampening of the expression of BMAL1 and BMAL2 in total leukocytes in 17 PD patients. The expression pattern of other canonical clock genes (Per1, Clock and Dec1) was not affected. Although the sampling span of those two studies was limited to 12 hrs, their results nevertheless suggest that the molecular machinery of the peripheral clock is altered, and could therefore account for a variety of behavioral and physiological desynchronise in PD (see above). The contribution of the circadian mechanisms in sleep disturbances in PD was also explored recently by assessing the association between a single nucleotide polymorphism of the thyrotroph embryonic factor (Tef) gene, a circadian clock-controlled gene, and sleep abnormalities in patients with PD (Ding et al., 2011; Hua P et al., 2012). This polymorphism was found to be an independent risk factor in the severity of nocturnal sleep problems in PD. Besides this finding, nothing is known about the association between clock gene variants and various symptom phenotypes of circadian and sleep disturbances in PD. Other studies on this subject are warranted if we aim to clarify the role of clock genes on the general clinical picture of PD.

Collectively, the above data show that the circadian system is affected in PD and could constitute a potential cause of many non-motor symptoms in PD and contribute to a decrease in the quality of life PD patients.

Any circadian rhythm is triggered as a consequence of an internal coherent synchronization of a hierarchical inter-related multi-oscillator network (**Figure 1**). The alignment of this rhythm with the light-dark cycle is mediated by photic inputs from the retina to the central SCN. By using both neuronal and humoral signals, SCN neurons transduce the rhythmic signal to many regions (Kramer et al., 2001; Saper et al., 2005a; Antle and Silver, 2005; Kraves and Weitz, 2006). These output pathways are responsible for an appropriate phase relationship between various hormonal, physiological and behavioral rhythms and between all of these and the

SCN. This network organisation of the circadian system implies that any circadian disorder may arise as a consequence of a dysfunction in one or multiple hubs of the network or in the connecting pathways of the network. The dissection of the status of distinct circuit elements of the circadian system in PD and its evolution over disease progression constitute the first need for the development of any therapeutic strategy aiming to reverse sleep and circadian disturbance in PD.

3-Dissection of the circadian system in PD; Insights from De novo untreated patients:

Pathologically, PD is characterized by a deposition of α -synuclein immunopositive lewy neurites and lewy bodies in multiple brain areas with a very specific pattern (Braak H et al., 2004) starting in lower brainstem nuclei in earlier preclinical stages and ascending gradually to cortical areas in later advanced stages (Figure 5). Although multiple hypothalamic nuclei are affected by the same pathology, the SCN is relatively spared (Langston and Forno, 1978) suggesting that the central pacemaker is not affected by the same neurodegenerative process that affects other centers in PD brain. Indeed, previous studies investigating melatonin secretion in drug naïve PD patients have demonstrated a daily profile similar to that of age-matched controls (Fertl et al., 1993; Bordet et al., 2003). Thus, it appears from those results that up to the emergence of motor symptoms in PD patients, the SCN is still functioning normally. By comparison, in Alzheimer's disease (AD) where the pathological deposition of β -amyloid neuritic plaques and hyperphosphorylated tau neurofibrillary tangles have been observed in the SCN (Stopa et al., 1999), alterations in the 24-hrs melatonin profile have been detected during the preclinical phase suggesting an early pathophysiological contribution of the central synchronizer of the circadian system to the disease (Wu et al., 2003).

Recently, and confirming the conclusions of Bordet and Fertl, Aziz et al investigated the diurnal secretion profiles of multiple physiological output rhythms in de novo untreated PD patients (Growth hormone, thyroid-stimulating hormone, prolactin, leptin, adiponectin and resistin). The authors found no indications of altered diurnal rhythmicity in all parameters investigated in PD patients compared to controls (Aziz et al., 2011). Taken together, all the above mentioned evidence shows that up to the early pre-treatment stages of PD, SCN dysfunction does not constitute a primary cause of rhythmic abnormalities in PD and hence these abnormalities may emerge as secondary consequences of multiple factors acting during advanced stages on the SCN or other elements of the circadian network system.

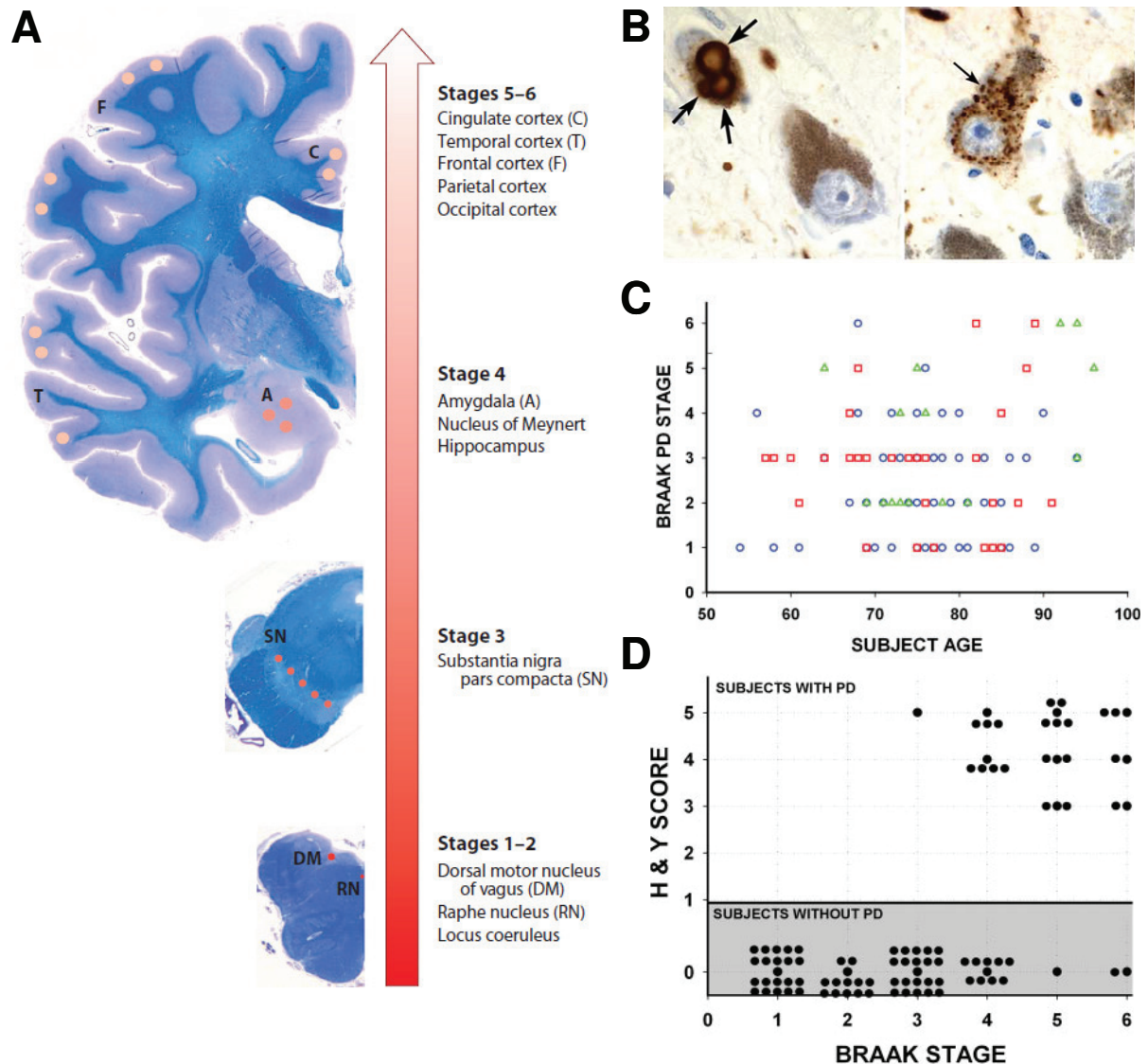


Figure 5: Progression of Parkinson's disease (PD) pathology according to Braak et al (Braak H et al., 2004). (A) Schematic outlining the major stages of PD pathology, as proposed by Braak et al., In stages 1–2, Lewy neurites and Lewy bodies (B) are found within the medulla and pons. Only in stage 3 does aggregated α -synuclein affect the substantia nigra. Later stages (4–6) involve the supratentorial compartment in a graded fashion, which ultimately leads to substantial neocortical pathology in stage 6. The accuracy of the model is still highly debated. For example, figure C depicts the relationship between subject age at time of death and Braak Parkinson's disease (PD) stage in 116 individuals without dementia or parkinsonism. The data presented are from Braak et al., (2004) (circles; $n=67$), Parkkinen et al., (2005) (squares; $n=32$), and Bloch et al., (2006) (triangles; $n=17$). There is no relation between age at death and Braak stage ($r=0.09$, not significant). Braak stages 1 to 6 synuclein pathology is observed in neurologically unimpaired individuals who live decades beyond the average life expectancy at birth for industrialized nations. (D) Relationship between Braak Parkinson's disease (PD) stage and Hoehn and Yahr (H&Y) PD score at the time of death in 96 individuals. The 68 individuals with a Hoehn and Yahr score of zero are the same individuals as shown in C, with the single exception that Braak Case 105, with a diagnosis of Alzheimer's disease, is excluded in C. Among 29 patients with PD (Hoehn and Yahr score=1), there is no relation between

Hoehn and Yahr score and Braak stage ($r=0.2$, not significant). Data appear to be clustered into two populations: those with PD and those without.

The first potential factor of these was reported by [Fertl et al., \(1993\)](#) that compared daily melatonin secretion in De novo unmedicated, L-Dopa receiving PD patients and age-matched controls. The authors found that the nycthemeral secretion patterns of melatonin were very similar between the 3 groups except for a phase advance of the nocturnal melatonin elevation in the treated group. These results lead the authors to conclude that the misalignment of melatonin rhythm in L-Dopa receiving Parkinson's patients was possibly due to a central nervous dopaminergic effect elicited by L-Dopa administration and not inherent to PD per se. Although the cerebral structure involved in the phase shift induced by increased DA neurotransmission is unknown, the pineal gland is unlikely to be responsible given its peripheral localisation outside the blood-brain barrier ([Møller et al., 1978](#)) and that peripheral effects of L-Dopa are largely blocked by co-administration of benserazide with L-Dopa ([Wooten, 1987](#)). In addition pineal histopathological structure was found to be unremarkable in parkinsonian patients without pathological intracytoplasmatic lewy bodies ([Critchley et al., 1991](#)). All these results suggest that the output structure (Pineal gland) of the neural circuitry involved in the circadian control of melatonin secretion is unaffected both by disease processes and L-Dopa treatment and thus not responsible for a disturbed melatonin rhythm in treated PD patients. Recently, studies using DNA microarray technology have identified expression of D1 ([Weiner DM et al., 1991](#)), D2 receptors ([Panda S et al., 2002](#)) and $\alpha 1$ adrenergic receptors ([Weiland and Wise, 1987](#)) in mouse SCN. Furthermore, pharmacological administration of haloperidol, a non-selective D2 receptor antagonist induced mPer1 gene expression both in vivo and in cultured SCN cells ([Viyoch et al., 2005](#)). Therefore a plausible mechanism of L-Dopa inducing phase shift of melatonin rhythm would be a direct action of the by-products of in situ L-Dopa conversion (Dopamine and/or norepinephrine) on SCN neurons.

Because melatonin production occurs during the dark phase and is acutely suppressed by light, it is considered as an endogenous signal of darkness ([Lewy et al., 2006](#)). Melatonin exerts many of its physiological actions through activation of MT1 and MT2 receptors that are expressed widely in the central nervous system (SCN, hippocampus, cerebellar cortex, prefrontal cortex, basal ganglia, SN, VTA, nucleus acumbens and retina) and in the majority of peripheral organs (blood vessels, liver, Kidney and bladder, ovary, testis, prostate,

gastrointestinal tract, mammary gland, skin and the immune system) (Pandi-Perumal et al., 2008). Through its action on those receptors, melatonin plays a major role in the regulation and synchronization of the phase of circadian rhythms (reviewed in Pandi-Perumal et al., 2008). Indeed melatonin administration has been shown to shift circadian rhythms in both rodents (Redman et al., 1983) and humans (Arendt and Skene 2005; Arendt 2006; Cardinali et al., 2006) and when administered daily during normal bedtime hour, it entrains free running circadian rhythms of most blind subjects and improves their sleep-wake behavior (Arendt and Skene 2005; Lockley et al., 1995; Sack et al., 2000). Additionally, melatonin has been used successfully in treating various circadian rhythm disorders such as delayed sleep phase syndrome (Dahlitz et al., 1991), shift-work sleep disorders (Folkard et al., 1993; Burgess et al., 2002; Srinivasan et al., 2006), blindness or pathophysiological states of delayed/advanced sleep phase syndromes (Hagan and Oakley, 1995; Zisapel, 2001; Arendt and Skene 2005; Lewy et al., 2006; Cardinali et al., 2006). Given this critical role of melatonin in internal rhythmic synchronization, the disruption of melatonin rhythm in PD by L-Dopa treatment may constitute another secondary factor exacerbating and generalizing the internal desynchrony in central and peripheral physiological functions in PD patients.

Non motor symptoms and their inevitable worsening with disease progression have also a significant effect on the quality and the organisation of circadian parameters. Although many of these NMS have the potential to affect the circadian system in PD, only few of them have been studied in relation to nycthemeral abnormalities in PD. By comparing the daily organisation of rest-activity rhythm in hallucinating vs non hallucinating PD patients, Whitehead et al showed that independently of many clinical factors, such as cognitive impairment, L-Dopa treatment and severity of motor fluctuations, hallucinating patients showed lower inter-daily stability, greater activity levels at night time and a lower amplitude than their non-hallucinating counterparts (Whitehead et al., 2008). The pattern of rest-activity behavior was less predictable across days in hallucinators compared to non hallucinating patients (Whitehead et al., 2008). Hallucinations are not the only neuropsychiatric symptom in PD. Dementia also occurs in PD with incidence of about six times higher than that in healthy individuals (Emre, 2003). Dementia and cognitive deteriorations are an ongoing progressive process in the course of PD (Maetzler et al., 2009). Therefore, their effects on circadian organisation of sleep-wake behavior are likely to be substantial in advanced stages compared to early stages. In PD, there is no studies that investigated the independent effect of cognitive problems on the circadian system; However, a recent correlational study investigating the

relationship between cognitive performance and characteristics of sleep-wake rhythm assessed by NPCRA in elderly population showed a significant positive correlation between the two, implying that the deterioration of cognitive abilities may be a predictor of more fragmentation of rest-activity rhythm (Oosterman et al., 2009; Schlosser Covell et al., 2012). Depression and levodopa-related motor complications (LDRMCs) have also been shown to influence the nycthemeral variation of, respectively, core body temperature and melatonin secretion pattern in PD patients (Bordet et al., 2003; Suzuki K et al., 2007). PD patients with depression showed lower amplitude of core body temperature and higher minimum rectal temperature related to those of patients without depression (Suzuki K et al., 2007). Similarly, the daytime area under the curve (AUC) of melatonin secretion rhythm was significantly increased in patients with LDRMCs leading to a decreased night time AUC/ daytime AUC ratio of melatonin secretion (Bordet et al., 2003). Finally, the disruption of sleep-wake behavior as a non motor symptom is a significant potential factor with clear negative effects on the circadian system in PD. As detailed above, sleep is highly deteriorated in both its quality and quantity. Additionally, several studies have assessed the progression of general aspects of sleep in PD over many years of follow-up and showed an increase of sleep-related symptoms prevalence with disease duration (Maetzler et al., 2009).

In recent years, a number of studies in both humans and animals have established the reciprocal interactions that relate the sleep and the circadian systems. Scheduling of sleep can synchronize the circadian rhythms of some blind persons (Klerman et al., 1998). Sleep deprivation in humans in dim light conditions modulates the phase of the melatonin rhythm (Cajochen et al., 2003). Similarly, sleep deprivation without exercise in the Syrian hamster induces a phase shift of circadian rhythms and suppresses expression of immediate-early gene *c-fos* (Antle and Mistlberger, 2000). The most compelling evidence of a feed-back interaction of sleep-wake behavioral state onto the clock has been obtained in the lab of Meijer by simultaneous recording of cortical EEG activity and multi-unit activity from the SCN in the rat (Deboer et al., 2003). This study showed that during NREM sleep with high values of SWA, MUA was low. However, during REM sleep and wakefulness, MUA was high. These associations persisted during selected SWS or REM deprivations, indicating that the EEG correlates of NREM sleep, REM sleep and wakefulness are influencing MUA in the clock. Therefore the disruption of sleep in PD and its worsening during disease progression is likely to later the firing rates of SCN neurons and hence account as a neurophysiological mechanism of circadian disruptions in late stages of PD.

The same mechanism may also account for the secondary influences of motor abnormalities on the circadian system in PD. Recently, the same team of Meijer et al., by using in vivo SCN MUA recordings in combination with video-scored behavioral activity in mice, examined how the SCN electrical activity responded to spontaneous and induced activity (Van Oosterhout et al., 2012). Spontaneous behavioral activity was consistently associated with a decrease in SCN electrical activity. The magnitude of suppression depended on the type of behavior and correlated positively with the intensity of behavioral activity. These results indicate that SCN rhythm amplitude can be boosted by a consolidated and proper timing of behavioral activity. Inversely, and given the same bidirectional influences operate in diurnal animals, fragmented activity and the occurrence of activity during uncommon phases of the circadian cycle like in PD patients would result in lowered SCN amplitude, leading to the disruption of circadian rhythms under the control of the SCN. This hypothesis is supported by the finding that the nycthemeral pattern of melatonin secretion was much more disturbed in PD patients with LDRMCs compared to patients without LDRMCs (Bordet et al., 2003). Notably, behavioral activity has a synchronizing effect on the human circadian clock (Miyazaki et al., 2001; Barger et al., 2004; Atkinson et al., 2007; Yamanaka et al., 2010). In the elderly and in people suffering from Alzheimer's disease or affected disorders, increased daytime exercise leads to improvement of sleep-wake rhythm, mood and performance (Van Someren et al., 1997; Mistlberger and Skene, 2005; McCurry et al., 2005; Youngstedt, 2005; Teri et al., 2003; 2011). Collectively then, the worsening of the motor symptoms over disease progression in PD patients has a great pathological potential to disturb and deepen the amplitude of multiple circadian rhythms.

The complexity of the underlying pathophysiology of circadian problems in PD is strengthened by the interlocking relationship of all these factors that impact on the circadian system. For example sleep disruption is known to dampen melatonin rhythm and elevate circulating cortisol levels (Spiegel et al., 2009). Reversely, increased cortisol secretion promotes sleep fragmentation (Rodenbeck and Hajak, 2001). The same bidirectional relation exists also between sleep disruption and cognitive performances (Oosterman et al., 2009; Havekes et al., 2012). This suggests that non-motor disruptions may lead to a vicious cycles contributing to the augmentation and progression of circadian dysfunctions in PD.

The normal functioning of the SCN during the prodromal and the early symptomatic stages up to the start of dopamine medication implies that any abnormalities of the timing of circadian rhythms during these stages are not due to a dysfunctional central biological clock. Rather

their underlying pathophysiology may rest on or reside in other components outside the circadian system. For example, the well-documented fragmentation of behavioral sleep-wake cycle during the premotor stage of PD would result from the degeneration of Orexinergic neurons in the lateral hypothalamus (Fronczec et al., 2007, Thannickal et al., 2007). These neurons have a crucial role in the consolidation of wakefulness during the appropriate phase of the day (Sakurai, 2007). Similarly, the daily alterations of the cardiovascular control of blood pressure and heart rate variability would result from a dysfunction of sympathetic and parasympathetic defects in the cardiovascular regulation (Pursiainen et al., 2002). Indeed, it's mainly SD1 of the Poincaré, a variable that describes the magnitude of the beat-to-beat variability reflecting vagal modulation of the heart rate and the sympathetic morning peak that lose the circadian modulation; Accordingly, a marked decrease of TH immunoreactivity within nerve fibers in the heart tissue of incidental lewy body disease cases (considered as premotor stage of PD) was observed (Ghebremedhin et al., 2009).

Since the intrinsic period of oscillation of the SCN pacemaker is not exactly 24 hours, its synchronization to the 24 hrs day is an important process that optimises the timing of the body's physiology and behavior with the environment (Pittendrigh, 1960; Berson et al., 2002). This process known as photoentrainment is assured by a non-visual pathway from the retina to the SCN via a monosynaptic pathway called the retinohypothalamic tract (RHT). This tract originates from a small population of a recently discovered photosensitive retinal ganglion cells (ipRGCs). ipRGCs uses Melanopsin as a photopigment to transmit informations about environments irradiance to the SCN (Moore et al., 1995; Berson et al., 2002). By using Melanopsin knockout mice and mice with genetically ablated ipRGCs (Opn4aDTA/aDTA), recent exciting studies have shown that in addition of ipRGCs, rod and cones partially contribute to circadian photoentrainment and PLR (Güler et al., 2008; Altimus et al., 2008). However, ipRGCs constitute the principal link for rod-cone input to this non-image forming retinal functions (Güler et al., 2008).

It has been speculated that the ageing-related increase in crystalline lens light absorption and decrease in pupil area leading to decreased retinal illumination could contribute to the development of insomnia, depression, flattening of hormonal profiles and cognitive difficulties (Turner and Mainster, 2008). Therefore, and given ageing is the only well-established risk factor for developing PD, the same mechanism may account at least partially for sleep and circadian (e.g. melatonin rhythm) disruption in PD. In addition, several studies have documented a reduction in the average thickness in nerve fibre layer of the retina in PD

patients (Inzelberg et al., 2004; Hajee et al., 2009; Cubo et al., 2010; Moschos et al., 2011; Tian et al., 2011; Kirbas et al., 2012; but see Archibald et al., 2011; Tsironi et al., 2012) suggesting that a decrement in ipRGCs may be intrinsic to PD. However, this decrease in retinal nerve fiber layer thickness may arise as a consequence of degeneration of ganglion cells belonging to the photic pathway of the retina. Whether ipRGCs are affected also in PD remains to be elucidated.

Neurochemical evidence for dopamine deficiency in the retina of PD patients has also been reported (Nguyen-Legros, 1988; Harnois and Di Paolo, 1990). This DA deficiency was related to impaired visual acuity and abnormal contrast sensitivity commonly reported in PD patients (reviewed in Archibald et al., 2009) given the finding that contrast sensitivity improves after L-Dopa administration (Bulens et al., 1987; Hutton et al., 1993). Recently, Jackson et al have investigated the overall role of retinal dopamine in vision by using a mouse model in which the expression of tyrosine hydroxylase, the rate-limiting enzyme for DA synthesis, is selectively disrupted in the retina (Jackson et al., 2012). In addition of confirming human data regarding the pivotal role of retinal DA to maintain normal visual acuity (action through D1 receptors), and contrast sensitivity (action through D4 receptors), the study showed that DA deficiency induced a complete loss of circadian regulation of the light adapted ERG. This effect was not a consequence of the stoppage of the retinal circadian clock, rather it was due to either altered coupling of retinal oscillators or altered coupling of oscillators to functional rhythms.

On the basis of all these findings, it is clear that the only organ through which light synchronizes the circadian system is affected by multiple abnormalities in PD. However, unless the non-image forming pathway of the retina is proven to be affected in PD, the contribution of all these abnormalities to circadian dysfunction in PD is tertiary. For example diminished visual acuity and contrast sensitivity were linked to visual hallucinations, impaired cognition and impaired activities of daily living in elderly and PD patients (Glass, 2007; Archibald et al., 2009; Moes and Lombardi, 2009; Diederich et al., 2009). Through the mechanisms discussed above, the emergence of these secondary dysfunctions will impact negatively on the circadian system (**Figure 6**).

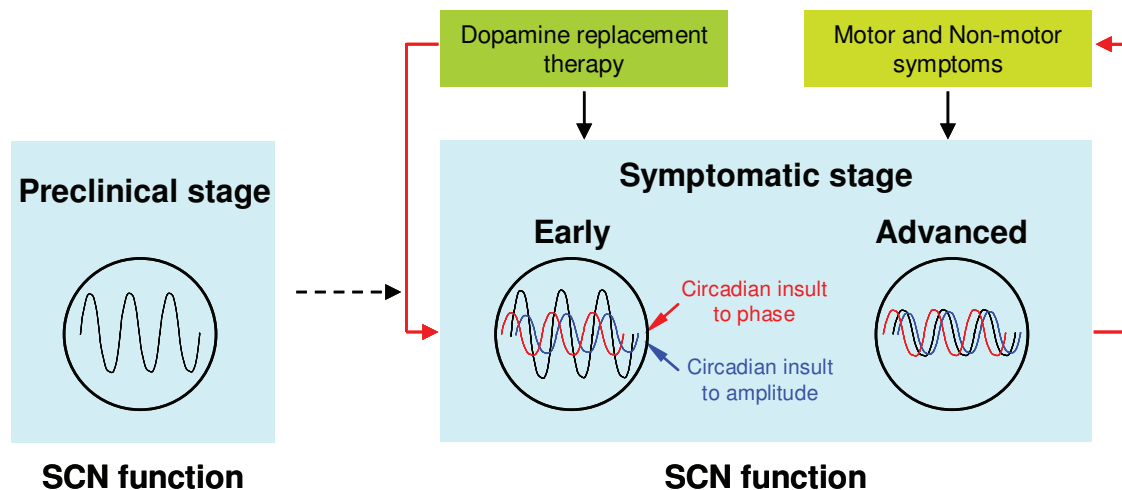


Figure 6: The evolution of the SCN function over ageing in PD. Although timing-related behavioral disturbances occur during preclinical stage of PD, the SCN function seems unaltered. The emergence of early signs of motor disorders and especially the initiation of dopamine medication constitute the first insult to the phase of physiological rhythms. The effect of worsening of both motor and multiple non-motor symptoms over disease progression on the circadian clock will add up on the top of the normal ageing-related insult to the amplitude of clock oscillation in the SCN. As a result, a self-sustained (red arrows) internal desynchrony is generated leading to impairment of the general quality of life in PD patients.

4-The entraining and masking effect of light on behavioral rhythms:

In mammals, a wide variety of physiological and behavioral rhythms are under the control of an endogenous biological clock located in the SCN of the anterior hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972; Edgar et al., 1993). These rhythms are sensitive and could be synchronized to the daily 24h cycle by a variety of environmental cues. In nature, multiple environmental factors oscillate over the daily cycle, including light and darkness, temperature, humidity, food availability, and social cues. Some of these factors can function as zeitgebers (timegiver). The most consistent environmental time cue is the 24h cycle of light and darkness, and almost all circadian rhythms can be entrained to LD cycles. The photoreceptive system leading to this synchronization involves a separate photic pathway; the retinohypothalamic tract (RHT) (Pittendrigh, 1960; Berson et al., 2002). This photic pathway is completely independent from the classical visual projections used for image formation of objects (vision) (Moore et al., 1995). Light information is perceived primarily via intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina, which express the photopigment Melanopsin. This population of retinal ganglion cells is unique in neurochemical phenotype (containing pituitary adenylate cyclase-activating peptide (PACAP) as well as Melanopsin), has the appropriate morphology and project directly to the SCN and multiple other non-image

related brain structure (Hattar S et al., 2006; **Figure 7**). These cells send photic information directly to the SCN via the RHT (**Figure 8**). The monosynaptic RHT fibers terminate in the ventrolateral part of the SCN, directly onto neurons that express vasoactive intestinal polypeptide (VIP). Light stimulation of the retina during the subjective night leads to the release of the neurotransmitters glutamate (Glu) and PACAP at the terminal synapses of the RHT, and the signal is then propagated to the SCN (**Figure 8**) (reviewed in Ecker et al., 2010). This leads to the activation of several signalling pathways that evoke chromatin remodelling and the induction of immediate early genes and clock genes (reviewed in Golombek and Rosenstein, 2010). As a consequence, the circadian clock phase is changed, and this alteration can be readily observed (e.g., a change in the onset of wheel running activity in rodents, reviewed in Antle et al., 2009).

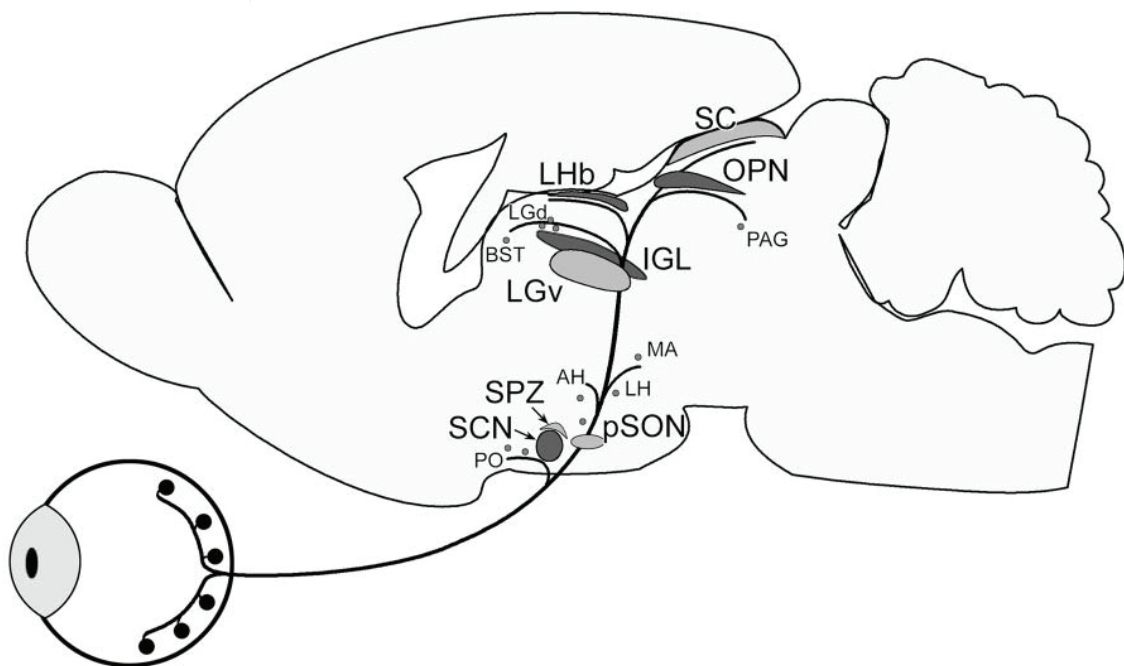


Figure 7: Schematic drawing illustrating nuclear targets of melanopsin RGCs. Principle targets are shown in dark gray and secondary targets in light gray. Minor targets are indicated by small lettering and dots. (BST: bed nucleus of the stria terminalis; IGL: intergeniculate leaflet; LGd: lateral geniculate nucleus, dorsal division; LGv: lateral geniculate nucleus, ventral division; LH: lateral hypothalamus; Lhb: lateral habenula; MA: medial amygdaloid nucleus; MHb: medial habenula; OPN: olivary pretectal nucleus; PO: preoptic area; pSON: peri-supraoptic nucleus; SC: superior colliculus; SCN: suprachiasmatic nucleus; SPZ: subparaventricular zone; PAG: periaqueductal gray matter (From Hattar S et al., 2006).

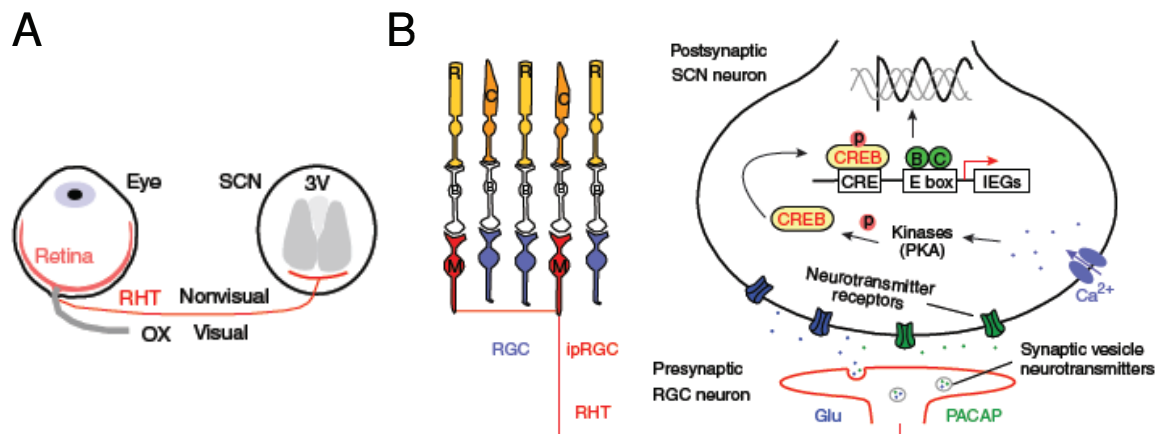


Figure 8: External synchronization of the SCN to light-dark cycles. **(A)** Photic inputs from the retina to the ventral SCN. The SCN is situated in the hypothalamus immediately above the optic chiasm (OX) on either side of the third ventricle (3V). A small population of retinal ganglion cells (RGCs; red) form the retinohypothalamic tract (RHT) in the OX, which projects to the neurons in the ventral SCN. **(B)** Retinal photoreception. Melanopsin (M)-containing RGCs are intrinsically photosensitive, and these ipRGCs also receive light input from the rods (R) and cones (C) of the retina through bipolar (B) and amacrine cells (not shown). Their axons form the RHT and project to the SCN. Photic transmission to the SCN and intracellular signal transduction. Glutamate (Glu) and PACAP are the main neurotransmitters released from the presynaptic RGCs. During the process of light input at night, glutamate and PACAP bind to the respective receptors, resulting in membrane depolarization and an influx of Ca^{2+} in targeted SCN neurons. Within postsynaptic SCN neurons, changes in intracellular Ca^{2+} and cAMP levels lead to activation of multiple kinases and ultimately phosphorylation of CREB; phospho-CREB (CREB-p) acutely activates immediate early genes (IEGs) such as *Per1* via CRE, leading to establishment of a new circadian phase (depicted in black) (From Liu et al., 2007b).

The overriding function of circadian rhythms is to provide an internal estimate of the external local time, i.e., to allow the organism to program its activities to occur at an appropriate phase relationship, or phase angle, to the daily environmental cycle. This process called “Entrainment” constitutes one of the fundamental properties of circadian rhythmicity (Pittendrigh, 1960; Pittendrigh, 1981b). The consequences of entrainment are that the period of the biological rhythm becomes equal on average to that of the entraining stimuli with a stable phase relationship (\approx phase angle) between the entraining and entrained oscillations.

Which aspect of an LD cycle is responsible for this entrainment of biological rhythms? Is it the dawn and dusk spectral quality (i.e., color) of light, the continuous presence of light during the daytime, or some other factor? (Roenneberg and Foster, 1997). Two major classes of models have been proposed to explain the mechanism(s) by which circadian clocks are entrained to environmental cycles: the discrete (also called nonparametric or phasic) and the

continuous (also called parametric or tonic) (Aschoff, 1960; Bruce, 1960; Daan, 2000; 1977; Daan and Aschoff, 2001; Pittendrigh, 1966; 1981a; 1981b; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976b; Swade, 1969). The continuous entrainment model has been based on the observation by Aschoff that the FRP is dependent upon light intensity, and suggests that light has a continuous action on the clock to entrain it to the LD cycle. One mechanism that has been suggested is that the acceleration and deceleration of FRP (i.e., angular velocity) by daily changes in light intensity could allow the circadian pacemaker to continuously adjust its cycle length to that of the environment. The discrete model advances that an entrained circadian pacemaker is in equilibrium with an LD cycle consisting of repetitive light pulses (the zeitgeber). That equilibrium is achieved when each light pulse falls at a phase so as to elicit a phase shift that is equal to the difference between the FRP and the period of the entraining cycle (T). In nature, the zeitgeber is the dawn and dusk transitions, which can be mimicked in the laboratory by brief light pulses. Because the effective action of light is considered to be due essentially to discrete time cues, e.g., in nature at dawn and/or dusk, this mechanism of entrainment has been called the discrete or nonparametric model. The simplicity of this model lies in its excellent predictive properties based on only two elements of information: the FRP and the map of phase-dependent resetting called the phase response curve (PRC) (Aschoff, 1965a; DeCoursey, 1960; Johnson, 1999; Pittendrigh, 1960; Pittendrigh and Minis, 1964).

In addition of its entraining effect on circadian rhythms, light also exerts a profound acute effect on biological overt rhythmic output independently of the effect on the central pacemaker. This phenomenon is called “Masking” and has a role in immediate adjustment of the rhythm to the lighting conditions (Mrosovsky, 1999; Redlin, 2001). For this reason, it is believed to complement and fine-tune the control by the circadian system. The immediate reduction of the melatonin release at night-time and the inhibition of locomotor activity in nocturnal rodents after a light pulse are well-known examples (**Figure 9**). The existence of this direct masking effect of light implies that accurate reading and assessment of the endogenous components of a circadian rhythm should be conducted in constant conditions where environmental stimuli (i. e. light/dark cycle) with potential entraining or/and masking influence on the overt rhythm are absent. In natural conditions, habitat like caves and deep abysses of the ocean would be example of such constant conditions. In the laboratory, constant conditions are modelled by constant darkness or constant light regimes with ad libitum or randomly distributed feeding schedules.

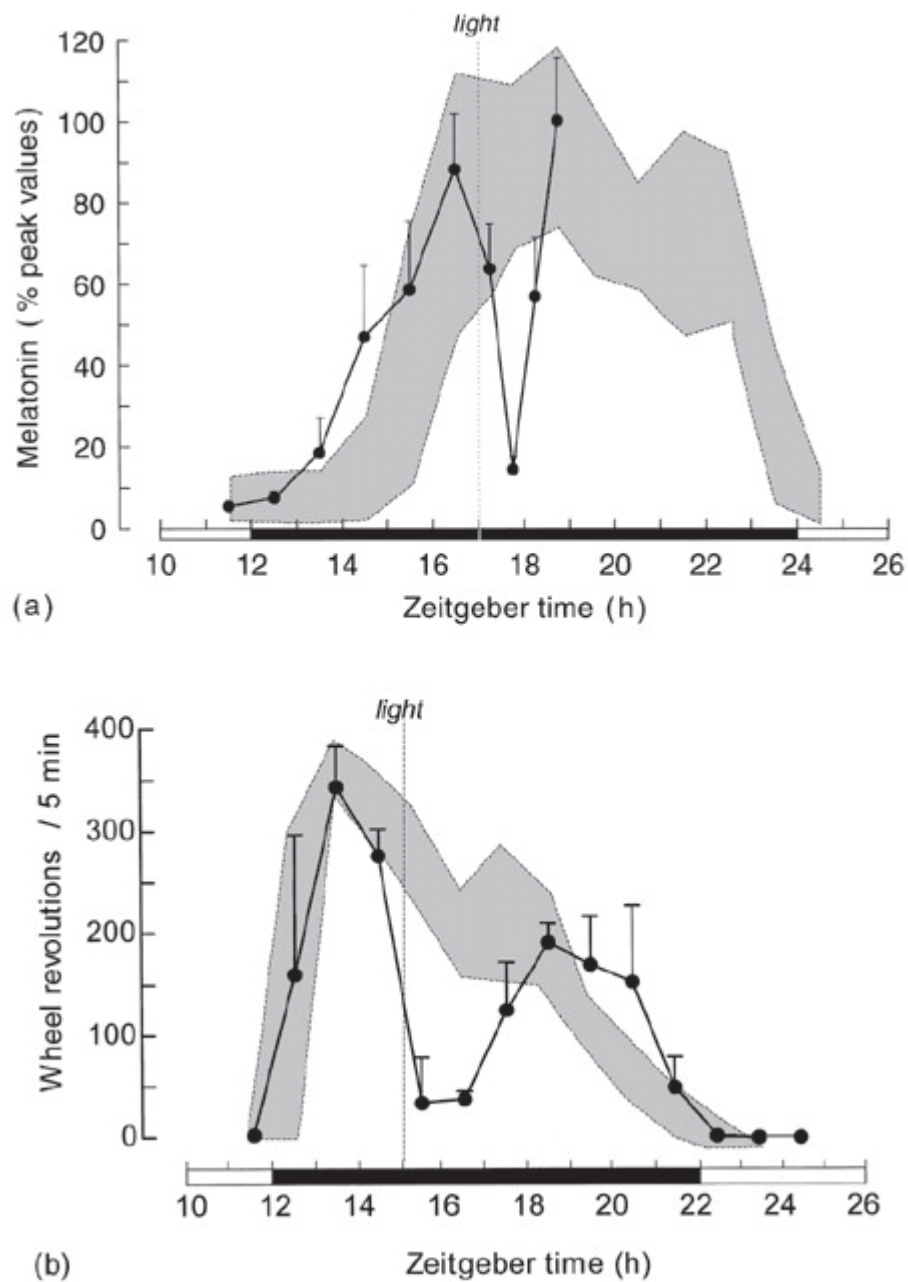


Figure 9: (a) Inhibition of melatonin after a 1-minute light pulse at zeitgeber time 17 (ZT17) in rats. Closed symbols show effect of light pulse on melatonin release measured by transpineal microdialysis ($n=7$). Shaded area indicates the mean data of melatonin concentration in dialysate from rats not exposed to light. (From [Kalsbeek et al., 1999](#)) (b) Inhibition of wheel running after a 15-minute light pulse (ca. 500 lux) at ZT15 in Syrian hamsters. Shaded area indicates wheel running (mean \pm SD) of the same hamsters not exposed to light ($n=12$) (From [Redlin, 2001](#)).

5-MPTP based mice and Non-Human Primate Models:

While investigating the mode of action of reserpine on the central nervous system, Carlsson and colleagues observed that reserpine and haloperidol systemic administration to rodents and rabbits leads to an akinetic and sedative state resembling Parkinsonian symptoms in humans (Carlsson, 2001). This observation was followed by what the authors described as a “Rosetta stone” which would provide the key to the chemical neurotransmission of the brain. The discovery was that the Parkinsonian-like symptoms induced by reserpine were reversed by the administration of L-Dopa. Indeed, in addition of providing the direct evidence for a paradigm shift from electrical to chemical signalling between nerve cells in the brain (Carlsson, 2001). Those observations opened the door to an area where animal models of PD were used to both investigate the pathophysiological of PD and establish the basis of symptomatic treatment 10 years later (Carlsson, 2001).

Multiple toxin-based animal models of PD have been developed using a variety of toxins including; haloperidol (Sanberg, 1980), 6-OHDA (Ungerstedt, 1968; Bevan, 1983; Deshaies et al., 1984), MPTP (Davis et al., 1979; Burns et al., 1983; Langston et al., 1983; 1984; Jenner et al., 1984), Rotenone (Betarbet et al., 2000), Paraquat and Maned (Ascherio et al., 2006; Costello et al., 2009). Several detailed reviews on those models have been recently published (Duty and Jenner, 2011; Bezard et al., 2012; Blesa et al., 2012). Since during my PhD research, we focused on MPTP as a toxin of choice, I will therefore provide an overview of this model, describing its key features and the rationale of its use in both mice and non-human primate.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is the only neurotoxin that produces a syndrome in humans that resembles PD (Davis et al., 1979; Langston et al., 1983). Its discovery goes back to early 1980s, when young drug abusers in California inadvertently self-administered MPTP and rapidly developed a syndrome clinically undistinguishable from advanced PD in humans (Davis et al., 1979; Langston et al., 1983; Kopin and Markey, 1988).

MPTP is a highly lipophilic molecule with an octanol/water partition coefficient of 15.6. As a consequence, after its systemic administration, it readily crosses the blood-brain barrier and accumulates in the brain (Markey et al., 1984). The oxidation of MPTP to 1-methyl-4-phenylpyridinium (MPP⁺) via monoamine oxidase B generates the neurotoxin form, which is, due to its low octanol/water partition coefficient (0.09) (Riachi et al., 1989), trapped in the intracellular compartment. Within the cytosol, MPP⁺ interacts with cytosolic enzymes and

concentrates within the mitochondria where it impairs oxidative phosphorylation and leads to ATP deficit by inhibiting complex I of the mitochondrial electron transport chain (Kopin and Markey, 1988). The other mechanism of cellular toxicity of MPP⁺ is the formation of free radicals (Kopin and Markey, 1988). There are evidences that those two mechanisms instead of killing directly the cells trigger cell-death related molecular pathways which lead to the demise of the intoxicated neurons (Kopin and Markey, 1988; Dauer and Przedborski, 2003). Inside neurons, MPP⁺ can binds also to vesicular monoamine transport-2 (VMAT-2) and be translocated into the lumen of synaptic vesicles (Liu et al., 1992). This sequestration appears to play a neuroprotective role against cellular neurodegeneration since MPTP neurotoxicity is enhanced in VMAT2-deficient mice (Staal and Sonsalla, 2000; Guillot and Miller, 2009). The specificity of MPTP-induced toxicity toward dopaminergic neurons have been explains by the selective uptake of MPP⁺ by dopaminergic neurons through their specific expression of the dopamine transporter (DAT) (Javitch and Snyder, 1984; Javitch et al., 1985). While it's true that MPP⁺ binds with high affinity to DAT (Javitch and Snyder, 1984; Javitch et al., 1985), subsequent studies have revealed that other neurotransmitter systems such as serotonergic and noradrenergic systems are affected (Burns et al., 1983; Gupta et al., 1984 ; Namura et al., 1987; Seniuk et al., 1990) though to a lesser extend compared to dopaminergic system. This involvement of non-dopaminergic system in MPTP toxicity may be explained by the non-specific penetration of MPTP to the entire brain parenchyma and/or the fact that MPP⁺ binds also to other carrier systems such as that for serotonin (Heikkila et al.,1985), norepinephrine (Javitch and Snyder, 1984; Javitch et al., 1985) and histamine neurons (Gasser et al., 2006).

The mouse and the non-human primate model generated by the administration of MPTP are probably the most commonly used models in the Parkinson's disease field. In following chapters, I will discuss in more detail the strengths of those two models and the rational of their use in our studies aimed at revealing the potential contribution of the circadian system impairments in the circadian and sleep abnormalities in PD.

5-1) MPTP Mouse Models of Parkinsonism:

The MPTP mouse model of Parkinsonism is probably the most commonly used Parkinson model today. Several factors contribute to the widespread use of this model including the availability and relatively low cost of mice and the speed with which studies can be completed. Although MPTP-treated mice recapitulate the hallmark neuropathological feature of PD, that is the loss of dopamine neurons in the SN and the subsequent degeneration of

dopamine terminals in the striatum, mice fail to show any behavioral abnormalities. Only a short-term and reversible reduction of spontaneous motor activity, for which the equivalence with clinical symptoms in PD patients is unknown, has been repeatedly reported (Dauer and Przedborski, 2003; Jackson-Lewis and Przedborski, 2008). Nevertheless, the MPTP mouse model has been proven highly valuable as a tool to elucidate the mechanisms of the different molecular pathways of dopaminergic neurons degeneration (Dauer and Przedborski, 2003; Przedborski and Vila, 2003). The vast majority of MPTP studies in mice have used either an acute regime of administration (4ij of 20mg/kg given at 2hrs intervals) or a sub-acute paradigm of 2 injections (20-30mg/kg per day for 5-10 days) (Schneider et al., 2008). Recent refinement of the MPTP regimen of administration which consists of continuous systemic infusion of MPTP using osmotic minipumps has shown a progressive nigral cell loss paralleled with a progressive and persisting behavioral abnormalities.

In the present work, both acute and chronic MPTP treated mouse models of PD were used to investigate the evolution of locomotor, circadian and cognitive abilities over a 1 year study. The rationale of the choice of the mouse as a model in the context of our studies was the possibility of a full and detailed investigation of the molecular clockwork machinery in both the central structure and peripheral organs in the case of the development of daily and/or circadian dysfunctions.

5-2) MPTP primate model:

The discovery that MPTP induces Parkinsonism in human was followed immediately with its systemic administration in non-human primates. A wide range of species were used, including the squirrel monkey (*Saimiri sciureus*) (Langston et al., 1984), cynomolgus monkeys (*Macaca fascicularis*) (Mitchell et al., 1985), rhesus macaque (*Macaca mulatta*) (Burns et al., 1983; Chiueh, 1984b; Markey et al., 1984), Japanese macaque (*Macaca fuscata*) (Crossman et al., 1985; Jenner et al., 1986), bonnet monkey (*Macaca radiata*) (Freed et al., 1988), owl monkey (*Aotus trivirgatus*) (Collins and Neafsey, 1985), baboon (*Papio papio*) (Hantraye et al., 1993; Moerlein et al., 1986), African green monkey (*Cercocebus aethiops*) (Taylor et al., 1994), pigtail macaque (*Macaca nemestrina*) (Chefer et al., 2007), and common marmoset (*Callithrix jacchus*) (Jenner et al., 1986).

Given the multitude of similarities with human beings in physiology, cognitive capabilities, neuroanatomy, social complexity, reproduction and development, NHP serve as an indispensable bridge in neuroscience research (Capitanio and Emborg, 2008) linking the

phylogenetic continuum between rodents and humans. Differences in the anatomical organisation and the physiology of the SNc exist between primates and rodents (Hirsch et al., 1997; Damier et al., 1999b). Such differences are for example the presence of neuromelanin in NHP/humans (McCormack et al., 2004; Purisai et al., 2005) but not in rodents and the different anatomical organisation of the DA system between NHPs and rodents (Björklund and Dunnett, 2007). Such differences in cellular organisation are likely to translate into different mechanisms of cell death after MPTP insult (Hirsch et al., 1997; 1999). Therefore, to approach any pathology (i.p. PD) related to the complex intrinsic organisation of the human SNc (Damier et al., 1999a; 1999b), it is more rational to use a NHP rather than a rodent model (German et al., 1983). Additionally, rodents display little or modest age related changes in nigrostriatal DA (Pasinetti et al., 1992; Dawson et al., 2002) and do not display age-related loss of DA phenotype (TH-immunoreactivity) in nigral cells (McNeill and Koek, 1990). In contrast, monkey aging faithfully replicates the progressive and simultaneous loss of DA phenotype with motor activity worsening seen in human ageing (Irwin et al., 1994; Emborg et al., 1998; Roth et al., 2004; Levy et al., 2005). Taken together, all these features render the NHP a valuable species that fulfils the crucial requirements of any reliable model of a particular pathology; that is the ability to easily correlate the behavioral and anatomical outcome measures and confidently draw inferences concerning the human counterpart of the model.

Indeed, MPTP-treated NHPs present major strengths over MPTP-treated mice including the display of Parkinsonian features that closely resemble the human condition with the classical tetrad of motor symptoms; bradykinesia, postural instability, freezing and rigidity. The robust response to dopaminergic replacement therapy and the manifestation of levodopa-induced motor complications including dyskinesia and wearing-off which was very useful in studies aiming to elucidate the pathophysiology of the complication and to eventually develop novel therapeutic strategies. Furthermore, the pathoelectrophysiological consequences of DA depletion within basal ganglia structures was one of the principal areas of research that has greatly benefited from the use of the MPTP NHP model (Fox and Brotchie, 2010). The large body of data gathered from such studies has given rise to multiple models of parkinsonian pathophysiology (Rubin et al., 2012). The exploration of these finding has triumphed with the development of deep brain stimulation in multiple site of the basal ganglia in attempts to treat Parkinsonism (Wu and Frucht, 2005; Wichmann and DeLong, 2006).

Given all the above discussed advantages and strengths of the MPTP NHP model of PD, we undertook a longitudinal behavioral and physiological assessment of multiple components of the circadian system after MPTP treatment in 2 macaque species.

6-Circadian and Homeostatic regulation of sleep-wake behavior:

Sleep/wake behavior is regulated by a complex neural circuitry involving widespread specific brain structures. Based on the functional role these structures play in the regulation of sleep and wakefulness states, they are subdivided into 3 categories; (i) wake promoting areas; these structures form a large network of highly neurochemically diverse neural population with widespread projection to the entire brain (Datta and Maclean, 2007; Brown et al., 2012). These wake-promoting cell groups are: 1) noradrenergic (NE)-synthesizing cells in the locus coeruleus, 2) serotonin (5-HT) synthesizing cells in the raphe nuclei, 3) acetylcholine (ACh)-synthesizing cells in the pedunclopontine tegmentum and the basal forebrain 4) glutamate (Glu)-synthesizing cells in the midbrain, 5) dopamine (DA)-synthesizing in the substantia nigra compacta and ventral tegmental area, 6) histamine (His)-synthesizing cells in the tuberomammillary nuclei in the posterior-hypothalamus, 7) and hypocretin-synthesizing cells in the lateral hypothalamus. Activation of these systems results in arousal, thus preventing an organism from falling asleep. (ii) Slow-wave sleep promoting area which consist of GABA-containing neurons in the Pre-optic area (POA) of the hypothalamus (Nauta, 1946; McGinty and Serman, 1968; Sherin et al., 1996). These neurons promote sleep by inhibiting many arousal systems towards which they project via monosynaptic connections (Sherin et al., 1998). (iii) REM-sleep promoting network; this network is composed of multiple REM sleep sign-generators, each one is involved in the generation of a particular physiological sign of REM sleep (Datta and Maclean, 2007). The reciprocal-interaction model of MacCarley and Hobson (McCarley and Hobson, 1975) postulates that the activation of these REM sleep sign-generators results from a decrease of aminergic tone and an increase of cholinergic tone in each of these generators leading to the expression of the specific REM sleep sign (McCarley and Hobson, 1975; Pace-Schott and Hobson 2002). Inspired by Cliff Saper's hypothalamic flip-flop switch model for the sleep cycle control (Saper et al., 2001; 2005a; 2005b), REM-NREM sleep switch was proposed by Lu et al. (Lu et al., 2006; Saper et al., 2010), in which a mutually inhibitory relationships between GABAergic neurons in both VIPAG and sublateralodorsal region (SLD) are responsible for transitions between REM and NREM sleep.

Models of sleep regulation (Borbély, 1982; Dijk and Czeisler, 1994; Borbély and Achermann, 1999; Dijk and Lockley, 2002) have emphasized two distinct processes for the regulation of the timing of sleep: a sleep-control mechanism or sleep homeostat (or process S), and a circadian oscillator (or process C) (**Figure 10**). The circadian oscillator is responsible for the tendency to sleep during specific windows of the 24-hour cycle and by opposing process S gates consolidated bouts of sleep and wakefulness. The sleep homeostat is responsible for monitoring and adjusting the duration and intensity of sleep depending on prior amounts of sleep or wakefulness.

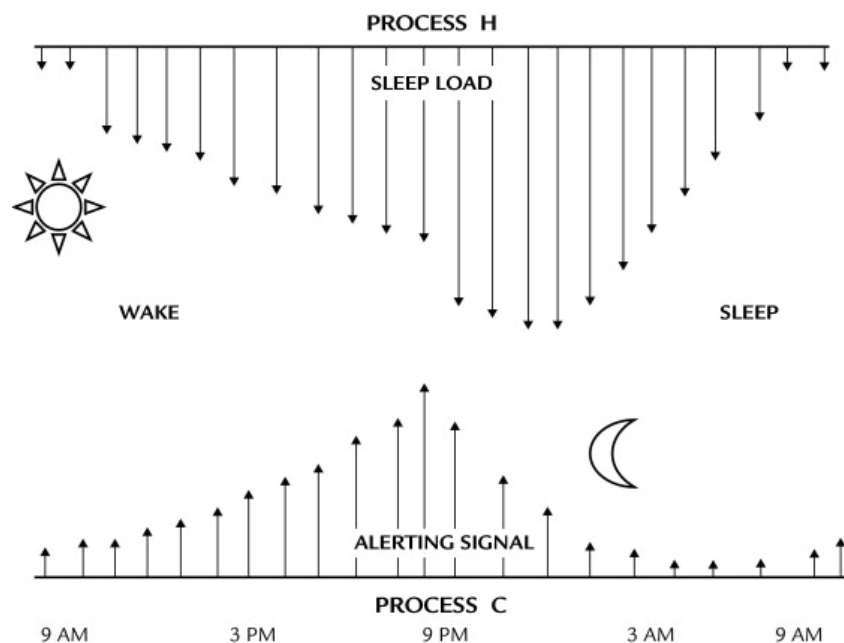


Figure 10: A schematic representation of the “opponent process” model. Alternation of wakefulness and sleep results from the interaction of homeostatic (H) and circadian (C) process. Process H represent sleep load, which is determined by the temporal sequence of behavioral states. Process H increases during waking and declines as sleep ensues. In contrast, Process C is regulated by the suprachiasmatic nucleus, the circadian pacemaker, without regard to behavioral state. The solid line represents the resulting course of sleepiness as a function of time during the day. This figure was created based on data from previously published studies (From Dijk and Edgar, 1999; Rama et al., 2006).

In contrast to our advanced molecular and mechanistic understanding of the circadian system and the potential neural pathways by which the central clock regulates the timing of sleep-wake behavior (Mistlberger, 2005; Takahashi et al., 2008), the brain structures and mechanisms that regulate the homeostatic sleep drive remain unclear. However, there is increasing evidence that a build-up of a substantial number of sleep-initiating metabolic

factors (i.e, adenosine, GABA...etc) in specific brain regions (especially the basal forebrain) provides the molecular basis for sleep propensity during wakefulness (Zeitzer et al., 2006; Wigren et al., 2007; Krueger et al., 2008; Landolt, 2008). The production of these sleep-inducing endogenous metabolites during wakefulness is proportional to the duration and intensity of wakefulness. These metabolites, when reaching a critical level, act by slowing down wake-promoting structures leading to the initiation of sleep behavior (Datta and Maclean, 2007).

Until recently, circadian and homeostasis regulation of sleep-wake cycles were thought to act independently on the neural circuitry of sleep-wake behavior (Yasenkov and Deboer, 2012). In recent years however, a number of studies have provided some evidence that sleep feeds back to the clock. Scheduling of sleep can synchronize the circadian rhythms of some blind persons (Klerman et al., 1998). Sleep deprivation in dim light modulates the phase of the melatonin rhythm, and this modulation is significantly larger than predicted from models for the effect of light on the human circadian pacemaker (Cajochen et al., 2003). Gradual shifts of the timing of sleep within an episode of near darkness leads to small changes in the phase of circadian markers such as body temperature and melatonin (Danilenko et al., 2003). Similarly, sleep deprivation without exercise in the Syrian hamster induces a phase shift and suppresses expression of the immediate early gene c-fos (Antle and Mistlberger, 2000). The most direct evidence that the homeostatic sleep components can modify circadian pacemaker function was obtained from simultaneous recording of EEG activity from the cortex and MUA from the SCN in the rat (Deboer et al., 2003). Sleep states were found to affect activity of SCN neurons with decreasing firing rates during NREM sleep and increasing firing rate during REM sleep and wakefulness. Moreover, sleep loss affects the expression of clock genes in the cerebral cortex by upregulating both of Per1 and Per2 (Wisor et al., 2002; 2008; Franken et al., 2007) and reducing the DNA-binding of clock proteins (BMAL1, CLOCK, and NPAS2) to specific clock genes (Dbp, Per2) (Mongrain et al., 2011). Whether these modulatory effects of homeostatic sleep components on the electrical activity of the SCN also affect the molecular machinery of the clock, as it does in the cerebral cortex, remain to be established. Nevertheless, these data indicate that the sleep and circadian system are interrelated even more closely than was previously recognized and that the interactions between these systems are bidirectional.

Hypothesis and specific objectives of the thesis:

Study 1 (Chapter 2): Several lines of evidence implicate the Dopamine system in the regulation of cognitive, sleep-wake and circadian functions. In this chapter, our objective was to assess the emergence of these non-motor symptoms in an acute and in a chronic MPTP-treated mouse model of Parkinson's disease. The rationale behind using both acute and chronic regimes of MPTP treatments is the relatively high degree of construct and face validity of chronic vs acute MPTP treatment. The objective in this study was to examine whether MPTP treatment contributes to accelerate age-related behavioral changes in a mouse model. Almost all previous studies have only examined the effects of MPTP on a short term basis. For this we followed the daily and circadian rhythms of the two mouse models over nearly one year. We expected to observe, even in the absence of Parkinsonism, an increased deterioration of circadian parameters in treated vs control animals.

Study2 (Chapter 3): As discussed in the introduction, Non Human primates (NHP), given their close phylogeny to humans, represents multiple features that render it the gold standard model to understand specific human neurological pathologies. While numerous studies using animal models of Parkinson's disease (PD) have explored different aspects of these non-motor deficits, there is a lack of information concerning their overall orchestration with respect to the onset of classical clinical motor symptoms. We thus assessed the long term evolution of cognitive, motor and circadian functions after MPTP treatment. Behavioral activity recordings as well as motor and cognitive assessments were carried out continuously and in parallel before, during and for several months following MPTP-treatment for up to 56 weeks. We used chronic low-dose injections of MPTP to induce a Parkinson-like state that includes the development of compensatory mechanisms, thereby providing a model of low grade dopaminergic lesion and sub-threshold clinical states. We hypothesised that this model would replicate at least partially the multifaceted symptomatology of PD patients in terms of cognitive and circadian alterations of rest-activity behavior.

Study 3 (Chapter4): We showed in the second study that indeed, the MPTP-treated NHP models replicate multiple features of circadian disturbances observed in PD patients.

However, in Light/Dark cycles, in which we assessed those parameters, light exerts both acute and chronic influences on the daily pattern of behavior. These conditions prevent an accurate assessment of the characteristics of the circadian system after MPTP treatment. We thus evaluated alterations of circadian rhythms in a light dark cycle and in constant light conditions to assess the output of the endogenous circadian clock. In addition to behaviour, we studied hormonal rhythms of melatonin and cortisol and periodically used *in vivo* PET ($[^{11}\text{C}]\text{-PE2I}$) to evaluate the extent of dopaminergic degeneration. Post-mortem evaluations were performed to verify dopaminergic degeneration in the nigrostriatal system and other neuropeptide/neurotransmitter markers were used to measure possible anatomical alterations in the hypothalamus and in the retina. Using these approaches we aimed to determine whether alterations in rhythmic behavior and physiology in PD result from a deficit in the SCN endogenous clock itself or involve processes downstream from the clock.

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Chapter 2

Title

Lack of long term changes in circadian, locomotor and cognitive functions in acute and chronic MPTP mouse models of Parkinson's disease

Abbreviated title

Circadian and cognitive alterations in MPTP mice

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Abstract

In addition to the hallmark motor disorders in Parkinson's disease (PD) patients, non-motor symptoms have attracted increasing attention. Among the non-motor symptoms, sleep disturbances and cognitive deficits are frequently reported and contribute to a decrease in the quality of life. The pathophysiology of cognitive and sleep-wake abnormalities in PD is poorly understood partially due to the lack of appropriate animal models that fully replicate the entire pathological and behavioral spectrum of the disease. In this study, we undertook a long-term evaluation of circadian, locomotor and cognitive abilities in both acute and chronic MPTP treated mouse models. Activity rhythms and locomotor activity were assayed under light dark cycles, constant darkness or constant light, re-entrainment to shifts of the light dark cycle and a behavioral masking paradigm. Cognitive abilities were assessed using a radial water maze task. Although both acute and chronic treatment regimes induced 70% degeneration of dopaminergic neurons in the substantia nigra, neither circadian, nor cognitive alterations were observed even after nearly one year. During aging, there was a significant decrease of locomotor activity and of several circadian parameters without any exacerbation in MPTP treated animals. These results emphasize the limitations of the MPTP treated mouse as an animal model of non-motor symptoms of PD in addition to the already well documented inadequacy to replicate cardinal motor features of the disease.

Keywords. non-motor symptoms, mouse model, circadian entrainment, cognitive deficits, sleep, chronobiology, masking, radial water maze.

Introduction

Until recently, Parkinson's disease (PD) has been mainly considered as a motor disorder resulting from degeneration of dopamine (DA) synthesizing neurons in the substantia nigra. However, there is currently an increasing awareness that non-motor symptoms including cognitive deficits, depression and sleep-wake disturbances are also present at all stages of disease progression (Chaudhuri et al., 2006; Chaudhuri & Schapira, 2009). Sleep disturbances are a prominent feature and several studies have shown abnormal aspects of sleep in PD such as increased sleep latency, excessive daytime sleepiness, general hypersomnia and fragmented sleep/wake behavior (De Cock et al., 2008). Furthermore, some of those alterations may precede the emergence of motor symptoms of the disease, and inevitably deteriorate over time (Chaudhuri et al., 2006).

Several lines of evidence suggest that the DA system is involved in the regulation of sleep-wake and circadian functions. Dopaminergic D1 receptors are present in the suprachiasmatic nucleus (SCN), the central endogenous oscillator in mammals (Weaver et al., 1992; Ishida et al., 2002). Through D1 and D2 receptors, DA regulates clock gene expression in the dorsal striatum (Imbesi et al., 2009; Hood et al., 2010) and the administration of haloperidol, a D2 receptor antagonist, has been found to increase clock gene expression both *in vivo* and in cultured SCN cells (Viyoch et al., 2005). Lesion of the nigro-striatal system induces disruption of the daily pattern of rest-activity and clock gene expression in the striatum (Gravotta et al., 2011). In mice and non-human primates, DA loss following MPTP treatment is associated with a disruption of sleep efficiency, sleep architecture (Lima et al., 2007; Laloux et al., 2008; Barraud et al., 2009) and daily locomotor activity rhythms (Vezoli et al., 2011). The Dopaminergic system also plays a critical role in cognition and severe memory impairments have been reported in rats following DA depletion (Simon et al., 1986; Stam et al., 1989; Bubser & Schmidt, 1990; Da Cunha et al., 2002). Taken together, these findings provide a strong rationale to study the relationship between the Dopaminergic system and cognitive and circadian alterations associated with Parkinsonism.

Since sleep-wake regulation is governed by both homeostatic and circadian processes (Borbély & Achermann, 1999) alterations of one or both of these components can lead to disruption of daily rhythmic functions. Paradoxically, few studies have addressed the question of alterations of the circadian system in animal models of PD (Rye, 2010; Taylor et al., 2010; Chesselet & Richter, 2011). The majority of studies (Ben & Bruguierolle, 2000; Baier et al., 2006; Taylor et al., 2010) have mainly examined the expression of daily locomotor activity

under a light dark cycle, conditions in which both the entraining and masking effects of light operate to regulate rest-wake cycles of behavior (Mrosovsky, 1999; Redlin, 2001; Johnson et al., 2003). In a day-night cycle, light exerts strong influences on the daily pattern of behavior, often preventing expression of the underlying circadian rhythm controlled by the endogenous clock of the SCN (Johnson et al., 2003, Reppert & Weaver, 2002). Assessment of endogenous circadian rhythmicity requires exposure to constant conditions in which cyclic environmental cues, such as light, temperature and feeding are absent (Johnson et al., 2003). Hence, our current understanding of the possible dysfunction of the circadian timing system in PD remains incomplete.

MPTP is a neurotoxin that causes degeneration of dopaminergic neurons in the substantia nigra and the MPTP mouse model of Parkinsonism is probably the most commonly used model today. However, many controversies have emerged regarding the behavioral outcome of MPTP treatment in mice (Sedelis, 2001) and the reliability of the model to accurately recapitulate PD symptomatology has been questioned (Jackson-Lewis & Przedborski, 2008; Potashkin et al., 2010). Some studies have reported a reduction of spontaneous locomotor activity or some type of limb trembling for which the equivalence with PD bradykinesia and resting tremor is doubtful (Beal, 2001; Plowman & Kleim, 2011) whereas other studies have failed to find any behavioral consequences despite neurodegeneration of the nigrostriatal pathway (reviewed in Jackson-Lewis & Przedborski, 2008). The absence of Parkinsonism in mice is frequently attributed to the acute nature of the insult induced by the bolus injection regimes that are often employed (Beal, 2001). Recent advances in toxin administration methods have shown that chronic delivery of MPTP more reliably reproduces Parkinson-like features in mice, namely behavioral parkinsonism, development of Lewy body-like inclusions and degeneration of dopamine and non-dopamine neurons (Fornai et al., 2005). However, subsequent studies, while confirming the original anatomical findings, have been unable to replicate the behavioral deficits (Thomas et al., 2007; Cleren et al., 2008; Alvarez-Fischer et al., 2008; Fujita et al., 2009; Yang et al., 2009a, 2009b; Gibrat et al., 2009) reinforcing the controversies regarding the MPTP mouse as an appropriate model for behavioral evaluations of PD. A supplementary reason for concern is that behavioral assessments are often conducted hours or at best days after MPTP treatment and the evolution of the behavioral consequences of MPTP treatment with age, considered as a confounding factor in disease progression, is unknown. None of these studies using MPTP in mice examined possible alterations of daily or circadian rhythms.

In the present study, we investigated the evolution of general locomotor abilities, alteration of several aspects of circadian rhythms, entrainment and cognitive abilities over a 1 year study in both acute and chronic MPTP treated mouse models of PD to (1) compare non-motor behavioral outcomes and (2) evaluate the long term effects of the two treatments. The results show no significant alteration in MPTP treated mice compared to controls for the different behavioral assays. The well-known age related changes of locomotor activity and of the circadian timing system were confirmed, but no differences were found between MPTP treated and control groups. These results highlight the limitations of the MPTP mouse as a useful model for the study of circadian and cognitive alterations of PD.

Material and Methods

Animals and Ethics

Wild type males C57BL/6JRj mice (Janvier, France) 28 days old (22-30 gms, n=26) at the beginning of the experiments were used. Mice were kept individually in plexiglass cage in a temperature-controlled room (24-25°C) with free access to food and water.

All experiments with mice were in conformity with principles of animal care (Portaluppi et al., 2010), institutional (ethics committee of the University of Lyon), national (Decret No. 87-848) and European Community Council directives and regulations (86/609/EEC) for animal care, housing, breeding, animal experimentation and welfare. The specific protocols used in this study were approved by the Direction des Services Vétérinaires, approval DDSV #A690290402). Animals were sacrificed with a prior lethal dose of barbiturate to avoid any unnecessary suffering or distress.

MPTP acute and chronic treatments

Mice were treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) according to standard procedures (Jackson-Lewis & Przedborski, 2007) using two paradigms. A first group of mice (n=6) received acute daily intraperitoneal injections of 4 mg/Kg of MPTP (Sigma-Aldrich, France) in sterile saline for four days (Bezard et al., 1997). The second treatment consisted of a chronic infusion of 22 mg/kg/day of MPTP for 28 days using Alzet osmotic minipumps (Alzet model 1004, 0.11µl/h) implanted subcutaneously (n=8). As the volume flow rate for the osmotic pump model 1004 is 2.64µl/day, we used a concentration of

8.33 mg MPTP/kg/ μ l to obtain a dose of 22 mg/kg/day. Since the average weight of the mice was 26 g, the total amount required for each mouse is $8.33 \times 0.026 = 0.216$ mg/ μ l. The total quantity of MPTP in each pump was 0.216 mg/ μ l \times 100 μ l = 21.6 mg. Before surgery, all mice received a subcutaneous injection of 0.5 ml of 0.9%NaCl to insure adequate hydration. Surgical procedures were performed under continuous administration of 2.0% isofluran and an oxygen flow rate of 0.8 L/min. Each mouse was shaved on the implantation site and disinfected by alcohol. The incision site measured approximately 1 cm and the Alzet osmotic mini-pumps were implanted under the skin of the back. After that the skin was sutured. The total surgery duration was < 5 min and no adverse effects of surgery were observed. Control cohorts of mice received equivalent administrations of saline using the same protocols as the acute (n=5) and chronically (n=7) treated MPTP groups. The treatment period began when mice were 152 days of age. The overall time-line of the entire experimental protocol is shown in Fig. S1.

Assessment of behavioral rhythms

For monitoring locomotor activity, mice were housed individually in cages equipped with passive infrared motion captors placed over the cages and a computerized data acquisition system (Circadian Activity Monitoring System, INSERM, France). Activity records were analyzed with the Clocklab software package (Actimetrics, Evanston, IL). To study the effect of MPTP treatment on circadian photoentrainment and masking, we assayed entrainment, endogenous circadian activity, masking and phase shifting using a series of light regimes before and after MPTP treatments.

Activity recording began at 40 days of age. Mice were first exposed to a 12h light:12h dark cycle (12:12 LD, LD1) for 15 days. Mice were then released into constant darkness (DD1) to monitor free-running behavioral rhythms (21 days). Mice were then re-entrained to a 12:12 LD cycle (LD2, 15 days) after which the LD cycle was advanced by 6 hours to measure re-entrainment (16 days, Phase Advance 1). Subsequently, the animals were exposed to a 1hr light:1hr dark masking cycle (Masking 1, 13 days). Mice were then re-entrained to 12:12 LD cycle LD3 (32 days) prior to MPTP treatment.

Implant of the minipumps for chronic MPTP administration and the control group began one day prior to DD2 (43 days) and for acute treated and control groups by ip injections just

after lights on (ZT0-1) during the last 4 days of LD3 for safety handling reasons before being released into DD2.

Following MPTP treatments, the same general paradigm of lighting conditions was repeated: entrainment to 12:12 LD (LD4, 12 days), addition of an inversion of the light cycle (inversion, 13 days), 6 hr phase advance 2 (15 days), 1hr:1hr masking 2 (14 days), re-entrainment to 12:12 LD cycle (LD5, 13 days), addition of a period of constant light exposure (LL, 14 days), re-entrainment to 12:12 LD cycle (LD6, 13 days) and a final period of constant darkness (DD3, 41 days). The total behavioral recording period lasted 290 days, at which time the animals were 330 days old.

After each transition to a new light regime, the first 2-3 days were not included in the analysis in order to avoid transient effects of the previous light regime. In phase shift essays (6 hr phase advances and LD inversion), re-entrainment of the animals to new LD regime was considered complete when the variability of the new activity onsets was equivalent to variability of activity onsets in the previous LD condition.

Cognitive testing, Radial Arm Water Maze

The radial arm maze protocol used in this study was that of Alamed J et al (Alamed et al., 2006; Vorhees & Williams, 2006) and consisted of 3 days of testing, plus 1 day reversal learning testing. The test was conducted when the mice were 345 days old. The test has the spatial complexity and performance measurement simplicity of the dry radial arm maze combined with the rapid learning and strong motivation observed in the Morris water-maze. Similar to Morris water-maze testing, the objective of the task is to learn and remember the location of an escape platform that is submerged in a tank of opaque water. The maze has six arms radiating from an open center and visual cues placed on the walls of the testing room. For each mouse, the platform was consistently placed in the same arm area for the first 3 days (the platform arm was randomized across mice), whereas each mouse began every trial sequentially in a different arm to eliminate “chaining”. Each mouse received 15 trials per day in 3 blocks. During this period, mice were under an LD cycle and were tested during their active phase between ZT14 and ZT18. They were tested in groups of 7-8, such that mouse 1 would receive trial 1, then mouse 2 would receive trial 1, etc., repeated until all 7-8 mice in a group had received the trials of each block. On day 1 alternation between visible and hidden platform were made, on the other days of learning, only the hidden platform for all 15 trials was used. A rest period of 10 to 12 minutes was given for each mouse before its subsequent

trial within a block, with 2 hours in between blocks. If the mouse did not find the platform after 60 seconds, they were gently guided to the platform, where they were left for 30 seconds before being returned to their cage. A third day of testing was added in order to reach the criterion of one or fewer errors in the final blocks of three trials. Reversal learning consisted of a fourth day of testing with the mice receiving 15 additional trials using only a hidden platform in the arm of the maze opposite to that of the training sessions.

Tyrosine hydroxylase immunostaining

At the end of the study (390 days of age) mice were anesthetized using a lethal dose of pentobarbital (100 mg/kg) and perfused intra-cardially with 100 ml of 0.9% saline followed by 100 ml of 4% paraformaldehyde in PBS (pH 7.4). Brains were removed, postfixed overnight in the same fixative, and cryopreserved in 30% sucrose for 48h at 4°C. Forty micrometer-thick free-floating coronal sections were cut on a sliding microtome (Microm, France). The sections were rinsed and blocked in PBS containing 0.3% Triton X-100 (PBST) and 1% of BSA at 4°C for 1hr. Sections were then incubated in 1:1000 anti-tyrosine hydroxylase antibody (Millipore, California, USA), in PBST containing 1% BSA for 72hrs at 4°C and post-rinsed in PBST. Sections were incubated for 2hrs in the secondary antibody (1:200 biotin-labeled F(ab')₂ fragment goat-anti mouse, Vector laboratories, Burlingame, USA) in PBST, washed, and incubated in avidin-biotin-horseradish peroxidase complex (1:200, Sigma-Aldrich, St. Louis, USA) at 22-24°C. The immuno-reactive label was visualized using 3, 3'-diaminobenzidine as the chromogen with 0.5% ammonium nickel sulfate and 0.03% H₂O₂ in tris buffer. Sections received additional rinses, mounted on gelatinized slides, dried and dehydrated in graded ethanol, cleared in xylene and coverslipped with Depex.

Brains from all animals were included in the assay and 5-6 sections for each animal (1 of every 3 sections) from the substantia nigra were analyzed. Quantification was performed using densitometry analysis of immuno-reactive label using computer assisted image analysis (Biocom, Les Ulis, France). Optical density of label was measured bilaterally from digitized images of the substantia nigra. This previously validated method (Rieux et al., 2002; Dkhissi-Benyahya et al., 2000) is based on quantification of the total optical density of the labelling, defined as the integral sum of the surface area of all pixels in the area multiplied by their corresponding optical density value. The optical density of specific signal was calculated by subtracting the background density value determined from an adjacent brain area that did not

contain staining. The background for each animal was systematically measured and a threshold value was determined for detection of label significantly above the background level. The identity of the individual animals was unknown to the observer during the analysis.

Circadian data analysis and statistics

We used Chi-squared periodogram method (Sokolove & Bushell, 1978) to calculate the period of behavioral rhythm which is defined as the time elapsed for one complete oscillation or cycle (the time between two consecutive peaks (or troughs) of a recurring rhythm). The subjective day and the subjective night are defined as the segment of a circadian cycle during the free running state that corresponds to, respectively, the illuminated and the dark segment of the light-dark cycle. The activity onset which is a robust indicator of entrainment to LD cycle, corresponds to the average clock (or circadian) time of activity onset. Finally, Alpha corresponds to the segment of a circadian cycle during which the organism is active.

SigmaStat version 3.1 software package was used. Significant differences between groups were determined using 1-way analysis of variance (ANOVA, with groups as a factor) or a mixed-design analysis of variance (2-way repeated measure analysis of variance, with Groups and age as factors), followed, when significant ($P < 0.05$) by post-hoc Holm-Sidak test to evaluate the differences between groups (the same results were obtained with Bonferroni correction). The level of significance was $P < 0.05$. Results are expressed as means \pm SEM.

Results

Animals were exposed to an identical sequence of lighting protocols before and after MPTP treatment and control procedures to assess the effects of the loss of dopaminergic neurons on cognitive, circadian and locomotor abilities. These two phases will hereafter be referred to as pre-treatment and post-treatment periods. Continuous 24-hr recordings of locomotor activity during wake-rest cycles began at 6 weeks of age and lasted 290 days, at which time the animals were 330 days old. Typical locomotor activity behaviors for control and MPTP treated mice are illustrated in **Fig. 1** for the pre-treatment, treatment and post-treatment periods. Chronic MPTP treatment was administered during the period of constant darkness (DD2) and acute treatment during the last 4 days of LD3 just prior to DD2. This protocol allowed assessment of the changes in the animal's ability to entrain to a light dark (LD) cycle, to re-entrain to a shifted LD cycle, expression of the free-running endogenous

rhythm, masking responses to light and overall locomotor ability in each phase and throughout advancing age.

Entrainment of daily activity to a Light Dark Cycle

To determine the effect of MPTP on the light synchronization of activity under LD conditions, circadian parameters were compared in LD pre-treatment and post-treatment (for LD3 and LD5 as both had the same light regime history before the assays). There were no differences in the total mean activity per day between any of the groups within the pre- and post-treatment periods (**Fig. 2A**). Although there were similar decreases in overall locomotor activity in all groups of mice during the post-compared to pre-treatment period, these decreases were not statistically significant. The mean levels of total activity during the pre-treatment period were 3572 ± 388 and 3803 ± 520 counts/day for acute and chronic control groups respectively and 3820 ± 441 and 3678 ± 413 counts/day for the acute and chronic MPTP treated groups respectively. In the post-treatment period the means of total activity were 2569 ± 564 counts/day (acute control), 3027 ± 565 counts/day (chronic control), 3185 ± 518 counts/day (acute MPTP) and 3184 ± 544 counts/day (chronic MPTP).

Locomotor activity of the mice in an LD cycle was predominantly nocturnal during both pre- and post-treatment stages (**Fig. 2B**). All mice appeared to be slightly less nocturnal during the pre-treatment compared to the post-treatment period. In the pre-treatment period, the ratio of night-day activity was $75 \pm 5.1\%$ (acute control), $72.1 \pm 1.8\%$ (chronic control), $80.6 \pm 1.7\%$ (acute MPTP), and $73.1 \pm 2.5\%$ (chronic MPTP). There was a slight tendency for a consolidation of activity during the dark phase in all groups during the post-treatment period, with nocturnality increasing to 75.8-81.9% depending on the group (**Fig. 2B**). However, the difference was significant only for the chronic MPTP group ($F=8.4$, $P<0.05$).

Under the LD cycles, the mice always displayed robust entrainment of their locomotor activity. During the pre-treatment period, mice began their onset of nocturnal activity with a small phase delay in relation to lights-off between -3.5 ± 10.3 min and -11.2 ± 3.1 min, depending on the group (**Fig. 2C**). During the post-treatment period, mice showed a slight phase advance of activity onsets except for the chronic MPTP group that showed a slight, but non-significant delay (**Fig. 2C**). All groups of mice showed a similar tendency to increase the variability of activity onset during the post-treatment (from 14.2 ± 0.8 to 16.2 ± 1 min)

compared to the pre-treatment period (from 20.7 ± 0.8 to 27.4 ± 3 min) which was significant only for chronic control group ($F=15.1$, $P=0.001$; **Fig. 2D**).

Free running endogenous circadian rhythm in constant conditions

During the treatment period (DD2), subsequent to the insertion of minipumps and acute injections of MPTP, the animals were maintained in constant darkness and circadian parameters were assessed. No significant differences were found between any of the treated and the control groups in the free running periods (acute MPTP= 23.73 ± 0.04 hr, chronic MPTP = 23.74 ± 0.04 hr, acute control= 23.61 ± 0.04 hr, chronic control = 23.71 ± 0.04), the duration of the active period (*alpha*), variability of the onset of activity in DD or the total mean activity per day (data not shown).

When these same parameters in constant darkness were compared pre-treatment (DD1), during treatment (DD2) and post-treatment (DD3), no differences were found in total activity, variability of activity onset or period length between the treated and corresponding control groups during each DD test period (**Fig. 3**). Likewise, there were no differences in *alpha* between the groups for each DD period (data not shown). However, there were significant increases in onset variability in all groups ($F=45.8$, $P<0.01$), significant decreases in total activity ($F=11.7$, $P<0.05$) for most groups (**Fig. 3A-B**) but no differences in period length (**Fig. 3C**).

Locomotor activity under constant light (LL) was only assayed post-treatment (**Fig. 1**). During this assay, the free running period was longer than 24 hrs and was significantly greater ($F=215$, $P<0.001$) in all groups (acute control: 24.60 ± 0.20 hr; chronic control: 24.58 ± 0.08 hr; acute MPTP: 24.6 ± 0.07 hr; chronic MPTP: 24.52 ± 0.04 hr) compared to the free running periods in DD conditions (23.61-23.74hrs, see above). No significant differences in period length in LL were found between acute and chronic MPTP treated mice compared to their corresponding control groups.

Transient effects of MPTP treatment on circadian rhythms

To determine whether there were any acute or transient effects of the neurotoxic MPTP treatment we assessed circadian parameters of locomotor activity during the DD2 period following the acute administrations and during which the chronic treatment was performed. The 40 days of the DD2 phase was divided into 4 blocks of 10 days each (**Fig. S2**). No

differences were found in total activity (**Fig. S2A**), onset variability (**Fig. S2B**) and Alpha (not shown) between control and treated groups. Although there appeared to be a slightly longer period in the acutely treated animals this was not significantly different from controls (**Fig. S2C**).

Re-entrainment to a shifted LD cycle

A 6 hr advance of the LD cycle assay was conducted pre-and post-treatment (phase advance 1 and 2 respectively, shown in **Fig. 1**). Pre-treatment, the animals re-entrained to the new LD cycle within 10 ± 0.4 days (**Fig. 4A**). Post-treatment (**Fig. 4B, C**), there was a significant increase in the number of days required to achieve a complete re-entrainment for both the chronic MPTP group and their corresponding controls (chronic control: 12.3 ± 0.28 days; chronic MPTP: 11.6 ± 0.46 days; $F=24.5$, $P<0.05$) but not for the acute and control groups (acute control: 12.4 ± 0.6 days; acute MPTP: 11.8 ± 0.47 days).

Following MPTP treatments, animals were also subjected to an inversion of the LD cycle (**Fig. 1**). All groups of mice showed similar rates of re-entrainment. A Two way repeated measurement ANOVA was used to compare the mean number of days necessary for each group to reach entrainment following the inversion of the LD schedule. No significant differences were found between MPTP treated animals and the controls (data not shown).

Masking Effects of light in a 1hr light:1hr dark cycle

The inhibitory masking effects of light were assayed using a 1hr:1hr LD cycle pre- and post-treatment (*Masking 1 & 2*, **Fig. 1**). As has been previously shown for masking regimes in rodents (Mrosovsky, 1999; Redlin, 2001), mice maintained a free running circadian rhythm with less activity during the subjective day and higher levels of locomotor activity during subjective night (**Fig. 5**). During the subjective day (CT0-CT12) the 1-hr light periods had no significant effect on the levels of activity both pre-and post treatment in any of the groups (**Fig. 5A**). In contrast, during the active, subjective night phase (CT12-CT24), locomotor activity was consistently suppressed by light with equal efficiency in all control and MPTP treated mice (In pre-treatment assay; activity counts dark vs light: acute control: 1160 ± 149 vs 906 ± 101 , $P<0.05$; chronic control: 1210 ± 180 vs 874.4 ± 138 , $P<0.01$; acute MPTP: 987 ± 160 vs 609 ± 116 , $P<0.01$; chronic MPTP: 1180 ± 111 vs 832 ± 93 , $P<0.01$; $F=32.8$. In post-treatment assay; activity counts dark vs light: acute control: 872 ± 130 vs 584.2 ± 137 ,

$P < 0.05$; chronic control: 1062 ± 164 vs 757 ± 152 , $P < 0.01$; acute MPTP: 1265 ± 148 vs 636 ± 55 , $P < 0.001$; chronic MPTP: 1116 ± 166 vs 704 ± 130 , $P < 0.001$, $F = 71.5$). No significant differences in the ability to mask were found between MPTP mice and their corresponding controls pre and post-treatment (**Fig. 5B**). We also found under this regime a significant lengthening of the free running period in post-treatment compared to pre-treatment assay within all groups ($F = 22.8$, $P < 0.05$) with however, no significant difference between the groups (**Fig. 5C**).

Spatial Learning and Memory

In the version of radial arm water maze used, the goal arm is held constant throughout all trials, whereas a different starting arm is used on successive trials. Animals are required to use visuo-spatial cues to guide their choice and retain the learning over successive trials. Performance is evaluated as the number of incorrect arm entries (errors) and improvement in performance as a reduction of errors during learning. During the training and learning phases (days 1-3), we found no significant differences between control and MPTP treated groups. All mice learned the task with equal performance and eventually learned to attain the escape platform location with no errors by day 3 (**Fig. 6**). When the location of the arm was reversed on day 4, all groups of mice showed an increase of errors on the first block of trials, but rapidly relearned the new location on following trial blocks.

MPTP induced neurotoxicity

At the end of the test protocols, all animals were examined for dopaminergic cell loss in the substantia nigra. Consistent with a previous study using a similar acute method of MPTP administration ([Bezard et al., 1997](#)), we found that acute MPTP injections (four daily doses of 4mg/kg) caused a significant loss of dopaminergic neuronal staining in the substantia nigra as measured by optical density of TH-positive labelled neurons ($70.9 \pm 9.8\%$; $P < 0.05$; **Fig. 7**). Similarly, continuous MPTP infusion via the minipumps of 22 mg/kg/day during a period of 28 days induced an equivalent decrease of $68.8 \pm 6.4\%$ in TH-positive labelling in the substantia nigra ($F = 4.9$, $P < 0.05$, **Fig. 7**).

Effects of Age on locomotor and circadian parameters

Although there were no obvious differences between the control and MPTP treated mice, all groups showed similar tendencies for age related alterations for several parameters (total activity, onset of activity, phase angle, days required to re-entrain to a shifted LD cycle) with significant changes in total activity and onset variability in DD and period length in the masking regime. In order to determine whether there was a potential long-term age-dependent decline in locomotor and circadian functions following MPTP treatment, we pooled data from the two control and the two MPTP groups to increase the power of our statistical analysis and assessed several activity parameters from the ages of 8-13 weeks and 40-47 weeks in both DD and LD conditions.

There was a significant age-related decrease in total activity (**Fig. 8A**), and increases in period length and onset variability (**Fig. 8A-C**). We also found a significant difference in the number of days required to re-entrain to a 6 hr shift in the LD cycle (**Fig. 8D**; phase advances 1-2). However, no differences were found between control vs MPTP groups for any of these parameters at young or older ages. All these findings show that both MPTP and control mice had similar age-dependent changes of locomotor and circadian parameters suggesting that the loss of dopaminergic neurons did not lead to an age dependent aggravation of behavior in the MPTP treated mice.

Discussion

The main finding of this study is that although both acute and chronic MPTP treatment regimes induce a significant decrease of DA (as quantified by TH immunostaining) in the substantia nigra in mice, this loss fails to induce any significant circadian, locomotor or cognitive behavioral alterations. We had hypothesized that the dopaminergic neuronal loss, combined with the processes of ageing would eventually lead to an aggravation of behavioral symptoms. However, this was not the case and all mice showed similar tendencies for age related effects of locomotor, rest-activity and circadian parameters.

The quantification of DA loss using optical density differs from the more classical approach of stereological cell counts. In a previous study (Rieux et al., 2002) we have shown that integral OD is an accurate quantification of the amount of immunopositive label in a given structure compared to other methods of quantification. The OD technique has the advantage of taking into account the relative amount of protein expressed in each cell, but

does not differentiate between label in cell soma and cell processes. Stereological cell counts accurately reflect cell numbers but do not take into account differences in protein staining between cells. Thus our estimate of the extent of the DA lesion may not be directly comparable to those of other studies based on stereological cell counts.

Among the diversity of non-motor symptoms associated with PD, cognitive deficits and sleep disturbances are among the most debilitating especially in advanced forms of the disease (Chaudhuri et al., 2006; Chaudhuri & Schapira, 2009). The former are experienced as impairments in executive functions, language, visuo-spatial skills and memory (Bosboom et al., 2004) and the latter as increased sleep latency, excessive daytime sleepiness, general hypersomnia and a fragmented sleep/wake pattern (Chaudhuri et al., 2006; De Cock et al., 2008).

Alterations of the circadian system in neurotoxic based animal models of PD are still poorly understood (Taylor et al., 2010; Chesselet & Richter, 2011). In rats where severe lesion of the nigro-striatal system by local bi-lateral injections of 6-OHDA produces a selective deterioration of locomotor abilities, the profiles of the daily rhythms of locomotor activity and temperature remained largely intact (Ben & Bruguierolle, 2000; Baier et al., 2006). The only observed effect on daily locomotor activity rhythms was a reduction of overall activity and of the amplitude of the rhythm. Two limitations of these studies were that alterations of locomotor activity rhythms were only examined under nycthemeral conditions in which the light dark cycle exerts strong regulatory influences on behavior and neuropeptide regulation. Secondly, behavioral consequences were only evaluated short term (less than 1 month following treatment). Recently, Gravotta et al using the same 6-OHDA model under conditions of constant darkness, showed that treated animals were less active and had a disorganized pattern of activity over the 24hr period. However, these locomotor and rhythmic changes were only observed for the first few days following injection of the neurotoxin, and showed improvement over the following weeks. In addition, clock gene expression in the SCN was found to be normal (Gravotta et al., 2011). Similarly, sleep architecture in the MPTP mouse, although showing some transient changes immediately following MPTP treatments, gradually returns to a normal state over time (Lima et al., 2007; Laloux et al., 2008).

In the current study, we have conducted a detailed long-term investigation of circadian parameters in both LD, constant dark and constant light conditions by comparing two MPTP

treated mouse models, the first obtained by acute daily bolus injections and the second by chronic infusion of MPTP using osmotic minipumps. The later model has formerly been claimed to recapitulate both behavioral and cardinal pathological features of PD (Fornai et al., 2005). However, we failed to observe any significant disturbances of the circadian system following MPTP-induced DA neuronal degeneration. To our knowledge, few studies have investigated locomotor activity rhythms under circadian conditions (constant darkness) using either transgenic mouse models (Doi et al., 2006; Kudo et al., 2011; Pfeffer et al., 2012) or a toxin based-model (Rotenone; Lax et al., 2012). In accordance with our results, these studies have also shown that the fundamental properties of the circadian system (endogenous free running rhythm, LD entrainment, 6hr phase shifts) remain unimpaired, an observation that is further supported by the normal pattern of expression of clock genes in the suprachiasmatic nucleus of transgenic models of PD (Doi et al., 2006; Kudo et al., 2011; Pfeffer et al., 2012). However, Kudo et al (2011) in the transgenic overexpressing alpha-synuclein mouse model, have shown several alterations of circadian outputs including greater fragmentation in the wheel-running activity, alteration of temporal distribution of sleep and a reduced daytime firing rate of SCN neurons.

Some of these studies also report a continuous decline in the amplitude of the rhythm as secondary to the aggravation of motor symptoms with age (Kudo et al., 2011; Lax et al., 2012). The long-term assessment of circadian parameters revealed an age-dependent alteration of circadian rhythms in both control and MPTP treated mice as has been previously documented in normal mice (Valentinuzzi et al., 1997). Several parameters were affected, including a decline in total activity and increased onset variability both in LD and constant darkness conditions, increased number of days required for re-entrainment after 6hr phase advance and a lengthening of the free-running period under both masking protocol and constant darkness. Kudo et al also investigated changes of locomotor functions, circadian rhythms and behavioral sleep in a transgenic mouse overexpressing alpha-synuclein, a model of general synucleinopathy like PD. Compared to littermate controls, the transgenic mice showed a more severe age-related aggravation of both locomotor skills and the temporal distribution of sleep (Kudo et al., 2011). In our MPTP models, we found no differences in the rate of evolution of behavioral parameters with age between control and MPTP treated mice.

Using the radial water maze, a behavioral test that spans several days and allows for a memory consolidation to occur during overnight periods (Alamed et al., 2006; Vorhees & Williams, 2006) we showed that 70 % nigrostriatal DA depletion did not produce significant

deficits in working and consolidation of spatial memory. The majority of the studies that evaluated cognitive performances in MPTP treated mice conducted behavioral testing only hours or days after MPTP treatment (Tanila et al., 1998; Deguil et al., 2010; Dluzen & Kreutzberg, 1993; Yang et al., 2004). For example, using a T-maze 7 days after the last injection, Tanila et al showed unimpaired spatial working memory in easy version of the task and minor deficits when the difficulty of the task was increased (Tanila et al., 1998). Another study showed that 4 days after MPTP treatment, mice had altered performance in cued and spatial reference memory versions of the Morris Water Maze test (Deguil et al., 2010). In contrast, Pothakos et al (Pothakos et al., 2009) reported significant impairments in the cued but not the spatial reference version of water maze test 1 or 8 weeks after MPTP treatment. One possible explanation of the apparent discrepancy between these studies and our results is the timing of the behavioral assessment relative to the last MPTP treatment. In our study, the assessment of spatial memory was conducted more than 7 months after MPTP treatment, and compensatory mechanisms may have contributed to the normalisation of cognitive performances after DA depletion (Bezard et al., 2000a, 2000b). Although in our study we did not assess TH staining in the striatum, Bezard et al (2000b) using the same protocol showed that compensatory mechanisms (sprouting of TH terminals in the striatum) can account for normalisation of striatal DA content for daily MPTP treatments that do not exceed 10 daily injections. Other studies in MPTP treated mice have also demonstrated an increase in DA content and elevated homovanillic acid (HVA)/DA and 3,4-dihydroxyphenylacetic acid (DOPAC)/DA ratios indicating compensatory increase of DA turnover after MPTP injury (Pifl & Hornykiewicz, 2006). Much more severe memory impairment has been reported in rats 2-3 weeks following local DA depletions in the prefrontal cortex (Bubser & Schmidt, 1990), ventral tegmental area (Stam et al., 1989), substantia nigra (Da Cunha et al., 2002) and lateral septum (Simon et al., 1986) suggesting that an imbalance in the activity of various dopaminergic pathways is more detrimental to cognitive functions than a general decrease in all dopaminergic activity (Tanila et al., 1998). In transgenic mouse models, decline of cognitive abilities ranging from Y-maze performance, decreased novel object recognition and deficits in operant reversal learning have been reported in mice over-expressing α -synuclein (Fleming et al., 2008; Magen & chesselet, 2010), DJ-1 knockout (Pham et al., 2010) and *parkin* knockout mice (Zhu et al., 2007). In contrast to neurotoxic models, these genetic models may be more appropriate to study the pathophysiology of cognitive deficits.

It has been argued that the lack of PD-like symptomatology in toxin-based mice models is at least partially related to the acute mode of intoxication (Jackson-Lewis & Przedborski, 2008), which differs from the slow and progressive nature of neurodegenerative processes in PD (Maetzler et al., 2009). However, the lack of circadian and cognitive abnormalities reported here, even in the chronic MPTP infusion model, argues against this hypothesis and suggests that these limitations are inherent to the MPTP mouse as animal model rather than the mode of intoxication. Our results concerning non-motor symptoms reinforce previous conclusions concerning the absence of motor deficits in MPTP treated mice (Jackson-Lewis & Przedborski, 2008).

PD shares many molecular and functional aspects with normal ageing (Collier et al., 2011). Many hypotheses have proposed that PD occurs from processes that result in degeneration of DA as well as non-DA neurons that accelerate the normal course of age-related neuronal dysfunctions (Schapira & Jenner, 2011). Multiple agents have been proposed to contribute to this process (i.p. environmental insults, prenatal infections and genetic predispositions). In this perspective, toxins, such as MPTP, used to model PD are proposed to be the equivalent of those insults. Although a significant age-related alteration in locomotor and circadian parameters was found in our study, we did not observe any exacerbation of these parameters in our MPTP treated mice. This result constitutes another intrinsic limitation of the MPTP mouse model compared to certain transgenic mouse models or the non-human primate in which MPTP accelerates age-related decline in multiple motor and non motor functions (Collier et al., 2011; Fox & Brotchie, 2010; Kudo et al., 2011).

Taken all together, and given the central role that the circadian system plays in the regulation of sleep (Borbély & Achermann, 1999; Dijk & Lockley, 2002), one might conclude based on rodent models of PD that the circadian system is not affected and hence does not contribute to sleep abnormalities reported in PD. In PD patients however, a substantial number of studies have also shown multiple behavioral and physiological circadian alterations, for example, in rest-activity and the phase and amplitude of melatonin and cortisol rhythms (Videnovic & Golombek, 2012). The failure of all neurotoxic based models to replicate motor, sleep, cognitive symptomatology of PD (Taylor et al., 2010; Chesselet & Richter, 2011), circadian abnormalities revealed in the current study and their well known limitation in modelling the entire scope of PD brain pathology (Dawson et al., 2010; Beal, 2010) questions the relevance of conclusions based on this mouse model regarding human PD.

The MPTP mouse model has led to tremendous insights into the molecular mechanisms by which DA neurons in the substantia nigra degenerate (Przedborski & Vila, 2003) and in this area of research, the MPTP mouse model is still a valid model. However, studies investigating more integrative behavioral symptoms like circadian and cognitive abnormalities of PD will need to focus mainly on non-human primate model, which thus far constitutes the gold standard model of symptoms of PD (Fox & Brotchie, 2010), and in which we and others have reported both cognitive and circadian disturbances resembling their clinical counterparts in PD patients (Schneider et al., 1995; Barraud et al., 2009; Vezoli et al., 2011).

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Figures:

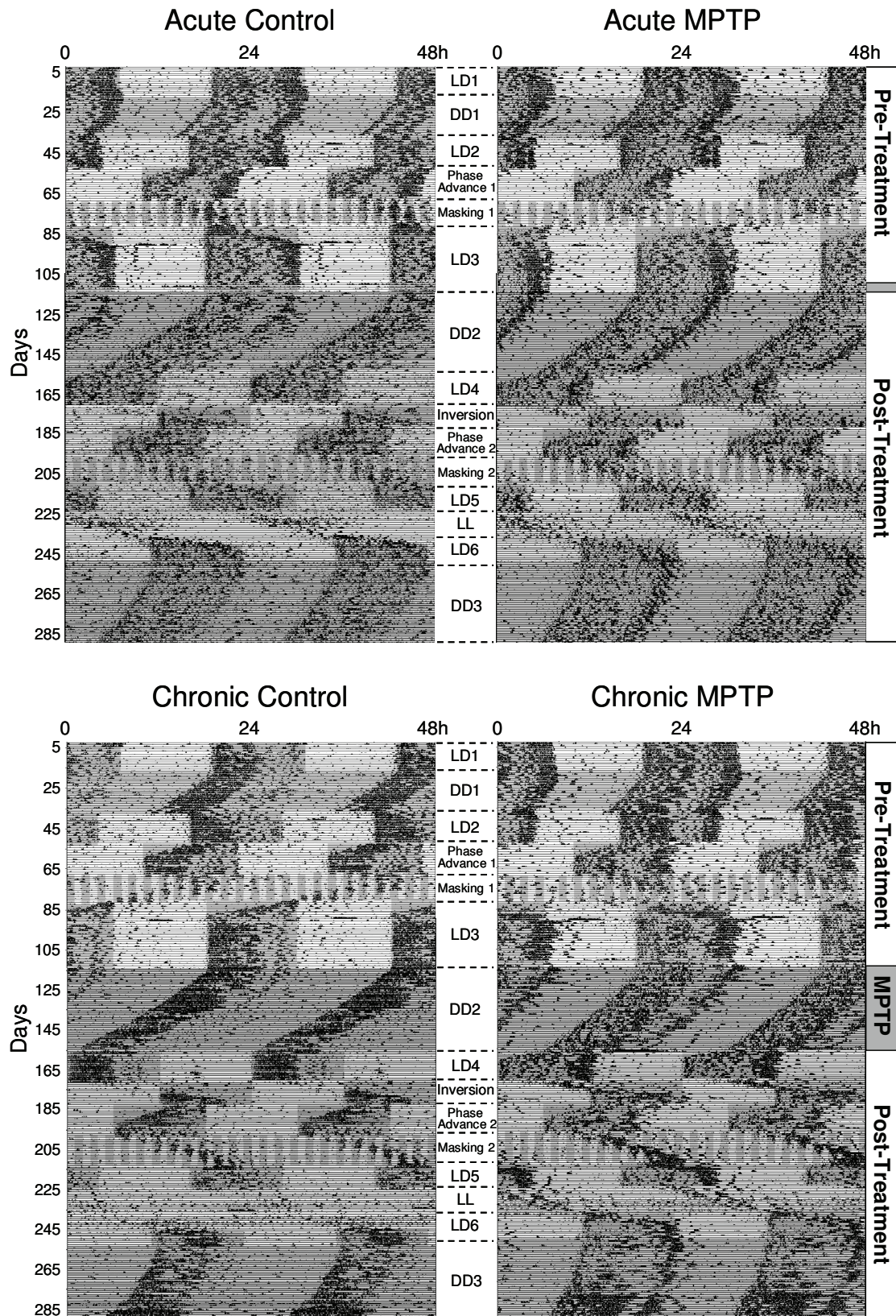


Figure 1. Representative double-plotted actograms of locomotor activity from an acute and a chronic MPTP treated mice with their corresponding controls (upper and lower panels, respectively) illustrating the complete experimental sequence of different lighting conditions. LD1-6: 12hr light:12hr dark cycle, DD1-3: constant darkness, Phase Advance 1-2: 6hr advance of 12:12 LD Cycle, Masking 1-2: 1hr: 1hr LD cycle, LL: constant light. Shading indicates the dark phases. The chronic infusion (osmotic minipump) was performed during the second period of constant darkness (DD2), whereas the acute administration (ip injections) was during the last 4 days of LD3 (grey bar on the right side of actograms).

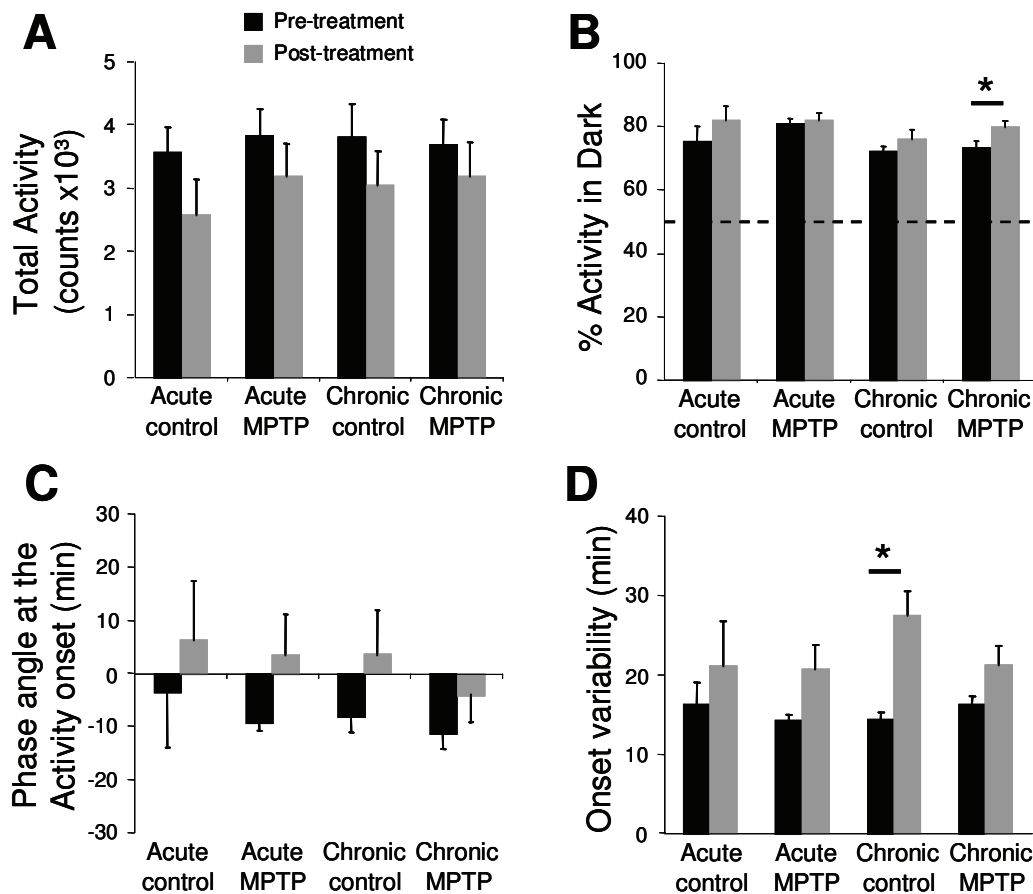


Figure 2. Comparison of different parameters of locomotor activity under a light dark cycle pre- (LD3) and post- (LD5) treatment periods in both acute and chronic MPTP treated mice and corresponding controls. (A) Comparison of the total amounts of locomotor activity (mean

counts per day). **(B)** Comparison of the percent of activity during the dark phase of the LD cycle (dotted line = 50%). **(C)** Phase angle differences between times of lights off and onset of activity. Negative values indicate a delayed and positive values an advanced activity onset. **(D)** Mean activity onset variability. There were no significant differences between control and MPTP treated mice either in pre- or post-treatment phases. There was a significant within increase group in the percent activity in the dark only in the chronic MPTP mice and for onset variability in the chronic controls. Acute MPTP, n=6, chronic MPTP, n=8, acute control, n=5, chronic control, n=7. Error bars: s.e.m. (*P<0.05).

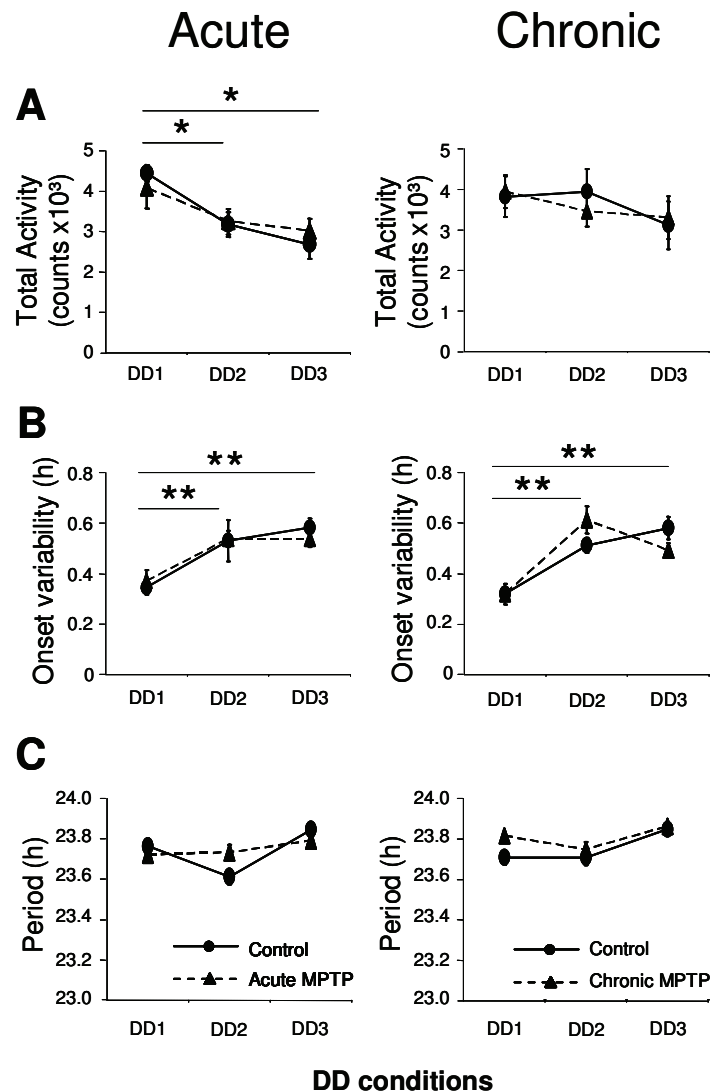


Figure 3. Change in mean total activity (A), onset variability (B) and endogenous circadian period (C) under constant darkness (DD) in both acute (n=6) and chronic (n=8) MPTP treated mice and their corresponding controls (n=5 and n=7, respectively). No significant differences were found between MPTP treated mice and controls within each DD phase although there was a significant decrease in total activity and increase in onset variability in most groups from DD1-DD3. Circadian period did not differ significantly between MPTP treated mice and controls. Error bars: s.e.m. (*P<0.05, **P<0.01).

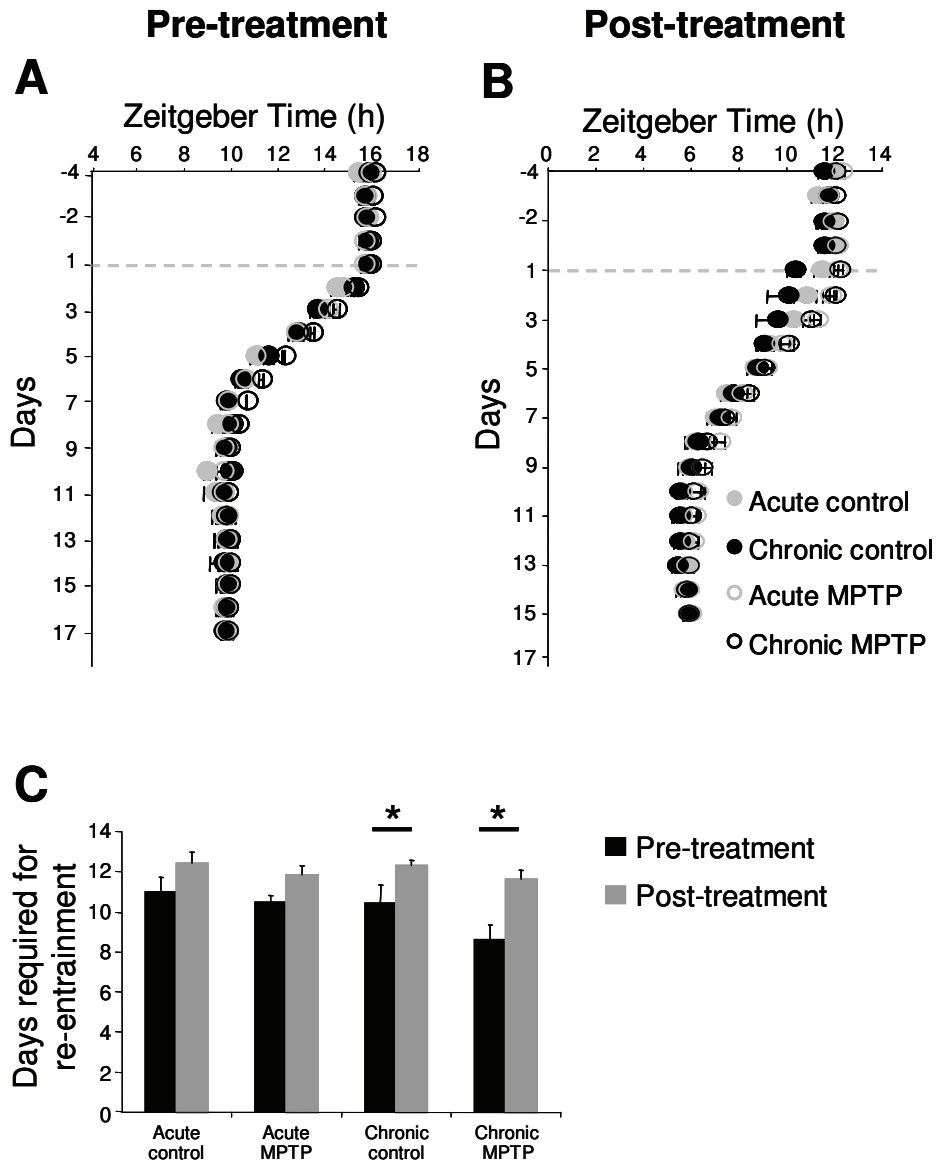


Figure 4. Mean daily activity onsets following a 6 hr advance of the LD cycle pre- (A) and post- (B) treatment. Day 1 corresponds to the first day of the shifted LD cycle. A significant increase in the number of days required to fully re-entrain to the new LD cycle during post-treatment compared to pre-treatment assay was seen for Chronic MPTP and the corresponding control without, however, any significant effect of MPTP treatment (C). Error bars, s.e.m. (* $P < 0.05$). Acute MPTP, $n=6$, chronic MPTP, $n=8$, acute control, $n=5$, chronic control, $n=7$.

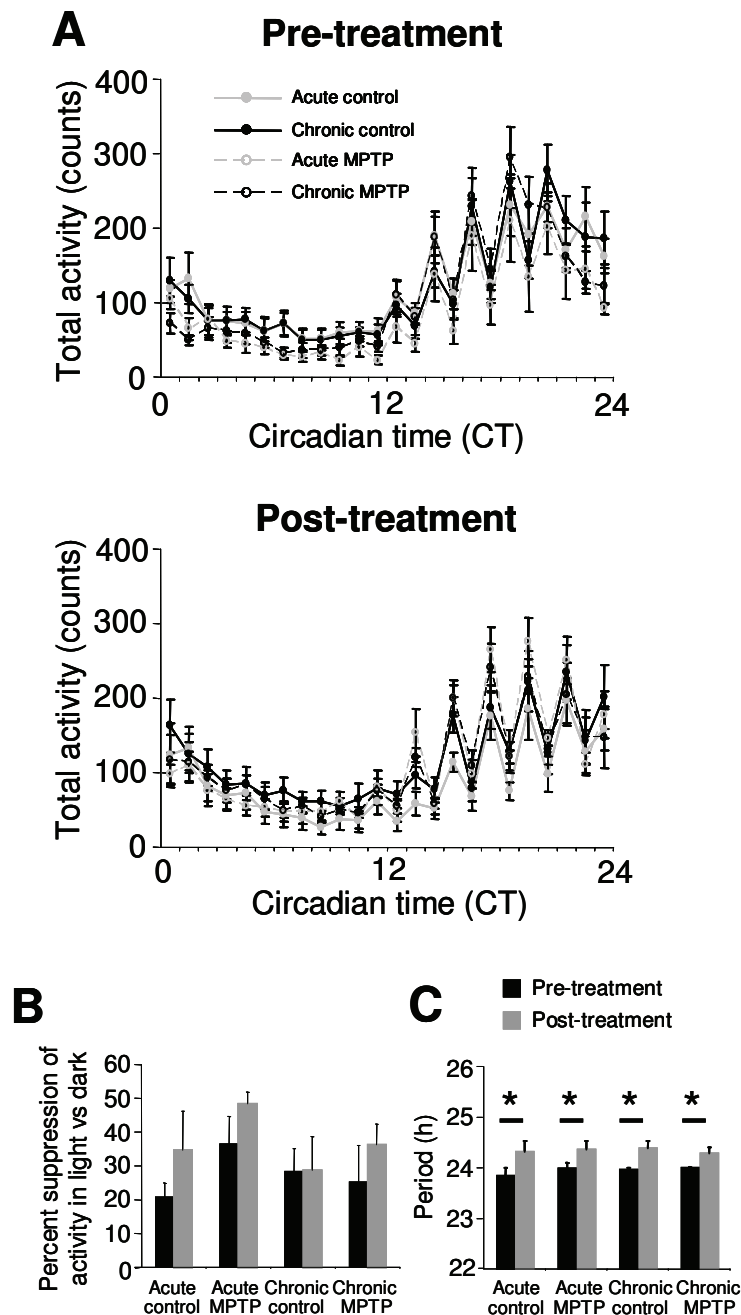


Figure 5. Masking responses to light are not impaired following MPTP treatment. Mean profiles of activity under the 1hr:1hr LD cycles for all groups pre- and post-treatment (**A**) plotted according to circadian time. A consistent decrease in locomotor activity was observed during the light compared to the dark phases in all groups (**B**). The percentage of suppression of activity in the light compared to activity in the dark portion of the 1hr:1hr LD cycle (CT12-24) and the circadian period (χ^2 periodogram) show no significant differences between groups (**B**, **C**). However, under the masking regime, a significant lengthening of the free running

period was observed pre-treatment and post-treatment within each group (C). Error bars: s.e.m. (* $P < 0.05$). Acute MPTP, $n=6$, chronic MPTP, $n=8$, acute control, $n=5$, chronic control, $n=7$.

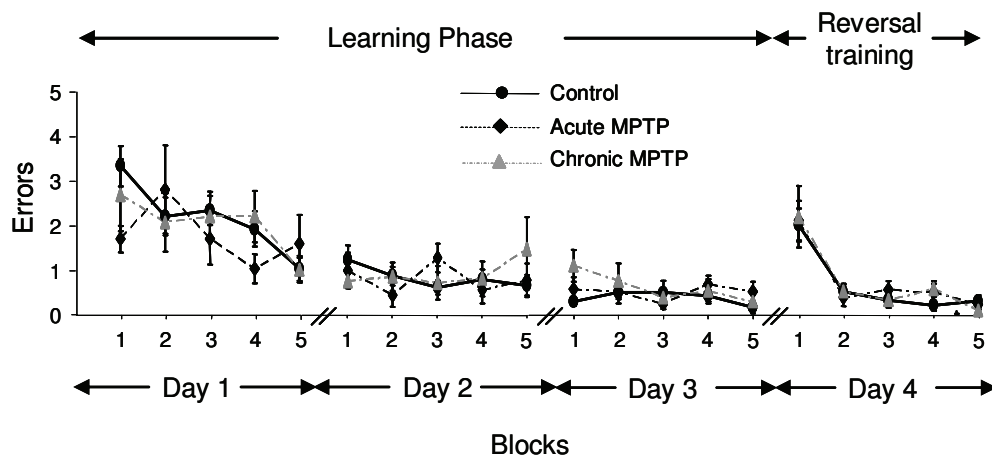


Figure 6. Learning performance in the Radial Arm Water Maze is not affected in acute and chronic MPTP treated mice. The graphs show the results of 4 successive days of testing in control and MPTP treated mice. All animals show an improvement in performance over the three training days ($P < 0.001$) reaching the learning criterion (< 1 error) by block 2-3 on day 2. There was no significant difference between control and MPTP treated mice in both learning performance (Days 1-3) and in reversal learning performance (Day 4). Data presented are mean \pm s.e.m. Acute MPTP, $n=6$, chronic MPTP, $n=8$, acute control, $n=5$, chronic control, $n=7$.

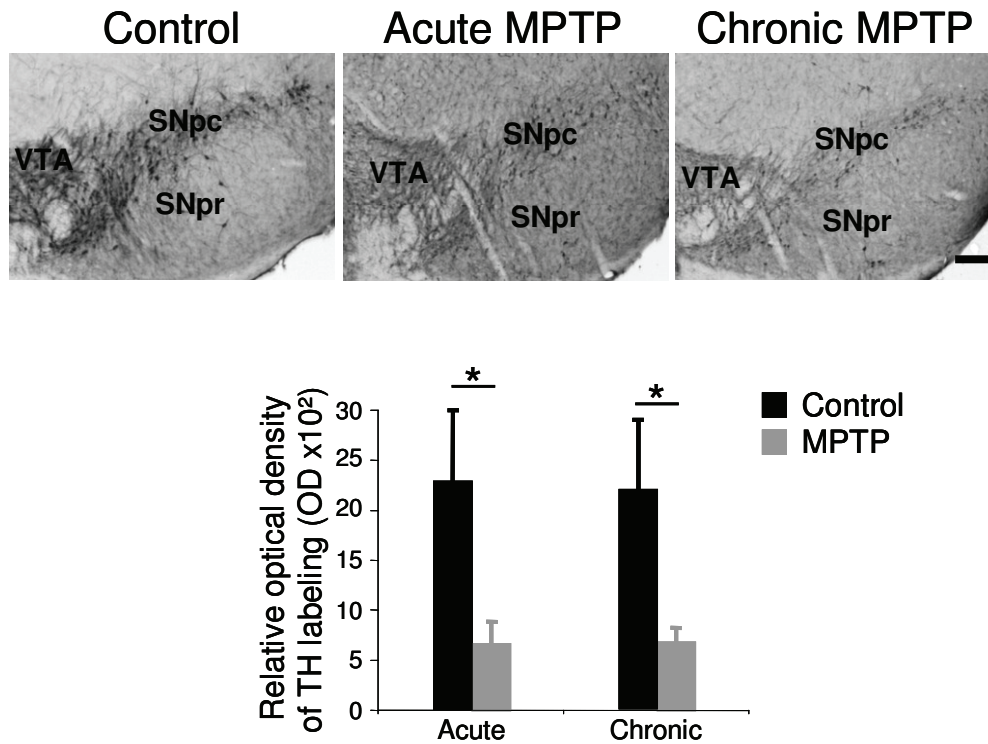


Figure 7. Dopaminergic-related neurotoxicity induced by acute and chronic MPTP administration. Photomicrographs showing representative frontal sections through the substantia nigra in a control, acute and chronic MPTP treated mice. Scale bar = 200 μ m. SNpc, compact part of the substantia nigra; SNpr, reticular part of the substantia nigra; VTA, ventral tegmental area. Histogram below: Measures of mean relative optical density (OD) of TH immunoreactivity (\pm s.e.m) in controls (acute and chronic grouped together), acute MPTP treated and chronic MPTP treated groups show a significant reduction (* P <0.05) in mean OD in MPTP mice. (Number of sections per animal = 5, number of animals: control = 12, acute = 6, chronic =8).

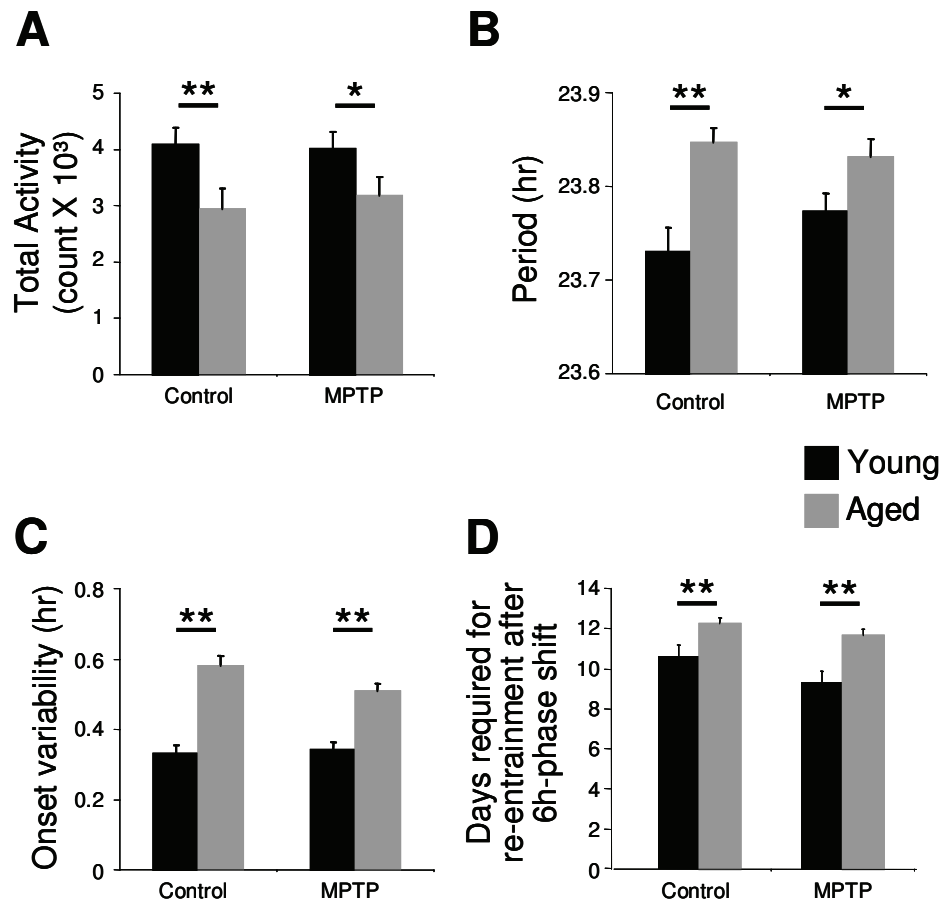


Figure 8. Effects of age on circadian and locomotor parameters. Data was pooled from all MPTP mice and compared to all controls at young (8-13 weeks) and older (40-47 weeks) ages. Both groups showed age dependent decreases in total activity (**A**), and increases in period (**B**), onset variability (**C**) and the number of days required to re-entrain to a 6 hr shift of the LD cycle (**D**). No differences were found between controls vs MPTP mice for any of the parameters at the young or older ages. Error bar: s.e.m. (Acute MPTP, n=6, chronic MPTP, n=8, acute control, n=5, chronic control, n=7. *P<0.05, **P<0.01).

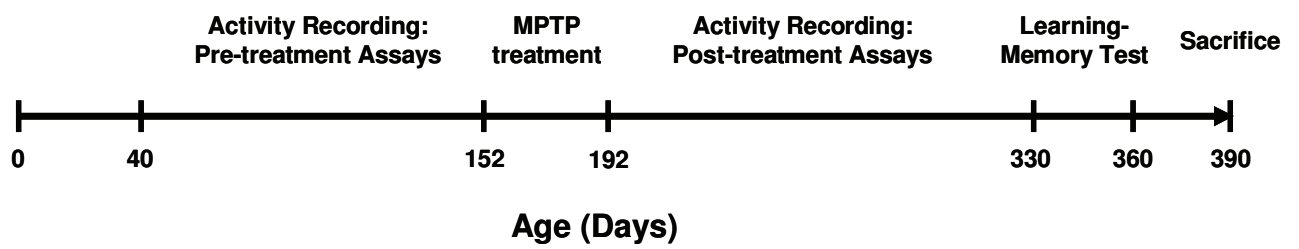
Supplementary Figure Legends

Figure S1. Time-line of the entire protocol of the experiment.

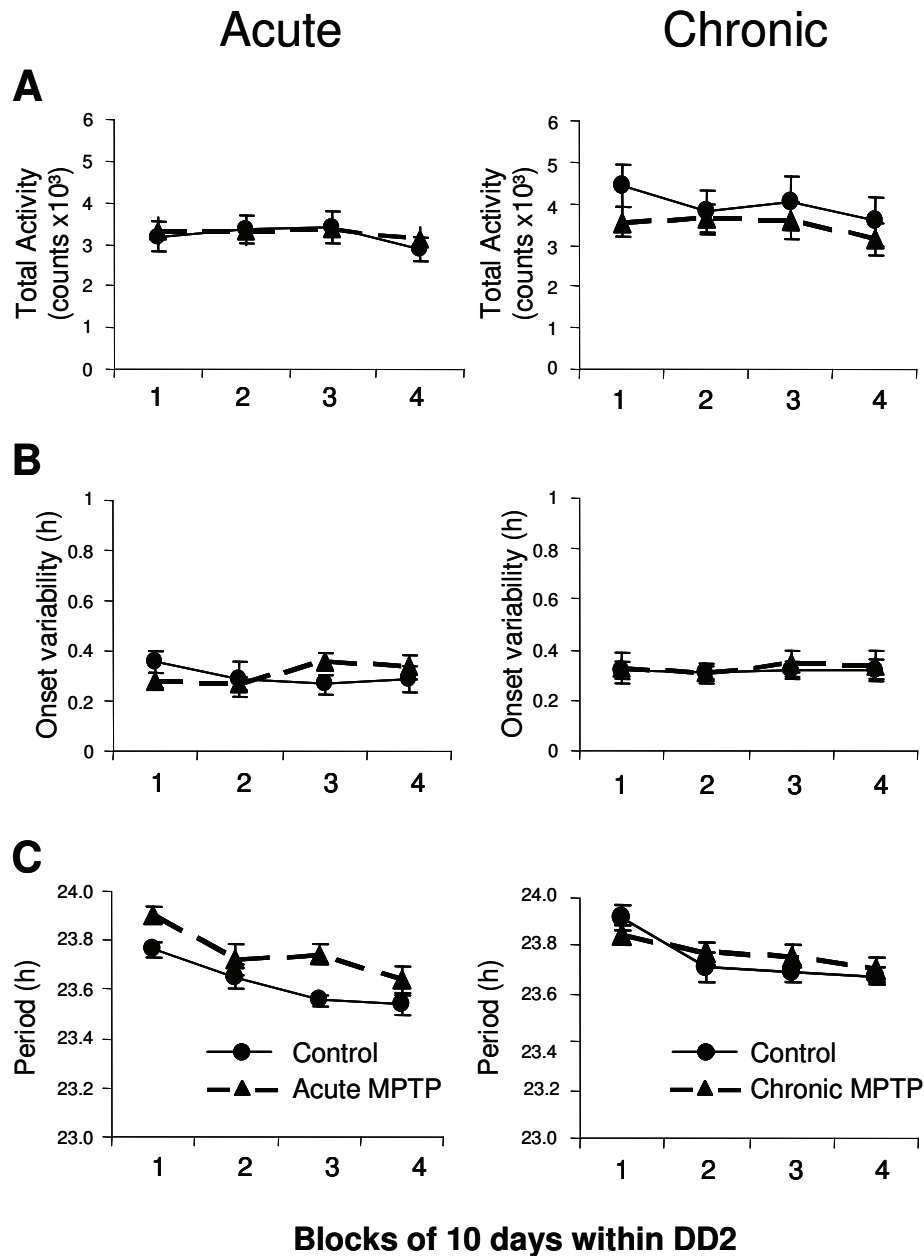


Figure S2. Change in mean total activity (A), onset variability (B) and endogenous circadian period (C) during the treatment period of the chronic MPTP mice (minipumps) under constant darkness (DD2). The acute mice were treated with MPTP immediately prior to DD2. The 40 days of DD2 are divided into blocks of 10 days (1-4). No significant differences were found between acute and chronic MPTP treated mice or between MPTP mice and controls within each of 10 day block of the assay. Error bars: s.e.m. Acute MPTP, n=6, chronic MPTP, n=8, acute control, n=5, chronic control, n=7.

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Chapter 3

Title**Early Presymptomatic and Long-term Changes of Rest Activity Cycles and Cognitive Behavior in a MPTP-Monkey Model of Parkinson's Disease.*****Running title***

Non-Motor Symptoms in Chronic Low-Dose MPTP Monkeys.

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Abstract

Background: It is increasingly recognized that non-motor symptoms are a prominent feature of Parkinson's disease and in the case of cognitive deficits can precede the onset of characteristic motor symptoms. Here, we examined in 4 monkeys chronically treated with low doses of the neurotoxin MPTP the early and long-term alterations of rest-activity rhythms in relationship to the appearance of motor and cognitive symptoms.

Methodology/Principal Findings: Behavioral activity recordings, motor and cognitive assessments were carried out continuously and in parallel before, during and several months following MPTP-treatment (12-56 weeks). Cognitive abilities were assessed using a task that is dependent on the functional integrity of the fronto-striatal axis. Rest-activity cycles were monitored continuously using infrared movement detectors of locomotor activity. Motor impairment was evaluated using standardized scales for primates. Results show that MPTP treatment led to an immediate alteration (within one week) of rest-activity cycles and cognitive deficits. Parkinsonian motor deficits only became apparent 3 to 5 weeks after initiating chronic MPTP administration. In three of the four animals studied, clinical scores returned to control levels after 5-7 weeks following cessation of MPTP treatment. In contrast, both cognitive deficits and chronobiological alterations persisted for many months. Levodopa treatment led to an improvement of cognitive performance but did not affect rest-activity rhythms in the two cases tested.

Conclusions/Significance: Present results show that i) changes in the rest activity cycles constituted early detected consequences of MPTP treatment and, along with cognitive alterations, characterize the presymptomatic stage; ii) following motor recovery there is a long-term persistence of non-motor symptoms that could reflect differential underlying compensatory mechanisms in these domains; iii) the progressive MPTP-monkey model of presymptomatic ongoing parkinsonism offers possibilities for in-depth studies of early non-motor symptoms including sleep alterations and cognitive deficits.

Introduction

Parkinson's syndrome (PS) was initially characterized by motor symptoms including rigidity, akinesia and tremor. However, it is increasingly recognized that these motor deficits are accompanied by symptoms affecting cognitive performance, neuropsychiatric states, sleep, autonomic, and sensory domains occurring in up to 88% of PS patients and are important factors contributing to physical and mental disability [1,2,3,4]. These non-motor symptoms have important consequences on performance and contribute to a decline in the quality of life. The causes of non-motor manifestations of PS are multifactorial and could be related to widespread neurodegenerative processes of both dopaminergic and non-dopaminergic systems [5].

While numerous studies using animal models of Parkinson's disease (PD) have explored different aspects of these non-motor deficits, there is to date a lack of information concerning their overall orchestration with respect to the onset of classical clinical disease. We have therefore conducted a longitudinal study to simultaneously evaluate the temporal patterns of alterations in rest-activity cycles and cognition in the MPTP monkey model of PS.

The compensatory mechanisms and the concealed or partially concealed deficits that appear early on in PS, when the brain is undergoing major alterations during the pre-symptomatic period, are still not fully understood [6]. A long-standing literature has established that a low-dose MPTP regime in the monkey can induce long-lasting cognitive deficits resulting from fronto-striatal dysfunction, independent of the manifestation of any clinical signs of motor dysfunction [7,8,9,10]. Other studies have shown that high doses of MPTP that rapidly engender severe clinical motor symptoms also influence biological rhythms and sleep [11]. However, it is not known if the impact of the dopaminergic lesion on biological rhythms occurs independently of either motor or cognitive effects during the so called pre-symptomatic period in which cognitive deficits are typically reported [12,13].

While Parkinsonism is currently viewed as a complex network disorder [14], there is need for a more complete understanding of the spectrum of changes in PS that precede the diagnosis of classical motor deficits. This information is crucial for developing more effective and specific means of detection of the disease onset as well as designing more effective therapeutics for the full range of symptoms. It follows that long term multi-dimensional survey of neurobiological states during the early stages of development of PS would be a valuable prerequisite for gaining a better understanding of the diagnostic features and the sequence of onsets of the different physiopathological components.

We therefore used continuous, long-term recordings in a non-human primate model of PS to analyze in parallel the changes in clinical scores, cognitive task performance and locomotor rest-activity patterns . We used chronic low-dose injections of MPTP to induce a Parkinson-like state that includes the development of compensatory mechanisms, thereby providing a model of low-grade dopaminergic lesion and sub-threshold clinical states [6]. Importantly, injections were ceased when monkeys reached a specific clinical threshold thereby favoring spontaneous motor recovery. However, analysis of behavioral parameters before, during and after chronic low-dose MPTP treatment demonstrated clear dissociations between clinical manifestations of motor dysfunction and both rest activity cycles and cognitive performances.

Materials and Methods

Ethics Statement. All procedures were carried out according to the 1986 European Community Council Directives (86/609/EEC), the French Ministère de l'Agriculture et de la Forêt, French Commission of animal experimentation, the Department of Veterinary Services (DDSV Lyon, France). This experiment was also carried out according to guidelines published in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Under local rules, authorization is granted to the laboratory for a range of experiments, rather than for specific studies. Specific authorization covering this study was delivered by the "Préfet de la Région Rhône Alpe" and the "Directeur départemental de la protection des populations" under Permit Number: #A690290402, including approved protocols in NHPs (#047, #048, #0198, #0199, #0200). All procedures complied with guidelines for animal welfare in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research". The nature of the measures and interventions described below required individual housing of monkeys during the experiment. This is also a welfare measure – symptomatic MPTP-treated monkeys would be at risk of persecution in a group-housing situation. Given this, however, all efforts were made to provide an enriched and social environment for the animals in a colony room with other familiar monkeys. All monkeys were closely monitored on a regular basis throughout each day, by several researchers as well as animal care staff, in order to ensure that levels of health and welfare were maintained. This was particularly the case during the MPTP period. As required, adaptations to housing and feeding procedures were made in direct response to

individual symptoms in the MPTP phase, for example adaptations to water provision to ensure monkeys were able to drink *ad libitum* throughout.

Animals and housing. Four monkeys were studied: three female *Macaca fascicularis* (monkeys J, F and L; aged respectively of 15, 12 and 11 years old) and a 13-year old male *Macaca mulatta* (monkey T), weighting 6, 5.5, 4.7 and 12kg, respectively. Animals were housed in a room dedicated to MPTP experiments, had free access to water and received food twice a day. Illumination was provided by white fluorescent tubes (Phillips Master, 15 Watts) mounted on the ceiling above the animal's cage providing 450-500 lux depending on the animal's position and gaze inside of the cage. The lighting schedule was clock-controlled for 14 hours light - 10 hours dark (14:10 LD) for monkeys J and T and 12 hours light - 12 hours dark (12:12 LD) for monkeys L and F. Mean room temperature during data collection was maintained at approximately 24-26°C.

MPTP intoxication. A protocol of chronic injections at low-doses of 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) was employed based on previous studies [15] in order to obtain a slow onset of symptoms. CLD-MPTP treatment consisted of intramuscular injections of MPTP-HCl (Sigma M0896) diluted in sterile water at a concentration of 0.2 mg/kg. Injections were performed every 3 to 4 days in the home cage, without prior sedation. Pre-diluted doses of MPTP were prepared and stored in single-dose syringes (Myjector, Terumo®) at -80°C. The storage period did not exceed 2 months since MPTP can become unstable after this period [16]. NIH safety guidelines were observed for housing of treated-animals, MPTP preparation, storage, injection, and elimination. Injections of sterile water alone were performed previously to MPTP administration to ensure the absence of effects on measured behavioral parameters. As these animals were part of an ongoing study, they were not sacrificed at the end of the recordings and thus the extent of induced lesion on the aminergic system could not be verified. In one case (L), the levels of DA transporters was evaluated in-vivo in a follow-up PET study using a specific radioligand ([11C]-PE2I, [17]). Moreover, cumulated doses used here (>2mg/kg) were superior to minimal cumulated dose used in previous studies that resulted in significant insult to the DA system [6,18].

Clinical Motor Evaluation A clinical rating scale: the Parkinsonian Monkey Rating Scale (PMRS), adapted from the Unified Parkinson's Disease Scale (UPDRS) used for patients and compatible with rating scales used for monkeys [19], was employed to define

the onset of the symptomatic period and to cease MPTP injections at the appropriate level of symptoms. We used the subtotal score of the PMRS corresponding to previously described scoring criteria in monkeys [20]: a score of 0 defined the asymptomatic stage; a score between 1 and 5 defined the pre-symptomatic period; a score above 5 defined the symptomatic stage. Once monkeys entered the symptomatic stage, injections were halted in order to gain information about possible spontaneous recovery. Clinical symptoms were assessed daily and the total score for freezing (0-3), resting tremor (0-3, for right and left side), rigidity (0-3), posture (0-2), bradykinesia (0-3), and ability to manipulate food (0-3, for right and left side) was used to frame injections (maximum total score of 23).

Cognitive Behavioral task. Cognitive performance was monitored 5 days a week using a behavioral test previously used to assess fronto-striatal functions and post-MPTP cognitive deficits [7,8,10] : Object Retrieval Detour Task (ORDT, **Figure S3**). The task was presented in the animal's home cage and prior to injections during the MPTP period. Monkeys had to retrieve within a delay of 1 min a piece of fruit placed in a transparent box with an opening for access placed on only one side. Animals were tested for detour trials (see **Figure S3**) in which the open side was not facing the animal (either right or left side), requiring inhibition of the tendency to reach directly toward the reward and required a strategy of a detour reach. However, each testing session contained a third of trials with the open side facing the animal so that the reach was straight forward, in order to avoid automatic responses. Performance was evaluated by measuring successes and errors on detour trials. Successes (retrieval of reward on the first reach) were expressed as percentage of total number of trials. Errors (barrier hits i.e. hitting a transparent side of the box) were expressed as percentage of responses observed (there could be several responses per trial except in the case of success). A maximum of 18 trials per session were presented randomly to the animal each day. Animals initially received a short period of training to become accustomed to the presence of the experimenter and to learn the task; performance was then evaluated.

Rest-wake activity profiles. Locomotor activity was recorded in the home cage (actimetry), continuously throughout the entire duration of the study (up to 16 months). Animal movements were detected using passive infrared captors placed above the cage and a monitoring system developed in the laboratory (Circadian Activity Monitoring System, CAMS, Inserm; see [21]). The captors detect the animal's displacements in the cage but are not sensitive to small tremor. Signals from the captors were recorded continuously and stored

in 1 min bins (counts/min). The quantified activity thus represents spontaneous locomotor displacements throughout the rest-activity cycle. Monkeys were maintained in a 14:10 or 12:12 light: dark cycle. Maximum light intensity was 400 lux (Fluorescent white light 4000K) during the daytime period and 0 lux at night.

L-Dopa treatment. A short-term dopatherapy was scheduled in cases J and T in order to investigate effects on: i) cognitive performances, and ii) circadian expression of locomotor rhythms post-MPTP. During the period of these tests (3-4 weeks), monkeys were treated twice-daily with oral administration of levodopa (Sinemet®, levodopa:carbidopa - ratio 10:1) at a dose designed to produce a full reversal of the parkinsonian condition (15-20 mg.kg⁻¹ of body weight; [22]). This was not carried out on cases L and F because they entered a cellular therapy protocol that will not be described here.

Data analysis. We segmented MPTP and recovery periods into 4 equal epochs, 5 quantiles were then calculated from the beginning of each period using a 1-D linear interpolation method (MatLab interp1 function, de Boor, C. (1978) A Practical Guide to Splines. Springer-Verlag). These quantiles allowed a comparison of the time course of the variations of different parameters across an equivalent number of epochs for all subjects. This method of segmentation thus compares the stages of progression of processes linked to the aminergic lesion according to the behavioral outcomes rather than based on an absolute time-scale. Non-parametric circadian rhythm analysis (NPCRA) was used to extract actigraphic variables from diurnal activities [23]. These included measures of (1) intra-daily stability (calculated as the variance of the average 24-hr pattern of activity around the mean and overall variance, indicating the consistency of day to day activity or the strength of coupling to the LD cycle i.e. the synchronizer) (2) intra-daily variability (indicating the frequency of transitions between rest and activity periods, corresponding to the fragmentation of activity) and (3) relative amplitude (ratio between the acrophase and the nadir of the rhythm, representing the ratio between the amplitude of activity in the light and dark phases). When the rest-activity cycle is stable, intra-daily stability is high, intra-daily variability is low (low fragmentation), and relative amplitude is high. Cognitive and clinical scores were also analyzed for the same quantile epochs using the same method to facilitate comparison between these three sets of scores.

Statistics. Results are presented as means \pm standard errors. Each monkey's weekly performance along the protocol was compared with control by a treatment-contrast test using control measures as the first level (estimated standard errors and z-ratio were computed using GLM fit and contrast result was given by two-tailed p-values corresponding to z-ratio based on a Student t-test). Significance was considered for $p < 0.05$. Statistical analyzes were computed using the R software (R Foundation for Statistical Computing, Vienna, Austrian <http://www.R-project.org>) and the summary function (Chambers, J. M. and Hastie, T. J. (1992) *Statistical Models in S*. Wadsworth & Brooks/Cole).

Results

Clinical score, cognitive performance in a detour reaching task, and rest-activity cycles were followed continuously before, during and after MPTP-treatment for a period of up to 16 months in four macaque monkeys. While there was considerable variability in the sensitivity of individual animals to the chronic low-dose MPTP treatment, the repeated sub-acute injections of MPTP reproduced non-motor features typical of PS. The relative timing of the changes in rest activity and cognitive symptoms with respect to the onset of clinical motor symptoms were similar among all cases. In one case motor deficits persisted after MPTP treatment cessation and here cognitive and chronobiological deficits could not be dissociated. However, we observed long lasting non-motor symptoms in all cases studied and importantly in the three cases where there was recovery from motor deficits.

Motor symptoms.

All monkeys presented a clinical motor score of 0 in control conditions (Parkinsonian Monkey Rating Scale – PMRS, see materials and methods section) and after injections of sterile water. **Figure 1** represents changes in clinical scores of all cases studied aligned at the end of MPTP-treatment. Despite the different durations of MPTP intoxication required to attain clinical threshold and the different scores attained after MPTP-Off, the three monkeys that spontaneously recovered (cases J, T and F) presented highly similar dynamics of motor recovery (within 5-7 weeks). In case J the first clinical manifestation was observed 3 weeks after the onset of MPTP treatment and symptoms progressively increased up to 7 weeks when threshold (clinical score >5) was reached defining a pre-symptomatic period of 6 weeks, for a total cumulative dose of 2.2 mg/kg (**Figure 2A, left**). For case T, the onset of

clinical symptoms occurred at 4 weeks and threshold was achieved at 28 weeks so that the pre-symptomatic period lasted considerably longer than in other cases (**Figure 2A, right**). In case T, we observed a marked but small peak in the clinical score at week 6 of treatment consisting of slight resting tremor (low amplitude and only intermittently present in the upper limbs). Given the weak response to MPTP in this case, doses were increased 2 fold for the last 3 weeks (generating a total cumulative dose of 10.4 mg/kg). In case F, clinical symptoms also appeared 5 weeks after the beginning of MPTP-treatment and remained stable and low (i.e. score < 5) for the 4 following weeks (**Figure 3A, left**), here MPTP treatment was therefore increased from week 10 onwards, which led to a progressive increase of symptoms until week 12, when the clinical threshold was reached. The pre-symptomatic period in this case lasted 11 weeks for a total cumulative dose of 5.4 mg/kg. In case L, clinical symptoms emerged 5 weeks after initiation of treatment and increased continuously to reach threshold at week 9 (**Figure 3A, right**) for a total cumulative dose of 4.4 mg/kg and a pre-symptomatic period of 8 weeks, note that here the regimen of MPTP-injection was also increased in the last 3 weeks of intoxication.

Clinical symptoms emerged in the following order: action and resting tremor, bradykinesia, rigidity, stooped posture and freezing for cases J and L; action tremor, bradykinesia, stooped posture, resting tremor and freezing for case F; action tremor, bradykinesia, stooped posture, freezing and resting tremor for case T. The symptomatic period defined by a clinical score ≥ 5 lasted 17 days for case J, 24 days in case F and 6 days for case T. In case L, the symptomatic period lasted more than 12 weeks i.e. this animal remained symptomatic after MPTP-Off with no spontaneous recovery. In this case, we confirmed that the lesion induced by MPTP progressed further than the critical threshold of 70-80% of striatal denervation [24], as shown by a separate study of DA transporter using PET-imaging with a specific radioligand ([¹¹C]-PE2I, [17]). This showed that striatal denervation was >80% as estimated by binding potential calculation [25] two months after MPTP cessation (data not shown).

For the other cases (J, F and T) spontaneous recovery was consistently observed with clinical scores decreasing progressively after 1 to 5 weeks of symptomatic state (**Figure 2A, 3A**). In case J some minor, transient bouts of motor symptoms were also observed around weeks 20 and 33 post-treatment. Following the last MPTP injection, recovery was observed in 6 weeks in case J, 5 weeks in case F and 7 weeks in case T.

Case J presented a persistent but slight action tremor of the upper limbs, which is not taken into account in PMRS scoring. This action tremor in case J was present throughout the pre-symptomatic period, stress enhanced and clearly visible during precise grasping movements. Levodopa treatment alleviated this condition by reducing action tremor amplitude and occurrence in the first week and making it rare to undetectable during the following weeks of therapy. It reappeared one week after levodopa offset. Case T presented a clinical score of 0 and no clear action tremor from week 35 onwards. In case T the only abnormality observed was a depressed-like behavior the week following levodopa offset (with little or no motivation to work for rewards). Case F presented a persistent slight bradykinesia throughout the rest of the protocol with a score of 1 (**Figure 3A, left**); L-Dopa was not tested in this case (see the material and methods, L-Dopa treatment section for details).

The clinical score variations for all cases when calculated according to quantile segmentation (see below), showed a highly similar sequential pattern over the intoxication and recovery periods and this despite the individual differences in sensitivity to MPTP, in respect to both duration of effects and dose response.

Cognitive Performances.

Following one month of initial training, control performances in the detour reaching task (testing capacity to inhibit erroneous actions, see methods for further details) were averaged over the week preceding MPTP injections. Monkeys made relatively few errors and attained success rates of 60-80% (*Mean±SD*; Case J: success rates 57±36%, error rates 17±15%; case T: success rates 69±25%, errors rates 30±23%; Case F: success rates 81±7%, error rates 23±8%; Case L: success rates 68±9%, error rates 24±10%; **Figure 2B,C and Figure 3B,C**).

For case J, MPTP performance was significantly worse than in the control conditions starting from the 2nd week of injections ($p < 0.05$, with $Z\text{-value} < -2.4$ for success and $Z\text{-value} > 2.3$ error rates; **Figure 2B,C left**). The balance between error and success rates observed during the control period gradually reversed to an absence of success and an large increase of errors (more than 50%) at the end of MPTP intoxication (week 6). These poor performance levels, reminiscent of frontal-like behavior, remained constant even after the injections were terminated ($p < 0.05$, $Z\text{-value} < -2.1$ for success rate and $p < 0.001$, $Z\text{-value} > 3.4$ for error rate; **Figure 2B,C left**).

For case T, performance in the first week of intoxication up to week 7 was significantly worse than during the control period ($p < 0.001$, Z -value < -3.8 for success rate and $p < 0.05$, Z -value > 2.1 for error rate; **Figure 2B,C right**). During the remaining weeks of intoxication (week 8-27) performance continued to be globally worse than during the control period (**Figure 2B,C right**). After MPTP injections were terminated, case T recovered a control level of performance, except during the first week following MPTP-Off ($p < 0.01$, with Z -value < -3.4 for success and Z -value > 5.2 for error rates).

For case F, the first week of intoxication led to an abrupt decrease of performance and a return to control values in the 2nd week (**Figure 3B,C left**). Subsequently performance deteriorated significantly with respect to the control condition and we observed again a reversal of the balance between success and errors. Success rate decreased and error rate increased progressively to reach maximum values at week 9 ($p < 0.001$, with Z -value < -4.9 for success and Z -value > 4.6 for errors). During the course of the MPTP-treatment, monkey F was sometimes reluctant to work for rewards and this was identified as a lack of motivation since generally monkey F would reach for fruit when offered outside the context of the task. Performance remained significantly worse than in the control condition for the entire period following MPTP-treatment cessation (12 weeks; $p < 0.05$, with Z -value < -1.4 for success and Z -value > 0.7 for errors; **Figure 3B,C left**).

For case L, performance deteriorated significantly from the 5th week of injections ($p < 0.001$, with Z -value < -3.5 for success and Z -value > 2.7 for errors); but was noticeably altered beginning with the 1st week of MPTP injections (**Figure 3B,C right**). In case L in the last weeks of MPTP-treatment the balance between success and errors was totally reversed (success rates $31 \pm 10\%$, error rates $59 \pm 12\%$). Because of a persistent, mild-parkinsonian state in case L, trial number per session for behavioral tests was reduced following MPTP-Off but was sufficient to show that performance remained constantly worse than in the control period ($p < 0.01$, with Z -value < -2.1 for success and Z -value > 1.9 for errors).

For case J, levodopa therapy improved performance (success rate $> 30\%$, error rate $< 50\%$; **Figure 2B,C left**) but did not allow recovery up to control level. Performance for case T during levodopa therapy was better than during control (zero errors and 100% success rate throughout the 3 weeks of levodopa treatment; **Figure 2B,C right**).

Quantile comparison of error rates revealed similar dynamics across animals of the increasing difficulties to perform the task throughout the MPTP intoxication phase (see **Quantile Analysis section**). During the recovery period, these difficulties persisted for the

three cynomolgus monkeys (error remained consistently higher than control levels for cases J, F and L) even though clinical scores had returned to non-symptomatic levels for cases J and F. However, for the rhesus (case T) the error rate rapidly returned to control levels.

Rest-activity rhythms.

During the initial control period all monkeys presented rest-activity rhythms that were highly structured and precisely synchronized with the light-dark (LD) cycle. Animals typically presented an immediate but progressive onset of activity at lights-on and an abrupt offset of activity at lights-off. Almost all activity ($95.4\pm 2\%$) was confined to the light phase (**Figure 4A,C and Figure S1A,C**). For case J, the mean \pm SD activity was 240.6 ± 7.8 counts/hour during the day (light phase) and 12 ± 0.6 counts/hour at night (dark phase); for case T, 617.4 ± 17.4 counts/hour during the day and 48.6 ± 1.2 counts/hour at night; for case F, 260 ± 23.1 counts/hour during the day and 23.4 ± 1.8 counts/hour at night and for case L, 413.9 ± 25.6 counts/hour during the day and 18.3 ± 3.5 counts/hour at night (**Figures 2D, 3D**).

The rest-wake activity patterns during MPTP-intoxication are illustrated on the averaged profiles (**Figures 4B,D and Figure S1B,D**). All cases showed an abrupt and immediate change in rest-activity patterns from the first days of treatment, except in case L in which alterations were observed after one week of MPTP intoxication (**Figures 2D, 3D**). In case J, these changes consisted of a sustained and abnormally elevated night-time activity throughout the period of MPTP treatment (**Figures 2D, left**). This was also the case for case L during the first 6 weeks of MPTP treatment (**Figures 3D, right**). For cases T, F and L, a progressive decrease in daytime activity was observed. The curves consistently showed a slight regain of daytime activity levels around the last third of the whole MPTP treatment period, just preceding a sharp decrease in activity that accompanied the invalidating clinical symptoms (**Figures 2A,D, and 3A,D**). During the recovery period, rest-activity rhythms remained abnormally low for cases T, F and L for prolonged periods and failed to fully return to baseline levels throughout the entire time course of the observations (>11 months; **Figures 2D right, 3D, 4B,D and S1B,D**), long after spontaneous recovery from clinical symptoms. In case L, which failed to recover from motor symptoms, night time activity increased dramatically after MPTP-Off (**Figures 3D right, and S1C,D**).

In the two cases tested with levodopa (cases J and T), rest-activity rhythms were not affected in contrast to effects on cognitive performance (**Figures 2D and 4A-D**).

Quantile Analysis: Inter-individual comparison of behavioral changes.

Since the four animals showed different vulnerabilities to the MPTP treatment resulting in large differences in the time required to display clinical motor symptoms (6, 8, 11 and 28 weeks), we analyzed several parameters according to subdivisions of time periods (quantiles) in order to disclose any similar trends in the appearance of the non-motor symptoms. This was assayed by dividing each phase (MPTP and post-MPTP) in an equal number of epochs. When normalized in this way, the evolution of the clinical scores during treatment and recovery exhibits a similar pattern (**Figure 5A**), even though the time required to attain the critical clinical score of 5 differed significantly between animals (**Figure 1**). There was a similar trend in the increase of errors during treatment. However, during recovery cases J, L and F showed no post-treatment improvement while case T showed a sustained reduction in errors (**Figure 5B**).

All cases showed severely altered values for rest-activity parameters by the end of the first quantile of the treatment period, when clinical scores were still at the baseline. Analysis of the intra-daily stability, the relative amplitude and the intra-daily variability of rest-wake activity revealed that all cases showed similar patterns of alteration during and after MPTP treatment (**Figure 5C-E**), with the exception of case L that showed more severe alterations. Intra-daily stability of the rest-activity cycle (that represents the strength of coupling of the rest-activity cycle to the external light-dark cycle [26]) decreased immediately after initiation of MPTP treatment, and continued to decrease throughout treatment. For cases J and T stability only returned to baseline levels several months after MPTP treatment ended while in cases F and L there was no recovery (case J 8 months and case T 3 months, **Figure 5C**). The relative amplitude represents the strength of the rhythmic oscillation of motor activity and is obtained by comparing the difference between the most and least active periods. Relative amplitude was also found to evolve in a similar manner during recovery for the 3 monkeys (J, T, F; **Figure 5D**) only returning to baseline levels months after treatment (and not at all for monkey L). Intra-daily variability, a parameter that reflects the fragmentation of activity (i.e. frequency and extent of transitions between activity and rest) consistently increased during and after MPTP treatment (**Figure 5E**). Thus, this method of comparison showed similar outcomes during and after MPTP treatment for the spontaneously recovered cases. For case L, that continued in a symptomatic state, all parameters remained dramatically affected.

Discussion

We have designed a protocol for chronic multidimensional characterisation of non-motor aspects of PS. Despite inter-individual differences, monitoring a range of behavioural parameters, our results clearly show that repeated exposure to low-doses of MPTP induces early and long-term non-motor features of idiopathic PS (see Table S1 for a resume of main results). Furthermore, all cases exhibited very early changes in both rest-activity cycles and cognitive performances, virtually immediately after the first MPTP administration and prior to clinical motor symptoms.

A key finding is the demonstration that the MPTP induced changes in chronobiological parameters constitute a precocious and consistent feature of the pre-symptomatic phase that precedes or coincides with measurable cognitive decline (i.e. after the 1st MPTP injection in case J and L, the second MPTP injection in case T and the 6th injection in case F). Identification of the temporal sequence of behavioral events (chronobiology, cognitive, motor) subsequent to MPTP intoxication benefited from several factors. The within subject experimental design allowed each animal to serve as its own control, decreasing variability and increasing the sensitivity of the measures. The long-term, continuous 24h monitoring of behavior permitted a fine grain analysis of behavioral decline for several chronobiological parameters.

Cognitive deficits. The cognitive decline induced by MPTP-intoxication was typically pre-symptomatic (i.e. before the onset of characteristic parkinsonian motor symptoms) and persisted after full clinical recovery. The detour task exploited here is very simple to learn, easy-to-test, has been used in numerous protocols and is considered to be dependent on the integrity of frontal cortex, the dopaminergic system and DA innervation of frontal cortex. For example, these tests were used by Diamond and Goldman-Rakic in the 1980's to demonstrate that human infants and infant monkeys show a clear developmental progression of cognitive abilities that accompanies maturation of the DA system (see [27] for a review). Cognitive operations, crucial for planning and problem solving, are known to be impaired in both parkinsonian patients and CLD MPTP-monkeys [9,10,28,29]. In monkeys, cognitive and complex motor planning abilities under MPTP have previously been tested using various protocols including extra- and intra-dimensional shifting tasks, delayed response tasks, Go No-Go tasks, context dependant movement selection (automated version of the ODRT), multiple choice retrieval tasks, and the object detour retrieval task [9,10,13,30]. These

investigations have shown that cognitive impairments can occur without clinical symptoms when using low-dose MPTP injections with, in some cases, maintenance of deficits several weeks (e.g. [31]) or years after MPTP exposure [32]. Here we show that the detour task revealed deficits within one to five weeks of low dose treatment. Previous investigations also reported cognitive deficits at various delays depending on the injection protocol [10,13,30]. Inter-individual variability of the onset is frequently reported. Our day by day examination of cognitive performance showed the first significant decline to be detectable after 1st injection at day 2 in case T, 4th injection at day 11 in case J, 2nd injection at day 4 in case F and after the 5th injection at day 19 in case L.

The classical DA levodopa therapy protocol clearly improved performance in cases J and T. In case J the error rate decreased and the success rate improved to levels that were not significantly different to those prior to MPTP treatment. In case T, DA levodopa therapy led to 100% success rates and zero failure rates, rates that were actually superior to those encountered prior MPTP treatment. These results indicate that poor cognitive performances can be imputed to a dopaminergic deficit in the brain.

Our results thus confirm previous observations and indicate that the detour task allows detection of early presymptomatic frontal behavior deficits during CLD-MPTP and their possible maintenance after clinical recovery.

Chronobiology. Following the MPTP treatment and despite a recovery from motor symptoms, the rest-activity profiles did not return to a completely normal state throughout the duration of the experimental observation period. These findings differ from previous studies of daily activity patterns in MPTP monkeys that mainly showed (i) a decrease in the overall amount of activity without any obvious modification of the day-night activity profiles and (ii) changes in rest-activity uniquely when clear motor symptoms were present [33]. This latter finding differs from our results where changes in activity profiles are found in the presymptomatic stage. These differences with our results may be due to the mode of intoxication (i.e. intravenous injections of MPTP with twice the concentration we used), the age of monkey used (3 years old i.e. juvenile) and the lack of pre-intoxication control measures from the same monkey. Intravenous MPTP-treatment, corresponding to an acute intoxication is known to be much more offensive towards the DA system and induces a different topography of the DA lesion within basal ganglia [34]. It has also been shown that older monkeys are more suitable to reproduce the characteristic features of this age-related

disease [35,36]. Our results are consistent with those of Barraud et al. (2009) [11] that showed a long-term disruption of sleep–wake architecture and a reduced sleep efficiency that can be observed years after MPTP administration in monkeys suffering from chronic clinical motor symptoms following MPTP administration.

The present findings extend the observations of others by showing that alterations of the locomotor activity rhythm can persist in the absence of stable clinical symptoms and are not necessarily correlated with the degree of motor disability [11,33]. The persistence of activity alteration is particularly evident in the quantile analyses. The quantile analysis allows a representation of the behavioral effects during the different periods according to the experimental stages rather than on their durations and provides a means to equate the behavioral changes independent of the time required to attain the clinical criteria. This analysis shows that despite the different effects on day and night time activities, following motor recovery, all cases presented similar dynamics in the deterioration of the stability, amplitude and variability of the rest-wake cycle.

Alterations in sleep, including excessive daytime sleepiness, sleep fragmentation and rapid eye movement (REM) sleep deregulation are observed in MPTP treated monkeys [11] and in PD patients during early and symptomatic stages of the disease [37]. In humans, actigraphy (motor activity) is routinely used to detect circadian rhythm disorders [38]. The technique has also been validated against polysomnography to assess the quality and fragmentation of sleep and wakefulness [39,40]. Although in monkeys actigraphy has not been compared to polysomnography, it is likely that, as in humans, an increase in motor activity during the night time is a marker of sleep disturbance, and that a decrease in activity during the daytime could be a sign of daytime sleepiness, in agreement with Barraud et al. (2009) [11]. These two changes in activity that we observed during both the presymptomatic and symptomatic phases, could therefore mimic the patterns of sleep fragmentation and excessive daytime sleepiness observed in PD patients [41].

The origin of these rest-activity (or sleep) disturbances in MPTP monkeys is still a matter of speculation. In MPTP treated monkeys some studies e.g. [42] have reported loss of dopaminergic as well as other monoaminergic cell populations (noradrenalin, serotonin) whereas others report only dopamine degeneration e.g. [11] or together with increased levels of serotonin [43]. PD patients show multiple neuronal system degenerations [44]. Furthermore, it is unknown whether the observed chronobiological alterations can be attributed to direct or indirect consequences of a breakdown of dopamine homeostatic

regulation affecting the sleep-wake system and/or the circadian oscillator system. At least some outputs of the circadian control system appear to be conserved, since the concentration and rhythms of melatonin, cortisol and prolactin secretion reportedly remain largely intact [45].

Inter-individual variability. The four monkeys showed a differential vulnerability to the MPTP treatment, in agreement with previous studies [6,7,18]. All three female cynomolgus showed highly similar early and long-lasting non-motor alterations during and after CLD-MPTP treatment. Despite inter-individual difference we clearly show that all cases exhibited very early changes in both rest-activity cycles and cognitive performances, virtually immediately after the firsts MPTP administration and prior to clinical motor symptoms.

In case T the period of intoxication required to reach clinical significance was nearly 3 to 5 times longer than for other cases. The lower sensitivity of case T to MPTP at the doses and frequency employed here (0.2 mg.kg^{-1} every 3-4 days) may have allowed specific compensatory mechanisms that were distinct from those in other cases, which however did not differentially affect the expression of non-motor symptoms (here rest-activity cycle alterations and cognitive deficits). In case T, cognitive difficulties in the detour reaching task were stable during the MPTP treatment but were practically undetectable after spontaneous clinical recovery. We observed that case T learned the detour reaching task more rapidly than other cases. In view of these observations case T was also trained to a more complex delayed response task (spatial working memory task with a delay of 10sec, **Figure S2**) that revealed stable cognitive deficits throughout the presymptomatic period and persistence after full clinical recovery. These results are in agreement with those showing that the level of cognitive deficits depends on task difficulty, in both parkinsonian patients [46] and rhesus monkeys treated with MPTP [13]. The variability between subjects could originate from various factors ranging from individual genetic susceptibility to the treatment, to age, species or gender differences [6,18,47]. Although several species (macaques, vervet monkeys) and both genders have been used in previous experiments no comprehensive comparative studies have been made with non-human primates. The issue of variability is also illustrated by the fact that fully clinically recovered animals can present DA cell loss that is equivalent to those observed for severe symptomatic cases [6]. Similarly, asymptomatic-recovered monkeys

show similar changes in subcortical/cortical aminergic levels compared to those observed for symptomatic cases [48].

Here we show that the use of quantile analyses to equate the different durations of each phase overcomes some aspects of individual variability and allows a more rationalized behavioral evaluation. Nevertheless the inter-variability observed in response to treatment or for post-treatment recovery in the MPTP model is clearly of value since it may help to better understand the variability of human PS.

Compensatory mechanisms. The use of low dose MPTP models potentially allows determination of the functional basis of the compensatory mechanisms leading to or accompanying spontaneous motor recovery and possibly involving more than one aminergic system [6,42,49]. Our finding that chronobiological and cognitive symptoms persist in motor recovered animals poses a number of questions concerning the physiological status of motor-recovered animals. Along with previous studies that have shown cognitive deficits during the presymptomatic period the present findings emphasize that the full spectrum of deficits during this stage is more extensive than previously thought. This kind of protocol (longitudinal and using the CLD-MPTP model) aims at reaching deeper insights into the presymptomatic period and should be further used to determine clearly if neurophysiological markers of sleep structure [11] or of performance monitoring [50] can ultimately be used as reliable diagnostic tools for ongoing PS in humans.

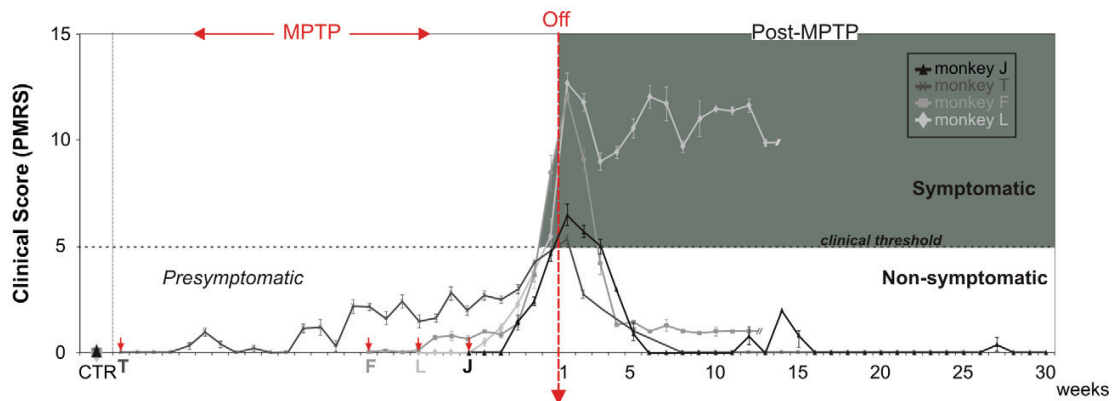
Figures:

Figure 1. Progression of Clinical Scores in MPTP treated monkeys. Weekly clinical measures (mean \pm SEM) for all monkeys aligned on MPTP treatment cessation (MPTP-Off). The shaded area corresponds to the symptomatic stage. During chronic low dose MPTP intoxication (MPTP) and prior to attaining the clinical threshold of 5, monkeys are considered as presymptomatic. After MPTP-Off, 3 monkeys spontaneously recovered to a non-symptomatic stage within 1-4 weeks while 1 monkey remained in a mild-symptomatic stage. Arrowheads in red indicate the time of MPTP onset for each animal.

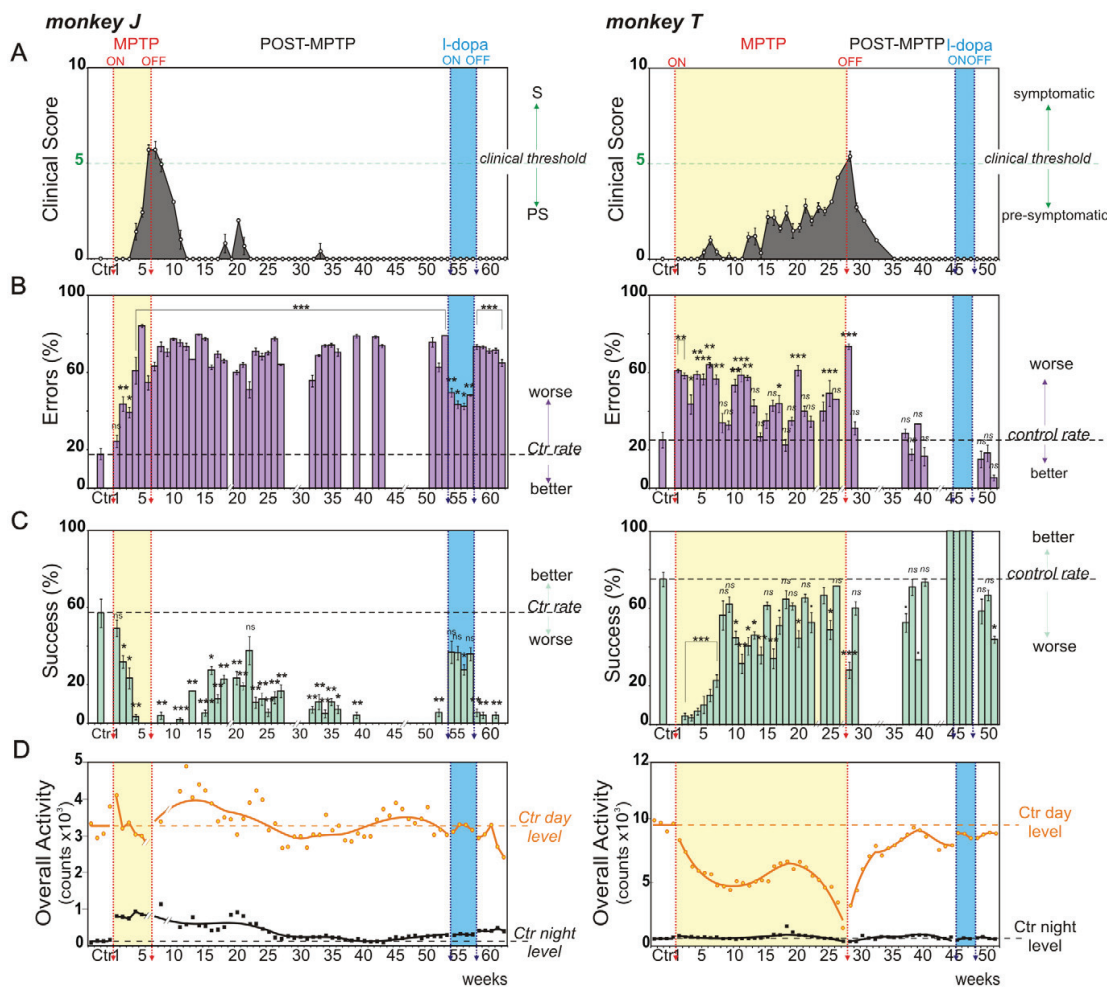


Figure 2. Evolution of behavioral parameters throughout the entire protocol. Weekly behavioral measures (mean \pm SEM) for monkey J, left and monkey T, right. **A.** Clinical score variations from PMRS. Note that a score above 5 defines the symptomatic stage (clinical threshold, green line). **B.** Percentage of errors (barrier hits, purple) and **C.** percentage of successes (direct reach, blue) for the detour task (detour trials, see Figure S3). Performances (successes, errors) during and after MPTP-treatment are compared to control measures (horizontal dashed lines). **D.** Total daytime (orange circles) and night time (black squares) locomotor activity counts and associated curves of activity smoothed with the rloess method (span=0.5). Baseline levels are represented by dotted lines for each behavioral parameter (Ctr day, Ctr night). Stars refer to statistics applied on Success and Error rates. ns: non-significant, \cdot : $p < 0.1$, $*$: $p < 0.05$, $**$: $p < 0.01$, $***$: $p < 0.001$.

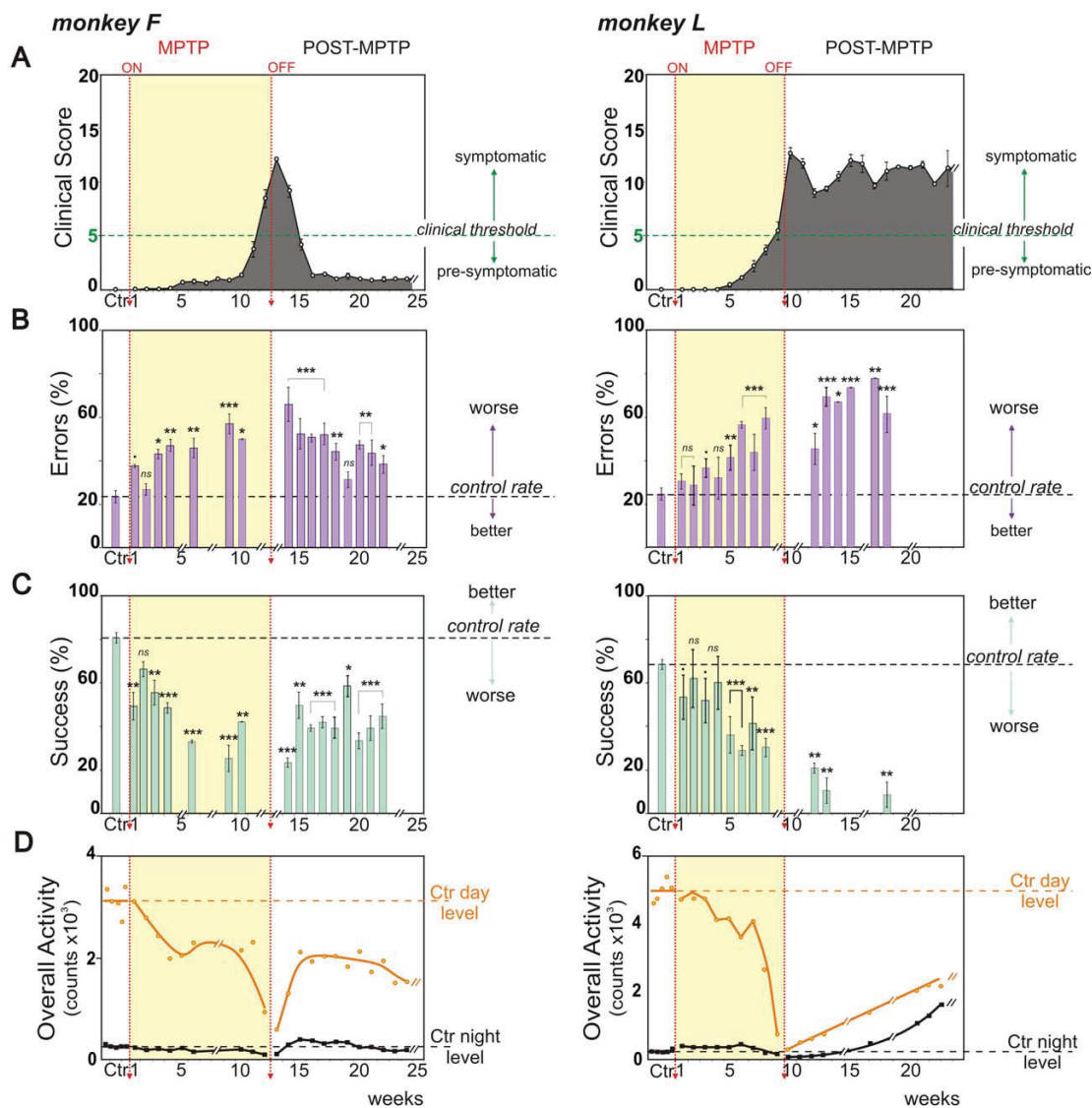


Figure 3. Changes of behavioral parameters over MPTP and Recovery periods. Weekly behavioral measures (mean±SEM) for monkey F, left and monkey L, right. **A.** Clinical score variations from PMRS. **B.** Percentage of errors (barrier hits, purple) and **C.** Percentage of successes (direct reach, blue) at the detour task. **D.** Total daytime (orange circles) and night time (black squares) locomotor activity counts and associated curves of activity smoothed with the rloess method (span=0.5). Conventions as for Figure 2.

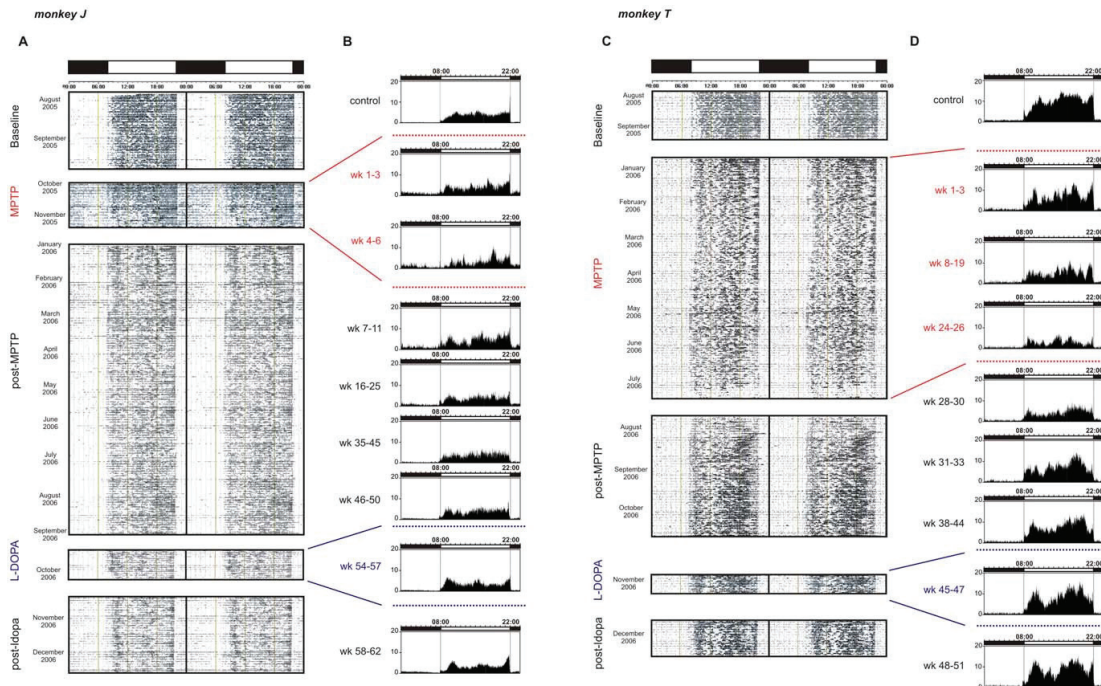


Figure 4. Locomotor activity rhythms (monkey J): **A.** 48h double-plot actogram: activity presented over consecutive days (each line is 48h) for the entire protocol. Daytime and night time periods are delimited by white and black rectangles, respectively (above actogram). Note the strong synchronization of activity at lights-off. **B.** 24h average locomotor activity profiles of 3 representative weeks taken over different time-windows of the protocol (time-window periods indicated on the left of profiles). Note the clear increase of night-time activity during MPTP and the progressive decrease after MPTP-Off.

Locomotor activity rhythms (monkey T): **C.** 48h double-plot actogram and **D.** 24h average locomotor activity profiles (average over 3 representative weeks within the period indicated on the left). Note the clear fragmentation and amplitude decrease of daytime activity with MPTP intoxication and, the progressive return to near baseline after MPTP-Off.

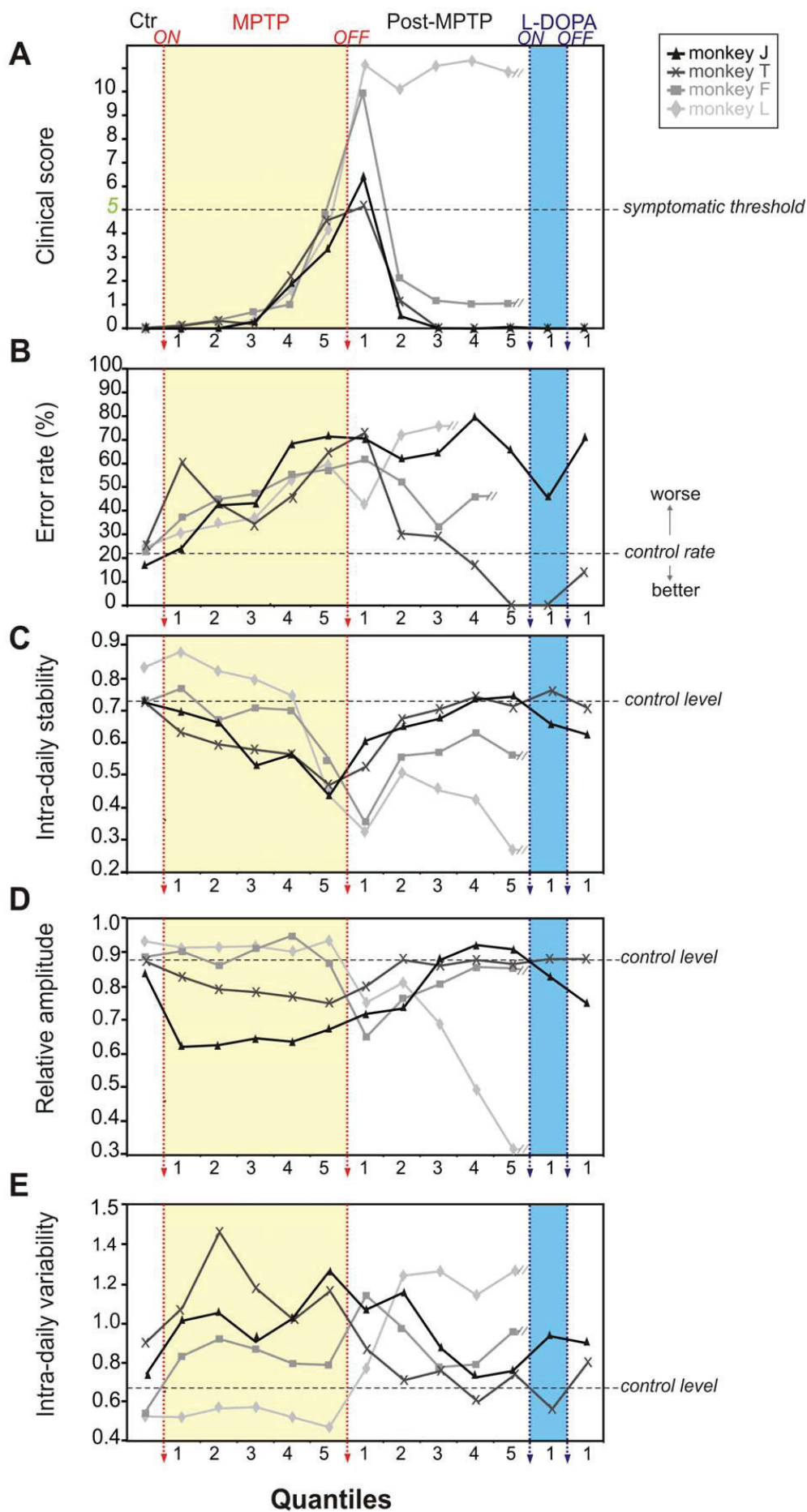


Figure 5. Quantile Analysis. Clinical Scores (**A**), behavioral performance (**B**, error rate) and measures on rest-activity rhythms representing intra-daily stability (**C**, strength of coupling), relative amplitude (**D**, rhythm intensity) and variability (**E**, fragmentation) for case J (black), case T (dark grey), case F (grey) and case L (light grey).

Supporting Table

	monkey J	monkey T	monkey F	monkey L
Duration (in weeks)				
Presymptomatic state	6	27	11	8
Symptomatic state	2	1	3	>12
Recovery period	6	7	5	-
Total cumulated dose (in mg/kg)	2.2	10.4	5.4	4.4
ORDT (errors %, mean \pmSD)				
CTR	<i>17 \pm15</i>	<i>25 \pm20</i>	<i>23 \pm8</i>	<i>24 \pm10</i>
Presymptomatic state	51 \pm 21	46 \pm 12	43 \pm 10	41 \pm 12
Symptomatic state	68 \pm 7	73 \pm 7	65 \pm 18	66 \pm 11
Recovery period	74 \pm 3	31 \pm 17	51 \pm 0.7	-
ORDT (success %, mean \pmSD)				
CTR	<i>57 \pm36</i>	<i>75 \pm19</i>	<i>81 \pm7</i>	<i>68 \pm9</i>
Presymptomatic state	18 \pm 20	40 \pm 23	46 \pm 14	46 \pm 13
Symptomatic state	2 \pm 3	28 \pm 21	23 \pm 6	7 \pm 8
Recovery period	0.5 \pm 1	60 \pm 17	44 \pm 6	-
Overall activity, in average counts $\times 10^3$				
Day counts/night counts (Day/Night, in % of Total)	<i>3.3/0.1 (97/3)</i>	<i>7.9/0.5 (94/6)</i>	<i>3.1/0.3 (92/8)</i>	<i>4.9/0.2 (96/4)</i>
CTR				
Presymptomatic state	3.3/0.8 (81/19)	4.5/0.5 (90/10)	2.4/0.2 (92/8)	4.1/0.3 (92/8)
Symptomatic state	3.4/1.1 (75/25)	2.7/0.3 (91/9)	1.0/0.2 (84/16)	1.2/0.5 (69/31)
Recovery period	4.6/0.5 (90/10)	5.7/0.4 (93/7)	2.0/0.4 (84/16)	-

Table S1. Summary table of results. Behavioral data and MPTP cumulated dose are shown for each monkey (columns) and for the major periods of the follow-up study: control (CTR), presymptomatic state (PMRS score <5), symptomatic state (PMRS score >5) and recovery period. -: not available.

Supporting Figures Legends

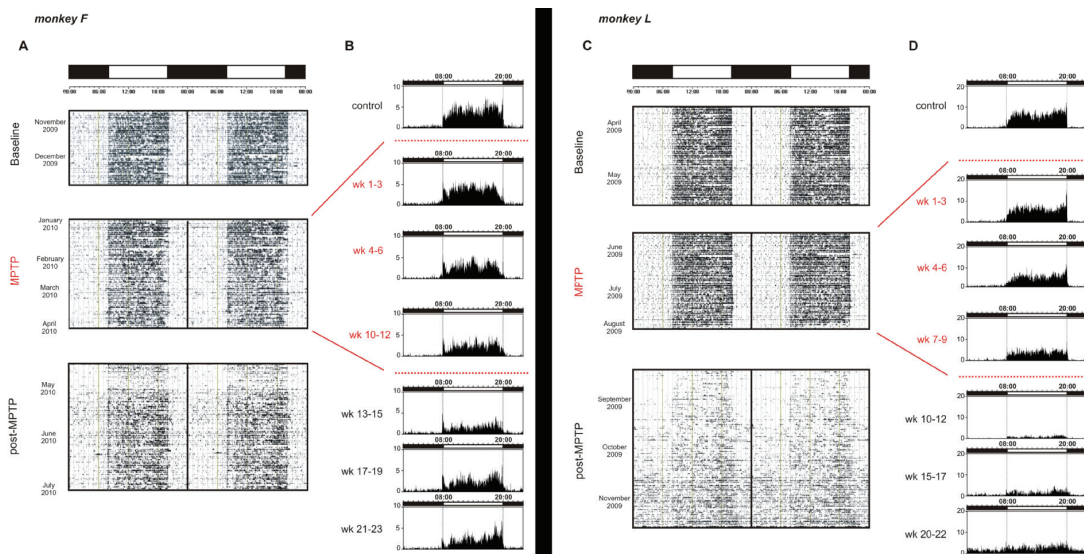


Figure S1. Locomotor activity rhythms (monkey F): **A.** 48h double-plot actogram: activity presented over consecutive days (each line is 48h) for the entire protocol. Daytime and night time periods are delimited by white and black rectangles, respectively (above actogram). Note the strong synchronization of activity at lights-off and fragmentation of daytime activity with MPTP treatment. **B.** 24h average locomotor activity profiles of 3 representative weeks taken over different time-windows of the protocol (week periods indicated on the left of profiles).

Locomotor activity rhythms (monkey L): **C.** 48h double-plot actogram and **D.** 24h average locomotor activity profiles (average over 3 representative weeks within the period indicated on the left). Note the clear fragmentation and amplitude decrease of daytime activity with MPTP intoxication. While we can observe a progressive return of daytime activity after MPTP-Off, this is far from baseline levels and moreover accompanied by a strong increase in night time activity.

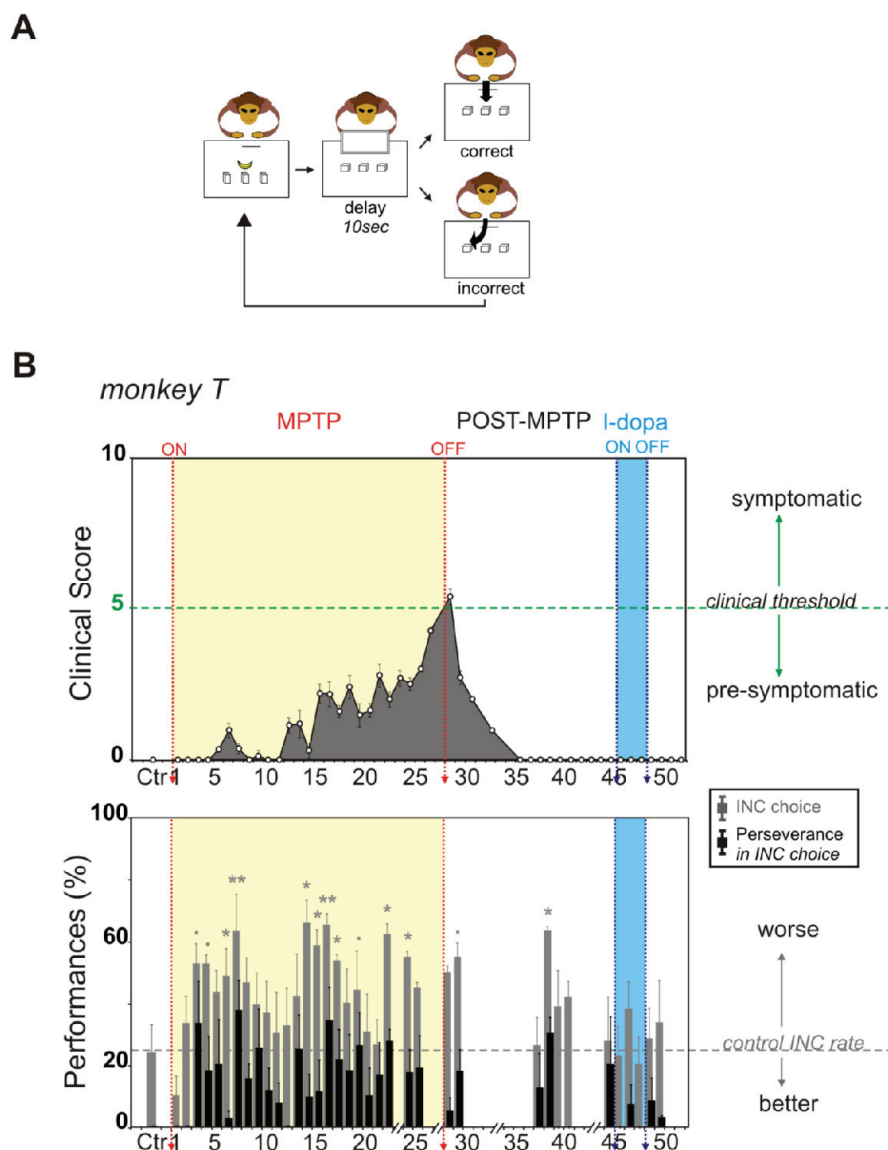


Figure S2. Spatial memory task.

A. Spatial delayed retrieval task paradigm. The monkey is facing 3 wells with opaque cover; first, wells are opened so that the monkey can see which one contains the reward. Then an opaque screen is placed between animal and wells during 10sec; after the delay the monkey had to reach the correct well to obtain the reward. In case of error, the trial start again until reward is found. Perseverance in incorrect choices is carefully noted: incorrect trials for which the monkey makes the same incorrect choice as in the previous trial. This task evaluates the integrity of short term spatial memory. **B.** Clinical score (up) and performances (down) of monkey T along the entire protocol. Note the clear expression of frontal-like behavior (perseverance, black bars) with MPTP intoxication and persistence after MPTP-off. *In B.* stars correspond to statistical results of contrast with control values (see material and method section); *: $p < 0.05$, **: $p < 0.01$, †: $p < 0.07$ i.e. marginally significant.

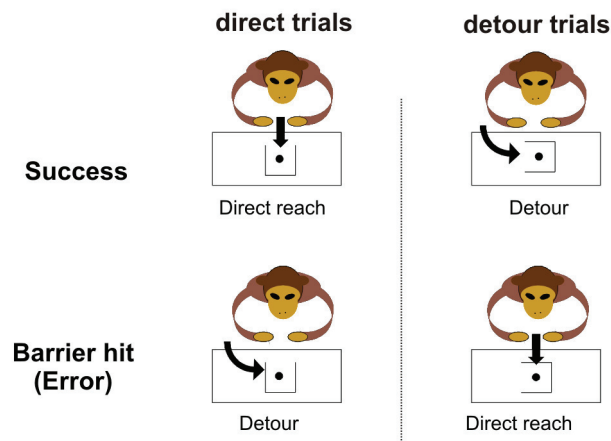


Figure S3. Detour Task paradigm.

In this task monkeys have to reach a piece of fruit placed in a transparent box with only one opened window. In direct trials (left), the opening is facing the animal so that reach is straightforward; in detour trials (right), the opening is not facing the monkey so that a detour has to be planned before reaching the reward. This easy-to-learn and easy-to-test task evaluates lack of inhibition, behavior dependent on the integrity of frontal cortex, the dopaminergic system and DA innervation of frontal cortex.

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Chapter 4

Title**Loss of Circadian Rhythmicity in an MPTP non-human primate
model of Parkinson's disease****Running title****Circadian alterations in MPTP Monkeys****Authors and Affiliations****Karim Fifel^{1,2}, Julien Vezoli^{1,2,3}, Bruno Claustrat^{1,2}, Vincent Leviel^{1,2}, Henry Kennedy^{1,2}, Emmanuel Procyk^{1,2}, Ouria Dkhissi-Benyahya^{1,2}, Claude Gronfier^{1,2}, Howard M Cooper^{1,2*}**

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Abstract

Disturbances of the daily sleep/wake cycle are common non-motor symptoms of Parkinson's disease (PD). However, the impact of dopamine (DA) depletion on circadian rhythms in PD patients or non-human primate (NHP) models of the disorder have not been investigated. We thus evaluated alterations of circadian rhythms in NHP following MPTP lesion of the dopaminergic nigro-striatal system. In a light:dark cycle, control and MPTP-treated NHP both exhibit rest-wake locomotor rhythms, although DA depleted NHP show reduced amplitude, decreased stability and increased fragmentation. In all animals, 6-sulphatoxymelatonin peaks at night and cortisol in early morning. When the circadian system is challenged by exposure to constant light, controls retain locomotor rest-wake and hormonal rhythms that free-run with stable phase relationships whereas in the DA depleted NHP, locomotor rhythms are severely disturbed or completely abolished. The amplitude and phase relations of hormonal rhythms nevertheless remain unaltered. Use of a light-dark masking paradigm shows that expression of daily rest-wake activity in MPTP monkeys requires the stimulatory and inhibitory effects of light and darkness. *In vivo* PET ($[^{11}\text{C}]$ -PE2I) and post-mortem TH and DAT quantifications showed that the extent of dopaminergic degeneration was not an accurate predictor of the loss of circadian rhythmicity. These results suggest that following DA lesion, the SCN clock remains intact but, in the absence of environmental timing cues, is unable to drive downstream rhythmic processes of striatal clock gene and dopaminergic functions that control locomotor output. These findings emphasize that appropriate light exposure is essential for optimal circadian function in PD patients.

Keywords

dopamine / chronobiology / sleep / orexin / retina / melanopsin / masking

Introduction

Disturbances of the sleep-wake timing are a prominent feature of non-motor symptoms of Parkinson's disease (PD), expressed as insomnia, excessive daytime sleepiness and fragmentation of sleep architecture (1, 2). While the loss of dopaminergic neurons in the nigro-striatal pathway is the main neuropathological feature and cause of motor disabilities in PD (3), dopamine (DA) is also involved in several aspects of sleep regulation and is an important wake promoting factor across species (4, 5). Recently, DA has been also shown to be closely linked with circadian clock function. Almost all aspects of dopaminergic transmission exhibit daily rhythms of expression (6-8) that are both light and clock dependent (9, 10). Inversely, DA has been shown to modulate clock gene expression both *in vivo* and *in vitro* (8, 11, 12) and lesion of the nigro-striatal system disrupts the daily and circadian rhythms of rest-activity and striatal clock gene expression (12).

Given the association between DA and circadian processes and the fundamental role of the circadian system in the determination of sleep timing (13, 14), we explored the alterations of daily and circadian rest-wake activity and hormonal rhythms in the DA depleted MPTP-treated non-human primate (NHP). The MPTP-treated NHP is considered a highly appropriate model of PD since, in addition to cognitive and motor deficits, it closely replicates several features of sleep and wake abnormalities, including excessive daytime sleepiness, REM sleep disturbances and altered day-night activity patterns. Many of these precede the appearance of motor symptoms and have been proposed as early biomarkers of the premotor phase of PD (2, 15, 16). Alteration of hormonal rhythms has been reported in both MPTP monkeys (17) and in PD patients (18-21). However, an important limitation of these studies is that investigations were conducted under a light/dark cycle that exerts strong acute and chronic effects on daily physiology and behavior patterns (22, 23). None of these animal studies nor the observations in PD patients have been performed under constant conditions. Because the absence of cyclic environmental time cues is a prerequisite to determine the impact of DA neurodegeneration on circadian functions (24) we have examined the rest:activity rhythms in lighting conditions that challenge the circadian timing system in the MPTP monkey model of PD.

Our findings show that in constant conditions, rhythmic rest-wake activity after MPTP treatment is strongly attenuated or abolished whereas hormonal rhythms are conserved. These results argue that the circadian clock remains intact but, in the absence of environmental light-dark cues is unable to drive locomotor rhythms, suggesting that the

circadian component of the sleep-wake disturbances observed in PD is more profoundly affected than previously assumed.

Results

MPTP-treated non-human primates show a loss or reduction of circadian rhythmicity under constant conditions. Under a daily 12h/12h light/dark cycle (LD), control (n=4) experimental animals prior and post-MPTP treatment (n=7) expressed a clear diurnal pattern of activity, although in the treated monkeys certain behavioral parameters were less precise (Fig. 1, S1), as has been previously reported (16, 25). Compared to controls, MPTP animals showed a reduced activity during the light phase ($91.5 \pm 0.6\%$ vs $86.0 \pm 1.3\%$, $P < 0.01$) and a decreased amplitude of the activity rhythm ($\chi^2 = 7087 \pm 534$ vs 4553 ± 385 , $P < 0.001$). In MPTP animals activity onsets were less precise (Fig. S2A) and the rest-activity cycle was more fragmented and less stable (Fig. S2E-F). However, activity offsets and the phase angles of both activity onsets and offsets were not significantly affected after MPTP treatment (Fig. S2B-D).

When control animals were released into constant conditions (LL) they expressed a robust free running circadian rhythm with a mean period close to 24h (23.99 ± 0.04 h; Fig. 1A) with $89.4 \pm 0.7\%$ of activity consolidated during the subjective day and a mean amplitude (χ^2 periodogram) of $4,983 \pm 256$. In contrast, 3 out of 7 MPTP-treated animals showed a complete loss of circadian rhythmicity (Fig. 1B, S1B-C) with locomotor activity homogeneously distributed throughout the 24h period. The remaining treated animals showed weak, low amplitude circadian rhythms (examples in Fig. S1D-F) with less precise activity onsets (Fig. S2H). Compared to controls, MPTP-treated animals showed a slight but insignificant relative decrease in the mean overall 24h activity in LL compared to LD (control: $0.58 \pm 5.8\%$; MPTP: $-16.70 \pm 6.24\%$) with the notable exception of one arrhythmic animal that significantly increased activity ($+151 \pm 5.6\%$; $P < 0.001$; Fig. S1B). Upon the transition from LD to LL conditions, the 4 rhythmic animals showed longer, but not significant, phase angle delays of activity onset compared to controls (Fig. S2I) indicating that entrainment under LD conditions was not altered after MPTP treatment. When re-exposed to LD conditions, both rhythmic and arrhythmic animals regained rhythmic behavior similar to that expressed in LD before LL (Fig. 1B, S1).

Circadian rest-wake rhythmicity is more disturbed than daily rhythms. It is well known that in both PD patients and in MPTP-treated NHP there is a high degree of variability between individuals in the expression and severity of motor symptoms (26). We therefore

examined the relationship between the clinical state of the animals and the amplitude of activity rhythms under LD and constant LL conditions. In an LD cycle, both control and MPTP animals maintained significant amplitudes of day-night rhythms with activity concentrated during the light phase regardless of the severity of parkinsonian motor symptoms (Fig. 1E). Under LL conditions, controls showed a robust circadian rhythmicity similar to that in LD (χ^2 amplitude; 5252 ± 500). Following MPTP treatment, animals in non-symptomatic and mildly symptomatic states showed distinct free-running rhythms but with a significantly reduced amplitude compared to controls (non-symptomatic, $\chi^2 = 2890 \pm 447$, $P < 0.001$; mildly symptomatic, $\chi^2 = 2831 \pm 361$, $P < 0.001$). One notable exception was a non-symptomatic animal assessed 7.3 months after MPTP treatment that displayed a robust day-night rhythmicity in LD conditions but a complete loss of circadian rhythm when exposed to LL (Fig. S1B, 1E). Animals in a highly symptomatic state showed a marked dampening or a loss of rhythmicity in LL (Fig. 1E). In the MPTP animals that conserved a significant circadian rhythm under constant conditions, there was no significant change in the period of the endogenous rhythm compared to controls (Fig. S2E). We plotted the amplitude of the rhythm as a function of motor clinical score in order to explore in greater detail the relation between expression of circadian rhythms and severity of motor symptoms (Fig. 1F). In an LD cycle, there is an inverse correlation between χ^2 amplitude and clinical score of the animals ($P < 0.0001$) with the most severely affected animals showing the lowest amplitude (Fig. 1F). In constant light, this correlation is weaker ($P < 0.01$) and clinical score was not an accurate predictor of the loss of rhythm. For example, arrhythmia was seen not only in symptomatic animals, but also in one completely non-symptomatic animal (Fig. 1F).

The extent of dopaminergic degeneration was evaluated in a longitudinal follow-up study using PET scans of [^{11}C]-PE2I as a marker for visualization of DA transporters (DAT; 26, 27) and post-mortem histological examination of TH and DAT protein levels in the substantia nigra, VTA and striatum. At the end of MPTP treatment, PET scans ($n=4$) revealed an overall mean decrease of striatal [^{11}C]-PE2I binding potential of $83 \pm 1.7\%$ (Fig. 1G). The most affected structures were the putamen and the caudate nucleus (total reduction of $88\% \pm 1.1$) compared to the nucleus accumbens that was less affected ($74 \pm 1.6\%$ reduction). The postmortem histological quantifications ($n=5$) showed a comparable amount of reduction in the striatum for DAT ($63 \pm 8.2\%$; PET-scan cases [$n=3$]: $71 \pm 11.5\%$) and for TH immunoreactivity ($55 \pm 6.7\%$; PET-scan cases [$n=3$]: $57 \pm 11.8\%$). Reduction in mesencephalic structures (SN and VTA) averaged $40 \pm 4.6\%$ for TH and $58 \pm 4.8\%$ for DAT staining. A loss of circadian rhythmicity in LL was not correlated with the degree of striatal DA loss. Of the 4 animals that showed a final 82-90% reduction of [^{11}C]-PE2I, 2 showed

arrhythmia while 2 maintained a circadian rhythm in LL although the rest-activity cycle became less consolidated with a $50.3 \pm 0.33\%$ reduction in amplitude. The clinical scores for these subjects ranged from 7 to 13. Furthermore, one monkey that had one of the lowest reduction in striatal DA (-46.6%, striatal DAT immunolabel) and no clinical symptoms (score =1) was arrhythmic in constant conditions but showed a normal rhythm in LD cycle.

Endogenous circadian hormonal rhythms remain intact in MPTP-treated monkeys. The disruption or the loss of rhythmicity seen in MPTP-treated monkeys suggests that the functional integrity of the SCN and/or the endogenous 24h rhythm might no longer be intact (28-29). To explore this possibility, we assayed the rhythms of 6-sulphatoxymelatonin (aMT6s, the major metabolite of melatonin) and cortisol, two hormonal gold-standard output markers controlled by the SCN endogenous clock. Under LD entrainment conditions, both control and MPTP-treated animals show significant 24h rhythmicity in aMT6s and cortisol (Fig. 2). In both controls and MPTP-treated animals the peak of aMT6s was observed as expected during the night and was in opposition of phase with the diurnal peak of activity, whereas the peak of cortisol occurred in early morning (Fig. 2A, C, E, G).

Under constant conditions, all control and MPTP-treated animals all showed a significant circadian rhythmicity of both aMT6s and cortisol (Fig. 2B, D, F). The phase relationships of activity and hormonal rhythms in controls and in rhythmic MPTP-treated animals were conserved as in LD. In both, the peak of aMT6s occurred during the subjective night in opposition of phase with activity while high cortisol levels were found during the early subjective morning (Fig. 2G-H). In arrhythmic animals, the times of subjective day and night could not be determined due to the lack of an activity phase marker. aMT6s and cortisol excretion nevertheless showed a significant circadian rhythm and retained normal phase relationships (Fig. 2F-H). The amplitudes of aMT6s and cortisol were not affected by MPTP treatment (Fig. 2I). Together, these results show that the endogenous rhythmicity of the SCN is unimpaired in MPTP-treated animals.

Masking effects of darkness on locomotor activity are unaltered following MPTP treatment. How can the maintenance of a daily rest-activity rhythm in an LD cycle in MPTP animals be explained given the absence of a circadian locomotor rhythm in constant conditions? In addition to entraining effects, light is known to exert an acute effect on locomotor activity known as masking (22, 23). In nocturnal rodents light strongly inhibits activity whereas in diurnal species, darkness is inhibitory (22, 23). We explored the possibility that masking effects play a role in the apparent synchronization of behavior to the

LD cycle by assaying activity in a 1h light/1h dark cycle (22, 23, 30). Both control and MPTP monkeys showed strong masking effects of darkness on activity (Fig. 3A-C). There were no significant differences in relative locomotor activity during the light phases (control= $73.1 \pm 1.6\%$, MPTP= $66.1 \pm 5.4\%$; Fig. 3D). The cosinor analysis showed a daily modulation of the average level of activity within both the light and dark phases in both control and MPTP animals with higher amplitudes in the controls (Fig. 3E, $P < 0.05$). The masking effect was observed for all treated monkeys regardless of the severity of motor symptoms and despite a significant reduction in both the amount of overall activity (control= 4040.6 ± 368.1 counts/24h, MPTP= 2156.4 ± 823.8 counts/24h; $P < 0.001$) and the amplitude of the rhythm (Fig. 3E).

Absence of alterations in the SCN, hypothalamus and retina following MPTP treatment. The conservation of hormonal rhythms and masking responses favors the hypothesis of an intact photic and circadian pathway, despite the disruption of circadian locomotor activity. We found no qualitative differences in the intensity of immunohistochemical staining or the morphology of vasoactive intestinal peptide (VIP) containing neurons in the SCN (Fig. S3A-B) between control and MPTP-treated animals. The hypothalamic orexinergic system plays a crucial role in sleep-wake regulation and recent findings have suggested a reduction of orexinergic neurons and orexin levels in ventricular CSF in PD patients (31, 32). However, in our study, we found no differences in the number, distribution or dendritic morphology of orexin-A immunoreactive neurons between control and MPTP-treated animals (Fig. S3C-G). In the retina, we found no significant differences in the density or dendritic morphology of TH stained dopaminergic amacrine interneurons and melanopsin ipRGCs following MPTP treatment (Fig. S4).

Discussion

The main finding of this study is that, in the absence of cyclic environmental day-night cues, DA depletion of the nigro-striatal pathway prevents the circadian system from effectively driving rhythmic rest-wake locomotor activity. This result was unexpected and suggested that the loss of rhythmicity results from a defect within the SCN endogenous clock. However, the conservation of normal amplitude and phase relations of hormonal rhythms in constant conditions suggests that the SCN clock is not dysfunctional and that the observed deficit resides in the downstream output control of rhythmic locomotor functions.

Following MPTP treatment, increased motor impairment caused a decrease in the total daily amount of motor activity and consequently a reduction in the amplitude of activity rhythms. As in previous studies of MPTP-treated monkeys in a light:dark cycle (15, 25, 16), rest-wake rhythms nevertheless remained synchronized in phase with the LD cycle, with activity predominantly confined to the light phase. However, compared to control or pre-treatment state, MPTP monkeys showed less precision in their daily rhythm expressed as decreased amplitude and inter-daily stability and increased variability of activity onsets at lights-on and intra-daily variability. In contrast, the time and variability of activity offset at lights-off was as precise in MPTP-treated as in the control animals.

The environmental light-dark cycle exerts a powerful synchronizing effect on the endogenous circadian clock of the SCN, thereby tightly controlling physiological and behavioral rhythms (29, 33, 34). The rhythmic control of the circadian clock on output functions can thus only be assessed in constant conditions where temporal influences of the light dark cycle are absent. Previous studies in PD patients and NHP models of the disease have not investigated alteration of locomotor and physiological rhythms under these conditions (15-21, 24,25). In LL, normal diurnal monkeys show robust circadian rhythms (present study; 35, 36) as do nocturnal rodents in constant darkness (37). We found that MPTP-treated monkeys exposed to constant light displayed highly disturbed circadian rest-activity rhythms, ranging from a poorly consolidated daytime activity of extremely low amplitude to complete loss of the circadian rhythm. Animals with greater motor deficits generally showed a reduced amplitude of rhythmicity in both LD and LL conditions compared to non- or mildly symptomatic states. However, a high clinical score or loss of DA function as measured by [¹¹C]-PE2I or DAT and TH in the SN were not reliable predictors of a complete loss of circadian locomotor rhythmicity. It has long been recognized that MPTP treated monkeys show large inter-individual variability in the severity of motor impairments in relation to DA loss, and in their vulnerability to neurotoxic treatment, possibly associated with differences in compensatory mechanisms (38, 39). In our study, one animal that showed

no clinical symptoms for several months following the end of MPTP treatment and a normal daily rhythm in LD conditions, nevertheless completely lacked a circadian rhythm in LL associated with a significant increase of continuous 24h activity. The loss of rhythmicity seen in our MPTP-treated animals is therefore not an epiphenomenon of MPTP-induced hypokinesia.

A loss of circadian expression of rhythmicity is a severe state that typically requires interference with, or impairment of, the core molecular clock mechanism in the SCN. Arrhythmia is typically observed following lesion of the SCN (28, 29) or invalidation of core clock genes (*Per*, *Cry*, *Bmal*, *Clock*; 29). Despite the absence of a functional clock in these cases, a daily rhythm of behavior can often be observed in the presence of a light-dark cycle, whereas upon release into constant conditions rhythmicity is abolished. Although in our NHP model we could not probe clock gene expression directly, a deficiency of the clock mechanism does not appear to be responsible for the loss of the activity rhythm. Indeed, the cytoarchitecture and main neuropeptide content (VIP) of the SCN is unaltered. Furthermore, hormonal rhythms of melatonin and cortisol are preserved with normal phase relationships in LD and LL, confirming that an endogenous rhythm is present in the SCN and is capable of driving these physiological outputs. These results are also consistent with previous studies that report little or no alteration of several hormonal rhythms in PD patients and MPTP-treated NHP (17-21).

Under LD conditions, light not only synchronizes rest-activity behavior through the circadian system, but also exerts an independent acute effect on locomotor activity known as masking (22, 23). Light strongly inhibits activity of nocturnal rodents whereas in diurnal species, darkness is inhibitory (22). In MPTP monkeys, the conservation of photic light detection and the ability of light to acutely affect behavior is confirmed by the normal morphology of melanopsin ipRGCs in the retina and the strong masking effects of darkness in parkinsonian monkeys, even in the highly symptomatic, arrhythmic cases. The robust inhibitory effect of darkness, coupled with the stimulating influence of light on activity in primates (22) is a plausible explanation for the maintenance of daily rhythms of activity in the LD cycle and is further exemplified by the abrupt offset of activity caused by lights off in the LD cycle even in the most affected animals.

Since the SCN oscillator controlling hormonal rhythms appears intact, the loss of circadian sleep-wake rhythmicity in the MPTP monkey likely lies downstream from the SCN, in the control of locomotor output behaviors. DA is a necessary component for normal motor behavior and is required to drive the expression of locomotor output activity (40). The primary deficit following MPTP treatment is the depletion of DA in the striatum. Virtually

all aspects of dopaminergic transmission (DA, TH, DA receptors, DAT transporter and the main DA metabolites DOPAC & HVA) show daily rhythms of expression in the striatum (6-8). Several of these components are also light and clock dependent since the diurnal rhythm is abolished or damped by lesion of the SCN (9, 10). Light also exerts an acute activating effect on DA in the substantia nigra and the striatum (41). Finally, *in vivo* and *in vitro* studies further suggest DA mediated regulation of clock genes in striatal neurons (8, 11, 12). The absence of DA rhythmicity in the rat striatum alters, but does not prevent a daily rhythm of activity whereas the circadian rhythm is abolished in constant darkness (7, 12). Per2 levels and cycling in the striatum but not in the SCN are also affected, suggesting a local role of DA in the regulation of both clock genes and behavioral output (12). Similar results have been described in DA deficient flies that show a decreased entrainment in LD but normal masking responses to light (42). These studies in rodents and flies, along with the present findings in NHP, support the idea that not merely DA per se, but rhythmic dopaminergic function is critical for the normal expression of rhythmic clock gene and control of rest-wake behavior. The importance of this circadian pattern of DA function is in accordance with observations that continuous infusion of DA medication during waking hours yields superior outcomes with fewer side effects compared to discontinuous or continuous 24h round-the-clock administration (43).

Our results suggest that in the neurotoxic MPTP treated NHP, the retinal input and core clock mechanism of the circadian timing system remain essentially intact but are unable to efficiently drive rhythmic locomotor activity. In normal animals, both the clock-dependent and the activating acute effects of light control the rhythmic expression of DA transmission that in turn drives rhythmic striatal gene expression and behavior. Following DA depletion, the combined stimulatory effects of light and inhibitory effect of darkness on the remaining dopaminergic and other systems (22) may be sufficient to sustain a daily rest-activity pattern in an LD cycle. However, in constant conditions these acute effects are lacking. We propose that the inability of the circadian system to drive rhythmic locomotor activity in the absence of environmental timing cues can be attributed to a downstream effect involving disconnection of the central circadian clock from its output target networks controlling locomotor function (see model in Fig. 4). As DA levels decrease following neurotoxicity, one can hypothesize that rhythmic dopaminergic neurotransmission falls below a hypothetical threshold necessary for overt locomotor rhythms. An alternative but not exclusive hypothesis is that DA may be required to synchronize the phase relationships of individual oscillatory neurons and to generate coherent systems level output of the striatal system, in which case

the function of DA in the striatum would be analogous to that of VIP as a coupling factor in the SCN (44).

The disconnection of circadian control of the dopaminergic system implies that exposure to a sufficiently luminous day-night cycle is critical for the maintenance of rhythmic dopaminergic function. This may be highly critical for elderly PD patients, particularly in health care facilities, as previous studies have shown that in elderly patients, increasing the ambient light levels during the day improves rest-activity rhythms, sleep quality, alertness and well-being (45). In northern latitudes where the circadian system is seasonally confronted with continuous day or night conditions it is tempting to speculate that rest-activity cycle and sleep disturbances in PD may be exacerbated during the summer or winter. Indeed, several epidemiological studies report increased occurrences of PD in northern compared to southern latitudes (46-48). Our results are of clinical importance and stress that sleep/wake disturbances associated with DA loss may be more severely affected than previously thought, in particular in sub-optimal lighting conditions. In the light of beneficial effects of long-term light therapy on motor and non-motor symptoms of PD (49), our study further emphasizes that the circadian component of PD related symptoms must be considered in therapeutic approaches along with classical DA treatments.

FIGURES

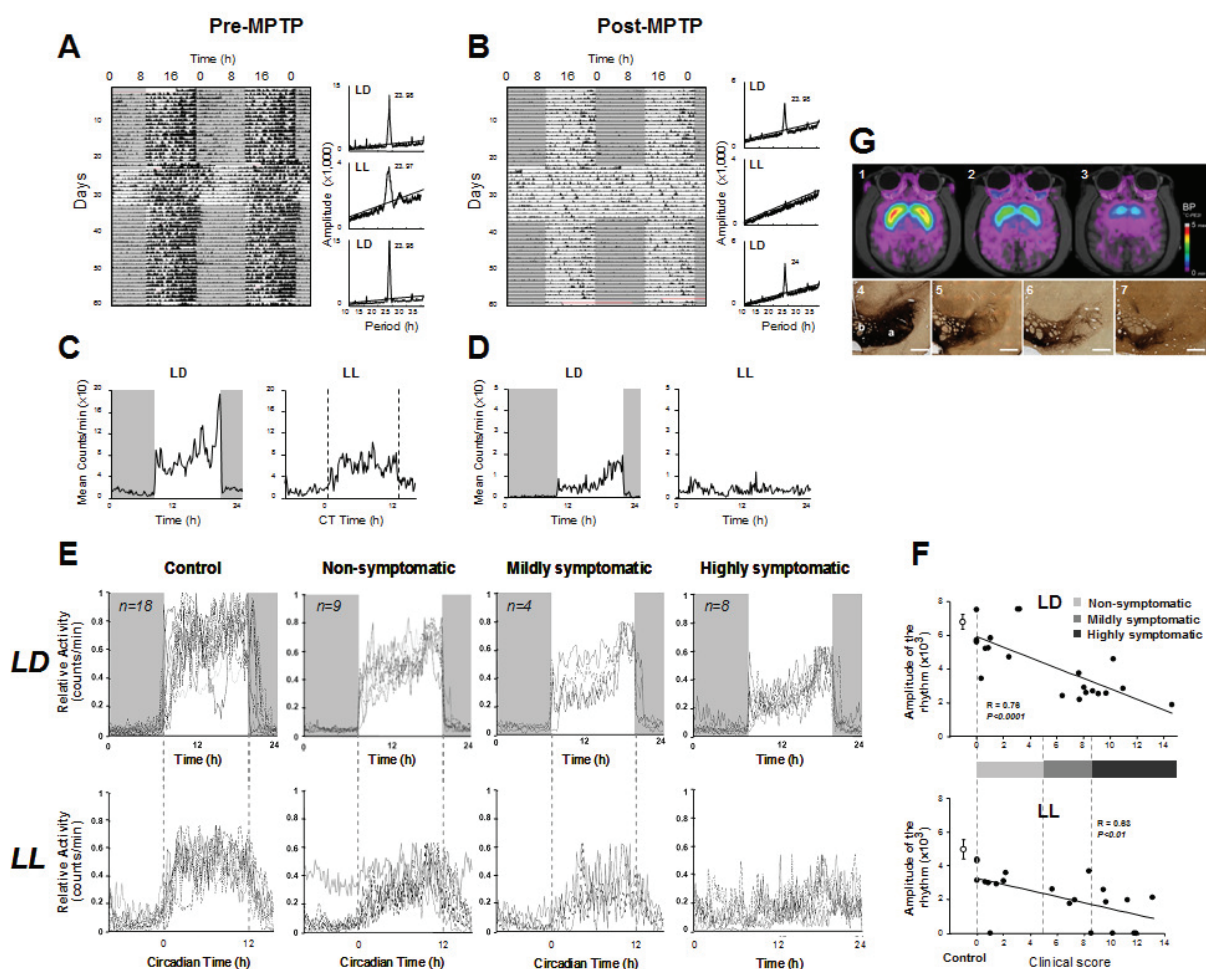


Figure 1. Following MPTP treatment, the circadian locomotor rest-activity rhythm is abolished under constant light. **(A)** Pre-MPTP treatment, daily locomotor activity is synchronized to the LD cycle (dark phase shown by shading) and in constant light (LL) the circadian rhythm free runs with a period of 23.97 hrs (Monkey Y). **(B)** Same case, 4.8 months post-MPTP treatment, rest-wake activity is synchronized to the LD cycle but becomes arrhythmic in LL. Compared to pre-treatment, periodogram analyses (panels to right of actograms) confirm a quantitative decrease in amplitude during LD and the absence of rhythmicity during LL. The averaged 24h locomotor activity profiles **(C-D)** illustrate the consolidated activity during the light phase in LD cycle both pre- and post-MPTP and during LL pre-MPTP but a complete loss of rhythmicity post-MPTP. **(E)** Averaged 24h locomotor rest-activity profiles for all control and MPTP-treated monkeys with individual traces for all recording periods ($n = 39$ cases) in LD (upper panel) and LL (lower panel) conditions. Data for LD always correspond to the recording period immediately preceding LL. Control (or pre-treatment) animals show a robust daily and circadian rhythmicity with a consolidated activity

during the light phase in LD and the subjective day in LL. In MPTP-treated animals, the daily rhythmicity of locomotor activity in the LD cycle is clearly evident but becomes progressively less robust in non-symptomatic, mildly symptomatic and highly symptomatic animals. When transferred to constant LL conditions, circadian rhythmicity deteriorates and highly symptomatic animals are arrhythmic with a continuous distribution of activity throughout the 24 h period. Note that the loss of rhythmicity is also recorded in one asymptomatic animal under LL (also shown in Fig S1B). Relationship between the amplitude of the rhythm (χ^2 periodogram) and clinical score in LD and LL (**F**). A complete loss of rhythmicity is mainly, but not exclusively observed for the highest clinical scores whereas some subjects with severe motor symptoms still maintained rhythmicity. Values are shown as mean \pm SEM.

MPTP treatment induced loss of dopamine neurons in the mesencephalon and dopaminergic depletion of the striatum (panels **G₁**, **G₇**). Examples of [¹¹C]-PE2I scans of the basal ganglia before (**G₁**) and after MPTP treatment showing 69% decrease in [¹¹C]-PE2I binding potential in the striatum (**G₂**) and 86% in a more extreme case (**G₃**). The reduction of TH immunostaining in the mesencephalon of MPTP-treated monkeys (**G₅**, 65%, **G₆**, 72%, **G₇**, >80%) compared to a control (**G₄**). a: Substantia nigra (SN); b: Ventral tegmental area (VTA), Scale bar: 1000 μ m.

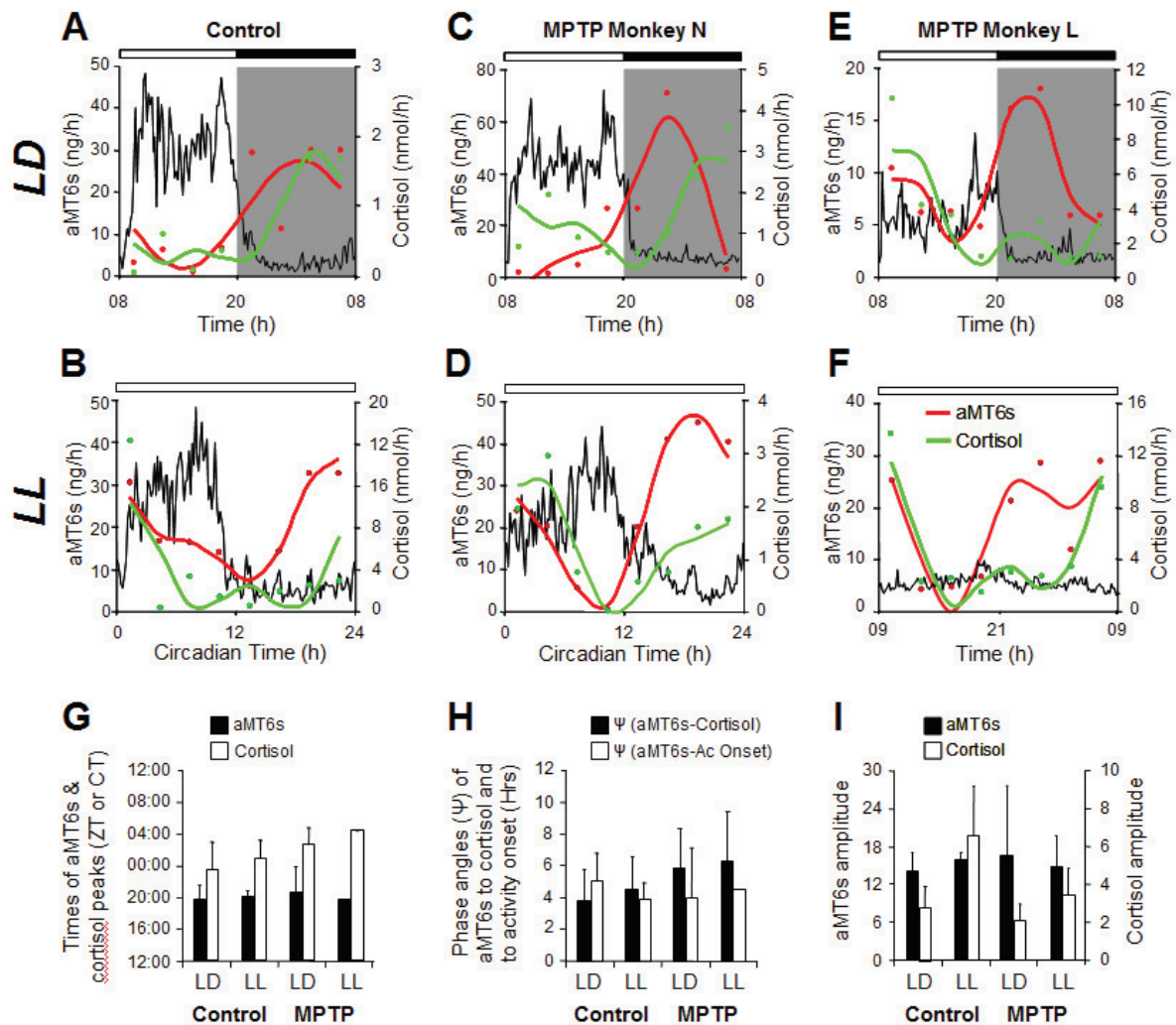


Figure 2. MPTP-treated monkeys maintain 24h hormonal rhythms of melatonin and cortisol in both entrained (LD) and constant conditions (LL). Representative individual 24h values and profiles (dual harmonic regression fit) of urinary concentration of 6 sulphatoxymelatonin (red circles, line) and cortisol (green circles, line) compared to the mean relative activity profile (black line). Gray shading indicates the dark phase. In LD, controls and MPTP-treated animals show clear rhythms of locomotor activity, aMT6s and cortisol (**A**, **C**, **E**). In LL, control (**B**), rhythmic (**D**) and arrhythmic (**F**) MPTP animals also show a clear circadian rhythmicity of aMT6s and cortisol. The times of the aMT6s and cortisol peaks (**G**), the phase angles of aMT6s to both cortisol and activity rhythms (**H**) and the amplitudes of aMT6s and cortisol rhythms (**I**) were similar in LD and LL in both controls and MPTP-treated subjects (controls $n=4$; MPTP $n=3$).

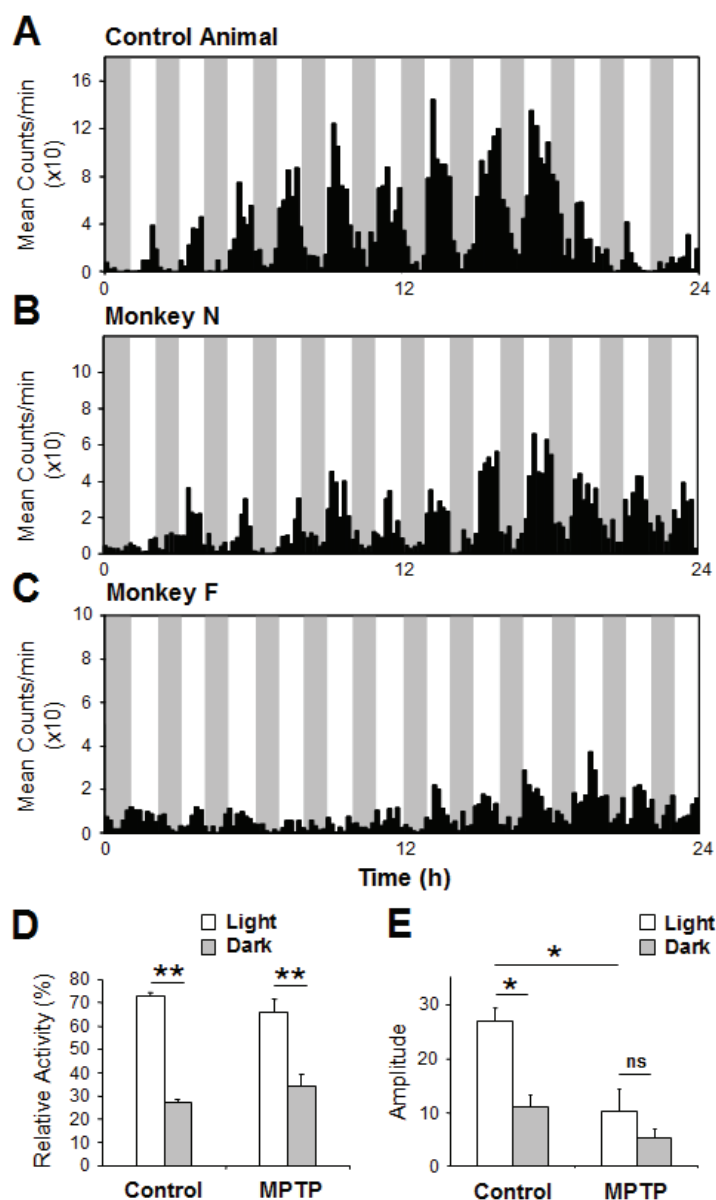


Figure 3. MPTP-treated monkeys show normal masking responses to darkness in a 1 h light–1 h dark regime. The control monkey (**A**) shows a clear 24h rhythmicity coupled with a strong masking effect of darkness on locomotor activity. MPTP-treated monkeys show an attenuated amplitude of the rhythm (**B**, mildly symptomatic, **C**, highly symptomatic) but nevertheless a strong masking effect of darkness proportionately similar to that of controls. (**D**) Mean relative total activity during light and dark phases. (**E**) Amplitude of the rhythm analysed separately for light and dark phases (controls $n=4$; MPTP $n=5$). * $P<0.05$, ** $P<0.001$.

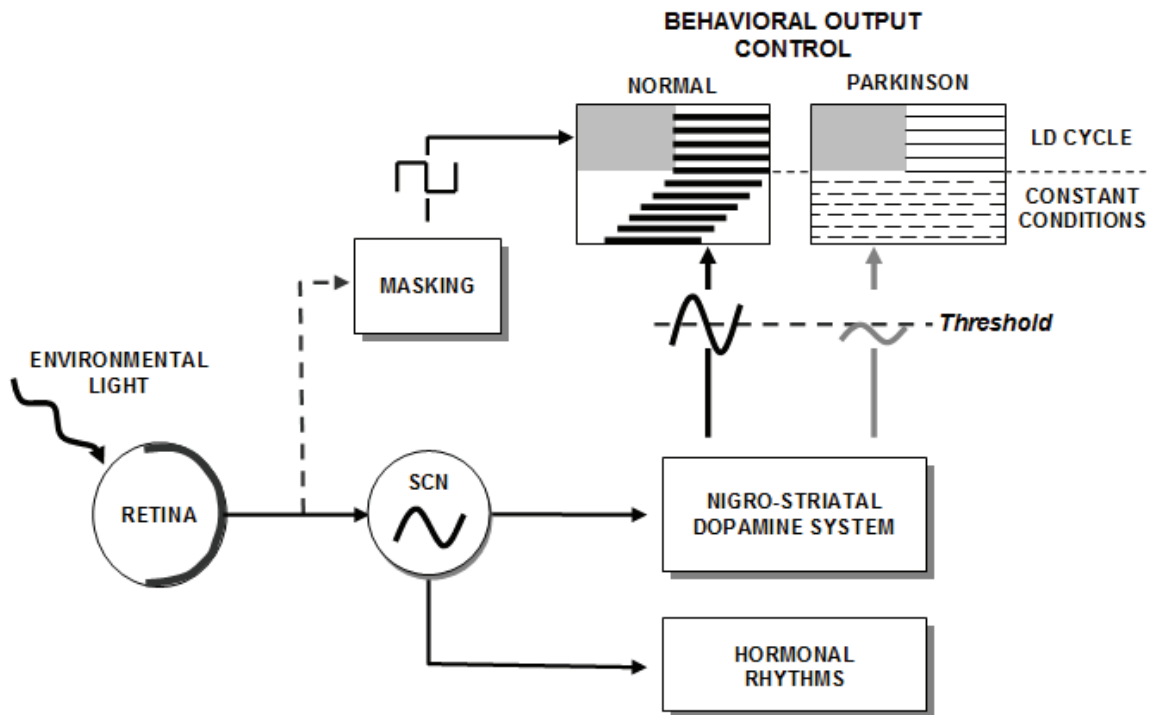


Figure 4. Model of the consequences of dopamine depletion in the nigrostriatal system. In healthy subjects the LD cycle synchronizes daily output rhythms (activity, hormones) and fine tunes these rhythms through acute masking effects. In constant conditions, rhythmic outputs free-run under the control of the circadian clock. In the case of dopamine depletion in the nigrostriatal system, the excitatory and inhibitory effects of environmental light and dark are sufficient to sustain expression of a daily locomotor rhythm. In constant conditions, the endogenous clock is intact and drives rhythmic hormonal outputs. In contrast, rhythmic locomotor behavior is abolished if the rhythmic dopamine function driven by the SCN falls below a hypothetical threshold.

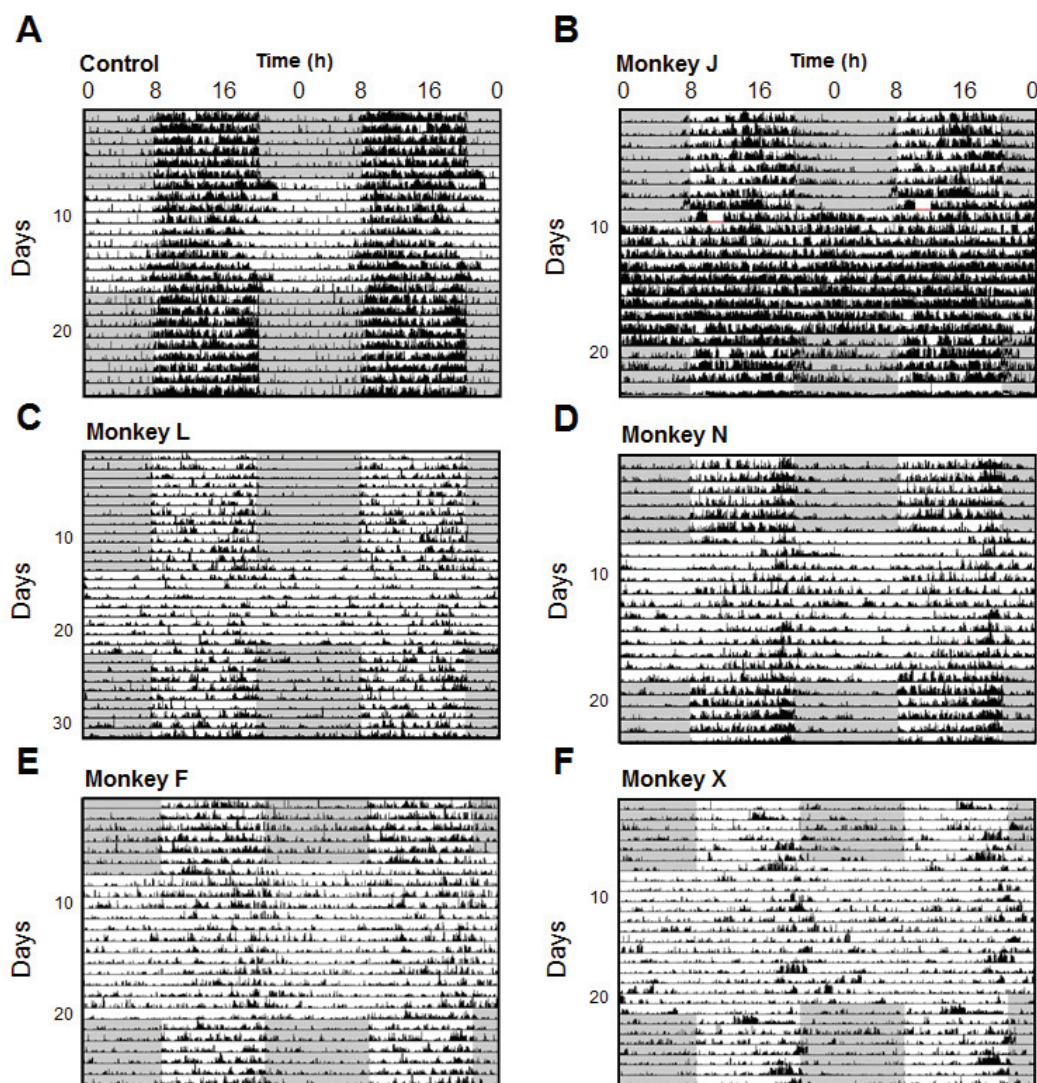
SUPPORTING INFORMATION**FIGURES**

Figure S1. Double-plotted actograms illustrating alteration of circadian expression of locomotor activity in constant light (LL) in an untreated control and in 5 MPTP treated animals in addition to that shown in Figure 1. Note that while daily locomotor rest activity rhythms are observed for all animals during the LD cycle preceding and subsequent to LL. In MPTP-treated animals the circadian rhythm of locomotor activity was strongly altered (D, E, F) or abolished (B, C) in LL. C and F were highly symptomatic cases, D and E were mildly symptomatic and B was asymptomatic. The dark phase is shown by shaded areas and light phase by light areas.

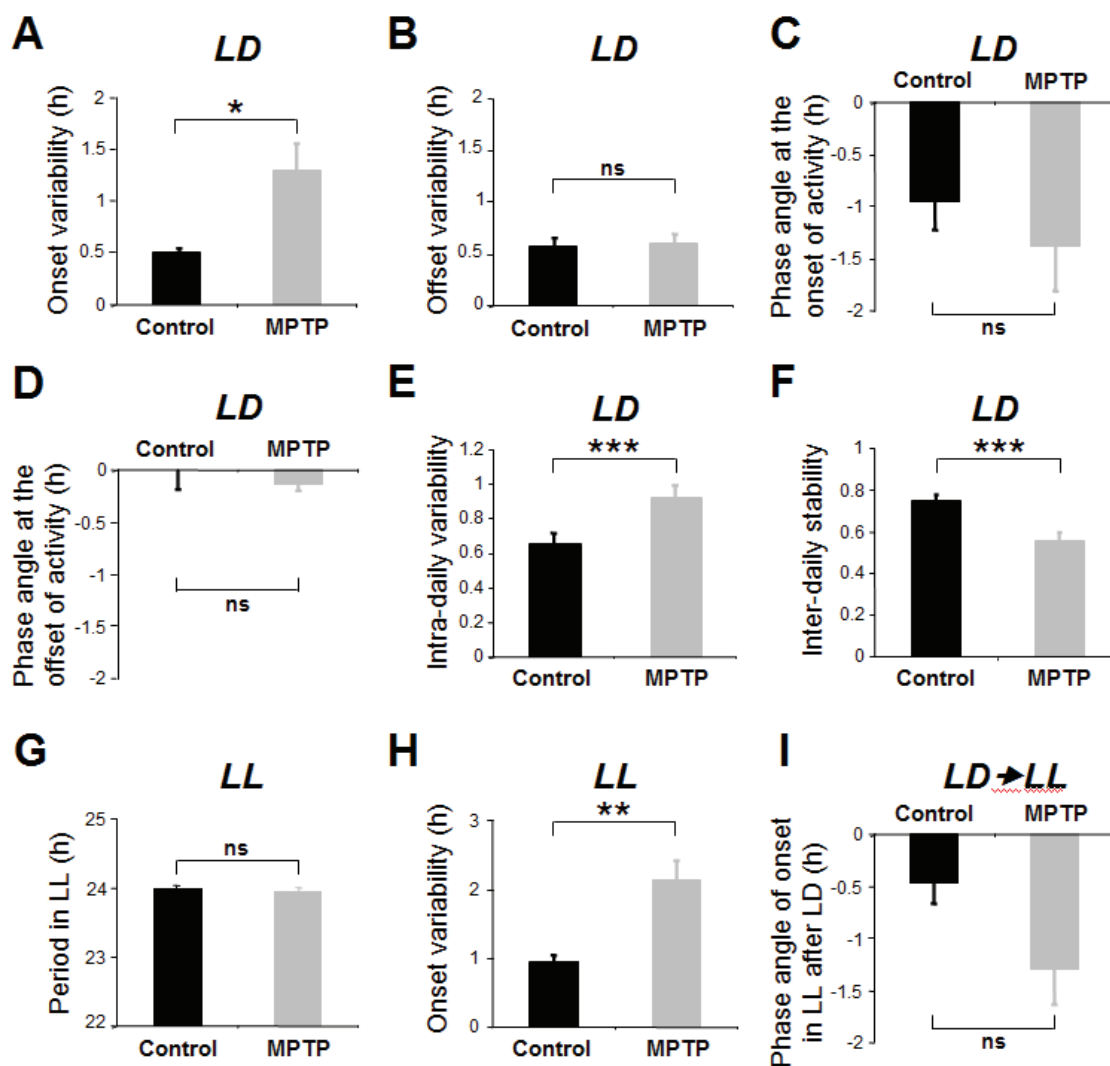


Figure S2. Variability of onsets, offsets, period, intra-daily variability, inter-daily stability and phase angle in control ($n=10$) and MPTP-treated animals ($n=7$). After MPTP treatment there was a significant increase of variability at the onset but not in the offset of locomotor activity in LD (**A**, **B**). Control and MPTP-treated animals show an approximately 1 hour phase delay in activity following lights on (**C**) and no phase angle difference in activity offset at lights off (**D**). The intra-daily variability increased (**E**) while the inter-daily stability decreased (**F**) after MPTP treatment. In LL the still rhythmic MPTP-treated animals showed an increased activity onsets variability (**H**), but with no significant difference in period (**G**). Upon release from LD to LL, MPTP animals had no significant difference in the phase angle of activity onset compared to controls (**I**) showing that the entrainment of activity rhythm under LD regime was not affected by MPTP. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

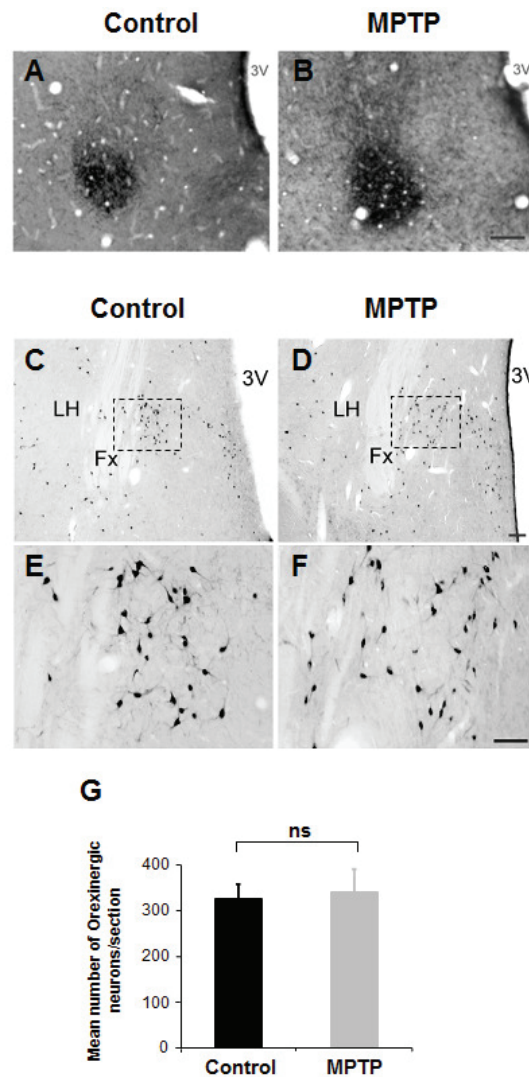


Figure S3. The orexinergic system in the lateral hypothalamic area and vasoactive intestinal peptide (VIP) containing neurons in the suprachiasmatic nucleus (SCN) are not affected after MPTP treatment. Example section in the hypothalamic SCN illustrating staining of VIP immunopositive neurons in a control (**A**) and an MPTP-treated animal (**B**). Scale bar= 100 μ m. Example sections of orexin-A immunopositive neurons in the lateral hypothalamus of a control (**C**, **E**) and MPTP-treated animal (**D**, **F**). The regions indicated dashed square in **C** and **D** are shown at higher magnification in **E** and **F**. (**G**) There was no significant difference in the number of orexin expressing neurons between control (n=1) and MPTP-treated animals (n=3). All orexin-A expressing neurons in the hypothalamus were counted on 5-7 sections per animal (distance between sections 150-200 μ m). Scale bar: **C**, **D**=200 μ m, **E**, **F**=100 μ m. LH = Lateral hypothalamus; Fx = Fornix; 3V = Third ventricle.

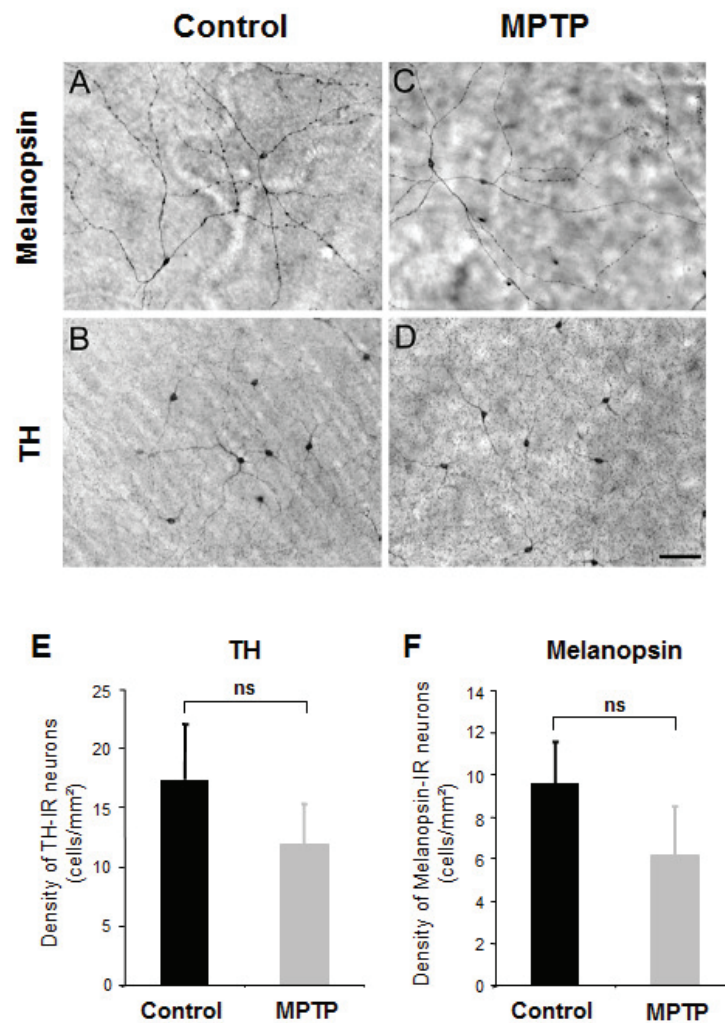


Figure S4. In the retina, the morphology of melanopsin and TH immunoreactive neurons appear normal after MPTP treatment. The photomicrographs are from representative flat-mounted retina immunostained for melanopsin (A, C) or TH (B, D) in control (A, B) and MPTP-treated animals (C, D). Scale bar: 100 μ m. E and F illustrate histograms of the densities of TH and melanopsin immunoreactive neurons. (Controls n=2; MPTP n=3).

Materials and Methods (All methods are described in more detail in [Supporting Information](#))

Animals and Housing. Eleven rhesus and cynomolgus monkeys (11-14 years) were used, housed in individual cages in light, temperature-controlled and sound-proofed rooms with free access to water and fed twice a day with pellets (Special Diets Services) supplemented with fruit. All procedures were carried out according to the 1986 European Community Council Directives (86/609/EEC).

MPTP intoxication. Monkeys were treated with intramuscular low dose injections (0.2 mg/Kg) of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP; Sigma, St Louis, Mo, USA) (16).

Clinical Motor Evaluation. Motor status was monitored 5 days/week by at least 2 trained observers using a semi-quantitative Parkinsonian Monkey Rating Scale (PMRS (50)).

Assessment of behavioral rhythms. Locomotor activity was continuously recorded throughout the entire study (8 to >24 months per individual) using a computerized data acquisition system (Circadian Activity Monitoring, INSERM (51)). Data were analysed using the Clocklab software package (Actimetrics). Animals were maintained under an LD cycle (12h light:12h dark; 450-500 lux) and were periodically exposed to conditions of constant light (LL, 450-500 lux) for 12-15 days or to a masking protocol (1h light/ 1h dark) for 4-5 days.

Functional [¹¹C]-PE2I Binding Potential Evaluation, PET scans using [¹¹C]-PE2I (N-(3-iodoprop-2 E-enyl)-2beta-carbomethoxy-3beta-(4-methylphenyl) nortropane) were periodically obtained to evaluate *in vivo* DA transporter binding. Specific procedures have been described in detail elsewhere (52).

Hormone radioimmunoassays (RIA). Profiles of aMT6s and cortisol excretion were studied from urine samples collected every 3h during 48h and hormone concentrations determined by radioimmunoassay as previously described (53).

Brain and Retinal Immunohistochemistry. Brain sections were reacted for immunohistochemical visualization of tyrosine hydroxylase (TH, Millipore #MAB318) DAT (Millipore, #MAB369), Orexin A (Phoenix Pharma #H-003-30), vasoactive intestinal peptide (VIP; generous gift of Prof. Jen Mikkelsen). Evaluation of the lesion in the SN-VTA

complex and striatal structures was performed using semi-quantitative immunohistochemistry (16). Retinal wholemounts were examined using antibodies against TH (1:1000, Millipore, #MAB369) or Melanopsin (generous gift of Prof. I. Provencio, USA).

Analysis and Statistics. Analysis of behavioral rhythms and amplitudes was performed using Chi-squared periodogram, cosinor analysis and a Linear mixed model in R. Hormonal rhythms were evaluated using a dual harmonic regression model. Behavioral parameters were analysed using a two-way analysis of variance ANOVA and *Post hoc* comparisons of interactions with the Bonferroni method. P values less than 0.05 were considered statistically significant.

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SUPPORTING INFORMATION

Material and methods

Animals and housing

Ethics Statement

All procedures were carried out according to the 1986 European Community Council Directives (86/609/EEC) which was the official directive at the time of experiments, the French Commission for animal experimentation and the Department of Veterinary Services (DDSV Lyon, France). Authorization for the study was delivered by the "Préfet de la Région Rhône Alpes" and the "Directeur départemental de la protection des populations" under Permit Number: #A690290402. All procedures were designed with reference to the recommendations of the Weatherall report, "The use of non-human primates in research". All monkeys were closely monitored on a regular basis throughout the day, by researchers and animal care staff, in order to ensure that levels of health and welfare were strictly maintained. As required, adaptations to housing and feeding procedures were made in direct response to individual symptoms in the MPTP phase.

Five adult rhesus monkeys (*Macaca mulatta*, 1 female and 4 males) aged between 5-11 years old and six adult female cynomolgus monkeys (*Macaca fascicularis*) aged between 11-14 years old were used. The animals were housed in individual cages in light, temperature-controlled (24-26 °C) and sound-proofed rooms dedicated to MPTP and circadian experiments. There was free access to water and animals were fed twice a day with pellets (Special Diets Services), supplemented with fruit. Food was supplied randomly twice a day in the light phase of the LD cycle or on the subjective day during constant conditions. Illumination was provided by white fluorescent tubes (Philips, 15 Watts) mounted on the ceiling above the animal's cage.

MPTP administration

The eleven drug-naïve monkeys were divided into a group of 4 monkeys used as untreated controls and 7 monkeys that were rendered Parkinsonian with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP; Sigma, St Louis, Mo, USA). MPTP injections were intramuscular using relatively low doses (0.2 mg/Kg). The neurotoxin was delivered initially every 3-4 days, and in some cases, towards the end of treatment, every 1-2 days as previously

described (1). The administration was continued until animals showed a significant clinical score of motor deficits varying from mildly to highly symptomatic states (see definitions below). The total cumulative doses ranged from 2.2 – 7.8 mg/kg and the duration of treatment ranged from 18 to 90 days. This mode of MPTP treatment has the advantage of producing behavioural symptoms that closely resemble the clinical features of idiopathic PD and the possibility of titrating the degree of lesion by adjusting the frequency of injections resulting in a range of clinical symptoms (2). In the text as well as in the analyses, control animals refer to the 4 untreated animals and the 7 treated animals prior to MPTP administration, unless otherwise specified.

Clinical Motor Evaluation

Motor status was monitored 5 days/week, by at least 2 trained observers, during 10-15 min observation periods using a semi-quantitative Parkinsonian Monkey Rating Scale (PMRS) adapted from the Unified Parkinson's Disease Scale (UPDRS) used for patients and compatible with rating scales used for monkeys (3). We defined three different states of motor symptom severity: a score of 0 to 5 defined the non-symptomatic stage, a score from 5 to 8.5 corresponded to a mild symptomatic state and a score of ≥ 8.5 to a severe symptomatic state, in accordance with previous criteria (4). The use of a score >5 to define the threshold for the symptomatic stage was applied as previously described (1, 3). The subdivision of the symptomatic stage into mildly and highly symptomatic stages was based on the fact that monkeys with a score >8.5 become increasingly bradykinetic, adopting a flexed posture with increased rigidity of the limbs and less accurate movements when for example reaching for food and with occasional episodes of freezing.

Assessment of behavioral rhythms

Locomotor activity was continuously recorded in 1 min intervals throughout the entire duration of the study using passive-infrared motion detectors mounted above each animal's cage. The motion captors were connected to a computerized data acquisition system (Circadian Activity Monitoring, INSERM, France; for details see 5). Data were analysed using the Clocklab software package (Actimetrics, Evanston, IL, USA).

We used Chi-squared periodogram method to calculate the period of behavioral rhythm which is defined as the time elapsed for one complete oscillation or cycle (the distance in time between two consecutive peaks or troughs of a recurring rhythm). The stability and the

consistency of the rhythm are reflected by the value of the periodogram amplitude which corresponds to the zenith of a periodogram curve. The subjective day and the subjective night are defined as the segment of a circadian cycle during the free-running state that corresponds to, respectively, the light and dark segments during entrainment by a LD cycle. Finally, activity onset, which is a robust indicator of entrainment to LD cycle, corresponds to the average clock (or circadian) time of activity onset while activity offset to the average clock (or circadian) time of activity offsets. Phase angle is the difference between the time of the activity light onset or offset compared to lights on-lights off.

Non-parametric circadian rhythm analyses (NPCRA) were used to estimate the strength and fragmentation of rest-activity rhythms (6). These included measurements of the inter-daily stability (calculated as the ratio between the variance of the average 24-hour pattern around the mean and the overall variance and gives an indication of the strength of coupling between the rest-activity rhythm and LD cycle) and the intra-daily variability (calculated as the ratio of the mean squares of the difference between successive hours and the mean squares around the grand mean and gives an indication of the frequency of transitions between rest and activity periods, corresponding to the fragmentation of the rhythm). When the rest-activity rhythm is stable, inter-daily stability is high and the intra-daily variability is low.

Lighting protocols

Animals were recorded for periods of 8 months to more than 24 months. Prior and after MPTP administration, the animals were maintained under an LD cycle (12h light:12h dark) of approximately 450-500 lux during the light phase and 0 lux during the dark phase. The animals were periodically exposed for 12-15 days to conditions of constant light (LL) of 400 lux. On a few occasions continuous dim light (0.5-5 lux) was examined, but as no differences were found between the two lighting conditions, data was grouped together for analysis. The average time between the end of MPTP treatment and the first exposure to LL was 30-210 days (avg = 96 days) and for subsequent exposures was 60-367 days thereafter. Between two LL periods, the animals were always re-entrained to an LD cycle for at least 4 weeks. Behavioral masking responses were investigated using a 1h light/ 1h dark lighting regime for 4-5 days.

Functional [¹¹C]-PE2I Binding Potential Evaluation

To evaluate the *in vivo* DA function, images from PET scans using [¹¹C]-PE2I (N-(3-iodoprop-2 E-enyl)-2beta-carbomethoxy-3beta-(4-methylphenyl) nortropane) were obtained at different periods throughout the entire protocol. PE2I specifically binds with high affinity and selectivity to DAT ($K_i = 17\text{nM}$) and is considered to provide an index of the integrity of the DA pathway that has been used for PD diagnosis (7-9). PET studies were performed with an ECAT Exact HR+ tomograph (Siemens CTI), in 3D acquisition mode, covering an axial distance of 15.2cm. The transaxial resolution of the reconstructed images was about 4.1mm full-width and half maximum in the center. Transmission scans were acquired with three rotating ⁶⁸Ge sources and were used to correct the emission scans for the attenuation of 511 Kev photonrays through tissue and head support. The specific procedures of the experimental methods employed at the CERMEP imaging facility (Lyon) ([¹¹C]-PE2I labeling, subjects PET examinations, PET Evaluation and modelling) have been described in detail elsewhere (10).

Hormone radioimmunoassays (RIA)

Nycthemeral and circadian profile of aMT6s and cortisol excretion were studied in control and MPTP-treated animals in both LD and constant conditions. Previous reports have shown that aMT6s assays correlate significantly with daily plasma melatonin profiles and can be used as non-invasive methods to study melatonin secretion (11-14). Urine samples were collected at 3h intervals for 48h. In the end of collection, urine samples were filtered, aliquoted and stored at -20°C until assayed. Urinary aMT6s and cortisol levels were determined by radioimmunoassay as previously reported (12). Quantity rates (quantities/time span) were calculated for each parameter. This resulting expression takes into account a possible dilution of urine related to the hydration of animals. Of the 27 series of assays, 9 were excluded due to an insufficient number of samples during the 48h sampling period. Day-night concentration ratios were determined for the remaining 18 assays, and 13 of these had a sufficient number of successive samples over a 24h window within the 48h sampling period for the dual harmonic regression analysis.

Brain Immunohistochemistry

Anesthesia was induced with Ketamine after premedication with chlorpromazine hydrochloride (Largactil™) followed by a lethal dose of pentobarbital sodium (60mg.kg⁻¹,

i.p.) before animals were transcardially perfused with saline (0.9% with procaine) and 4% paraformaldéhyde and 0.05%-glutaraldehyde in phosphate buffer (PB). Cryoprotection was ensured by sucrose gradients (10-30%) perfusion post fixation. Brains were removed, kept in cryoprotecting liquid overnight and coronal 50µm thick sections were cut on a freezing microtome. Free floating sections were rinsed briefly in PB (0.01 M, pH7.4) containing 0.9% saline, 0.3% triton X-100 and 0.1% sodium azide (PBSTA) and blocked with 1.5% normal serum. Sections were then incubated with antibodies against Orexin A (1: 50.000, rabbit, Phoenix Pharma #H-003-30) tyrosine hydroxylase (TH; 1:1000, mouse, Millipore #MAB318), DAT (Millipore, #MAB369), or vasoactive intestinal peptide (VIP; 1:10.000, rabbit, a generous gift of Prof. Jen Mikkelsen) at 4°C for 72h. After rinsing twice in PBSTA, sections were incubated with biotinylated secondary antibodies for 2h (1:200, Vector) and in an avidin-biotin-horseradish peroxidase complex (1:200, Sigma-Aldrich) at 22-24°C. The reaction product was visualized with 3,3'-diaminobenzidine as the chromogen with 0.5% ammonium nickel sulphate and 0.03% H₂O₂ in TRIS buffer. Sections received additional rinses, were mounted on gelatinized slides, dried and dehydrated in increasing gradients of ethanol, cleared in toluene and were coverslipped with Depex.

Semi-quantitative immunohistochemistry. Evaluation of the extent and amplitude of the lesion in the SN-VTA complex and the striatal structures was obtained with semi-quantitative immunohistochemistry (15). The mean optical density (O.D.) of TH and DAT immunoreactive regions were computed on at least 5 sections (distance between sections 150µm) per animal (range; 5-20) from 8bit images. All hypocretin-1 expressing neurons in the hypothalamus were counted on 5-7 sections per animal (distance between sections 150-200µm).

Retinal Immunohistochemistry

Following the perfusions, the retina was gently dissected from the eyecup, and post-fixed for 24h in 4% buffered paraformaldehyde. To digest the vitreous and enhance penetration, the retinas were pre-treated with a mixture of collagenase-hyaluronidase in TBS/CaCl₂ (10mM) at 37°C for 30 min, and then rinsed with TRIS buffer (0.05M). Endogenous peroxidase activity was suppressed by treatment with a solution of 50% ethanol in saline with 0.4% H₂O₂ for 1h. After a blocking step (1.5% normal serum, 0.3% triton X-100, 0.1% sodium azide in saline, 1h, 4°C), tissue was incubated for 3 days in primary antibodies against TH (1:1000, Millipore, #MAB369) or Melanopsin (1/4000, a generous gift

of Prof. I. Provencio, USA) at 4°C. After two PBST washes (10min, 20°C), flat mounts were incubated for 2 hours with biotinylated secondary antibodies diluted 1:200. Thereafter, the avidin-biotin-peroxidase reaction (1:200, Sigma-Aldrich) was carried out using diaminobenzidine as the chromogen. After a final 2 washes in TBS (10min, 4°C) the flat-mounts were mounted in gelatinized slides and were coverslipped with Depex. The density of melanopsin and TH immunopositive neurons was evaluated in the flat mounted retinas by counts of the average number of cells in sampling areas of 0.38 mm² spaced 3 mm apart.

Analysis and Statistics

The calculation of a significant behavioral rhythm was performed using Chi-squared periodogram method (16). For analysis of behavioural amplitudes at different clinical stages, cosinor analysis and a Linear mixed model (lme4 package) in R (version 2.12.2) were used. For analysis of hormonal rhythms, the dual harmonic regression model was used to estimate the amplitude and the acrophase of the rhythms (Sigmaplot 12, Systat Software Inc.). Behavioral parameters were analysed using a two-way analysis of variance ANOVA (with treatments and light conditions as factors). *Post hoc* comparisons of interactions were performed with the Bonferroni correction. For analysis of the TH, melanopsin and orexin-A positive cell, a one-way analysis of variance ANOVA was used. P values less than 0.05 were considered statistically significant.

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Discussion and Conclusion

Discussion and Conclusion:

Parkinson's disease is first and foremost considered as a motor control disorder. However, over the last 3 decades substantial evidence from numerous studies has revealed a large range of non-motor symptoms and several reviews have focused on the description, quantification and management of these symptoms (Chaudhuri et al., 2006; Chaudhuri & Schapira, 2009). My thesis aimed to contribute to our knowledge of the pathophysiology of two of the most common and important non-motor symptoms of PD, namely, cognitive dysfunctions and mainly circadian disturbances.

As discussed in the previous chapters, many aspects of these abnormalities were replicated in both rodent and non-human-primate models of PD. In this last chapter of the thesis, I will confront our findings with the symptomatology described in PD patients and critically discuss the validity of our interpretations and some of the shortcomings related to use of animal models. The first part of this general discussion will consider cognitive functions while the circadian aspects will be tackled in the second part. In the third section, I will discuss some implications of our results in regard to emerging therapeutic strategies for PD patients and finally some perspectives will be presented.

1-Cognitive dysfunctions in Parkinson's disease

Cognitive dysfunctions are among the most well established non-motor symptoms of PD. The prevalence of cognitive deficits in people with Parkinson's disease varies between 40% to 93%, a rate more than 10 times higher than that observed in age-matched healthy individuals (Dubois and Pillon, 1997; Emre, 2003). Such high rates of dementia have also been reported by Hely and colleagues in their long term (15-18 years) follow-up study of patients with Parkinson's disease (Hely et al., 2005).

Clinically, cognitive dysfunctions in PD are characterized by impairment in visuospatial abilities, memory and executive functions (Apaydin et al., 2002; Aarsland et al., 2004). The impairment of executive functions represents a core feature of neuropsychological deficits in PD (Pillon et al., 2003). Executive functions refer to the mental processes involved in the elaboration and control of cognitive and behavioral responses to challenging situations (Crucian GP et al., 2000; Pillon et al., 2003). Deficits in the planning, sequencing and execution of such complex goal-directed behaviors have been described in PD. In particular,

these patients have difficulties in the maintenance of new sequence patterns after the shift from a previously learned sequence of movement (Brown and Marsden, 1990).

Executive dysfunctions can be investigated with tasks requiring internally guided behavior. In PD patients, multiple tasks have been used including; Tower tasks for problem-solving, Wisconsin and California Card Sorting Man Out Tests for set-shifting and Verbal fluency and Stroop test for set-maintenance and inhibition of interference (Dujardin et al., 2001; Pillon et al., 2003).

In our Monkey study, we used the object retrieval detour (ORD) task to measure inhibitory control in order to assess the effects of MPTP treatment on executive function in macaques. Performance on the ORD task has been found to be sensitive to impairments of the frontal cortex (Dias et al. 1996; Jentsch and Taylor, 1999; Roitberg et al., 2002) and the dopaminergic system (Schneider and Roeltgen, 1993; Taylor et al., 1990). The task requires retrieval a food reward placed in a transparent box that has one open side. Successful performance required the subject to suppress a tendency to reach directly at the reward while orientation of the open side and the position of the reward in the box were manipulated in order to vary the cognitive and motor difficulty of the trial (Taylor et al., 1990).

Our results show a decline in the performance of MPTP-treated monkeys in ORD task during MPTP treatment and persisted even after full motor recovery. Interestingly, deterioration of cognitive abilities was brought on by MPTP treatment even before reaching the clinical threshold that determines symptomatic state (motor score = 5). These results confirm previous studies showing an independent emergence of frontal-like cognitive deficits in chronically MPTP treated NHP (Schneider and Kovelowski, 1990; Schneider et al., 2008). By using low doses of MPTP (0.05 to 0.15 mg/Kg, i.v) Schneider et al (1999) developed a model characterized by cognitive deficits with minimal or no gross Parkinson-like motor deficits. Under these conditions, the MPTP based NHP models of PD have been considered as a reliable model of cognitive impairments of early-stage PD.

When our MPTP-treated animal was treated with L-DOPA, we observed a rapid and significant improvement in performances in the ORD task. Given the dependence of successful performances in the ORD task on the integrity of frontal cortex and especially on DA innervation of frontal cortex (Diamond, 1996), the improvement of performance during the period of L-DOPA treatment suggests that the worsening of this kind of cognitive abilities

can be attributed to dysfunctional fronto-striatal connections consequent to the loss of DA innervation.

The same pathophysiological mechanisms may also account at least partially for executive dysfunction in PD patients. A beneficial effect of L-DOPA therapy on some aspects of cognition has been observed in non-demented PD patients, particularly on intentional resources and frontal lobe-like functions (Cooper et al., 1992; Growdon JH et al., 1998). If dopamine loss is the pathophysiology mechanism of cognitive deficits in PD, two main pathways may be involved. Firstly, the loss of nigrostriatal dopaminergic fibres is likely to impair the function of the corticostriatal loops subserving cognitive function (Alexander and Crutcher, 1990), which relay mainly in the caudate nucleus, with a final output towards the frontal lobes via the thalamus. However, more recently, it has been suggested that the loss of the direct mesocortical dopaminergic innervation of the frontal lobe (Javoy-Agid et al. 1981) might also play a role in the cognitive deficit found in PD patients. Both hypotheses have received support from functional imaging studies. In summary, studies of the role of dopaminergic pathways suggest that a decline in caudate dopamine-modulated basal ganglia outflow contributes to brain activation changes in corticostriato-pallidal-thalamocortical loops at early Parkinson's disease stages, while mesocortical degeneration mediated increases of inefficient DLPFC activation (Cools et al., 2002; Mattay et al., 2002) may be a feature of more advanced disease stages only.

The dopamine hypothesis of cognitive dysfunctions in PD does not however, account for the entire spectrum of cognitive abnormalities in PD (Calabresi et al., 2006). By using imaging techniques, Owen et al. (1999) performed a series of studies that established the functional correlates of impaired performance in the *Tower of London task*, as one paradigm of higher executive function. Spatial planning in the task is related to cortical activation in frontal and parieto-occipital areas in healthy controls and moderately advanced Parkinson's disease patients. However, in the patients, an abnormal decrease in pallidal blood flow was noted during spatial planning. Subsequently, impaired activation within the prefrontal cortex bilaterally and the right caudate nucleus was identified during the Tower of London task at increasing levels of performance in moderately advanced patients (Dagher et al., 2001). To assess the contribution of the Dopaminergic system on the performance of the task, Cools et al (2002) studied the performance on the task and its related brain activation during the off-levodopa and on-levodopa state. No significant mean effect of levodopa on task performance

was detected, but levodopa-induced decreases in abnormally elevated activation of the right dorsolateral prefrontal cortex (DLPFC) and the right occipital lobe were identified. Similarly, Mattay and colleagues (Mattay et al., 2002) observed a task-related decline in a working memory paradigm (N-Back task). Working memory challenge was related to activation of the prefrontal cortex (Brodmann Area 9/10) (Mohr et al., 1992, Peppard et al., 1992; Brooks, 1993) pericingulate cortex, anterior cingulated (Brodmann Area 24/32), as well as parietal areas (Brodmann Area 7, 39/40) during the ON-levodopa and the OFF-levodopa state. No significant mean effect of levodopa on task performance was observed.

The lack of a clear performance improvement with L-DOPA (Cools et al., 2002) despite a normalization of task-specific activation also suggests that degeneration of DA system may not be the sole cause of cognitive deficits in PD. Indeed multiple findings imply several non-dopaminergic neuronal systems such as the cholinergic, noradrenergic and serotonergic neurons in the pathophysiology of cognitive dysfunctions in PD (Calabresi et al., 2006).

Patients with Parkinson's disease usually develop abnormalities of the neural circuitries that underlie central cholinergic transmission. Both cortical and subcortical cholinergic systems are impaired and the neuronal loss correlates with the decline of cognitive functions (Dubois et al., 1983). The basal forebrain cholinergic cell groups, including the nucleus basalis of Meynert, undergo degeneration in Parkinson's disease (Perry et al., 1985) and the neurodegenerative process is also reported in the pedunculopontine tegmental nucleus (Hirsch et al., 1987). Similar post-mortem evidence of severe damage of noradrenergic neurons in the locus coeruleus and serotonergic neurons in the raphe which result in the reduction of cortical and forebrain concentration of noradrenaline and serotonin have been reported (Scatton et al., 1983). Both of these two monoaminergic systems play a crucial role in the regulation of cognitive functions.

Pharmacological evidence for the involvement of non-DA system in cognitive functions of PD patients are also compelling; for example, blocking cholinergic transmission with anticholinergic drugs consistently results in learning and frontal lobe-like deficits in patients with PD (Dubois et al., 1990). Attentional control is increased by a selective noradrenergic alpha-1 agonist, which partly reverses attentional deficits, whereas an alpha-2 agonist improves spatial working memory performance in PD (Pillon et al., 2003).

The neurochemical basis of cognitive dysfunction associated with Parkinson's disease appears therefore to be highly complex and involves both DA and multiple non-dopaminergic

systems. In MPTP-treated non-human primates however, a similar template of dysfunction has not been documented and many non-DA pathology (loss of 5HT neurons in the raphe and cholinergic neurons in the PPN and the basal forebrain) appear to be lacking.

Taken together, these studies suggest that the cognitive abnormalities observed in the MPTP-treated NHP are part of much more diverse and complex spectrum of cognitive dysfunctions of Parkinson's disease and that the current MPTP based primate models are unlikely to advance both our understandings of the pathophysiology of cognitive dysfunctions in PD and the therapeutic strategies aimed to alleviate these pathologies.

2-Circadian dysfunctions in Parkinson's disease

As discussed in the introduction, behavioral, physiological and molecular evidence for circadian disturbances in PD patients are compelling. By using actigraphy as a technical tool to assess circadian rhythm disturbances, we firstly investigated rest/activity behavior under nyctemeral conditions in MPTP-treated non-human primate model of PD. This technique is routinely used to study circadian rhythm disorders in human ([Morgenthaler et al., 2007](#)).

Nonparametric analysis of actigraphic data in our MPTP-treated monkeys revealed a similar pattern of alteration of rest/activity behavior as in PD patients ([Van Hilten et al., 1993](#); [Whitehead et al., 2008](#); [Santiago et al., 2010](#); [Niwa et al., 2011](#)). Inter-daily stability of the rest-activity cycle decreased immediately after the initiation of MPTP treatment and continued to decrease throughout MPTP treatment period. The relative amplitude of rest-activity rhythm also evolved in a similar way as inter-daily stability. Regarding the intra-daily variability, a parameter that reflects fragmentation of the rest/activity cycle, as soon as MPTP treatment is started, it increased consistently all along MPTP treatment periods. These results show that the MPTP-NHP model replicates, in addition to the cardinal motor symptoms of PD, the abnormalities related to the temporal distribution of rest-activity behavior displayed by PD patients. Our results do extend however these findings by showing the independence of circadian abnormalities from motor disabilities. Circadian alterations indeed persisted even after full recovery of motor symptoms and showed no correlation with the degree of motor disability.

The non-human primate model of PD model we used is obtained by intramuscular administration of the neurotoxin MPTP. The discovery of this toxin dates back to 1976 when Barry Kidston, a chemistry graduate student that had been synthesizing a heroin-like

compound known as MPPP for his own use. The drug however was contaminated with MPTP that caused in both Kidston's case and in later outbreaks among drug users severe Parkinson's disease-like motor symptoms (Davis et al., 1979; Langston et al., 1983; Kopin and Markey, 1988). The administration of MPTP was subsequently shown to induce PD-like dopaminergic degeneration in both mice and primates. So far, MPTP-treated NHP constitute the gold standard model because of the replication of the cardinal clinical signs of PD (*See Introduction Section 5*).

By using the MPTP-treated NHP model, we evaluated daily and circadian rest-activity rhythmicity by exposing the animals respectively to 12h/12h LD cycle and to constant light (LL) conditions. We made two unexpected observations. First, compared to control animals (before MPTP treatment) that express a robust free running rhythm when released to constant LL conditions, following MPTP treatment, the treated animals displayed severely disturbed circadian rhythms with highly reduced amplitude and poorly consolidated daytime activity as was evident from the increased intra-daily variability and activity onset variability. Some animals showed a striking complete loss of circadian rhythmicity during which locomotor activity became homogeneously disturbed throughout the 24h period. Second, when re-exposed to nycthemeral LD conditions, the animals regained a synchronized rhythmic behavior of 24h periodicity. Their activity onsets coincided with lights on and 86% of their activity was re-consolidated to light phase of LD cycle.

In order to assess whether the disruption or the loss of rhythmicity in MPTP-treated monkeys is a consequence of an impairment of the functional integrity of the endogenous clock, we assessed simultaneously locomotor activity and the excretion pattern of cortisol and 6-sulphatoxymelatonin (aMT6s, the major metabolite of melatonin), two hormonal rhythms controlled by the SCN endogenous clock. Unexpectedly, both rhythmic and arrhythmic MPTP treated animals had a normal daily and circadian rhythms of both cortisol and melatonin. The phase angle relationships of aMT6s to both cortisol and activity were conserved. All these results suggest that the endogenous rhythmicity of the SCN is unimpaired in treated animals and therefore does not explain the disruption of behavioural rhythms experienced by animals after MPTP treatment. How can the maintenance of a daily rest-activity rhythm in an LD cycle in MPTP animals be explained given the absence of a circadian locomotor rhythm in constant conditions?

Since in diurnal mammals, light triggers activity whereas darkness is inhibitory (Borbely, 1978; Gander and Moore-Ede, 1983; Rajaratnam and Redman, 1999; Kas, 1999; Aschoff, 1999), we hypothesised that masking effects of lightness and /darkness could be responsible for the apparent synchronization of behavior during an LD cycle. To explore this possibility, we released the animals into a 1h light/1h dark cycle, a regime that elicits masking responses to light/dark transitions (Aschoff, 1999; Redlin, 2001). As hypothesised, MPTP treated animals showed similar robust masking effects of darkness on activity compared to the control animals. The conservation of strong masking effects of darkness in parkinsonian monkeys, even in the highly symptomatic, arrhythmic cases demonstrates that this response is unaffected. We therefore conclude that the robust inhibitory effect of darkness, coupled with the stimulating influence of light on activity in primates could be a plausible explanation for the maintenance of daily rhythms of activity in the LD cycle.

Our results suggest that in MPTP-treated NHP, the core clock mechanism of the circadian timing system remains essentially intact but is unable to efficiently drive downstream rhythmic locomotor activity. We proposed a model in which DA plays a coupling role between SCN and the output network controlling locomotor function (**Figure 4 in chapter 4**). As Dopamine levels decrease following neurotoxicity, rhythmic dopaminergic neurotransmission is dampened and may fall below a hypothetical threshold level leading to a disconnection of the central circadian clock output from the locomotor network output necessary for rhythmic locomotor behavior. This hypothesis is consistent with the idea that under LD conditions, the robust inhibitory effect of darkness, coupled with the stimulatory influence of light on activity in primates (Borbely, 1978; Gander and Moore-Ede, 1983; Rajaratnam and Redman, 1999; Kas, 1999; Aschoff, 1999) act to maintain daily rhythms of activity. In constant conditions, the absence of these acute effects combined with a disconnection between the central clock (SCN) and the motor system downstream lead to behavioral arrhythmia.

Although we have found no data in the literature to determine whether PD patients experience the same disruption of circadian rest-activity rhythms under continuous or forced-desynchrony protocols, our findings suggest that the same abnormalities would be expected in PD patients.

1-In our study we assessed whether MPTP treatment induced a neurodegeneration of hypocretin neurons which could account for the more fragmented rest-activity behaviour

seen in our MPTP-Treated NHP. No alterations were found in the number, the distribution or the dendrite morphology of hypocretin neurons after MPTP treatment. In contrast, two independent studies have reported significant (50%) hypocretin loss in post-mortem hypothalamus of patients with Parkinson's disease (Fronczek et al., 2007; Thannickal et al., 2007) and the presence of lewy bodies in some hypocretin-producing cells. The loss of hypocretin cells was correlated with disease severity (Bourgin et al., 2008). Since the hypocretin system plays an essential role in the consolidation of wakefulness by excitation of multiple wake promoting brain areas (Saper et al., 2005; Sakurai, 2007). Loss of hypocretin might, therefore lead to a more destabilisation and fragmentation of sleep-wake behavior in Parkinson's patients.

2-In arrhythmic MPTP treated NHP, both the amplitude and the phase angle of melatonin and cortisol secretion were normal. Given the entraining role of melatonin of multiple physiological rhythms and sleep-wake behavior (Pandi-Perumal et al., 2008; Pevet and Challet, 2011) even at low physiological doses (Lewy, 2001; 2002), the impairment of the circadian pattern of melatonin production in PD patients (See Introduction section 2) could contribute as an additional pathophysiological factor to the alterations of sleep-wake behavior in PD patients.

3-We found no correlation between the loss of circadian rest-activity rhythmicity and both the clinical score and the extent of dopaminergic neurodegeneration in our MPTP-treated NHP. The only common feature between the animals that developed behavioral arrhythmicity was the fact that they received a protracted long term MPTP treatment; this mode of intoxication has two advantages over acute treatment protocols (Schneider et al. 2008). First, it more closely approximates the clinical conditions of PD (slow onset of symptoms, long lasting duration of symptoms, slow onset of dyskinesia after L-DOPA treatment and development of many non-motor symptoms of PD); Second, it more closely replicates the chronic slow neurodegenerative process seen in PD (such as slow and maintained neurodegeneration of DA neurons, development of eosinophilic inclusions reminiscent of Lewy bodies in PD brains and affection of non-dopaminergic systems (Forno et al., 1993; Schneider et al. 2008).

The cumulative additive contributions of these factors, in addition to the primary loss of DA neurons could potentially worsen the circadian disruption of rest-activity rhythms and makes

it more likely that at least similar behavioral arrhythmia as found in MPTP treated NHP would emerge under forced desynchrony protocols in PD patients.

3-Light therapy in Parkinson's disease, state of the art and unresolved questions

With the recognition of the complex multifaceted nature of Parkinson's disease syndrome, therapeutic strategies aiming to alleviate non-motor symptoms are still key unresolved clinical issues (Meissner et al., 2011). The majority of these non-motor symptoms are not responsive to dopaminergic treatment (Chaudhuri and Schapira, 2009). Rather dopaminergic medication constitutes a factor that potentially contributes to the exacerbation of these non-motor disorders particularly in advanced stages (Chaudhuri and Schapira, 2009). Recently, very few drug developments have been undertaken to manage the non-motor symptoms of PD and efficacious treatments are lacking and are therefore a priority (Meissner et al., 2011). In view of the diversity and common occurrence of non-motor symptoms of PD, the inevitable limitation of multiple medication intake (even after the development of efficacious drugs) would be polypharmacy which is a well established risk factor for adverse drug reactions in older people (Lai et al., 2010; 2012; Chang et al., 2012). A positive correlation between polypharmacy and the risk of PD have been reported in older people (Lai et al., 2011).

Given the role of melatonin in the internal synchronization of circadian and sleep-wake rhythms (Pandi-Perumal et al., 2008; Pevet and Challet, 2011) it has been used to decrease sleep initiation difficulties and night time activities in older adults (MacFarlane et al., 1991; Garfinkel et al., 1997). In PD, only two studies using melatonin have been undertaken in PD patients with disturbed sleep. In the first study (Dowling et al., 2005), melatonin was administered 30 min before bedtime for a period of 2 weeks and a doses ranging from 5 to 50 mg/day. The second study used 3 mg of melatonin, 1h before bedtime and for 4 weeks (Medeiros et al., 2007). Although both studies reported a significant improvement in the subjective quality of sleep, the objective architecture of sleep abnormalities assessed by PSG remained unchanged; In addition, motor dysfunction was not improved by the use of melatonin (Medeiros et al., 2007; 2008).

As an alternative to polypharmacy, light therapy was introduced as an adjuvant to DA therapy in PD. Exposure to light of 1000-1500 lux intensity 1h prior to bedtime for a period of 1-1.5h has been shown to improve bradykinesia and rigidity in 12 PD patients (Willis and Turner,

2007); A recent retrospective study administered polychromatic light at a dose of 4000-6000 lux daily for up to 8 years in 129 PD patients. A surprising finding of this study was the significant and persistent improvement in both motor and non motor functions (Insomnia and depression) as long as the patients remained in the program (Willis et al., 2012). Whether the light therapy benefits resulted from interference with the neurodegeneration process or not remains to be determined. The recovery rate was dependent on the variables, depression and insomnia responded quickly within the first month, while the improvement of the primary motor manifestations was slow but constant over several months to years. The underlying mechanisms for the therapeutic effect of bright light are also unknown. Light could act by resetting the phase of abnormal circadian rhythms; evidence for such mechanism came from a study showing that light presented to PD patients 1h after wakening does not improve bradykinesia and insomnia (Paus et al., 2007) while the application of the same light 1h before bedtime, when it is expected to have a correcting effect on the advanced rhythm of PD patients, does improve both motor and sleep abnormalities (Willis and Turner, 2007; Willis et al., 2012).

Although these results constitute an experimental demonstration that light plays an important role as a therapeutic strategy which may open a new direction for amelioration of both motor and non motor symptoms of PD, the field is in its infancy and many questions remain unanswered. For example, the evolution of light therapy efficiency with disease progression is unknown, especially considering the previously discussed retinal abnormalities that develop with disease progression (*see Introduction*). Second, the modulation of spectral properties of light to optimize the therapeutic effects has not been examined. Until now, only polychromatic light has been employed. A monochromatic blue light or a polychromatic with a blue enriched light would be expected to trigger a more efficient effect particularly if the effect of light is at least partially mediated through melanopsin retinal ganglion cells. Additionally, the general mechanisms of the beneficial effects of light are still unknown. No study has specifically assessed the mechanism of light therapy on molecular or physiological rhythms to verify whether light exerts effects by correcting the misalignment of multiple physiological rhythms in PD.

Our limited knowledge of the state of the circadian system in PD also constitutes an intrinsic limitation of a potential broad application of light therapy. A genuine assessment of circadian parameters should be performed under constant conditions in which environmental time cues

(light/dark transition, scheduled feeding) are absent. In PD, no circadian-related investigations or observations have been performed under these conditions and studies are typically conducted under a nycthemeral Light/Dark (LD) cycle. Under LD conditions, light and dark exert, in addition to its chronic entraining effect, acute excitatory and masking effects. These effects influence the daily pattern of behavior and often prevent expression of the underlying circadian rhythm controlled by the endogenous clock of the SCN (Johnson et al., 2003, Reppert and Weaver, 2002). While practically and ethically challenging, there is a need to explore the potential disruptions of the circadian system in PD patients in constant or forced-desynchrony conditions.

Holistic approaches for normalization of the circadian system in PD

Parkinson's disease remains an incurable progressive neurodegenerative disease (Lees et al., 2009). After its introduction 50 years ago, Levodopa treatment in combination with a peripheral dopa decarboxylase inhibitor remains the most effective therapy and the first treatment option for motor symptoms of the disease (Lees et al., 2009). The therapeutic effect of L-Dopa, however, wanes over time and on the average, 5 years after initiation of treatment, motor fluctuations and adventitious involuntary movements re-emerge. Moreover, additional adverse effects such as excessive daytime sleepiness have been associated with Dopamine medication (Arnulf and Leu-Semenescu, 2009). To overcome these side effects of DA medication, the development of surgical deep brain stimulation (DBS) has led to remarkable results on reduction of both intrinsic motor abnormalities (bradykinesia, rigidity and resting tremor) and long term DA intake related side effects. This remarkable achievement was tempered by the discovery of behavioural problems related to DBS such as abulia, depression, reduced verbal fluency, weight gain and difficulty with social adjustment (Krack et al., 2003). Rare fatalities such as intracerebral haemorrhage and suicide have also been reported (Voon et al., 2008). Additionally, both DA replacement medication and DBS do not alleviate the wide spectrum of non-motor symptoms including the circadian dysfunctions that dominate the clinical picture of the disease in advanced stages (Chaudhuri et al., 2006; Chaudhuri and Schapira, 2009; Fasano et al., 2012; Lozano and Lipsman, 2013). Currently, therapeutic strategies that would target NMS along with neuroprotective or disease-modifying therapies remain a critical unsettled clinical need (Meissner et al., 2011). These limitations highlight the difficulties we face and the need for a holistic therapeutic approach in which the benefits of

light therapy as an adjuvant to DA therapy alongside a shift from a sedentary towards an active lifestyle of PD patients. In this section, we will discuss evidence-based results in support of this approach.

With the limited treatment possibilities of the wide spectrum of non-motor symptoms in PD, other symptom management approaches are highly warranted. Here we discuss a dual approach with long-term well scheduled bright light stimulation and relatively long bouts of active exercise. The retrospective study of Willis et al (2012) showed that a dose of 4000 to 6000lux one hour prior to bedtime significantly improved symptoms related to insomnia, depression and multiple motor symptoms. No studies have yet evaluated the influence of light therapy on other non-motor symptoms in patients with PD. However, it is reasonable to expect that patients with PD would experience similar benefits for cognitive functions as those seen in the demented elderly, for which recent, large scale randomized controlled trials demonstrated that bright light exposure attenuated cognitive and functional decline and positively influenced mood (Riemersma-van der Lek et al., 2008). Interestingly, the study by Willis et al (2012) showed that light therapy significantly reduced and/or stabilized the amount of L-Dopa required to maintain therapeutic efficacy compared to a 17% increase observed in non-exposed controls over the period of Bright light therapy (BLT). This result suggests that light therapy improves, by an as yet unknown mechanism, Levodopa efficacy. A possible mechanism of this effect might be the well established enhancing effect of light of dopaminergic neurotransmission. Indeed, light has been shown to exert an acute activating effect on the DA system in both substantia nigra and the striatum (Nieoullon et al., 1977). In humans there is only limited evidence on daily light-dependent changes in dopamine neurotransmission. However, light may also be activating for the human DA system since dopamine is higher during the light phase of the day than at night (Poceta et al., 2009). An upregulatory effect of light is also consistent with seasonal increased endogenous dopamine function during summer compared to winter (Eisenberg et al, 2010; Tsai et al., 2011).

A second complementary approach that has been proposed to improve the quality of life in individuals with PD is physical activity and exercise. A recent review by Speelman et al (2011) listed 10 potential reasons why an active lifestyle might benefit patients with PD. Interestingly, among the symptoms that responded positively to exercise and general physical training are the previously evoked BLT-sensitive symptoms (cognitive functions, depression, sleep, motor performances, Levodopa efficacy and dopaminergic signalling). In addition to

these symptoms, osteoporosis, cardiovascular function, constipation and fatigue also improved with exercise. Given the brief duration of the bouts of exercise (maximum 2h) evaluated in the current studies, it is likely that the beneficial effects of physical activity on both motor and non-motor symptoms act via adaptive neuroplasticity; that is the brain's capacity to adjust through dynamic neuronal reorganisation (Hillman et al., 2008). Another, yet not systematically investigated mechanism of therapeutic effect of exercise in PD and in the elderly in general is the non-photic entrainment of the circadian clock by locomotor activity as a non-photic stimulus. Numerous studies have established the potency of timely scheduled locomotor exercise to entrain circadian functions in both nocturnal and diurnal (including humans) species (reviewed in Hughes and Piggins, 2012).

Interestingly, behavioral studies on the interactions of photic and locomotor induced-activity stimuli on circadian rhythms following a misalignment between the circadian system and the environmental LD cycle suggest a synergistic relationship between photic and non-photic cues. For example, in rodents the latency to reentrainment to an 8h phase advance of LD cycle (which simulate the jetlag encountered by humans following transmeridian air travel) can be dramatically reduced to 2-3 days by 3h of locomotor activity at the beginning of the dark phase on the first day in the shifted LD cycle instead of ~8 days under normal conditions where light is the only entraining factor (Mrosovsky and Salmon, 1987; 1990; Van Reeth and Turek, 1987; 1989a; 1989b). In vitro pharmacological studies on SCN-containing brain slices also support the beneficial and additive effects of photic and non-photic stimuli on SCN oscillations (Biello et al., 1997). taken together, these data, along with evidence of the general resemblance of the PRC to exercise for humans to that of both nocturnal and diurnal species, delay shifts occur during the late night and advances are reported during the late day and into the early evening (Buxton et al., 1997a; 1997b; 2003; Mistlberger and Skene, 2005; Van Reeth et al., 1994). It could be expected that scheduled exercise in the early morning combined with light therapy just before bedtime would most efficiently correct the abnormally advanced circadian system in PD patients (Fertl et al., 1993; Bordet et al., 2003).

The inexpensive and non-invasive nature of both light therapy and exercise renders this approach a highly attractive therapeutic strategy to postpone the onset of parkinsonism or even to prevent the manifestations of multiple non-motor symptoms. This possibility is all the more pertinent given the emerging strategies used to identify people who are at high risk of developing PD: individuals (and especially family members of individuals with PD) who have

a reduced sense of smell, individuals with fragmented sleep, daytime sleepiness and rapid eye movement sleep behavior disorders, people with chronic constipation and individuals who carry a mutation in a PD susceptibility gene (Stephenson et al., 2009). Applying a dual light therapy-exercise approach in such individuals as a pre-emptive strategy could potentially confer beneficial results and due to its non-invasive nature, does not face the ethical issues of chemical-based neuroprotective therapies with potential long-term side effects (Obeso et al., 2010). The potential pre-emptive nature of this dual approach is further supported by studies in mouse models of PD that have highlighted the neuroprotective and neurorestorative effects of exercise and light therapy (Xu et al., 2010; Willis et al., 2012).

Perspectives:

A significant outcome of my thesis is the hypothesis of a potential role of dopamine as a chemical signal to drive the circadian expression of DA related functions such as locomotor activity. This hypothesis is consistent with numerous studies showing that almost all aspects of DA neurotransmission exhibit daily rhythms of expression (Castaneda et al, 2004; Hood et al, 2010) that are both light and clock dependent (Sleipness et al, 2007). Inversely, DA has been shown to modulate clock gene expression both *in vivo* and *in vitro* (McClung et al, 2005, Hampp et al, 2008) and lesion of the nigro-striatal system disrupts the daily and circadian rhythms of rest-activity and striatal clock gene expression (Gravotta et al, 2011). Furthermore, Dopamine is also considered a potent wake promoting factor across species (Wisor et al., 2001; Ueno et al., 2012).

The logical follow-up of our series of studies is to assess the validity of these hypotheses regarding the critical role of dopamine in circadian and sleep processes. One hypothesis is that decreased DA levels will result in rhythmic dopaminergic neurotransmission falling below a hypothetical threshold level necessary for the expression of overt locomotor rhythms (**Figure 1**). An alternative but not exclusive hypothesis is that DA may be required to synchronize the phase relationships of individual oscillatory neurons and to generate coherent systems level output of the striatal system. In this case, the function of DA in the striatum would be analogous to that of VIP as a coupling factor in the SCN (Lucassen et al., 2012; Maywood et al., 2006; 2011). Indeed; in the retina DA is recognized to play a synchronizing role for both physiological and clock functions.

In general only sparse informations are available concerning the role of DA neurotransmission in the modulation of sleep and circadian functions. This is surprising given the availability and characterization of several DA receptor knockouts and compelling evidence suggesting an involvement of DA in sleep and circadian functions. Several appropriate knockout and transgenic models are currently available and can provide promising tools for the study of the relationships between DA, sleep, circadian clock and clock driven functions.

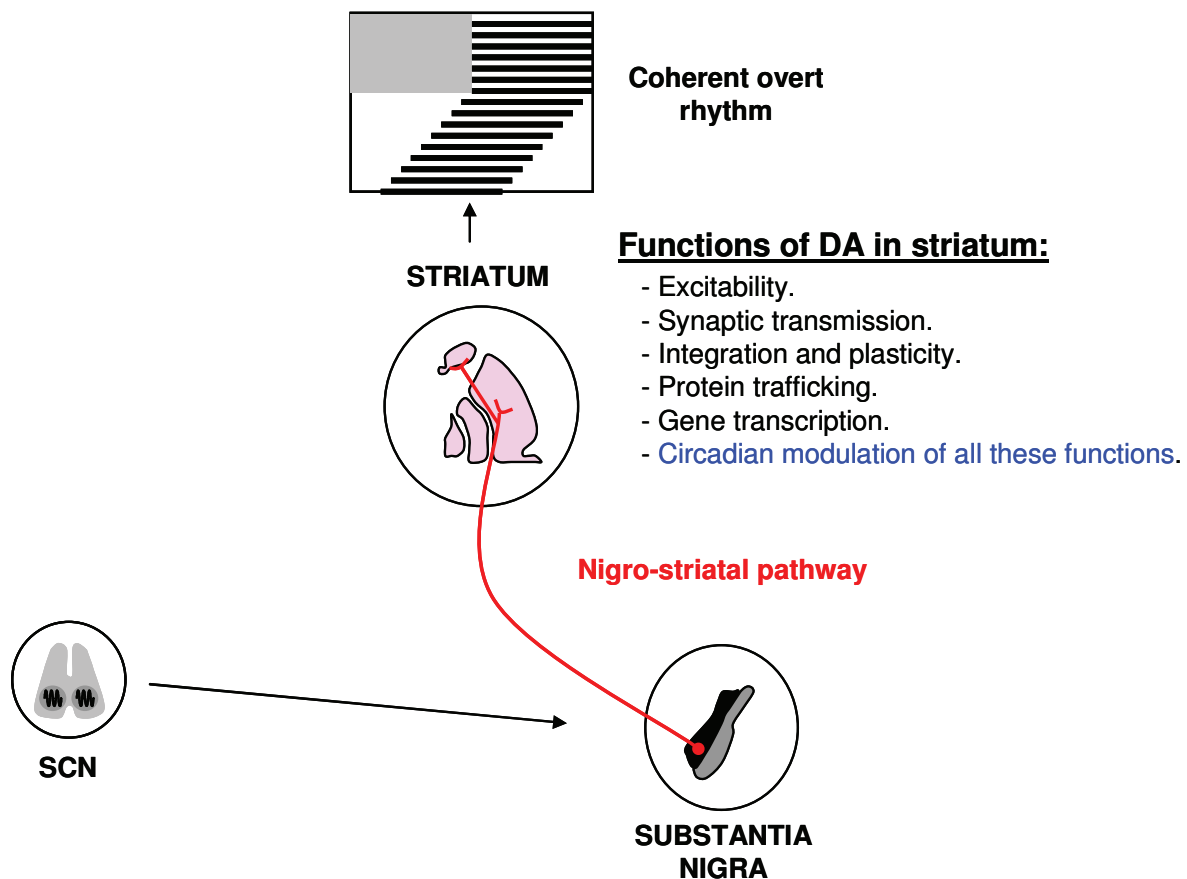


Figure 1: Role of DA in driving the circadian component of striatal related functions: The results of our studies suggest that in addition to the well known regulatory roles of DA in the striatal system (Tritsch & Sabatini, 2012) DA neurotransmission may also constitute a synchronizing factor that translates circadian output signals from the SCN to rhythmic functions of the striatum in order to assure a coherent rhythmic behavioral rhythm.

A number of key questions can be addressed at the molecular level. Do clock genes and/or individual cells continue to oscillate normally in DA deficient animals? How is coherent clock gene expression affected by the presence or absence of DA? Is DA capable of synchronizing individual cellular oscillators in the striatum and if so, what receptor channels are involved?

In vivo electrophysiology is also an essential approach to elucidate the role of DA in the modulation of 1) electrical activity of SCN clock neurons, 2) the rhythmic electrical activity of striatal neurons and 3) sleep architecture. Simultaneous recordings could be made of multiunit activity of the SCN, of the striatum and of EEG Polysomnography. These combined approaches can be employed to explore the hypotheses that DA is required to drive coherent clock gene expression in the striatum, to synchronize electrical activity of the neuronal population and to effectively drive overt rhythmic behaviours.

In order to further clarify the role of DA as a synchronizer of striatal neuronal activity, the use of *in-vitro* electrophysiological recordings in brain slices of striatum would be a tool of choice. The circadian modulation of electrical activity of DA neurons and medium spiny neurons in the striatum in normal mice and in DA deficient mouse models can be characterized using multiunit recordings at different stages of DA degeneration. DA receptor agonists and antagonists can be applied to define the specific receptor channels involved in this regulation and the alterations in electrical and clock gene expression. The ability of DA administration to restore synchronized neuronal activity can also be assessed.

As discussed in the introduction, Parkinson diseased patients may suffer from a direct and indirect desynchrony of the circadian clock network, not only in terms of daily locomotor rhythms but also for functions in diverse peripheral tissues. The evidence of such internal desynchrony is evident in PD patients as well as in a number of other neurodegenerative diseases. A critical question for better understanding the underlying pathophysiology of this desynchrony requires assessment of the circadian topology of clock gene oscillatory patterns of both the central nervous system (CNS) and peripheral tissues.

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