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Defensive freezing links Hypothalamic-Pituitary-Adrenal-axis activity and internalizing symptoms in humans

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ABSTRACT

The Hypothalamic-Pituitary-Adrenal (HPA)-axis plays an important role in the expression of defensive freezing. Adaptive freezing reactivity, characterized by an immediate increase in acute stress and timely termination upon threat offset or need to act, is essential for adequate stress coping. Blunted HPA-axis activity in animals is associated with blunted freezing reactivity and internalizing symptoms. Despite their potential relevance, it remains unknown whether these mechanisms apply to humans and human psychopathology. Using a well-established method combining electrocardiography and posturography, we assessed freezing before, immediately after, and one hour after a stress induction in 92 human adolescents. In line with animal models, human adolescents showed stress-induced freezing, as quantified by relative reductions in heart rate and body sway after, as compared to before, stress. Moreover, relatively lower basal cortisol was associated with reduced stress-induced freezing reactivity (i.e., less immediate freezing and less recovery). Path analyses showed that decreased freezing recovery in individuals with reduced cortisol levels was associated with increased levels of internalizing symptoms. These findings suggest that reduced freezing recovery may be a promising marker for the etiology of internalizing symptoms.

1. Introduction

Freezing is one of the main defensive responses to stressful situations, widely observed across species. During threat exposure, activation of the parasympathetic and sympathetic branches of the autonomic nervous system with their respective cholinergic and catecholaminergic neurotransmitters facilitates distinct defensive reactions, including freezing and fight-or-flight responses. Freezing is the result of parasympathetic dominance over sympathetic activity and serves as a break on the sympathetically-driven fight-or-flight actions (Fanselow, 1994). It is characterized by heart rate deceleration (bradycardia) and bodily immobility (Fanselow, 1984; Schenberg et al., 1993) and facilitates selection of appropriate coping responses by enhancing perception, risk assessment, and action preparation (Blanchard et al., 2011; Gladwin et al., 2016; Lojowska et al., 2015). Although those immediate stress-induced freezing responses are considered adaptive stress responses, prolonged freezing and reduced flexibility to shift to active fight-or-flight responses may signal maladaptive coping (for reviews see Buss and Larson, 2000; Hagenaars et al., 2014). Animal research suggests that the Hypothalamic-Pituitary-Adrenal (HPA)-axis plays an important role in the expression and timely termination of these defensive reactions (de Kloet et al., 1999; Oitzl et al., 2010; Sherman and Kalin, 1988). Despite the potential relevance of these mechanisms for stress coping, it remains unknown whether they apply to humans and human psychopathology.

Research with animals consistently has shown that the HPA-axis with its end-product corticosterone (cortisol in humans) plays an important role in the expression of defensive freezing responses to stress. Removal of the adrenal glands in rats, for instance, resulted in disruption of freezing behavior, whereas renewed daily administrations of corticosterone in the same animals restored adaptive freezing responses to stress (Takahashi and Rubin, 1993). Furthermore, pharmacological stimulation and blockage of the HPA-axis at various levels increased and decreased stress-induced freezing, respectively (Corodimas et al., 1994; Kalin et al., 1988; Roozendaal et al., 1996; Sherman and Kalin, 1988). Corticosterone is not only important for the expression of freezing, it is also relevant for the timely termination of the freezing response. For instance, pharmacological stimulation of the

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HPA-axis not only facilitates onset of the freezing response, it also facilitates rodents' freezing recovery after a shock (Sherman and Kalin, 1988). This observation fits the role of the HPA-axis in the onset and timely termination of behavioral and neural stress responses in animals (de Kloet et al., 1999; Oitzl et al., 2010; Sherman and Kalin, 1988) and humans (Hermans et al., 2014).

Acute stress activates large scale neural (salience) networks—at the cost of an executive control network —that facilitate amygdala and brainstem driven defensive responses including freezing (Hermans et al., 2014). Following the natural course of stress recovery approximately one hour after acute stress, the balance between the salience and executive control networks reverses, allowing the body to return to homoeostasis and stress responses such as freezing to recover (Hermans et al., 2014). However, if the stress system remains activated, it may be detrimental for stress coping and eventually for mental and physical health (McEwen and Gianaros, 2011). Similarly, if freezing persists and parasympathetic dominance prevents flexible responding to environmental changes, it may signal maladaptive stress coping and may lead to chronic stress symptoms and internalizing psychopathology (for reviews see Hagenaars et al., 2014; Kozlowska et al., 2015). For example, Bovin et al. (2008) found that individuals who reported increased immobility during a traumatic event showed increased anxiety symptoms afterwards. Also, a pioneering study in humans showed that children with relatively higher basal and reactive cortisol levels displayed longer freezing episodes during threat exposure (Buss et al., 2004). Together, the reported studies suggest that immediate freezing in response to acute stress may be adaptive, while prolonged freezing may be maladaptive. Despite the potential relevance of altered defensive responses for internalizing symptoms in humans, to the best of our knowledge, no existing study objectively has quantified immediate versus delayed freezing responses to stress in humans, or the association with HPA-axis functioning and internalizing symptoms.

Therefore, the goal of this study was to investigate human freezing behavior before, immediately after, and one hour after a social-evaluative and physical stress test in adolescents. Adolescence is a period of increased stress sensitivity and vulnerability for the development of internalizing symptoms (McLaughlin and King, 2015; Romeo, 2010), making it an especially important time period to study the associations among stress-induced freezing, HPA-axis functioning, and internalizing symptomatology. To assess adolescent's freezing behavior, we used a well-established and objective measure that combines electrocardiographic and posturographic recordings (Azevedo et al., 2005; Niermann et al., 2015; Roelofs et al., 2010). We hypothesized: (i) that stress-induced freezing can be observed in human adolescents, (ii) that HPA-axis activity—both basal and reactive—is associated with a reactive freezing pattern (in the form of immediate stress-induced increases in freezing followed by successful recovery), and (iii) that decreased freezing recovery is associated with higher levels of internalizing symptoms.

2. Method

2.1. Participants

Participants were 96 adolescents (47 females, M_{age} = 17.19 years, SD = 0.15, age range: 16.83–17.70 years). Four did not complete the tasks following the stress induction and were excluded from the analyses, resulting in a sample of 92 participants for the current analyses (43 females, M_{age} = 17.19 years, SD = 0.15, age range: 16.83–17.70 years). Two participants were excluded from the body sway analyses due to technical problems, and one was excluded from the symptomatology analysis because of missing questionnaire data. The participants were recruited as part of the 10th measurement wave of the Nijmegen Longitudinal Study (Niermann et al., 2015; van Bakel and Riksen-Walraven, 2002). All of the 116 participants who are currently still participating in this longitudinal study were approached for participation. The current sample size reflects the number of participants agreeing to participate. This sample size can be considered large for a within-subject design in the stress literature (Dickerson and Kemeny, 2004). Participants took part in a 5-h protocol that included a stress induction procedure as well as an unrelated fMRI session (see Fig. S1 in Supplementary Material for an overview of the tasks). Because of the known effects of menstrual cycle on stress hormones, the female participants (28 of them were on oral contraceptives) were tested outside the menstrual phase. The protocol was approved by the regional medical ethics committee (CMO Arnhem-Nijmegen, The Netherlands). Prior to testing, participants and their parents gave informed assent and informed consent, respectively. Participants were financially reimbursed for their participation.

2.2. Procedure

To obtain reliable saliva assessments, participants were asked to refrain from eating or drinking (except for water) 45 min before the start of the experiment, and to reduce smoking and the consumption of coffee, tea, soft drinks, chocolate, and alcohol as much as possible on the day of testing. Additionally, participants were asked to not perform any heavy physical exercise for at least 2 h prior to the experiment. All participants were tested after noon, with 28 participants starting at 12 pm and 64 starting at 5 pm. All participants consumed a standardized meal and glass of water approximately 30 min prior to the first basal physiological measurements (blood pressure and heart rate), and subjective and saliva measurements. After assessment of these first basal stress measurements, participants were instructed to stand on the stabilometric force-platform for the first administration of the Emotional Face Task (EFT; pre). The second and third administrations of the EFT followed immediately after a physical and social-evaluative stress test (Post1) as well as at approximately +55 min (Post2; see Fig. 1A) when the acute stress responses should be back to baseline (Hermans et al., 2014). Physiological and subjective stress measurements were assessed again prior to and immediately after the stress induction, as well as at approximately +19, +30, +40, and +55 min after stress onset. Saliva samples were collected at the same time points, except immediately after the stress induction to ascertain that participants remained in an acute stress state during the second EFT (Post1). Participants also performed a set of decision-making tasks before and after the stress induction (see Fig. S1 in Supplementary Material). The behavioral part lasted approximately 2.5 h. Within one to two weeks after participation, participants and their parents completed online questionnaires of internalizing and externalizing symptomatology at home.

2.3. Emotional face task

To assess adolescents' freezing behavior, we used a well-established Emotional Face Task (EFT: Niermann et al., 2015; Roelofs et al., 2010) administered on a stabilometric force-platform that measures spontaneous fluctuations in body sway during passive picture viewing of angry, happy, and neutral faces. Electrocardiographic (ECG) recordings were collected simultaneously.

Participants were instructed to stand quietly on the stabilometric force-platform and to passively look at happy, angry, and neutral faces. We presented the faces in three blocks, each consisting of 20 face stimuli from the same emotional category (presentation time of each face was 3 s). Block and stimuli orders were randomized between participants. Within-participant block order was maintained across the presentation of the 3 tasks. Before the first EFT, participants completed a short (1 min) practice block presenting letters instead of faces. For a detailed description of the instructions and the emotional face-viewing paradigm, see Niermann et al. (2015). One participant felt dizzy during the first EFT (Pre), and five participants reported some dizziness during the second EFT (Post1). After a short break, they repeated the task; for

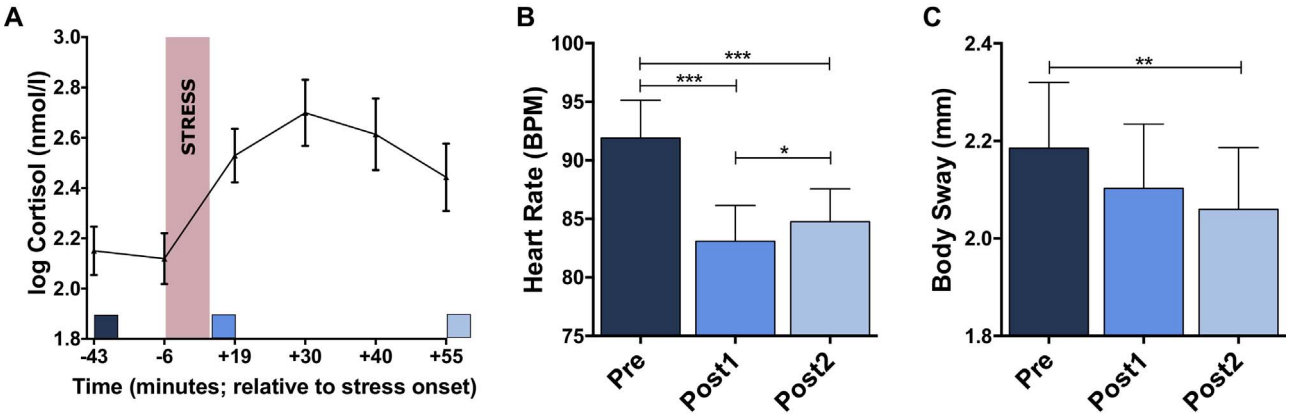

Fig. 1. Panel A: Stress induction by the Maastricht Acute Stress Test led to significant increases in cortisol. The timing and duration of the stress induction is shown in the figure by a red bar, including the instruction/anticipation and stressor phase. The timing of the freezing assessments relative to stress onset is shown by means of blue squares. Panels B and C: Display of mean heart rate (in BPM) and body sway (in mm) of all participants before (Pre), immediately after (Post1), and +55 min after stress (Post2). Stress resulted in decreases in heart rate after stress compared to before, which was accompanied by relative reductions in body sway. Error bars represent 95% confidence intervals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these participants, the data from the repeated task were used in the analyses.

2.4. Stress induction and measurements

We used the standardized Maastricht Acute Stress Test (MAST; Smeets et al., 2012), administered by an unfamiliar male experimenter, to induce moderate levels of stress. The protocol employs physical and social-evaluative stressors and consists of a 5-min instructional and anticipation phase followed by a 10-min stress induction phase, in which participants were instructed to immerse their left hand into ice-cold water (0.2–2.1 °C; 5 trials of either 60 or 90 s), alternated by trials in which they were instructed to count backwards in steps of 17 from 2043 or a similar high number determined by the experimenter (4 trials of either 45, 60, or 90 s). At all times, participants were allowed to remove their hand from the water and/or stop the entire stress induction procedure (12 participants stopped the procedure, but continued with the ensuing tasks; these participants were included in all data analyses). 82% of our participants showed a cortisol increase of at least 1.5 nmol/l in response to the stress induction (Miller et al., 2013), which falls in the normal range of responding (Quaedflieg et al., 2016). After the stress induction, participants were allowed to put their hand into lukewarm water for approximately 1 min to warm up their hand, which also served to prevent additional hand movements during the following task. Two participants requested something to eat because they felt some weakness during the experiment; they received a small sweet to remedy this. Although physiological and behavioral data did not show any differences between these two participants and the other participants, we checked whether results remained the same when they were excluded, which was the case.

2.5. Stress measurements

To ascertain successful stress induction, physiological (blood pressure and heart rate), hormonal (cortisol and alpha-amylase), and subjective measures were collected throughout the behavioral assessment. Saliva samples were collected six times by passive drool of approximately 1 ml (see Fig. 1A). They were frozen and stored at −20 ° Celsius until analysis. Salivary concentrations were measured using commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany). The intra and inter-assay coefficients for cortisol were below 8%. One participant had no reliable cortisol sample at −43 min. See Appendix S1 in Supplementary Material for a detailed description of the remaining measurements.

2.6. Internalizing symptoms

Internalizing symptoms were assessed with the self- and parent-report Social Anxiety Scale for Adolescents (SAS; La Greca and Stone, 1993; $M = 39.03$, $SD = 11.13$, range: 18–75 and $M = 31.70$, $SD = 11.87$, range: 18–67, respectively; 17% and 8% of our participants, respectively scored in the clinical range [cutoff = 50]; observed α was 0.92 and 0.94, respectively) and the parent-report internalizing subscale of the Child Behavior Checklist (CBCL 4–18 years; Achenbach, 1991; $M = 5.92$, $SD = 6.16$, range: 0–29; 19% of our participants scored in the subclinical to clinical range [cutoff T-score = 60]; observed α was 0.88). As the total scores on these three scales were positively correlated (rs ranging from 0.26 to 0.61; $ps < 0.05$) and in order to reduce the number of comparisons, we computed an overall internalizing score ($M = -0.00$, $SD = 0.80$, range: −1.23–3.02; $\alpha = 0.95$, based on all items of the three scales) by averaging the standardized total scores (higher scores indicating higher levels of internalizing symptoms). One participant did not fill out the self-report SAS. For this participant, we computed the average of the other two available scales. Externalizing symptoms were assessed (see Appendix S2 and S6 in Supplementary Material) to explore a possible association of freezing with aggression-related behavior.

2.7. Data analysis

2.7.1. Posturography during EFTs

Using Brainvision Recorder, a time series of deviations from participants' center-of-pressure (COP) in both the anterior-posterior (AP) and mediolateral (ML) direction was recorded by four force sensors (one in each corner) of the stabilometric force-platform (dimensions: 50 cm × 50 cm; sampling frequency: 5000 Hz, down-sampled to 200 Hz prior to analysis; 1 mm accuracy).

Posturographic analyses were conducted in MATLAB (MathWorks, Natick, MA, USA) using a 10 Hz low-pass and a 0.1 Hz high-pass filter. As an indicator for postural (im)mobility, we determined participants' variability in body sway during each 1 min presentation block of facial emotional expressions from the same emotional category by computing the standard deviation of the COP in the AP direction (SD-AP; see Niermann et al., 2015 for a detailed description).

2.7.2. Heart rate during EFTs

Mean heart rate in beats-per-minute (BPM) was determined with Brainvision (Analyzer 2.0) separately for each participant for each of the three emotional blocks of each EFT viewing.

2.7.3. Cortisol

Prior to analyses, individual cortisol values were log-transformed to reduce skew. Basal cortisol was determined by taking the mean cortisol value for each participant of the two assessment points before stress (untransformed $M = 8.55$, $SD = 5.67$). One participant did not provide a reliable saliva sample at −43 min prior to stress, therefore only the cortisol value immediately prior to stress was used as an indicator of this participant's basal cortisol level. Another participant showed an unexpected increase in cortisol at −6 min (z-score > 3). Prior to the calculation of mean basal cortisol, the impact of this outlier was reduced by estimating his cortisol level based on the expected percentage of increase/decrease in the corresponding assessment point for his gender. We determined stress-induced cortisol levels by calculating the area under the curve with respect to increase (AUC; Pruessner et al., 2003) for the assessment points at −6, +19, +30, +40, and +55 min. As indicated, one participant showed an unexpected increase in cortisol at −6 min, and another an unexpected decrease in cortisol at +40 min (z-score > 3). Prior to the calculation of AUC, the impact of these outliers was reduced as reported.[1]

2.7.4. Statistical analyses

All analyses were conducted in R (version 3.3.2; R Core Team, 2015). The main analyses used a linear mixed-effect models approach with the *lmer* function (*lme4* package; version 1.1.10; Bates et al., 2015). The repeated-measures nature of the freezing data (i.e., indicated by heart rate and body sway changes during the EFT viewings) was taken into account by including a per-participant random intercept and by modeling all within-subject predictors (i.e., categorical variables of Time [Pre, Post1, Post2], Emotion [happy, angry, neutral faces], and of Block order [1, 2, 3]) not only as fixed effects but where appropriate also as random slopes varying across participants (all possible random correlation terms were also included). This represents a "maximal" random effects structure as recommended by Barr et al. (2013) to avoid inflated Type-1 errors. *P*-values were determined using Likelihood Ratio Tests, using the function *mixed* of the package *afex* (Singmann et al., 2015). Linear mixed-effect models were also used to determine the success of the stress induction. Correlations were run to test whether stress-induced freezing behavior was associated with HPA-axis activity and internalizing symptoms.

3. Results

3.1. Stress manipulation checks

The stress induction was successful as indicated by significant increases on all stress measurements (cortisol, subjective stress, blood pressure, heart rate, and alpha-amylase) at time points after stress induction compared to before (see Fig. 1A for cortisol). A linear mixed-effect model with linear and quadratic effects of Time, Gender (males, females on oral contraceptives, females not on oral contraceptives), and Time-of-day (early, late) as additional predictors showed linear, $\chi^2(1) = 24.85$, $p < 0.001$, and quadratic, $\chi^2(1) = 51.72$, $p < 0.001$, effects of Time on cortisol. Results for the other stress measurements are displayed in Appendix S3 in the Supplementary Material.

3.2. Stress-induced freezing

Stress resulted in increased freezing behavior as indicated by reductions in heart rate and body sway. A linear mixed-effect model predicting heart rate from fixed effects of Time (Pre, Post1, Post2), Emotion (angry, happy, neutral faces), and Block order (1, 2, 3), all possible two-way and three-way interactions, and all possible random effects, showed main effects of Time, $\chi^2(2) = 87.44$, $p < 0.001$, and Block order, $\chi^2(2) = 138.94$, $p < 0.001$, and a Time × Block order interaction, $\chi^2(4) = 31.73$, $p < 0.001$. Tukey-corrected post-hoc comparisons of the main effect of Time indicated that heart rate decreased from Pre to Post1, followed by a slight increase at Post2 (see Fig. 1B and Appendix S4 in the Supplementary Material for results of the full model as well as the post-hoc comparisons of the Time × Block order interactions). This suggests that stress induced a relative reduction in heart rate. To investigate whether such a decrease was indeed a common pattern among our participants, we computed how many participants showed a reduction of at least 1 BPM: We found that 92% of our participants showed such a reduction in heart rate from Pre to Post1, indicating that it was a common pattern. In addition, 54% of these participants showed an increase in heart rate (> 1 BPM) from Post1 to Post2, while 46% of these participants showed a further decrease (> 1 BPM) or no change (< 1 BPM) in heart rate from Post1 to Post2. Fig. S6 in Appendix S5 in the Supplementary Material shows each participant's individual heart rate trajectory across the three time points (Pre, Post1, Post2).

Similar stress effects were found predicting body sway: Time: $\chi^2(2) = 10.64$, $p = 0.005$; Block order: $\chi^2(2) = 7.43$, $p = 0.024$; Time × Block order: $\chi^2(4) = 16.94$, $p = 0.002$. Tukey-corrected post-hoc comparisons of the main effect of Time demonstrated that body sway decreased steadily from Pre to Post2 (see Fig. 1C and Appendix S4 in the Supplementary Material).

3.3. HPA-axis activity, freezing, and internalizing symptoms

To investigate whether stress-induced freezing and its recovery were associated with HPA-axis activity and internalizing symptoms, we first calculated two difference scores indicating *immediate stress-induced freezing* and *freezing recovery*, respectively. Immediate stress-induced freezing was calculated by subtracting heart rate at Post1 from the same measurement before stress (Pre; see Fig. 1B), with higher immediate stress-induced freezing scores indicating stronger freezing responses immediately after stress induction compared to before. Freezing recovery was calculated by subtracting heart rate at Post1 from heart rate at Post2, with higher freezing recovery scores indicating stronger recovery one hour after stress induction, compared to immediately after stress. For body sway, the same difference scores were calculated.

Correlational analyses (see Table S2 and S3 for the full correlation matrix) showed that basal cortisol was positively associated with immediate stress-induced freezing ($r = 0.22$, $p = 0.032$, 95% CI [0.02, 0.41], Fig. 2B)[2] and freezing recovery ($r = 0.34$, $p = 0.001$, 95% CI [0.15, 0.51], Fig. 2C). Internalizing symptoms were negatively associated with freezing recovery ($r = -0.21$, $p = 0.042$, 95% CI [−0.40, −0.01]), but not with immediate stress-induced freezing ($r = 0.03$, $p > 0.250$, 95% CI [−0.18, 0.24]). These correlational results for stress-induced freezing were observed for heart rate, but not for body sway. No such results were found for reactive cortisol (AUC, see Appendix S6 in Supplementary Material). Control analyses (see Appendix S7 in Supplementary Material for results of all control analyses) showed that internalizing symptoms varied by Gender (male, female; $t(81.42) = 3.30$, $p = 0.001$; 95% CI [0.21, 0.85]), whereas basal cortisol varied by Time-of-day (early, late; $t(51.86) = 2.46$, $p = 0.017$, 95% CI [0.04, 0.42]). Importantly, the associations between freezing recovery and internalizing symptoms as well as between stress-induced freezing and basal cortisol remained significant when controlling for Gender and Time-of-day respectively (see Appendix S7 in Supplementary Material).

Based on these correlational results, we ran a path analysis within

[1] Similar results for the analyses regarding basal and stress-induced cortisol were found when these outliers were not adjusted.

[2] The positive association between immediate stress-induced freezing and basal cortisol remained when 5 multivariate outliers were excluded ($r = 0.27$, $p = 0.011$, 95% CI [0.06, 0.46]).

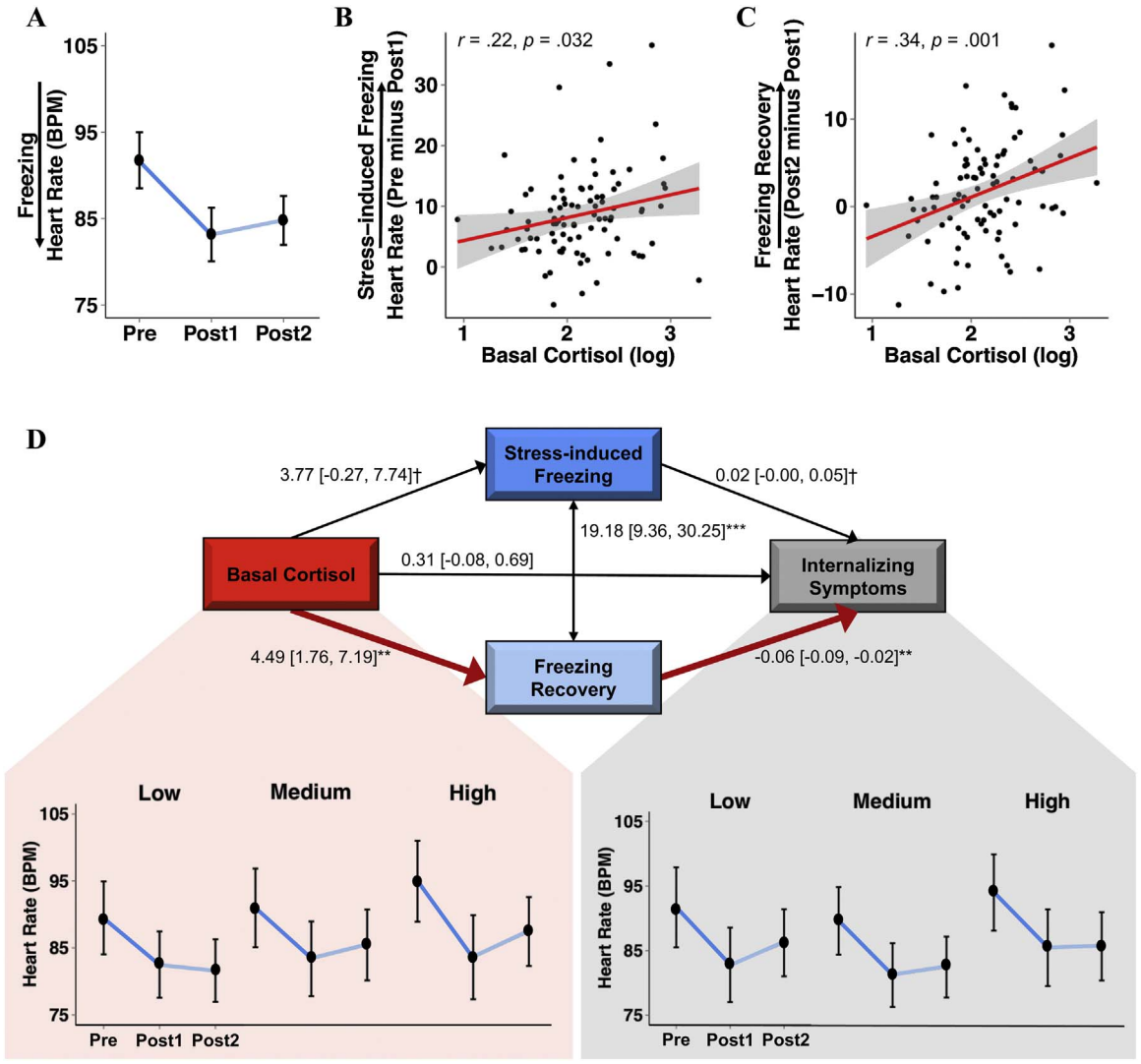

Fig. 2. Panel A: Mean heart rate (in BPM) is separately displayed for all participants ($N = 91$) before (Pre), immediately after (Post1), and +55 min after stress (Post2). Panel B: Positive correlation between basal cortisol levels (log-transformed) and immediate stress-induced freezing (calculated by subtracting heart rate assessed at Post1 from the same measurement before stress [Pre]). Panel C: Positive correlation between basal cortisol levels and freezing recovery (calculated by subtracting heart rate assessed at Post1 from the same measurement at Post2). Panel D: The model illustrates an indirect mediation path, suggesting that lower basal cortisol levels were associated with less freezing recovery, which in turn was associated with increased levels of internalizing symptoms. For illustrative purposes only, results have been displayed separately for groups of low, medium, and high levels of basal cortisol on a red background underneath the corresponding red block of the mediation model. Likewise, groups of low, medium, and high levels of internalizing symptoms have been displayed on a gray background expanding from the gray block in the mediation model. The three lines on a red background show that heart rate reactivity increased (both the decrease in heart rate from Pre to Post1 as well as the recovery from Post1 to Post2) when basal cortisol levels went from low to high. The three lines on a gray background show that only heart rate recovery (from Post1 to Post2) decreased when internalizing symptoms went from low to high. Error bars represent 95% confidence intervals. Unstandardized regression coefficients, with bootstrapped 95% confidence intervals in parentheses, are shown for each path ($^{\dagger}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the *lavaan* package (Yves, 2012)[3] to examine whether freezing behavior, as indicated by heart rate, showed an indirect pathway between basal cortisol and internalizing symptoms. The model explained 10% of the variance in internalizing symptoms and revealed an indirect mediation path via freezing recovery (indirect: $b = -0.25$, $z = -2.27$, 95% CI [−0.46, −0.03]), but not via immediate stress-induced freezing (indirect: $b = 0.09$, $z = 1.02$, 95% CI [−0.08, 0.26]). This path indicated that basal cortisol was positively associated with freezing recovery, which in turn was negatively associated with internalizing symptoms (see Fig. 2D). Individuals with lower levels of basal cortisol showed decreased freezing recovery, which in turn was associated with more internalizing symptoms. This indirect mediation path remained significant when Gender (male, female; $b = -0.25$, $z = -2.40$, 95% CI [−0.44, −0.04]) and Time-of-day (early, late; $b = -0.26$, $z = -2.35$, 95% CI [−0.46, −0.04]) were included separately as control variables. Please note that we did not observe a direct association between basal cortisol and internalizing symptoms, neither when they were tested separately ($r = 0.08$, $p > 0.250$, 95% CI [−0.13, 0.28]) nor when taking the dynamical aspects of freezing reactivity into account (Fig. 2D).

4. Discussion

The aim of this study was to investigate whether stress-induced freezing can be observed in human adolescents and whether freezing reactivity is associated with HPA-axis activity and internalizing symptoms. Similar to findings in animals, we observed stress-induced freezing in adolescents, quantified by a relative reduction in heart rate and body sway after stress compared to before. Additionally, basal

[3] As recommended by Shrout and Bolger (2002), we determined bootstrapped standard errors and bootstrapped 95% confidence intervals.

cortisol levels were positively associated with immediate freezing as well as with subsequent freezing recovery as reflected in heart rate. Importantly, a path analysis indicated that individuals with lower levels of basal cortisol showed less freezing recovery and in turn increased levels of internalizing symptoms. These results support the notion that HPA-axis activity plays a crucial role in the expression of stress-induced freezing behavior in humans, which may be a promising marker for internalizing symptoms.

This is the first study establishing a potential association between HPA-axis functioning and an objective measure of stress-induced freezing in humans: relatively higher levels of basal cortisol were associated with relatively increased freezing reactivity (i.e., increased immediate stress-induced freezing followed by successful recovery). This result is consistent with findings from research in animals, suggesting an important role of the HPA-axis in the expression of freezing behavior (Corodimas et al., 1994; Kalin et al., 1988; Roozendaal et al., 1996; Sherman and Kalin, 1988; Takahashi and Rubin, 1993). The fact that cortisol was not only related to the acute expression of freezing, but also to its timely termination, supports the notion that the HPA-axis plays a critical role in the optimization of behavioral responses as part of adequate stress coping (de Kloet et al., 1999; Oitzl et al., 2010).

The importance of flexibility in the stress system—characterized by an immediate response to acute stress and timely termination of the stress response (Hermans et al., 2014; McEwen and Gianaros, 2011)—was further supported by the observation that reduced freezing recovery but not immediate freezing was associated with increased levels of internalizing symptoms. Most interestingly, freezing recovery formed an indirect pathway from HPA-axis activity to internalizing symptoms. This finding supports the potential role of flexibility in defensive stress reactions for adequate stress coping and is particularly interesting in light of clinical research suggesting an association between freezing and the development of posttraumatic stress symptoms (Bovin et al., 2008; Hagenaars et al., 2014; Kozlowska et al., 2015). Posttraumatic stress disorder has been associated consistently with reduced basal cortisol levels (Jacobson, 2014; Zaba et al., 2015) and with reports of prolonged immobility reactions to stress (Bovin et al., 2008; Kozlowska et al., 2015). Although immediate freezing is generally considered adaptive, reduced freezing recovery may reflect individuals' reduced flexibility to adequately cope with the immediate stressor (Buss and Larson, 2000; Hagenaars et al., 2014; Kozlowska et al., 2015). Together, these findings suggest that less freezing recovery combined with lower basal cortisol levels may increase individuals' vulnerability to stress and anxiety-related behavior problems.

A few interpretational issues should be discussed. First, our observed decreases in heart rate were relatively large compared to previous studies in which freezing was elicited by more subtle threats such as angry faces (Roelofs et al., 2010). For example, Roelofs et al. (2010) found mean heart rate reductions of 4.49 BPM in response to angry versus happy faces compared to the mean heart rate reduction of 8.60 BPM (Fig. 2A) in response to the stress induction in the current study. Interestingly, in our main analyses we observed no effects of emotional facial expressions on freezing behavior, neither before nor after stress. It is tempting to speculate that the absence of these effects might result from stress anticipation and the subsequent experience of stress, wiping out the usually subtle effects of face valence. Although such mechanisms may have suppressed emotion effects at the group level, additional correlational results demonstrate that individual differences in threat-related reduction in heart rate were correlated to individual differences in threat-related reduction in body sway in response to angry versus neutral faces before the stress induction (see Appendix S8 in Supplementary Material), as was observed previously (Niermann et al., 2015).

Second, our stress manipulation induced reductions in both heart rate and body sway, while only heart rate reductions were associated with basal cortisol levels and internalizing symptoms. Interestingly, Gladwin et al. (2016) also found that heart rate was more sensitive to threat manipulations than body sway, suggesting that heart rate might be more sensitive to individual differences in HPA-axis activity and symptomatology than body sway.

Third, research in animals (De Boer et al., 1990; Kalin et al., 1998) has suggested that both basal and stress-induced cortisol levels are associated with freezing behavior. Basal activity of the HPA-axis has been shown to be regulated by mineralcorticoid receptors (MR), setting the threshold for onset of a stress response, while glucocorticoid receptors (GR), which bind with a 10-fold lower affinity, have been shown to facilitate the offset of a stress response (de Kloet et al., 2005; Reul and de Kloet, 1985). The finding that basal but not stress-induced cortisol levels were associated with stress-induced freezing and freezing recovery may suggest that our cortisol associations are largely MR mediated, though future investigations using specific MR and GR blockers are needed to test the relative contribution of MR and GR in human freezing reactions. To the best of our knowledge, only one other human study addressed the associations among freezing, basal, and stress-induced cortisol levels: Buss et al. (2004) found that increased signs of children's freezing behavior to a threatening situation (a stranger approaches the child) were associated with basal and stress-induced cortisol levels. In our study, we only observed an association between stress-induced freezing and basal cortisol levels. Several methodological differences might account for these different findings, including the participant sample (children vs. adolescents), freezing assessment (observation vs. heart rate/body sway), and stress induction (approaching stranger vs. social-evaluative and physical stressor). Thus, although the role of the HPA-axis is well-established in freezing behavior in animals, future replication studies are needed to investigate the exact role of distinct HPA-axis functions in freezing behavior in humans.

Fourth, because our study was part of a longitudinal study we could not include a non-stress condition in half of the participants. Therefore, we should consider the alternative interpretation that the observed changes in freezing were a consequence of simple task repetition rather than an effect of stress manipulation. We regard this as highly unlikely because there was no monotonic decline in heart rate as a function of the three task administrations, arguing against a simple task repetition effect. The drop in heart rate only occurred immediately after the stressor (i.e., between task administrations 1 and 2), but not after a subsequent non-stress delay (i.e., between task administrations 2 and 3; there was no further decrease, but an overall increase; Fig. 2A). Nevertheless, future studies should include a non-stress condition to control for order effects.

Fifth, the participants in this study were adolescents. Adolescence is characterized by a number of neuroendocrine and social-emotional changes (Blakemore et al., 2010; Romeo, 2010) and therefore considered a sensitive period for the development of internalizing symptoms (McLaughlin and King, 2015). However, we cannot make any inferences regarding the specificity of our results for adolescence as we cannot compare them to another age group. Future studies should repeat the current study in other age groups to investigate whether the observed effects are specific for adolescence.

Sixth and finally, it would be interesting for future studies to assess changes in perceptual sensitivity associated with freezing. Research with both humans and rodents suggests that freezing facilitates the perceptual system to detect threatening stimuli (Blanchard et al., 2011; Lojowska et al., 2015). Such a mechanism may modulate the association between freezing recovery and internalizing symptoms (Hermans et al., 2013). Prolonged freezing may enhance individuals' vigilance for threat cues, promoting a vicious circle in which attention is drawn to negative stimuli. Although speculative, it has been argued that such a threat bias in turn may facilitate appraisal and encoding of adverse events as well as further enhancement of sensory vigilance which is characteristic of internalizing psychopathology (Mathews and MacLeod, 1994; Williams et al., 1996).

In conclusion, this study provided a novel setup that enabled us to show that humans, like animals, display stress-induced freezing behavior. Lower levels of basal cortisol were associated with reduced freezing reactivity, both in terms of reduced immediate stress-induced freezing and reduced recovery. Individuals with lower levels of basal cortisol showed decreased freezing recovery, and in turn increased levels of internalizing symptoms. These findings support the notion that HPA-axis activity plays a crucial role in the expression of human freezing behavior, and that less freezing recovery may be a promising marker for the etiology of internalizing symptoms. As this is the first study investigating the relation between human freezing, HPA-axis activity, and internalizing symptoms, the current results should be seen as tentative until replicated. Nevertheless, the current setup clearly opens the way for testing mechanistic accounts of stress-induced freezing responses in human psychopathology.

Contributions

All authors contributed to the study concept and design. Data collection was done by H. C. M. Niermann and A. Tyborowska. H. C. M. Niermann performed data analysis and interpretation under the supervision of B. Figner, K. Roelofs, and A. H. N. Cillessen. H. C. M. Niermann drafted the paper and all others provided critical revisions. All authors approved the final version of the paper prior to submission.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2017.05.001. Data are available upon request from the Data Archiving and Networked Services (DANS): http://dx.doi.org/10.17026/dans-xuy-mjs8.

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