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**Relationship between post-awakening salivary cortisol and melatonin secretion in
healthy participants.**

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Abstract:

We report the relationship between patterns of post-awakening salivary melatonin and cortisol secretion in healthy participants (n=51; mean age 21.6 ± 5.0 years). Saliva samples were collected within the domestic setting, at 0, 15, 30 and 45 min post-awakening on 2 consecutive typical weekdays. Analyses were undertaken on data with electronically verified sample timing accuracy (< 5 min delay between awakening and the start of saliva sampling). Melatonin secretion declined linearly by an average of 29% within the first 45 minutes post-awakening. In contrast there was a marked 112% surge in cortisol, characteristic of the cortisol awakening response. No day-differences in melatonin or cortisol secretion were observed but melatonin concentrations were lower with later awakening. Despite contrasting post-awakening changes in these hormones there was a lack of relationship between overall levels or patterns of melatonin and cortisol during this period.

Circadian rhythm disruption is a common feature in aging, mental and physical ill-health (Jagannath et al., 2013; Karatsoreos, 2012; Wulff et al., 2010), which has led to intense investigation of the mechanisms linking circadian function and health (Menet & Rosbash, 2011; Pezuek et al., 2012). Circadian rhythms exhibit distinct diurnal and nocturnal states with an abrupt switch-like transition between sleep and waking; initiating a more gradual change in function to prepare for the day ahead: biological dawn (Morris et al., 2012; Wehr et al., 2001). Biological dawn is frequently measured by examination of post-awakening cortisol secretion, an index of circadian functioning of the HPA axis. Indices include the rise in cortisol (cortisol awakening response: CAR) and overall cortisol concentrations (e.g. area under the curve with reference to ground: AUCg) in the first 30-45 minutes following morning awakening. The CAR is a discrete aspect of the circadian pattern of cortisol secretion (Clow et al., 2010) and is believed to play a role in preparation for the day ahead (Adam et al., 2006; Fries et al., 2009; Stalder et al., 2009). In contrast melatonin, the dominant hormone of the night-time neuroendocrine system, is low during the day (Morris et al., 2012). Both hormones are regulated by the suprachiasmatic nucleus and are responsive to light; melatonin secretion is suppressed and the CAR enhanced (Morris et al, 2013; Scheer and Buijs, 1999). Although a relationship between the daily dynamics of salivary cortisol and melatonin secretion has been demonstrated (Corbalan-Tutau et al., 2014; Lang et al., 1986) a detailed examination of post-awakening melatonin secretion and its relationship with cortisol has not been reported. This is important as if the hormones are related underlying changes in melatonin may contribute to some of the effects attributed to cortisol.

The demonstration that melatonin is stable in saliva samples, with a circadian pattern reflecting serum (Vakkuri, 1985; Voultsios et al., 1997), together with the introduction of commercially available sensitive assay systems enables simultaneous determination of both cortisol and melatonin in the same saliva samples. The aim of the current study was to

simultaneously study post-awakening cortisol and melatonin secretion from saliva samples on 2 days within the domestic setting in a population of healthy participants. As sample timing accuracy is critical for accurate CAR measurement (Smyth et al., 2013; Stalder et al., In press) electronic monitoring of awakening and sampling times were employed. Using a within-subjects study design it was hypothesised that the fall in melatonin concentrations would be related to the increase cortisol concentrations in the immediate post-awakening period.

Methods

Participants

Fifty-one healthy participants (41 females) were recruited from within the academic community on the basis they had no diagnosed illness and taking no prescribed medication. Ages ranged from 18-39 (21.6 ± 5.0) years. Participants received no financial incentive to take part in the study but students received course credit. The University of Westminster ethics committee approved the protocol and all participants gave their informed written consent.

Materials

Participants were provided with full standardised written instructions, a saliva sampling kit consisting of two Zipoloc bags labelled Day 1 and Day 2, each containing four coded Eppendorf tubes labelled tube 1-4 and a record sheet to record their awakening and saliva sampling times. Participants were also provided with wrist-worn activity-recording devices (Cambridge Neurotechnology, Cambridge, or Philips Respironics, UK) to monitor awakening times. To monitor saliva sampling times the straws used for passive drool into the Eppendorf's were stored in a medication event monitoring (MEM) bottle and participants

asked to remove a straw at each specified sampling time and drool saliva into a pre-labeled tube. The actiwatch and MEMs devices were used in combination to identify delays between verified awakening and sampling times.

Procedure

Participants attended a one-to-one 20-30 minute induction session during which they received full instructions on procedures, including use of electronic monitoring devices and techniques for collecting and recording times of saliva samples. Participants were instructed to collect saliva samples on 2 typical weekdays (Tuesday, Wednesday or Thursday) via passive drool (recommended by Salimetrics as necessary for the accurate assessment of melatonin from saliva samples), using the Eppendorf tube, immediately on awakening and 15, 30 and 45 min later. Participants were instructed to awake in their usual way and to avoid food and drink (apart from water) or brush their teeth during the sampling period. Participants were asked to complete a record sheet with their awakening and saliva sampling times. Samples were initially stored in a domestic freezer until returned to the laboratory, where they were stored at -20°C until assayed.

Cortisol and melatonin assessment and assay

Saliva samples were thawed and centrifuged for 10-15 minutes at 3,500 rpm. Cortisol and melatonin concentrations were determined in duplicate by enzyme linked immune-sorbent assays (Salimetrics LLC, USA). The lower limit of sensitivity for cortisol is <.01638 nmol/l and for melatonin 1.8 pg/ml. Intra- and inter-assay variations were below 10% for both hormones.

Statistical analysis

Hormone concentrations ranged between 2.58 -116.21 pg/mg for melatonin and 0.24-41.82 nmol/l for cortisol. Data for both hormones were both positively skewed; square root

transformations normalised sample distributions. There were 15 days in which either actigraph or MEMs data was missing; these were excluded from the analyses. Delays between awakening and collection of the first sample greater than 5-minutes were excluded from analyses (26 days). Thus analysis included data, fully verified as accurate, from 61 days (from 37 participants). Mean (SD) awakening time was 6:44hrs (1.05hrs).

Mixed regression modelling (MRM) of growth curves was used to examine patterns of hormone secretion. First-order autoregressive covariance structure provided optimal data modelling. Simple linear effects were investigated in model A, a quadratic term was added in model B. Within-person variables, including study day and awakening time and the simple interaction of each with sample time were explored in model C. Final models presented here involved backward elimination of non-significant effects.

In further analyses composites of melatonin and cortisol were calculated. Standardized area under the curve with respect to ground (AUCg) was computed to estimate total secretion of both hormones. For cortisol, the CAR was calculated as the standardized mean increase (MnInc) of the second, third and fourth samples from the first awakening sample. For melatonin the standardized mean decrease (MnDec) was calculated as the mean decrease from the first sample. The relationships between melatonin and cortisol composites were investigated using MRM methods.

Results

The results of model A (see Table 1) indicate a linear decline in melatonin over the post-awakening period ($F = 23.362$, $df = 193.556$, $p < 0.001$). Model B indicated no quadratic effect for melatonin ($F = 2.812$, $df = 172.200$, $p = .095$). In model C a significant effect of awakening time on melatonin was observed ($F = 11.788$, $df = 228.830$, $p < 0.005$) such that

later awakening times were associated with lower melatonin. The decline in melatonin is illustrated in Figure 1a. There were no day differences in melatonin over the two study days and no evidence for modulation of its time course (i.e. no significant interaction) by study day or awakening time.

Insert Table 1 about here

As expected there were both linear ($F = 132.740$, $df = 205.583$, $p < 0.001$) and quadratic ($F = 12.539$, $df = 180.725$, $p < 0.001$) effects for cortisol (See Table 1, models A and B). Cortisol showed the typical linear rise followed by a smaller quadratic component. Figure 1b shows the linear rise component followed by a quadratic (curvilinear) component reflecting negative acceleration towards a peak. There were no day differences in cortisol and no effect of awakening time. There were also no significant two-way interactions (modulatory influences) of day or awakening time with sample point.

Insert Figure 1 about here

Modelling indicated that there were no relationships between indices of cortisol and melatonin: AUCg ($F = 0.112$, $df = 151.993$, $p = .739$) and Mninc/MnDec ($F = 0.368$; $df = 149.740$, $p = .546$). For total secretion of AUCg, the slope coefficient was -0.046 indicating that the amount of total cortisol secretion variance explained by total melatonin secretion (or vice versa) is estimated at 0.2%. The equivalent coefficient for MnInc / MnDec was 0.073, estimating only 0.5% shared variance.

Discussion

Here we report the relationship between the pattern of post-awakening melatonin and cortisol secretion in healthy participants using saliva samples collected within the domestic setting. Using data with verified sample timing accuracy melatonin secretion declined by an average of 29% within the first 45 minutes post-awakening. Over the same period there was a marked 112% surge in cortisol secretion, characteristic of the CAR. Both hormones showed consistency across the 2 study days. Further analyses demonstrated a lack of relationship between concentrations or patterns of melatonin and cortisol across this period. We conclude that post-awakening cortisol and melatonin are discrete and distinctive aspects of neuroendocrine function.

Within the circadian pattern of neuroendocrine function cortisol and melatonin are both regulated by the suprachiasmatic nucleus and perform complementary roles as endogenous synchronisers with cortisol dominant during daytime activities and melatonin dominant during night-time sleep. Further both are affected by bright light (Scheer & Buijs, 1999; Thorn et al., 2004; Claustrat et al., 2005; Wehr et al., 2001). Despite these complementary mechanisms and functions data from this study suggest no direct relationship between the hormones in the post awakening period; relationships later in the day were not explored. It may be that the complex multi-synaptic pathway from the SCN to the pineal does not lend itself to rapid changes in concentration of melatonin. In contrast to melatonin, the more direct route of cortisol syntheses via the HPA axis facilitates more rapid responding. Indeed the CAR is enhanced by a light-sensitive, extra-pituitary mechanism, involving a direct neural projection to the adrenal zona fasciculata that accelerates the rate of cortisol secretion in the post-awakening period (Buijs et al., 2003; Clow et al., 2010).

It was interesting to note that secretion of melatonin was consistent across the 2 study days, as for cortisol. The within-subject study design presented here did not enable analysis of between-subject differences in melatonin, which is something that deserves further

exploration. The study did however demonstrate that melatonin secretion was related to awakening time with lower melatonin secretion observed with later awakening. This finding is consistent with the underlying circadian pattern of declining melatonin secretion from its night-time peak (Voultsios et al., 1997). Surprisingly, in contrast to other reports (Edwards et al., 2001) cortisol secretion was not associated with awaking time in this study, which may be related to the relatively small range of awakening time observed.

A comparable diurnal rhythm of melatonin in serum and saliva was first demonstrated in 1985, with (as is found for cortisol) concentrations in saliva being lower than in serum (Vakkuri, 1985). It was concluded that salivary melatonin reflects biosynthesis of melatonin from the pineal and that such sampling was a simple and non-invasive way of studying melatonin in human participants. Further work demonstrated no effect of change in body posture or naturalistic saliva flow rate on salivary melatonin levels (Voultsios et al., 1997). Like for cortisol, melatonin is known to bind to blood proteins and it is the free, biologically active fraction (~30%) that is measured in saliva samples (Vakkuri, 1985; Voultsios et al., 1997). These studies have authenticated the use of self-collection of saliva in the determination of the diurnal pattern of melatonin, as used here.

Strengths of the study include two consecutive study days with four post-awakening samples at 15 minute intervals. Electronic monitoring of awakening and sampling times enabled use of a strict cut-off criteria for adherence (Smyth et al., 2013; Stalder et al., In Press). However, this resulted in a small sample. Further limitations included an unequal number of males and females and that seasonal variation in the relationship between the hormones could not be explored. We have demonstrated that the best estimates of association between the hormones are close to zero (i.e. no relationship) for both total secretion and dynamic change. We conclude that underlying changes in melatonin secretion are unlikely to be associated with effects linked to the CAR.

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Declaration of interests:

The authors report no conflict of interests.

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