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ORIGINAL ARTICLE

Adult attention-deficit hyperactivity disorder is associated with alterations in circadian rhythms at the behavioural, endocrine and molecular levels

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Attention-deficit hyperactivity disorder (ADHD) in adults is associated with impaired sleep, and it has been postulated that this impairment may contribute to the psychopathology of this common condition. One key driver of sleep/wake cycles is the circadian system, which at the molecular level consists of a series of transcriptional feedback loops of clock genes, which in turn produce endocrine, physiological and behavioural outputs with a near 24 h periodicity. We set out to examine circadian rhythms at the behavioural, endocrine and molecular levels in ADHD. Adults with ADHD as well as age- and sex-matched controls were recruited. Circadian rhythms were measured by means of actigraphy for the determination of gross motor patterns, by self-sampling of oral mucosa for assessment of rhythmic expression of the clock genes *BMAL1* and *PER2*, and by estimation of salivary cortisol and melatonin levels. Actigraphic analysis revealed significant diurnal and nocturnal hyperactivity in the ADHD group, as well as a significant shorter period of best fit for the locomotor circadian rhythm in ADHD. *BMAL1* and *PER2* showed circadian rhythmicity in controls with this being lost in the ADHD group. Cortisol rhythms were significantly phase delayed in the ADHD group. These findings indicate that adult ADHD is accompanied by significant changes in the circadian system, which in turn may lead to decreased sleep duration and quality in the condition. Further, modulation of circadian rhythms may represent a novel therapeutic avenue in the management of ADHD.
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Introduction

Attention-deficit hyperactivity disorder (ADHD) is a psychiatric condition that can affect both children and adults, with an estimated prevalence of 3.4% in adults.[1] The disorder is characterised by behavioural and attention difficulties, which can lead to secondary problems such as drug addiction and delinquency in adults.[2] Sleep deficits are a prominent characteristic of the disorder, with up to 83% of adult with ADHD reporting problems with sleep.[3] Actigraphy and polysomnography studies of sleep disturbance in ADHD have demonstrated significant association of sleep disturbance with both childhood and adult ADHD, including delayed sleep onset and difficulties in awakening, increased nocturnal activity, reduced sleep efficiency and reduced percentage of Rapid Eye Movement (REM) sleep.[4–7] Furthermore, the core symptoms of ADHD, inattention, impulsivity and restlessness, are known characteristics of sleep deprivation.[8] Chronic sleep-onset insomnia has also been shown to be associated with childhood and adult ADHD.[9,10] ADHD is also associated with changes in diurnal preference towards greater eveningness.[4]

The circadian clock is responsible for the generation of rhythms of behaviour and physiology on a near 24 period base, and has a key role in determining the rhythm of the sleep/wake cycle.[11] The master clock is in the suprachiasmatic nuclei (SCN) of the hypothalamus and it is entrained to the 24 h day via retinal photic input with further semiautonomous oscillators present throughout the brain and periphery.[12] SCN output is responsible for driving circadian rhythms in a number of hormones, including melatonin and cortisol. Abnormal rhythms of melatonin secretion has been associated with childhood and adult ADHD.[9,10] Cortisol circadian profiles have been reported to be either unaltered in ADHD[12] or altered in association primarily with the hyperactive subtype of ADHD.[13,14] The molecular basis of such circadian

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rhythm generation consists of positive and negative transcriptional/translational feedback loops of 'clock' genes and their protein products, and single-nucleotide polymorphism in clock genes have been associated with ADHD.[15–17] Given these indications that the circadian clock may be compromised in ADHD, we have examined for the first time circadian rhythmicity at the molecular, endocrine and behavioural levels in adult ADHD.

Materials and methods

Subjects and ethics

Adult ADHD patients were recruited from the Adult ADHD clinic at Cefn Coed Hospital, Swansea, Wales, ($n = 13$) and age- and gender-matched healthy controls ($n = 19$) were recruited from the university staff, hospital staff and a student population. Each participant was assessed by structured clinical interview according to the international diagnostic criteria (DSM-IV SCID-RV), in addition to ADHD questionnaires and psychometric tests (WHO's Adult ADHD Self Report Scale), Conners scales (CAARS-Self-Report Long Version and CAARS-Observer Long Version) and the Wender–Utah Rating Scale for retrospective information on childhood ADH (group scores in Supplementary Table 1). Information regarding habitual sleep habits (bed time, time to fall asleep, wake time and sleep duration), alcohol and substance use was also gathered. Only control subjects who did not show evidence of suffering from a current psychiatric or sleep disorder were included in the study. As ADHD in adults is associated with high levels of psychiatric comorbidities, subjects with current or lifetime mild comorbid conditions were included to allow for a representative sample. Participants with histories of severe psychiatric illness were excluded, as were those who work night shifts. Details of the included participants can be found in Table 1. Chronotype was assessed by Horne–Oestberg Morning–Eveningness questionnaire. Informed consent was obtained from all participants, and the study was approved by the Local Research Ethics Committee for the NHS in Swansea.

Table 1 Characteristics of the study participants

	Controls (n = *19*)	*ADHD* (n = *13*)
Age in years, mean ± s.d.	32.3 ± 13.3	31.3 ± 11.7
Male	12 (63.1%)	8 (61.5%)
Current depression	0 (0%)	1 (7.7%)
Previous depression	1 (5.3%)	7 (53.8%)
Current anxiety disorder	0 (0%)	4 (30.7%)
No current comorbidities	19 (100%)	7 (53.8%)
Stimulant medication	0 (0%)	3 (23.1%)
Atomoxetine	0 (0%)	1 (7.7%)
Alcohol use >20 units per week	2 (10.5%)	1 (7.7%)
Habitual time to fall asleep >1 h	0 (0%)	7 (53.8%)

Abbreviation: ADHD, attention-deficit hyperactivity disorder.

Actigraphy

Subjects wore an ActiWatch Light (Cambridge Neurotech, Cambridge, UK) on their nondominant wrist for at least 7 days, the last day of which was when mucosal/saliva samples were collected. Data were collected in 1-min epochs. Measures of light exposure were also collected via the actiwatches. Data analysis excluded the first and last days of data collection. Analysis of actigraphic data was via nonparametric circadian rhythm analysis for at least 5 consecutive days[18] using the Actiwatch Sleep and Circadian Rhythm Analysis Software (version 5.54, Cambridge Neurotech). Objective measures of sleep parameters (for example, sleep onset, sleep duration, sleep efficiency and wake time) were also calculated from the actigraphic data. Circadian period from actigraphic data was calculated via χ^2 periodograms for data of at least 5 consecutive days. Details of the nonparametric measures of circadian rhythms used and what these represent can be found in the Supplementary Materials and methods.

Clock gene analysis

Buccal samples were collected every 4 h over a 24 h period using foam-tipped swab, and the material was stored on FTA cards (Whatman International, Kent, UK). RNA was extracted using the Magmax viral RNA isolation kit (Ambion, Warrington, UK). The quality and quantity of total RNA isolated was confirmed using the Experion automated electrophoresis system (Bio-Rad, Hertfordshire, UK). There was no evidence of variations of RNA quality according to time of sampling. One-step quantitative real-time PCR was performed using the Light Cycler 3.5 (Roche Diagnostics, Mannheim, Germany) with SYBR-Green I dye amplimer detection. Details of primers used and PCR protocol are in the supplementary Materials and methods. Normalisation was to glyceraldehyde-3-phosphate dehydrogenase.[19] Subjects were reminded by short message service text message to take their samples at the specified time, and *post-hoc* analysis of the actigraphic activity record was used to confirm the presence of an activity bout for at the nocturnal sampling time points. Subjects collected samples first during the day and then the night, so as that disturbed sleep caused by sampling would not impact on daytime values of gene expression.

Melatonin and cortisol analysis

Saliva samples were collected every 4 h over a 24 h period (at the same time periods as buccal sampling), by chewing on a cotton swab (Bühlmann Laboratories, Schönebuch, Switzerland) and subsequently stored at −20 °C before analysis. Subjects were asked to collect nighttime samples under dim illumination. Both the levels of salivary cortisol and melatonin were assayed by ELISA (IBL International, Hamburg, Germany).

Statistical analysis
Group-wise comparisons were conducted by Mann–Whitney U tests or *t*-tests. Clock gene expression and hormone data were fitted by the method of single cosinor analysis to determine whether significant circadian rhythms were present (Chronolab software, Universidad de Vigo, Vigo, Spain).[20] Briefly, this entails fitting data via least squares regression to a cosine curve with a 24 h period. Cosinor analysis produces measures of the rhythm peak-to-trough amplitude (the fit was deemed significant if the 95% confidence interval for the fitted amplitude did not include zero), the acrophase (time of the peak of the rhythm) and the MESOR (the middle value of the cosine wave). The regression fitting also produces an R-squared statistic, which is then used to compute the percentage of variance in an individuals time-series data that is accounted for by the fitted 24-h curve. For between-group comparisons of population chorometrics derived by this method, we used the Bingham test,[21] which compares the amplitude, MESOR, acrophase and amplitude/acrophase pair between groups. Clock gene and hormone data are presented as standardised Z-scores, to allow for the direct inter-variable comparison of amplitudes between genes and hormones measured in different units.[22] The hormone data were Z-scored from raw data, the clock gene data were Z-scored from glyceraldehyde-3-phosphate dehydrogenase-normalised data. Data were also examined by the above methods before Z-scoring, with no meaningful differences observed between the two methods. Correlations between the DSM ADHD index score and chronometric parameters were by the Pearson's product moment correlation.

Results

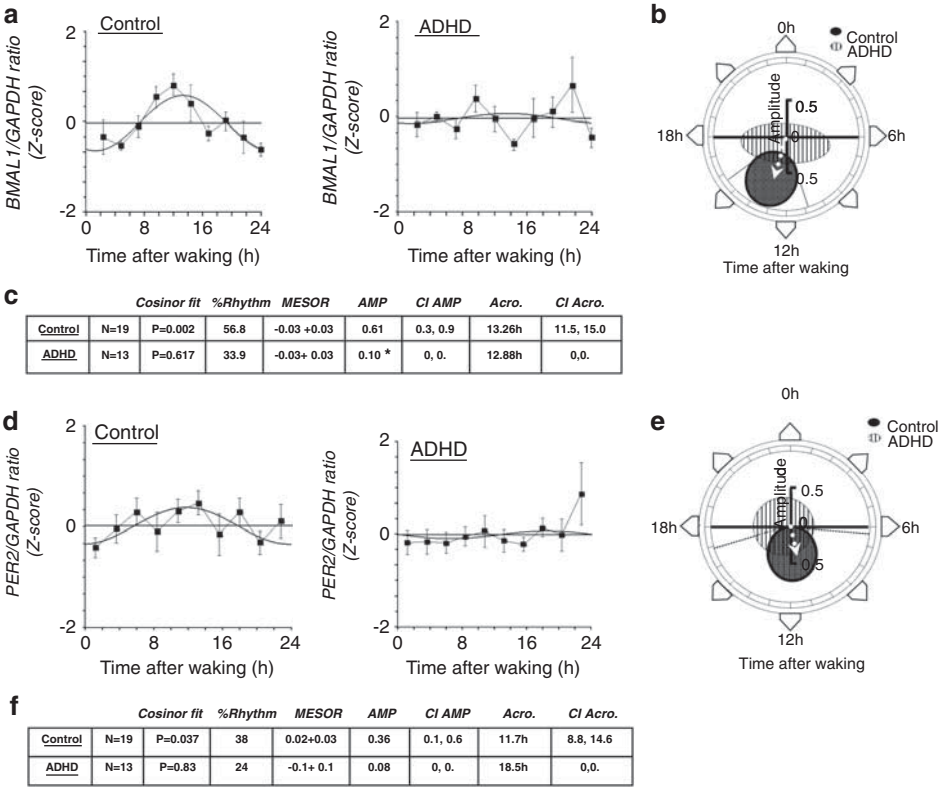
Actigraphy and diurnal preference
Analysis of actigraphic parameters is shown in Table 1. The amount of activity in the least active 5 h, L5, is significantly increased in the ADHD group, as is the amount of activity in the most active 10 h period, M10, and this in turn leads to an increase in the rhythm amplitude in the ADHD group (also Supplementary Figure 1). Analysis of the period shows that the ADHD group displayed significantly shorter period than the controls (Table 1). There was a significant increase in the average illumination in the 6 h between midnight and 0600 hours in ADHD subjects compared with controls (Supplementary Table 2). Morning–Eveningness questionnaire data showed that the ADHD subjects scored significantly higher than controls, indicating a shift to eveningness (Table 1). There were significant inverse correlations between the DSM index ADHD scores and the Morning–Eveningness questionnaire and the actigraphic period ($r = -0.619$ and -0.534, respectively, both $P < 0.005$). Objective measures of sleep indicate differences in the amount of sleep and sleep efficiency in the ADHD group compared with the controls (Supplementary Table 3). Self-reported sleep parameters indicate that 53% of the ADHD group habitually took longer than 1 h to fall asleep after bedtime, while no controls reported this (Tables 1 and 2), suggesting the presence of sleep-onset insomnia in a significant portion of the ADHD group.

Table 2 Actigraphic and diurnal preference measures in control and ADHD groups

	Controls (n = *19*)	*ADHD* (n = *13*)
Interdaily stability	0.579 ± 0.03	0.546 ± 0.03 *(NS)*
Intradaily variability	0.785 ± 0.05	0.707 ± 0.05 *(NS)*
M10	19620 ± 1156	25302 ± 1430 *($P < 0.001$)*
M10 o (h)	9.52 ± 0.32	10.8 ± 1.01 *(NS)*
L5	841 ± 98	1647 ± 295 *($P < 0.001$)*
L5 o (h)	2.2 ± 1.1	4.45 ± 1.6 *(NS)*
Amplitude	18785 ± 1156	23696 ± 1273 *($P < 0.001$)*
Relative amplitude	0.904 ± 0.015	0.879 ± 0.015 *(NS.)*
Period (h)	23.95 ± 0.07	23.76 ± 0.14 *($P < 0.001$)*
Acrophase (h)	14.62 ± 0.29	14.83 ± 0.66 *(NS.)*
Wake time (h)	7.96 ± 0.27	8.61 ± 0.47 *(NS.)*
MEQ score	52.6 ± 1.4	41.5 ± 2.9 *($P < 0.001$)*

Abbreviations: ADHD, attention deficit/hyperactivity disorder; MEQ, Morning–Eveningness Questionnaire; M10, activity counts in the 10 most active hours; M10 o, time of onset of the 10 most active hours; L5, activity counts in the 5 least active hours; L5 o, time of onset of the least active 5 hours; NS = not significant.

Clock gene and hormone rhythms
BMAL1 exhibited rhythmic expression in the control group, but not the ADHD group (Figures 1a and b). The amplitude and amplitude/acrophase relationship was found to be significantly different between the control and ADHD groups (Figures 1b and c). Rhythmic circadian expression of *PER2* was found in the control group, but the ADHD group did not display a significant circadian rhythm in *PER2* (Figure 1d), although between-groups comparisons of chronometrics did not reveal any significant differences (Figure 1f). Melatonin was strongly rhythmic in the control group and the ADHD group displayed a rhythm that appeared to be broadly similar, but with a dampened amplitude that meant the rhythm was not significant (Figures 2a and b). Between-groups comparisons did not reveal significant differences in any chronometrics (Figure 2c). For salivary cortisol, the control group showed a robust circadian rhythm with a peak in expression ~1 h after waking (Figures 2d and e). The ADHD group also showed rhythmic cortisol, although the peak of secretion was phase delayed relative to wake time and occurred ~3 h after waking. This difference in acrophase was found to be significant as was the amplitude/acrophase relationship (Figure 2f). When chronometrics from individual profiles were



	Cosinor fit	%Rhythm	MESOR	AMP	CI AMP	Acro.	CI Acro.
Control	N=19	P=0.002	56.8	-0.03 +0.03	0.61	0.3, 0.9	13.26h	11.5, 15.0
ADHD	N=13	P=0.617	33.9	-0.03+ 0.03	0.10 *	0, 0.	12.88h	0,0.

	Cosinor fit	%Rhythm	MESOR	AMP	CI AMP	Acro.	CI Acro.
Control	N=19	P=0.037	38	0.02+0.03	0.36	0.1, 0.6	11.7h	8.8, 14.6
ADHD	N=13	P=0.83	24	-0.1+ 0.1	0.08	0, 0.	18.5h	0,0.

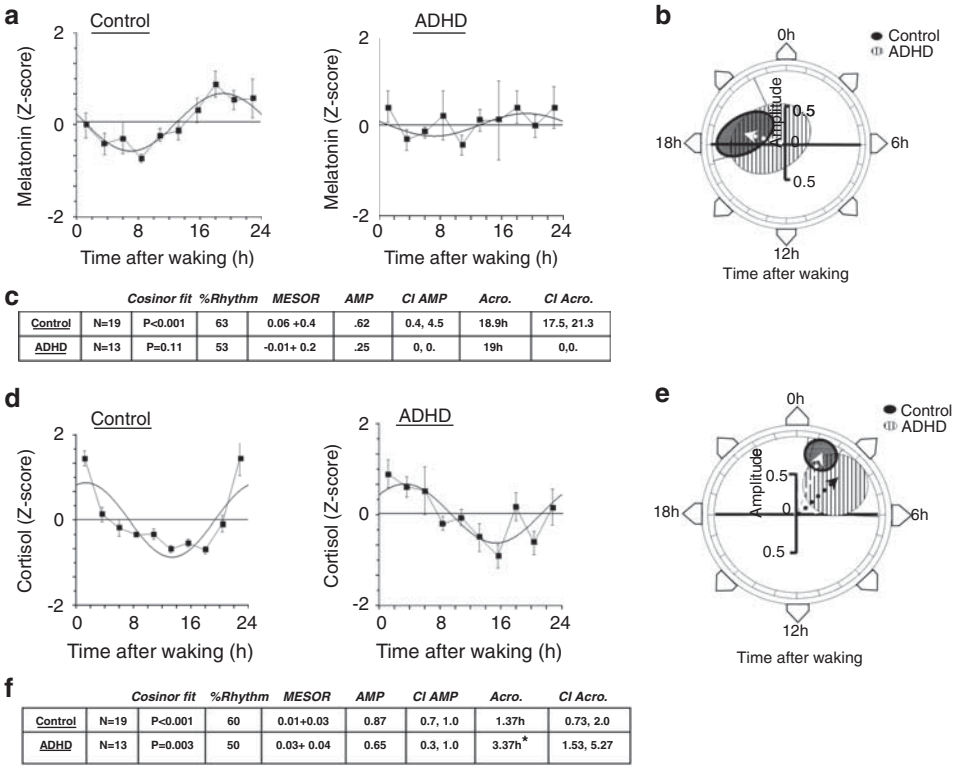
Figure 1 Circadian rhythms in *BMAL1* and *PER2* expression in oral mucosa in control and ADHD groups. (**a**) Chronograms for the expression of *BMAL1* in the oral mucosa of controls and the ADHD group. The fitted dark line represents the cosine wave of best fit with a 24 h period. The horizontal line represents the MESOR. (**b**) Polar plot illustrating the phase/amplitude relationship for *BMAL1* expression in the oral mucosa of controls (black ellipse) and ADHD subjects (hatched ellipse). Length of the dotted white vector indicates amplitude of circadian rhythm and the orientation of this vector represents the acrophase for the control group. Ninety-five percent confidence interval for the combined amplitude and acrophase are represented by elliptical areas around the vector tips, indicated by lightly dashed line. Note that the error ellipse for the ADHD group covers the zero point of amplitude, indicating a nonsignificant circadian fit for the observed data, while the error ellipse of the control group does not overlap the zero point, indicating a significant cosinor fit. (**c**) Chronometrics for circadian *BMAL1* expression in the control and ADHD groups. % Rhythm refers to the percentage of variance in the population data that is explained by regression with the 24-h cosine wave of best fit. AMP is the amplitude of the rhythm, CI AMP is the 95% confidence interval for the amplitude, Acro. is the time of the peak of the rhythm, CI Acro. is the 95% confidence interval for the amplitude. * indicates $P < 0.05$ by Bingham test between groups. (**d**) Chronograms illustrating the rhythmic expression of *PER2* in the oral mucosa of controls and the ADHD group. The fitted dark line represents the cosine wave of best fit with a 24 h period. The horizontal line represents the MESOR. (**e**) Polar plot illustrating the phase/amplitude relationship for *PER2* expression in the oral mucosa of controls (black ellipse) and ADHD subjects (hatched ellipse). (**f**) Chronometrics for circadian *PER2* expression in the control and ADHD groups.

examined across the control group it was found that, in agreement with the population profiles, there were significant differences in the amplitude of the BMAL1 rhythms between the control and ADHD groups, as well as a significant difference in the acrophases of the two groups for the cortisol rhythm (Supplementary Figure 2). We examined correlations between the DSMIV-index ADHD score in participants in the control and ADHD groups: in the control group we only found a significant correlation between PER2 acrophase and ADHD scores, while in the ADHD group we found strong inverse correlations between the ADHD index score and a number of chronometrics (Figure 3). We also report significant correlations between actigraphic measures of circadian behaviour and clock gene chronometrics (Supplementary Figure 3).

Discussion

Actigraphy and diurnal preference

Actigraphic measures demonstrate hyperactivity of the ADHD patients across the circadian cycle in the current study, while Boonstra *et al*.[7] report increases in M10 (but not L5) in adult ADHD and no significant differences in IS, IV or RA, similar to the present results. We also report significant shortening of the activity rhythm period in the ADHD group, one possible explanation for which is altered entrainment to external Zeitgebers in ADHD, as has been postulated for the phase delay of the dim-light melatonin onset.[10] In our data set, there is a strong positive correlation between the ADHD clinical score and period length in the ADHD group, suggesting that the



c

		Cosinor fit	%Rhythm	MESOR	AMP	CI AMP	Acro.	CI Acro.
Control	N=19	P<0.001	63	0.06 +0.4	.62	0.4, 4.5	18.9h	17.5, 21.3
ADHD	N=13	P=0.11	53	-0.01+ 0.2	.25	0, 0.	19h	0,0.

f

		Cosinor fit	%Rhythm	MESOR	AMP	CI AMP	Acro.	CI Acro.
Control	N=19	P<0.001	60	0.01+0.03	0.87	0.7, 1.0	1.37h	0.73, 2.0
ADHD	N=13	P=0.003	50	0.03+ 0.04	0.65	0.3, 1.0	3.37h*	1.53, 5.27

Figure 2 Circadian rhythms in salivary melatonin and cortisol in control and ADHD groups. (**a**) Chronograms for salivary melatonin of controls and the ADHD group. The fitted dark line represents the cosine wave of best fit with a 24 h period. The horizontal line represents the MESOR. (**b**) Polar plot illustrating the phase/amplitude relationship for melatonin of controls (black ellipse) and ADHD subjects (hatched ellipse). (**c**) Chronometrics for salivary melatonin in the control and ADHD groups. (**d**) Chronograms for the secretion of salivary cortisol in the control and the ADHD groups. The fitted dark line represents the cosine wave of best fit with a 24 h period. The horizontal line represents the MESOR. (**e**) Polar plot illustrating the phase/amplitude relationship for salivary cortisol in controls (black ellipse) and ADHD subjects (hatched ellipse). (**f**) Chronometrics for circadian cortisol secretion in the control and ADHD groups.

period of the activity rhythm would shorten with the severity of the disorder. It is worth sounding a note of caution in the interpretation of any actigraphic data, in that it assesses the gross rhythm in motor output, and does not delineate between endogenous circadian processes and environmental factors, and the interaction between these, and thus provides limited mechanistic insight.

Clock gene rhythms

An increasing number of laboratories are utilising molecular circadian clocks in peripheral and easily accessible tissues in order to gain insight into the clock gene cycles under various conditions.[19,23,24] In the present study, we developed a novel, noninvasive technique of sampling of oral mucosa for monitoring of clock gene expression. The advantage of the protocol used in this study is that it allows for self-sampling, and so does not require laboratory-based sampling and thus, when applied with actigraphy allows for a more naturalistic monitoring of circadian processes. This study is, to the best of our knowledge, the first to examine regulation of circadian clock gene expression in ADHD. We report a significant rhythm of *BMAL1* expression in the oral mucosa of control subjects with significant alterations in ADHD, while *PER2* expression exhibits a significant rhythm in controls, which is lost in ADHD. Furthermore, the DSMIV–ADHD score is inversely correlated with the amplitude and percentage rhythm of *PER2*, indicating a relationship between the strength of clock gene rhythmic expression and clinical ADHD ratings. Thus, a role for dysfunction in the entrainment of the circadian clock in ADHD may be tentatively postulated, although it is prudent to sound a number of notes of caution. First, we sampled one particular peripheral circadian clock (as sampling of the master SCN clock is obviously not possible in such studies), and there is a paucity of information available in relation to the regulation of the oral mucosal clock and how it sits in the overall integrated circadian system. Second, alteration in circadian processes in peripheral pacemakers as noted here may not necessarily reflect changes in central processes (the key regulators of behaviour) and thus we make no claims that the oral mucosa is a proxy for the SCN. Having said this, it is worth noting that the characteristics of molecular rhythms in skin fibroblasts[25] and in hair follicles[23] do correlate with behavioural measures of the subjects from whom the samples were derived, as

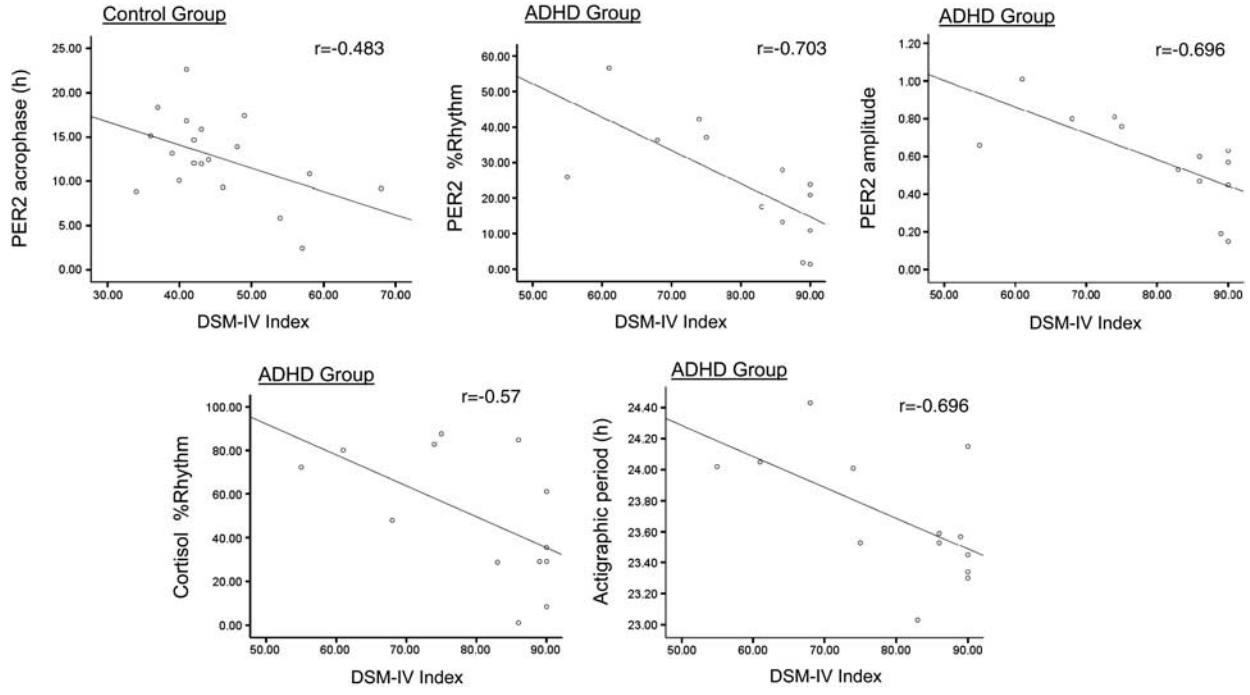


Figure 3 Correlations between chronometrics and DSM-IV ADHD index scores. Significant inverse correlations between DSM-IV ADHD index score and PER2 acrophase in controls, as well as with percentage of PER2 variance accounted for by a 24 h rhythm, PER2 rhythm amplitude, cortisol percentage rhythm and actigraphic period in the ADHD group.

they do in our present study (correlations between clock gene chronometrics and actigraphic period).

Melatonin and cortisol

Our data demonstrate a robust significant rhythm of melatonin in the healthy control group as expected, and a loss of significant rhythmicity in adult ADHD. This loss of rhythmicity is probably due to the reduced amplitude of the melatonin rhythm, although peak melatonin levels occurred at approximately the same time as the control group. Previous studies have reported an association of altered phasing of the melatonin rhythm with both childhood ADHD[9] and adult ADHD.[10] In these cases, an abnormal rhythm of melatonin secretion is associated with the ADHD where it is comorbid with sleep-onset insomnia, and a delayed sleep phase and delayed dim-light melatonin onset is observed. As we did not measure the dim-light melatonin onset in the current study, a direct comparison between our findings and those noted above cannot be made. A simple explanation for the dampened amplitude of melatonin secretion in ADHD is that as melatonin secretion is inhibited by light, an altered sleep–wake rhythm involving more nocturnal activity, and thus increased exposure to light, could suppress the secretion of melatonin. This hypothesis is supported by the actigraphy data, which demonstrate that the ADHD group is exposed to a significantly greater amount of nocturnal light than the control group. We report a significant rhythm of cortisol secretion in adult ADHD, although the rhythm of cortisol is phase delayed by $\sim$2 h in ADHD. Eveningness might be associated with a phase delay of the cortisol rhythm,[26] consistent with our present results demonstrating that ADHD is associated with more eveningness and a phase delay of the cortisol rhythm. The inverse correlation between ADHD score and the percentage of variance of the cortisol rhythm suggests that greater robustness of the cortisol rhythm is associated with ADHD, which is further supported by group-wise comparisons. As the rhythm in cortisol is known to be centrally driven via the SCN master clock,[27] the phase delay observed in our study may further reflect a deficit in the entrainment of the master circadian clock in adult ADHD to appropriate environmental and social stimuli. Seemingly normal diurnal rhythms of cortisol secretion have been reported in adult ADHD,[28,29] but as these studies did not undertake chronometric analysis, these findings may simply reflect the preservation of the rhythmic cortisol levels in adult ADHD (as reported here) but would not be able to detect the alterations in the phasing of the rhythm that we report. Altered neuroendocrine signalling by melatonin or cortisol may affect the entrainment of peripheral clocks, such as the oral mucosa.

There are a number of important caveats to consider in the interpretation of our data set. ADHD is a very heterogenous condition with high levels of lifetime psychiatric comorbidities. Given that affective conditions, as are often comorbid with ADHD, are also associated with altered circadian timekeeping,[30] it

will be difficult to associate any circadian alteration directly with ADHD *per se*. The presence of sleep-onset insomnia with ADHD is another factor to be considered.[10] A further complication is the unknown effects of stimulant and non-stimulant medications used in ADHD management on circadian parameters. While it would be desirable to be able to control for all of these potentially confounding factors, there is equally an argument to be made for the assessment of a clinically representative sample, as examined in the current study.

Conclusions

We have demonstrated that in ADHD there are significant disturbances not only in the rhythmic secretion of endocrine factors that are key outputs and regulators of the master circadian clock, but also in circadian clock gene expression of a peripheral oscillator and in actigraphic measures of circadian organisation of gross behaviour. Future work should examine chronotherapuetic approaches in ADHD and explore the effect of ADHD medications upon the circadian clock. Further research into modulation of circadian deficits in ADHD may prove beneficial in understanding the underlying causes of the symptomology and may serve to aid in appropriate and efficacious treatment of the disorder.

Conflict of interest

JT has received financial support from various pharmaceutical companies, including AstraZeneca, Bristol-Meyer Squibb, Janssen, Lilly, Lundbeck, MEDICE, Merz, Novartis, Pfizer and Servier, some of which manufacture medication used in the treatment of ADHD. The other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)