Supplementary Online Content

Breen DP, Vuono R, Nawarathna U, et al. Sleep and circadian rhythm regulation in early Parkinson disease. *JAMA Neurol*. Published online March 31, 2014. doi:10.1001/jamaneurol.2014.65.

This supplementary material has been provided by the authors to give readers additional information about their work

Methods

Clinical assessments

Patients were recruited to the PICNICS study, a community-based epidemiological study designed to identify all new cases of Parkinson's disease (PD) in the county of Cambridgeshire, UK. Patients were recruited from primary and secondary care. In order to ensure that the cohort was representative, assessments took place either at the John van Geest Centre for Brain Repair or the patient's own home. All patients met UK Brain Bank criteria for the diagnosis of PD. Patients completed the Pittsburgh Sleep Quality Index (PSQI), Epworth Sleepiness Scale (ESS) and Parkinson's Disease Questionnaire (PDQ-39); and underwent a range of other assessments including the Beck Depression Inventory (BDI), Addenbrooke's Cognitive Examination (ACE-R), Apathy Evaluation Scale (AES and AES-companion), Neuropsychiatric Inventory (NPI), Cambridge Behavioural Inventory (CBI) and Unified Parkinson's Disease Rating Scale (MDS-UPDRS). Motor phenotype (tremor dominant (TD) or non-tremor dominant (non-TD)) was calculated based on tremor, gait and postural instability subscores from the MDS-UPDRS.[1] Levodopa equivalent daily dose (LEDD) was calculated using the formula proposed by Tomlinson and colleagues[2], and other concurrent medication conditions and treatments were recorded.

Following their baseline assessment, 30 consecutive PICNICS patients were invited to take part in the intensive sleep study (regardless of whether or not they had sleep problems). Every PICNICS patient was asked to participate until the target recruitment number was reached. Exclusion criteria were patients with evidence of an atypical parkinsonian disorder; patients unable to give informed consent (including those with significant cognitive impairment); patients without a working knowledge of the English language; and patients not able to stay in hospital unaccompanied overnight. We also recruited 15 healthy age and sex-matched controls via local advertising. Participants who were (or had been) shift workers, and PD carers/bed partners, were not eligible for inclusion in the study. Patients underwent further subjective sleep assessment, actigraphy assessment, polysomnography and circadian rhythm analysis.

Actigraphy assessment

These uniaxial accelerometers measure peak intensity of movement each second, gauged by the voltage generated by the accelerometer, which is then expressed as an activity count. The actiwatches were set to a medium sensitivity, with 40 counts defined as "awake". Epoch length was 30 seconds. Patients were asked to firmly press the indented circle on the upper side of the activity monitor to denote 'lights out' and 'lights on'. When this marker was missing or late, a judgement was made by the study investigator, taking into account the self-reported bed times (taken from the sleep diary completed during the same two-week period) and activity level. Daily actigraphy data was downloaded, averaged and analysed using specialist software (Actiwatch and Sleep Analysis 5.51; Cambridge Neurotechnology, Cambridge, UK).

Actigraphy was used to calculate the following nocturnal measures – sleep start (derived automatically from the marked 'lights off' time), sleep end (derived automatically from the marked 'lights on' time), sleep period (time elapsed from sleep start to sleep end), total sleep time (assumed sleep minus wake time), sleep latency (time between 'lights off' and sleep onset), sleep efficiency (percentage of time spent asleep whilst in bed), and movement and fragmentation index (the addition of percentage of time spent moving and immobility phases of one minute) which is used as an indicator of restlessness.

Non-parametric circadian rhythm activity analysis was subsequently carried out using the same software to calculate general activity/rest measures – M10 count (average activity during the most active 10 hours), L5 count (average activity during the last active five hours), relative amplitude (difference between M10 and L5), intra-daily variability (quantifies the degree of fragmented motor activity during the 24-hour period), inter-daily stability (quantifies the degree of resemblance between activity patterns on individual days), light:dark ratio, average count during light, and average count during dark.

Polysomnography

Patients were not allowed to nap during the day before the sleep study, nor were they allowed to take vigorous physical activity or have caffeinated drinks. Electroencephalography was carried out using the standard international 10-20 electrode placement system, together with electrooculography, submental chin and bilateral anterior tibialis electromyograms, an electrocardiogram, pulse oximetry, chest and abdominal movement detector, nasal airflow detector, thermistor and snoring sensor.

The following polysomnography measures were captured – sleep period time (time elapsed from sleep onset to sleep offset), total sleep time (actual sleep time in a sleep period), sleep latency (time between lights out and sleep onset; defined as three epochs of stage 1 or one epoch of any other sleep stage), REM latency (time between sleep onset and first epoch of REM sleep), proportion of sleep period spent in each sleep stage, sleep efficiency (ratio of total sleep time to sleep period time), arousals index (AI) (number of arousals per hour during sleep period), periodic limb movement index (PLMI) (number of sleep-related periodic limb movements per hour), apnoea-hypopnoea index (AHI) (number of apnoeas or hypopnoeas per hour), desaturation index (DI) (number of desaturation episodes per hour greater than 4%) and minimum nocturnal oxygen saturation.

During the day, patients underwent Multiple Sleep Latency Testing (MSLTs). This consisted of five nap opportunities performed at two-hourly intervals, during which the lights were turned off and the patient was asked to relax and close their eyes. Daytime sleep latency time (time of first epoch of greater than 15 seconds of cumulative sleep on a 30 second epoch) was recorded. If the patient fell asleep, the nap was allowed to continue for a further 15 minutes. If no sleep was observed after 20 minutes, the nap opportunity ended. Mean Sleep Latency (MSL) was calculated by averaging across all nap opportunities.

Diagnostic criteria for primary sleep diagnoses

REM Sleep Behaviour Disorder (RBD) was defined as the presence of REM sleep without atonia and a history of injurious, potentially injurious or disruptive behaviour associated with sleep and/or documentation of abnormal behaviours observed on time synchronized infrared video recording during those periods of REM sleep without atonia. Significant Periodic Limb Movements of Sleep (PLMS) was defined as a periodic limb movement index exceeding 15/hour. A diagnosis of Restless Legs Syndrome (RLS) relied on the patient describing the characteristic symptoms (typically unpleasant sensations in the legs that occur in the evening and are relieved by movement). Obstructive Sleep Apnoea (OSA) and Central Sleep Apnoea (CSA) were diagnosed according to established criteria (9), with severity of OSA defined according to the apnoea-hypopnoea index: mild (5.1-15/hour), moderate (15.1-30/hour) or severe (>30/hour). Excessive Daytime Sleepiness (EDS) was defined as a mean sleep latency (MSL) of less than eight minutes.

Circadian rhythm analysis

Hormone analysis

ELISA was carried out using the IBL International GMBH and R&D Systems according to the manufacturer's instructions. For the quantitative measurement of melatonin, each sample was first passed through a C18 reversed phase column, then extracted with methanol, evaporated to dryness and reconstituted with water. Following this, each sample was added to the corresponding well of a microtiter plate coated with the goat-anti-rabbit antibody. The unknown amount of antigen present in the sample and the fixed amount of enzyme-labelled antigen competed for antibody binding sites. After incubation for one hour at room temperature, the wells were washed to stop the competition reaction. After adding the p-nitrophenyl phosphate substrate solution, the concentration of melatonin was inversely proportional to the optical density measured at 405nm. Optical densities were measured using a µQuan microplate spectrophotometer (Biotek, Winooski, VT, USA). Melatonin standards were used to construct a calibration curve against which the concentration of unknown samples was calculated.

The cortisol assay was based on the competitive binding technique in which cortisol present in a sample competes with a fixed amount of horseradish peroxidase-labelled cortisol for sites on a mouse monoclonal antibody. Following incubation, the wells were washed to remove excess conjugate and unbound sample, and a substrate solution was added to the wells to determine the bound enzyme activity. The concentration of cortisol was inversely proportional to the optical density measured at 450nm.

Based on hormone concentrations at each time point, we determined the acrophase (maximum concentration) and nadir (minimum concentration). Amplitude was defined as half the difference between acrophase and nadir. Hormone onset time was defined as the first sustained rise of two standard deviations (SD) above baseline levels recorded between 13:00-16:00 (melatonin) or 20:30-23:30 (cortisol). Hormone offset time was defined as the first sustained fall of two standard deviations below acrophase. We also calculated the total 24-hour hormone production (area under curve calculated using the trapezoid rule).

Clock gene analysis

Total cellular RNA was isolated from samples of peripheral blood mononuclear cells using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. On-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen) to avoid residual genomic DNA contamination. RNA concentrations were measured using the Nanodrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA quality was checked by ensuring that the A260/280 ratio was greater than 1.8. The qRT-PCR was performed on a Light Cycler 480 Instrument using the one-step RealTime Ready RNA Virus Master Kit and the RealTime Ready Probes (Roche, Mannheim, Germany). 50ng of RNA per reaction and 10μM of gene specific assays were used. Cycling parameters were 8 minutes at 50°C (reverse transcription), followed by a pre-incubation at 95°C for 30 seconds and 45 cycles at 95°C for 1 second, 60°C for 20 seconds, and 72°C for 1 second. The specificity of PCR products was confirmed using melting curve analysis. The relative abundance of messenger RNA was calculated using a standard curve method. The constitutively expressed non-rhythmic *β-actin* gene was chosen after screening a panel of 19 human housekeeper genes using the RealTime Ready Human Reference gene panel.

Statistical analysis

Data was examined for normality using visual histograms and the Kolmogorov-Smirnov test. Means were compared using unpaired t-tests or ANOVA (normally distributed data), and Mann-Whitney (non-normally distributed data). Chi-squared or Fisher's Exact tests were used to compare proportions between groups. Pearson or Spearman's rank correlation coefficients were calculated to assess bivariate associations. Positive and negative predictive values were used to evaluate the usefulness of screening questionnaires for predicting certain primary sleep disorders. Linear regression (continuous dependent variables) and logistic regression (ordinal dependent variables) models were used to control for covariates (sex and age unless stated otherwise). Effect sizes were calculated using Glass's delta. Clock gene expression data was normalised by calculating z-scores at each time point. Hormone and normalised clock gene data was then analysed using a repeated-measures 2-way ANOVA (adjusted for age and sex). Mauchly's test was performed to examine for any violations of the assumption of sphericity, and, if present, the degrees of freedom were corrected using the Greenhouse-Geiser method. Cosinor analyses were subsequently applied to all normalised circadian data to define the phase (mesor of the fitted curve) and rhythmicity (how well the sine curve fitted the data) of each individual circadian profile. The threshold of significance was set at $P<0.05$. Statistical analyses were performed using SPSS version 21 (SPSS, Chicago, IL, USA).

Online-only References

1. Stebbins GT, Goetz CG, Burn DJ, et al. How to identify tremor dominant and postural instability/gait difficulty groups with the movement disorder society unified Parkinson's disease rating scale: comparison with the unified Parkinson's disease rating scale. *Mov Disord* 2013;28(5):668-670.
2. Tomlinson CL, Stowe R, Patel S, et al. Systematic review of levodopa dose equivalency reporting in Parkinson's disease. *Mov Disord* 2010;25(15)**:**2649-2653.

eTable 1. Clinical characteristics of intensive sleep assessment cohort versus overall PICNICS cohort

Variable	PD sleep (n=30)	PICNICS (n=239)	P-value
Male sex (%)[a]	53%	62%	0.388
Age at motor symptom onset (years)[b]	62 (8)	67 (9)	<0.001*
Age at diagnosis (years)[b]	63 (8)	68 (9)	0.009*
On dopaminergic therapy (%)[a]	80%	42%	<0.001*
LEDD in treated patients (mg)	312 (157)	316 (209)	0.710
MDS-UPDRS part III[c]	24 (6)[a]	32 (13)	0.001*
Hoehn and Yahr stage[a]	1.27 (0.45)[d]	1.80 (0.77)[e]	<0.001*
ACE-R	92 (4)	90 (7)	0.263
Global PSQI	5.0 (2.9)	6.0 (3.9)	0.302
ESS	8.4 (4.6)	6.6 (3.8)	0.086

Results expressed as mean (SD) unless stated otherwise
*Significant difference at 0.05 level
LEDD=Levodopa Equivalent Daily Dose, MDS-UPDRS=Unified Parkinson's Disease Rating Scale, ACE-R=Addenbrooke's Cognitive Examination, PSQI=Pittsburg Sleep Quality Index, ESS=Epworth Sleepiness Scale
[a]Chi-squared test; [b]Unpaired t-test; otherwise Mann Whitney test used; [c]Based on MDS-UPDRS assessments performed within the last six months; [d]Range=1-2; [e]Range=1-4

eTable 2. Clinical characteristics of good versus poor sleepers

Variable	Domain	Good sleepers (n=98)	Poor sleepers (n=94)	P-value
Male sex (%)	Gender	63	60	0.599[a]
Age at diagnosis	Age	68 (9)	68 (10)	0.879[b]
BDI	Depression	5.8 (4.3)	8.8 (5.5)	<0.001*
ACE-R	Cognition	91 (6)	88 (6)	<0.001*
AES	Apathy	19 (15)	25 (15)	0.002*
AES-companion[c]	Apathy	18 (15)	25 (17)	0.007
NPI[c]	Neuropsychiatric	2.0 (5.7)	3.6 (5.1)	0.009
MDS-UPDRS part I	Non motor ADL	6.6 (3.6)	8.9 (3.6)	<0.001*
MDS-UPDRS part II	Motor ADL	8.4 (5.2)	9.8 (5.1)	0.010
MDS-UPDRS part III	Motor impairment	29 (11)	33 (12)	0.007
Hoehn and Yahr	Motor milestones	1.7 (0.7)	1.9 (0.8)	0.241[a]
TD phenotype (%)	Motor phenotype	33	18	0.018[a]
CBI[c]	Behavioural	15 (17)	21 (23)	0.075
ESS score	Daytime sleepiness	6.1 (3.8)	7.3 (3.8)	0.026
On dopaminergic therapy (%)	Medication	65	55	0.140[a]
LEDD (mg)	Medication dose	282 (184)	323 (209)	0.243
Dopamine agonist (%)	Dopamine agonist	10	20	0.053[a]

Results expressed as mean (SD) unless stated otherwise
*Significant difference at 0.05 level, after adjustment for multiple comparisons using Bonferonni method
BDI=Beck Depression Inventory, ACE-R=Addenbrooke's Cognitive Examination, AES=Apathy Evaluation Scale, NPI=Neuropsychiatric Inventory, MDS-UPDRS=Unified Parkinson's Disease Rating Scale, TD=Tremor dominant, CBI=Cambridge Behavioural Inventory, ESS=Epworth Sleepiness Scale, LEDD=Levodopa Equivalent Daily Dose
Non-completion of questionnaires – BDI (n=14), AES (n=2), AES-companion (n=28), NPI (n=52), CBI (n=34) and ESS (n=22)
[a]Chi-squared test; [b]Unpaired t-test; otherwise Mann-Whitney test used; [c]Questionnaires completed by companion as instructed

eTable 3: Rest/activity (actigraphy) measures in PD versus controls

Parameter	PD (n=29)[a]	Controls (n=15)	P-value (univariate)	P-value (multivariate)[c]
Sleep start	23:36 (00:51)	23:01 (00:53)	0.060	0.018*
Sleep end	07:05 (00:50)	06:59 (00:44)	0.843	0.532
Sleep period	07:28 (00:48)	07:57 (00:59)	0.165	0.081
Total sleep time (mins)	06:40 (00:44)	06:55 (00:40)	0.257[b]	0.274
Sleep latency (mins)	00:04 (00:05)	00:04 (00:06)	0.170	0.580
Sleep efficiency (%)	88.5 (4.8)	86.9 (5.7)	0.360	0.275
Fragmentation index	44.4 (20.3)	39.4 (14.9)	0.612	0.421
Amplitude	14280 (8989)	18435 (6304)	0.017*	0.113
M10 count	15238 (9250)	19581 (6518)	0.013*	0.109
L5 count	959 (559)	1145 (693)	0.473	0.378
Intra-daily variability	0.92 (0.16)	0.82 (0.16)	0.044[b]*	0.018*
Inter-daily stability	0.51 (0.10)	0.54 (0.09)	0.236[a]	0.273
Light:dark ratio	3.02 (0.85)	3.86 (1.03)	0.006[b]*	0.002*
Average during light	113 (71)	145 (51)	0.018*	0.123
Average during dark	39 (22)	39 (14)	0.407	0.913

Results expressed as mean (SD) unless otherwise stated
*Significant difference at 0.05 level
[a]Actigraphy not completed in one PD patient, therefore this data excluded from the analysis; [b]Unpaired t-test; otherwise Mann-Whitney test used; [c]Linear regression used, adjusting for age and sex

eTable 4. Polysomnography findings in PD patients with and without OSA

Parameter	No OSA (n=14)[a]	OSA (n=15)	P-value (univariate)	P-value (multivariate)[c]
Sleep start (hrs:min)	22:27 (00:46)	20:38 (05:44)	0.102	0.163
Sleep end (hrs:min)	06:45 (00:29)	07:04 (00:27)	0.102	0.117
Total sleep time (mins)	381 (50)	432 (76)	0.045*	0.040*
Sleep latency (mins)	12 (13)	15 (14)	0.603	0.693
REM latency (mins)	111 (61)	122 (128)	0.220	0.883
Sleep efficiency (%)	76 (12)	79 (9)	0.394	0.302
AI (arousals per hour)	19 (6)	23 (7)	0.201	0.060
PLMI (events per hour)	23 (35)	12 (21)	0.310	0.202
AHI (events per hour)	1 (1)	20 (19)	<0.001*	0.020*
DI (events per hour)	1 (1)	14 (17)	0.010*	0.017*
Minimum O_2 sats (%)	90 (4)	85 (4)	0.001*	0.003*
MSL (mins)[d]	12 (5)	10 (5)	0.234	0.195
% time in sleep stages				
Awake	23 (12)	19 (11)	0.310	0.289
Stage 1	8 (4)	12 (6)	0.105	0.163
Stage 2	49 (10)	49 (9)	1.000	0.833
Stages 3 and 4	9 (6)	8 (6)	0.652	0.801
REM	11 (6)	13 (7)	0.422	0.324

Results expressed as mean (SD) unless otherwise stated
All severities (mild, moderate and severe) included in the OSA group
*Significant difference at 0.05 level
AI=Arousals Index, PLMI=Periodic Limb Movement Index, AHI=Apnoea-Hypopnoea Index, DI=Desaturation Index, MSL=Mean Sleep Latency
[a]Not possible to distinguish EEG wakefulness from individual sleep stages in one PD patient, therefore this data excluded from the analysis; [b]Unpaired t-test; otherwise Mann-Whitney test used; [c]Linear regression used, adjusting for age and sex (and, in the case of AHI, the effect of BMI); [d]In two PD patients, MSL based on four rather than five MSLT nap opportunities

eTable 5: Effect of dopaminergic medications on sleep in PD

Parameter	None (n=10)[a]	Dopamine agonist (n=8)[b]	Levodopa (n=11)[c]	Both (n=1)[d]	P-value[e]
Total sleep time (mins)	425 (87)	400 (78)	402 (49)	362 (-)	0.465
Sleep latency (mins)	16.8 (18.7)	9.2 (13.5)	12.8 (7.5)	24 (-)	0.293
REM latency (mins)	118 (118)	93 (71)	103 (54)	425 (-)	0.833
Sleep efficiency (%)	79.1 (9.4)	78.8 (12.1)	76.8 (10.5)	64.0 (-)	0.566
AI (arousals per hour)	22.1 (8.5)	23.3 (6.5)	18.5 (4.9)	23.6 (-)	0.459
PLMI (events per hour)	24.3 (30.0)	0.3 (0.7)	25.2 (34.6)	0 (-)	0.408
AHI (events per hour)	6.8 (4.1)	18.4 (24.9)	8.6 (16.7)	19.3 (-)	0.957
DI (events per hour)	3.5 (3.0)	13.4 (20.7)	5.7 (12.4)	1.2 (-)	0.808
Minimum O_2 sats (%)	87.2 (4.2)	87.7 (3.1)	87.7 (5.5)	82 (-)	0.364
MSL (mins)	11.9 (4.7)	7.9 (6.0)	12.5 (3.6)	11.2 (-)	0.914
% time in sleep stages					
Awake	19.0 (11.3)	21.2 (12.1)	20.7 (10.8)	36.0 (-)	0.483
Stage 1	9.0 (4.9)	10.9 (6.8)	10.4 (4.8)	8.4 (-)	0.713
Stage 2	48.5 (9.6)	48.6 (12.3)	49.2 (8.5)	46.1 (-)	0.640
Stages 3 and 4	10.4 (8.2)	7.6 (3.3)	7.8 (6.0)	4.3 (-)	0.115
REM	13.1 (7.6)	11.7 (6.4)	11.3 (5.5)	5.1 (-)	0.364

Results expressed as mean (SD)
AI=Arousals Index, PLMI=Periodic Limb Movement Index, AHI=Apnoea-Hypopnoea Index, DI=Desaturation Index, MSL=Mean Sleep Latency
[a]Patients also taking rasagiline (n=3) and amantadine (n=1); [b]Patients also taking rasagiline (n=1) and amantadine (n=1); [c]Patients also taking rasagiline (n=1) and amantadine (n=2); [d]No SD reported because only one patient in this subgroup; [e]Linear regression used, taking into account any effects of age, sex and disease duration

eTable 6. Melatonin parameters in PD patients versus controls

Parameter	PD (n=30)	Controls (n=15)	P-value (univariate)[a]	P-value (multivariate)[b]
Acrophase	35.9 (30.3)	43.5 (30.7)	0.176	0.296
Nadir	3.2 (3.0)	6.7 (4.1)	0.005*	0.004*
Amplitude	17.0 (15.3)	18.1 (13.1)	0.503	0.633
Onset time[c]	20:58 (04:23)	17:30 (09:29)	0.627	0.119
Offset time	06:06 (05:11)	05:06 (01:19)	0.914	0.526
Peak duration[c]	6.4 (2.4)	5.8 (1.5)	0.429	0.450
Area under curve[d]	19693 (11267)	28937 (17497)	0.071	0.048*

Results expressed as mean (SD)
*Significant difference at 0.05 level
[a]Mann-Whitney test used; [b]Linear regression used, adjusting for age and gender; [c]Absence of sustained rise in melatonin in 2 PD patients and 2 controls, therefore melatonin onset time and peak duration could not be determined; [d]Area under curve calculated using the trapezoid rule (pg/ml*minute)

eTable 7. Cortisol parameters in PD patients versus controls

Parameter	PD (n=30)	Controls (n=15)	P-value (univariate)	P-value (multivariate)[c]
Acrophase	109.4 (27.8)	78.4 (31.4)	0.002[a]*	0.001*
Nadir	8.2 (5.2)	6.0 (4.1)	0.169[a]	0.101
Amplitude	50.6 (13.4)	36.2 (15.4)	0.003[a]*	0.002*
Onset time[d]	04:11 (02:28)	04:06 (02:04)	0.910[a]	0.631
Offset time[d]	08:18 (01:59)	08:43 (01:28)	0.721[b]	0.505
Peak duration[d]	4.6 (2.4)	4.4 (1.8)	0.759[a]	0.876
Area under curve[e]	57204 (16028)	37277 (5857)	<0.001[a]*	<0.001*

Results expressed as mean (SD)
*Significant difference at 0.05 level
[a]Unpaired t-test; [b]Mann-Whitney test; [c]Linear regression used, adjusting for age and gender; [d]Peak duration could not be determined in 7 PD patients and 2 controls due to the absence of sustained cortisol onset (n=3 and n=0 respectively) or cortisol offset (n=4 and n=2 respectively); [e]Area under curve calculated using the trapezoid rule (ng/ml*minute)