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Are you gonna leave me? Separation anxiety is associated with increased amygdala responsiveness and volume

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The core feature of separation anxiety is excessive distress when faced with actual or perceived separation from people to whom the individual has a strong emotional attachment. So far little is known about the neurobiological underpinnings of separation anxiety. Therefore, we investigated functional (amygdala responsiveness and functional connectivity during threat-related emotion processing) and structural (grey matter volume) imaging markers associated with separation anxiety as measured with the Relationship Scale Questionnaire in a large sample of healthy adults from the Münster Neuroimaging Cohort ($N = 320$). We used a robust emotional face-matching task and acquired high-resolution structural images for morphometric analyses using voxel-based morphometry. The main results were positive associations of separation anxiety scores with amygdala reactivity to emotional faces as well as increased amygdala grey matter volumes. A functional connectivity analysis revealed positive associations between separation anxiety and functional coupling of the amygdala with areas involved in visual processes and attention, including several occipital and somatosensory areas. Taken together, the results suggest a higher emotional involvement in subjects with separation anxiety while watching negative facial expressions, and potentially secondary neuro-structural adaptive processes. These results could help to understand and treat (adult) separation anxiety.

Keywords: adult separation anxiety; fMRI; voxel-based morphometry; amygdala

INTRODUCTION

The core feature of separation anxiety is excessive distress when faced with actual or perceived separation from people to whom the individual has a strong emotional attachment. Moreover, people with separation anxiety strongly worry about being alone and abandoned. In diagnostic classification systems such as the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV-TR, separation anxiety (disorder) was described primarily as a childhood disorder requiring an onset before the age of 18 as criterion. This age of onset requirement has recently been dropped in the DSM V, hence separation anxiety turned to a diagnosable disorder for adults. This is supported by several empirical studies, which argued that adult separation anxiety is more common than suggested by the DSM-IV-TR and might constitute a clinical category on its own (Ollendick *et al.*, 1993; Manicavasagar *et al.*, 1997; Cyranowski *et al.*, 2002; Shear *et al.*, 2006; Bögels *et al.*, 2013). In addition, the clinical importance of separation anxiety as a risk factor for mental disorders has often been underestimated. Separation anxiety has been discussed to create a strong vulnerability for a number of affective and anxiety disorders, and clinicians should be more sensitive to the presence of separation anxiety (Manicavasagar *et al.*, 1998; Lewinsohn *et al.*, 2008; Silove *et al.*, 2010).

So far little is known about the neurobiological underpinnings of adult separation anxiety and to the best of our knowledge, there is no study that investigated adult separation anxiety with neuroimaging techniques. However, one study reported that for healthy adolescents, increased amygdala activity was highly related to separation anxiety and concerns about being separated from parents and family (Killgore and Yurgelun-Todd, 2005).

Behavioral studies indicate that children with separation-anxiety disorder show negative emotional hyper-reactivity, deficits in emotion regulation, were found to interpret ambiguous situations as more threatening and seek more help from others instead of solving problems by themselves than healthy children (Dadds *et al.*, 1996; Bögels and Zigterman, 2000; Carthy *et al.*, 2009). In contrast to other subcategories of anxiety disorders, subjects with separation anxiety disorder in history recorded more severe symptoms of depression, anxiety and stress in adulthood (Silove *et al.*, 2010).

However, the generation of hypotheses regarding functional and/or structural aberrations in adult subjects with high levels of separation anxiety is limited by the lack of pre-existing data. Nevertheless, there is some evidence that separation anxiety shares common features with other domains of anxiety (Bögels *et al.*, 2013). Hence, we suggest that a common element of separation anxiety might be a hyperresponsiveness to negative social signals (faces). Up to now, hyperactivity or reactivity in a limbic circuit with the amygdala as a key structure has been observed during negative emotional processing in patients with social anxiety disorder (Stein *et al.*, 2002; Straube *et al.*, 2005; Phan *et al.*, 2006), specific phobia (Schienle *et al.*, 2005; Straube *et al.*, 2006; Schweckendiek *et al.*, 2011), panic disorder (van den Heuvel *et al.*, 2005; Pfleiderer *et al.*, 2007), and post-traumatic stress disorder (Shin *et al.*, 2005; Francati *et al.*, 2007) as well as in healthy but high-anxious subjects (Vrticka *et al.*, 2008; Pejic *et al.*, 2011; Sehlmeyer *et al.*, 2011; Laeger *et al.*, 2012; Abraham *et al.*, 2013) and healthy subjects with a history of childhood maltreatment (Dannlowski *et al.*, 2013; Dannlowski *et al.*, 2012).

However, to understand the complex function of the amygdala in the context of emotion processing, its functional interplay with other brain areas should be taken into account. Functional connectivity (Friston, 1994) is one possibility to identify networks of brain regions

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showing patterns of co-activation throughout the time course of a task. During emotion processing, the amygdala was reported to show tight functional coupling to several prefrontal, temporal and occipital regions, as well as to hippocampal and thalamic areas, which are suggested as important for several neurocognitive domains, including emotion regulation, associative learning processes, stimulus evaluation, visceral responses and attentional processing (Banks *et al.*, 2007; Robinson *et al.*, 2010; Bzdok *et al.*, 2012), see Davis and Whalen (2001) for a review. Regarding subjects with anxiety disorder, increased functional connectivity of the amygdala to prefrontal and occipital areas have been reported, which was implicated to be associated with dysfunctional emotion regulation and increased vigilance and attentional processes for anxiety-relevant stimuli (McClure *et al.*, 2007; Kim *et al.*, 2011; Strawn *et al.*, 2012).

Regarding structural abnormalities, the literature on other anxiety disorders is less consistent with few studies, however, suggesting reduced amygdala volumes in paediatric patients with anxiety disorder (Milham *et al.*, 2005) and adult patients suffering from panic disorder (Hayano *et al.*, 2009).

In this study, we sought to uncover functional (amygdala responsiveness to emotional faces) and structural (grey matter volume) imaging markers associated with adult separation anxiety in a large sample of healthy subjects, carefully screened for psychiatric conditions. We hypothesized that healthy adults reporting higher degrees of separation anxiety would show amygdala hyper-responsiveness to negative social stimuli including an abnormal functional coupling of amygdala and sensory visual areas. Regarding structural aberrations, we speculated that higher separation anxiety could be associated with decreased amygdala grey matter volumes. We further hypothesized that these associations are independent from general measures of (unspecific) anxiety traits.

METHODS

Participants

The complete data set comprised 320 right-handed healthy volunteers. Data were collected in the context of a large ongoing study (Münster Neuroimaging Cohort) investigating the neurobiology of emotional processes. For all analyses, 14 subjects had to be excluded due to anatomical abnormalities leaving 306 subjects (154 women, mean age: 38.3, s.d. = 11.5 years; 152 men, mean age: 36.9; s.d. = 11.2 years). Participants were recruited by public notices and newspaper announcements. All subjects had no history of psychiatric illness, according to the Structured Clinical Interview for DSM-IV (SCID)-Interview (Wittchen *et al.*, 1997), had no neurological conditions, were free of psychotropic medication, had normal or corrected-to-normal vision, and had adequate knowledge of German and cognitive abilities [verbal IQ >80; multiple-choice vocabulary intelligence test MWT-B (Lehrl, 2005)].

Subjects were screened for imaging safety concerns, and informed, written consent was obtained following the Declaration of Helsinki (World Medical Association, 1991). The experimental procedure was approved by the ethics committee of the Medical Faculty at the University of Münster. Handedness was defined by the Handedness Questionnaire (Raczkowski *et al.*, 1974). For detailed sample characteristics, see Table 1.

Table 1 Sociodemographic questionnaire and behavioral data of study participants

Age	37.6 ± 11.4 (18–59)
Years of education	15.3 ± 2.2 (9–21)
Sex (M/F)	152/154
Verbal intelligence (MWT-B)	116.7 ± 12.3 (92–145)
STAI-T	31.8 ± 6.6 (20–54)
HAMA	0.7 ± 1.3 (0–8)
BDI	1.9 ± 2.5 (0–13)
RSQ separation anxiety	24 ± 6.2 (12–64)
Percentage of correct shapes	97.7 ± 1.1 (82–100)
Percentage of correct faces	98.0 ± 1.1 (69–100)
Mean RT shapes (ms)	866.3 ± 146.0 (451–1497)
Mean RT faces (ms)	1051.2 ± 207.3 (642–2367)

$n = 306$ representing the final sample included in the morphometry analysis; mean ± SE (range). M, male; F, female; STAI-T, State-Trait Anxiety Inventory-Trait version; HAMA, Hamilton Anxiety Rating Scale; BDI, Beck Depression Inventory; RSQ, Relationship Scales Questionnaire; MWT-B, Mehrfachwahl-Wortschatz-Intelligenztest (multiple choice vocabulary test); RT, reaction time.

Questionnaire measures

The Relationship Scales Questionnaire (RSQ; Griffin and Bartholomew, 1994; Steffanowski *et al.*, 2001) was applied to assess separation anxiety. RSQ scores have shown temporal stability in longitudinal studies with adults so that they appear to measure stable traits of personality in adulthood (Scharfe and Bartholomew, 1994; Scharfe and Cole, 2006). Inter-item analysis of the separation anxiety scale showed acceptable internal consistency estimates of reliability in the whole sample (Cronbach's $\alpha = 0.75$). The questionnaire items were rated on a 1 (not at all like me) to 5 (very much like me) scale. Subjects had to indicate the extent to which they believe each of the statements best describes their feelings about close relationships. Ten items measured separation anxiety (e.g. 'I worry about being abandoned', 'I want to merge completely with another person', 'I worry about being alone').

In order to control for effects of unspecific trait anxiety, the State-Trait Anxiety Inventory (STAI-trait version; Spielberger *et al.*, 1970) was administered as self-evaluation questionnaire. Additionally, the Hamilton Rating Scale of Anxiety (HAMA; Hamilton, 1959; Maier *et al.*, 1988) was conducted by a clinical interviewer as an objective anxiety measure. The Beck Depression Inventory (BDI; Beck and Steer, 1987; Hautzinger *et al.*, 1994) was used to assess the presence of depressive symptoms. RSQ scores were positively associated with STAI-trait scores ($r = .29$) and BDI scores ($r = .15$), but not with HAMA scores ($r = .08$).

Stimulus materials and procedure

A robust paradigm for eliciting amygdala responsiveness that has been used in several previous imaging studies was applied as experimental fMRI paradigm (Hariri *et al.*, 2002; Dannlowski *et al.*, 2011, 2012; Domschke *et al.*, 2012). A set of negative (angry and fearful) faces was used. The paradigm consisted of five blocks of a sensorimotor control task alternating with four blocks of a face-processing task. During the face-processing task, participants viewed a trio of faces and selected one of the two faces (bottom) that was identical to the target face (top). Each face-processing block consisted of six images, balanced for target gender. During the sensorimotor control blocks, the participants viewed trios of geometric shapes (circles and ellipses) and selected one of the two shapes (bottom) that was identical to the target shape (top). Each sensorimotor control block consisted of six shape trios. All blocks were preceded by an instruction ('Match faces' or 'Match shapes' in German) that lasted 2 s. In the face-processing blocks, each of the six face trios was presented for 4 s with a variable inter-stimulus interval of 1.5–5.5 s (mean, 3.5 s), for a total block length of 47 s. In the sensorimotor control blocks, each of the six shape trios was presented for 4 s with a fixed inter-stimulus interval of 1.5 s, for a total block length of 35 s. The total task time was 363 s. Participant performance (accuracy and reaction time) was recorded.

fMRI data acquisition and analysis

T2* functional data were acquired using a 3 T scanner (Gyroscan Intera 3T, Philips Medical Systems, Best, NL), using a single-shot

echoplanar sequence, with parameters selected to minimize distortion in the region of central interest, while retaining adequate a signal-to-noise ratio (S/N) and T2* sensitivity. Volumes consisting of 34 slices were acquired (matrix 64×64, resolution 3.6 mm $\times$ 3.6 mm $\times$ 3.6 mm; TR = 2.1 s, TE = 30 ms, FA = 90°). The slices were tilted 25° from the AC/PC line in order to minimize drop out artifacts in the mediotemporal and orbitofrontal region.

The paradigm presentation was projected to the rear end of the scanner (Sharp XG-PC10XE with additional HF shielding). During the experiment, subjects lay supine in the MRI scanner with the response box in their right hand. The head position was stabilized with a vacuum head cushion.

Data were analyzed using statistical parametric mapping software (SPM8, Welcome Department of Cognitive Neurology, London, UK; http://www.fil.ion.ucl.ac.uk/spm). Functional data were pre-processed, including realignment using a set of six rigid-body transformations determined for each image, and normalization of each participant's functional images to the Montreal Neurological Institute International Consortium (MNI) for Brain Mapping template. Images were smoothed with a Gaussian kernel of 8-mm full-width at half-maximum (FWHM).

Twenty-four further subjects had to be excluded from the fMRI analyses due to excessive head movement (exclusion criterion 3 mm/3°) and/or due to technical problems with the functional sequence, leaving 282 subjects for functional data analyses.

The onsets and durations of the experimental conditions (faces and shapes) were modelled using a canonical hemodynamic response function in the context of the general linear model, and the model was corrected for serial correlations. A high-pass filter of 128 s was used to remove low-frequency noise. Movement parameters were entered as nuisance regressors. For each subject, one contrast image, contrasting negative faces with the shapes baseline, was generated in each individual first-level analysis. Then, second-level-group analyses were performed, regressing separation anxiety scores on brain activation to emotional faces.

At first, in order to address our hypotheses regarding amygdala responsiveness, region of interest (ROI) analyses of the bilateral amygdala were performed. The mask for bilateral amygdala was created by means of the WFU PickAtlas (Maldjian *et al.*, 2003) by dilating the defined mask according to the AAL-Atlas (Tzourio-Mazoyer *et al.*, 2002) by 1 mm. In addition, the anatomy toolbox (Eickhoff *et al.*, 2006, 2005) was applied to evaluate the affected amygdala substructures. For exploratory reasons, an additional whole-brain analysis, with a cluster threshold of $k = 20$ voxels, was conducted. For both, amygdala ROI and whole-brain analyses, rigorous family-wise error (FWE) correction were applied on the voxel level, with a corrected statistical threshold of $P < .05$.

Second, we explored whether other variables influenced or confounded the association of neural activation and separation anxiety. Therefore, for each subject, the resulting contrast values of the resulting peak voxel from the second-level analysis were extracted and further analyzed by using SPSS Statistics 21 (IBM, Armonk, New York). We performed a linear multiple regression predicting amygdala responsiveness by separation anxiety scores, age, gender, total education time (in years), verbal intelligence, STAI scores, HAMA scores and BDI scores. To cover for multicollinearity, we additionally performed a linear regression model appertaining a collinearity analysis, regressing the STAI-T scores onto amygdala activations and in a second step regressing the RSQ scores to the residuals of the first model.

Third, we performed a functional connectivity analysis to characterize alterations associated with separation anxiety scores in the functional coupling between the amygdala and other brain areas. The methods for functional connectivity analyses of the amygdala have been described previously (Dannlowski *et al.*, 2009). Briefly, for each subject, the signal time course of the entire right amygdala (defined as 'seed' region) was extracted and then entered into a new first-level model of the same subject predicting brain activity by the amygdala time series. The experimental conditions were entered as nuisance regressors. These resulting contrast images now represent functional connectivity maps of the amygdala, corrected for the experimental conditions (i.e. co-activation by the task). On the basis of these images, we performed a second-level whole-brain regression analysis on amygdala functional connectivity with separation anxiety scores as predictor, again using a cluster threshold of $k = 20$ voxels and a statistical threshold of $P < 0.05$, FWE corrected for the entire brain.

The anatomical labelling was performed by means of the AAL-Toolbox (Tzourio-Mazoyer *et al.*, 2002), and the Brodmann areas were identified with the Talairach Daemon atlas (http://www.talairach.org). The sub-structural amygdala labelling was performed by means of the anatomy toolbox (Eickhoff *et al.*, 2006, 2005).

Voxel-based morphometry acquisition and analysis

T1-weighted high-resolution anatomical images were acquired with a 3D fast gradient echo sequence ('Turbo Field Echo', TFE), TR = 7.4 ms, TE = 3.4 ms, FA = 9°, two signal averages, inversion prepulse every 814.5 ms, acquired over a field of view of 256 (FH) $\times$ 204 (AP) $\times$ 160 (RL) mm, phase encoding in AP and RL direction, reconstructed to cubic voxels of 0.5 mm $\times$ 0.5 mm $\times$ 0.5 mm. As described in our earlier work (Baune *et al.*, 2012a,b), the voxel-based morphometry 8-toolbox (VBM8-toolbox) (http://dbm.neuro.unijena.de/vbm) was used for preprocessing the structural images with default parameters. Images were bias-corrected, tissue-classified, and normalized to MNI-space using linear (12-parameter affine) and non-linear transformations, within a unified model (Ashburner and Friston, 2005) including Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL)-normalization. Grey matter segments were modulated only by the non-linear components in order to preserve actual GM values locally (modulated GM volumes). As mentioned earlier, 14 subjects showing anatomical abnormalities or strong artifacts were identified and excluded. The remaining $n = 306$ images were clear of such problems. The modulated grey matter images were smoothed with a Gaussian kernel of 8 mm FWHW. Group statistics were calculated using exactly the same analysis strategy as described earlier for functional data by using SPM8, including a ROI analysis of the bilateral amygdala and a whole-brain approach. Again, to test for multiple comparisons, a rigorous FWE correction of $P < .05$ was applied. The resulting contrast values of the peak voxel of significant clusters from these second-level analyses were extracted for each subject for further analyses regressing out potential confounders, as already described earlier for the functional data.

RESULTS

Behavioral performance in the fMRI experiment

The mean accuracy rate in the shape condition was 97.7% (s.d. = 1.1%). The mean accuracy rate for the face condition was 98.0% (s.d. = 1.1%). The average reaction times for whole group were 866.3 ms (s.d. = 146 ms) for shapes and 1051.2 ms (s.d. = 207 ms) for faces. According to paired *t*-tests, correct responses between both conditions were not significant ($P = 0.09$), whereas there has been significant shorter reaction times to shapes than to faces ($P < 0.001$). Separation anxiety was not significantly correlated with reaction time or response hit rate controlling for age ($P > 0.19$).

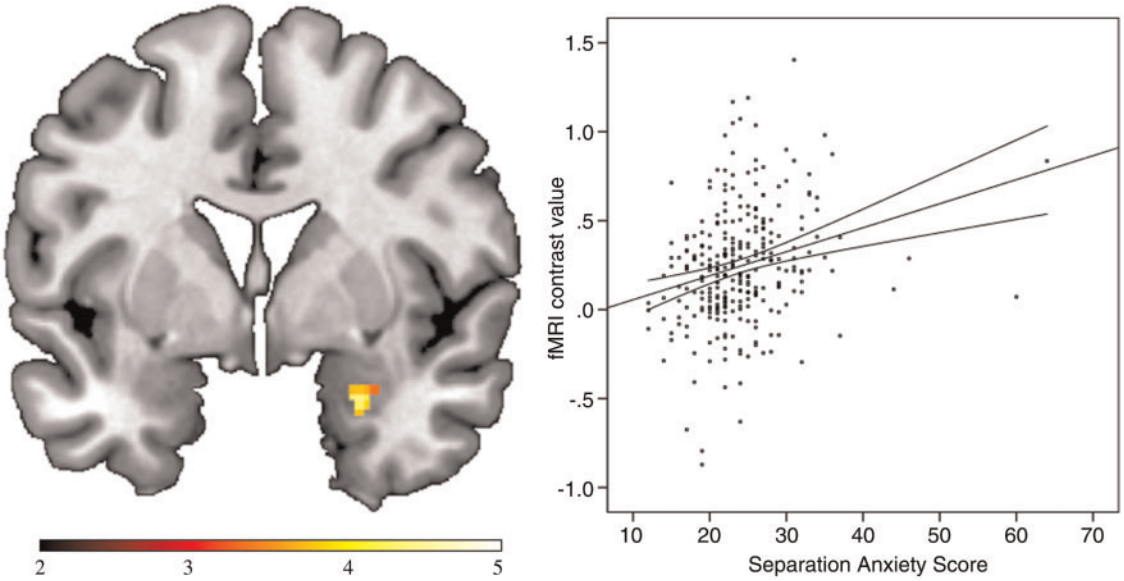

Fig. 1 Separation Anxiety is positively associated with right-amygdala responsiveness to negative faces. Left: Coronar view ($y = 2$) depicting association of separation anxiety scores and amygdale responsiveness to negative faces. Colour-bar, *t*-value. Right: Scatter plot depicting the positive correlation of the peak voxel contrast values (left panel) and the separation anxiety scores ($r = 0.26$, $P < 0.001$). Contour lines, mean confidence interval. fMRI, functional magnetic resonance imaging.

fMRI activation and connectivity analysis

The regression analysis indicated a strong positive association of right amygdala responsiveness and separation anxiety scores ($x = 26$, $y = 2$, $z = -24$; $t(280) = 4.44$, $P_{\text{FWE-corrected}} = 0.001$; $r = 0.26$, cluster size $k = 33$). When using the anatomy toolbox, a virtually identical cluster ($x = 24$, $y = 0$, $z = -24$; $t(280) = 4.40$, $P_{\text{FWE-corrected}} = 0.001$, $k = 28$) within the basolateral parts of the amygdala was found (Figure 1). Excluding outliers (subjects with values more than 4 s.d. above the mean) did not affect the significance of these results ($r = 0.27$; $P < 0.001$). The subsequent multiple regression analysis predicting the mean activation of this significant cluster by separation anxiety score, age, gender, total education time, verbal intelligence, STAI score, HAMA score and BDI score, confirmed the strong association between separation anxiety and amygdala responsiveness, which remained nearly unchanged ($\beta = 0.23$, $t(274) = 3.69$, $P < 0.001$). Also a semi-partial correlation analysis between RSQ scores and amygdala function controlling for the STAI yielded significant semi-partial correlations of $r_p = 0.24$ ($P < 0.001$). The collinearity analysis yielded highly tolerable values [tolerance > 0.68; variance inflation factor (VIF) < 1.54], which mean that multicollinearity did not inflate the variances of the parameter estimates. Thus, the association of separation anxiety and amygdala responsiveness to emotional faces was not significantly influenced by general measures of anxiety, depression level or sociodemographic factors, and separation anxiety decisively contributed to the explanation of variance of amygdala responses beyond the effects of other variables like trait anxiety, A non-parametric correlation (Spearman's rho) between right amygdala responsiveness and separation anxiety scores yielded similar values ($\rho = 0.28$; $P < 0.001$).

The whole-brain analysis indicated that no other brain area showed any significant association with separation anxiety scores in this task at this rigorous threshold.

The functional connectivity analysis revealed a significant positive correlation between separation anxiety scores and functional connectivity between the right amygdala and several occipital areas including the lingual gyrus, the middle occipital gyrus, the cuneus extending to the superior occipital gyrus, the postcentral area, the supplementory motor area and the precuneus. No negative correlations between separation anxiety scores and functional coupling of the amygdala with other brain areas were observed. For details, see Table 2.

Voxel-based morphometry

The analysis of the bilateral amygdala revealed a significant positive association of amygdala grey matter volume and separation anxiety scores in the right amygdala ($x = 33$, $y = 2$, $z = 14$; $t(304) = 3.76$, $P_{\text{FWE-corrected}} = 0.008$; $r = 0.21$, $k = 29$) as well as a small cluster in the left amygdala ($x = -24$, $y = -2$, $z = 12$; $t(304) = 3.24$, $P_{\text{FWE-corrected}} = 0.042$; $r = 0.18$, $k = 4$) within the superficial parts of the amygdala labelled by using the anatomy toolbox. Excluding subjects with values >4 s.d. above mean does not decisively affect the significance of the structural results ($r = 0.19$; $P < 0.001$). The subsequent multiple regression analysis predicting the mean grey matter volume of the significant cluster in the right amygdala by separation anxiety score, age, gender, total education time, verbal intelligence, STAI score, HAMA score and BDI score, the association between separation anxiety and amygdala volume remained significant, albeit slightly weaker ($\beta = .014$, $t(298) = 2.44$, $P = 0.015$). The semi-partial analysis between RSQ scores and amygdala volume controlling for STAI scores also yielded significant semi-partial correlations ($r_p = 0.2$; $P = 0.001$) as well as the non-parametric correlation between RSQ scores and amygdala volume ($\rho = .19$; $P = .017$). The collinearity analysis yielded tolerable values (tolerance > 0.63; VIF < 1.57). Again, our whole-brain analysis yielded no other brain area revealing an association of separation anxiety scores and grey matter structure outside the amygdala using our corrected statistical threshold. Bivariate correlation analyses between functional and structural data yielded no significant correlations (all $P > 0.367$), controlling for age.

Table 2 Results of whole-brain functional connectivity regression analysis of separation anxiety scores on right amygdala functional connectivity at $P_{FWE\text{-}corrected} < 0.05$, $k = 20$ voxels

Anatomical region	BA	Cluster size (k)	*P*-value (FWE-corrected)	*x*	*y*	*z*	Side	*t*-value
Lingual gyrus	30, 18	50	<0.001	18	−70	4	R	6.30
Calcarine gyrus			0.001	10	−60	4	R	5.75
Postcentral gyrus	2, 3, 40, 1	53	<0.001	48	−36	62	R	6.26
Supplementary motor area	6	24	<0.001	4	18	66	R	6.11
Middle occipital gyrus	19	22	<0.001	−32	−84	34	L	5.92
Postcentral gyrus	3	23	0.001	54	−16	54	R	5.82
			0.002	44	−20	60	R	5.55
Cuneus, superior occipital gyrus	7, 31, 19	41	0.002	18	−78	30	R	5.60
Precuneus	7	22	0.003	−6	−48	52	L	5.50

BA, Brodmann area; R, right, L, left.

DISCUSSION

To our knowledge, this is the first study investigating neural correlates of separation anxiety in adult subjects with neuroimaging methods. In line with our hypothesis, the main result of this study was a stronger reactivity to negative faces in subjects with higher separation anxiety. Additionally, the functional connectivity analysis revealed a positive association between separation anxiety and the functional coupling of the amygdala to occipital, somatosensory and supplementary motor areas.

Amygdala hyperactivity to threatening faces has been observed in subjects with higher trait anxiety (Stein *et al.*, 2007), subclinical anxious subjects (Blackmon *et al.*, 2011; Laeger *et al.*, 2012), as well as in patients with anxiety disorders (Etkin and Wager, 2007; Klumpp *et al.*, 2010). Corresponding to these findings, we found a strong reactivity to negative faces also in subjects with higher scores of separation anxiety. This apparent similarity may emerge due to the fact that separation anxiety shares common neurobiological features with other types of anxiety.

In social life, facial expressions are important cues for the evaluation of social contexts, and it is assumed that facial expressions of joy, sadness or threat act as a discriminative stimulus that an aversive or appetitive reaction may follow (Adolphs, 1999, 2001; Hooker *et al.*, 2006). Presumed that for high separation-anxious people it is more important to assess, explain and predict other peoples' intentions because of an exaggerated anxiety to get abandoned or forsaken, the hyper-reactivity of the amygdala could be associated with an automatically increased rapid emotional reaction due to higher individual relevance of facial, in particular to negative facial expressions that may hint a cue for the threat of leaving.

Our functional connectivity results, including the association between separation anxiety and functional coupling of the amygdala to occipital and somatosensory areas, seem to support the notion of an increased interplay between the amygdala and areas modulating attention and emotional salience, and in turn an increased attention load for social cues in subjects with separation anxiety. These areas have in common, that they are related to vigilance and emotional attention processing, especially when they are self-related (Straube and Miltner, 2011; Vuilleumier, 2005). The limbic and visual systems are extensively interconnected, and recent research has begun to reveal the neural processes by which attention and visual processes can be modulated by stimuli with a high individual affective significance. For this modulation, the amygdala seems to have a crucial hub function and projects readily top-down signals on sensory (including extrastriate and striate visual areas) pathways, which in turn influence the representation and emotional value, of (in particular threat-related) emotional events (Pessoa and Adolphs, 2010; Tamietto and de Gelder, 2010; Vuilleumier, 2005). This top-down modulation is assumed to generate saliency signals that modulate perceptual and motor processes to regulate adaptive behavior appropriately (Said *et al.*, 2011; Pourtois *et al.*, 2013). These networks have been recently confirmed, demonstrating that the functional connectivity between amygdala and sensory perceptual areas is modulated by vigilance for threatening facial features (Miyahara *et al.*, 2013).

In contrast to our hypothesis, our morphometric analysis yielded a positive association between separation anxiety and amygdala volume that also seems to contrast previous reports regarding decreased amygdala volume in anxiety disorders (Milham *et al.*, 2005; Hayano *et al.*, 2009). Hence, complementary to the discussed functional findings, it is imaginable that the increased amygdala volume may not necessarily reflect a deficit in subjects with separation anxiety but potentially a compensatory structural process in our—psychiatrically healthy—study sample. A recently published study reported a positive association of amygdala volume with the size and complexity of social networks in adult humans (Bickart *et al.*, 2011). This link between social network size and amygdala volume is further supported by neuroanatomical studies in non-human primates (Lewis and Barton, 2006). Therefore, the increased amygdala volume could result from a relative greater social network size of people with high separation anxiety given their distress resulting from being alone. However, these assumptions remain speculative and need further research.

Taken together, the positive association of separation anxiety and amygdala volume, hyper-reactivity and functional coupling between amygdala and occipital and somatosensory areas to emotional faces might be driven by a higher involvement, more detailed processing and correspondingly, an increased attentiveness particularly to social stimuli such as emotional faces. However, this interpretation remains speculative given that our paradigm was not suited for differentiating between social and non-social stimuli or different emotion types, and thus should be taken with care. Future studies should investigate samples with pathological forms of adult separation anxiety and compare these findings with other forms of anxiety disorders in order to provide neurobiologically informed arguments for or against the postulation of a distinct illness category in adults.

LIMITATIONS

Due to the fact that this is the first study that investigated separation anxiety with neuroimaging methods, conclusions and interpretations must be treated with caution and some methodological limitations should be acknowledged. First, we applied a frequently used face-matching paradigm for eliciting amygdala responsiveness that only displayed negative facial expressions. The study design did not allow separating effects of the response to faces in general and the response to

(specific) threat-related facial expressions. It would be insightful whether the reported associations are specific to negative faces, or if they also apply to emotional stimuli in general. Future studies should address this limitation using different stimuli contrasting social threat with non-social threat. Further, the task demanded implicit emotion processing using faces of strangers rather than explicit emotion processing including facial stimuli from persons with close personal relationships to the participants. Potentially stronger results might be expectable for faces of people who play significant roles in the participants' lives. In addition, we did not administer a measure of subclinical social anxiety besides the SCID ruling out a clinical diagnosis. Hence, potentially confounding effects by subclinical social anxiety should be addressed in future studies.

Second, the reported correlations were generally rather low possibly due to the fact that a healthy sample was used. If the assumption of a continuum of separation anxiety is right, even stronger results should also emerge in pathological forms of separation anxiety, e.g. in children with separation anxiety disorder or psychiatric patients suffering from disorders such as some personality disorders in which separation anxiety constitutes a diagnostic criterion. However, we used a questionnaire not providing cutoff scores for pathological forms of separation anxiety, probably due to the fact that adult separation anxiety disorder is currently not included in clinical classification systems. Therefore, it remains to be clarified whether our findings also apply for subjects suffering pathological forms of adult separation anxiety.

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