Original article

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The cortisol awakening response—an exploration of intraindividual stability and negative responses

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Objectives The purpose of the present analysis was to examine the prevalence and intraindividual stability of negative awakening responses in a healthy study population. Furthermore, it was of interest to elucidate the extent to which self-reported stress, sleep disturbances, or delay between awakening and the first salivary sample could explain the negative awakening response.

Methods Altogether 142 participants, 75 women and 67 men, were monitored during 3 workdays and 1 weekend day. On each day, the participants collected saliva at awakening, 30 minutes after awakening, 8 hours after awakening, and at 2100.

Results The daily prevalence of negative awakening responses varied between 19% on a workday and 38% on a day off. Altogether, 26% of the awakening responses were negative. Most of the participants had a mixture of positive and negative responses. The difference between positive and negative responses could not be explained by self-reported awakening time, subjective stress, or sleep disturbances. A delay between awakening and the first sample was more prevalent in cases of negative response, but it was also observed in cases of positive response.

Conclusions Most people seem to exhibit one or more negative awakening responses occasionally. Essentially, therefore, the awakening response cannot be considered stable for one person. Although the negative awakening responses were not found to be clearly linked to self-reported awakening time, the actual time of awakening may influence the awakening response.

Key terms diurnal pattern; stability.

When exposed to stressful situations, the human organism may react through activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn leads to the release of cortisol. The release of cortisol follows a robust circadian rhythm, with peaking levels in the morning following awakening (the awakening response) and decreasing levels thereafter throughout the day. The awakening response comprises two different elements, the overall cortisol secretory activity during a period after awakening and the dynamic of the response measured as the change in concentrations between awakening and the peak level (1). The awakening response has been suggested to be under a distinct regulatory influence, different from the rest of the diurnal cortisol secretory cycle (1). One reason for this suggestion is that the dynamics of the awakening response have been found to be unrelated to the mean underlying level of cortisol concentrations during the rest of the day (2). Furthermore, a genetic influence on the awakening response has also been found, but not on the remaining diurnal profile (3). A high awakening response has been suggested to be related to a higher degree of work stress (4).When measured repeatedly and with reference to awakening, as opposed to being measured in a single assessment at a fixed time, the awakening response has been considered a reliable biological measure of adrenocortical activity (5, 6).

Different research groups have used differing approaches for measuring salivary cortisol. In some studies, cortisol has been sampled at several fixed time points over the day (7, 8), sometimes in combination with sampling with reference to awakening time (3, 9, 10). Others have measured cortisol responses to various experimental challenges or real life situations (11–15). A wide range of measures of the awakening response has been used in different studies. Most studies

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applied a procedure involving sampling immediately at awakening, and then one or several subsequent samples with a 10-, 15-, 20- or 30-minute interval within the first hour after awakening (2, 5, 16–22). The awakening response has typically been reported to be about 50–160%, the peak occurring about 30 minutes after awakening (1, 2, 5, 6, 23, 24). The intraindividual stability of the free cortisol awakening response has shown a mean of r = 0.55 across studies (6).

Previous studies have shown that some participants have an inverted cortisol response upon awakening, that is, their cortisol concentrations decrease from the first to the second sample. A previous study reported that 18% of a study group showed a decrease between awakening and 20 minutes after awakening (24). Wust et al (6) found that, over 2 days, a mean of 23.2% of a study group was classified as "nonrespondents". Nonrespondents were defined as having less than a 2.5-nmol increase from the basal level (corresponding to one "secretory episode") within the first 45 minutes after awakening. In addition, in previous studies, certain portions of participants have not followed the "expected" diurnal rhythm when saliva samples have been collected at fixed time points. In such studies the expected diurnal pattern is a negative slope from the first sample (not collected immediately upon awakening); hence deviation from this negative slope has been noted as a "flat cycle" (25). A study covering 2 days of saliva sampling showed that 51% of the participants had the typical decline during both days, 17% did not show the diurnal pattern on both days, and 31% had a different pattern during the 2 days (25). A replication of this study, including four different studies, concluded that at least 10% had "no significant diurnal cycle" (26). The reason for these inconsistent patterns is not known, although some studies have aimed at identifying certain traits or specific demographic characteristics of the persons with "flat cycles" or "nonresponse" (25) .

The purpose of our present analysis was to examine the prevalence and stability of negative awakening responses in a healthy study population. Furthermore, it was of interest to clarify the extent to which self-reported stress, sleep disturbances, or delay between awakening and the first salivary sample could explain the negative awakening response. Our study is a re-analysis of data collected for other purposes (Carlsson et al & Persson et al. submitted manuscripts).

Study population and methods

Study population

The participants in our study were recruited as either cases (N=86) or reference persons (N=56) in a study of environmentally related annoyance. All of the participants were healthy as confirmed by a thorough medical check-up, and they showed normal patterns of cortisol secretion. There were no differences between the "cases" and referents in regard to everyday cortisol secretion (27), and the total group of participants was used in the present study. The participant group consisted of 142 persons, 75 (53%) women and 67 (47%) men. Their mean age was 44 (SD 9, range 22–57) years.

Design

Cortisol sampling. The participants were monitored during 3 ordinary workdays and 1 weekend day in the following sequence: Wednesday, Sunday, Tuesday, and Thursday. On each of these days, they collected saliva at the following four prespecified time points: at awakening, 30 minutes after awakening, 8 hours after awakening, and at 2100.

Logbook. The participants also filled out a logbook three times each day (at awakening, 8 hours after awakening and at 2100). In the morning notes, they specified their awakening time. The logbook contained the 12-item stress–energy inventory (28), which measures feelings of arousal in two dimensions: stress and energy (6 items per dimension). The stress dimension, which was included in the present study, covers a range of negatively valued, high-activity states to positively valued, low-activity states. The Karolinska Sleep Diary (KSD) was also included in the logbook (once each day, in the morning) to assess the day-to-day variability of several sleep parameters (29). In our present study, the disturbed sleep score and the awakening score were used as outcome measures. The disturbed sleep score was calculated as the mean score of four items asking whether the person during the night had (i) difficulties falling asleep, (ii) disturbed or restless sleep, (iii) repeated awakenings, and (iv) premature awakenings. Higher scores represented increasingly disturbed sleep (score range 1–5). The awakening score was calculated as the mean score for three items concerning (i) the ease of awakening, (ii) whether the sleep was refreshing, and (iii) exhaustion at awakening, for which higher scores represented a more satisfactory awakening (score range 1–5).

Procedure

Before the sampling period, the participants were carefully instructed and trained in how to sample salivary cortisol. They were told to collect saliva samples by placing the cotton swab from the sampling tube in the mouth until hydrated, but no longer than 5 minutes. To ensure the participants' correct understanding further, we issued each person written information together with four kits of saliva sampling tubes (Salivette®, Sarstedt

Ltd., Leicester, UK). Then to ensure a high quality of saliva samples, we instructed the participants to refrain from brushing their teeth after awakening until they had obtained the second saliva sample. This procedure was primarily initiated to avoid swabs being contaminated by microbleeding from the gums. The participants were also instructed to refrain from smoking and eating heavy meals 1 hour prior to the saliva sampling. To facilitate the collection of saliva tubes, each participant received prestamped envelopes to be returned after each day of sampling. In case of hindrance, they were instructed to store the samples in the refrigerator (at home or at work) until they were able to mail them. On arrival at the clinic, the samples were immediately frozen until analysis.

Measurement of cortisol in saliva

The assay used for determining cortisol in saliva was competitive radioimmunoassay (RIA) (Spectria Cortisol Coated Tube RIA) purchased from Orion Diagnostica, Espoo, Finland. The assay was designed for quantitative in vitro measurement of cortisol in serum, plasma, urine, and saliva. A method evaluation of certified reference material in water showed no bias of the method, with 97% recovery (95% CI 94–100.9%). The limit of detection (LOD) was 1.59 nmol/l. The between-run coefficient of variation (CV) was 19% at 11.5 nmol/l and 16% at 49.2 nmol/l (30). To show equivalence between different runs, we used natural saliva samples at two concentrations (11.5 and 49.2 nmol/l) as control materials and analyzed them together with the samples. Westgard control charts were used to monitor and control variation and ensure that the analytical methods remained stable. The performance of the methods was further evaluated by participation in interlaboratory comparison schemes (30–32).

Definition of variables

A positive awakening response was defined as an increase in cortisol concentration between the first and the second morning sample. A negative awakening response was defined as a decrease in the cortisol concentration between the first and second morning samples. The information about delay between awakening and the first saliva sample was based on a comparison between the self-reported awakening time noted in the logbook and the time point noted on the first saliva sample. The stress scores were calculated as the mean score of the six items comprising the stress dimension. The disturbed sleep score was calculated as the mean score of four items asking whether, during the night, the person had (i) difficulties falling asleep, (ii) disturbed or restless sleep, (iii) repeated awakenings, and (iv) premature awakenings. Higher scores represented increasingly disturbed sleep (score range 1–5). The awakening score was calculated as the mean score for the following three items: (i) ease of awakening, (ii) whether the sleep was refreshing, and (iii) exhaustion at awakening, for which higher scores represented a more satisfactory awakening (score range 1–5).

Statistics

Statistical computations were made with SPSS 11.0 (SPSS Inc, Chicago, IL, USA, 2001). Student's t-tests, Fischer's exact test, and the Mann-Whitney U-test were used for group comparisons. The level of significance was set at $P \leq 0.05$. Descriptive data, as well as group-wise comparisons, are presented for the workdays (3 days collapsed) and day off separately.

Results

Negative awakening responses were significantly ($P<0.001$) more prevalent on the day off than on the workdays. The daily prevalence of negative awakening responses varied between 19% (Wednesday) and 38% (day off) (table 1). Altogether 26% of the awakening responses were negative. Forty-one participants delivered a dry cortisol sample on at least one of the four mornings, and the calculation of the awakening response in these cases was impossible. Of the remaining 101 participants with complete morning samples on all four mornings, 34 participants had a positive awakening response on all 4 days, whereas two persons had a negative response on all 4 days. The remaining participants (N=70) had a mixture of positive and negative responses (table 2).

Table 1. Proportion of negative responses.

Day	N (total)	Negative responses (%)	Negative responses (valid %)	Dry or missing [a] (%)
Workday 1	142	19.0	21.8	12.7
Workday 2	142	23.2	25.4	8.5
Workday 3	142	22.5	25.2	10.6
Day off	142	38.0	43.2	12.0

[a] Due to one or two dry or missing morning samples.

Table 2. Number of days with negative responses.

Number of days with negative responses	N	%
0	34	23.9
1	35	24.6
2	32	22.5
3	3	2.1
4	2	1.4
Dry or missing [a]	41	25.4
Total	142	100

[a] Due to dry or missing morning samples on any of the mornings.

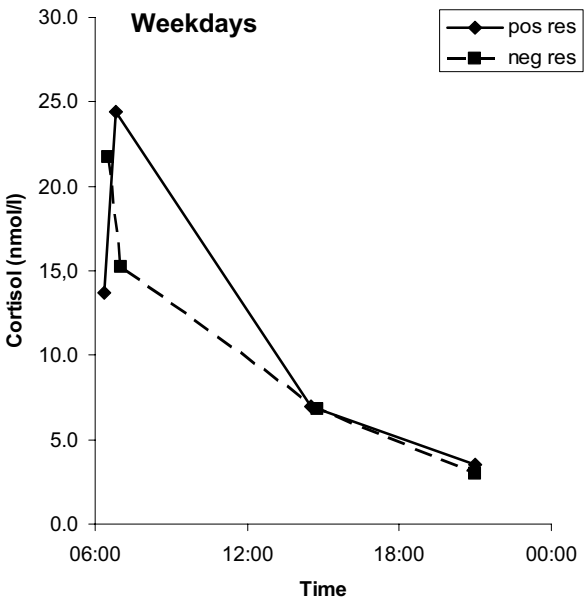

Figure 1. Cortisol profile for the workdays for the participants with positive (N=289) and negative (N=92) awakening responses—median cortisol concentrations. (pos res = positive response, neg res = negative response)

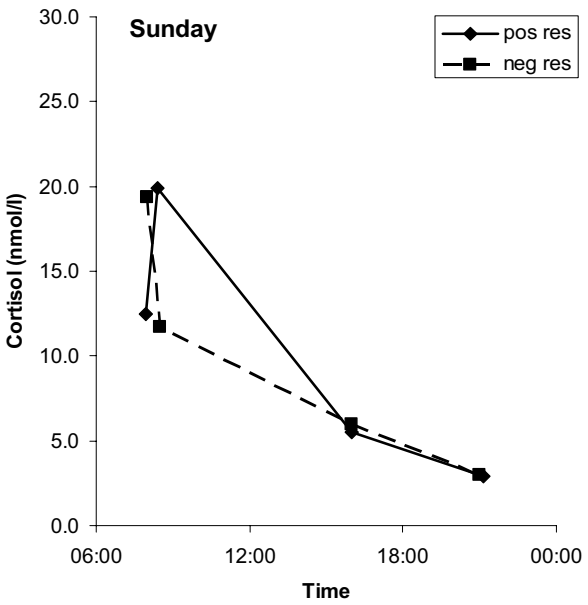

Figure 2. Cortisol profile for the day off for the participants with positive (N=71) and negative (N=54) awakening responses—median cortisol concentrations. (pos res = positive response, neg res = negative response)

Table 3. Descriptives of the negative and positive awakening responses on the workdays.

	Negative response (N=92)					Positive response (N=289)				
	Mean	SD	Median	10th percentile	90th percentile	Mean	SD	Median	10th percentile	90th percentile
Increase (%)	–28.1	21.6	–23.1	–60	–3	95.7	91.0	72.0	14	212
Increase (nmol/l)	–11.3	31	–4.8	–18	–0.4	11.8	9.9	9.0	2	23
Time of awakening sample	0629	0111	0630	5.3	7.25	0629	0106	0620	0525	0800
Awakening sample (nmol/l)	30.4	41.4	21.7	12.1	36.0	15.0	6.9	13.7	8.0	24.4
Sample 30 minutes after awakening (nmol/l)	19.1	17.6	15.2	7.4	25.0	26.8	12.3	24.4	14.6	40.0
Mean morning concentration (nmol/l)	24.8	27.8	19.3	10.9	30.1	20.9	8.6	19.5	11.6	31.2
Delay between self-reported awakening and time for first sample (minutes)	13	28	0.0	0	40	4	13	0.0	0	15
Stress score (awakening)	2.7	0.9	2.5	1.5	4.1	2.7	0.9	2.5	1.7	4.0
Disturbed sleep score	2.0	0.8	1.8	1.0	3.0	1.9	0.8	1.8	1.0	3.0
Awakening score	2.7	0.9	2.7	1.7	4.0	2.7	0.8	2.7	1.7	3.7

The day profiles for the persons with positive and negative awakening responses are shown in figures 1 and 2. There were no significant differences between the sample time for the first (or second) saliva samples between the negative and positive responses on either the workdays or the day off (first sample: P=1.00 for workdays and P=0.21 for day off; second sample: P=0.93 for workdays and P=0.21 for day off) (tables 3 and 4). The estimated delay between the self-reported awakening time and the time of the first saliva sample was significantly greater for the negative responses than for the positive responses during both workdays (P=0.002) and the day off (P=0.02) (tables 3 and 4). A significantly larger proportion of the negative respondents reported a delay between awakening and the first sample even though a delay was reported also in connection with positive responses (table 5).

There were no differences in the stress scores (P=0.87 for the workdays and P=0.74 for the day off), the "disturbed sleep" scores (P=0.10 for the workdays and P=0.45 for the day off), or the "ease of awakening" scores (P=0.84 for the workdays and P=0.51 for the day off) between the participants with negative and positive awakening responses (tables 3 and 4).

Table 4. Descriptives of the negative and positive awakening responses on the day off.

	Negative response (N=92)					Positive response (N=289)				
	Mean	SD	Median	10th percentile	90th percentile	Mean	SD	Median	10th percentile	90th percentile
Increase (%)	–30.2	20.9	–26.3	–57	–4	64.3	55.8	54.1	9	142
Increase (nmol/l)	–6.4	5.3	–4.9	–15	–1	7.8	7.1	5.6	2	18
Time for awakening sample	0811	0114	0800	6.42	9.55	0753	0124	0755	6.00	9.44
Awakening sample (nmol/l)	20.2	7.7	19.4	12.1	32.1	14.0	8.5	12.5	8.6	20.6
Sample 30 minutes after awakening (nmol/l)	13.8	6.2	11.7	7.3	30.0	21.8	12.7	19.9	12.3	30.3
Mean morning concentration (nmol/l)	17.0	6.4	15.4	10.5	26.9	17.9	10.2	16.3	10.8	24.5
Delay between self-reported awakening and time for first sample (minutes)	22	56	0.0	0	60	5	14	0.0	0	15
Stress score (awakening)	2.2	0.8	2.0	1.4	3.5	2.3	0.8	2.2	1.5	3.3
Disturbed sleep score	1.9	0.8	1.8	1.0	3.0	1.8	0.7	1.8	1.0	2.7
Awakening score	2.4	0.8	2.3	1.1	3.6	2.5	0.8	2.7	1.3	3.6

Table 5. Share of participants with 0 minutes, 1–10 minutes, and more than 10 minutes of self-reported delay between awakening and the first salivary sample.

Delay	Workdays					Day off				
	Negative response		Positive response		P-value	Negative response		Positive response		P-value
	N	%	N	%		N	%	N	%	
0 minutes	60	65.2	228	78.9	0.12 [a]	33	61.1	55	77.5	0.05[a]
1–10 minutes	9	9.8	22	7.6	0.36 [b]	4	7.4	4	5.6	0.71[b]
> 10 minutes	23	25.0	39	13.5	0.008 [c]	17	31.5	12	16.9	0.54[c]
Total	92	100	289	100	..	54	100	71	100	..

[a] Fishers exact test, 1–10 minutes versus 0 minutes.
[b] Fishers exact test, >10 minutes versus 0 minutes.
[c] Fishers exact test, 0 minutes versus total.

Discussion

The results showed that most of the participants exhibited one or more negative awakening responses when measured on four different days within 2 weeks. Essentially then, the awakening response cannot be considered to be stable within the individual. Thus any characterization of the participants into "trait-like" categories based on one, or just a few, awakening responses is likely to lead to a high degree of misclassification. This probability warrants caution as to the use of a single cortisol awakening response as a means of determining a negative respondent at the individual level.

We found that the total proportion of negative responses on workdays was comparable with the result from a previous study using the same definition of negative response (24). In contrast to the previous study, our current study measured awakening responses on several workdays and a day off for the same participants rather than once per participant. However, the proportion of negative responses that has previously been found in studies including only 1 day was still seen when several days were included in the analysis and thus could not be regarded as some kind of coincidence. In our present study, negative awakening responses were more prevalent on the day off (38%) than on the workdays. Although the negative awakening responses were not linked to self-reported sampling time (which was supposed to be to the same as the awakening time), the actual time of awakening may have influenced the awakening response. A delay between awakening and the collection of the first sample was more frequently reported in connection with the negative responses than with the positive responses, and the mean delay was higher in connection with the negative responses. However, delays were reported in both groups; therefore, delays could not completely explain the negative responses. One important factor may also be the definition of awakening. Because of difficulties in deciding when the actual awakening occurred, there may be a delay between awakening and the sampling that the participants were not aware of. A previous study approached this issue in a slightly different way; the participants were classified according to self-reported delay between awakening and the first sample, dichotomized as <10-minute delay and >10-minute delay. The diurnal rhythm in the group with a >10-minute delay was similar to what we saw in the "negative responses"

in our present study (33). A similar classification of this material, however, did not show the same results, although the awakening response was slightly attenuated among those with a >10-minute delay.

The impact of different possible confounding factors is somewhat contradictive. Yet we tried to control for some of the factors that possibly influence cortisol secretion. Accordingly, the participants were instructed to refrain from heavy meals, smoking, and teeth brushing for at least 30 minutes prior to the sampling (1).

The procedure of participants collecting salivary samples at home following verbal directions and written instructions relies heavily on participant adherence. Participant adherence is known to be an important factor for correct measures of awakening response in particular (20, 34, 35). It has been argued that even small deviations from the protocol may have substantial effects on the obtained values of the awakening response (1). A previous study reported attenuated awakening responses in noncompliant versus compliant participants, the status of compliance being based on self-reported delay between awakening and the first salivary sample (20). Electronic devices have been used to track when participants actually access the cotton swab, and they showed that 74% of the participants correctly followed the protocol, while 26% failed to take at least one out of six samples. Of the noncompliants, 55% failed to take the second morning sampling correctly after 30 minutes. The participants who were not informed that their sampling was being tracked were significantly less compliant than the informed participants (35). Another study examining participant adherence found that 71% of the participants who were unaware that they were being monitored correctly followed the protocol, although the self-reported compliance was 93%. Among the persons aware of being monitored, the objective compliance was 90%, consistent with the self-reported compliance of 93%. (34). In both studies, the nonadherent participants had significantly lower morning cortisol values than the adherent participants. This issue has also been examined in direct relation to the occurrence of negative awakening responses. A previous cortisol study also included electrocardiography, and motility recording showed that, among the participants presenting a negative awakening response, as many as 77% had actually awakened earlier than reported. Among those with the expected "positive" awakening response only 13% showed a lack of correspondence between self-reported awakening and actual awakening (36).

We did not have the opportunity to track participant adherence electronically, but the general compliance appeared to be good, with very few missing samples and only two dropouts during the study period. A further problem in this kind of study may be dry samples (ie, samples not containing enough saliva with which to perform the chemical analysis). In the verbal instructions prior to the sampling period, we emphasized the importance of keeping the cotton swab in the mouth until hydrated (although no longer than 5 minutes). The relatively low share of dry samples, showing that the participants followed this guideline, further indicates good participant compliance. In the verbal instructions, we also strongly emphasized the importance of taking the first sample immediately upon awakening. Still, it is almost impossible to know how good the compliance was, but the importance in this study is whether there are reasons to believe that the groups would have been unequally compliant. The information about delay between awakening and the first sample indicated a greater delay in association with the negative awakening responses. The definition of delay was based on information from the logbook, where the participants noted their awakening time, compared with the reported time point for the first morning sample. This procedure may not be the best way to detect delay between awakening and saliva sampling, but the difference between the negative and positive responses indicate that the delay may have influenced the awakening response. This possibility is also in accordance with what has previously been shown (36). It is still interesting to note that the difference in delay between the positive and negative responses was only 9 minutes on the workdays and 17 minutes on the day off. This finding supports the previous findings that even small deviations from the protocol may affect the results (1). Still, the difference in delay did not fully explain the difference in the cortisol awakening response.

The main message from this study is that, when monitored by salivary sampling, not all persons show an increase in salivary cortisol following awakening. These negative awakening responses are not traits, but occur in almost all persons when measured on four different days. A delay between awakening and the first salivary sample may partially explain the profile, while the actual time of the sampling, or self-reported stress scores, disturbed sleep, or ease of awakening scores appeared to have no influence on the awakening response profile.

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