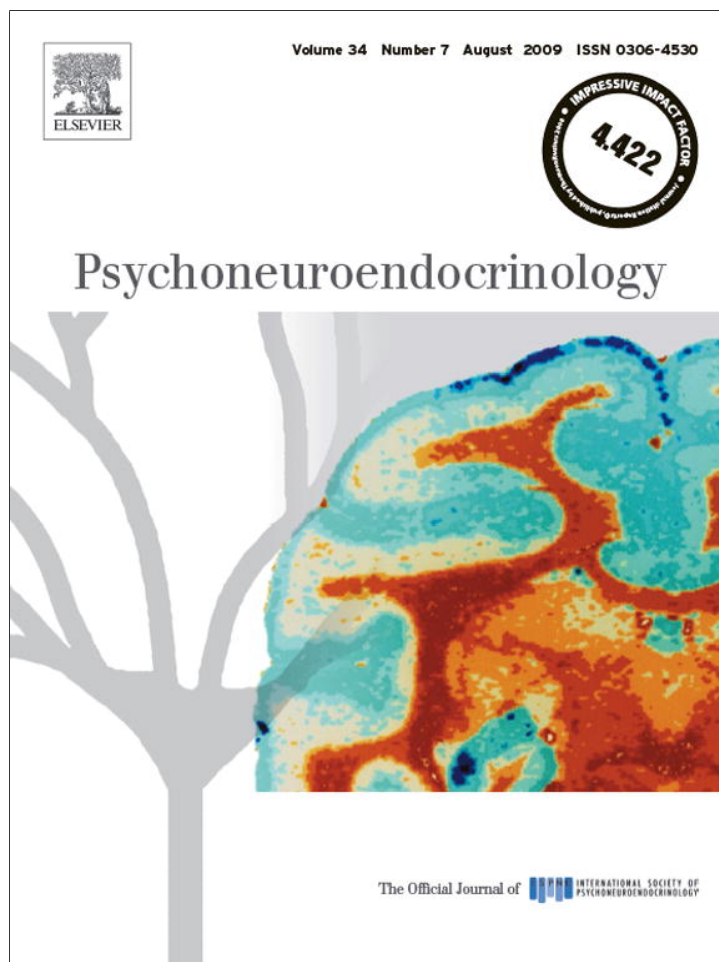


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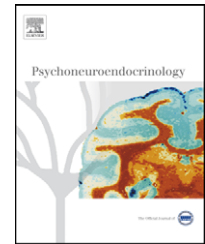


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Neuroendocrine and psychometric evaluation of a placebo version of the 'Trier Social Stress Test'

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Summary The "Trier Social Stress Test" (TSST) is one of the most prominent laboratory stress paradigms. It is often used to investigate the effects of stress on cognitive or affective parameters. Such studies need a non-stress control condition. However, control conditions currently employed are often rather ill defined and do not parallel important modulating variables, e.g., physical or cognitive load of the TSST. We here introduce a placebo version of the TSST, which contains a free speech and a simple mental arithmetic task without uncontrollability and social-evaluative threat. In two studies, this control condition was evaluated using salivary markers of stress reactivity (cortisol and alpha-amylase) and a questionnaire for anticipatory cognitive stress appraisal (PASA). In experiment 1 participants who were treated with the placebo condition showed no cortisol response and a small, but significant salivary alpha-amylase (sAA) response. Both responses were significantly smaller than those of TSST-treated participants. The placebo-treated participants also rated the treatment situation as less stressful. In experiment 2 a crossover study with the use of an intercom to instruct the participants and ensure their compliance was conducted. Again there was a strong cortisol response to the TSST, which differed significantly from the cortisol levels observed during the placebo condition. Importantly the cortisol response was not influenced by treatment order (TSST or placebo first). However, in this study we found similar reactions between TSST- and placebo-treated participants with regard to sAA-response. We suggest that the introduced placebo protocol for the TSST is a promising tool for future psychobiological research. The exact procedure for a given experiment should be tailored to the specific needs of the empirical question studied.

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1. Introduction

Studies on psychological stress effects have used different types of stress tasks, like emotion induction procedures, public speaking tasks, cognitive tasks, noise exposure and tasks which combine public speech and cognitive tasks (Biondi and Picardi, 1999). Already Cannon (1935) empha-

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sized the importance of psychological and emotional stimuli in activating the “fight-or-flight-response”. Mason (1968a,b) referred to Cannon's concept of stress and assumed that psychological variables such as novelty, unpredictability, anticipation of negative outcome and ego-involvement are factors that most commonly define a stressful situation. Nearly 40 years later Dickerson and Kemeny (2004) delivered a quantitative summary of the empirical evidences for Mason's assumptions by meta-analytically reviewing more than a hundred laboratory stress studies. They found *uncontrollability* and *threat to the social self and the self-esteem* to be especially effective for inducing a significant cortisol response and being implemented into several psychosocial laboratory stressors. The combination of an evaluated public speech and a cognitive task integrates these factors and reliably stimulates the hypothalamus–pituitary–adrenal (HPA) axis in the laboratory (Linden et al., 1998; Biondi and Picardi, 1999; Dickerson and Kemeny, 2004; Kudielka and Kirschbaum, 2005)

One prominent laboratory stress procedure is the “Trier Social Stress Test” (TSST), published by Kirschbaum et al. (1993). This active performance task consists of a public speech and a mental arithmetic task (see description below). The participants' self-esteem is threatened by a committee that pretends to evaluate the participants' performance without any signs of social support. Thus the participant does not know whether his/her behaviour is accurate, which leads to feelings of uncontrollability. This procedure was designed to be in accordance with Mason's assumptions (1968a,b) and is quite effective in activating the HPA and the sympathetic nervous system (SNS; e.g. Kirschbaum et al., 1999; Schommer et al., 2003; Kern et al., 2008).

Studies using the TSST can be divided into two experimental approaches. One strategy is to use the TSST to investigate the neuroendocrine stress response (e.g. cortisol response) and to compare the stress responsivity between certain groups of interest, e.g. women versus men (Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005), young versus old (Kudielka et al., 2004a), ‘normal’ versus diseased (Stones et al., 1999; Buske-Kirschbaum et al., 2002; Gaab et al., 2002; De Vente et al., 2003; Rohleder et al., 2003; Ahrens et al., 2008) or whites versus blacks (Chong et al., 2008). In these studies the cortisol stress response is the dependent variable of interest and the stress response is assessed by comparing poststress cortisol levels with a pre-stress baseline measure.

Another approach is to use the TSST to induce stress and to test the effects of stress and its biological responses on cognitive or affective outcome measures (e.g. Kuhlmann et al., 2005; Schoofs et al., 2008) or on physiological measures (e.g. Nater et al., 2006; Rohleder et al., 2006a). In these studies the TSST is used for the creation of the independent variable (stress versus no stress). In the latter design the stress condition has to be contrasted with a non-stress control condition. Although the TSST has been widely used, there is a lack of a standardized control condition or a “placebo version”. When researchers make use of an appropriate control condition the internal validity and the statistical conclusion validity increases, due to the exclusion of confounding variables (Cook and Campbell, 1979; Krauth, 2000). Thus an appropriate control condition helps to eliminate alternative explanations for

a detected causal relationship between an independent and a dependent variable. In psychoneuroendocrine research this is especially important in studies which investigate the effects of stress on cognitive or affective variables since these are vulnerable towards subtle changes in physical and cognitive demands. In the case of the TSST a control condition would be needed in order to demonstrate that observed effects are indeed caused by the stress response induced by the TSST and are not just secondary to the physical or cognitive demands of the task (e.g. giving a speech or calculating).

According to Shapiro and Morris (1978) placebo treatment is identical to the intended treatment, except its specific psychological or physiological effective factors. So, the appropriate control situation for the TSST must be equal to it except of its effective factors, namely the social evaluative component and the uncontrollability, according to the theory of Dickerson and Kemeny (2004). However, typical control conditions used for this treatment in past studies had often been quiescent and uneventful circumstances, in which the participants of the control group usually stayed alone in a room reading a magazine or completing questionnaires (e.g. Kirschbaum et al., 1993; Wolf et al., 2001; Domes et al., 2002, 2004; Nater et al., 2006, 2007a; Rohleder et al., 2006a). These control conditions differed from the TSST not only in their stressfulness, but also in the physical and cognitive load they impose on the participants. Those factors might influence neuroendocrine, affective and cognitive measures taken during or after the control condition. Body posture may be one factor that needs to be similar, since an orthostatic response may influence SNS parameters (Lake, 1979; Januszewicz et al., 1982; Goldstein, 1987; Carnethon et al., 2002). Thus, an appropriate placebo version of the TSST should require the participants to stand in an upright posture. Doing so, the physiological load of the participants of the control group would be comparable to that of the TSST group. Furthermore, the placebo version of the TSST should include tasks leading to a cognitive load comparable to the TSST, such as speaking aloud and/or performing mental arithmetic. In contrast to that, the participants must under no conditions perceive these tasks as stressful.

In our laboratories we have already started using a standardized placebo version of the TSST (e.g. Kuhlmann et al., 2005; Schoofs et al., 2008). We created a condition which is similar in physical and mental demand (speech and math task) to the TSST, but in which the stress inducing negative social evaluation component of the TSST is lacking. The participants of the placebo group are usually alone in a room and complete the tasks by themselves (see description below). Until now there is no neuroendocrine and psychological evaluation of the practicability of this standardized control condition of the TSST. Thus, the aims of the present set of two studies are as follows:

1. To evaluate a standardized placebo version of the TSST using neuroendocrine and psychometric stress measures.
2. To find out whether there is a difference in neuroendocrine parameters if an intercom is used to control the participants' compliance during the placebo version.
3. To test for carryover effects within the use of crossover design with the TSST and its placebo version.

We predicted that the placebo TSST would not lead to a HPA stress response. With respect to the SNS (indirectly assessed via alpha-amylase) we expected that the placebo TSST might lead to a modest response due to the physiological demands of the task. With respect to the intercom we speculated that it might activate thoughts of being controlled and evaluated by the experimenter. Thus, we predicted that an intercom might induce some neuroendocrine stress responses (modest HPA and/or sAA increases). With regard to carryover effects within the crossover design, we predicted that there would be no effect of treatment order (TSST first, placebo on the second trial or vice versa) with regard to cortisol- and sAA-levels. This would be in line with results from former studies of our laboratory (e.g. Kuhlmann et al., 2005). Finally, in accordance to the findings of Gaab et al. (2005) we predicted that participants treated with the TSST will show more negative anticipatory cognitive stress appraisal than participants treated with the placebo version of the TSST.

2. Experiment 1: neuroendocrine evaluation of the 'placebo TSST' using a between subjects design

The aim of experiment 1 was to evaluate the standardized placebo version of the TSST in general using a randomized, between subject comparison study design. For this purpose we compared participants treated with TSST with participants treated with its placebo version with regard to salivary cortisol, sAA-levels and anticipatory cognitive stress appraisal. We predicted that placebo-treated participants show lower cortisol and sAA-levels on average and lower ratings of stress appraisal than participants treated with the TSST.

2.1. Methods

2.1.1. Participants

Participants were recruited among students of the University of Bielefeld within the context of a series of studies on the effect of stress on memory. Results of the male part of the sample are already published elsewhere (Schoofs et al., 2008). The total sample consisted of 84 participants, with 40 men and 44 women participating. The participants underwent a brief medical and psychological examination prior to testing. The following exclusion criteria were selected: (a) BMI below 19 or above 26 kg/m², (b) younger than 18 or older than 35 years of age (Kudielka et al., 2004a), (c) acute or chronic somatic and/or psychiatric disease, (d) intake of medication, (e) current psychotherapy treatment, (f) acute or chronic self-reported stress, and (g) female participants with an irregular menstrual cycle or intake of oral contraceptives (Kirschbaum et al., 1996a, 1999). All female participants were tested in the luteal phase of their menstrual cycle (self-report). Participants refrained from smoking, physical exercise, meals, alcoholic beverages and stimulating drinks (e.g. coffee or tea) at least 1 h prior to testing. Participants received detailed information about the study, provided written consent and were paid for their participation. The study protocol was approved by national ethics committee of the German Psychological Society (DGPs).

2.1.2. Experimental protocol

Single experimental sessions took about 90 min and were conducted between 1000 h and 1230 h. After arrival at the laboratory, participants rested for 30 min. The first saliva sample (baseline) was obtained 1 min prior to treatment (-1 min). Each participant was assigned randomly to the TSST or its placebo version. Both treatments lasted 15 min. At the beginning of the TSST or its placebo version the participants completed a questionnaire on stress appraisal (see below). After completion of the TSST or its placebo version the next saliva sample (+1 min) was obtained. Saliva sampling was repeated again after 10 and 25 min. At the end participants were debriefed by the experimenter.

2.1.2.1. TSST. The TSST was performed similarly to the description provided by Kirschbaum et al. (1993). The participant was told to introduce him-/herself to a selection committee. After an initial preparation period of 5 min during which the participants filled out the questionnaire on stress appraisal (PASA; see below) participants had to give a free speech. During the speech (5 min duration) he or she had to convince the committee that he/she was the perfect applicant for a vacant position (his or her 'dream job'). The committee was dressed in white coats and was introduced as consisting of psychologists who are specially trained to monitor and analyze verbal and nonverbal behaviour. Furthermore it was announced that the participant's performance was recorded on the video-cassette-recorder to later analyze the interview and the nonverbal behaviour. If the participant finished his/her speech in less than 5 min, standardized questions were used. Then the participant was asked to serially subtract the number 17 from 2043 as fast and as accurately as possible within 5 min. On every failure the psychologist interfered and the participant has to start again at 2043. Both members of the committee acted in a very cold and reserved manner.

2.1.2.2. Placebo TSST. The standardized control version for the TSST (the 'Placebo TSST') was designed to be as similar as possible to the TSST without being stressful for the participants. At first the experimenter led the participant into an empty room where he/she was asked to talk for 5 min aloud about a movie, a novel, or a recent holiday trip. He/she was told that there is a preparation time of 5 min. In this period he/she should think about the topic he/she will present. Before preparing the speech the participants were asked to complete the PASA. After 5 min the experimenter entered the room and asked the participant to stand up, take position somewhere in the room and start to talk loudly about the chosen topic. Five minutes later the experimenter entered the room again and asked the participant to start adding up the number 15 starting at 0. This second task also lasted 5 min. After the second task the experimenter led the participant back to the waiting room. The experimenter controlled the compliance of the participant by asking the participant about the number he/she had reached while counting forwards in steps of 15. The placebo TSST was performed in the same room as the TSST but all 'stressing' elements of the TSST (committee, video camera, and microphone) were removed prior to the start of it. This procedure was expected to eliminate the main effective factors of the TSST, namely the social evaluative threat and the uncontroll-

ability, according to the theory of Dickerson and Kemeny (2004). It also fits to Mason (1968a,b) assumptions on factors that define stressful situations. Ego-involvement is minimized by performing a speech on a superficial, not self-relevant topic. This is also the case for the factor novelty, because people are rather used to talk on such topics than on their personality traits. Because of a lack of an audience the factor unpredictability and anticipation of a negative outcome is reduced as well.

2.1.3. Measures

2.1.3.1. Saliva sampling and biochemical analysis. Saliva was collected to obtain free cortisol levels (Kirschbaum and Hellhammer, 1989, 1994) as well as sAA-activity (Rohleder and Nater, 2009) as markers of HPA and SNS activity, respectively. The samples were obtained using Salivette sampling devices (Sarstedt, Nümbrecht, Germany) 1 min before treatment and 1, 10 and 25 min after treatment. Free cortisol levels were measured using a commercially available immunoassay with chemiluminescence detection (IBL-Hamburg, Germany). sAA-activity was measured by using a quantitative enzyme kinetic method, as described elsewhere (Rohleder and Nater, 2009). Inter- and intra-assay coefficients of variation were below 15%.

2.1.3.2. Measurement of anticipatory cognitive stress appraisal. Participants' anticipatory cognitive stress appraisal at the beginning of the TSST or its placebo version was measured using the German version of "Primary Appraisal–Secondary Appraisal (PASA)" questionnaire which was developed by Gaab et al. (2005). This questionnaire consists of 16 items and was developed in accordance to the model of Lazarus and Folkman (1984). The questionnaire assesses four anticipatory cognitive appraisal processes: "Threat", "Challenge", "Self Concept of Own Abilities" and "Control Expectancy". Each scale of the PASA comprised four items with a 6-point scale ranging from "Strongly disagree" to "Strongly agree". All items were directly related to the treatment situation (TSST or placebo) before. For the placebo TSST the items of the scale "Control Expectancy" were altered, because of inappropriate wording. The word "interview" was replaced by the word "situation" and the description "experts' judge" was left out. In addition two items were excluded because of inappropriate content with regard to the placebo TSST ("It mainly depends on me whether I manage this situation successfully." and "My Success in this situation is a consequence of my effort and personal commitment"). For each of the four scales sum scores were calculated and averaged.

2.1.4. Statistical analysis

Demographic and descriptive variables were investigated by Pearson's Chi-square-test and Student's *t*-test. The results of the questionnaire were analyzed using Mann–Whitney-*U*-test, because of ordinal level of measurement. Pearson's correlation was used due to investigate the relationship between the results of the stressed participants in the questionnaire and their actual neuroendocrine reaction, using the method of area under the curve (AUC, Pruessner et al., 2003). AUC was calculated with respect to ground, as a measure of the total neuroendocrine reaction, and with respect to baseline, for the increase of the neuroendocrine

variables, as a measure of their reactivity. Mixed model analyses of variance (ANOVA) for repeated measures were performed on the results of cortisol and sAA to reveal possible effects of time, treatment and sex. The sAA data were tested for normal distribution with Kolmogorov–Smirnov-test (*K–S*-test), because sAA-data are usually positively skewed distributed and ANOVA premises normal distribution. In case of a significant result the sAA-data were log-transformed and the ANOVA was performed with the transformed data. Greenhouse-Geisser adjusted *p* values are reported in case of violated sphericity assumption. Overall level of significance was defined as $p < .05$. Differences of interest between the groups within a particular variable and a specific sampling time were evaluated post hoc by using Student's *t*-test for independent samples. In this case level of significance was Bonferroni-adjusted. Statistical analyses were performed by SPSS 13 for MAC OS X.

2.2. Results

2.2.1. Description of the sample

The total sample of experiment 1 consisted of 84 participants with a mean age of 24.8 yrs ($\pm .43$ S.E.M.) and a mean BMI of 22.7 kg/m² ($\pm .27$ S.E.M.). The experimental group consisted of 19 male and 23 female participants. The control group consisted of 21 women and 21 men. There was no significant group difference in distribution of sex, age, BMI, or smoking status ($ps > .10$). Because of missing data (sample contamination or insufficient saliva volume) one participant of the control group had to be excluded from the analysis of cortisol and two participants of the control group from the analysis of sAA, respectively. See Table 1 in the supplementary material for details.

2.2.2. Salivary cortisol

As expected, the TSST caused an activation of the HPA system as shown in Fig. 1. While placebo-treated participants showed decreasing cortisol levels. A three-way ANOVA revealed a significant effect of time ($F_{(2.05/161.99)} = 10.99$; $p < .01$), an interaction between treatment and sex ($F_{(3/79)} = 4.78$; $p < .01$), and between time, treatment and sex

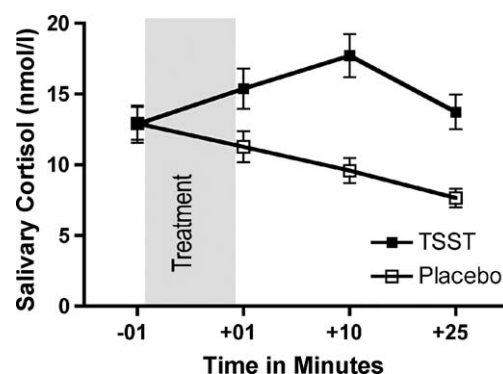


Figure 1 Salivary cortisol concentrations (mean and S.E.M.) of participants exposed to the TSST or the placebo TSST. As expected participants under the TSST condition showed elevated cortisol concentrations 10 min after the TSST. Participants, who were treated with the placebo version of the TSST showed a decline during the course of the experiment.

($F_{(6.15/161.99)} = 7.94$; $p < .01$). The cortisol response defined as the difference between the baseline level and the time of the expected cortisol peak (+10') was calculated for both sexes. Although not significant ($t_{40} = 1.04$; $p > .05$), male participants displayed on average a stronger cortisol increase (6.3 nmol/l; ± 8.93 S.D.) in response to the TSST than female participants (3.6 nmol/l; ± 7.77 S.D.). This difference disappeared when participants of the TSST group showing a cortisol response of at least 2.5 nmol/l (difference in cortisol concentration between baseline and time of expected cortisol peak) were compared (9.61 nmol/l; ± 8.35 S.D. (female) vs. 9.33 nmol/l; ± 5.57 S.D. (male)). There was no difference between the sexes during the placebo TSST (data not shown).

2.2.3. Salivary alpha-amylase

The results for sAA are shown in Fig. 2. K-S-tests indicated that sAA data were skewed ($ps \leq 0.009$). Thus, sAA data were log-transformed to approach a normal distribution. Three-way ANOVA revealed a significant effect of time ($F_{(2.4/191.8)} = 28.3$; $p < .01$), treatment ($F_{(1/80)} = 4.75$; $p < .05$), and the interaction between these two factors ($F_{(2.4/191.8)} = 5.1$; $p < .01$). There was no effect of sex on these results. The groups did not differ at baseline ($t_{81} = -0.88$; $p > .05$). Placebo-treated participants showed a small, but significant increase in sAA immediately after the treatment ($t_{40} = -3.24$; $p < .01$). The stressed participants showed a nearly twofold increase 1 min after the treatment ($t_{41} = -5.27$; $p < .01$).

2.2.4. PASA

The two groups differed on all four scales of the PASA (data not shown). Wilcoxon-U-tests revealed significant differences for threat ($p = .01$), challenge ($p = .007$), self-concept of own abilities ($p = .007$), and control expectancy ($p = .02$). Stressed participants felt more threat and challenge, and rated their own abilities and control expectancy lower than participants who had been exposed to the placebo TSST. As shown in Table 2 of the supplementary material there was a significant negative correlation between control expectancy and the total cortisol levels (AUC_{ground}) within the stressed group. Neither for cortisol nor for sAA did any

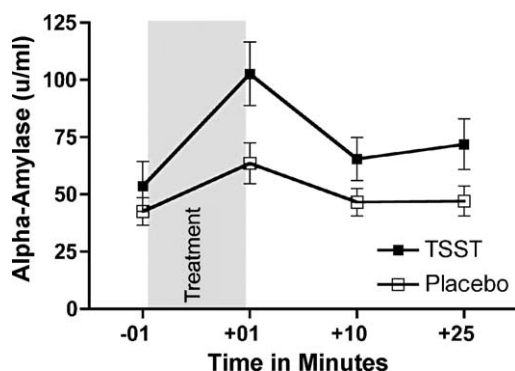


Figure 2 Salivary alpha-amylase (SAA) concentrations (mean and S.E.M.) of participants exposed to the TSST or the placebo TSST (not logarithmized data used for display purposes). While the stressed group showed a strong sAA increase in response to the TSST, the control group showed only a minor increase in response to the placebo TSST.

additional correlation reach the uncorrected threshold of $p < .05$.

2.3. Discussion of experiment 1

The neuroendocrine and psychometric data of experiment 1 suggest that the placebo version of the TSST is a non-stressful and thus a well-suited control condition. While the stressed participants showed the expected increase of cortisol in response to the TSST with peak levels reached 10 min after the cessation of the stressor (Kirschbaum et al., 1993, 1999; Kudielka and Kirschbaum, 2005), the placebo-treated participants showed a continuous decline in cortisol. The missing HPA response in the control condition reflects the fact that the effective component of the TSST, i.e. uncontrollable, social evaluative threat (Dickerson and Kemeny, 2004) was successfully eliminated in the placebo version.

The results for sAA are similar to the results for cortisol. While the control group showed only a small increase in sAA-activity, the stressed participants displayed the expected elevation of sAA-activity as already reported in recent studies (e.g. Rohleder et al., 2004; Nater et al., 2006). The slight, although significant increase within the control group 1 min after the treatment probably reflects the physical activity since participants had to take an upright posture in the room and had to talk loudly.

In line with the neuroendocrine measures were the PASA results. Participants of the control group felt less threat and challenge (primary appraisal), and had a greater self-concept of positive abilities and control expectancy (secondary appraisal) than TSST-treated participants. The negative correlation between control expectancy and overall cortisol output indicates that reduced control expectancy is accompanied by a higher overall cortisol output. This is in accordance with the social self-preservation theory of Dickerson and Kemeny (2004). The higher primary and the lower secondary anticipatory cognitive appraisals in participants immediately prior to stress exposure are in line with previous studies using this questionnaire (Gaab et al., 2005).

In sum, this first experiment indicated that the placebo TSST appears to be a promising standardized control condition for the TSST. Its lack of uncontrollable socio-evaluative threat appears to prevent an HPA response. For sAA, only a moderate increase occurs, which suggests that the physical demand and/or the somewhat unusual task of speaking alone in a room lead to a slight SNS response, which however is substantially weaker than the sAA response to the TSST.

3. Experiment 2: neuroendocrine evaluation of the placebo TSST using a within subjects design

The aim of experiment 2 was to find out whether a cross-over design, which is quite common in psychoneuroendocrine research, can be used with the TSST and its placebo version. In other words, we wanted to test whether initial exposure to the placebo TSST might influence the response to the TSST and vice versa. Furthermore we asked if there is an effect on the reactivity of the HPA and the SNS when

experimenters use an intercom to instruct the participants for the placebo version of the TSST and to control their compliance (to control that they actually spoke aloud and counted aloud). For this purpose we invited participants into our laboratory twice. On the first day participants were treated either with TSST or its placebo version, and on the second day they received the opposite treatment. Again salivary cortisol, sAA and the PASA were used to evaluate the effects. We predicted that there is no effect of treatment order. Furthermore, we assumed that the intercom might affect the neuroendocrine stress response, especially the response of sAA, which appears to be far more sensitive to subtle emotional challenges (van Stegeren et al., 2008).

3.1. Methods

3.1.1. Participants

The participants of this experiment were recruited among students of the Technical University of Dresden. Similar to experiment 1, the volunteers underwent a brief medical and psychological examination prior to testing, using the same inclusion and exclusion criteria except the menstrual cycle and intake of oral contraceptives. Female participants were asked to document their menstrual cycle in order to calculate their cycle phase. Again, all participants refrained from smoking, physical exercise, meals, alcoholic beverages and stimulating drinks (e.g. coffee or tea) at least 1 h prior to testing. Participants received detailed information about the study, provided written consent and were paid for their participation. The study protocol was approved by the local ethics committee.

According to its purpose, experiment 2 was based on a crossover design, with 2 days of experimental investigation and repeated measures of salivary cortisol, alpha-amylase and anticipatory cognitive stress appraisal. The assignment of treatment order (TSST/placebo or placebo/TSST) was randomized, with similar distribution of both sexes in session orders. The second trial was conducted at least 14 days after the first testing.

3.1.2. Experimental protocol

Both experimental sessions took about 90 min and were conducted between 1400 h and 1800 h. After arrival at the laboratory, participants were allowed to rest for 20 min. The TSST was conducted as described in Section 2.1.2.1. The placebo TSST was similar to experiment 1 (Section 2.1.2.2), except, we here used an intercom which was turned on during the whole placebo TSST in order to communicate with the participants and control their compliance to the tasks of the placebo TSST. The intercom use was announced to the participants during the instructions but they were told not to be recorded or evaluated. Like in experiment 1, the participants had to complete the PASA during the preparation phase of both treatments. Finally, participants were debriefed.

3.1.3. Measures

3.1.3.1. Saliva sampling and biochemical analysis. Saliva samples were obtained 1 min before treatment and 1, 15 and 30 min after treatment. Biochemical analyses were conducted using the same assays as described in experiment 1.

3.1.3.2. Measurement of stress appraisal. The anticipatory cognitive stress appraisal was again assessed by the PASA (see experiment 1 for description) within the preparation time of the TSST and the placebo. For the placebo phase we used the same modified PASA as in experiment 1.

3.1.4. Statistical analysis

Statistical analyses were performed as similar as described for experiment 1, except that this time procedures for repeated measurement designs were applied. Thus paired *t*-tests with adjusted α -levels were used to evaluate differences at particular sampling times. Analyses of variance (ANOVA) for repeated measures were employed with *time* and *treatment* as within subject factors. Treatment order and sex were used as between subject factors. Overall level of significance was defined as $p < .05$.

3.2. Results

3.2.1. Description of the sample

The total sample consisted of 47 participants (28 women and 19 men) with a mean age of 23.2 years ($\pm .42$ S.E.M.) and a mean BMI of 22.5 kg/m² ($\pm .43$ S.E.M.). The two groups with opposite treatment order (TSST first versus placebo first) did not differ with respect to sex, age, BMI, smoking, intake of oral contraception and menstrual cycle phase ($ps > .10$). Twenty-four participants performed on the TSST first, while 23 participants started with the placebo TSST. Because of missing data (sample contamination or insufficient saliva volume), three participants had to be excluded from the analysis of cortisol and eight participants from the analysis of sAA, respectively. See supplementary material (Table 1) for details.

3.2.2. Salivary cortisol

Results for salivary cortisol are presented in Fig. 3.

Fig. 3 (top) displays the results for the entire group, while middle and bottom display the findings for the two different treatment orders separately. ANOVA with the factors time, treatment, treatment order and sex revealed a significant effect of time ($F_{(1.84/73.74)} = 23.97$; $p < .001$), and treatment ($F_{(1/40)} = 10.99$; $p < .01$). Furthermore, there was a significant time \times treatment interaction ($F_{(1.58/63.08)} = 13.13$; $p < .001$). There was no main-effect of sex ($F_{(1/40)} = 1.40$; $p = .24$), but the order of treatment ($F_{(1/40)} = 6.85$; $p = .012$) was significant. Treatment order did not interact with any of the other factors (time and sex). The average cortisol response for each group of treatment order was assessed by computing the differences between baseline and cortisol levels obtained 15 min after the treatment. While the participants with the placebo/TSST treatment order showed on average lower baseline cortisol levels (Fig. 3 bottom) their response to the TSST (6.01 nmol/l \pm 1.42 S.E.M.) was very similar to the participants of the TSST/placebo group (8.04 nmol/l \pm 2.05 S.E.M.). Thus, the two treatment orders did not differ significantly in their cortisol response to the TSST ($t_{42} = .81$; $p > .10$). The difference between the cortisol levels 1 min prior and 1 min after the placebo treatment was significant ($t_{44} = 3.27$; $p < .01$), reflecting a small but significant increase of cortisol level within this treatment condition.

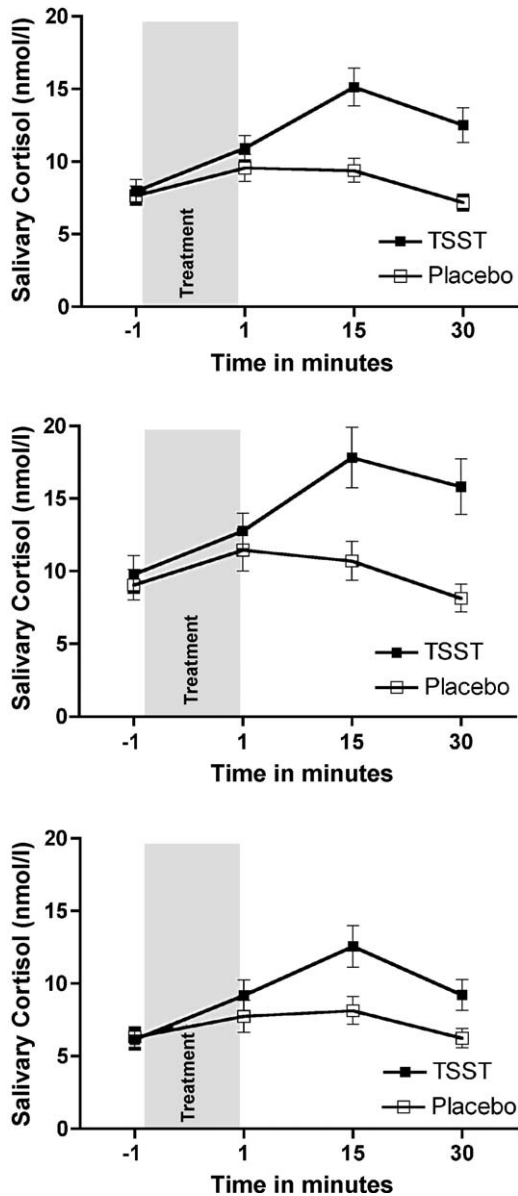


Figure 3 Salivary cortisol concentrations of participants exposed to the TSST or its placebo version in a within subject crossover design. The figure on top shows both treatment orders combined, the figure in the middle displays participants who were first exposed to the TSST and the figure on the bottom displays participants who were first exposed to the placebo TSST. Irrespective of treatment order participants showed a robust cortisol increase in response to the TSST, which peak levels obtained 15 min after stress cessation.

3.2.3. Salivary alpha-amylase

The results on the salivary alpha-amylase for each condition are shown in Fig. 4.

Fig. 4 (top) displays the results for the entire group, while middle and bottom display the findings for the two different treatment orders separately. K-S-test revealed a significant result for three sampling times ($ps \leq .05$). ANOVA with the factors time, treatment, treatment order and sex revealed only a significant effect of time ($F_{(2,1/73,54)} = 6.44; p < .01$). However, there was neither an interaction with treatment,

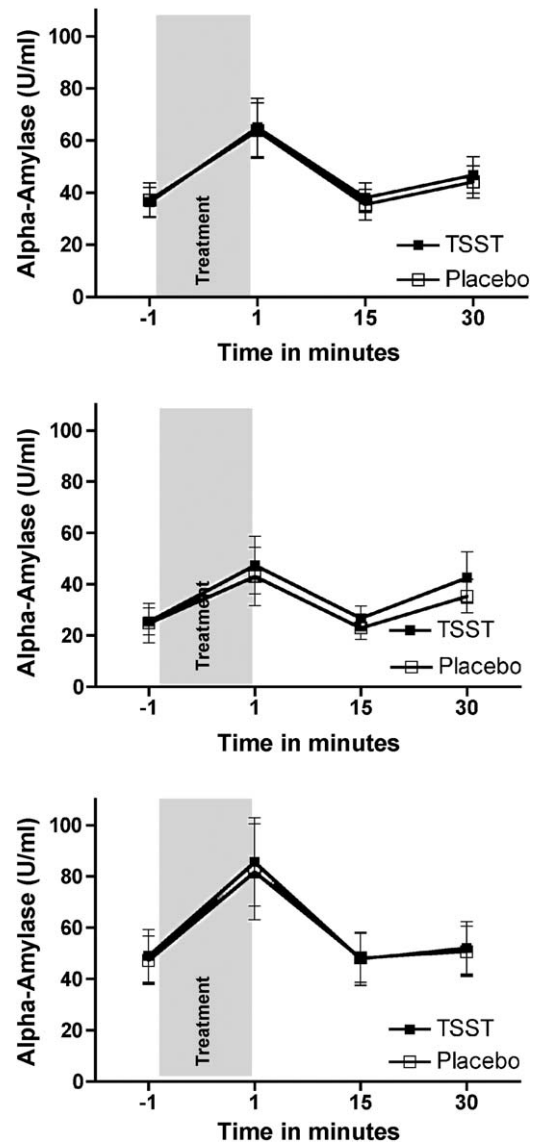


Figure 4 Salivary alpha-amylase (sAA) concentrations of participants exposed to the TSST or its placebo version in a within subject crossover design. The figure on top shows both treatment orders combined, the figure in the middle displays participants who were first exposed to the TSST and the figure on the bottom displays participants who were first exposed to the placebo TSST. In each experimental condition participants showed an increase of sAA in response to the treatment (not logarithmized data used for display purposes). There was no difference between TSST and placebo treatment.

treatment order, nor sex. These results indicate that the sAA response to the TSST and placebo TSST did not differ from each other. In addition, there was a main effect of sex ($F_{(1/35)} = 4.8; p < .05$). Male participants were found to display higher levels of sAA during both experimental conditions (data not shown).

3.2.4. PASA

Results on the PASA revealed that TSST-treated participants scored higher on items of primary appraisal (i.e. threat and

challenge), and lower on items of secondary appraisal (i.e. self-concept and control expectancy) than control participants (data not shown). Wilcoxon-signed ranks test revealed significant differences between the experimental conditions on threat, challenge and self-concept ($ps < .0001$). However, there was no significant difference for the scale control-expectancy ($p = .51$). Table 3 of the supplementary material reports a significant positive correlation between the scale threat and the reactivity of cortisol (AUC_{increase}) during the TSST condition (both treatment orders combined). Neither for cortisol nor for sAA did any additional correlation reach the uncorrected threshold of $p < .05$.

3.3. Discussion of experiment 2

The results of experiment 2 revealed significant differences in salivary cortisol responses between the TSST and the placebo condition irrespective of treatment order. Within the TSST condition we observed enhanced cortisol levels with a maximum at 15 min after the treatment. The significant differences between the conditions indicate a stronger HPA response to the TSST compared to the placebo TSST. Again, the placebo version of the TSST was a useful control condition with respect to the HPA stress response. Within the placebo condition the participants displayed a small but significant cortisol increase, this contrasts findings of the first experiment and might, at least in part, reflect the use of the intercom.

The intercom might lead to cognitions of being monitored or evaluated. Due to the spatial separation of the experimenter the participants had no knowledge about her/his behaviour. In addition, the PASA results for this experiment showed no difference in control expectancy for the two conditions. This might be secondary to the use of the intercom, since participants can not control whether or not the experimenter is listening to the content of their speech or not. This might lead to a slight, but significant threat of the drive to preserve the social self and keep control, due to keep or increase the basic goal of self-esteem (Dickerson and Kemeny, 2004; Grawe, 2004, 2007). These cognitions might have resulted in a slightly enhanced HPA activity in some individuals.

The differences on the PASA scales threat, challenge and self-concept of own abilities are in accordance with our expectations and indicate a higher stress appraisal within the TSST condition in contrast to the placebo TSST condition. The positive correlation between the scale threat and the cortisol increase due to the TSST condition is in line with the findings of Gaab et al. (2005). It indicates that a higher level of threat appraisal with regard to a particular situation is accompanied with a stronger cortisol increase.

We found no evidence for a carryover effect. Independent of which kind of treatment participants received first, their cortisol response to the TSST was very similar. This is in line with a former study of our laboratory (Kuhlmann et al., 2005) in which we used the TSST and its placebo version also in a crossover manner. However, we found a significant main effect of treatment order reflecting differences in baseline levels between the two treatment orders.

For sAA we found no differences between the two treatment conditions. Thus, our results indicate that TSST as well as the placebo TSST with the intercom activated the SNS system to a similar degree. The presence of the intercom

most likely induced an increased emotional arousal. This effect was obviously strong enough in order to abolish any differences between the two treatment conditions for sAA. Future studies should use additional markers of SNS activity (heart rate, heart rate variability, etc.) in order to monitor SNS activity during the placebo TSST.

A second finding of interest with respect to sAA is a significant main effect of sex. Male participants on average displayed higher sAA-activity throughout the experiment than women. A similar sex-difference in sAA basal activity was recently reported in a laboratory study by van Stegeren et al. (2008). However, no sex differences in sAA were observed in a circadian day profile study (Nater et al., 2007b). In the general discussion the issue of sex differences in sAA-activity will be discussed in some more depth.

4. General discussion

In the present study we evaluated a placebo version of the TSST. While the TSST is frequently used in psychoneuroendocrine research, a standardized control condition is often missing. Considering the social self-preservation theory of Dickerson and Kemeny (2004) we developed a placebo version of the TSST by eliminating its effective components: the uncontrollability and socio-evaluative threat to the self-esteem. In two studies we evaluated this placebo treatment with regard to neuroendocrine measures (salivary cortisol, sAA) and subjective ratings. The first study employed a between subject design. The second study was done to answer the question whether crossover designs can be used with the TSST and its placebo version. In addition we tested the impact of the use of an intercom for monitoring the compliance of participants during the placebo version.

4.1. Effects of the placebo TSST on the HPA axis

In both experiments strong differences between the TSST and its placebo version were observed for salivary cortisol levels. Thus, the placebo TSST does not activate the HPA axis, making it an appropriate control condition in studies interested in the HPA response to the TSST. The obtained cortisol levels of control participants were similar to those of former studies using resting and quiet control conditions (Kirschbaum et al., 1993, 1999; Rohleder et al., 2001, 2003; Kudielka et al., 2004b). Unexpectedly we found a slight enhancement of HPA activity within the control condition of experiment 2 as indicated by a small increase in cortisol levels. We suggest that this effect is caused by the use of an intercom in experiment 2. This issue will be discussed later. In general our results on the effect of the introduced version of placebo TSST on the HPA axis accord to the results of former studies of our laboratory (Kuhlmann et al., 2005; Schoofs et al., 2008), which already used this placebo treatment. Our findings support the conclusions of Dickerson and Kemeny (2004) who observed enhanced HPA activity only in situations with the experience of uncontrollability and social evaluation.

4.2. Effects of the placebo TSST on sAA

In experiment 1 the TSST led to the expected strong rise in sAA-activity, similarly to previous studies (Rohleder et al.,

2004; Nater et al., 2006). This rise was significantly stronger in the TSST condition than in its placebo version. For the placebo group however we also obtained a more modest, but significant enhancement of sAA-activity immediately after treatment. This is most probably due to physical activity (e.g. upright posture, talking) as already reported elsewhere (e.g. Goldstein, 1987). Thus while the TSST leads to a stronger sAA response than the placebo TSST, a sAA increase does still occur in the placebo TSST. This is in contrast to studies comparing the TSST with resting control conditions (e.g. Rohleder et al., 2004; Nater et al., 2006). Thus, the benefit of controlling for the physical and cognitive demands of the TSST with the placebo TSST comes at a certain cost, namely the modest activation of the SNS system. However, using the placebo TSST without an intercom enables the researcher to characterize the specific amount of sAA activation that is caused by the physical and cognitive demands of the TSST situation. In other words, the researcher has the opportunity to characterize the amount of sAA activation that is specifically caused by the psychological factors of the situation.

In experiment 2 we found similar sAA-levels in the TSST condition and in the placebo condition, which suggests comparable SNS activities in both treatment conditions. Experiment 2 differed from experiment 1 in the use of an intercom, which was introduced to control the participants' compliance within the placebo treatment and to instruct them. Thus, we conclude an arousing effect of intercom usage resulting in equally enhanced sAA-levels in both conditions. In addition, the intercom led also to a slight, but significant enhancement of HPA activity within the control condition of experiment 2 and nearly similar ratings of control expectancy with regard to the PASA. How this arousing effect might occur will be discussed in the next section. The finding that the intercom effect is more obvious for sAA fits to the general notion that the SNS is more responsive to emotional arousal and effort, whereas the HPA only gets activated in uncontrollable situations of more serious and continuous threats (e.g. distress; see Frankenhaeuser et al., 1978; Lundberg and Frankenhaeuser, 1980; Wortsman, 2002; Schommer et al., 2003; van Stegeren et al., 2008).

4.3. Effects of intercom use

The observed intercom effect could be explained by the social facilitation theory (Zajonc, 1965) as well as the theory of objective self-awareness (Duval and Wicklund, 1972; Wicklund, 1979). In terms of the social facilitation theory, the intercom could lead to arousal, similar to a performance in front of an audience. In terms of the theory of objective self-awareness, one might think that our participants were confronted by the intercom with a discrepancy between their actual performance and their self-expectations. This could lead to cognitive dissonance which causes additional arousal within the SNS and HPA activity (e.g. Elkin and Leippe, 1986; Losch and Cacioppo, 1990; Etgen and Rosen, 1993; Harmon-Jones and Harmon-Jones, 2007). Alternatively the intercom effect can be explained by the social self-preservation theory of Dickerson and Kemeny (2004) and by Grawe's consistency theory (2004, 2007). In terms of Dickerson and Kemeny (2004) intercom can be seen as an uncontrollable, socio-evaluative threat to one's social status. This would lead, in terms of

Grawe (2004, 2007), to a frustration of two basic goals, namely the need for control and orientation in life and for self-enhancement and self-esteem. Frustration of at least one of these goals leads to stress and psychological disorders (Grawe, 2004, 2007; Grosse Holforth et al., 2006).

Thus, while it is certainly desirable to control the compliance of study participants during the placebo TSST the usage of an overt intercom abolishes between condition differences for sAA. It awaits to be shown whether similar results would be obtained with other SNS markers. Future studies might want to explore alternative compliance measures. One possibility is to use a hidden camera (with debriefing afterwards) as was done in a previous stress study (Kern et al., 2008).

4.4. Sex differences in alpha-amylase

Male participants displayed higher sAA-levels than women at all sampling times in experiment 2. This finding is in line with a current study of van Stegeren et al. (2008). Interestingly, this difference was not seen in experiment 1. These conflicting results are typical for the issue of sex differences in sAA-levels (e.g. Kivlighan and Granger, 2006; van Stegeren et al., 2006; Yamaguchi et al., 2006; Nater et al., 2007b). Interestingly, significant sex-differences are reported in studies with female samples without any restriction on menstrual cycle or intake of oral contraceptives. In contrast to this, experiment 1 was performed with female participants all being in the luteal phase of the menstrual cycle. In experiment 2, we investigated female participants with and without the intake of oral contraceptives and during different phases of menstrual cycle. The impact of the menstrual cycle is relatively well documented for the HPA axis (Kirschbaum et al., 1995a, 1999; Rohleder et al., 2001; Kudielka and Kirschbaum, 2005), but an in-depth view of similar effects on sAA is still missing. In addition, other potential confounders like time of day, age, diseases or the exact sampling procedure need to be evaluated (Bosch et al., 1996; Nagler et al., 2000; Nater et al., 2007b; Rantonen and Meurman, 2000; Kivlighan and Granger, 2006; Rohleder et al., 2006b; Harmon et al., 2008).

4.5. Effects of the placebo TSST on anticipatory cognitive stress appraisal

With respect to anticipatory cognitive stress appraisal we found that stressed participants felt more threat and challenge and estimated their own abilities to get through the TSST as being lower, indicating a negative anticipatory cognitive stress appraisal. Interestingly, presence of the intercom leads to similar ratings of control expectancy. These findings are in line with previous studies (Gaab et al., 2005; Wirtz et al., 2006, 2007). Thus, similar to the HPA and SNS measures the subjective ratings support the general conclusion that the placebo version of the TSST is appropriate as a tool for psychoneuroendocrine research. Having said this it must be acknowledged that the PASA was developed for the assessment of appraisal processes occurring during the preparation period of the TSST. The questionnaire was therefore not ideally suited for the placebo version and had to be modified by us, especially the content and meaning of the scale control expectancy was problematic.

Even though not the primary goal of our current study we also searched for associations between the PASA and the cortisol as well as sAA stress response. For cortisol we found some associations with the PASA (with the scales control expectancy and threat), but the strength of these associations was smaller than previously reported (Gaab et al., 2005; Wirtz et al., 2006, 2007). For the sAA response no association were detected suggesting, that the constructs assessed with the PASA are closer linked to HPA than to SNS activity.

4.6. Carryover effects

The second experiment of this study was performed to evaluate the use of the TSST and the placebo TSST in a crossover design, which often is more powerful than a two-group comparison study. We found no carryover effect for salivary cortisol, sAA, or anticipatory cognitive stress appraisal. Treatment order also did not interact with any of the measures of interest. Thus, a previous experience with the placebo TSST has no substantial impact on the HPA response to the TSST, which is in line with a previous study from our group (Kuhlmann et al., 2005). This is in contrast to the strong habituation of cortisol stress responses observed if the TSST is presented a second time to the same participants in identical settings (Kirschbaum et al., 1995b; Pruessner et al., 1997; Schommer et al., 2003).

4.7. Limitations and outlook

The two studies presented in this report have several limitations which have to be acknowledged. The studies were conducted at different times of the day (experiment 1 in the morning and experiment 2 in the afternoon). Even though unlikely in our opinion, we cannot exclude the possibility that some of the differences observed between these experiments are secondary to this factor. In both experiments only salivary neuroendocrine stress markers were obtained. Especially in light of the findings for sAA (small increase in experiment 1 and similar increase as in the TSST in experiment 2) additional psychophysiological measures of SNS activity should be obtained in future studies. In addition, the present study only employed a single questionnaire in both studies. Additional mood measures obtained in parallel in both studies would have been desirable.

Another limitation is the fact that in both studies only one baseline (pre treatment) salivary sample was obtained. Future studies should consider taking more baseline samples in order to exclude with more power a priori group differences.

Furthermore, it would have been interesting to contrast the placebo TSST with the naturalistic diurnal course of cortisol and sAA, obtained by a resting control condition. This third treatment leg would have allowed us to test whether or not the cortisol decrease observed during the P-TSST in study 1 is similar or smaller than the decrease occurring during a rest condition.

Finally, we want to emphasize that the introduced placebo version of the TSST is one paradigm among several possible options. Although not explicitly intended, Dickerson et al. (2008) showed another possibility how a placebo treatment for the TSST could look like. In this study partici-

pants delivered a speech on why they would be a good job applicant. In one of the experimental conditions the participants had to deliver their speech in the presence of a research assistant who worked on a computer in the participants' line of vision but who did not look at or acknowledged the participant. It was observed that the mere presence of another person did not lead to HPA activation in this public speaking paradigm, which is in line to our findings reported here. However no SNS markers were obtained in this study.

Future methodological studies might want to develop alternative standardized control conditions along some of the lines suggested in this discussion. In the meantime we propose that the introduced placebo version of the TSST is a useful standardized control condition for researchers interested in controlling some of the non-specific effects of the TSST (physical and cognitive demand) on their outcome measure of interest.

Role of the funding sources

The funding source (German Research Foundation; DFG) had no further role in the design of the study, and in the collection, analysis and interpretation of the data. In addition, it had no role in the decision to submit the paper for publication.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.psyneuen.2009.02.008](https://doi.org/10.1016/j.psyneuen.2009.02.008).

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