Stressor-specific sex differences in amygdala-frontal cortex networks

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**Abstract:** Females and males differ in stress reactivity, coping and in the prevalence rates of stress-related disorders. According to a neurocognitive framework of stress coping, functional connectivity between the amygdala and frontal regions (including dorsolateral-prefrontal cortex (dlPFC), ventral anterior cingulate cortex (vACC) and medial-prefrontal cortex (mPFC)) plays a key role in how people deal with stress. In the current study we investigated the effect of sex and stressor type in a within-subject counterbalanced design on resting-state functional connectivity (rsFC) of the amygdala with these frontal regions in 77 healthy participants (40 females). Both stressor types led to changes in subjective ratings with decreasing positive affect and increasing negative affect and anger. Females showed a higher amygdala–vACC and amygdala-mPFC rsFC for social exclusion than for achievement stress, and than males: while higher amygdala-vACC rsFC points to activation of emotion processing and coping, higher amygdala–mPFC rsFC indicates feelings of reward and social gain, pointing to positive effects of social affiliation. Thus, for females, feeling socially affiliated might be more fundamental than for males. Our data indicate interactions of sex- and stressor in amygdala-frontal coupling which translationally contribute to a better understanding of sex differences in prevalence rates and stress coping.

**Keywords:** resting-state functional connectivity; sex differences; stress; social exclusion; achievement stress; Cyberball; MIST

1. Introduction

In our daily lives we are confronted with psychosocial and physiological stressors. Both can elicit a typical physiological stress response which is activating the hypothalamo-pituitary-adrenal (HPA)-axis and thus releases cortisol, which happens especially in situations that are uncontrollable and threatening to the social self [1]. In an MRI environment, psychosocial stress is often induced using the Cyberball task [2] or the Montreal Imaging Stress task (MIST, [3]). In the Cyberball task, participants are actively excluded from a ball tossing game by peers, creating a threat to the basic social need of being included [4] and represents interpersonal, social stress. Contrarily, the MIST elicits achievement stress by using mental arithmetics including also social evaluation. While the single correlates of neural stress research have been investigated frequently (for a meta-analysis see e.g., [5]), models on connectivity in neural stress research also exist but have gained less attention.

De Raedt and Hooley (2016) [6] put forward a neurocognitive framework for stress coping including three main regions: (1) the amygdala, (2) the dorsolateral prefrontal cortex (dlPFC), and (3) the ventromedial-frontal region, including the ventral anterior cingulate cortex (vACC) and the (ventro)medial prefrontal cortex ((v)mPFC). The framework suggests that the amygdala, a region central in emotion processing [7] plays a key role in stress coping. The dlPFC, a brain region involved in attentional control, executive function, and reappraisal of negative experiences [8], downregulates amygdala activity, thereby improving stress coping. In contrast, activation of the ventromedial-frontal region, involved in stress processing [9,10], attentional emotional processing [11] and in reward processing [12], upregulates amygdala activity, resulting in a lower ability for stress coping. Thus, this framework specifies effects to stress via brain connectivities.

Resting-state functional connectivity (rsFC) is of particular interest when investigating interactions between brain areas. Therewith, rsFC can be used to determine anticipatory effects and the aftermath of stress in neural networks, including those described by the framework by de Raedt and Hooley [6]. Previous studies already reported increased rsFC between amygdala–dlPFC and amygdala–vACC resulting from psychosocial stress (including both achievement and social stress components) (30min after stress induction) in a mixed sample of females and males [13]. In a male-only sample, increased amygdala–vmPFC rsFC has also been reported one hour after psychosocial stress induction [10]. Additionally, cortisol responders showed a reduced amygdala–dlPFC rsFC and an increased amygdala–mPFC rsFC compared to non-responders, suggesting less effective coping with psychosocial stress in cortisol responders [13], and the administration of hydrocortisone to raise cortisol levels increased task-based amygdala–mPFC coupling in a dynamic facial expression task in a male-only sample [14]. In contrast to psychosocial stress, in the immediate aftermath of social exclusion brain networks seem to shift towards a more vigilant state through an increase in rsFC of the default mode network with the salience network, thereby increasing the allocation of neural resources to deal with the social exclusion and potentially mobilise energy for a fight-or-flight response [4].

Sex differences are well known in terms of stress reactivity to psychosocial stress (achievement stress with a social component) and their coping mechanism. While females seem to be more susceptible to social stress [15], males seem to be more sensitive to achievement-related stress [16]. Sex differences are also apparent in the function and regulation of the HPA-axis, with conflicting studies reporting higher cortisol levels after stress induction in females and others reporting them in males (for review see [17]). These sex differences in stress reactivity and coping might contribute to differences between sexes in the prevalence of stress-related diseases [18], where females have a higher risk of suffering from affective disorders, such as depression and anxiety [19,20], while males more likely suffer from substance misuse [20,21]. To assess sex differences in the coupling of brain regions, rsFC is of specific interest, as during resting-state, neural connectivity can be characterized beyond task-based activation, which is of particular interest when it comes to differences in abilities or preconditions between females and males. Concerning sex differences in rsFC of the amygdala with frontal regions independent of stress reactivity, several studies reported significant findings. In adolescent boys increased left amygdala–mPFC rsFC was reported, whereas girls showed an increased right amygdala–mPFC rsFC [22]. In adults, higher amygdala–vmPFC rsFC emerged in males compared to females [23] and negative associations of cortisol with rsFC of the amygdala with the dlPFC and the subgenual ACC (sgACC), a subpart of the vACC, in females but positive in males were reported [24]. So far, to the best of our knowledge, no study specifically examined sex differences in rsFC in situations of social exclusion or achievement stress.

Taken together, previous studies indicate that amygdala-frontal neural networks seem to be modified by diverse stressors in specific manners and additionally are differently wired in females and males. With the current study, we set out to investigate the effect of sex and stressor-type on networks between the amygdala and frontal rsFC, following the assumption that the amygdala takes a crucial part in stress processing and interacts with the frontal cortex in terms of stress coping [6]. Thus, we compare amygdala-frontal rsFC before and after two different psychosocial stressors and additionally investigate sex differences. We hypothesise that both stressors affect amygdala-frontal rsFC, but differently in females and males. More specifically, we expect higher amygdala–mPFC rsFC in males than females in general [23] and an increase in rsFC pre- to post-stress at least in males [10,14], while we cannot conclude anything on females, as this was not assessed in female participants so far. Due to task-related sex differences, with females being more affected by social exclusion [15] and males by achievement stress [16], we additionally expect sex differences in rsFC depending on stressor type. Additionally, we hypothesise that a stress-induced cortisol change is associated with an increase in amygdala–mPFC rsFC in both sexes [10,13] and a decrease in amygdala–dlPFC and amygdala-vACC rsFC especially in females [24].

2. Materials and Methods

2.1. Sample

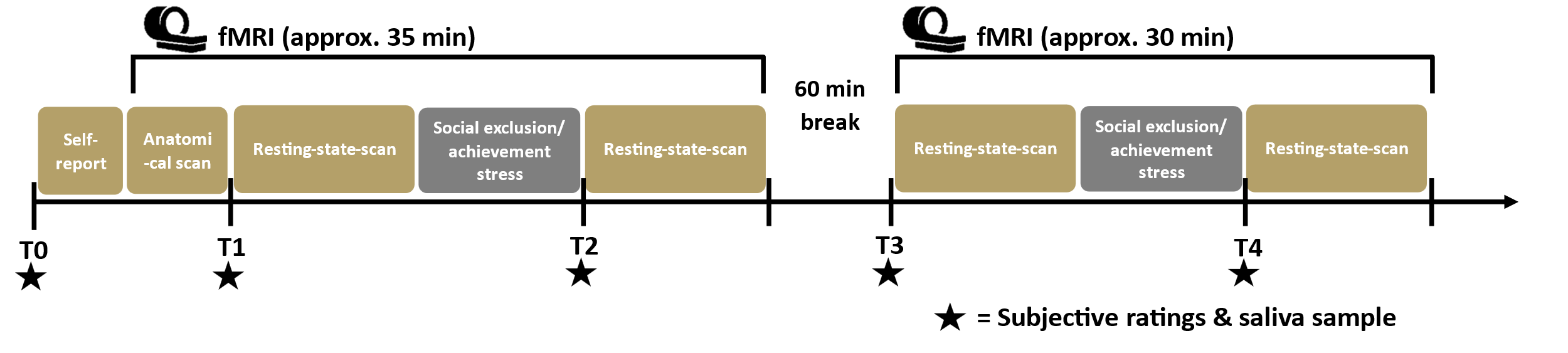
For this study, 77 healthy, non-smoking, right-handed university students (40 females) were included. Sample size was calculated using G\*Power [25] (alpha error rate 0.05, power 0.95, f=0.18), which calculates a total sample size of n=68 for a repeated-measurements, within-between-subjects-design. Considering dropouts, we targeted to include 80 participants, 40 females and 40 males, in the study. Recruitment was done through advertisements posted at the Medical University of Vienna and the University of Vienna, Austria, as well as through various online student platforms. Psychology students were excluded due to their potential familiarity with the stress induction tasks. Exclusion criteria were a history of neurological or mental disorders (assessed via a structured clinical interview, SCID, [26]), chronic illnesses, drug or hormone intake including hormonal contraception, alcohol abuse or addiction, working night shifts, engaging in competitive sports, recent or current pregnancy, premenstrual dysphoric disorder, allergic asthma, and MRI incompatibility (i.e., metal parts in the body, etc.). Participants were asked to refrain from alcohol consumption and exercise 24hrs prior to testing, medication, caffeine, and drug intake on the day itself, as well as food or drink intake (except water) two hours before arriving at the lab. We included female participants over the whole menstrual cycle to include a variety of female hormonal profiles. Cycle phase was assessed through documentation of the previous three cycle phases and confirmed via baseline measurements of estradiol and progesterone with saliva samples on the day of testing. Females and males were matched for age.

Written informed consent was obtained from all participants and the study was approved by the Institutional Review Board of the Medical University of Vienna. All participants were treated according to the Declaration of Helsinki (1964). At the end of the test day, participants were fully briefed on the aims of the study and received financial compensation.

2.2. Procedure

All measurements were done at the MR Centre of Excellence at the Medical University of Vienna, Austria, between 1:30pm and 6:30pm to control for circadian hormone rhythms. After arrival, participants received detailed instructions, filled in questionnaires pertaining to social demographics (i.e. sex, age, education status), verbal intelligence (Mehrfachwortschatztest-B, MWTB, [27]), social support in the last 10 days, rejection sensitivity (Rejection Sensitivity Questionnaire, RSQ, [28]), stress coping (Stressverarbeitungs-Fragebogen, SVF, [29]), and subjective ratings (positive and negative affect scale, PANAS, [30]; emotional self-rating, ESR, [31]). Figure 1 illustrates the procedure of fMRI and saliva sample data collection.

First, participants provided a first saliva sample for hormone analysis (T0; approximately 15min after arrival and before scanner entry) to analyse estradiol, progesterone, testosterone, and cortisol. Participants then underwent two fMRI sessions, with two separate tasks (achievement stress and social exclusion, described below in more detail and see also [32,33]) applied in a randomised order across participants. Before and after each task, a resting-state (rs) scan was conducted (i.e., four rs-scans in total; Figure 1). Anatomical images were assessed at the beginning of the first fMRI session. To examine effects of the tasks on subjective ratings and hormonal stress reactivity, before the first and third rs-scan and after each task, participants again completed the PANAS and ESR and provided saliva samples (Figure 1, T1-T4). Between both tasks a break of at least 60min was spent outside the scanner.



**Figure 1.** Study design. After arrival and self-report questionnaires, two fMRI sessions consisting of an anatomical scan (only before the first session), two rs-scans per session, a stress induction task with either the Cyberball (for social exclusion) or the modified MIST (for achievement stress) were administered to participants, with a 60 min break in-between. Saliva samples and subjective ratings were collected at dedicated time points. Social exclusion and achievement stress were administered in a randomised order, counterbalanced between females and males.

2.3. Stress tasks

2.3.1. Modified Montreal Imaging Stress Task - MIST

For achievement stress, we used a modified version of the MIST [3], in which participants had to solve mental arithmetic problems. The task’s difficulty was adapted to each participant’s performance and participants received feedback on their accuracy on the arithmetic tasks. The modified MIST consists of two conditions: (1) In the control condition, participants performed arithmetic tasks without time pressure, and (2) in the stress condition, participants’ success rate was kept at 20-45% by increasing difficulty and diminishing available time. For the current study, the MIST was modified to exclude the social component of this task by giving no negative social feedback nor feedback about performance of an average group of peers. This was necessary to compare social exclusion and achievement stress between females and males in the current study design. In total, the task lasted 10 min, with two control conditions and two stress conditions of 70 sec each, repeated twice. For a more detailed description of this task, see [32].

2.3.2. Cyberball

For social exclusion, we used a modified version of the Cyberball task [2], a virtual ball tossing game played with two other players (1 male, 1 female; modified version according to [34]). Participants were instructed that they would engage in a ball tossing game with other participants and that they were not to meet the other players before the game to avoid first impressions influencing gameplay. To increase credibility that other players were “real”, participants were told that they would be allowed to meet the other players after the game. Thus, participants didn’t know that the two other players were computer-determined. Participants had the choice of pressing one of two buttons to toss the ball to either of the two other players. The players were represented by silhouettes from a pre-recorded video of real people. The participant was represented with two hands at the bottom of the screen. The task consisted of three conditions: (1) in the technical exclusion condition, a message appeared that the network connection was not working properly; (2) in the social exclusion condition, participants did not receive any passes, with a message assuring them that network connection was working properly; and (3) in the inclusion condition, participants received a third of the passes. The task started with a technical exclusion block, followed by three inclusion blocks, five exclusion blocks and finished with two more inclusion blocks (30-40sec/block). Each block consisted of approximately 12 passes. Inter-block intervals with a fixation cross lasted 1-3sec. Total duration of the task was approximately 10min. For a more detailed description of this task, see [33].

2.4. Saliva samples

To assess hormone concentrations, saliva samples were obtained at five different time points (T0-T4), four times (pre- vs. post-stressor samples) while participants were lying in the MRI scanner (see Figure 1 and paragraph 2.2). The samples were stored at -20°C until shipping to the analysis laboratory (SwissHealthMed, Aying, Germany), where they were frozen overnight at -20°C again, thawed, and centrifuged. Hormone concentrations of cortisol, testosterone and progesterone were assessed for all time points through competitive luminescence immunoassay (LUMI) kits, while estradiol levels were assessed with enzyme-linked immunoassay (ELISA) kits (SwissHealthMed, Aying, Germany), as they show a better sensitivity than the LUMI kits for estradiol. Estradiol was only assessed in females at arrival (T0), as it merely served to determine menstrual cycle phase. Reliable and valid measurements were achieved for the four hormones with these kits (cortisol: intra-assay coefficient of variability (CV)<4% and inter-assay CV<5%, testosterone: intra-assay CV<4% and inter-assay CV<7%, progesterone: inter-assay CV<4% and inter-assay CV<5%, estradiol: intra-assay CV<8% and inter-assay CV<4%).

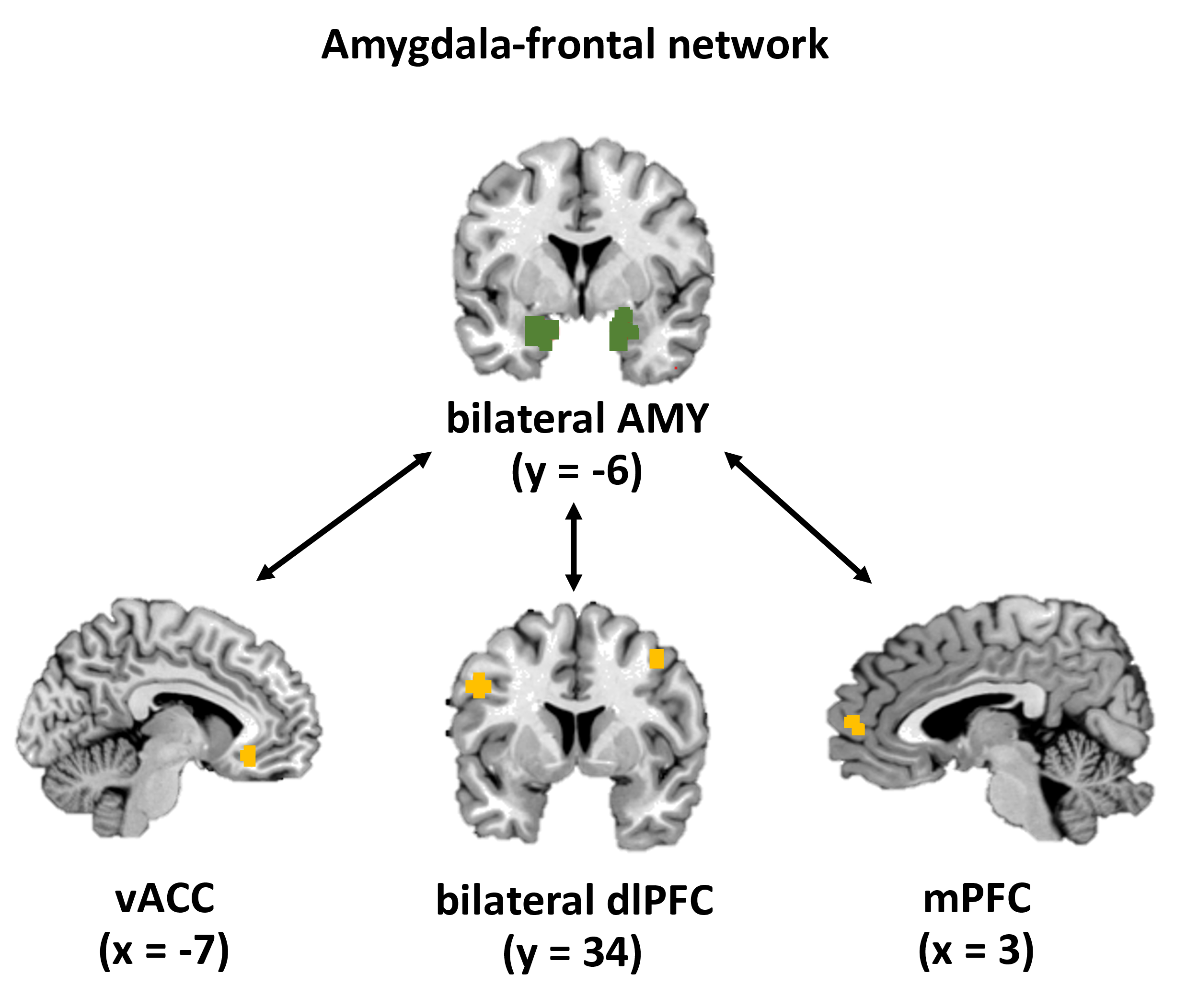
2.5. Data and statistical analysis of behavioural and hormonal data

Statistical analysis of sex differences in demographic, subjective, and hormonal data was done with IBM SPSS statistics for Windows, version 27 (IBM Corp., Armonk, NY, USA). For demographic data used for the sample description (see paragraph 3.1), we tested assumptions for parametric testing and t-tests or non-parametric equivalents were performed. For subjective (positive and negative affect, anger) and hormonal (cortisol) stress reactivity, four separate three-factor ANCOVAs with two within-subject factors (task [achievement stress/social exclusion], time [pre-/post-stress]), one between-subjects factor (sex [female/male]) and a covariate-of-no-interest (order of task presentation), to account for a potential effect of the order of task presentation, were performed. The significance level for all statistical tests was set at p<.05. ANCOVAs were Bonferroni-corrected.

2.6. rsFC analyses

2.6.1. Definition of regions-of-interests

To assess amygdala-frontal rsFC, we used the left and right amygdala [35] and assessed their connectivity with the following frontal areas as regions-of-interest (ROIs). Based on previous literature the unilateral vACC [36], the unilateral mPFC [37] and the left and right dlPFC [36] were chosen. As done previously, ROIs were created by adding a 6-mm sphere around the MNI-coordinates [38] (see Figure 2 and Table 1 for ROIs and coordinates).



**Figure 2.** Amygdala-frontal network: representation of the ROIs. For the analyses we assessed rsFC of the amygdalae (in green) with one of the other ROIs (in orange), namely the ventral anterior cingulate cortex (vACC, unilaterally), the medial prefrontal cortex (mPFC, unilaterally) or the dorsolateral prefrontal cortex (dlPFC, bilaterally).

**Table 1.** MNI coordinates of each ROI.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **MNI coordinates** |  | **X** | **Y** | **Z** |
| Amygdala(bilateral) | R | 26 | -6 | -14 |
|  | L | -24 | -6 | -14 |
| vACC (unilateral) |  | -7 | 29 | -12 |
| mPFC (unilateral) |  | 3 | 54 | 6 |
| dlPFC unilateral | R | 37 | 34 | 35 |
|  | L | -37 | 44 | 37 |

**Note**. Abbreviations: vACC = ventral anterior cingulate cortex, mPFC = medial prefrontal cortex, dlPFC = dorsolateral prefrontal cortex.

2.6.2. Acquisition, pre-processing and calculation of rsFC

Functional and anatomical data were acquired on a 3T TIM Trio Scanner (Siemens Medical Systems, Erlangen, Germany) at the Medical University, Vienna. Anatomical images were acquired using an MPRAGE sequence (3D Magnetisation Prepared Rapid Gradient Echo: 1x1x1.1 mm resolution, TR=2300 ms, TE=4.21 ms, flip angle 9°, inversion time 900 ms, 160 sagittal slices). For the rs-scans, a gradient-echo EPI sequence (with distortion correction; 23 interleaved slices, TE/TR=38/1800 ms, voxel size 1.5x1.5x3 mm, 90° flip angle; bandwidth=1446Hz/pixel, 1.8 mm slice gap) was applied, acquiring 167 images in an axial plane for each subject. These settings lead to a high spatial resolution, which, in combination with low slice thickness, helps avoid signal dephasing in the ventral brain [39,40]. Thus, the applied parameters lead to high sensitivity and specificity, especially around the amygdala.

Functional data was processed using SPM12 (Wellcome Trust Centre for Neuroimaging, London, http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) implemented in Matlab (Version R2019a; Mathworks Inc., Sherborn, MA, USA). The first four volumes were discarded for each participant before further processing. Then images were realigned to the initial image followed by alignment to the mean EPI. The resulting mean EPI image of each subject was then spatially normalized to the ICBM-MNI152 template for each participant [41] with a “unified segmentation” approach [42]. The deformation resulting from this method was applied to the individual EPI volumes which were further resampled to 2mm isotropic. Smoothing was done by a 5mm full-width-at-half-maximum Gaussian kernel to enhance signal-to-noise-ratio as well as to compensate for anatomical variations. The time-course of each ROI was extracted for each participant by computing the first eigenvariate of all voxels within a 6mm sphere around the respective ROI-coordinate, as done previously [38]. These time-courses were further denoised (to reduce spurious correlations, [43]), by excluding variance explained by the following variables: (a) the six motion parameters derived from the image realignment and (b) their first derivatives, as well as (c) white matter (WM) and cerebral blood flow (CBF) intensity (each tissue-signal-class related signal separately). All these nuisance variables entered the model as first and second order terms to increase sensitivity and specificity of the analyses as well as to detect valid correlations and anti-correlations during rest [44]. Data was band-pass filtered with a cut-off frequency of 0.01 and 0.08Hz and rsFC of each participant was adjusted for the effect of age via a regression analysis to account for even subtle changes in brain architecture (see also [5,24]). Linear (Pearson) correlation coefficients of the resulting time-series between the time-series of the left and right amygdala and the time-series of the four frontal ROIs (unilateral vACC and mPFC, bilateral dlPFC, see Table 1) were calculated to quantify rsFC, resulting in eight rsFC assessments. This was done for each of the four rs-scans (T1-T4). The resulting correlation coefficients were Fisher z-transformed for building an approximately normal distributed variable for further statistical analyses.

2.6.3. Statistical analyses of rsFC

For this data analysis, all further statistical analyses were performed with IBM SPSS 27 (IBM Corp., Armonk, NY, USA). RsFC of both amygdalae with frontal ROIs were analysed in a seed-to-seed analysis method, excluding rsFC between frontal ROIs or between the amygdalae, as the framework of de Raedt and Hooley (2016) [6] does not include interactions between these rsFCs. First, all eight included rsFCs (bilateral amygdala with unilateral vACC and mPFC and bilateral dlPFC), for each of the four rs-scans (T1-T4), were tested for significant deviation from zero by one-sample t-tests. Only rsFC values significantly different from zero in at least one of the four rs-scans (T1-T4) were included for further analyses. This procedure excluded rsFC of the right amygdala with the dlPFC bilaterally (all ps >.077), as well as rsFC between the left amygdala and the right dlPFC (ps >.068). For each of the remaining five rsFCs (bilateral amygdala with unilateral vACC and mPFC; left amygdala with left dlPFC), separate three-factor ANCOVAs with two within-subject factors (task [achievement stress/social exclusion], time [pre-/post-stress]), one between-subjects factor (sex [female/male]) and a covariate-of-no-interest (order of task presentation), to account for a potential effect of the order of task presentation, were performed. Sticking to de Raedt and Hooley’s framework [6], no dependencies between rsFCs are assumed, thus for our analyses testing this specific framework we assume independence of connectivities. Nevertheless, to account for repeated testing due to laterality, Bonferroni correction was applied for bilateral amygdala analyses (bilateral amygdala with unilateral vACC and unilateral mPFC), with an α-value set to .025. All ANCOVAs were Bonferroni corrected. Partial eta-squared as effect size, reflecting the proportion of variance due to a certain parameter or set of parameters in a model relative to the variance in a simpler, nested model [45], is reported for ANCOVAs.

*2.6.3.1 Exploratory regression analyses*

To further exploratorily assess associations of stress-induced changes in cortisol reactivity and subjective ratings with changes of amygdala–frontal rsFC before vs. after each task, we ran multiple regression analyses. Therefore, the change from pre-stress to post-stress of amydala-frontal rsFC, cortisol, positive affect, negative affect, and anger for each stressor (achievement stress/social exclusion) were calculated. For each amygdala-frontal rsFC, exploratory multiple regression analyses (with forced entry) were performed. For the following predictors, we ran separate regression analyses with change in amygdala-frontal rsFC from pre-stress to post-stress as dependent variable, separately for each stressor: change in (a) cortisol, (b) positive affect, (c) negative affect and (d) anger from pre- to post-stress. For each analysis, sex and order of task presentation were included as additional predictors. One exception was change in cortisol, where we observed a significant sex difference (see paragraph 3.2 and Figure 4C). We therefore added the interaction of sex and cortisol as a predictor to the regression and found it to be significant (p=.021) and thus performed this regression analysis separately for females and males. For all regression analyses, outliers were identified and excluded using a Cook’s distance above 0.5 as cut-off. Significance levels for all regression analyses were Bonferroni corrected for laterality, as explained above.

3. Results

3.1. Sample description

Groups were matched for movement parameters (FD, DVARS, RMS, [44,46]), removing four male participants from the analysis, leading to a final sample of 73 participants (40 females). After matching, females and males did not differ in their mean movement (all ps >.56). Females and males did not differ significantly in age, verbal intelligence, amount of social support in the last 10 days, rejection sensitivity or in positive coping strategies (see Table 2). Besides expected sex differences in progesterone and testosterone values, with higher progesterone in females and higher testosterone in males, there was a significant difference in negative coping strategies, with females scoring higher (i.e., they are using more negative coping strategies) than males. Almost half of the females were tested during menstruation in their early follicular phase (day 1-5, n=19), two were tested during their ovulation (day 12-14, n=2) and the rest was tested during their midluteal phase (day 18-23, n=19). With regards to order of task presentation, 19 females and 15 males first performed the achievement stress task vs. 21 females and 18 males first went through social exclusion (χ2(1)=0.03, p=.862, φ=0.02). Please see Table 2 for sample description.

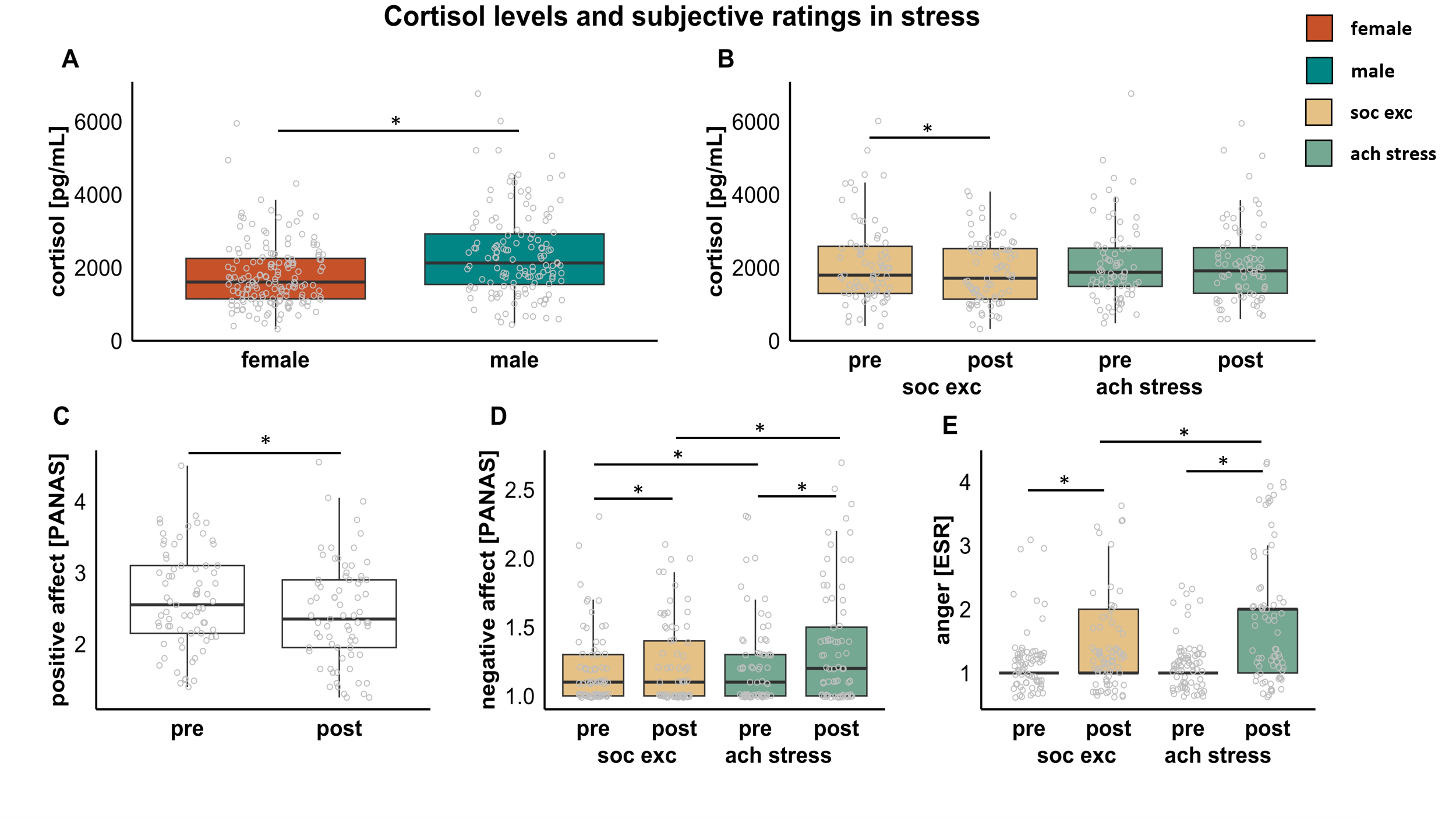
**Table 2.** Sample description including details on age, questionnaire data and hormonal concentrations.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Females (n=40)** | | **Males (n=33)** | |  |  |
|  | **Mean** | **SD** | **Mean** | **SD** | **Statistical parameters** | ***p*-value** |
| **Age (in years)** | 24.7 | 3.8 | 24.0 | 3.0 | t(71)=0.755 | .453 |
| **Verbal intelligence** | 26.7 | 4.0 | 27.4 | 3.5 | t(701)=0.875 | .385 |
| **Social support in last 10 days** | 19.3 | 20.5 | 20.2 | 19.2 | t(71)=0.201 | .841 |
| **Rejection sensitivity** | 8.4 | 3.3 | 8.1 | 2.9 | t(71)=0.412 | .681 |
| **Positive stress coping strategies** | 2.2 | 0.4 | 2.3 | 0.3 | t(71)=0.298 | .767 |
| **Negative stress coping strategies** | 2.0 | 0.6 | 1.7 | 0.6 | t(71)=2.080 | **.041\*** |
| **Estradiol levels at arrival [pg/mL]** | 4.58 | 4.07 | n/a | n/a | n/a | n/a |
| **Progesterone levels at arrival [pg/mL]** | 71.97 | 57.61 | 42.65 | 29.48 | t(691)=2.582 | **.012\*** |
| **Testosterone levels at arrival [pg/mL]** | 25.99 | 19.07 | 87.93 | 60.62 | t(71)=6.114 | **<.001\*** |

**Note**. Abbreviations: SD = standard deviation. \* indicates significance of p<.05. 1 : datapoint missing for one or two participants.

3.2. Associations between stress and cortisol.

The three-factor ANCOVA (within-subject factors: task [achievement stress/social exclusion], time [pre-/post-stress]; between-subjects factor: sex [female/male]; covariate-of-no-interest: order of task presentation) showed a main effect of sex (F(1,70)=9.085, p=.004, ηp2=0.115) with higher cortisol levels in males than in females (Figure 3A). A task-by-time interaction was found (F(1,70)=5.301, p=.024, ηp2=0.070) and post-hoc t-tests showed significantly lower cortisol levels post- compared to pre-social exclusion (t(71)=2.579, p=.012), which was not seen post- compared to pre-achievement stress (Figure 3B). Directly comparing pre and post values between the two tasks, however, did not yield significant differences (all ps>.077). Please see Figure 3A and 3B and Table A1 for detailed cortisol levels.



**Figure 3.** (A) Significantly higher cortisol level in females compared to males; (B) Cortisol levels for each stressor show a decrease post- vs. pre-social exclusion which is not apparent for achievement stress; (C) Positive affect decrease pre- to post-stress in both tasks; (D) Negative affect is higher in achievement stress compared to social exclusion and pre- compared to post-stress; (E) Increased anger rates post-stress are seen in both tasks, with higher ratings post-achievement stress compared to post-social exclusion. \* indicates significance of p<.05. Abbreviations: PANAS = positive and negative affect scale, ESR = emotional scale rating, soc exc = social exclusion, ach stress = achievement stress.

3.3. Associations between stress and subjective rating.

For each of the three subjective rating measures positive affect, negative affect and anger a three-factor ANCOVA (within-subject factors: task [achievement stress/social exclusion], time [pre-/post-stress]; between-subjects factor: sex [female/male]; covariate-of-no-interest: order of task presentation) was performed.

*Positive affect.* We observed a significant main effect of time (F(1,71)=21.035, p<.001, ηp2=0.229), with lower positive affect ratings post-stress compared to pre-stress. No other main or interaction effect reached significance (all ps>.386, see Figure 3C and Table A1).

*Negative affect.* A main effect of task (F(1,71)=5.896, p=.018, ηp2=0.077) , with higher negative affect for achievement stress than for social exclusion, and time (F(1,71)=10.358, p=.002, ηp2=0.127), with higher negative affect post-stress compared to pre-stress was found. No other main or interaction effect was seen (all ps>.071, see Figure 3D and Table A1).

*Anger.* Anger showed a main effect of task (F(1,71)=11.849, p<.001, ηp2=0.143), with higher ratings for achievement stress compared to social exclusion, and time (F(1,71)=45.125, p<.001, ηp2=0.389), with higher ratings post-stress compared to pre-stress. Further, a task-by-time interaction could be seen (F(1,71)=14.689, p<.001, ηp2=0.171). Post-hoc t-tests showed that anger was significantly higher after both social exclusion (t(72)=-2.819, p=.006) and achievement stress (t(72)=-6.158, p<.001), but was higher after achievement stress than after social exclusion (t(72)=-4.125, p<.001). No significant effect was seen before the tasks, nor was there an effect of sex or other interaction effects (all ps>.085, see Figure 3E and Table A1).

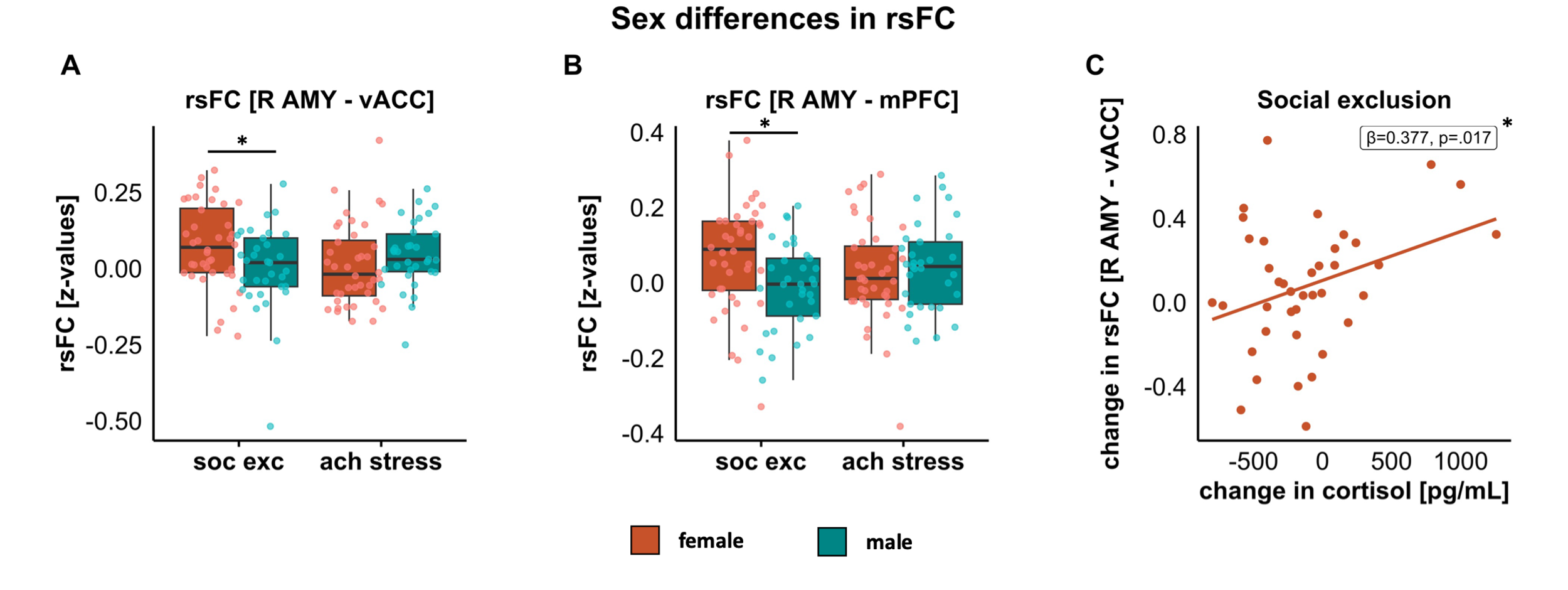
3.4. Associations between stress and rsFC

For each of the five retained rsFCs, a three-factor ANCOVA (within-subject factors: task [achievement stress/social exclusion], time [pre-/post-stress]; between-subjects factor: sex [female/male]; covariate-of-no-interest: order of task presentation) was conducted.

3.4.1. Amygdala–vACC.

*Right amygdala-vACC*. The ANCOVA showed a significant task-by-sex interaction (F(1,69)=5.533, p=.022, ηp2=0.074). Post-hoc t-tests demonstrated higher rsFC for social exclusion than for achievement stress in females (t(39)=2.210, p=.033), while such a difference was not apparent in males (p=.200). Furthermore, females exhibited higher rsFC than males for social exclusion (t(70)=-2.068, p=.042, see Figure 4A) which was not apparent for achievement stress. No other main effect or interaction was significant (all ps>.126).

*Left amygdala-vACC*. No significant effects emerged considering Bonferroni correction (all ps>.039, cut-off α=.025).



**Figure 4.** Sex differences in rsFC of right amygdala for both paradigms with vACC (A) and mPFC (B). In (C), association of change in cortisol with change in amygdala-vACC rsFC for social exclusion in females. \* indicates significance of p<.05. Abbreviations: vACC = ventral anterior cingulate cortex, AMY = amygdala, mPFC = medial prefrontal cortex, soc exc = social exclusion, ach stress = achievement stress.

3.4.2. Amygdala–mPFC.

*Right amygdala-mPFC.* A task-by-sex interaction (F(1,69)=6.093, p=0.016, ηp2=0.081) emerged. Post-hoc t-tests indicated higher rsFC for social exclusion in females than males (t(71)=-2.543, p=.013, see Figure 4B). No significant sex difference appeared for achievement stress and no task differences appeared within sexes (all ps>.051). No other main effect or interaction was significant (all ps>.079).

*Left amygdala-mPFC*. Here, a main effect of time was observed (F(1,69)=6.542, p=.013, ηp2=0.087), with higher rsFC pre-stress compared to post-stress. No other main effect or interaction was significant (all ps>.069).

3.4.3. Amygdala–dlPFC.

For the rsFC of the left amygdala with the left dlPFC, no significant effects emerged (all ps>.081).

3.5. Cortisol and subjective ratings as predictors for rsFC change.

To assess the impact of subjective and physiological stress reactivity on rsFC, we conducted exploratory multiple regression analyses. Therefore, the change in subjective ratings and cortisol levels from pre- to post-stress was calculated for each paradigm separately and used as predictors for the change in rsFC from pre- to post-stress. Sex and order of task presentation were additionally added as predictors. Please see Table 3 for the values of change for cortisol and subjective ratings that were used for analysis.

3.5.1. Predictor: Change in cortisol from pre- to post-stress.

For change in cortisol from pre- to post-stress, we ran separate analyses for females and males, as we see differences in cortisol levels between sexes in the current sample and the interaction of sex with cortisol turned out to be significant in the regression (predictors: change in cortisol from pre- to post-stress and order of task presentation).

*Females, right amygdala–vACC; social exclusion.* For social exclusion, cortisol change was significantly positively associated with change in rsFC (R2=0.204, F(2,36)=4.624, p=.016). A decrease in cortisol during social exclusion was associated with a decrease of rsFC between right amygdala and vACC (β=0.377, t=2.513, p=.017; see Figure 4C). No other significant association emerged (all ps>.066).

Change in cortisol from pre- to post-stress did not predict changes in any other rsFC neither for social exclusion nor for achievement stress in any of the two sexes (all ps >.102).

**Table 3.** Changes in cortisol and subjective ratings due to social exclusion and achievement stress.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Social exclusion** | | | | **Achievement stress** | | | |
|  | **Females (n=40)** | | **Males (n=33)** | | **Females (n=40)** | | **Males (n=33)** | |
|  | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | |
| Change in cortisol [pg/mL] | -115.05 | 437.71 | -373.09 | 1021.32 | 124.15 | 630.81 | -152.91 | 909.76 | |
| Change in positive affect (PANAS) | -0.26 | 0.55 | -0.24 | 0.57 | -0.11 | 0.61 | -0.21 | 0.58 | |
| Change in negative affect (PANAS) | 0.03 | 0.25 | 0.05 | 0.25 | 0.19 | 0.51 | 0.11 | 0.30 | |
| Change in anger (ESR) | 0.18 | 0.64 | 0.24 | 0.61 | 0.80 | 1.11 | 0.76 | 1.06 | |

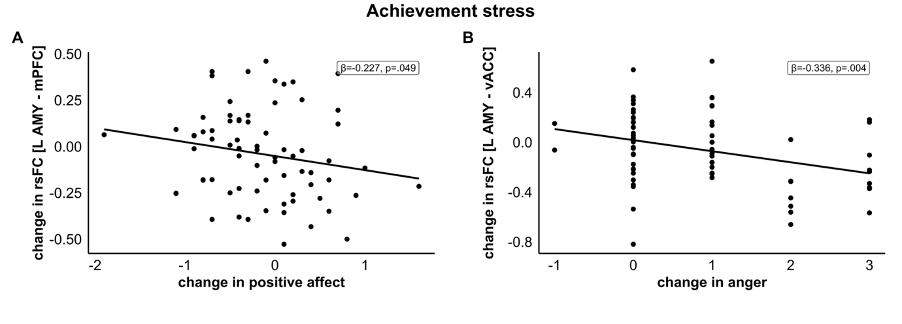
**Note**. Abbreviations: SD = standard deviation; PANAS = Positive and Negative Affect Scale; ESR = Emotional Scale Rating.

3.5.2. Predictor: Change in positive affect from pre- to post-stress.

For change in positive affect from pre- to post-stress, multiple regression analyses were performed with the predictors: change in positive affect, sex and order of task presentation.

*Left amygdala–mPFC, achievement stress.*Changes in positive affect had a significant negative relationship with changes in rsFC (model R2=0.132; F(3,69)=3.497, p=.020) for achievement stress across both sexes. A decrease in positive affect due to achievement stress was associated with an increase in rsFC (β=-0.227, t=-2.003, p=.049, see Figure 5A).

Change in positive affect from pre- to post-stress did not predict changes in any other rsFC neither for social exclusion nor for achievement stress (all ps >.051).



**Figure 5.** (A) Association between change in rsFC of the amygdala and the mPFC and change in positive affect from pre- to post-achievement stress, and (B) association between change in rsFC of the amygdala and the vACC and change in anger from pre- to post-achievement stress. Change values from before to after social exclusion or achievement stress, respectively. Abbreviations: vACC = ventral anterior cingulate cortex, AMY = amygdala, mPFC = medial prefrontal cortex.

3.5.3. Predictor: Change in negative affect from pre- to post-stress.

For change in negative affect from pre- to post-stress, regression analyses were performed with the predictors: change in negative affect, sex and order of task presentation. Change in negative affect from pre- to post-stress did not predict changes in any rsFC neither for social exclusion nor for achievement stress (all ps>.045, cut-off α=.025).

3.5.4. Predictor: Change in anger from pre- to post-stress.

For change in anger from pre- to post-stress, regression analyses were performed with the predictors: change in anger, sex and order of task presentation.

*Left Amygdala–vACC, achievement stress*. The change in anger was negatively associated with changes in rsFC (model R2= 0.139; F(3,69)=3.699, p=.016), with increased anger, a decreased rsFC was seen for achievement stress across both sexes (β=-0.336, t=-3.005, p=.004), see Figure 5B.

Change in anger from pre- to post-stress did not predict changes in any other rsFC neither for social exclusion nor for achievement stress (all ps>.083).

4. Discussion

The aim of the current study was to investigate the effect of sex and stressor type on rsFC of the amygdala with frontal regions. This selection of regions was based on a recent neurocognitive framework on stress coping by de Raedt and Hooley [6]. In the following we will discuss our results.

4.1. Sex differences in stress reaction on amygdala-frontal stress networks

We observed significant sex differences in the amygdala-frontal network, with higher amygdala-vACC and amygdala-mPFC rsFC for social exclusion in females that was not seen in males. Additionally, in females rsFC of the amygdala with the vACC was significantly higher for social exclusion than for achievement stress. Furthermore, in an exploratory multiple regression analysis, females showed an association between change in cortisol values from pre- to post-stress with the rsFC between the amygdala and the vACC. Hence, social exclusion seems to exhibit a sex-specific effect on the amygdala-frontal network. This fits nicely with previous reports that females have been shown to be more susceptible to social affiliation and exclusion [16,47]. For the current results, besides directly experiencing social exclusion, females more than males might also be triggered by anticipatory effects of an upcoming social, interpersonal stress situation as participants were told that they will participate in a ball tossing game. In previous studies, increased amygdala-vACC connectivity has been found in trauma-exposed females at rest and during affective processing [48], during implicit processing of sad facial stimuli in healthy females and males [49], and following a conflict task using emotional stimuli [50]. This supports the assumption from the neurocognitive framework [6] that activation of the ventromedial-frontal region, which is involved in stress [9,10] and attentional emotional processing [11] upregulates amygdala activity, further resulting in a lower ability for stress coping. The vACC is further closely related to stress processing after social exclusion [9], and social decision making. Importantly, the vACC coordinates used in the current study (based on [36]) are more specifically located in a part of the vACC also known as subgenual ACC (sgACC) [51]. In previous studies, sgACC was often associated with mood disorders such as depression, showing a reduced gray matter volume and higher activation of sgACC in depressed compared to healthy participants [52], and with processing of sadness [53,54]. Heightened amygdala-vACC rsFC in social exclusion compared to achievement stress in females is in line with the aforementioned higher susceptibility of females to social exclusion and suggests more affective processing in socially stressful situations than in achievement-related stress in females. In our exploratory multiple regression analyses we additionally saw a positive association between change in cortisol and change in amygdala-vACC rsFC in females for social exclusion, which was reported previously in a mixed sample for psychosocial stress (thus, achievement stress including a social component) [13].

Taken together with previous literature, the current results show that rsFC between the amygdala and the vACC is elevated in stressful situations, when females are exposed to emotionally or socially stressful contexts, such as social exclusion, and they therewith must engage in emotion processing. As females are more susceptible to social affiliation and social exclusion, social stress might be particularly salient for them. Thus, our findings corroborate the assumption that females are engaging more coping mechanisms and are more affected by socially stressful situations than males, and they are also more triggered in socially stressful situations than in achievement situations.

Moreover, in the current results we saw a higher coupling between the amygdala and the mPFC for social exclusion in females compared to males. Increased amygdala–mPFC rsFC was previously observed in a male-only sample after psychosocial stress (achievement stress also including a social component) [10], in reward learning during wins compared to losses in both sexes [12] and has been associated with lower anxiety [55]. Thus, an elevated rsFC between the amygdala and the mPFC is present in situations of positive gain, and in achievement-related stress situations at least in males. Together with the current results, this suggests that females are possibly experiencing social gain from interactions although in stressful situations. Indeed, reward anticipation, as in this case the social interaction for females, could even buffer the subjective and hormonal stress reactivity [56].

In addition to this, the observed sex difference with higher amygdala-mPFC rsFC in females compared to males is partly in contrast to a previous study, describing heightened amygdala-mPFC rsFC in males compared to females but worth mentioning independent of an applied task and rather in the connectivity between the laterobasal amygdala and the rectal gyrus [23]. Furthermore our results also emphasise the importance of setting up study designs specifically investigating sex differences and of including both females and males, as male-only samples suggest heightened amygdala-mPFC rsFC in a facial expression task [14] and during the stress-recovery phase one hour after stress [10]. Taken together with the current results, this shows that neglecting females in research biases the outcome interpretation. Thus, investigating sex and stressor specific differences and interactions is still in its infancies and more studies on sex differences in stress specifically must be performed.

In our current study, we propose that heightened rsFC of the amygdala with the vACC and mPFC before and after social exclusion in females compared to males and compared to achievement stress might indicate that females are feeling rewarded by social engagement and are experiencing social gain from interactions. Additionally, females seem to be more susceptible to social stress, which might be particularly salient for them, and they are therefore more affected by socially stressful situations and need to engage more coping mechanisms within these situations than males.

The assessment of sex differences are particularly important with regards to different prevalence rates of mental disorders, as women are more susceptible to stress-related disorders such as depression and anxiety [20], while men are more prone to suffer from substance misuse [20,21]. These prevalence rates might be affected by different stressor types such as social exclusion or achievement stressors in sex-dependent, distinct ways. Sex-specific reactions to different stressors might partly explain sex-specific prevalence rates in mental disorders [18].

4.2. Sex-independent effects in stress reaction on amygdala-frontal stress networks.

Stressor type seems to be influential not only regarding sex differences, but general differences also appeared in both females and males for social exclusion and achievement stress. Cortisol levels decreased after stress induction in social exclusion, but not following achievement stress. Anger showed a stressor-specific effect with higher anger ratings post-achievement stress not present post-social exclusion. Further, while bearing in mind that our multiple regression analyses were merely exploratory, we found negative associations of positive affect and anger with amygdala-frontal rsFC for achievement stress: decreased positive affect was associated with increased amygdala–mPFC rsFC and decreased anger was associated with increased amygdala–vACC rsFC.

Although we were expecting a cortisol increase after the stressors and did not see it, this lacking cortisol increase has been reported repeatedly in other studies after stress induction in an fMRI environment ([57], for a review see [58]). In itself, the fMRI environment can be stressful and lead to high cortisol levels preceding the scan, which then normalise after the scan and in subsequent scans [59]. Generally, within the circadian cortisol rhythm, levels decrease during the course of the day, referred to as the diurnal cortisol slope [60]. This diurnal decrease was not apparent for achievement stress in the current study, speaking for some (weak) cortisol reactivity. Interestingly, we further did not see any change in amygdala-dlPFC rsFC in social exclusion or in achievement stress, which might be explained by the missing cortisol increase in our participants. Based on the neurocognitive framework by de Raedt and Hooley, increased connectivity between the amygdala and the dlPFC is associated with successful stress coping. Nevertheless, Quaedflieg and colleagues [13] reported reduced amygdala-dlPFC coupling only in cortisol responders compared to non-responders, suggesting worse stress coping in cortisol responders than non-responders. Thus, coupling between the amygdala and the dlPFC might be connected to hormonal changes such as cortisol expression [24]. Nevertheless, even with a missing cortisol increase, subjective parameters, such as elevated anger and negative affect as well as decreased positive affect clearly indicate an increase in stress and negative emotions. Especially anger showed a stressor-specific increase, with higher ratings post-achievement stress compared to post-social exclusion. Together with the exploratory result of decreasing amygdala-vACC rsFC going along with increasing anger, this might suggest that achievement stress negatively, subjectively affects participants. Higher rsFC of the amygdala with the vACC was previously found in situations of achievement stress with an additional social component [10,13], but not with achievement stress alone. Further, we found that decreased positive affect was associated with increased amygdala–mPFC. Taken together with the literature showing amygdala-mPFC rsFC to be present after psychosocial stress (achievement stress with a social component) in a male-only sample [10] as well as in male and female cortisol responders compared to non-responders [13], Quaedflieg and colleagues suggested that this points towards less effective stress coping, which fits to the framework of De Raedt and Hooley, as connectivity between the ventromedial frontal regions with the amygdala suggests less effective stress coping.

Taken together, achievement stress is subjectively perceived as more stressful than social exclusion, corroborated by increased anger, negative affect and (according to the circadian rhythm) a missing decrease in cortisol levels. Higher anger and lower positive affect is connected to a lower amygdala-vACC and higher amygdala-mPFC rsFC, showing an association between subjective parameters and amygdala-frontal coupling.

4.3. Strengths, limitations and future directions

Within the current study we separately investigated two different stressors, namely social exclusion and achievement stress, in a within-subject design within the same individuals. We investigated rsFC before and after each of these stressors, enabling us to investigate changes of coupling between the amygdala and frontal regions due to social exclusion and achievement stress. As most studies on stress reactivity so far investigated male-only samples or did not investigate sex specific analyses in mixed samples, in the current study we not only included a fair amount of females and males but additionally specifically assessed sex differences in social exclusion and achievement stress as well as in rsFC.

Previous studies showed that menstrual cycle and hormonal contraceptive use affects stress reactivity and rsFC [61,62], which should be considered in future studies. For the current study, the sample size was too small to additionally include menstrual cycle effects in the analyses. However, as females had significantly higher values in negative stress coping strategies (see Table 2), we exploratorily performed a regression analysis to investigate whether menstrual cycle phase predicted negative stress coping strategies. The linear regression analysis with menstrual cycle phase as predictor (early follicular/ovulation/midluteal phase) and negative stress coping strategies as the dependent variable yielded no significant association between negative coping strategies and menstrual cycle phase (R2=0.059, F(1,38)=2.380, p=.131). Negative stress coping strategies reported by the females in this study therefore do not seem to be associated with their menstrual cycle phase.

For reasons of homogeneity, the current sample was comprised of young university students. For generalisability of the results, and regarding stress-related mental disorders, future studies should assess more heterogenous samples including different education levels throughout the lifespan.

Further, for the current study we relied on rsFC between the amygdala and frontal regions as suggested in the neurocognitive framework for stress coping by De Raedt and Hooley to assess the impact of different stressors on neural stress-networks. However, as previous studies have shown that analyses of amygdala connectivity on a whole brain or network level can also help to better understand the impact of stress and sex on rsFC [24,63], it would be of additional interest for future studies to create whole brain functional connectivity maps for the amygdala and specifically investigate the impact of different stressors and sex differences on amygdala rsFC.

Finally, we want to acknowledge that we investigated sex differences and did not take gender into account. While sex is usually categorized into female and male, defined primarily on the basis of biological attributes, gender refers to the socially constructed identity of female, male and other gender-diverse people (SAGER guidelines, [64]). It is of importance to include a higher gender diversity in studies to make them more broadly generalisable, as sex as well as gender, and their interaction (i.e., in transgender people), can have an influence on health and well-being in a variety of ways [64].

5. Conclusions

With the current study, we set out to investigate the effect of sex and stressor-type on networks between the amygdala and frontal regions with rsFC, following a neurocognitive framework for stress coping with the assumption that the amygdala takes a crucial part in stress processing and interacts with the frontal cortex in terms of stress coping. We observed significant sex differences in the amygdala-frontal network, seen in a higher rsFC of the amygdala with the vACC as well as with the mPFC for social exclusion in females than in males. This speaks for higher emotion and affect regulation in socially stressful situations in females compared to males, which might be attributed to higher social gain in females compared to males.

Thus, socially stressful situations have a higher impact on neural stress-networks in females than in males and neural stress-networks seem to be modified for diverse stressors in a sex-specific manner. The current results depict neural basic principles of sex differences in differently stressful situations, where females are triggered more intensely by social situations and might see social interaction as more rewarding, but also more effortful in terms of engaging coping resources than males. From a translational perspective, the results represent neural basics of the interaction between sex and stressor on stress reaction and contribute to a better understanding of this interaction which affects sex-specific prevalence rates. This knowledge is important to understand stress-related mental disorders showing differences in prevalence rates for females and males. Understanding and disentangling sex differences in acute stressful situations might help to further discern long-term stress effects and their influence on mental disorders specified for females and males.

**Author Contributions:** Zoé Bürger: Writing – original draft, Formal Analysis, Visualization; Veronika. I. Müller: Resources, Software, Writing – review & editing; Felix Hoffstaedter; Resources, Software, Writing – review & editing; Ute Habel: Conceptualization, Funding acquisition, Project administration, Writing – review & editing; Ruben C. Gur: Conceptualization Funding acquisition, Writing – review & editing; Christian Windischberger: Conceptualization Funding acquisition, Writing – review & editing; Ewald Moser: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing; Birgit Derntl: Writing – review & editing, Supervision, Conceptualization, Funding acquisition, Investigation, Project administration, Formal analysis; Lydia Kogler: Writing – review & editing, Supervision, Data curation, Formal analysis, Validation

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**Appendix A**

**Table A1.** Hormonal levels and subjective ratings pre- and post-stress.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Social exclusion** | | | | **Achievement stress** | | | |
|  | **Females (n=40)** | | **Males (n=33)** | | **Females (n=40)** | | **Males (n=33)** | |
|  | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | |
| Cortisol [pg/mL] |  |  |  |  |  |  |  |  | |
| pre-stress | 1767.90 | 807.39 | 2518.82 | 1351.30 | 1826.48 | 922.10 | 2475.12 | 1249.39 | |
| post-stress | 1672.45 | 806.46 | 2145.73 | 936.72 | 1950.62 | 1010.68 | 2322.97 | 1200.20 | |
| Positive affect [PANAS] |  |  |  |  |  |  |  |  | |
| pre-stress | 2.62 | 0.78 | 2.66 | 0.77 | 2.56 | 0.80 | 2.71 | 0.72 | |
| post-stress | 2.36 | 0.83 | 2.42 | 0.88 | 2.45 | 0.76 | 2.51 | 0.75 | |
| Negative affect [PANAS] |  |  |  |  |  |  |  |  | |
| pre-stress | 1.19 | 0.29 | 1.17 | 0.25 | 1.22 | 0.35 | 1.21 | 0.25 | |
| post-stress | 1.23 | 0.30 | 1.23 | 0.33 | 1.41 | 0.48 | 1.32 | 0.36 | |
| Anger [ESR] |  |  |  |  |  |  |  |  | |
| pre-stress | 1.08 | 0.35 | 1.24 | 0.56 | 1.10 | 0.30 | 1.12 | 0.33 | |
| post-stress | 1.25 | 0.54 | 1.48 | 0.80 | 1.90 | 1.13 | 1.88 | 1.05 | |

**Note**. Abbreviations: SD = standard deviation; PANAS = Positive and Negative Affect Scale; ESR = Emotional Scale Rating.

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