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Running title: Time course of the CAR

Detailed time course of the cortisol awakening response in healthy participants

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Abstract

The cortisol awakening response (CAR) can be assessed from saliva samples collected at home, which confers ecological validity but lacks researcher oversight. Participant non-adherence to requested saliva sampling regimes leads to inaccurate CAR estimates. Moderate sampling delays of just 8 (5-15) min between awakening and commencement of saliva sampling are reported to result in over-estimated CAR magnitude and earlier peaking. This has been attributed to an observed 'latent' period in which cortisol secretion does not increase for up to 10-min after awakening. Replication of this finding is essential as the findings have considerable implications for CAR research. Healthy participants (n=26) collected saliva samples at 5-min intervals for 60 min on 2 consecutive typical weekdays. Full electronic monitoring of awakening and sampling enabled exclusion of non-adherent data (i.e. delays of greater than 5 min between awakening and collection of the first sample). In the 0-15 min post awakening segment of the CAR a quadratic effect was observed, with no difference between the awakening and 5 and 10 min samples. Moderate sampling delays will shift assessment of the CAR just sufficiently along the time axis to not impact upon measurement of the first sample but to remove the immediate post-awakening latent period from CAR estimates - whilst retaining later estimates of elevated cortisol secretion. The implication from these results is that accurate CAR measures can only be determined from data with strict adherence to commencement of saliva sampling following awakening.

Keywords

Cortisol, saliva, CAR, awakening, timing, adherence

1. Introduction

Morning awakening elicits a marked surge in cortisol secretion (Pruessner et al., 1997; Wilhelm et al., 2007), known as the cortisol awakening response (CAR). Investigation of the CAR provides insight into the circadian rhythm of neuroendocrine function (Clow et al., 2010; Fries et al., 2009) disruption of which features in many psychiatric and neurodegenerative diseases (Jagannath et al., 2013; Menet & Rosbash, 2011). The CAR can be assessed from saliva samples collected at home, which confers ecological validity but lacks researcher oversight. Participant non-adherence to requested saliva sampling regimes leads to inaccurate CAR estimates. For example, delays of greater than 15 min between awakening and commencement of sampling cause significant under-estimation of the CAR (DeSantis et al., 2010; Dockray et al., 2008; Griefahn & Robens, 2011; Okun et al., 2010).

We recently demonstrated that in healthy participants moderate sampling delays of just 8 (5-15) min between awakening and commencement of saliva sampling resulted in over-estimated CAR magnitude and earlier peaking (Smyth et al., 2013). This finding was explored in a pilot study on healthy participants measuring cortisol at 5-min intervals in the first 30-min after awakening. Inaccurate CAR assessment was attributed to an observed 'latent' period in which cortisol secretion did not differ up to 10-min after awakening relative to the awakening sample (Smyth et al., 2013). The finding challenges the assumption of a linear increase in cortisol secretion from awakening to 15 or 30 min later. Errors in CAR measurement derived from moderately delayed (5-15 min) samples was claimed to result from the assessment period being shifted along the time axis sufficiently to not impact upon measurement of the first sample but to remove the immediate post-awakening 'latent' period from CAR estimates.

Replication of this pilot study is essential as the findings have considerable implications for those studying the CAR in that accurate measurement would require strict adherence to commencement of saliva sampling following awakening (Smyth et al., 2013). In an extension of the original study, reported above, healthy participants collected saliva samples at 5-min intervals for 60-min post-awakening. The pattern of cortisol secretion was determined in 4 segments: 0-15, 15-30, 30-45 and 45-60 min post-awakening. It was hypothesized that there would be a quadratic effect between awakening and 15-min post-awakening in line with Smyth et al. (2013). We expected significant linear effects for the 15-30 min segment (rise) and for the 45-60 min segment (fall). We expected no significant effects for the 30-45 min segment as there would be variability in the timing of the cortisol peak.

2. Method

2.1. Participants

An equal number of male and female participants, aged 22.4 ± 5.5 years, associated with the University of Westminster (N = 26) were recruited on the basis that they were healthy non-smokers, not suffering from any medical or psychiatric illness or taking prescribed medication. Of these 21 were students and 5 were members of the research community. Students either received course credits or a £25 shopping voucher. Non-student-participants received a £25 shopping voucher. Two participants provided insufficient saliva volume and were excluded from all analyses. Participants either received course credit or a £25 shopping voucher for their participation in the study. The University of Westminster ethics committee approved the protocol.

2.2. Materials

The study pack included full written instructions, a saliva sampling kit containing two Ziploc bags labelled day 1-2, each containing 13 coded salivettes (Sarstedt Ltd., Leicester, England), labelled S1 (awakening sample) - S13 (60-min sample). Participants were provided with a record sheet to record their awakening and saliva sampling collection times. Participants were given wrist-worn actiwatch devices (Actiwatch, Philips Respironics, UK) and track caps (e.g. Medication Event Monitoring: MEM cap) which contained the swabs from the salivettes for electronic monitoring of awakening and sampling time of S1, respectively (see Smyth et al., 2013 for a detailed description).

2.3. Procedure

Participants attended a detailed 20-min one-to-one induction session during which they provided informed consent, received full verbal and written instructions on procedures, and practiced the techniques for collecting and recording times of saliva samples. Participants were informed about the need to adhere to the strict sampling regime relative to awakening time and were informed that their awakening and sampling times would be monitored electronically. Participants were instructed to collect saliva samples on two consecutive typical weekdays immediately on awakening (S1) and every 5-min for the first 60-min post-awakening period (S2-S13). When participants are informed that their adherence is electronically monitored the principal delay in sampling is between awakening and collection of S1 (Smyth et al., 2013). To identify this delay, participants were instructed to open the MEMs when collecting their first saliva sample. To reduce participant burden participants' self-reported sampling times were used for samples 2-13. Participants were asked to wake up in their usual way, to refrain from brushing their teeth and exercising and take nil-by-mouth except water during the sampling period. Participants completed a record sheet on each day entering their awakening and saliva sampling times.

Samples were stored in a domestic freezer until they were returned to the laboratory at the University of Westminster to be stored at -20°C until assayed. Cortisol concentrations were determined by enzyme linked immuno-sorbent assay developed by Salimetrics LLC (USA). Intra and inter-assay variations were both below 10%.

2.4. Statistical analysis

Cortisol concentrations ranged between 0.20 and 51.04 nmol/l and values were moderately positively skewed. A square root transformation was performed to normalise sample distributions for inferential analyses. Awakening times were assessed in relation to electronic estimates of awakening derived from actigraph measures on each study day and commencement of sampling was assessed by opening of the MEM-caps. There were 7 days in which either actigraph or MEM-cap data were missing; these were excluded from the analyses. In line with Smyth et al. (2013) delays between awakening and collection of S1 greater than 5-min were excluded from analyses (10 days). This resulted in 31 days with verified delay of less than 5-min between awakening and initiation of sampling that were included in the analysis.

Growth curve modelling was used to examine patterns of cortisol secretion in the 0-15, 15-30, 30-45 and 45-60 min post-awakening segments. Linear effects were explored in model A with linear and quadratic effects in model B. When significant quadratic effects were observed in model B a minus two log likelihood (-2LL) change was calculated in order to compare the models. In this small but intensive study we did not have the power to model effects of day, EM-wake time or sex.

3. Results

Results are presented in Table 1 and the raw data is illustrated in Figure 1. Growth curve modeling demonstrated a linear effect and the hypothesized quadratic effect in the 0-15 min phase of the CAR (Figure 1, segment A). Further analyses showed that cortisol secretion did not differ between the 0-5 or 0-10 min sampling points ($p > 0.05$, in both cases). However, differences between the 5-10 and 10-15 min samples were observed ($p = 0.013$ and $p = 0.005$, respectively). A significant linear effect was observed for the 15-30 min phase indicating a rise in cortisol secretion in this phase (Figure 1, segment B). There was no linear or quadratic effect observed for the 30-45 min phase representing a period of no significant change (Figure 1, segment C). A significant linear decline was demonstrated for the 45-60 min phase (Figure 1, segment D).

Insert Table 1 and Figure 1 about here

4. Discussion

The main finding was that during the first 15-min post-awakening there were significant linear and quadratic effects. There was no increase between 0-5 min followed by an increase between 5-10 min post-awakening. Further analyses revealed that there was no difference in cortisol concentration between 0-10 min post-awakening. During the second segment of the CAR, 15-30 min post-awakening, there was the expected linear rise in cortisol secretion. This was followed by a period of no change between 30-45 min post-awakening. In this mixed sex sample, a broad band of maximal cortisol secretion was observed, rather than a discrete CAR peak at any one-time point. This finding may be attributed to individual differences in the timing of the CAR peak (Pruessner et al., 1997) and deserves

further investigation. The final 45-60 min post-awakening segment was characterized by the expected linear decrease in cortisol secretion.

The study has replicated and extended a pilot study (Smyth et al., 2013) of the detailed time course of post-awakening salivary cortisol secretion in healthy participants. Data used in the analyses was derived from electronically verified adherent sampling days: less than 5-min delay between awakening and commencement of sampling. The analyses were designed to examine the (usually invisible) pattern of cortisol secretion between samples collected 0, 15, 30, 45 and 60 min post-awakening. The finding of a latent period in cortisol secretion up to 10 min post-awakening may be related to its underlying ultradian rhythm: pulses of cortisol secretion that occur approximately once an hour (Lightman, 2008; Windle et al., 1998) and the synchronizing stimulus of awakening (Clow et al., 2010).

In conclusion, this study has confirmed that initiation of the rise in cortisol secretion which characterizes the CAR is delayed by up to 10-min following awakening. This latent period can account for overestimation of CAR magnitude and apparent earlier peaking when analyzing samples from moderately non-adherent days (delays of 5-15 min between awakening and initiation of sampling) as reported in in Smyth et al. (2013). Such delays shift assessment of the CAR just sufficiently along the time axis to not impact upon measurement of the first sample but to remove the immediate post-awakening latent period from CAR estimates - whilst retaining estimates of elevated cortisol secretion at a later stage in the CAR. The implication from these results is that accurate CAR measures can only be determined from data with strict adherence to commencement of saliva sampling following awakening.

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