Storm in a coffee cup: caffeine modifies brain activation to social signals of threat

Jessica E. Smith,[1] Andrew D. Lawrence,[2] Ana Diukova,[2,3] Richard G. Wise,[2,3] and Peter J. Rogers[1]

[1]School of Experimental Psychology, University of Bristol, [2]School of Psychology and [3]Cardiff University Brain Research Imaging Centre (CUBRIC), Cardiff University, UK

Caffeine, an adenosine A_1 and A_{2A} receptor antagonist, is the most popular psychostimulant drug in the world, but it is also anxiogenic. The neural correlates of caffeine induced anxiety are currently unknown. This study investigated the effects of caffeine on brain regions implicated in social threat processing and anxiety. Participants were 14 healthy male non/infrequent caffeine consumers. In a double-blind placebo-controlled crossover design, they underwent blood oxygenation level-dependent functional magnetic resonance imaging (fMRI) while performing an emotional face processing task 1 h after receiving caffeine (250 mg) or placebo in two fMRI sessions (counterbalanced, 1-week washout). They rated anxiety and mental alertness, and their blood pressure was measured, before and 2 h after treatment. Results showed that caffeine induced threat-related (angry/fearful faces > happy faces) midbrain-periaqueductal gray activation and abolished threat-related medial prefrontal cortex wall activation. Effects of caffeine on extent of threat-related amygdala activation correlated negatively with level of dietary caffeine intake. In concurrence with these changes in threat-related brain activation, caffeine increased self-rated anxiety and diastolic blood pressure. Caffeine did not affect primary visual cortex activation. These results are the first to demonstrate potential neural correlates of the anxiogenic effect of caffeine, and they implicate the amygdala as a key site for caffeine tolerance.

Keywords: amygdala; anxiety; fMRI; medial prefrontal cortex; periaqueductal gray; social threat

INTRODUCTION

Caffeine, prized for its psychostimulant effects, is consumed more often than any other drug worldwide, with an estimated 80–90% of adults reporting regular consumption of caffeine-containing drinks and foods (Fredholm et al., 1999; Heatherley et al., 2006a). Despite its popularity, caffeine has the adverse effects of increasing anxiety and raising blood pressure. This has been demonstrated in animal models of anxiety and in studies in humans (Bhattacharya et al., 1997; Rogers et al., 2008, 2010). Two human studies found that caffeine increased self-ratings of anxiety along with vigilance for social threat words (e.g. hated and lonely) (Rogers et al., 2006) and threat-related facial expressions (i.e. angry and fearful faces) (J.E. Smith et al., unpublished data). Notably, the anxiogenic effect of caffeine in humans is observed when administered at moderate doses relevant to dietary intake (equivalent to the caffeine present in 1–3 cups of ground coffee, Heatherley et al., 2006a) and in the form of coffee (Botella and Para, 2003), although the effect might be diminished with frequent caffeine intake owing to tolerance (Evans and Griffiths, 1992; Rogers et al., 2010).

These behavioral and physiological effects of caffeine occur primarily through antagonism by caffeine of the action of endogenous adenosine, at adenosine A_1 and A_{2A} receptors (Fredholm et al., 1999). Both receptors are considered to be modulators of glial function, neuronal communication and activity, and to be involved in anxiety and other mood disorders (Deckert et al., 1998; Ribeiro et al., 2002; Hohoff et al., 2010). In mice, genetic knockout of adenosine A_1 and A_{2A} receptors has been linked to increased anxiety (Ledent et al., 1997; Johansson et al., 2001), and recent studies in humans have discovered an association between adenosine A_{2A} receptor gene variation and caffeine induced anxiety (Alsene et al., 2003; Rogers et al., 2010). Moderate to high levels of adenosine and adenosine A_1 and/or A_{2A} receptors have been identified in the amygdala, medial prefrontal cortex (mPFC) and midbrain structures such as the periaqueductal gray (PAG) (Brass et al., 1986; Svenningsson et al., 1997; Rosin et al., 1998). These brain regions are considered to be crucially involved in threat processing, fear and anxiety, and to carry out these functions within a distributed neurocircuitry that involves top-down regulation orchestrated by the mPFC (Phelps and LeDoux, 2005; Price, 2005). More specifically, there is evidence from animal and human studies that the functions of specific subregions of these neural substrates of threat processing are threat-type specific (Keay and Bandler, 2001; Wager et al., 2009). This evidence suggests that the dorsolateral PAG (dlPAG), basolateral complex of the amygdala (BLA) and medial wall areas (areas 10, 25 and 32) of the mPFC (mPFC wall) are particularly associated with

activity related to social threat (An et al., 1998; Keay and Bandler, 2001; Wager et al., 2009). Effects of caffeine linked to anxiety in these brain regions have been observed in rats, including effects on neuronal activity (Singewald and Sharp, 2000; Singewald et al., 2003; Hale et al., 2010) and adenosine receptor function (Svenningsson et al., 1999). Together these findings suggest that the PAG, amygdala and mPFC wall may be neural correlates of the anxiogenic effect of caffeine; however, this hypothesis remains untested in humans.

Accordingly, this study investigated the effects of caffeine on activation in these brain regions to social signals of threat in healthy human volunteers. Participants were administered a single oral dose of caffeine (250 mg) or placebo in two blood oxygenation level-dependent (BOLD) functional magnetic resonance imaging (fMRI) sessions. They were assessed with fMRI while they performed an emotional face processing task with angry, fearful and happy faces (Hariri et al., 2002). This task has been used frequently in human fMRI studies to investigate neural correlates of anxiolytic and anxiogenic drugs (reviewed in Patin and Hurlemann, 2011). A region of interest (ROI) approach based on maximum probability maps (Eickhoff et al., 2006) was employed to test the primary hypotheses of this study. These were that caffeine would increase activation to social signals of threat relative to social signals of non-threat (angry and fearful faces > happy faces) in the midbrain in the region of the PAG (Wager et al., 2009), and in the amygdala, in particular the BLA, while it would decrease threat-related activation in the mPFC wall, a brain region implicated in emotion regulation (Bishop et al., 2004a; Kim et al., 2011). It was also expected that caffeine would increase self-rated anxiety and raise blood pressure.

Non/infrequent caffeine consumers were selected to minimize possible confounds of tolerance and withdrawal effects; nonetheless, relationships between caffeine effects on the ROIs and level of dietary caffeine intake were tested. To test for the existence of global pharmacological effects of caffeine on the BOLD signal, effects of caffeine on primary visual cortex (V1) activation were examined using a simple visual task.

MATERIALS AND METHODS
Participants
Fourteen healthy male volunteers participated in the present study. Suitability was assessed via questionnaire and followed up in a face-to-face interview. Key inclusion criteria were: male, right-handed dominance, aged between 18 and 65 years, good physical and mental health, which included no current or past medical or psychiatric illness, in particular anxiety/stress disorders, hypertension and/or heart disease, and not currently receiving medication (apart from occasional aspirin or paracetamol), no MRI contraindications, current non/infrequent dietary caffeine intake (see below), willingness to attend test sessions that would include MRI scanning and the consumption of caffeine, being a

non-smoker or a light smoker (≤ 5 cigarettes or equivalent a day), and blood pressure within normal range (cut-offs for systolic and diastolic blood pressure were ≥ 140 and ≥ 90 mmHg, respectively). Information on participants' caffeine intake over recent weeks (the 8 weeks preceding testing) was recorded during the week preceding testing using a caffeine intake questionnaire that assessed the frequency of consumption of teas, coffees, colas, other caffeine-containing drinks (e.g. Red Bull®) and products (e.g. Pro-Plus®), and chocolate (Heatherley et al., 2006b). Weekly caffeine intake was calculated from these self-report data using dietary and manufacturers' information on caffeine content (Heatherley et al., 2006a); for example, instant coffee 54 mg, ground coffee 105 mg, tea (bags, loose leaf, instant and green) 40 mg. These intakes were confirmed with participants on arrival at each fMRI session. Participants were restricted to individuals reporting no extreme sensitivity to caffeine (i.e. marked and distressing anxiety), but having a dietary caffeine intake of <280 mg per week, as the results of a prior study indicate this level of intake as around the threshold for withdrawal effects and tolerance (Rogers et al., 2010). Individuals reporting to have never consumed caffeine were excluded. Participants' trait anxiety was assessed using the trait version of the Spielberger State-Trait Anxiety Inventory (STAI) (Spielberger, 1983). The current investigation was limited to males to avoid any potential confounds associated with the menstrual cycle. The study was approved by the local research ethics committee, and all participants provided written consent prior to taking part.

Design and procedure
This was a double-blind crossover study, in which participants received 250 mg of caffeine or placebo in two fMRI sessions. Participants were previously asked to be sure to abstain from caffeine-containing products and not to consume alcohol from 9.00 p.m. on the evening before each session. They consumed a light (caffeine-free) snack 90 min prior to dosing. This dose of caffeine is equivalent to the caffeine present in 2–2.5 cups of ground coffee (Heatherley et al., 2006a), and has previously been found to modulate anxiety and blood pressure, particularly in non/infrequent caffeine consumers (Rogers et al., 2008, 2010). The treatment (caffeine/placebo) order was counterbalanced across participants, and the two fMRI sessions were spaced one week apart at the same time of day. The sessions began at 12.30 p.m. and participants received caffeine or placebo 30 min later. In both sessions, 1 h after treatment, participants performed a variant of the Emotional Face Processing Task (EFPT) (Hariri et al., 2002), then a simple visual task (SVT), while in the scanner. The timing of the fMRI session was designed to match the expected time window for peak plasma levels and psychoactive effects of caffeine (Fredholm et al., 1999; Rogers et al., 2010). Participants' current emotional states, alertness and blood pressure were assessed outside the scanner twice: before treatment (baseline) and 2 h after receiving

caffeine or placebo (post-treatment). The behavioral data described here are self-rated anxiety and mental alertness assessed using items (single or groups of descriptors, e.g. I feel anxious/worried/nervous; I feel mentally alert/attentive/observant) taken from the Mood, Alertness and Physical Sensations Scales (MAPSS) (Rogers et al., 2010). See supplementary material for full details of these scales. The study also included an fMRI simulation session completed within a mock MRI scanner. The primary aim of this was to alleviate anxiety related to the MRI procedure. See supplementary material for full details of the fMRI simulation session.

Drug administration

Caffeine BP (Caffeine anhydrous powder; Direct Food Ingredients Limited, Macclesfield, Cheshire, UK) and placebo (cornflour) were administered in white size 1 cellulose capsules (Capsuline, Pompano Beach, Florida, FL, USA). These caffeine and placebo capsules were identical in appearance, and were swallowed with 50 ml of room temperature water.

fMRI paradigm

The EFPT and SVT have been used frequently in previous pharmacological fMRI studies (reviewed in Patin and Hurlemann, 2011). The EFPT, adapted for this study, comprised a block-design involving incidental processing of social fear and anger. In brief, participants viewed a trio of faces with angry, fearful, happy or neutral expressions, derived from a standard validated set of faces [The Karolinska Directed Emotional Faces (KDEF), Lundqvist et al., 1998], and indicated which of two probe faces (bottom, left or right) was identical to a target face (top, centre). All three faces were of the same gender, and an equal number of male and female faces were presented. Each face was included in each of the four facial expression conditions once, as a target or probe. This task design allowed for isolation of activation in the ROIs specifically to social signals of threat (angry and fearful faces) relative to social signals of non-threat (happy faces), which differed only in the degree of threat signal they conveyed. For full explanation for the allocation of certain facial expressions to the threat and non-threat categories see supplementary material.

The task comprised a total of: 16 blocks of matching faces (as described above), with 4 blocks of each of the four facial expression conditions, interleaved with 8 blocks of matching simple shapes and 8 blocks of matching pictures of doors, similar to the instructions above, and 4 blocks during which participants viewed a fixation cross overlaid on a black screen. Each block consisted of 6 sequential 4-s matching trials. The blocks were split equally over two runs, each with a total scan time of 8.3 min (Figure 1). Participants responded during the task by pressing the left or right button on a button box using the index or middle finger of their dominant hand, respectively. These responses provided a measure of face-matching accuracy and reaction time. See supplementary material for additional details of the task.

The SVT consisted of alternating ON-OFF 20-s blocks of a fixation cross overlaid on a reversing visual checkerboard (frequency 8 Hz) (ON) or a black screen (OFF). Participants were instructed to fixate on the cross presented in the centre of the screen for the duration of the task (3.3 min). This task was employed to activate the visual cortex in order to test for global pharmacological effects of caffeine on the BOLD signal.

Image acquisition

Whole-brain T2*-weighted BOLD fMRI data were acquired using a gradient-echo echo-planar pulse sequence with a General Electric Excite-HDx 3T MRI scanner [repetition time (TR)/echo time (TE) = 3000/35 ms, flip angle = 90°, acquisition matrix = 64 × 64, field of view = 205 mm, in plane resolution 3.2 mm]. The volumes covered the whole brain in 46 slices (thickness 3 mm) and were acquired in line with the anterior commissure/posterior commissure line. Magnetic-field (B0) maps were acquired to correct image distortion caused by magnetic field inhomogeneities. Fieldmaps were calculated from the phase information of two 3D FSPGR images (TR/TE = 20/7 and 9 ms). After BOLD fMRI, a high-resolution T1-weighted FSPGR anatomical image (TR/TE = 7900/3 ms, inversion time = 450 ms, flip angle = 20°, 1 mm isotropic voxels) was acquired to assist in registration of functional images to standard Montreal Neurological Institute (MNI) space.

Data analysis

Preprocessing and statistical analysis of functional images was performed using FMRIBs Expert Analysis Tool (FEAT). A complete description of the preprocessing procedures is reported in supplementary material. At the first level of analysis, a separate general linear model was computed for each participant for each of the two runs of the EFPT they performed under caffeine and under placebo using FMRIBs Improved Linear Model (FILM) with local autocorrelation correction (prewhitening) (Woolrich et al., 2001). Experimental block conditions were modeled as boxcar regressors representing the occurrence and duration of each block. Brain activation to social signals of threat relative to social signals of non-threat was investigated using linear contrasts modeled as BOLD signal during angry/fearful face processing minus happy face processing (threat minus non-threat contrast). Reasons for including angry and fearful faces in one threat category and for restricting the non-threat category to happy faces are reported in supplementary material. To compute an average statistical map for the threat minus non-threat contrast for caffeine and for placebo, individual contrast maps of the appropriate runs were meaned in a second level fixed-effects model using FMRIBs Local Analysis of Mixed Effects (FLAME), by forcing the random effects variance to zero (Beckmann et al., 2003).
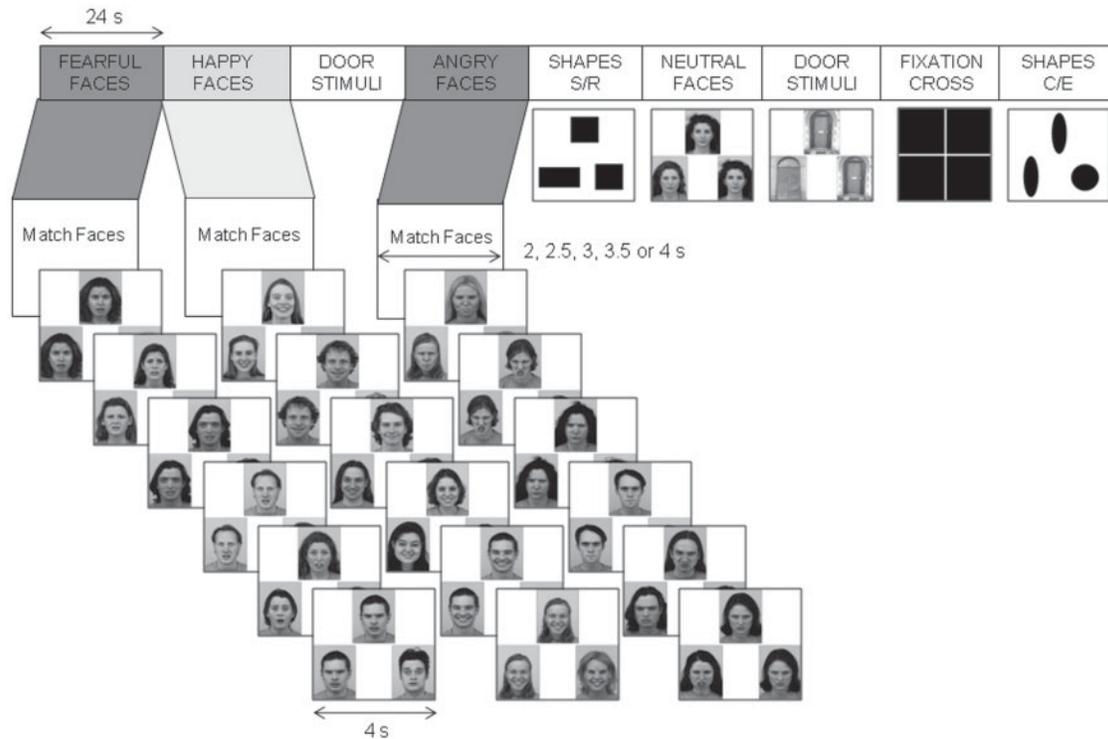
Fig. 1 Schematic diagram of the Emotional Face Processing Task (Hariri *et al.,* 2002). The diagram shows the first 9 blocks of one of the task runs. Each 24-s block consisted of 6 (4-s) matching trials, and for each trial participants were required to indicate which of two probes presented at the bottom of the image (left or right) was identical to the target (centre top) image. Duration of the 'match instructions' was jittered between 2 and 4 s (2, 2.5, 3, 3.5 or 4 s). The contrast of interest was angry/fearful face processing minus happy face processing. Examples of the stimuli presented in the blocks of matching neutral faces, simple shapes (C/E, circles/ellipses; S/R, squares/rectangles) and doors are shown. The fixation cross is also shown.

The ROIs included the midbrain-PAG, amygdala, in particular the BLA, and mPFC wall, which were defined using anatomical ROIs based on maximum probability maps derived from the Harvard–Oxford Cortical and Subcortical Structural Atlases (Harvard Centre for Morphometric Analysis) (midbrain-PAG and mPFC wall) and the Juelich Histological (cyto- and myelo-architectonic) Atlas (amygdala) (Amunts *et al.,* 2005). Defining ROIs where a change in activation is hypothesized reduces the problem of multiple comparisons since fewer simultaneous comparisons are required to test the hypothesis. Therefore, the tests that are conducted are more sensitive (Eickhoff *et al.,* 2006). Anatomical ROIs confer the added benefit of unbiased estimates of effect size in the ROIs since they are independent of the functional data analyzed (Vul *et al.,* 2009; Kriegeskorte *et al.,* 2010). Further, maximum probability maps define the most likely area each voxel of the reference space is assigned to, allowing for anatomical localization of non-overlapping clusters of activated voxels (Eickhoff *et al.,* 2006). Accordingly, this probabilistic anatomical ROI approach has proved feasible, reliable and sensitive in several other fMRI studies (e.g. Eickhoff *et al.,* 2006; Hurlemann *et al.,* 2008; Onur *et al.,* 2009), especially in comparison to other ROI approaches (e.g. spheres, boxes, etc, Eickhoff *et al.,* 2006). Moreover, this approach is recommended as an optimal method for pharmacological fMRI studies (e.g. Mitsis *et al.,* 2008), and is considered the most appropriate approach to the study of functional-anatomical correlations (Vul *et al.,* 2009). Thus, the present study employed this hypothesis-driven approach. See supplementary material for details of a supplementary whole-brain analysis conducted. The extent and location of the midbrain-PAG, mPFC wall and BLA ROIs are shown in Figures 2A, B and 3A, respectively. Full details of the ROIs are described in supplementary material. Using FSLs Featquery tool, the probabilistic maps of the ROIs were overlaid onto the average statistical map for caffeine and for placebo to determine the regional probability-weighted mean percent BOLD signal change for the threat minus non-threat contrast. This yielded a BOLD signal change value for each participant under caffeine and under placebo in each ROI. These data were then analyzed off-line using *t*-tests. Signal changes under placebo in the ROIs were analyzed using one-sample *t*-tests (test value = 0) to test for effects of threat. Effects of caffeine were assessed using paired *t*-tests (caffeine *vs* placebo). Correlation analysis (Pearson's *r*) was performed to test whether effects of caffeine calculated for the ROIs (signal change for the threat minus non-threat contrast for caffeine minus that for placebo) were related to level of dietary caffeine intake.

The existence of global pharmacological (e.g. vascular) effects of caffeine on the BOLD signal were assessed by examining V1 activation during visual stimulation ON minus OFF using the methods described above (see supplementary material for full details). Bayesian analysis, using the Jeffreys–Zellner–Siow (JZS) Bayes-factor paired t-test (Rouder et al., 2009), was conducted to quantify support for and against the null hypothesis that there was no effect of caffeine on V1.

Change from baseline data were calculated for self-rated anxiety and mental alertness, and (systolic and diastolic) blood pressure, by subtracting the baseline data from the post-treatment data. These data were analyzed for effects of caffeine using paired t-tests (caffeine vs placebo). Effects of caffeine (caffeine vs placebo) and threat (angry/fearful faces vs happy faces) on face-matching accuracy (percentage correct) and reaction time (ms) were also assessed with paired t-tests.

Statistical significance was set to $P < 0.05$, and all t-test and correlation analyses described above were conducted using SPSS 16.0.1 for Windows. Directional hypotheses (see 'Introduction' section) were tested using one-tailed tests. Where applicable, the 95% within-subject confidence interval (WSCI) (Loftus and Masson, 1994) or the 95% confidence interval for Pearson's correlation, based on Fisher's r–z transformation, was computed. In addition, the bootstrap 95% confidence interval for Pearson's r was computed based on 5000 iterations (Efron, 1988). Effect sizes (Pearson's r) for the ROI analyses were calculated, and by convention $r = 0.10$ indicates a small effect, $r = 0.30$ indicates a medium effect and $r = 0.50$ a large effect (Rosnow and Rosenthal, 2003).

RESULTS

Participants

The participants were aged between 18 and 55 years (mean $\pm$ SD $= 26.4 \pm 9.1$ years). They were nonsmokers, and all had consumed caffeine as part of their diet. Their weekly dietary caffeine intake ranged from 0 to 88 mg (29.1 ± 36.8 mg/week). Scores on trait anxiety ranged from 27 to 50 (37 ± 9). These scores are similar to published norms for this age group (36 ± 10) (Spielberger, 1983).

Behavioral and blood pressure data

Caffeine vs placebo increased anxiety [$t(13) = 4.58$, $P = 0.0005$] and diastolic blood pressure [$t(13) = 3.56$, $P = 0.002$], but did not affect systolic blood pressure ($P > 0.1$). Face matching was performed with high accuracy (>90%). Neither caffeine nor threat (angry/fearful faces vs happy faces) had an effect on face-matching accuracy or reaction time, and caffeine did not affect mental alertness (all P's > 0.1). Behavioral and blood pressure data are reported in Supplementary Table S1.

fMRI data

Caffeine induced threat-related (angry/fearful faces > happy faces) midbrain-PAG activation that did not exist under placebo [$t(13) = 2.13$, $P = 0.0265$, $r = 0.27$] (Figure 2). Threat-related mPFC wall activation was observed under placebo, which was abolished by caffeine [$t(13) = -2.28$, $P = 0.020$, $r = 0.29$] (Figure 2). There was modest threat-related amygdala activation under placebo that reached trend level significance, in particular in the BLA ROI ($P = 0.06$, small-to-medium effect size $r = 0.21$). For all results on the effects of threat under placebo see Supplementary Table S2. There was no overall effect of caffeine on threat-related amygdala activation (for BLA, CMA and SFA P's > 0.1, rs = 0.01, 0.06 and 0.02, respectively). However, correlation analysis revealed individual differences in the extent to which caffeine induced threat-related amygdala activation that were negatively correlated with level of dietary caffeine intake [$r(12) = -0.71$, $P = 0.005$] (Figure 3). This correlation was specific to the BLA ROI (for the other ROIs P's > 0.1).

V1 activation to visual stimulation was confirmed ($P < 0.001$, $r = 0.87$). No effect of caffeine on this was found ($P > 0.1$, $r = 0.002$) (Supplementary Figure S1), and this did not vary as a function of level of dietary caffeine intake ($P > 0.1$). The JZS Bayes-factor supported this; the null hypothesis that there was no effect of caffeine was found to be five times more probable than the alternative hypothesis.
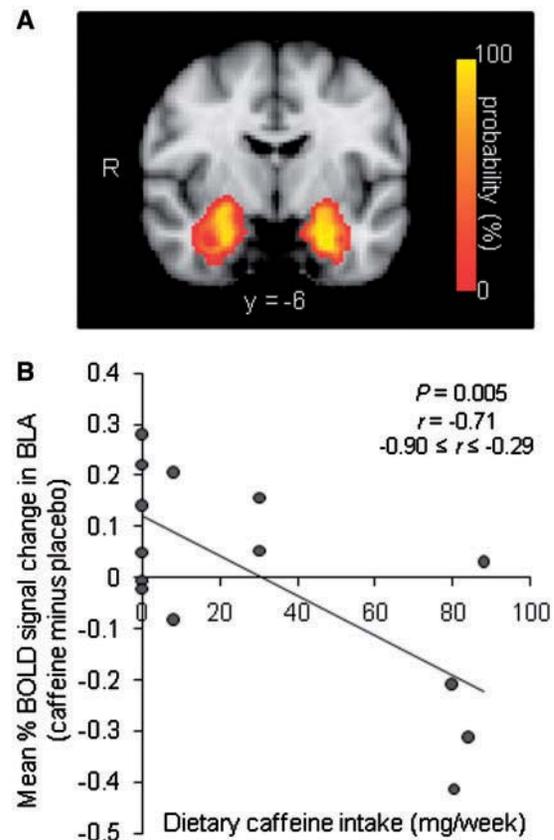
DISCUSSION

The present results provide novel evidence that caffeine modifies brain activation to social signals of threat in the human brain. Furthermore, they show that this can occur at a dose typically consumed during a single day. Specifically, caffeine induced threat-related midbrain-PAG activation and abolished threat-related mPFC wall activation. The extent to which caffeine induced threat-related amygdala activation was negatively related to level of dietary caffeine intake. The observed changes in threat-related brain activation were accompanied by increased self-rated anxiety and blood pressure, as was expected from previous studies (Rogers et al., 2008, 2010). These results are consistent with converging lines of evidence from animal and human studies demonstrating involvement of the PAG, amygdala and mPFC wall in threat processing and anxiety (Keay and Bandler, 2001; Price, 2005; Wager et al., 2009). There is evidence that suggests these brain regions form part of a functional neurocircuit dedicated to social threat processing (An et al., 1998; Keay and Bandler, 2001; Hoffman et al., 2007; Wager et al., 2009). Furthermore, pharmacological fMRI studies in humans investigating the neural correlates of other drugs or agents that modulate anxiety have found comparable effects on these brain regions using the same emotional face processing task (reviewed in Patin and Hurlemann, 2011). Thus, the results of this study fit well with the anxiogenic effect of caffeine and implicate several brain regions as neural correlates of caffeine induced anxiety.
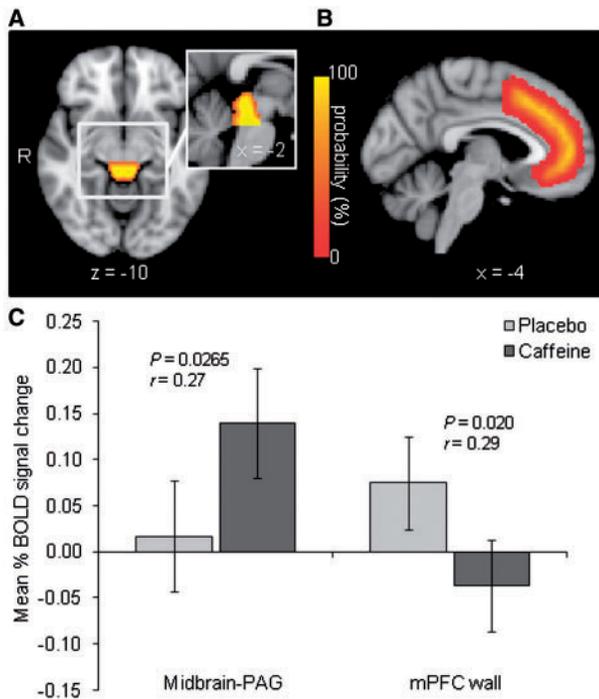
Fig. 2 Colored regions represent the probabilistic maps of (**A**) the midbrain-PAG ROI displayed on an axial and sagittal slice and (**B**) the mPFC wall ROI displayed on a sagittal slice. All slices are T1-weighted MNI templates and all coordinates are in MNI space. Colorbar represents probability levels coded by different colors; yellow color marks those voxels that are included in the ROI with 100% probability (**C**) Effects of caffeine on mean percent (%) BOLD signal change for the threat (angry/fearful faces) minus non-threat (happy faces) contrast in a priori ROIs. Error bars show 95% within-subject confidence intervals (Loftus and Masson, 1994). Effect size is indicated by *r* (Rosnow and Rosenthal, 2003).

Fig. 3 (**A**) Colored region represents the probabilistic map of the BLA ROI displayed on a coronal slice of a single T1-weighted template in MNI space (MNI $y = -6$). Colorbar represents probability levels coded by different colors; yellow color marks those voxels that are included in the ROI with 100% probability (**B**) Scatterplot of effects of caffeine on mean percent (%) BOLD signal change for the threat (angry/fearful faces) minus non-threat (happy faces) contrast in the a priori BLA ROI as a function of level of dietary caffeine intake. Data are signal change meaned across the left and right BLA ROIs. The 95% confidence interval for *r* based on Fisher's *r*–*z* transformation is displayed. The bootstrap 95% confidence interval (5000 iterations) for *r* was $-0.90 \le r \le -0.17$ (Efron, 1988).

The finding that caffeine induced threat-related midbrain-PAG activation is consistent with studies in rats that showed that caffeine, similar to other anxiogenic drugs, induced c-Fos expression, a marker of neuronal activity, in the PAG (Singewald and Sharp, 2000), and enhanced anxiety-like behaviors evoked by stimulation of this region (Jenck *et al.*, 1995). The dlPAG especially was found to be involved in these effects of caffeine (Jenck *et al.*, 1995; Singewald and Sharp, 2000) and, interestingly, it is this region of the PAG that is implicated, by other animal research, in responding preferentially to social threat (Keay and Bandler, 2001). It may be that threat-related midbrain-PAG activation was not present under placebo in the present study because this region is considered to respond under heightened threat conditions (Price, 2005; Mobbs *et al.*, 2007).

The effect of caffeine in abolishing threat-related mPFC wall activation is consistent with other studies demonstrating a role for this region in the regulation of anxiety, and in emotion regulation processes more generally. For example, high state and trait anxiety have been found to be negatively correlated with mPFC activation to threat (Bishop *et al.*, 2004a; Mathews *et al.*, 2004) and altered structural and functional connectivity between the mPFC and amygdala

(Kim and Whalen, 2009; Kim *et al.*, 2011). Clinical anxiety disorders, including post-traumatic stress disorder, are associated with reduced mPFC activation to threat (reviewed in Kim *et al.*, 2011), and notably a recent study showed reduced activation in the mPFC during social interaction (a trust game) in social anxiety disorder (SAD) (Sripada *et al.*, 2009). Conversely, increased mPFC activation to threat has been observed in individuals with better emotion regulation abilities (Drabant *et al.*, 2009) and during instructed emotion regulation (McRae *et al.*, 2010).

The precise regulatory mechanisms supported by the mPFC are currently unclear, but it is likely that this region contributes to several aspects of emotion regulation (Etkin *et al.*, 2011), beyond just regulation of amygdala activity. Regulation of systems such as the PAG by the mPFC is considered critical for maintaining context-appropriate visceral responses and behavior (Price, 2005) and, notably, studies of the anatomy of mPFC-PAG projections in rat and monkey

brains have discovered that the mPFC wall projects exclusively into the region of the PAG linked to social threat processing (An *et al.,* 1998; Floyd *et al.,* 2000; Price, 2005). Anatomical tracing studies also suggest the BLA is involved in regulating activity in that particular region of the PAG (An *et al.,* 1998). Concurrent with these anatomical data and the present results, Fos immunochemistry studies have revealed that, along with the PAG, putative rat homologues of the mPFC wall areas and the BLA are strongly targeted by caffeine and other anxiogenic drugs (Bennett and Semba, 1998; Singewald *et al.,* 2003; Hale *et al.,* 2010).

Taking this evidence together, it would appear from the present results that caffeine acts to increase threat-related activation in the midbrain-PAG and associated anxiety directly and/or by decreasing mPFC wall 'control' over this threat-related activity. Regarding the primary target(s) of caffeine, it may be that the mPFC functions as a gate to activity in other regions, including the PAG and amygdala, but there is also evidence of bidirectional influences (An *et al.,* 1998; Price, 2005).

Turning to the present results for the amygdala, although threat-related activation in this region under placebo was not statistically significant, there were trend significance levels with modest effect sizes. This is perhaps to be expected given the healthy sample used in this study (e.g. Ewbank *et al.,* 2009). Electrophysiological and fMRI studies in monkeys have shown that, at best, there is a very modest amygdala processing bias in favor of threatening faces in the context of greater activation to faces in general, and this bias appears to vary across individuals (Gothard *et al.,* 2007; Hoffman *et al.,* 2007). By no means do all human fMRI studies in healthy individuals find enhanced amygdala activation to threat (angry and fearful) *vs* non-threat (neutral and happy) faces (e.g. Onur *et al.,* 2009; Santos *et al.,* 2011). Part of the reason for this may be that within healthy populations there are marked individual differences in the magnitude of amygdala activation to threat, which are strongly influenced by both state and trait anxiety (Bishop *et al.,* 2004b; Ewbank *et al.,* 2009). In addition, previous pharmacological fMRI studies, including one that used the probabilistic maps of the amygdala employed in this study, have found that anxiogenic drugs may be required to induce a clear effect of threat on amygdala activation (Onur *et al.,* 2009). Such studies have also identified individual differences in the extent to which anxiolytic and anxiogenic agents modulate this effect (Cools *et al.,* 2005). These differential responses may be due to differences in underlying personality traits (e.g. Cools *et al.,* 2005) and/or drug intake due to tolerance (e.g. Kobiella *et al.,* 2011). Consistent with this, caffeine induced threat-related BLA activation, but primarily in those individuals with lower levels of dietary caffeine intake. Effects of caffeine on the BLA are in agreement with animal studies that found acute caffeine-induced c-Fos expression specifically in this region (Bennett

and Semba, 1998; Hale *et al.,* 2010). Furthermore, the present result may indicate a tolerance effect, given that the BLA is characterized as a key site of plasticity in fear (including social fear) learning (Fanselow and LeDoux, 1999; Johnson *et al.,* 2009). Further support for this comes from a study in rats that linked the development of tolerance to stimulatory effects of caffeine, including those associated with fear/anxiety, with alterations in adenosine (A_1) receptor function in the BLA (Svenningsson *et al.,* 1999). There is also strong evidence that tolerance to the effect of caffeine on self-rated anxiety develops in humans (Evans and Griffiths, 1992; Rogers *et al.,* 2010).

What is striking about the present result is that it occured in a sample of non/infrequent caffeine consumers, where differences in tolerance might not be expected from previous research (e.g. Rogers *et al.,* 2010). It may instead be argued that variation in the ability of caffeine to induce threat-related BLA activation reflects trait differences in vulnerability to caffeine induced anxiety that modulate caffeine intake. However, prior findings indicate that caffeine intake is little affected by the tendency of caffeine to increase anxiety, at least in part, because substantial tolerance develops to this effect even at modest levels of dietary intake and even in susceptible individuals (Rogers *et al.,* 2010). Notwithstanding this strong argument for a tolerance effect, a longitudinal study is required to discriminate definitively between the two possible explanations discussed (cf. Evans and Griffiths, 1992; Richardson *et al.,* 1995).

At the neuropharmacological level, together animal and human research has identified moderate to high levels of adenosine, adenosine A_1 and/or A_{2A} receptor binding sites in particular in the PAG, BLA and equivalents of mPFC wall areas (Brass *et al.,* 1986; Glass *et al.,* 1996; Rosin *et al.,* 1998; Svenningsson *et al.,* 1997). Furthermore, electrophysiological studies in rats found that adenosine activity modulated synaptic transmission in the PAG and BLA (Bagley *et al.,* 1999; Heinbockel and Pape, 1999). Combined with studies implicating adenosine receptor antagonism in caffeine induced anxiety (Alsene *et al.,* 2003; Rogers *et al.,* 2010), these data support the effects of caffeine on threat-related brain activation in the present study. Nonetheless, further studies are necessary to elucidate the exact neuropharmacological mechanism(s) underlying caffeine effects.

It is unlikely that the changes in threat-related brain activation in this study reflect global pharmacological effects of caffeine on the BOLD signal, since the results did not reveal an effect of caffeine on V1 activation to a simple non-threatening visual stimulus. Nor did this result vary as a function of level of dietary caffeine intake. They do not appear to reflect changes in mental alertness and/or task performance either, as no effects of caffeine on these measures were found. Absence of mentally alerting and cognitive performance-enhancing effects of caffeine in non/infrequent caffeine consumers has also been reported previously (reviewed in Rogers and Smith, 2011).

The results of studies investigating global effects of caffeine on the BOLD signal are mixed (e.g. Mulderink et al., 2002; Laurienti et al., 2003; Liau et al., 2008; Chen and Parrish, 2009). One argument is that caffeine may act universally to enhance the contrast of the BOLD signal by decreasing baseline cerebral perfusion (Mulderink et al., 2002). However, the relationship between baseline cerebral perfusion and the BOLD signal is complex, with studies showing that it is not possible to consistently increase BOLD signal change by decreasing baseline cerebral perfusion with caffeine (Laurienti et al., 2003; Liau et al., 2008). Furthermore, most of the studies that have investigated global effects of caffeine have not accounted for level of dietary caffeine intake, which the results of one study suggest may be an important modulatory factor (Laurienti et al., 2002). Specifically, that study compared the effects of caffeine on BOLD signal change in V1 induced by visual stimulation (ON-OFF blocks of a flashing checkerboard) in frequent and infrequent caffeine consumers (reported to have a mean daily dietary caffeine intake of 648 and 41 mg, respectively). It would appear from the results that an enhancing effect of caffeine on BOLD signal contrast is limited to individuals with higher levels of dietary caffeine intake. Indeed, the results of the present study do not show a general caffeine induced increase or decrease in BOLD signal change, and the lack of an effect of caffeine on BOLD signal change in V1 is probably due to the carefully selected sample of non/infrequent caffeine consumers. Therefore, it would seem the null results in this study support the notion that the effects of caffeine on the midbrain-PAG, BLA and mPFC wall are regionally selective and specific to threat processing.

The present results may also be relevant to the heightened sensitivity to the anxiogenic effect of caffeine reported in clinical populations such as panic disorder and SAD (Hughes, 1996; Nardi et al., 2009). Hyper- or hypoactivity in the brain regions found to be affected by caffeine in this study has been implicated in these and other anxiety disorders (e.g. Graeff and Del-Ben, 2008; Sripada et al., 2009; Kim et al., 2011; Tuescher et al., 2011). Perhaps interactions between this neural alteration and effect of caffeine on these brain regions explain the increased anxiogenic effect of the drug in such populations. More generally, the results might be relevant to future face processing studies in healthy and clinical populations, suggesting they take into account both acute and chronic caffeine intake.

This study should be considered preliminary, with some limitations that need to be acknowledged. First, the assignment of activation sites was probabilistic; therefore, it is not possible to conclude definitively that activation was limited to the ROIs. It is also worth noting that current fMRI is unable to resolve subregions of the PAG. Secondly, the selection of non/infrequent caffeine consumers is a possible source of bias in this study. It might be argued that this selection bias may have produced atypical effects of caffeine. It does mean that the results may not be generalizable to typical frequent caffeine consumers, who, as discussed previously, develop substantial tolerance to the anxiogenic effect of caffeine (Rogers et al., 2010). Nonetheless, they are instead relevant to individuals vulnerable to anxiety and/or consuming caffeine for the first time or after an extended period of caffeine abstinence, because they indicate neural correlates of anxiety per se and specifically anxiety increased by caffeine. The results may also be relevant to the development of tolerance to caffeine-induced anxiety. It should also be emphasized that all participants in this study had consumed caffeine as part of their diet, and those that had discontinued consuming caffeine reported that this was not due primarily to its anxiogenic effect. Furthermore, their trait anxiety scores did not differ from population norms.

In conclusion, the present results are the first demonstration in humans of modification by caffeine of brain activation to social signals of threat in brain regions implicated in anxiety. Specifically, there was a pronounced and selective impact of caffeine on threat-related activation in the midbrain-PAG and mPFC wall. Also, effects of caffeine on extent of threat-related amygdala activation were related to level of dietary caffeine intake, which may reflect the well-known plasticity of this region, suggesting it as a site for caffeine tolerance. Thus, the findings provide potential neural correlates of the anxiogenic effect of caffeine in humans and the development of tolerance to this effect.

SUPPLEMENTARY DATA

Supplementary data are available at SCAN online

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