β-Adrenergic blockade reduces cerebral blood flow in stress-related brain regions during acute stress

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The stress response is one of the most important mechanisms governed by the neuroendocrine system. Nonetheless, the effect that stress hormones have on the brain remains understudied in humans. We investigated state changes in neuronal activity in a young, healthy male population, during a situation of stress. We measured cerebral blood flow (CBF) with a specialised neuroimaging technique, arterial spin labelling. Using a double-blind, placebo-controlled paradigm, we tested the effects of β-adrenergic receptor blockade and cortisol synthesis inhibition. Salivary cortisol and α-amylase measures confirmed the effectiveness of our pharmacological manipulation. We found specific CBF reductions in response to β-blockade in the right amygdala, and in a cluster spanning the superior medial frontal gyrus/anterior cingulate cortex (sMPF/ACC). In addition, we found a whole brain reduction in CBF in response to β-blockade. We did not find a CBF effect of cortisol inhibition. Our study provides evidence for involvement of the β-adrenergic system in neuronal modulation of the entire brain, with a focus on the amygdala and sMPF/ACC during stress.

Keywords: amygdala; anterior cingulate cortex; arterial spin labelling; cerebral blood flow; norepinephrine; glucocorticoid; stress.

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Stress in an organism is a transitory state, which can be induced by any event that disturbs its homeostasis or threatens to do so, evoking the need for a means to neutralise the stressor and return to a state of homeostasis. The stress response as it is in humans, shaped by millions of years of evolution, enables us to effectively escape, learn from, and avoid danger. Even today, it is still very much entwined with everyday well being, and disturbance of the system can lead to psychiatric diseases (de Kloet, Joels, & Holsboer, 2005; Lupien, Maheu, Tu, Fiocco, & Schramek, 2007). Stress therefore remains one of the most active fields of study within cognitive neuroscience. Although much progress has been made, there are still important gaps in our understanding of the mechanisms by which hormones and the brain work together to create and maintain a stress response. The sites and mechanisms of interaction of stress hormones and the brain deserve further investigation.

We know that there are two principal hormonal systems involved in the stress response: the sympatho-adrenergic system, with its main effectors epinephrine and norepinephrine (NE), and the slower-responding hypothalamus-pituitary-adrenal (HPA) axis, with glucocorticoid release by the adrenal cortex as its hallmark (Ulrich-Lai & Herman, 2009). These hormone systems both have a specific role in the initiation and maintenance of a state of stress. When the brain perceives a stressor, specific areas in the brainstem and the hypothalamus become active, and the sympathetic nervous system releases epinephrine into the bloodstream, via its terminals in the medulla of the adrenal glands (and some NE, both can be classified as catecholamines). The locus coeruleus increases its activity, and thereby supplies NE to a large number of different brain centers. There are two main types of cellular receptors for (nor)epinephrine: the α- and the β-adrenercic receptors. Both are present in the brain. Kvetnansky, Sabban, & Palkovits (2009) have written an extensive overview of the central sympathetic system. Simultaneous with sympathetic activation, the paraventricular nucleus of the hypothalamus releases arginine-vasopressin and corticotrophin-releasing hormone into the circulation of the median eminence. These releasing factors reach the anterior pituitary, which releases adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH, when it reaches the adrenal cortex, induces a release of glucocorticoids into the blood (cortisol in man, corticosterone in rodents; Ulrich-Lai & Herman, 2009). The HPA axis works much slower than the sympatho-adrenergic system, partly because it activates via hormones in the peripheral blood stream.

Catecholamines are hydrophilic, and therefore do not cross the blood-brain barrier, but peripheral increase in catecholamines can exert its effects on the brain via activation of the sensory vagus nerve. This signals the nucleus of the solitary tract, which stimulates the locus coeruleus and other noradrenergic brainstem nuclei. These have their projections to almost the entire brain, meaning that via this route, peripheral catecholamines can influence neuronal activity over the entire brain also (Roozendaal, Okuda, De Quervain, & McGaligh, 2006).

Glucocorticoids are secreted in a circadian rhythm, with a peak right after awakening, after which blood levels gradually decrease (VanCauter, Leproult, & Kupfer, 1996). In contrast to the catecholamines, glucocorticoids are lipophilic in nature. This means that glucocorticoids in the blood can cross the blood-brain barrier to affect the brain. The cellular receptors for this hormone fall into two classes: the mineralocorticoid receptor has a very high affinity for glucocorticoids, and it remains highly occupied under normal conditions, even during the trough of the circadian rhythm. The glucocorticoid receptor on the other hand, has a lower affinity, and it becomes progressively more activated with cortisol increases tied to the morning-rise or to stress (de Kloet et al., 2005).

The amygdala is one of the key players in the brain's response to stressful situations, both in animals and humans (see for a recent and detailed review Ulrich-Lai & Herman, 2009). Its role in memory modulation is especially well studied. For instance, activation of the amygdala enhances emotional memory retention via projections to the hippocampus (LaBar & Cabeza, 2006). The amygdala has also been marked as one of the principal loci for an interaction between NE and glucocorticoids in the brain. Glucocorticoids have been shown to enhance the intracellular noradrenergic signal cascade in this region. They also seem to increase stress-induced NE release into the amygdala, and their effects on memory are dependent on noradrenergic activation of β-adrenoceptors in the basolateral amygdala (Roozendaal et al., 2006). It has been shown that infusion of NE into the mouse amygdala activated

fos, an immediate early gene, indicating that NE release into this region has prolonged effects in the form of changes in genetic activity (Stone, Zhang, Hiller, Simon, & Hillman, 1997).

Thus it seems that during the state of stress, the (nor)epinephric and glucocorticoid systems feed back on the amygdala to upregulate activity in a co-dependent manner. However, empirical data showing this effect in humans is still limited. Some consistent data stems from event-related functional magnetic resonance imaging (fMRI) studies (Strange & Dolan, 2004; van Stegeren et al., 2007). Despite this, most of our knowledge is still based on animal studies. In addition, the applicability of some of this human data is limited because of specificity in methodology. Studies that work with blood-oxygen level dependent (BOLD) fMRI cannot effectively pick up slow variations in signal, due to problems with signal drift (Smith et al., 1999). In addition, some investigations make use of stress induction using social-evaluative methods (such as the Trier Social Stress Test, TSST; Kirschbaum, Pirke, & Hellhammer, 1993) often involving mental arithmetic or similar non-stress related tasks (see e.g. Young, Abelson, & Cameron, 2005). The disadvantage is that this is far removed from actual traumatic situations that might arise (such as crime situations or a war scenario). More specifically, in task-induced stress methods, the stress effects are entangled with an unrelated cognitive operation, and stress is purely driven by a social-evaluative context. This limits the usefulness of the results in explaining brain function during trauma situations, and psychiatric disorders that relate to them, such as Post-Traumatic Stress Disorder (PTSD). Thus, it remains an open question how prolonged activation of the amygdala in response to acute stress is dependent on the (nor)adrenergic and cortisol systems in humans.

Here, we investigated the effects of (nor)adrenaline and cortisol on the amygdala directly after a stressful experience. We followed up with a more explorative analysis of the whole brain, where we tried to identify other regions whose activity under stress depends on these systems. By inhibiting either hormone using a pharmacological manipulation, we were able to investigate their neuronal influence. We used a β-adrenergic receptor blocker, propranolol, to suppress the noradrenergic system, and a cortisol synthesis inhibitor, metyrapone, to suppress the cortisol system.

Because hormones act on the brain on a relatively long timescale (minutes to hours), one needs to utilise a technique that is able to pick up state changes in brain activity (tonic rather than phasic changes). In this study, we utilised the fMRI technique known as arterial spin-labelling (ASL; Williams, Detre, Leigh, & Koretsky, 1992), which can measure cerebral blood flow (CBF). The technique makes use of arterial blood as an endogenous tracer, by inverting the spins in the carotid arteries. ASL has been shown to have good reproducibility over time and between subjects, and it does not suffer from temporal drift, as classic BOLD fMRI does (J. J. Wang et al., 2003). It has recently been shown that most of the variance in CBF during resting-state is caused by trait rather than state variations (Hermes et al., 2009). These characteristics make ASL well-suited for our purposes. For inducing stress in our population, we use a new and effective technique, employing a more "ecologically" valid design, with a stressor that is not linked to a cognitive task. Instead, by presenting participants with a combination of four stressors designed to resemble a traumatic scenario, we elicit moderate stress that is similar in nature to a traumatic experience. The study was designed to test the prediction that NE receptor blockade using propranolol will lower CBF in the human amygdala under conditions of acute stress. We further tested the prediction that inhibition of the cortisol system by metyrapone would also lower CBF, but not more than propranolol, because cortisol effects on the amygdala depend on noradrenergic activation. Our third prediction was that there would be other brain areas which show CBF reduction or increase with our pharmacological manipulation.

Methods

Participants

For this study, we recruited 41 healthy, non-smoking, right-handed males (age 18 – 35, mean age 22.6). The study was approved by the ethics committee of the University Medical Centre st. Radboud, Nijmegen, and was conducted in accordance with the declaration of Helsinki. All participants were required to sign informed consent prior to inclusion. Inclusion criteria were a body mass index between 18.5 and 30 kg/m^2, and normal hearing and (corrected to) normal vision. Respondents were excluded if they reported an average alcohol consumption of more than 3 beverages per day, or psychotropic medication or

recreational drugs more than once a week. Prior to participation, subjects did not use alcohol for 24 hours, and no psychotropic medication or recreational drugs for 72 hours. Further exclusion criteria were: regular use of corticosteroids, irremovable metal parts in or around the body, current or past psychiatric treatment, neurological treatment, endocrine treatment, or autonomic failure, current parodontitis, claustrophobia, intense daily physical exercise, and irregular sleep/wake rhythm. In addition, we applied a number of criteria in order to avoid risks related to the medication used in the present study, as follows: a history of heart related disease, A-V block, sinus bradycardia, hypotension, history of obstructive respiratory disease, chronic renal failure or adrenal dysfunction, hyperthyroidism, diabetes mellitus, Raynaud's syndrome, oversensitivity to β-blockers, history of cortisol-related dysfunction, plasma cortisol levels outside the range of 150 – 700 mmol/l, active peptic or duodenal ulcers, and active inflammatory disease. The participants were randomly divided into three groups, with different pharmacological treatment, as detailed below. There were no differences among groups for any medical or psychological variable (see results section for details). We administered a non-selective β-adrenergic antagonist to the first group (40 mg of propranolol), and a cortisol synthesis inhibitor to the second group (metyrapone, 2*750 mg). The third group acted as a control, and received only placebo. Drug administration was double-blind.

Materials and Apparatus

An electrocardiogram (ECG) was taken with a 12-lead ECG machine (CardioTouch 3000, Bionet America, Inc., Tustin, CA, US), and blood pressure was measured with a wrist blood pressure monitor (Braun BP 1600 VitalScan Plus, Braun GmbH, Kronberg, Germany). Blood samples were taken in order to asses blood cortisol levels. Participants filled out a questionnaire about their medical history, and Dutch versions of the Spielberger State-Trait Anxiety Inventory (STAI; van der Ploeg et al., 1980), the Beck Depression Inventory (BDI; Beck, Erbaugh, Ward, Mock, & Mendelsohn, 1961), the NEO-Five Factor Inventory (NEO-FFI; Costa and McCrae, 1992), and the Positive And Negative Affect Schedule (PANAS; Crawford & Henry, 2004). Saliva samples were collected using Salivette sampling devices (Sarstedt, Nümbrecht, Germany), stored at -20º C, and then transported to the laboratory for analysis.

Subjects were scanned on a Siemens (Erlangen, Germany) Avanto 1.5 Tesla MRI scanner, with an 8-channel head coil. Subjects could give feedback with a 4-button response device, controlled with four fingers of the right hand (not the thumb). Electrical stimulation was applied using a Digitimer DS7A stimulator (Digitimer LTD, Letchworth, England), with Ag/AgCl finger electrodes applied to the left index and middle finger.

Procedures

We invited participants for a pre-screen in advance of the test day. During this pre-screen, after informed consent was signed, we tested the participants for all inclusion and exclusion criteria by way of questionnaires, measured base heart rate (60 sec.) and blood pressure. We took a blood sample for assessing blood cortisol level, and the subjects filled in the STAI, BDI, NEO-FFI, and PANAS as a baseline control.

A typical test day would start at 8:00 in the morning (t = 0), and last until 19:15. Starting time varied between 7:00 and 10:00. On the test day, subjects were first informed of the planning of the day. The schedule for drug application, saliva sampling, questionnaires, and scanning can be reviewed in figure 1.

Ten saliva samples were taken: two baseline measures, one day before the test day, which the participants took themselves, at home, 15 minutes before lunch and 15 minutes before dinner, and one each on the test day at t = 5 min., 1h30, 2h50 (shortly prior to scanning), 3h45 (during scanning), 4h40 (immediately after scanning), 9h30, 10h15, and 11h00. Saliva was collected to obtain a measure of salivary cortisol, and salivary α-amylase (sAA). Salivary cortisol has been shown to be a reliable measure of blood free cortisol levels (Riadfahmy, Read, Walker, & Griffiths, 1982). Increases in salivary α-amylase have been shown to correlate with NE increases, independent of salivary flow rate (Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004; Rohleder, Wolf, Maldonado, & Kirschbaum, 2006). A recent review confirmed its validity as a measure for sympathetic activation, although its direct correlation with blood levels of catecholamines was found to be somewhat weak (Nater & Rohleder, 2009). Simultaneously with each saliva sample, except the sixth and seventh, participants reported their current affect by way of a PANAS.

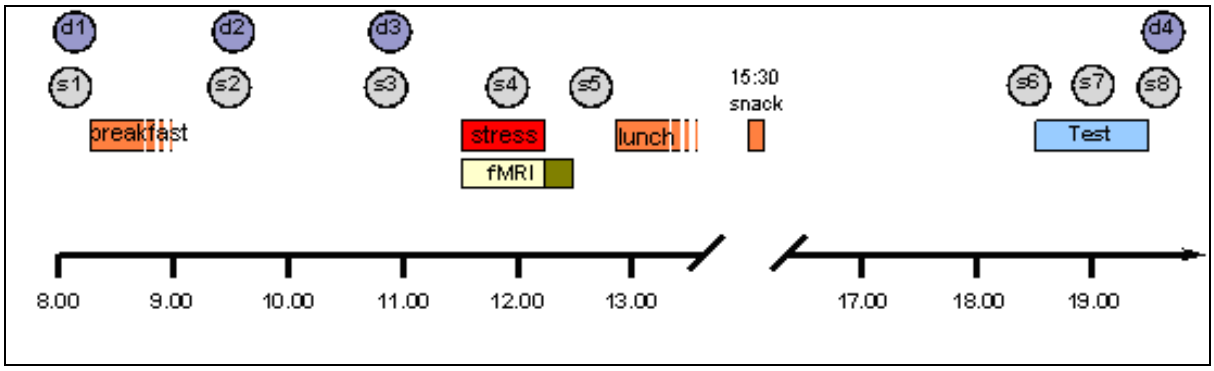

Figure 1. Timeline of a test day. d1 – d4: instances of placebo/drug administration. d1 could be placebo or 750 mg metyrapone, d2 could be placebo or 40 mg propranolol, d3 equal to d1, d4 could be 1mg dexamethasone or placebo. s1 – s8: saliva samples. A Positive And Negative Affect Scale PANAS was administered at s1 – s5, and at s8. breakfast: subjects were allowed to have a restricted meal after the first drug administration. stress: time of stress induction. fMRI: functional magnetic resonance imaging. Light yellow block represents event-related scanning, dark block represents arterial spin labelling and T1 scanning. Lunch, snack: respective times at which the subjects had their lunch and a snack. Test: time at which subjects received the memory tests.

There were four time points at which drug or placebo was administered. Participants received their first round of medicine immediately after the first saliva sample. First medicine was 750 mg of metyrapone for the metyrapone group, or placebo for the other groups. The second saliva sample was immediately followed by a dose of propranolol (40 mg) for the propranolol group, or a placebo for the other groups. The third saliva sample was followed by the second dose of metyrapone (750 mg) or placebo, as appropriate. Finally, just before leaving medical supervision, at t = 11h05, participants in the metyrapone group received a dose of 1 mg of dexamethasone, in order to restore glucocorticoid availability and to avoid acute adrenal insufficiency. Other groups received placebo. Similar procedures have been used successfully in the past (see e.g. Lupien et al., 2002; Maheu, Joober, Beaulieu, & Lupien, 2004).

To ensure that salivary measures were not influenced by food intake, we scheduled the intake of food for our participants on the test day. We requested that they eat or drink nothing but water for two hours prior to study, and that they did not floss, brush their teeth, or take chewing gum. After the first saliva sample and drug time point, subjects were given the opportunity to have breakfast, with the restriction that they take no coffee or large amounts of carbohydrates or fat. Immediately after the fifth saliva sample (which followed the scanning session), subjects had their lunch, and two hours before the sixth saliva sample, we provided a small snack (candy bar or the like).

Participants were placed into the MRI scanner in a head-first, supine position. In order to reduce head motion artifacts, the subject's head was secured in the head coil with foam pillows. Participants received ear-plugs and a set of head-phones to reduce scanner noise and to enable communication from outside the scanner room.

During fMRI scanning, we applied a combination of four methods of stress induction. First, participants were shown 111 pictures taken from the International Affective Picture System (IAPS), in random order, with high negative valence and arousal. These pictures were also part of a subsequent memory paradigm (data reported elsewhere). Secondly, the picture viewing was intermixed with 4 short movie clips involving extreme violence (taken from the movie Irréversible, by Gaspar Noé). The timing of movies and pictures was as follows: at t = 3h30 (beginning of scanning), they were presented with the first movie clip (2 min. 20 sec.), followed by a first set of 37 pictures, a second movie clip (1:30), another 37 pictures, a third movie clip (1:30), a last set of 37 pictures, and the fourth and final movie clip (1:30). Third, stress was enhanced by application of an aversive auditory input, consisting of a repetitive sound fragment. Fourth, participants were told they might receive a random number of electrical shocks (maximum 4) during the scanning session. In order to avoid shock habituation, we instructed subjects that any potential following shock would have a higher intensity than the last. The intensity was graphically depicted on the presentation screen. Electrodes

were applied to the left index and middle finger. Two short electrical pulse trains were actually applied, the first one consisting of five 2ms stimulations, 18 ms apart, which was applied on the 12th minute of scanning. The second one consisted of ten pulses with the same duration and spacing, and was given on the 30th minute after beginning of scanning. The digitimer was set to a maximum of 1.75 mA. Apart from our four stress induction methods, it has been shown that fMRI scanning in itself can be a stressful experience for participants, and it can induce an increase of cortisol levels (Raz, Lieber, Soliman, Peterson, & Posner, 2005; Tessner, Walker, Hochman, & Hamann, 2006).

During the stress induction methods, subjects were scanned with an echo-planar imaging (EPI) sequence, and we applied a classical event-related fMRI paradigm (data presented elsewhere). The EPI was interrupted after the first picture set (13th minute of scanning), in order to collect salivette #4, and to enable the participant to fill in a PANAS, using the buttonbox. After the event-related paradigm, and after all stress induction methods had ceased, we scanned subjects for 7 minutes with a continuous ASL (CASL) sequence, and after that, we recorded a 5-minute T1-weighted anatomical scan. During these scans, participants were instructed to lie still, with eyes closed, and to think of nothing in particular. Participants were taken out of the scanner 1 hour after the beginning of the first EPI sequence.

Scanning parameters

The parameters for our CASL sequence were as follows: We acquired 120 volumes, interleaved with and without labelling. Labelling time was 2000 ms, and an 800 ms delay was inserted between labelling and image acquisition. Acquisition was done with a gradient-echo 2D EPI readout, field of view (FoV) 224*224 mm, axial slices had an in-plane resolution of 3.5*3.5 mm, and 5.0 mm slice thickness. There was a 2.5 mm slice gap, echo time (TE) 11 ms, total repetition time (TR) for one volume 3500 ms, flip angle 90º. Fifteen axial slices were acquired in an interleaved ascending order. See for a description of the original sequence protocol J. J. Wang et al. (2005). The T1-weighted scan was acquired with a 3D magnetisation-prepared rapid gradient echo volumetric scan. FoV 256*256 mm, resolution 1.0*1.0*1.0 mm, 176 contiguous sagittal slices, TE 2.95 ms, TR 2250 ms, inversion time 850 ms with 15º flip angle.

Data processing

Laboratory analyses on the saliva samples were performed as reported in Rohleder et al. (2006). Salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Concentration of α-amylase in saliva was measured by an enzyme kinetic method: Saliva was processed on a Genesis RSP8/150 liquid handling system (Tecan, Crailsheim, Germany). First, saliva was diluted 1:625 with double-distilled water by the liquid handling system. Twenty microliters of diluted saliva and standard were then transferred into standard transparent 96-well microplates (Roth, Karlsruhe, Germany). Standard was prepared from ''Calibrator f.a.s.'' solution (Roche Diagnostics, Mannheim, Germany) with concentrations of 326, 163, 81.5, 40.75, 20.38, 10.19, and 5.01 U/l α-amylase, respectively, and bidest water as zero standard. After that, 80 ml of substrate reagent (a-amylase EPS Sys; Roche Diagnostics, Mannheim, Germany) were pipetted into each well using a multichannel pipette. The microplate containing sample and substrate was then warmed to 37°C by incubation in a waterbath for 90 s. Immediately afterward, a first interference measurement was obtained at a wavelength of 405 nm using a standard ELISA reader (Anthos Labtech HT2, Anthos, Krefeld, Germany). The plate was then incubated for another 5 min at 37ºC in the waterbath, before a second measurement at 405 nm was taken. Increases in absorbance were calculated for unknowns and standards. Increases of absorbance of diluted samples were transformed to α-amylase concentrations using a linear regression calculated for each microplate (Graphpad Prism 4.0c for MacOSX, Graphpad Software, San Diego, CA). Inter- and intra-assay variation was below 10%. Salivary free cortisol concentrations were measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/ml (IBL, Hamburg, Germany).

The CASL imaging time series were preprocessed using MatLab 7.5 (The MathWorks, Natick, Massachusetts) and the SPM5 software package (Wellcome Department of Cognitive Neurology, London, UK, http://www.fil.ion.ucl.ac.uk/spm/software/spm5/). The first five volumes were discarded to allow for stabilisation of the magnetisation. Then, the imaging series were realigned to correct for head motion, using a sum-of-squared-differences minimisation algorithm, and a 6 parameter rigid-body transformation. Realignment was done in two passes, where in the second pass, the images were

realigned to the mean image from the time series of the first pass. Time series mean images were then coregistered to their corresponding anatomical image using a mutual information algorithm with rigid-body transformation. The resulting transformation parameters were subsequently applied to all images in the series. Images were then spatially smoothed using a Gaussian kernel, 8mm full width at half-maximum. We used the ASL data processing toolbox (ASLtbx, http://www.cfn.upenn.edu, see also Z. Wang et al. (2008) for the next steps in the analysis. First, images were converted into floating-point format. The toolbox contains an algorithm which picks out the voxel with the highest measured value. The algorithm then masks out all the voxels that change less than 20% of this value. This way, a head-mask is created, so that the background is not taken into the calculation. After applying this algorithm, each labelled image was subtracted from its preceding control image, using the pair-wise subtraction method, as described by Z. Wang et al. (2008), and CBF was quantified using the model published by J. J. Wang, Zhang et al. (2005). We estimated the labelling efficiency at 71%. We should note here that this has not yet been verified in our lab. Therefore, it is possible that the absolute CBF values that we calculated are not in line with current literature. However, as we are interested in the differences between conditions in this instance, we do not need the absolute values. The relative differences should not be influenced by a flaw in the estimation of the labelling efficiency, as it is a linear transformation. After calculation of the CBF time series, the CBF maps were averaged across time for each subject. The individual anatomical images were normalized to a Montréal Neurologic Institute (MNI) space T1 template image, using a full affine and non-linear transformation in SPM5, and these transformations were applied to the averaged CBF images. We selected an anatomically defined region of interest (ROI) of the left and right amygdala, based on the WFU Pickatlas (Maldjian, Laurienti, Kraft, & Burdette, 2003). We tested these ROIs with the general linear model implemented in SPM5, using an initial threshold of $p = 0.005$, and following up with cluster-level statistical analyses. In addition to this, we searched for whole-brain relevant results using the same initial threshold of $p = 0.005$, followed by whole-brain corrected cluster-level statistics. To test for the effects of global changes in CBF, we extracted the mean brain CBF value of the average of the time series for each participant. These were entered into a one-way ANOVA with between-subjects factor drug, followed by planned contrasts between each of the drugs vs. placebo.

Analysis of PANAS and Salivary measures

All PANAS and salivary measures, as well as the tests for population differences between our drug groups were performed with the SPSS 15.0 for Windows software package (SPSS Inc., Chicago, Illinois, US). We used a significance criterion of $\alpha = 0.05$. We corrected all PANAS scores from the test day with their associated baseline measure taken at the pre-screen. The resulting baseline-corrected PANAS scores were further analysed by way of an ANOVA with time as a within-subject factor (6 levels) and drug as between-subject factor. Positive affect was analysed separately from negative affect, and any main effects or interactions of factors were followed up by T-tests between individual time points.

Results

Test for group difference in biological or psychiatric background

We performed separate ANOVAs to verify that there were no inherent differences between the populations in each pharmacological group concerning age, scores on BDI, STAI, NEO-FFI trait neuroticism, base systolic and diastolic blood pressure, and base heart rate. We found no significant differences between our populations (all p-values > 0.4).

Positive and Negative Affect

We performed a repeated-measures ANOVA on the baseline-corrected PANAS scores, entering the factor time, with six levels, and drug as between-subjects factor to see if there was an effect of our stress induction on positive or negative affect. First, we tested positive affect. The factor time had a significant effect ($F(5,34) = 4.773$, $p = 0.002$). This was further analysed with single contrasts. We found that at the first time point of the day, positive affect was significantly higher than the third, fifth, and sixth time points ($p = 0.027$, $p < 0.001$, and $p = 0.027$, respectively). In addition, positive affect at time point five (directly after scanning) was significantly less than at time point one, two, three, and four, but not six ($p < 0.001$, $p = 0.024$, $p = 0.040$, $p = 0.001$, and $p = 0.277$, respectively). Drug had no effect on positive affect ($F(2,38) = 1.460$, $p = 0.245$) and neither did the interaction between drug and time ($F(10,70) = 1.058$, $p = 0.406$). This suggests that there were no effects of our drug manipulation on positive affect.

We performed the same test on the negative affects scores. There was an effect of time ($F(5,34) = 9.751$, $p < 0.001$), which could be traced back to the fact that time point 4 and 5 differed from all other measures ($p \leq 0.001$ for all individual contrasts of 4 and 5 with other measures). This suggests that stress was accompanied by a higher negative affect, which lasted at least until directly after the stress induction. There was no effect of drug on overall negative affect ($F(2,38) = 0.606$, $p = 0.551$), and the factors time and drug did not show an interaction ($F(10,70) = 0.283$, $p = 0.983$), which suggests that our drug manipulations had no effect on the negative affect of our participants.

Salivary measures

The data from our salivary cortisol measurements is plotted in figure 2. To test whether our stress induction was accompanied by a cortisol rise, we calculated the area under the curve (AUC) from time point 3 (just before stress induction) to time point 5 (just after), setting the measurement from time point 3 as our baseline (as indicated in the plot). First, we tested whether the increase was significantly greater than zero in our placebo group via a one-sample T-test. This turned out to be the case ($t(13) = 2.209$, $p = 0.046$). We next performed an ANOVA with between-subjects factor drug, to test whether the increase was different between the pharmacological groups. There was a significant effect ($F(2,38) = 4.453$, $p = 0.019$), which we further analysed by contrasting each of our two medicine groups with the placebo. The metyrapone group showed a lower mean than the placebo group ($t(22.07) = -2.379$, $p = 0.026$), while propranolol did not differ ($t(20.72) = 0.979$, $p = 0.339$). We also wanted to see if the drug had any effect on the main cortisol level over the day, regardless of stress. Therefore, we performed another ANOVA, this time analysing the AUC from time point 1 (before application of the first drug) to time point 5. We found a significant main effect of drug ($F(2,38) = 9.169$, $p = 0.001$), which could be pinned down to a larger cortisol decrease in the metyrapone group than in the placebo group ($t(19.15) = -3.785$, $p = 0.001$). Propranolol versus placebo yielded no significant difference ($t(23.41) = -1.072$, $p = 0.259$). To summarise, our stress induction went accompanied by a rise in cortisol in the placebo group, which was not matched by the metyrapone group, suggesting that metyrapone abolished the stress-related cortisol rise in our current study. Apart from this, metyrapone decreased the overall cortisol levels during the test day in our sample, which is in line with expectations.

We next examined the α-amylase measurements (plotted in figure 3). It is known that this measure has a diurnal cycle, with low levels in the morning, and higher levels in the evening (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007; Rohleder et al., 2004). This diurnal trend might inflate our effects (contrary to the diurnal trend of cortisol, which goes in the opposite direction). Therefore, in order to test cleanly, we tried to control for the diurnal rhythm by detrending the data. Detrending of the α-amylase data, as well as foregoing this procedure in the case of cortisol, can both be viewed as a conservative measure. We estimated the linear trend in sAA level across subjects from the two time points at which a sample was acquired at home, the day before the experiment. These measures averaged 52.91 U/l before lunch (estimated to correspond with time point four on the day of test, t = 3.5 h), and 67.56 U/l before dinner (estimated t = 10.25 h). From this, we deduced the average rate of increase to be 2.17 U/l/h. In the following analyses, the data is detrended by subtracting 2.17*(time from reference point in hours) from every sample. After detrending, we calculated the AUC from time point three to five, as we did for cortisol before. We tested whether this area was more than zero for the placebo group, using a one-sample T-test, but we found no significant result ($t(13) = 0.769$, $p = 0.456$). We tested for differences in this increase between groups using an ANOVA (between-subjects factor drug), but again we were not able to show significant differences ($F(2,37) = 0.274$). These results suggest that neither does our stress induction go accompanied by an increase in sAA levels, nor is there an influence of our pharmacological manipulation on sAA increases during stress. Our next interest was to see if there was a general effect of drug on sAA levels. To test this, we detrended the data from time point one to five, as described before, and then tested the AUC for these samples with an ANOVA. There was an effect of drug ($F(2,37) = 4.863$, $p = 0.013$), stemming from a difference between propranolol and placebo ($t(37) = -3.058$, $p = 0.004$), where sAA levels in the propranolol group were lower. The contrast between metyrapone and placebo was non-significant ($t(37) = -0.934$, $p = 0.356$). Summarising, stress did not induce a rise in sAA levels in any of our groups, but, as expected, propranolol induced an overall reduction in sAA levels over the day.

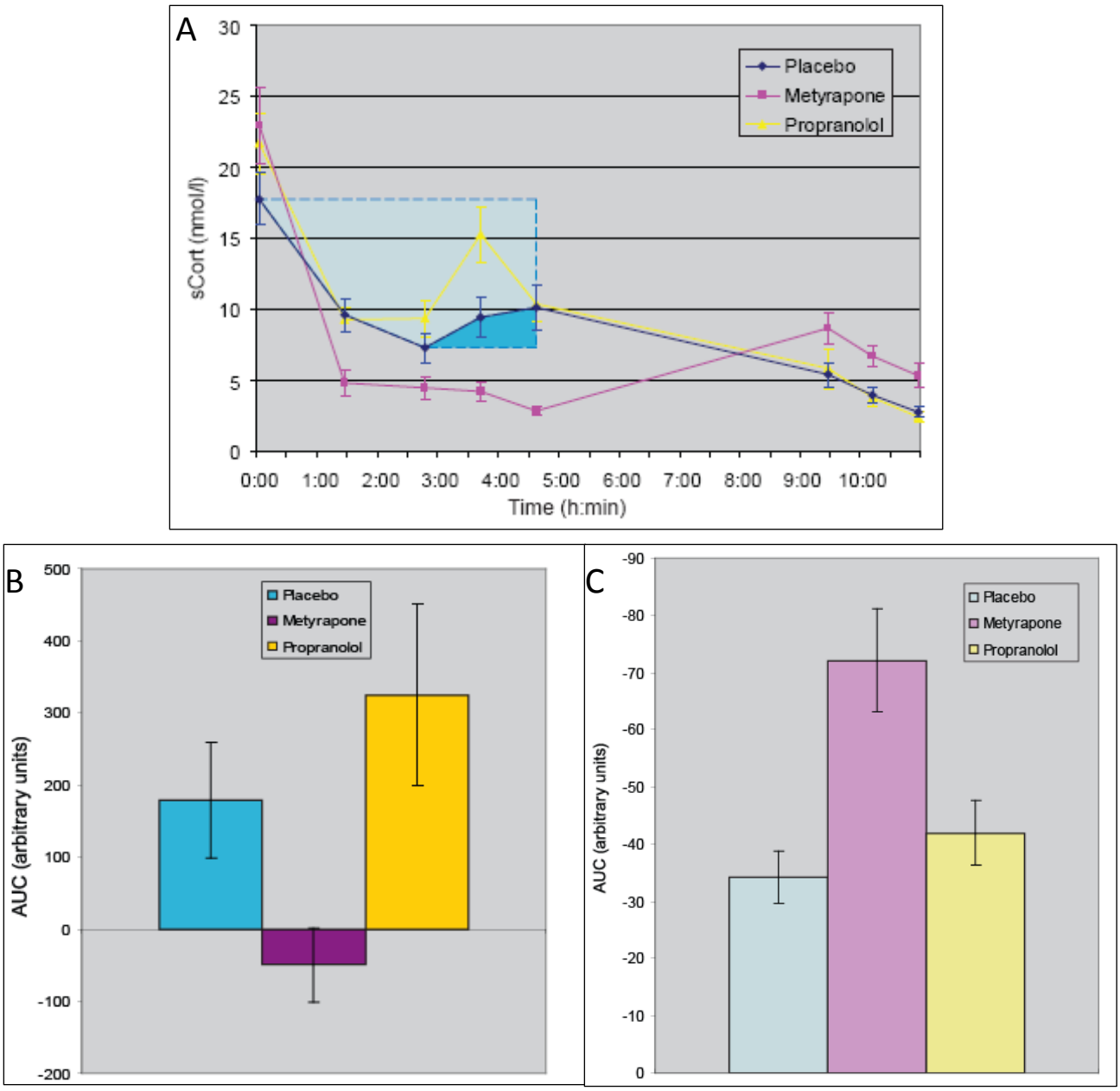

Figure 2. Salivary cortisol data from 8 time points on the testday. (A) Separate lines are shown for the placebo goup, the metyrapone group, and the propranolol group. The dark-blue shaded area under the placebo group indicates the area under the curve (AUC) which we tested to verify cortisol increase accompanying stress. The light blue area above the curve indicates the area we tested for drug influence on main cortisol levels. The same tests were performed on the other drug groups, but not shown for comprehensibility. (B) AUC data during stress (time point 3 to 5) extracted for each of the drug groups, as used to assess stress-induced increase. (C) AUC data from the start of the day to end of stress (time point 1 to 5), as used to assess whole-day levels. The scale is inversed for aesthetic reasons. sCort: level of salivary cortisol. All error bars represent standard error of the mean.

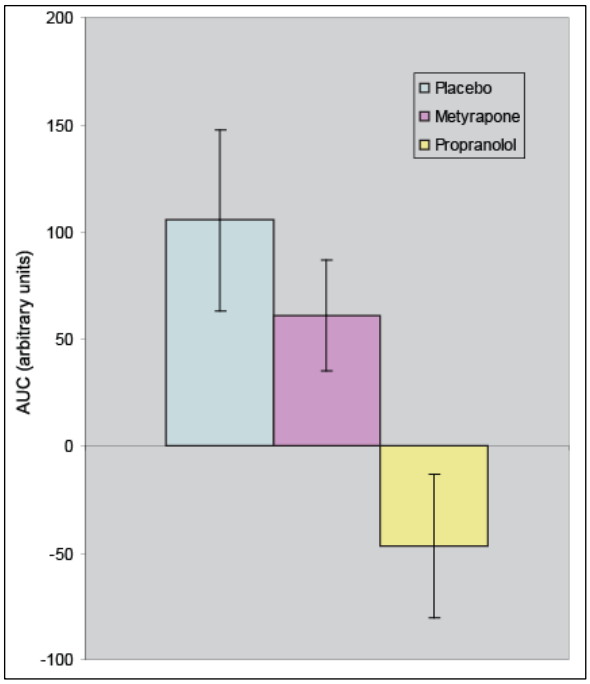

Figure 3. Salivary α-amylase (sAA) data. The bar graphs represent area under the curve (AUC) from the start of the day to end of stress (time point 1 to 5), as used to assess whole-day levels, analogous to the cortisol analysis.

CBF data

As we had a specific a priori hypothesis for the amygdala, we tested for an effect of propranolol on its perfusion using a small-volume correction, with an anatomical mask of the right amygdala and the left amygdala sequentially. In the contrast of propranolol vs. placebo, we found that right amygdala CBF was lower in the propranolol condition, as attested by a significant cluster, with a clustersize of 15 voxels, cluster-level p-vaue of 0.032, and the peak voxel at x = +28, y = -2, z = -18; peak t(38) = 3.15 (coordinates in MNI space, voxel size 2*2*2 mm). The left amygdala yielded no suprathreshold clusters. We tested for effects of metyrapone versus placebo in the same way, but found no significant clusters, even when we reduced the initial threshold to p = 0.01. See figure 4a for a graphical representation of the right amygdala contrast.

In order to test whether this effect was specific for the right amygdala or if there were more brain areas involved, we decided to do a whole-brain analysis with the same initial threshold of 0.005, but cluster-level corrected for whole brain search volume. We tested for whole-brain positive and negative effects of each of the drugs versus placebo. There was a large cluster which extended over the superior medial prefrontal gyrus (sMPF) and the anterior cingulate cortex (ACC) with decreased CBF in the propranolol condition when compared to placebo (i.e. same effect as in right amygdala). The cluster was mainly right-lateralised (clustersize: 707 voxels, with the peak voxel at x = +6, y = +50, z = 0) The peak t(38) = 4.24; cluster-level p < 0.001. The graphical

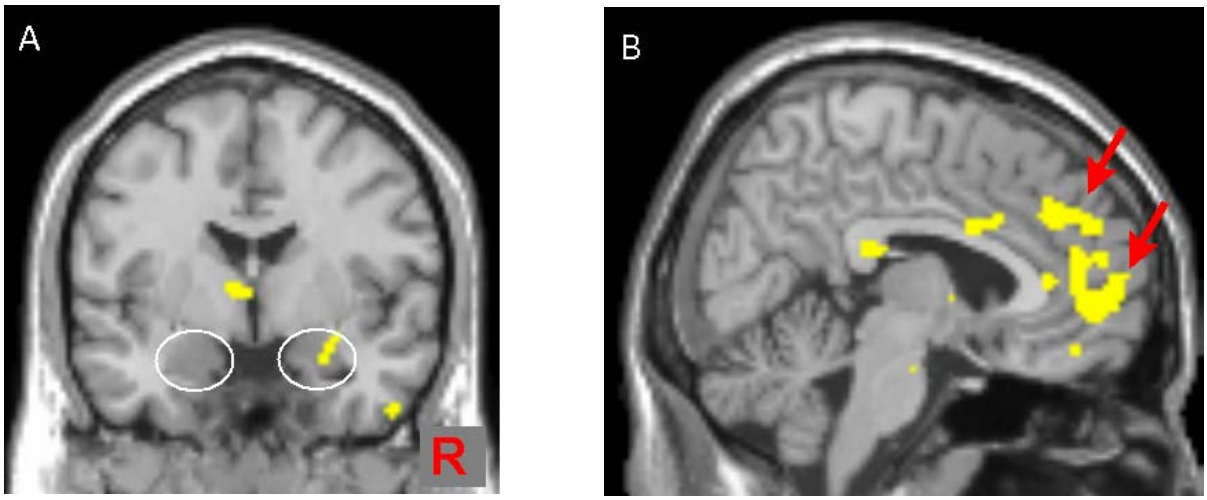

Figure 4. Cerebral blood flow (CBF) differences between drug and placebo groups. (A) Canonical single-subject anatomical image, coronal section at y = -2. Shown in yellow are clusters which show lower CBF in the propranolol condition than in the placebo condition, thresholded at p < 0.005 uncorrected for display purposes. The location of the amygdalae is indicated with a white ellipse. (B) Canonical single-subject anatomical image, sagittal section at x = +6 mm. Shown in yellow are clusters which show lower CBF in the propranolol condition than in the placebo condition, thresholded at p < 0.005 for display purposes. The twoapparent clusters indicated by red arrows are part of the same cluster. R = right.

depiction of this cluster is given in figure 4b. This suggests that the sMPF/ACC also experiences a reduction in CBF when propranolol is administered.

We went on to see if the overall perfusion, when averaged over the entire brain, was affected by drug. The overall mean CBF was computed for each subject and entered into a one-way ANOVA with between-subjects factor drug. The effect of drug showed a trend ($F(2,38) = 2.745$, $p = 0.077$), and the contrasts showed that the propranolol group had a lower perfusion than placebo ($t(38) = -2.166$, $p = 0.037$), while there was no difference between metyrapone and placebo ($t(38) = -0.286$, $p = 0.777$).

Discussion

Our aim in this study was to investigate the effects that the two main stress hormone systems, the (nor)adrenergic and the glucocorticoid system, have on the human brain, when they are produced endogenously in response to an ecologically plausible stressor. We tested the effects of β-blockade and cortisol synthesis inhibition on amygdala CBF during stress, and made an explorative analysis of the rest of the brain. We found that β-blockade reduces CBF in the right amygdala, and in a cluster that spans parts of the sMPF and ACC. There was a non-significant decrease in right amygdala CBF after cortisol synthesis inhibition also.

Effectiveness of stress induction

Our salivary measures, as well as the PANAS data we obtained, suggest that stress induction was successful. The fact that cortisol was raised during and after scanning in the placebo group gives an indication that the hormonal stress response was activated. At the same time, it seems that a significant increase of α-amylase could not be achieved, probably due to the large natural variability in this measure. On the other hand, we see that positive affect was lower at the time point directly after scanning (12 minutes after stress induction had ended), combined with a very strong increase of the negative affect during and directly after scanning. Taken together, it seems safe to conclude that our four-pronged stress induction method had the desired effect.

Amygdala and superior Medial Prefrontal Cortex/ Anterior Cingulate Cortex

Our analyses reveal that specific decreases in CBF are induced in the right amygdala and the sMPF/ACC when propranolol is applied to the stressed brain. It is clear from animal literature, that β(1)- and β(2)-adrenoceptors are abundantly present in the neurons of the basolateral amygdala, and that they exert cellular effects via a G-protein coupled intracellular signaling pathway (Qu, Guo, & Li, 2008). The fact that brain perfusion is attenuated with β-blockade suggests a tonic depression of neuronal activity within this region. What might this mean in terms of cognition? The basolateral amygdala projects to many brain regions involved in basic stress behaviour and higher-level cognition, directly and through the central nucleus of the amygdala (CeA; Rodrigues, LeDoux, & Sapolsky, 2009). A major portion of the literature on the amygdala links it to fear learning, in which the region seems to play a critical role (Rodrigues et al., 2009). We therefore propose that the β-adrenergic activation of the amygdala during a state of stress might have profound effects on the entire brain and its behavioural output, and may be particularly important in the encoding into memory of the stressful event.

Our whole-brain analysis yielded a fairly large cluster in the forebrain that was also less perfused after propranolol administration. Previously, another ASL study found a similar cluster in the ACC, which was more perfused during an eight-minute period directly after stress, compared to before-stress baseline (J. J. Wang, Rao et al., 2005). The methodology of this experiment was similar to our own (application of ASL, measurement of CBF during an after-stress period without task). This allows a direct comparison of the results, and it suggests that the frontal brain region that is active during the prolonged state of stress, is the same one in which we lowered CBF using β-blocker. In another article based on ASL, Gianaros et al. (2009) recently argued that increased resting-state CBF in a number of areas including the ACC predicts exaggerated blood pressure reactivity to stressors. These results imply that β-adrenergic activation of the sMPF/ACC plays an important role in stress. Other work might give us a hint as to this area's specific role within the stress response. A large body of evidence implicates the ACC in interpersonal emotional processing and emotional memory (Devinsky, Morrell, & Vogt, 1995). Disturbances in this region seem to lead to psychiatric disease. A meta-analysis of PTSD, a disorder in which stress is a major causative and

corollary, ACC volumes were found to be smaller in patients than in trauma-exposed controls (Karl et al., 2006). The same was true for amygdala volumes. If we combine the evidence, it seems that state changes in the activity of the sMPF/ACC have a role in the emotional and memory aspects of stress. These changes are likely dependent on β-adrenergic activity, and disturbance of the system seems to play a role in PTSD.

(Nor)adrenergic versus Glucocorticoid effects

The involvement of the two major stress hormone systems in the state of stress deserves some further attention. Though we did not detect significant CBF effects of cortisol synthesis inhibition, one cannot conclude that the system was not differentially involved. Notably, the salivary measurements strongly suggest that metyrapone lowered cortisol levels on the whole, as well as abolishing the cortisol reactivity to stress. It is possible that there were CBF effects which we could not pick up. This would be in line with our hypothesis that metyrapone induces similar effects as propranolol, but with an effect that should not be larger than that of propranolol. Improved techniques (higher field strength, ASL baseline-suppression method) might improve chances of picking up effects in the future. On the other hand, it remains well possible that acute stress effects are mostly carried by the NE system, and not by cortisol.

Another issue that we need to clear is a possible indirect effect of propranolol on brain perfusion, acting via an increase in cortisol. Previous research has reported that propranolol increases the cortisol response to exercise (Viru et al., 2007), and another paper reports a propranolol-induced ACTH increase during stress, on top of any stress-induced increase thereof (Oberbeck et al., 1998). It might be argued that our effects on brain perfusion are purely due to an increase in cortisol. However, it is clear from our data, that the stress-induced increase in cortisol is not higher in our propranolol group when compared to placebo, and nor is the overall level of cortisol. We therefore pose that it is very unlikely that any effect of β-blockade we measured stems from an indirect influence via cortisol.

Whole-brain effects of β-blockade

Besides specific regional brain effects of our pharmacological manipulation, we find that during the aftermath of stress, whole-brain perfusion is reduced in participants who receive propranolol, compared to their peers who receive a dose of metyrapone or placebo. A global decrease of CBF can have two plausible causes: First, there could be an effect of the drug on brain processes. This would mean that during stress, the blocking of β-adrenergic receptors makes many brain areas less active, decreasing perfusion overall. The alternative explanation would be that propranolol simply has a global physiological effect, which reduces perfusion of the brain without a neural cause. It is very important for our understanding of the mechanisms of (nor)adrenergic feedback on the brain that we try to elucidate which of the two explanations is the more likely.

We should first be clear on what it is that the CASL method actually measures. The ASL signal might come mainly from larger blood vessels, or it might arise mainly from the smaller vasculature and brain tissue. If the first is true, then if the effects of propranolol are mainly on the vascular system, we should see specifically pronounced effects in our contrasts exactly in those places where there are large blood vessels. The quantification of perfusion, which is implemented in the ASLtbx that we used for data analysis, comes from the model presented in J. J. Wang, Zhang et al. (2005). In this model, the contrast signal is assumed to arise from two compartments: the tissue compartment (gray and white matter) and the vascular compartment (aterioles, capillaries, and venules). At 1.5 T, with a 2000ms labelling time, and an 800ms post-label delay, it is reasonable to assume that all signal comes from these two compartments, with the largest proportion stemming from the tissue compartment. This is also in agreement with a recent publication by Wells et al. (in press), who tested the contribution to signal from these compartments at different labelling times and post-label delays (and additionally divided the tissue compartment in an intra- and an extracellular part). This means that any difference between conditions that we measure arises from the neural tissue and microvasculature. Therefore, a reduction in blood flow in the larger vessels would not have an influence on our data. This is unfortunate in our specific situation, because we cannot determine if the effects of β-blockade were purely vascular by comparing the perfusion pattern with the course of major blood vessels.

Since we cannot deduct from our own data which of the explanations is more probable, we need to turn

our attention to previous findings about the effects of propranolol on the body. Avram, Krejcie, Henthorn, & Niemann (2004) show that β-blockade reduces cardiac output, and increases systemic vascular resistance (the second being probably due to unopposed α-adrenergic reflex activation), both of which contributed to a decrease of perfusion to the (non-visceral) tissues. These results offer some support to the notion that our effects are largely caused by pure haemodynamic effects of β-blockade. On the other hand, Seifert, Rasmussen, Secher, & Nielsen (2009) report that mean arterial pressure, cardiac output, and velocity in the middle cerebral artery are not influenced by propranolol infusion (0.15 mg/kg) under resting conditions. This is very direct evidence against a pure haemodynamic hypothesis. If we want to determine whether it is likely that propranolol reduces overall neuronal activity, the picture gets somewhat complex. Starting with the basis, propranolol is highly lipophilic, which means that it can easily cross the blood-brain barrier to affect neuronal tissue (Mcainsh & Cruickshank, 1990). Several sources make clear that β-adrenergic receptors are located in many brain areas. Paschalis et al. (2009), for instance, show a wide distribution of β(1)-adrenoceptors throughout the brain of the rat. Also in the human brain, many areas have been shown to contain the β(1)- and β(2)-adrenoceptors (Joyce et al., 1992). An analysis by Rodriguez et al. (1995) has shown that there are low levels of messenger RNA present for β(3)-adrenoceptors in many brain areas in the human. Taken together, it is clear that propranolol has the potential to influence almost the entire brain, through the combined influence on all β-adrenergic receptors. The question remains whether this effect induces overall more activation or deactivation. In a large review article about the projections of the locus coeruleus, Samuels & Szabadi (2008) name a number of specific sites at which β-adrenoceptors have been found, with their probable excitatory or inhibitory effect. Parts of the basal forebrain are sensitive to β-adrenergic stimulation, and β activation works synergistically on α(1) activation. The basal forebrain, with its wide cholinergic projections, has a central role in sleep and arousal, so activation of this area puts many other parts of the brain into a more active state. The lateral hypothalamus, tuberoinfandibular area, trigeminal sensory nucleus, and the dorsal and ventral horn of the spinal cord are also named as being influenced by β-adrenergic input, mostly inhibitory (Samuels & Szabadi, 2008), but since these brain regions are very small, and the spinal cord is not measured in our experiment, we expect very little influence of this on the signal. To finish this part of the discussion, there is some direct evidence against a purely vascular effect of noradrenalin on total brain perfusion, while it seems likely that propranolol, by blocking β-adrenoceptors throughout the brain, induces an overall lower level of neuronal activity. We therefore cautiously conclude that our measured decrease in CBF is due to neuronal causes rather than vascular causes. Future research into the effects of β-adrenoceptor blocking on the brain might benefit from a control condition in which β-adrenoceptors are only blocked peripherally, for instance using a hydrophilic β-blocking agent such as atenolol, which does not easily cross the blood-brain barrier.

Global versus Local effects

Since we picked up a reducing effect of propranolol on perfusion of the whole brain, one might argue that our effects in the amygdala and sMPF/ACC are non-specific, and that they are due to an overall effect by which all brain areas have a reduced neuronal activity (and therefore perfusion). Though it remains hard to rule out this explanation entirely, we would like to argue that this is very unlikely, given the fact that we found only the one significant cluster when we performed whole-brain analysis. It seems more probable that the effects in the frontal cluster, together with activation of the amygdala, were carriers of the global effect.

Conclusion

In summary, we found specific β-blockade driven decreases in perfusion in the amygdala, and a cluster spanning the sMPF and the ACC. We also found that global levels of brain perfusion during the state of stress are lowered after β-blocking, likely due to decreases in neuronal processing of many brain areas. We were not able to pick up significant effects of cortisol synthesis inhibition on brain perfusion, even though this diminished overall levels of cortisol and abolished a stress-related increase thereof. We conclude that tonic activation of stress-related brain areas in response to acute stress is dependent on β-adrenergic activity, while a dependency on cortisol remains to be verified.

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