Fear bradycardia and activation of the human periaqueductal grey

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Animal models of predator defense distinguish qualitatively different behavioral modes that are activated at increasing levels of predation threat. A defense mode observed at intermediate threat levels is freezing: a cessation of locomotion that is characterized by a parasympathetically dominated autonomic nervous system response that causes heart rate deceleration, or fear bradycardia. Studies in rodents have shown that freezing depends on amygdalar projections to the periaqueductal grey (PAG). In humans, freezing-like behaviors are implicated in development and maintenance of psychopathology, but neural mechanisms underlying freezing or its characteristic autonomic response profile have not been identified. Here, we combined event-related blood oxygenation level-dependent functional MRI (BOLD-fMRI) with autonomic response measures in a picture viewing paradigm to probe activity and interconnectivity within the amygdala–PAG pathway and test for an association with parasympathetic activation. In response to negatively arousing pictures, we observed parasympathetic (bradycardia) and sympathetic (pupil dilation) autonomic responses, BOLD responses in the amygdala and PAG, and effective connectivity between these regions. Critically, BOLD responses in the PAG to negative pictures correlated on a trial-by-trial basis with bradycardia but not pupil dilation. This correlation with bradycardia remained significant when partialling out pupil dilation. Additionally, activity in regions associated with motor planning and inhibition mirrored the PAG response. Thus, our findings implicate the human PAG in a parasympathetically dominated defense mode that subserves a state of attentive immobility. Mechanistic insight into this qualitatively distinct defense mode may importantly advance translational models of anxiety disorders.

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Introduction

The coevolution of predators and prey has spawned a repertoire of qualitatively different behavioral defense strategies that animals deploy at various levels of predator threat (Blanchard et al., 2001; Fanselow, 1994). A passive defense mode activated at intermediate threat levels is freezing: a state of attentive immobility that serves to avoid detection by predators (Lang and Davis, 2006; Öhman and Wiens, 2002). It is well known that freezing behavior in both animals and humans is associated with heart rate deceleration, or fear bradycardia (Lang and Davis, 2006). This parasympathetically dominated autonomic response contrasts with the sympathetically dominated fight-or-flight response activated during imminent predation threat (Fanselow, 1994). In humans, freezing and its concomitant attentional focus on threat-related information is thought to contribute to a vicious cycle whereby emotional disturbance triggers an attentional bias for threat-related information, and vice versa (Fox et al., 2001; Hagenaes et al., 2012; Holmes et al., 2004; Marks, 1987; Roelofs et al., 2010). However, relatively little is known about the neural regulation of freezing in humans.

Rodent studies have shown that the amygdala plays a key role in orchestrating defensive behavior and transitioning between defensive modes (Davis and Whalen, 2001; Fendt and Fanselow, 1999; Gozzi et al., 2010; Haubensak et al., 2010). For instance, stimulation of the central nucleus of the amygdala produces freezing, bradycardia, and pupil dilation (Applegate et al., 1983), whereas lesions block both autonomic and behavioral manifestations of fear (Fendt and Fanselow, 1999; Kapp et al., 1979). Autonomic responses are mediated by downstream connections to the lateral hypothalamus, which controls sympathetic responses, and to medullar nuclei that control parasympathetic effects through vagal efferents (Schwaber et al., 1982). Behavioral manifestations of predator defense, however, depend on the periaqueductal grey (PAG; Ledoux et al., 1988), a midbrain region implicated in a host of homeostatic processes including fear, pain, and analgesia (Linnman et al., 2012; Neugebauer et al., 2009). In particular, lesions of the ventral (Liebman et al., 1970; Lyon, 1964), but not the dorsal (Kim et al., 1993)
PAG disrupt freezing. Human research on amygdala lesioned patients has produced findings that are roughly consistent with the animal literature (Bechara et al., 1995; Funayama et al., 2001; LaBar et al., 1995), but the anatomical specificity of this work is limited. Neuroimaging studies in humans have moreover shown that activity in amygdala–PAG circuitry varies with threat proximity (Mobbs et al., 2007, 2009, 2010). However, activity in these circuits has not been associated specifically with freezing or its accompanying parasympathetic autonomic response.

A well-established paradigm to study the autonomic psychophysiology of defense behaviors in humans is exposure to pictorial stimuli taken from the International Affective Picture System (Lang et al., 2005). Studies using this paradigm have shown that autonomic responses to affective stimuli, which vary on dimensions of valence and arousal, closely parallel autonomic responses that accompany defensive behaviors in rodents (Lang and Davis, 2006; Lang et al., 1998). For instance, negatively valenced and highly arousing pictures elicit sympathetic changes such as galvanic skin responses (Lang and Davis, 2006) and pupil dilation (Bradley et al., 2008). Notably, however, numerous studies have demonstrated that such stimuli also induce marked bradycardia (Bradley et al., 2008), and to observe increased neural activity in the amygdala–PAG pathway can be linked to freezing through an association with parasympathetically as opposed to sympathetically dominated autonomic responses.

To investigate this, we combined functional magnetic resonance imaging (fMRI) with autonomic response measures (heart rate change, pupil dilation) in a picture viewing paradigm. We expected to replicate previous findings of stronger bradycardia and pupil dilation to negatively arousing stimuli (Bradley et al., 2008), and to observe increased neural activity and connectivity within the amygdala–PAG pathway. Critically, we predicted a trial-by-trial correlation between PAG responses and parasympathetic (bradycardia) responses that is statistically independent of concomitant sympathetic (pupil dilation) responses.

Materials and methods

Participants

Eighteen (aged 19–31 years) male, healthy, right-handed volunteers were tested in a within-subject design. Exclusion criteria were history of head injury, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects CNS or endocrine systems, medical illness within 3 weeks before testing, self-reported mental or substance use disorder, daily tobacco use, current stressful episode or major life event, a score of 8+ on the Beck Depression Inventory (Beck et al., 1979), previous exposure to photographs used in the study, and regular exposure to violent movies or computer games. We excluded women in this study to reduce interindividual variance due to sex- and cycle-related confounds. The study was approved by the local institutional review board (CMO region Arnhem–Nijmegen, The Netherlands) in accordance with the declaration of Helsinki. All participants provided written informed consent.

General procedure and experimental paradigm

The design of this study is illustrated in Fig. 1. Participants were instructed not to use any recreational drugs for 3 days, and to refrain from drinking alcohol, exercising, and smoking for 24 h before each session. All completed Beck’s Depression Inventory (Beck et al., 1979) and were invited for scan sessions between 2:00 P.M. and 6:00 P.M.

Three different sets of 160 photographs were selected from the International Affective Picture System (Lang et al., 2005) and an additional set of newly rated pictures. New pictures were selected based on their emotionality and similarity to IAPS pictures, and were rated on a 1–9 score for subjective valence and arousal using the self-assessment manikin (SAM) scales (Bradley and Lang, 1994) by a separate group of 20 healthy male participants. SAM scales are standardized for use with the IAPS and consist of small pictograms depicting valence (using positive and negative facial expressions) and arousal (illustrated by small explosions at the level of the heart). Each picture set was used in six participants, and contained 80 aversive and 80 neutral pictures. Aversive photographs had moderate to high arousal scores (mean 5.5, SD .7) and negative valence (mean 3.1, SD .7). Neutral slides had low arousal scores (mean 2.5, SD .7) and neutral valence (mean 5.3, SD .3). The three different picture sets did not differ in arousal and valence ratings, amount of newly scored photographs, and chromatic features and complexity.

During functional MRI scanning, 160 pictures were shown in pseudorandom order (no more than two consecutive pictures from the same category) in three separate blocks of 12.5 min and presented for 5 s. An interstimulus interval varying randomly between 4 and 8 s was used, during which a fixation cross was shown. To ascertain that they processed the pictures attentively and to obtain an online indication of subjective affective ratings of the stimuli, participants were instructed to categorize their content as either aversive or neutral. Responses were given with right-hand button presses. Furthermore, participants were informed that their memory for the pictures would be tested after the scan session (reported elsewhere; Henckens et al., 2009).

In the scanner, participants wore ear plugs, and foam pads were used to restrict movement. Stimuli were back-projected onto a translucent screen positioned behind the participant’s head that was visible through a mirror mounted on the head coil. Scan sessions started with calibration of the eye-tracking system.

Physiological measurements

Heart rate was recorded throughout scanning using a 50 Hz finger pulse photoplethysmograph affixed to the left index finger. Data were processed using in-house software for interactive visual artifact correction and peak detection, resulting in time-series of interbeat intervals expressed in beats per minute (BPM). Data were baseline corrected using a −25 s to −1 s pre-stimulus onset average. We chose this relatively long baseline window because heart rate fluctuates with the respiratory cycle (i.e., respiratory sinus arrhythmia; De Geus et al., 1995), which causes a signal fluctuation that strongly affects baselines just prior to stimulus onset. Event-related responses were quantified as averaged heart rate change between 2 and 5 s post-stimulus onset. The 0–2 s response window was discarded because heart rate decelerates nonspecifically during this period (i.e., the orienting response). The average percentage of trials (and standard deviation across participants) that were valid (i.e., contained no artifacts) was 91.0 (11.1) and 91.3 (11.0) for negatively arousing and neutral pictures, respectively. Trial-by-trial measures of heart rate change were included in (hierarchical) parametric analyses of fMRI data (see below), and were tested for effects of picture category using a paired samples t-test.

Pupil dilation and eye movements were monitored using a 50 Hz iView system with MR-compatible MEyeTrack-LR camera mounted on the scanner bed (SensoMotoric Instruments, Teltow, Germany). Pupil dilation data were analyzed using in-house software implemented in Matlab 7.9 (The Mathworks, Natick, MA). Signal artifacts due to eye blinks were removed using linear interpolation (Siegle, 2003). Event-related pupil dilation responses were calculated by dividing averaged pupil dilation during the 1–5 s period after picture onset by the averaged 1 s prior to onset. Note that this baseline window differs from

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the one used for heart rate because pupil dilation is not confounded by low frequency fluctuations like respiration. The 1–5 s response window was chosen to avoid the confounding influence of the initial light reflex (a parasympathetically regulated pupil constriction) that starts upon stimulus onset and lasts about 1 s. Additionally, because pupil dilation varies approximately linearly with luminance (Bradley et al., 2008), luminance related variance in pupil dilation was removed on an individual trial-by-trial basis using linear regression of pupil dilation responses onto total luminance of the photographs. Event-related eye movement response curves were calculated using the sample-to-sample distance between gaze positions. For statistical analysis, eye movement responses were averaged across the same 1–5 s post-stimulus-onset time window. Trials were rejected if either the baseline or the response windows contained more than 50% interpolated data. Moreover, any trials with responses deviating more than three SDs from the individual’s mean pupil response were discarded. Eye-tracking data of three participants were lost due to excessive artifacts and/or apparatus failure, but these participants were included in all other analyses. For the remaining 15 participants, the average percentage of trials (and SD) that were not rejected due to artifacts was 79.3 (21.1) and 77.4 (24.9) for negatively arousing and neutral pictures, respectively. Luminance-corrected pupil dilation response magnitudes were used in (hierarchical) parametric analyses of fMRI data (see below), and were tested for effects of stimulus valence using a paired samples t-test.

To test whether heart rate and pupil dilation responses were correlated within subjects, we performed linear regression between these two measures separately within the two picture categories and for each participant on a trial-by-trial basis. The resulting regression slopes (for negatively arousing and neutral pictures, respectively) were then used to match images onto the MNI152 template. Finally, functional images were smoothed with a 4 mm isotropic FWHM Gaussian kernel (cf. Mobbs et al., 2007), and resliced into 2 mm isotropic voxels. Note that functional MRI with these parameters cannot resolve signal arising from medullar nuclei that control parasympathetic vagal outflow, which are substantially smaller than the PAG. T1 weighted images were resliced into MNI152 space with 5 mm isotropic voxel size and averaged across participants to serve as background for anatomical localization (Figs. 3–5). Statistical parametric maps were thresholded as indicated in figure legends and overlaid onto these images.

The first analysis aimed to identify regions expressing picture category main effects. In the first-level model, we included (for each of the three blocks) separate regressors for the two picture categories (negatively arousing and neutral, according to our a priori classification), which modeled event-related (5 s box function) responses to pictures. These task regressors were subsequently temporally convolved with the canonical SPM8 hemodynamic response function. Movement parameters (3 translations, 3 rotations) were included for each block. The analysis furthermore included high-pass filtering (1/128 Hz cut-off) and AR(1) serial correlations correction. Single subject parameter estimates from each condition and block obtained from first-level analyses were entered into a group-level random effects (RFX) analysis using a factorial ANOVA with picture category (negatively arousing versus neutral) and block (1–3) as within-subject factors. For this exploratory first analysis, alpha was set at .05, whole-brain family-wise error (FWE)

MRI scan acquisition

MRI scans were collected using a Siemens (Erlangen, Germany) TIM Trio 3.0 T MRI scanner equipped with an 8 channel head coil. The following scans were obtained: 3 (runs) * 345 gradient echo EPI T2* weighted blood oxygenation level-dependent (BOLD) images (TE/TR: 25/2180 ms, flip angle 90°, FOV: 212*212 mm, matrix 64*64, 3 mm slice thickness, .3 mm slice gap, 37 ascending slices. A relatively short TE was chosen to minimize signal dropout due to magnetic field inhomogeneity around air-tissue interfaces. The first five volumes of each run were discarded to allow for T1 equilibration. Structural scans were obtained using a Magnetization-Prepared RAdiofrequency GRE sequence combined with GeneRalized Autocalibrating Partially Parallel Acquisitions (GRAPPA; Griswold et al., 2002). The following parameters were used: TE/TR: 2.96/2300 ms, flip angle: 8°, FOV: 256*256*192 mm, voxel size: 1 mm isotropic, GRAPPA acceleration factor 2.

MRI scan analyses

Data were analyzed using SPM8 (http://www.filion.ucl.ac.uk/spm; Wellcome Department of Imaging Neuroscience, London, UK). Motion correction was performed on all functional scans using a rigid body transformation and sum of squared differences minimization. Mutual information maximization based rigid body registration was used to register functional and structural images. Structural images were segmented into grey matter, white matter, and CSF images using a unified probabilistic template registration and tissue classification method (Ashburner and Friston, 2005). Subsequently, images were registered with site-specific tissue templates (created from 384 structural scans acquired on the same system) using DARTEL (Ashburner, 2007). Affine transformations were then used to match images onto the MNI152 template. Finally, functional images were smoothed with a 4 mm isotropic FWHM gaussian kernel (cf. Mobbs et al., 2007), and resliced into 2 mm isotropic voxels. Note that functional MRI with these parameters cannot resolve signal arising from medullar nuclei that control parasympathetic vagal outflow, which are substantially smaller than the PAG. T1 weighted images were resliced into MNI152 space with 5 mm isotropic voxel size and averaged across participants to serve as background for anatomical localization (Figs. 3–5). Statistical parametric maps were thresholded as indicated in figure legends and overlaid onto these images.

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corrected using Gaussian Random Field Theory based methods. All further analyses were performed separately for negatively arousing and neutral items.

Second, we implemented a psychophysiological interaction (PPI) model (Friston et al., 1997) to assess effective connectivity of the bilateral amygdala. As a seed region, we used the bilateral amygdala clusters identified by the group-level main effect of picture category. The first eigenvariate of the extracted time courses was deconvolved (Gitelman et al., 2003), adjusted for task effects, and pre-whitened before computing the PPI regressor modeling differential connectivity during versus between picture presentations. After hemodynamic convolution, this interaction term and the seed region time course were added to each first-level design matrix for each block. This procedure was performed separately for negatively arousing and neutral images. Third, we set up a model to test for a linear association between event-related BOLD response magnitudes and heart rate change by adding a linear parametric modulation regressor to the first model (see Büchel et al., 1998). Finally, the fourth model was a hierarchical regression model which included both pupil dilation responses and heart rate changes as parametric modulators. Heart rate changes were orthogonalized with respect to pupil dilation responses to allow for assessment of the partial correlation between neural responses and heart rate changes controlling for pupil dilation. Relevant single subject parameter estimates from these regression models were entered into subsequent RFX analyses using factorial ANOVAs with block (1–3) as within-subject factor. Based on a priori hypotheses about the PAG and amygdala, and the results of whole-brain analysis of the main effects of picture category, associations between BOLD responses and physiological responses were tested using an alpha of .05 corrected for reduced search volumes using small volume corrections (SVC) using anatomical masks of the midbrain and bilateral amygdala (Lancaster et al., 2000).

For illustration of the time course of the BOLD response in the PAG to pictures in relation to heart rate changes and pupil dilation responses, we used median splits to divide, for each participant, all (negatively arousing and neutral) trials into four subgroups (high pupil dilation change/high heart rate change, high pupil dilation change/low heart rate change, low pupil dilation change/high heart rate change, and low pupil dilation change/low heart rate change). Subsequently, we used a finite impulse response model (a first-level model that makes no assumptions regarding the HRF shape) to estimate the pre-stimulus time histograms for all four trial subgroups within the negatively arousing and neutral picture categories. Response time courses to negatively arousing picture trials (averaged across participants) are shown separately for the four trial subgroups in Fig. 5D.

It can be argued that correlations between BOLD signal around the PAG and heart rate frequency changes may arise from alterations in the blood flow within large vessels nearby this region, because increased heart rate frequency would raise supply of oxygenated blood. It should be noted, however, that such correlations would have a sign that is opposite to the (negative) correlations with bradycardia. In Supplementary Fig. 1, we illustrate this by showing that BOLD signal change in response to pictures (collapsed across categories) is positively correlated with heart rate change in and around large vessels.

Results

Physiological responses to pictures

Event-related heart rate responses to pictures were calculated as changes in beats per minute during picture presentation relative to pre-stimulus baselines. As expected, heart rate change values were lower in response to negatively arousing than to neutral pictures ($t(17) = 4.80, P < .0005$; Fig. 2A). Heart rate dropped below baseline in response to negatively arousing pictures ($t(17) = 1.82, P < .05$, tested one-tailed), whereas heart rate increased after neutral pictures ($t(17) = 3.34, P < .005$).

Pupil dilation responses were calculated as the proportional change during picture presentation relative to pre-stimulus baselines (Fig. 2B). Responses correlated negatively with picture luminance ($t$-test across all participants’ regression slopes between picture luminance and pupil dilation: $t(14) = -8.19, P < .001$). Pupil dilation responses were therefore luminance-corrected in further analyses. Corrected pupil dilation responses remained stronger for negatively arousal than neutral pictures ($t(14) = 3.91, P < .005$).

Next, we investigated whether heart rate responses correlated negatively with pupil dilation responses on a trial-by-trial basis within the category of negatively arousing pictures. A one-sample $t$-test across within-subject regression slopes between these two measures did not yield evidence for such a correlation ($t(14) = 1.39, n.s.$). Also, we found no negative correlation between these two measures across subjects ($t(13) = -0.2, n.s.$). Similar null findings were observed for neutral images. Thus, although both heart rate and pupil dilation responses yielded robust effects of picture category, we found no systematic relation between the two variables within categories.

We furthermore confirmed that eye movement responses for the two picture categories did not differ significantly ($t(14) = 1.26, n.s.$).

Behavioral measures during scanning

Means (SDs) of the percentage of correct responses (i.e., subjective affective judgments corresponding to the a priori determined categories) for the picture judgment task performed during scanning was 92.8 (3.2) and 96.0 (3.7) for the negatively arousing and neutral pictures, respectively (difference: $t(17) = 2.26, P < .05$). Means (and SDs) of reaction times were 1364 (331) and 1388 (339) ms, respectively (difference $t(17) = .7, n.s.$).

Functional MRI

We first identified regions that were more responsive to negatively arousing than to neutral stimuli using a whole-brain analysis (Table 1). In line with expectations, such regions included bilateral (dorsal) amygdala, fusiform gyrus, middle temporal gyrus, right inferior frontal gyrus (rIFG), superior frontal gyrus, orbital frontal cortex, insula, preunecus, supramarginal gyrus, cerebellum, caudate nucleus, thalamus, and hypothalamus (Fig. 3A). Critically, we also found robust activation in the PAG (peak voxel [2, −30, −2]; $P < .001$, whole-brain corrected; Fig. 3B). Deactivations to aversive stimuli are also listed in Table 1. Further analyses focused on responses to negatively arousing pictures.

We first examined whether processing of negatively arousing pictures led to coupling between the bilateral amygdala (used as seed region) and PAG by including a psychophysiological interaction term (Friston et al., 1997) into our first-level model testing for differential connectivity during versus between presentation of negatively arousing images. This analysis confirmed our hypothesis (peak voxel in PAG: [−4, −30, −4]; $P = .025$, SVC; Fig. 4 and Table 2). Only one other region (thalamus) reached a whole-brain corrected threshold in this contrast.

Next, we addressed the main question of this study, namely, whether PAG and amygdala responses to negatively arousing pictures are associated with bradycardia. We therefore performed a focused analysis within the midbrain ROI in which trial-based measures of heart rate responses were included as linear parametric modulation regressors in first-level models (Büchel et al., 1998). Parameter estimates for these regressors were again tested in a second level RFX model. This analysis revealed that responses to negatively arousing pictures within the PAG were negatively associated with heart rate change (peak voxel [4, −28, −12]; $P < .01$, SVC, Fig. 5A; Table 2; see Supplementary Fig. 2 for whole-brain results). Similar effects were found in the right amygdala (peak voxel [30, −2, −18]; $P < .05$, SVC), and in rIFG, supplementary motor area (SMA),
caudate, putamen, anterior cingulate cortex (ACC), and parahippocampal gyrus (all \(P < .05\), whole-brain FWE corrected).

We then tested whether the correlation with PAG activity was specific to the parasympathetic bradycardia response. No significant trial-by-trial correlations were found for (sympathetically regulated) pupil dilation (all \(P > .05\), SVC, in both amygdala and PAG; Fig. 5B; no other regions reached a whole-brain corrected threshold). Finally, we used a hierarchical regression model to assess the correlation between PAG and amygdala activity and heart rate change while controlling for pupil dilation response magnitudes on a trial-by-trial basis. As can be seen in Fig. 5C and Table 2, this partial trial-by-trial correlation in the PAG remained significant (peak voxel \([-4, -28, -12]\); \(P < .05\), SVC), whereas the amygdala failed to reach statistical thresholds. Additionally, partial trial-by-trial correlations in SMA, rIFG, and putamen

![Fig. 2. Heart rate and pupil dilation responses to pictures. (A) Stronger decelerative heart rate responses to negatively arousing than to neutral pictures. (B) Stronger pupil dilation responses to negatively arousing than to neutral pictures. Pupil dilation drops below baseline because luminance is higher during picture presentation than during intertrial intervals. BPM, beats per minute; *, \(P < .005\); **, \(P < .0005\).](image)

Table 1

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Left hemisphere</th>
<th>Right hemisphere</th>
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<tr>
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<td>Region</td>
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<td></td>
<td>Amygdala</td>
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<td></td>
<td>Fusiform gyrus</td>
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Notes: all coordinates are defined in MNI152 space. All statistics listed are significant at \(P < .05\), whole-brain family-wise error corrected. MNI, Montreal Neurological Institute.
remained significant (P<.05, whole-brain FWE corrected). Thus, the trial-by-trial association between PAG and heart rate change cannot be explained by a concomitant sympathetic response.

All fMRI analyses were repeated for neutral images separately (Table 2). As expected, no effective connectivity between amygdala and PAG was observed within this category. Similar to negatively arousing pictures, amygdala responses were negatively associated with heart rate change (peak voxel [24, −8, −20]; P<.05, SVC). However, PAG responses to neutral pictures were associated with neither heart rate change nor pupil dilation.

Discussion

The aim of this study was to gain insights into the neural regulation of freezing-like behaviors in humans. Using variability in autonomic nervous system responses to pictorial stimuli, we show that neural activity within the amygdala–PAG pathway is specifically associated with a parasympathetically dominated response that is characteristic of freezing.

As expected, the present study replicates earlier work in humans showing that both the amygdala (Kober et al., 2008; Phan et al., 2004)
and the PAG (Buhle et al., 2012; Mobbs et al., 2007, 2009, 2010; Wager et al., 2009) activate in response to emotionally arousing stimuli. We could rule out an alternative explanation of the latter finding, namely that highly arousing images may elicit more eye movements and therefore lead to increased BOLD responses in the adjacent superior colliculus, by showing that the amount of eye movements did not differ between the two categories of stimuli. While most human functional neuroimaging studies to date have focused on the involvement of the amygdala and PAG increases while processing emotionally arousing stimuli. This finding is in line with known anatomical connections between these regions in both primates (Price, 2003) and rodents (Fendt and Fanselow, 1999). Studies on intrinsic functional connectivity in humans have moreover shown synchronized spontaneous activity in the amygdala and midbrain as part of a broader network involving frontoinsular and dorsal anterior cingulate cortices that is thought to regulate salience processing (Hermans et al., 2011; Kong et al., 2010; Roy et al., 2009; Seeley et al., 2007; van Marle et al., 2010). A recent study furthermore found that connectivity between these regions was higher during anticipation of threat than during a period of imminent threat (Mobbs et al., 2009). Thus, our findings converge with previous work showing that information flow within the amygdala–PAG pathway alters as a function of threat proximity.

Physiological responses to pictures robustly show the expected effects of greater sympathetic (pupil dilation; see Bradley et al., 2008; Henckens et al., 2009) and parasympathetic (bradycardia; see Hare et al., 1970; Hermans et al., 2007; Lang and Davis, 2006) responses to negatively arousing pictures compared to neutral ones. Notably, and in line with previous studies (Bernston et al., 1994; Bradley et al., 2008), responses of these two branches of the autonomic nervous system to negatively arousing pictures were uncorrelated both within and across participants. This finding is consistent with theories of autonomic nervous system function that postulate that parasympathetic and sympathetic branches do not necessarily activate reciprocally (Öhman and Wiens, 2002). Particularly, it has been argued that shifts in autonomic balance occur as a function of proximity of threat, with relatively independent activation, or even coactivation, of the two branches at intermediate (“post-encounter”) levels of threat that are also characterized by freezing behavior (Lang and Davis, 2006). In the present study, this relative independence of parasympathetic and sympathetic responses on a trial-by-trial basis allowed us to identify neural activity associated with a parasympathetically as opposed to sympathetically dominated autonomic response pattern.

Trial-by-trial regression analyses revealed that PAG responses were consistently stronger on trials in which participants showed decelerations of heart rate. This association could not be explained by a concomitant sympathetic response: even though we found robust pupil dilation responses to negatively arousing pictures, these did not correlate with PAG responses. Moreover, the negative association between PAG responses and heart rate change remained significant when partialling out variance explained by pupil dilation. Interestingly, the maximum of this partial correlation was located in the ventral part of the PAG. Although this finding should be interpreted with caution given the limited spatial resolution of BOLD-fMRI as used here, it is consistent with a functional segregation of the PAG described in the literature (Linman et al., 2012): stimulation of ventral (lateral) part of the rodent PAG evokes passive defense including freezing, hypotension, bradycardia, and opioid analgesia (Bandler et al., 2000). Lesions to this region moreover selectively disrupt freezing (Ledoux et al., 1988; Liebman et al., 1970; Lyon, 1964). In contrast, the dorsal (lateral) portion of the rodent PAG has been associated with active forms of defense, including flight-or-flight behavior, hypertension, tachycardia, and non-opioid analgesia. Consistent with these notions, one human study reported an association between BOLD activation in the PAG and tachycardia in response to an acute (socially evaluative) stressor, although this activity was not localized explicitly to a PAG subregion (Wager et al., 2009). Future work using higher resolution functional neuroimaging

Table 2

<table>
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<tr>
<th>Region</th>
<th>Left hemisphere</th>
<th>Right hemisphere</th>
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<tbody>
<tr>
<td></td>
<td>x (mm)</td>
<td>y (mm)</td>
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<tr>
<td>Aversive stimuli: amygdala connectivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>-4</td>
<td>-30</td>
</tr>
<tr>
<td>Periaqueductal grey</td>
<td>-8</td>
<td>-14</td>
</tr>
<tr>
<td>Aversive stimuli: bradycardia correlation</td>
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<tr>
<td>Periaqueductal grey</td>
<td>-8</td>
<td>-14</td>
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<tr>
<td>Midbrain/thalamus</td>
<td>-8</td>
<td>-14</td>
</tr>
<tr>
<td>Supplementary motor area</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>18</td>
<td>-42</td>
</tr>
<tr>
<td>Inferior frontal gyrus (triangular)</td>
<td>8</td>
<td>46</td>
</tr>
<tr>
<td>Anterior cingulate Cortex</td>
<td>36</td>
<td>-6</td>
</tr>
<tr>
<td>Putamen</td>
<td>36</td>
<td>-10</td>
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<tr>
<td>Neuron stimuli: amygdala connectivity</td>
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<td>Midbrain</td>
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<td>Neutral stimuli: bradycardia correlation</td>
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<tr>
<td>Midbrain/thalamus</td>
<td>-4</td>
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<td>Amygdala</td>
<td>24</td>
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Notes: all coordinates are defined in MNI152 space. *, P<.05, whole-brain family-wise error corrected. **, P<.01, small volume corrected for a priori region of interest (see Materials and methods); MNI, Montreal Neurological Institute.
techniques will therefore be needed to determine more conclusively whether a functional segregation similar to that in rodents exists within the human PAG.

Although a direct association between PAG activity and actual behavioral freezing cannot be established with the current paradigm, there are several indirect links between these two variables. Parallel to the PAG, we observed both main effects of picture category and correlations with bradycardia in the rIFG, SMA, and striatum. A large body of human neuroimaging work has consistently associated activity in rIFG and SMA with motor planning and motor inhibition (Aron, 2011). These findings have been confirmed in human lesion studies (Aron et al., 2003) and studies using transcranial magnetic stimulation (Chambers et al., 2006; Verbruggen et al., 2010). Moreover, withholding motor responses has been shown to yield a cardiac decelerative response which is associated with activity in the rIFG (Jennings et al., 2009). Our findings also converge with a number of recent studies that have used stabilometric force platforms to demonstrate reductions in postural sway in response to aversive photographs (Azevedo et al., 2005; Facchinetti et al., 2006; Hagnenaars et al., 2012; Stins and Beek, 2007) and to negative facial expressions (Roelofs et al., 2010). Three of these studies (Azevedo et al., 2005; Hagnenaars et al., 2012; Roelofs et al., 2010) reported decelerative heart rate responses in addition to reductions in postural sway and thus establish a direct link between freezing-like behavior in humans and bradycardia. Notably, such reductions in postural sway are accompanied by an increase in mean oscillation frequency (Azevedo et al., 2005), which is thought to reflect postural stiffness resulting from increased muscle tension (Stins et al., 2010). Together, these findings suggest that a freezing-like state is accompanied by a preparation of motor programs that remain inhibited from execution until an increase in threat proximity triggers their release.

A number of previous neuroimaging studies have investigated neural regulation of defense behaviors in humans by systematically manipulating the (spatio-)temporal proximity of noxious events (Mobbs et al., 2007, 2009, 2010). These studies demonstrated a shift of neural activity

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**Fig. 5.** Trial-by-trial correlations of BOLD responses with physiological responses to negatively arousing photographs. (A) Stronger PAG responses are associated with bradycardia (negative HR change; n = 18 participants). (B) No correlation between PAG responses and PD (n = 15). (C) Association between PAG response and bradycardia remains significant when partialing out trial-by-trial variance in PD (n = 15). For further illustration of this effect, the peristimulus time histogram in (D) was estimated using a categorical model in which four categories of trials were defined using median splits of trials based on heart rate changes (low HR/high HR) and PD responses (low PD/high PD). Time courses are averaged across a spherical region (6 mm radius) around the local maximum of the correlation effect shown in (A) and (B). Statistical parametric maps are thresholded at P < .001, uncorrected (see Table 2 for corrected inferential statistics) and overlaid onto the averaged T1 weighted image (sagittal slices at X = 4) of all participants. Coordinates are defined in MNI152 space (left = left). In (D), error bars depict standard errors of the mean and the shaded area indicates trial duration. PSTH, peristimulus time histogram; HR, heart rate; PD, pupil dilation; a.u., arbitrary units, MNI, Montreal Neurological Institute.
Acknowledgments

From the ventromedial prefrontal cortex, ACC, and amygdala towards the PAG as threat becomes more proximal. Although these findings are consistent with animal models, they do not distinguish different types of defensive behaviors. Such a distinction may be critical because animal work has shown that defensive behaviors vary qualitatively rather than quantitatively: depending on possibilities of escape from threat, animals switch between mutually exclusive strategies of passive (freezing) or active (flight-flight) defense (Eilam, 2005), and both of these have been shown to involve downstream connections from the central nucleus of the amygdala such as the PAG (Gozzi et al., 2010). Similarly, theoretical models of human autonomic nervous system function distinguish qualitatively different autonomic response patterns reminiscent of these two distinct defense modes (Lang and Davis, 2006). Our findings thus suggest that human models of neural regulation of defense behavior (e.g., Maren, 2007) should be extended to accommodate a similar distinction at the neural level, and highlight the potential of using autonomic nervous system measures to link behavioral and neural levels of analysis.

Finally, our findings may provide new directions for research into the pathophysiology of anxiety disorders. In animals, freezing behavior not only serves to avoid detection by predators, but also facilitates heightened attention to threat cues (Lang and Davis, 2006). A large body of evidence supports the notion that patients with anxiety disorders are similarly biased to excessively process threat cues relevant to their concerns (Williams et al., 1996). For instance, research into deployment of spatial attention has shown that threat cues not only draw attention more easily in anxiety patients, but also impair attentional disengagement when task demands require participants to reorient their attention away from such stimuli (Koster et al., 2004). Especially this latter process has been associated with freezing (Fox et al., 2001; Roelofs et al., 2010) and is thought to contribute to a vicious circle in which attention is captured by negative information, and vice versa, negative appraisals lead to a further enhancement of sensory vigilance (Mathews and MacLeod, 1994). In agreement, stronger freezing-like behavior was found in panic disorder patients (Lopes et al., 2009) and in patients with a history of aversive life events (Hagenaaars et al., 2012). Thus, our findings raise the question whether anxiety disorder patients may have abnormalities in the amygdala–PAG circuitry identified in the present study.

In conclusion, the present study links activation of the human PAG and regions involved in motor inhibition to a parasympathetically dominated autonomic response profile that is characteristic of freezing behavior in both animals and humans. Although these findings were obtained in men and therefore cannot readily be generalized to women, they suggest that the human PAG subserves a passive defense mode of attentive immobility that is qualitatively different from the flight-or-flight response. By identifying a potential neural substrate of freezing in humans, our findings open new perspectives for translational research into the neural underpinnings of freezing-like behaviors that are observed in various anxiety disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neuroimage.2012.10.063.

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