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ORIGINAL ARTICLE

Distinct phasic and sustained brain responses and connectivity of amygdala and bed nucleus of the stria terminalis during threat anticipation in panic disorder

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Background. Panic disorder (PD) patients are constantly concerned about future panic attacks and exhibit general hypersensitivity to unpredictable threat. We aimed to reveal phasic and sustained brain responses and functional connectivity of the amygdala and the bed nucleus of the stria terminalis (BNST) during threat anticipation in PD.

Methods. Using functional magnetic resonance imaging (fMRI), we investigated 17 PD patients and 19 healthy controls (HC) during anticipation of temporally unpredictable aversive and neutral sounds. We used a phasic and sustained analysis model to disentangle temporally dissociable brain activations.

Results. PD patients compared with HC showed phasic amygdala and sustained BNST responses during anticipation of aversive *v.* neutral stimuli. Furthermore, increased phasic activation was observed in anterior cingulate cortex (ACC), insula and prefrontal cortex (PFC). Insula and PFC also showed sustained activation. Functional connectivity analyses revealed partly distinct phasic and sustained networks.

Conclusions. We demonstrate a role for the BNST during unpredictable threat anticipation in PD and provide first evidence for dissociation between phasic amygdala and sustained BNST activation and their functional connectivity. In line with a hypersensitivity to uncertainty in PD, our results suggest time-dependent involvement of brain regions related to fear and anxiety.

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Key words: anterior cingulate cortex (ACC), anxiety, fMRI, insula, prefrontal cortex (PFC).

Introduction

Panic disorder (PD) is characterized by recurring, unexpected panic attacks involving extreme fear and strong physiological reactions like dizziness, sweating and chest pain (American Psychiatric Association, 2013). Besides actual panic attacks, PD is accompanied by a constant concern about future attacks (American Psychiatric Association, 2013). This anticipatory anxiety is based on the uncontrollability and aversiveness of panic-related events (Helbig-Lang *et al.* 2012) and commonly leads to avoidance behavior (Kessler *et al.* 2006; White *et al.* 2006). Indeed, the experience of anticipatory anxiety seems to be more strongly related to avoidance behavior than to the actual occurrence or frequency of panic attacks (Cox *et al.* 1991; Basoglu *et al.* 1994). Accordingly, Grillon *et al.* (2008) showed that the anxiety experienced when anticipating unpredictable aversive stimuli characterizes PD patients compared to healthy controls (HC).

A widely acknowledged neuroanatomical hypothesis attributes PD symptomatology to a low-threshold fear network (Gorman *et al.* 1989; Gorman *et al.* 2000) and is supported by neuroimaging studies pointing towards abnormalities in amygdala, hippocampus, frontal cortex, insula, thalamus and hypothalamus (for review see Dresler *et al.* 2013; Grambal *et al.* 2015). The few studies that investigated the neural basis of anticipatory anxiety in PD patients present inconsistent findings with both hyperactivation (Wittmann *et al.* 2011) and hypoactivation (Boshuisen *et al.* 2002; Tuescher *et al.* 2011) of amygdala, hyperactivation (Wittmann *et al.* 2011; Gorka *et al.* 2014; Wittmann *et al.* 2014) and hypoactivation (Boshuisen *et al.* 2002) of insula, and hyperactivation (Boshuisen

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et al. 2002) and hypoactivation (Tuescher *et al.* 2011) of anterior cingulate cortex (ACC), among other regions. Besides other methodological differences, these studies exhibit great variation regarding the duration of threat anticipation. While some studies used sustained anticipatory contexts (Boshuisen *et al.* 2002; Tuescher *et al.* 2011; Gorka *et al.* 2014), others studies used short anticipatory cues (Wittmann *et al.* 2011; Wittmann *et al.* 2014), likely explaining inconsistent findings among these studies. PD patients seem to show both phasic and sustained altered brain responses during anticipation of aversive stimuli, although the precise role and temporal characteristics of different brain regions are unclear.

In the context of anticipatory anxiety, the investigation of temporal characteristics of involved brain regions is important. Distinct regions have been shown to be involved in phasic and sustained responding during threat contexts (Grupe & Nitschke, 2013). While the amygdala is associated with short-term responses, the bed nucleus of the stria terminalis (BNST) shows sustained responding to anxiety states during unpredictable threat (Davis *et al.* 2010; Avery *et al.* 2016). The BNST, as part of the so-called extended amygdala, is a small but heterogeneous region with respect to nuclei and receptors (Lebow & Chen, 2016). It seems to play a central role in salient information processing and sustained threat monitoring (Mobbs *et al.* 2010; Somerville *et al.* 2010; Avery *et al.* 2016; Lebow & Chen, 2016). BNST functioning has been suggested to be highly relevant for pathological anxiety states (Lebow & Chen, 2016) and anxiolytic treatment (Hammack *et al.* 2009; Davis *et al.* 2010; Hazra *et al.* 2012). With regard to PD patients, BNST functioning has been associated with sustained anxiety (Dresler *et al.* 2013) and hypersensitivity to uncertainty (Grillon *et al.* 2008). Despite the fact that these are two cardinal features of the disorder (Helbig-Lang *et al.* 2012), studies on BNST responsivity in PD are completely lacking.

A dissociation of phasic amygdala and sustained BNST responding during threat anticipation has been reported by studies in healthy subjects (Alvarez *et al.* 2011; Grupe *et al.* 2013; Somerville *et al.* 2013; Herrmann *et al.* 2016) and specific phobia patients (Münsterkötter *et al.* 2015). The role of phasic and sustained brain responses during threat anticipation in PD patients is yet unknown and has not been investigated within one and the same experiment. Besides dissociable threat responses, amygdala and BNST are suggested to be embedded in distinct functional networks during anticipatory anxiety (McMenamin *et al.* 2014; Herrmann *et al.* 2016). While a few studies investigated amygdala connectivity in PD patients (Demenescu *et al.* 2013; Kircher *et al.* 2013; Lueken *et al.* 2013), there are no studies on connectivity patterns in PD patients during anticipatory anxiety, especially with regard to BNST.

The aim of the current fMRI study was to investigate phasic and sustained neural responses in PD patients during anticipation of temporally unpredictable aversive stimuli (human screams). Based on its potential relevance for PD pathophysiology, we were especially interested in BNST functioning and in delineating the time courses of activation in amygdala and BNST, due to their distinct roles during threat anticipation. We used two different analysis models to disentangle phasic and sustained brain responses and hypothesized that increased phasic amygdala activation and sustained BNST activation would be evident in PD patients relative to HC. We also expected anticipation of aversive *v.* neutral sounds to induce alterations in a typical emotion-processing network consisting of insula, ACC, and frontal cortex. Finally, we conducted psychophysiological interaction (PPI) analysis to elucidate functional connectivity patterns of amygdala and BNST.

Methods and materials

Subjects

In total 19 PD patients and 19 HC were recruited for the study through public advertisement and in collaboration with an outpatient clinic. Two patients had to be excluded, one due to technical problems during fMRI scanning and one because the experiment was aborted after the practice trials. The final sample consisted of 17 PD patients and 19 HC, matched for gender, age, and education (for characterization, see online Supplementary Table S1). Exclusion criteria were neurological disorders, traumatic brain injury, psychotic or bipolar disorder, and drug abuse or dependence within the past 10 years. PD patients with and without agoraphobia were included and were diagnosed with a structured clinical interview according to DSM-IV criteria (SKID; Wittchen *et al.* 1997). PD patients scored significantly higher than HC on the Panic and Agoraphobia Scale (PAS; Bandelow, 1997), Agoraphobic Cognitions Questionnaire (ACQ; Ehlers *et al.* 1993), Body Sensations Questionnaire (BSQ; Ehlers *et al.* 1993), Mobility Inventory for Agoraphobia (MI; Ehlers *et al.* 1993), Anxiety Sensitivity Index (ASI; Reiss *et al.* 1986), and the Beck Depression Inventory (BDI; Beck *et al.* 1996). Comorbidities of PD patients included depressive disorder ($n = 2$), eating disorder ($n = 1$), dysthymic disorder ($n = 1$), obsessive compulsive disorder ($n = 1$), generalized anxiety disorder ($n = 2$), social anxiety disorder ($n = 1$), somatization disorder ($n = 2$), and specific phobia ($n = 1$). Three PD patients took antidepressant medication

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and were stabilized on such medication, and three patients received therapy at the time of study participation. The study conforms to the Declaration of Helsinki and was approved by the local ethics committee. All participants gave written informed consent prior to the experiment.

Experimental design

During scanning, participants saw one of two cues (hash or percent sign) that announced the presentation of either an aversive or neutral sound. Cues were presented from trial onset until the end of sound presentation. Sounds were chosen from the International Affective Digitized Sounds database (IADS-2; Bradley & Lang, 1999) and consisted of human screams (#275, 276, 277) as aversive stimuli and water sounds (#172, 726, 377) as neutral stimuli. Such aversive stimuli are known to represent threat (Herrmann *et al.* 2016). The duration of the sound clips was shortened to a representative sequence of 4 s and the sound intensity was set to a constant level. For the purpose of familiarization, each of the six sounds was presented once before the start of the actual experiment. Additionally, participants were instructed about the assignment of the two cues to the aversive and neutral condition (counterbalanced across participants).

In total, the experiment consisted of 11 aversive and 11 neutral trials, which were presented in pseudorandomized order. Anticipation intervals were variable in length to keep the presentation of the sounds temporally unpredictable. The majority of anticipation intervals lasted 16 s (7 per condition) to allow for investigation of sustained brain responses, while there were four shorter intervals (2 × 3 s, 1 × 5 s, 1 × 10 s per condition). A similar study by Herrmann *et al.* (2016) used anticipation intervals of up to 35 s, reporting behavioral and neural correlates of anxiety in healthy volunteers. As demonstrated by Berns *et al.* (2006) anticipation becomes more aversive with increasing duration of the anticipation interval. To differentiate between HC and PD patients, known to be particularly sensitive to sustained uncertainty, our study thus used a threshold design with shorter anticipation intervals. Participants were told that the sounds could be presented at any time after cue appearance. After each trial, a white fixation cross was shown for 15 s before the subsequent trial started. In total the experiment lasted 11 min and 40 s.

After scanning, the participants had to rate the two cues as well as the six sounds with regard to valence (1 = very unpleasant, 9 = very pleasant, with 5 = neutral), anxiety (1 = not anxiety-inducing, 9 = highly anxiety-inducing) and arousal (1 = not arousing, 9 = highly arousing) on a nine-point Likert scale (Self Assessment Manikin; Bradley & Lang, 1994). Rating data were analyzed with mixed-model analyses of variance (ANOVAs) using IBM SPSS software (Version 22; IBM, Armonk, New York, USA), with group (PD *v.* HC) as between-subject factor and condition (aversive *v.* neutral sounds) as within-subject factor. *Post-hoc t* tests were used to resolve interactions where necessary. A p value of <0.05 was considered statistically significant.

FMRI

FMRI data were collected with a 3 T magnetic resonance scanner ("Magnetom PRISMA"; Siemens Medical Systems, Erlangen, Germany). Scanning began with a high resolution T1-weighted anatomical scan with 192 slices. Subsequently, functional data were acquired with a T2*-weighted echo-planar sequence (TE = 30 ms, flip angle = 90°, matrix = 92 × 92, FOV = 208 mm^2, TR = 2080 ms) consisting of 340 volumes with 36 axial slices (thickness = 3 mm, gap = 0.3 mm, in plane resolution = 2.26 × 2.26 mm).

Functional data were preprocessed and analyzed with BrainVoyager QX (Version 2.8; Brain Innovation, Maastricht, the Netherlands). To ensure adequate saturation, the first four volumes were discarded from each run. During preprocessing, data were corrected for slice time errors and movement artifacts. Anatomical and functional data were co-registered and normalized to fit Talairach space (Talairach & Tournoux, 1988). Finally, data were smoothed spatially [6 mm full-width at half maximum (FWHM) Gaussian kernel] and temporally (high pass filter: 10 cycles per run; low pass filter: 2.8 s; linear trend removal).

Statistical analysis consisted of multiple linear regression of the signal time course at each voxel. The expected blood oxygenation level dependent (BOLD) signal change for each condition was modeled with a canonical double-gamma hemodynamic response function (HRF). We calculated two separate general linear models (GLMs) for the anticipation interval. In the first GLM, the HRF was modeled over the whole anticipation interval (sustained response GLM). The second GLM modeled a phasic HRF as the HRF initiated by the onset (first second) of the anticipation interval (phasic response GLM). In both GLMs, sound presentation and six movement parameters were modeled as predictors of no interest. Afterwards, *z*-standardized predictor estimates based on voxel-wise statistical maps for each participant were calculated. Random effects analysis with adjustment for autocorrelation following a global AR(1) model across the individual predictor estimates for planned contrasts was performed.

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All analyses were small-volume-corrected for a-priori defined regions of interest (ROIs). ROIs for amygdala, insula, ACC, and PFC (lateral, medial) were defined on the basis of the Automated Anatomical Labeling (AAL) atlas included in the Wake Forest University (WFU) PickAtlas software (Tzourio-Mazoyer *et al.* 2002; Maldjian *et al.* 2003). MNI-coordinates were converted into Talairach space with ICBM2tal (Lancaster *et al.* 2007). The BNST ROI was defined based on an anatomical atlas (Mai *et al.* 1997; also see Herrmann *et al.* 2016) and was dilated by 1 mm to avoid missing relevant activation.

Because of their relevance for anticipatory anxiety, PPI analyses were conducted for significantly activated clusters in the amygdala and BNST as seed regions. Based on the contrast anticipation of aversive *v.* neutral sounds (psychological predictor) and the signal time course extracted from these seed regions (physiological predictor), we calculated a PPI GLM, which contained the signal time course of the seed region as well as the PPI predictor. Extraction of time courses and convolution with HRF were done with Neuroelf's (http://www.neuroelf.net) ComputeGLM method.

The cluster-level statistical threshold estimator plugin for BrainVoyager (Goebel *et al.* 2006) was used to correct for multiple comparisons. We used an initial voxel-level threshold of $p < 0.005$ to balance between Type I and II error rates (Lieberman & Cunningham, 2009) given the normally available patient sample sizes. Based on another study using the same anticipation paradigm in a different patient group (Brinkmann *et al.* 2017), we expected effect sizes of around $d = 1.2$ in the current study. With an initial threshold of $p < 0.001$, the estimated voxel-level Power to detect even such strong effects is close to chance level (0.60) in our study, while an initial threshold of $p < 0.005$ (also see Lieberman & Cunningham, 2009) raises the voxel-level Power to an appropriate level (0.80). Furthermore, we did not include whole-brain analysis to avoid possible inflation of false-positive clusters in parametric analyses due to inhomogeneity of spatial smoothness and spatial autocorrelations across the whole brain with resulting hot spots of false positive clusters in specific regions such as posterior cingulate cortex (Eklund *et al.* 2016). Our ROI approach did not include these critical regions and we restricted our analyses to a homogeneous search space, especially with regard to the BNST, guided by a hypothesis-driven approach. A mask based on predefined ROIs for amygdala, insula, ACC and PFC, and a separate mask consisting of bilateral BNST, were applied to the thresholded maps with an estimated FWHM for spatial smoothness (Forman *et al.* 1995) and an iterative procedure (Monte Carlo simulation) with 1,000 iterations. The minimum cluster size threshold with a cluster-level false positive rate of $p < 0.025$ (additionally corrected for number of masks) was applied to the statistical maps.

To investigate the influence of behavioral measures on differential brain responses and connectivity, ratings regarding valence, arousal and anxiety for the cues as well as symptom severity as measured by PAS scores (Bandelow, 1997), level of anxiety as measured by ASI scores (Reiss *et al.* 1986) and level of depression as measured by BDI scores (Beck *et al.* 1996) of PD patients were correlated with mean beta values for differential activation and connectivity clusters (anticipation of aversive – neutral sounds) resulting from ROI and PPI analyses (Bonferroni-corrected significance level; phasic model: $p \leqslant 0.0019$; sustained model: $p \leqslant 0.0045$).

Furthermore, we were interested in the development of brain responses over the course of the experiment within amygdala and BNST. Therefore, we differentiated between the first and second half of the experiment and calculated ANOVAs with differences between anticipation of aversive and neutral sounds for the first and second half as within-group factor and group (PD *v.* HC) as between-group factor. This analysis was conducted for differential brain responses within amygdala and BNST.

Results

Behavioral data

The ANOVAs on the ratings of valence, arousal and anxiety for the cues announcing aversive and neutral sounds (Fig. 1) yielded significant main effects for condition (valence: $F_{1,34} = 6.19$, $p = 0.018$; arousal: $F_{1,34} = 9.17$, $p = 0.005$; anxiety: $F_{1,34} = 7.09$, $p = 0.012$) and group (arousal: $F_{1,34} = 11.03$, $p = 0.002$; anxiety: $F_{1,34} = 6.30$, $p = 0.017$) as well as significant group × condition interaction effects (valence: $F_{1,34} = 9.36$, $p = 0.004$; arousal: $F_{1,34} = 5.77$, $p = 0.022$). *Post-hoc t* tests showed that PD patients rated the aversive cue as significantly more negative ($t_{34} = -3.02$, $p = 0.005$) and more arousing ($t_{34} = 3.78$, $p = 0.001$) than HC, while there were no significant group differences for the neutral cue. PD patients also rated the aversive cue as significantly more negative ($t_{16} = -3.49$, $p = 0.003$) and more arousing ($t_{16} = 3.20$, $p = 0.006$) than the neutral cue, while HC showed no differences.

The ANOVA for the aversive and neutral sounds (Fig. 1) resulted in significant main effects for condition (valence: $F_{1,34} = 142.33$, $p < 0.001$; arousal: $F_{1,34} = 139.33$, $p < 0.001$; anxiety: $F_{1,34} = 87.60$, $p < 0.001$) and group (valence: $F_{1,34} = 6.98$, $p = 0.012$; arousal: $F_{1,34} = 14.02$, $p = 0.001$; anxiety: $F_{1,34} = 10.45$, $p = 0.003$) as well as

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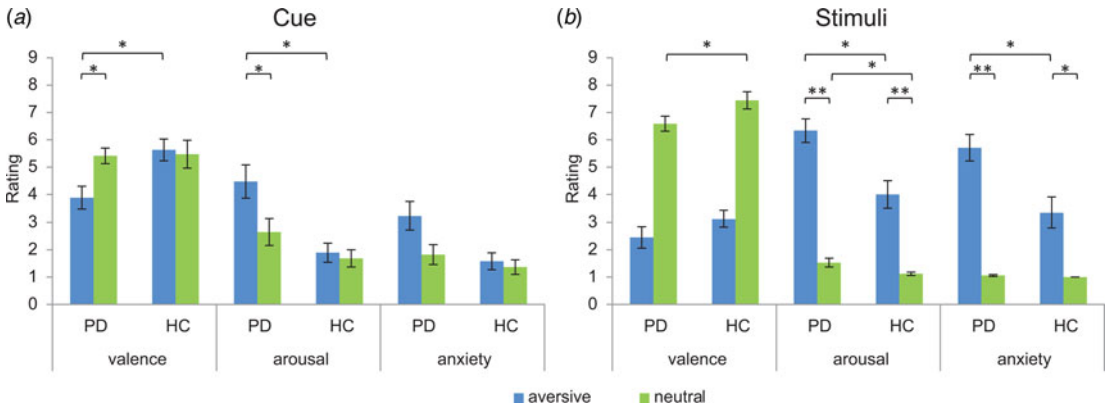

Fig. 1. Ratings for valence (1 = very unpleasant, 9 = very pleasant, with 5 = neutral), arousal (1 = not arousing, 9 = highly arousing), and anxiety (1 = not anxiety-inducing, 9 = highly anxiety-inducing) of panic disorder (PD) patients and healthy controls (HC) for the cue announcing aversive and neutral sounds (a) and for aversive and neutral sounds (b). $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.001$.

significant group × condition interaction effects (arousal: $F_{1,34} = 8.76$, $p = 0.006$; anxiety: $F_{1,34} = 9.43$, $p = 0.004$). *Post-hoc* analysis revealed that PD patients rated the aversive sounds as significantly more arousing ($t_{34} = 3.47$, $p = 0.001$) and more anxiety-inducing ($t_{34} = 3.15$, $p = 0.003$), and the neutral sounds as more arousing ($t_{34} = 2.47$, $p = 0.019$) than HC. Furthermore, PD patients and HC rated the aversive sounds as significantly more arousing (PD: $t_{16} = 11.13$, $p < 0.001$; HC: $t_{18} = 6.01$, $p < 0.001$) and more anxiety-inducing (PD: $t_{16} = 9.56$, $p < 0.001$; HC: $t_{18} = 4.22$, $p = 0.001$) than the neutral sounds.

FMRI data

ROI analysis

Phasic response. The phasic response GLM for the contrast anticipation of aversive *v.* neutral sounds resulted in several activation differences between PD patients and HC (Table 1). PD patients showed increased activation in right central and basolateral amygdala as compared with HC (Fig. 2). Furthermore, PD patients in contrast to HC showed hyperactivation in dACC, anterior, posterior and mid-insula, as well as dorsomedial PFC (dmPFC), ventromedial PFC (vmPFC), dorsolateral PFC (dlPFC), and ventrolateral PFC (vlPFC) (online Supplementary Fig. S1). Correlational analyses resulted in a significant negative correlation between valence ratings and phasic dmPFC activation ($r_{16} = -0.722$, $p = 0.001$), in that more negative ratings resulted in more activation. The ANOVA for the investigation of the development of brain responses within the amygdala over the course of the experiment yielded no significant effects.

Sustained response. For the sustained response GLM, PD patients compared with HC showed increased activation in right BNST during anticipation of aversive *v.* neutral sounds (Fig. 2). This contrast also led to increased activation in bilateral anterior insula as well as dmPFC, vmPFC, dlPFC, and vlPFC in PD patients compared with HC (Table 2, online Supplementary Fig. S1). The ANOVA for the investigation of the development of brain responses over the course of the experiment within the BNST resulted in a significant main effect of group ($F_{1,34} = 11.94$, $p = 0.001$).

PPI

The phasic GLM resulted in two activation clusters in the right amygdala, which served as seed regions for PPI analyses (Table 3, Fig. 3). The time course of the central amygdala seed was positively associated with phasic left amygdala, dACC, mid-insula, dmPFC, vmPFC, dlPFC, and vlPFC activation in PD patients compared with HC. Furthermore, PD patients in contrast to HC showed significant phasic hyperconnectivity between the basolateral amygdala seed and rostral ACC (rACC), as well as hypoconnectivity with anterior insula and dlPFC. Using the significant cluster in the right BNST that emerged from the sustained GLM as seed region, we observed positive sustained psychophysiological interaction of the time course in this region with rACC, dmPFC, vmPFC, and dlPFC as well as negative association with dlPFC (Table 3, Fig. 3). Correlational analyses resulted in a significant positive correlation between ASI scores and hypoconnectivity between basolateral amygdala and anterior insula ($r_{16} = 0.710$, $p = 0.001$).

Discussion

The aim of this study was to investigate neural correlates of unpredictable threat anticipation in PD

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Table 1. *Significant phasic activations during anticipation of aversive* v. *neutral sounds*

PD > HC						
Region	Hemisphere	*x*	*y*	*z*	*t*-value	mm^3
Amygdala	R	22	4	−11	3.78	88
	R	29	−3	−14	4.12	224
ACC	R/L	2	1	38	4.77	1272
	R	11	1	44	3.19	64
	L	−13	3	39	3.33	160
Insula	R	28	−9	15	3.87	120
	R	28	−30	17	4.02	112
	R	25	23	14	3.88	136
PFC						
dmPFC	R	12	43	39	3.67	248
	L	−8	47	35	3.33	104
vmPFC	R	10	43	14	3.83	152
	R	4	55	4	3.07	64
vmPFC/vlPFC	L	−15	55	4	4.58	1056
vmPFC/dmPFC	L	−16	59	23	3.35	72
dlPFC	R	23	29	38	3.16	64
	R	18	55	32	4.98	752
	L	−18	23	48	3.67	1168
	L	−35	23	31	3.47	528
vlPFC	R	34	37	14	3.61	192
	R	47	32	12	4.00	392
	R	22	53	−8	3.42	64
	R	22	29	−11	4.14	392
	L	−36	37	13	4.11	208
	L	−21	25	−15	4.43	448
	L	−44	33	−10	3.75	400
	L	−17	41	−10	5.36	696

PD, panic disorder; HC, healthy controls; ACC, anterior cingulate cortex; PFC, prefrontal cortex; dmPFC, dorsomedial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; vlPFC, ventrolateral prefrontal cortex; L, left; R, right; (*x*, *y*, *z*), Talairach coordinates of maximally activated voxel (activation threshold: $p < 0.025$ corrected).

patients, with a focus on the dissociation between amygdala and BNST. While the amygdala showed phasic responding during the first second of the anticipatory interval, the BNST showed a sustained response across the whole anticipatory interval. Furthermore, we observed phasic activation in dACC, insula, and medial and lateral PFC. The sustained model revealed increased activation in anterior insula and medial and lateral PFC. With regard to amygdala and BNST, PPI analysis demonstrated partly distinct phasic and sustained functional connectivity patterns.

In contrast to the phasic amygdala response, PD patients compared with HC showed sustained activation in BNST during threat anticipation. Animal research suggests that BNST activation reflects anxiety, heightened vigilance, and preparedness in contexts of sustained threat (Walker *et al.* 2009; Davis *et al.* 2010; Fox *et al.* 2015). This is in line with studies in healthy subjects that demonstrated increased BNST activation during sustained threat anticipation (Alvarez *et al.* 2011; Grupe *et al.* 2013; Somerville *et al.* 2013; Herrmann *et al.* 2016) and a relationship between BNST hyperactivation and temporal and physical proximity to threat (Mobbs *et al.* 2010; Somerville *et al.* 2010). So far, increased BNST activation during anticipatory anxiety has only been shown in specific phobia (Straube *et al.* 2007; Münsterkötter *et al.* 2015), while no such hyperresponsiveness has been reported for PD or other anxiety and stress-related disorders. The increased BNST activation in PD patients in the current study suggests exaggerated levels of anxious apprehension (Alvarez *et al.* 2011; Grupe *et al.* 2013; Somerville *et al.* 2013) and hyperreactivity to uncertainty during anticipation of aversive stimuli (Grupe & Nitschke, 2013).

Amygdala showed phasic responding during anticipation of aversive *v.* neutral sounds in PD patients

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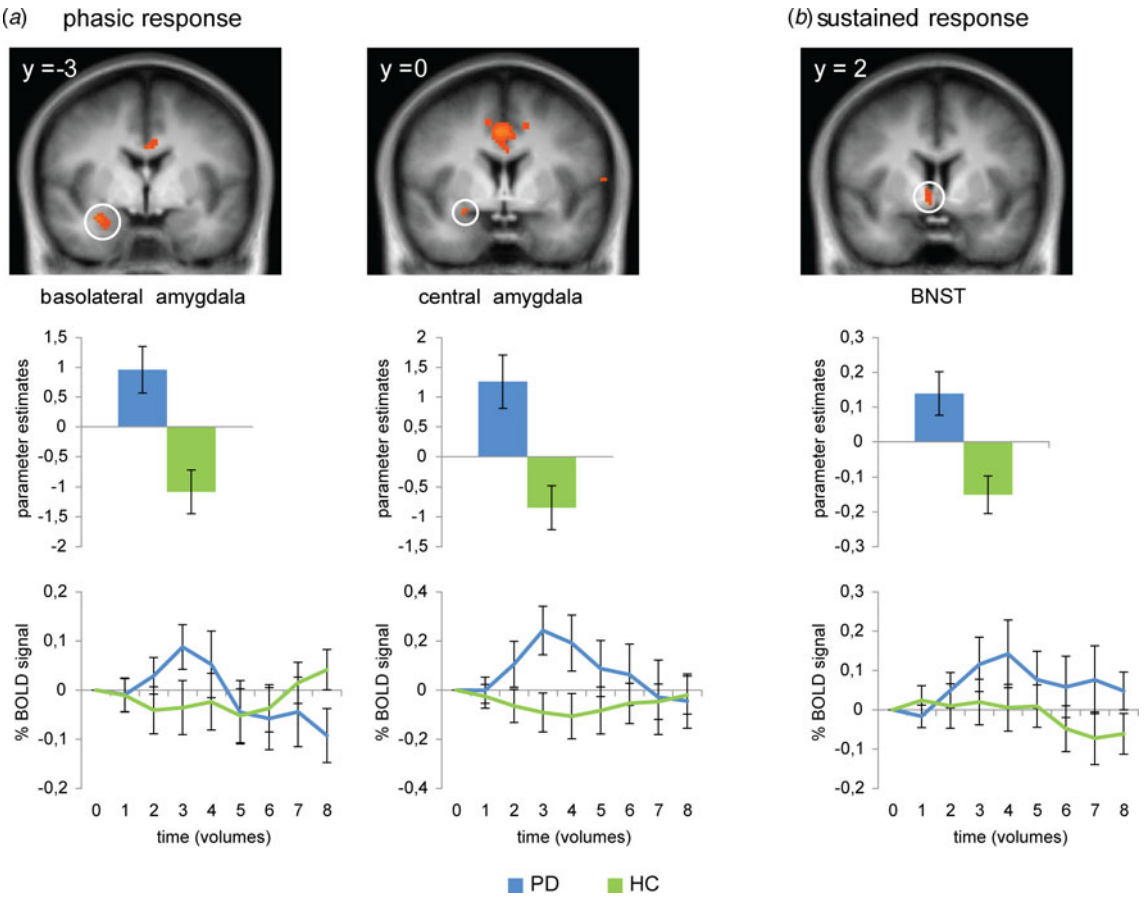

Fig. 2. Panic disorder (PD) patients compared with healthy controls (HC) showed increased phasic responses in right basolateral and central amygdala and an increased sustained response in right bed nucleus of the stria terminalis (BNST) during anticipation of aversive in contrast to neutral sounds. Statistical parametric maps are overlaid on an averaged T1 scan (radiological convention: left = right). Graphs in the middle row display contrasts of parameter estimates (anticipation of aversive *v.* neutral sounds; mean ± S.E. for activation cluster). Graphs in the bottom row display the relative blood oxygenation level dependent (BOLD) signal change over the anticipatory interval extracted from clusters of increased activation and averaged across time points of all trials with a duration of 16 s and across participants per group. Time points represent the contrast anticipation of aversive *v.* neutral sounds (mean ± S.E.).

compared with HC. Given its pivotal role in fear and anxiety (LeDoux, 2007), amygdala is also considered a central structure for PD (Gorman *et al.* 2000; Kim *et al.* 2012). Consistent with our results, Wittmann *et al.* (2011) found amygdala hyperactivation in response to briefly presented cues that indicated the appearance of agoraphobic or neutral pictures in PD patients with agoraphobia. However, contrary to our results, Boshuisen *et al.* (2002) found amygdala deactivation during anticipation of a panic attack in PD patients as compared with HC. This could be attributed to the fact that the study by Boshuisen *et al.* (2002) had only one anticipatory period that lasted 20 min and was analyzed as a whole. This is more comparable to our sustained analysis model, which revealed no amygdala activation. Other studies which only considered sustained anticipation of aversive stimuli also did not find an amygdala response (e.g. Straube *et al.* 2007; Alvarez *et al.* 2011; Grupe *et al.* 2013; Herrmann *et al.* 2016). However, as the current study shows, it is possible to detect amygdala hyperactivation in sustained anticipation paradigms by using a phasic analysis model (also see Alvarez *et al.* 2011; Grupe *et al.* 2013; Herrmann *et al.* 2016). Together, these findings support rapid threat processing in the amygdala (Alvarez *et al.* 2011; Grupe *et al.* 2013; Herrmann *et al.* 2016). The role of the amygdala in the deployment of attentional resources (Davis & Whalen, 2001; Grupe & Nitschke, 2013) indicates that PD patients showed enhanced vigilance as well as biased threat expectancies early during the anticipatory interval (Öhman & Mineka, 2001).

Furthermore, the current data provide evidence for characteristic functional connectivity patterns in

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Table 2. *Significant sustained activations during anticipation of aversive v. neutral sounds*

PD > HC						
Region	Hemisphere	*x*	*y*	*z*	*t*-value	mm^3
BNST	R	4	2	−2	3.17	64
Insula	R	36	21	6	3.28	168
	L	−31	22	−4	3.39	200
PFC						
dmPFC	R	17	17	53	5.06	1304
	L	−17	19	53	3.22	216
	L	−3	38	27	3.46	648
	R/L	0	49	36	3.49	328
vmPFC	L	−11	55	−5	3.20	128
	R/L	4	49	10	3.31	312
dlPFC	L	−27	43	22	3.36	488
vlPFC	R	47	24	−7	3.30	256

PD, panic disorder; HC, healthy controls; BNST, bed nucleus of the stria terminalis; PFC, prefrontal cortex; dmPFC, dorsomedial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; vlPFC, ventrolateral prefrontal cortex; L, left; R, right; (*x*, *y*, *z*), Talairach coordinates of maximally activated voxel (activation threshold: $p < 0.025$ corrected)

amygdala and BNST for PD patients. Our functional connectivity analysis revealed that phasic amygdala and sustained BNST responding are generally associated with activation in similar brain regions. This seems reasonable, as amygdala and BNST are closely interconnected and operate in similar networks (Walker *et al.* 2009; Fox *et al.* 2015). However, amygdala showed distinct functional coupling with insula. Although both amygdala and BNST showed positive association with ACC and PFC, they were related to distinct subregions within these areas. Therefore, amygdala and BNST seem to be embedded in partly distinct networks associated with phasic and sustained brain responses (McMenamin *et al.* 2014). Notably, the two seed regions in the amygdala corresponding to central and basolateral amygdala also showed partly diverging functional connectivity. Information is assumed to progress from basolateral to central amygdala (Davis & Whalen, 2001; LeDoux, 2007). Although both subregions are linked to threat processing and attentional processes (Etkin, 2010), lateral amygdala is assumed to integrate information (Phelps & LeDoux, 2005), while central amygdala is associated with emotional responding and modulation of vigilance (Davis & Whalen, 2001; LeDoux, 2007). Our findings are thus in line with partly different functions of amygdala subregions, engaging different networks within a phasic timeframe.

We found several clusters of activation in the insula, and amygdala showed altered functional connectivity with insula subregions in PD patients during aversive anticipation. Interestingly, our analysis revealed differential phasic activation in mid-, anterior and posterior insula, while sustained activation was only found in anterior insula. Posterior and mid-insula are implicated in the representation of affective bodily feelings and the integration of bodily information from internal and external sources, respectively (Craig, 2010). Anterior insula, on the other hand, is associated with subjective feelings as well as assessment of subjective value of situations and is suggested to regulate attentional processes (Craig, 2009; Menon & Uddin, 2010). Moreover, insula activation is considered to reflect increased reactivity to uncertainty during anxiety states and low perceived control (Sarinopoulos *et al.* 2010; Grupe & Nitschke, 2013; Alvarez *et al.* 2015), also in PD patients (Gorka *et al.* 2014). Thus, the current findings indicate hyperreactivity to temporally uncertain aversive stimuli in PD patients. Furthermore, PD patients seem to exhibit an early and phasic elevated focus on bodily feelings, as well as sustained and negatively biased generation of emotional responses, resulting in enhanced subjective feelings of threat (Singer *et al.* 2009; Grupe & Nitschke, 2013). Regarding functional connectivity, an amygdala-insula system has previously been implicated in anticipation of aversive stimuli (Sarinopoulos *et al.* 2010) and was associated with high trait anxiety (Carlson *et al.* 2011). Together with the current findings, amygdala and insula seem to exchange information during threat anticipation, possibly for the purpose of attention allocation.

Regarding ACC, our analysis revealed several clusters of phasic activation in dACC, while this region showed no sustained activation. Furthermore, we found increased functional connectivity between amygdala and dACC/rACC, and between BNST and rACC. DACC activation was also found in PD patients anticipating panicogenic symptoms (Boshuisen *et al.* 2002), while rACC hypoactivation was shown in PD compared with PTSD patients during threat of shock (Tuescher *et al.* 2011). However, these studies all used sustained anticipatory periods. Similar to our study, healthy subjects showed phasic ACC activation during unpredictable threat anticipation (Alvarez *et al.* 2011; Grupe *et al.* 2013; McMenamin *et al.* 2014; Herrmann *et al.* 2016). Converging evidence highlights a role of the dACC in the appraisal of negative emotion and threat (Etkin *et al.* 2011; Maier *et al.* 2012; Kalisch & Gerlicher, 2014). Thus, PD patients in the current study seem to exhibit rapid appraisal of the aversive anticipatory context. Increased functional connectivity of dACC with amygdala further suggests that these regions work in concert during initial aversive

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Table 3 *Significant functional connectivity differences between PD and HC during anticipation of aversive v. neutral sounds*

		PD > HC					HC > PD				
Region	Hemisphere	*x*	*y*	*z*	*t*-value	mm^3	*x*	*y*	*z*	*t*-value	mm^3
Seed: BNST											
ACC	R/L	−8	30	18	4.06	1816					
	R/L	2	25	−3	3.98	184					
dmPFC	R	5	51	30	3.36	192					
vmPFC	L	−7	53	−1	3.20	104					
dlPFC	R	36	27	25	3.66	144	31	4	33	3.90	392
	R						21	−7	66	3.40	320
	R						19	−11	49	4.25	160
	L	−17	37	37	4.11	464					
	L	−25	31	49	3.57	448					
Seed: central amygdala											
Amygdala	L	−26	1	−13	3.41	160					
ACC	R	6	15	35	3.92	248					
	R/L	3	3	33	3.28	264					
	R/L	−9	19	24	3.97	320					
Insula	L	−34	−9	14	4.11	120					
	L	−37	−1	−5	3.39	104					
dmPFC	R	5	35	31	3.42	120					
	L	−5	55	32	3.22	88					
vmPFC	R	16	36	−16	3.30	136					
	L	−13	26	−12	3.69	104					
dmPFC/vmPFC	L	−8	59	22	3.95	456					
dlPFC	R	19	4	52	3.71	176					
	L	−18	37	37	3.63	648					
vlPFC	R	39	28	−6	3.70	184					
	L	−28	35	−12	4.94	488					
	L	−29	55	−6	3.50	88					
	L	−32	29	−5	3.15	72					
	L	−56	11	9	3.93	216					
	L	−20	7	−16	4.02	120					
Seed: basolateral amygdala											
ACC	R/L	3	21	0	3.38	120					
Insula	L						−32	17	−4	3.29	120
dlPFC	R						33	11	42	4.42	88

PD, panic disorder; HC, healthy controls; ACC, anterior cingulate cortex; dmPFC, dorsomedial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; vlPFC, ventrolateral prefrontal cortex; L, left; R, right; (*x*, *y*, *z*), Talairach coordinates of maximally activated voxel (activation threshold BNST seed: $p < 0.025$ corrected; activation threshold amygdala seeds: $p < 0.0125$ corrected)

anticipation. In contrast, rACC is associated with the regulation of emotional responses generated in limbic regions (Etkin *et al.* 2011, 2015), which might be reflected in hyperconnectivity between amygdala as well as BNST and rACC in the current study.

More sustained regulatory functions and action preparation are generally also attributed to ACC (Shenhav *et al.* 2013; Vogt, 2014). Due to the absence of sustained ACC activation, the current results rather suggest that these functions are fulfilled by other prefrontal areas. We found phasic and sustained hyperactivation in medial and lateral PFC regions in PD patients as compared with HC during aversive anticipation. Medial and lateral PFC together with ACC have been implicated in emotion processing, appraisal, regulation, and expression of fear and anxiety (Etkin *et al.* 2011; Maier *et al.* 2012; Kalisch & Gerlicher, 2014; Duval *et al.* 2015). PFC regions are consistently implicated in anxiety disorders, although the picture is less clear for PD (Engel *et al.* 2009; Dresler *et al.* 2013). The multitude of hyperactivated PFC clusters in our study possibly reflects enhanced threat

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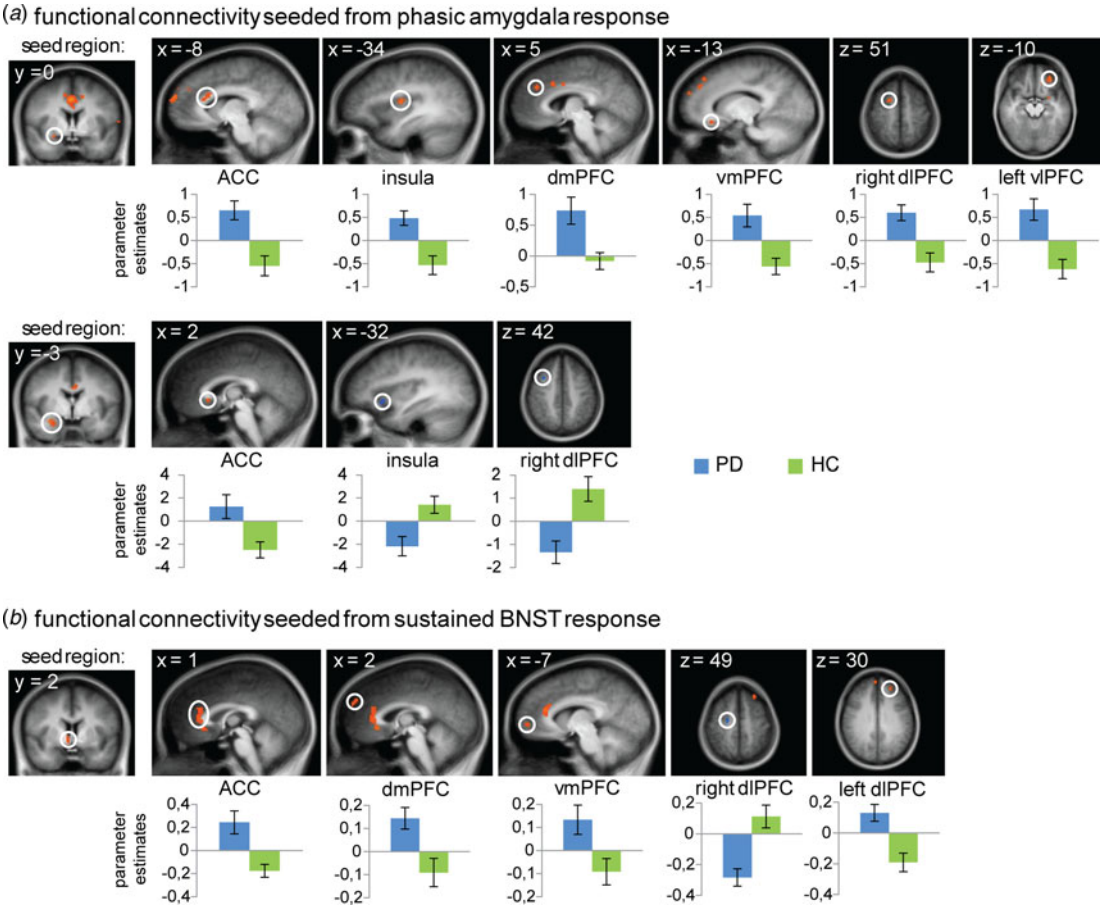

Fig. 3. Differential phasic activation in central amygdala showed increased functional connectivity with anterior cingulate cortex (ACC), insula, dorsomedial prefrontal cortex (dmPFC), ventromedial prefrontal cortex (vmPFC), right dorsolateral prefrontal cortex (dlPFC) and left ventrolateral prefrontal cortex (vlPFC) in panic disorder (PD) patients compared with healthy controls (HC) during anticipation of aversive in contrast to neutral sounds. Differential phasic activation in basolateral amygdala showed increased functional connectivity with ACC as well as decreased functional connectivity with insula and right dlPFC in PD patients compared with HC. Differential sustained activation in bed nucleus of the stria terminalis (BNST) showed increased functional connectivity with ACC, dmPFC, vmPFC, and left dlPFC as well as decreased functional connectivity with right dlPFC in PD patients compared with HC. For reasons of clarity, only the most significant activation cluster per brain region is displayed. Statistical parametric maps are overlaid on an averaged T1 scan (radiological convention: left = right). Graphs display contrasts of parameter estimates (anticipation of aversive *v.* neutral sounds; mean ± S.E. for activation cluster). R, right; L, left.

processing and the attempt to downregulate threat-related emotional responding in PD patients.

Evidence for a modulating influence of PFC regions also stems from our functional connectivity analysis. BNST showed increased connectivity with mPFC, while amygdala was positively associated with both medial and lateral PFC. Notably, amygdala and BNST both showed negative association with dlPFC. Enhanced connectivity between amygdala and mPFC/ACC regions was also shown in non-responders to cognitive behavioral therapy and might represent vulnerability for PD (Lueken *et al.* 2013). Additionally, anxiety symptom severity in PD patients was associated with amygdala-mPFC/ACC connectivity during processing of fearful faces (Demenescu *et al.* 2013). It has further been suggested that anxiety disorders are not necessarily based on a failure of mPFC to downregulate the amygdala, but that increased mPFC activation might also represent overcompensation (Duval *et al.* 2015) and lead to enhanced negative affect (Myers-Schulz & Koenigs, 2012). Keeping this in mind, the current results could indicate that amygdala-mPFC/ACC coupling during anticipatory anxiety increases negative experience in PD patients.

BNST and mPFC have been shown to be structurally and functionally connected (Avery *et al.* 2014; Torrisi

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et al. 2015). Accordingly, vmPFC lesions led to decreased BNST activation (Motzkin *et al.* 2015) and dmPFC showed functional connectivity with BNST under threat of shock (Kinnison *et al.* 2012). It is thus assumed that BNST interacts with mPFC to express aversive emotional states in contexts of threat anticipation (Avery *et al.* 2016). Functional connectivity has also been reported for amygdala and BNST with lateral PFC during anticipatory anxiety in HC (Herrmann *et al.* 2016). Assuming that lateral PFC is implicated in the integration of cognition and emotion (Pessoa, 2008; Duval *et al.* 2015), this functional connectivity might represent a pathway for the integration of information from multiple sources for evaluation and initiation of action (Pessoa, 2008).

Our findings should be considered in light of some limitations. A potential limitation is the restricted sample size. However, due to clear a priori hypotheses regarding amygdala and BNST, our findings should be considered a relevant contribution to the field. Furthermore, comorbidities constitute a possible confound of our study. Since PD was the main diagnosis and PD patients commonly present comorbidities (Kessler *et al.* 2006), the exclusion of patients with comorbid diagnoses would have limited the representativeness and generalizability of our findings. Future studies should test the specificity of our findings in larger samples, which would also allow for investigations of the moderating influence of specific variables, such as symptom severity, on brain responses. Additionally, it would be interesting to specifically investigate the influence of uncertainty on the neural correlates of PD patients during threat anticipation, for example by varying the probability of stimulus occurrence indicated by the cue.

To summarize, we provide first evidence for dissociation between amygdala and BNST in PD patients during unpredictable threat anticipation. While we found phasic amygdala and sustained BNST responding, the two regions were also embedded in partly distinct functional networks. Taken together, amygdala and BNST activations possibly mediate pathological fear and anxiety symptoms in PD. Furthermore, phasic and sustained activation was found in subregions of insula, ACC and PFC, suggesting different temporal and functional characteristics during threat anticipation. Our findings imply enhanced responding in emotion-processing regions in PD patients during unpredictable anticipation of aversive stimuli. Since maladaptive responding to uncertainty regarding the occurrence of panic attacks is one of the major burdens in PD (Bouton *et al.* 2001; Grillon *et al.* 2008), uncovering possible neural substrates for chronically increased fear and anxiety is particularly relevant and could provide valuable input for treatment.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0033291717001192

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Declaration of Interest

None.

Ethical Standard

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The study was approved by the ethics committee of the University of Muenster (reference number: 2012-294-f-S).

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