Acute stress modulates genotype effects on amygdala processing in humans

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Abstract

Investigating interactions between genetic and environmental factors is crucial for understanding the mechanisms leading to interindividual differences. Here, we investigated whether the current environmental context, by affecting the state of the brain, modulates genotype effects on brain function in humans. We manipulated the context by inducing acute stress which increases noradrenergic activity, and probed its effect on tonic activity and phasic responses in the amygdala. Results show that carriers of a deletion in ADRA2B, the gene coding for the α2b-adrenoceptor, display increased phasic amygdala responses under stress. In contrast, stress increased tonic activity in the amygdala for both genotypes. Thus, stress specifically increases phasic responses in deletion carriers. Our results demonstrate that genetic effects on brain operations can be dependent on the current environmental context.

Keywords: ADRA2B, acute stress, amygdala, fMRI, tonic and phasic activity, gene-environment interactions

Introduction

Interindividual differences in both normal and pathological brain function are caused by interactions between genetic and environmental factors. While the understanding of the effects of genetic variance on the brain has grown and multiple polymorphisms have been found to affect brain structure and function (*1, 2*), insight in the interactions between genetic and environmental factors remains limited. Investigating interactions is crucial for obtaining a complete understanding of the mechanisms leading to individual variation (*3*) and both genetic and environmental effects might not be uncovered without taking these interactions into account. It has been shown that the influence of genetic variation on brain activity (*4*) and risk for depression (*5*) is modulated by the accumulation of stressful life events. This supports the diathesis-stress theory, which predicts that genetic vulnerability interacts with stressful life events in determining psychological functioning (*6*). These effects are presumably mediated by epigenetic mechanisms that lead to long-lasting alterations in gene expression (7). However, the current environmental context, by affecting the state of the brain, might also modulate genetic influences on brain function. To investigate this, we designed an experiment in which we manipulated the environmental conditions to determine whether the current context modulates genetic effects on brain function in humans.

Animal models suggest that a state of acute stress modulates genotype effects on the brain (*8, 9*), underlining the importance of the stressful state. Indeed, stressful situations are evolutionary relevant and lead to a fundamental shift in the state of both the body and the brain. Acute stress is associated with a surge in vigilance, which enables the organism to respond quickly and adaptively to any threat to its homeostasis (*10*). This is

mediated by widespread noradrenergic projections from the locus coeruleus (*11*) which affect neuronal signaling via multiple receptors, among which the α2b-adrenoceptor (*12*). A common functional deletion in the gene coding for the α2b-adrenoceptor (ADRA2B), consisting of three glutamic acids (residues 301–303) in the third intracellular loop, leads to increased re-experiencing of traumatic memories (*13, 14*), which indicates that carriers of this deletion are more affected by stressful events. A key target of noradrenergic projections is the amygdala, the central structure for threat detection, vigilance regulation, and facilitation of memory for arousing experiences (*15, 16*). Moreover, vulnerability to stress has been shown to depend on amygdala predisposition, thereby indicating that the effects of stress on the amygdala are likely mediated by genotype (*17*). Therefore, we tested whether experimentally induced acute stress modulates the influence of ADRA2B variation on amygdala processing.

To this end, 41 healthy, young men participated in a functional Magnetic Resonance Imaging (fMRI) experiment with two sessions which were counterbalanced. Each subject was tested once in a stressful context and once in a control condition. Acute stress was induced by showing short movie clips with highly aversive content and a self-reference instruction that directly preceded and followed the task. This method of stress induction has been shown previously to elicit a measurable stress response (*18-20*). In the control condition, movie clips with emotionally neutral content were implemented. We investigated the effect of acute stress on amygdala reactivity using a task that robustly engages the amygdala (*18*), in which participants passively viewed photographed faces morphing dynamically into either happy or fearful expressions. This task was divided into two subparts which were presented in between the stressful or the neutral movie

clips. ADRA2B genotype was determined for each participant and 56% were carriers of the deletion variant (*14*). All participants were college students and the groups did not differ significantly in age ($p > .1$), trait anxiety ($p > .6$), and baseline levels of cortisol ($p > .8$) and α-amylase ($p > .2$) as measured the day before the experiment at home.

Results

To establish whether the stress induction was successful, heart rate was recorded throughout the stress and control condition and measures of salivary cortisol, salivary α-amylase, and subjective negative affect were taken before and after each experimental condition. A stress x time repeated measures ANOVA showed an interaction effect for heart rate ($F(2,37) = 34.79$, $p < .001$), with heart rate being higher before (mean beats per minute: 64.9 vs. 60.2), during (62.3 vs. 61.2), and after (66.1 vs. 60.0) the amygdala activation task in the stress condition than in the control condition. Similar stress x time interactions were found for salivary cortisol ($F(1,36) = 4.46$, $p = .042$), salivary α-amylase ($F(1,36) = 7.97$, $p = .008$), and negative affect ($F(1,39) = 20.43$, $p < .001$). Increases were observed during the stress condition (mean cortisol (nmol/l): from 7.68 to 8.32; mean α-amylase (U/l): from 47.77 to 55.30; mean negative affect: from 12.85 to 16.29), while decreases were observed during the neutral condition (cortisol: from 7.97 to 6.39; α-amylase: from 58.06 to 49.36; negative affect: from 13.23 to 12.55). Heart rate, salivary cortisol, salivary α-amylase, and negative affect did not vary as a function of ADRA2B genotype (heart rate: $p > .6$; cortisol: $p > .4$; α-amylase: $p > .1$; negative affect: $p > .07$). Together, these findings indicate that a state of acute stress was induced during the stress condition in deletion as well as non-deletion carriers.

Results obtained with conventional fMRI based on blood oxygen level-dependent contrast showed that our task elicited bilateral activation in a well-defined set of brain regions including the ventral visual stream ranging from the primary visual cortex to the fusiform gyrus and medial temporal lobe regions including the amygdala and hippocampus ($p < .001$ uncorrected) (Fig.1).

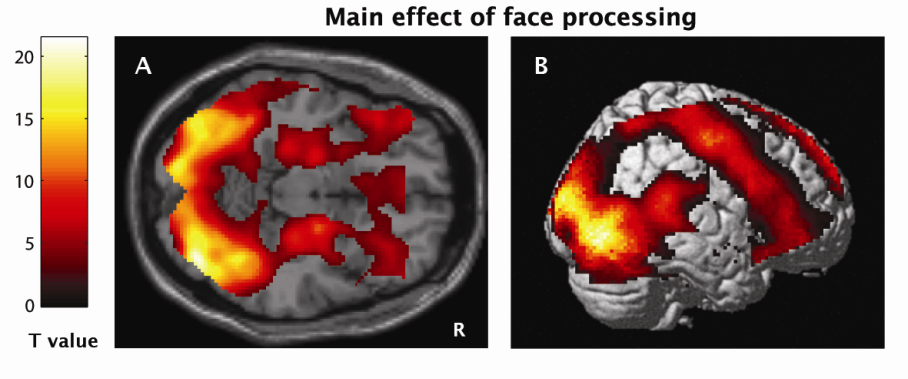

Fig.1. Statistical parametric maps illustrating the main effect of processing emotional faces superimposed on (A) a transverse slice through the amygdala and (B) a surface rendering ($p < .001$ uncorrected for visualization purposes, $z = -12$)

Importantly, the results revealed an ADRA2B genotype x stress interaction (Fig. 2a) in the right amygdala (MNI coordinates: [$x = 24$, $y = 0$, $z = -20$], $Z = 3.35$, $p = .013$ (SVC)). Tests for simple effects showed higher amygdala activity in deletion carriers as compared to non-deletion carriers (Fig. 2b) during the stress condition ([28, -2, -18], $Z = 3.26$, $p = .018$ (SVC)), whereas no significant difference between the groups was observed in the control condition ($p > .5$ (SVC)). Hence, this pattern of results demonstrates that the influence of ADRA2B variation on amygdala reactivity is dependent on the stress-related brain state as induced by an environmental context of acute stress.

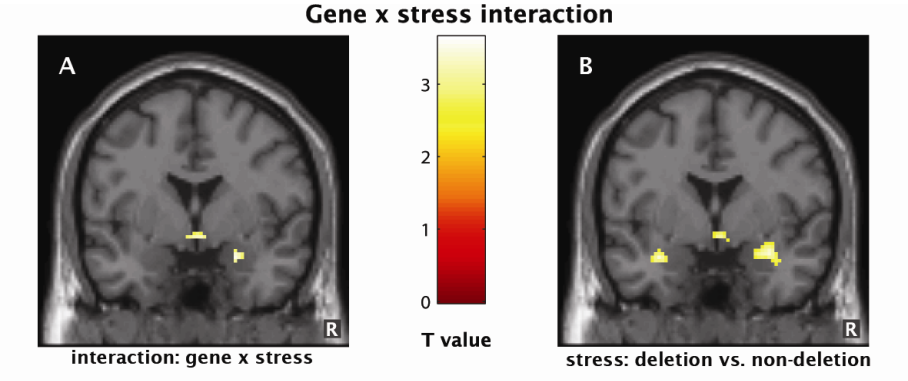

Fig.2. The influence of a common deletion in ADRA2B on amygdala responses is modulated by acute stress. (A) Significant interaction between ADRA2B genotype and acute stress in the right amygdala. (B) Larger fMRI amygdala responses in deletion carriers than non-deletion carriers in the acute stress condition. The figures show the statistical comparisons ($p < .005$ uncorrected for visualization purposes) superimposed on a single subject T1-weighted image ($y = 0$).

Conventional fMRI is a measure of phasic responses of neural activity induced by changing experimental conditions, but acute stress leads to more slowly modulated state changes and might therefore also affect tonic activity in the amygdala (*21*). To probe such stress-induced changes in tonic amygdala activity, we measured regional brain perfusion using another fMRI technique, continuous arterial spin labeling (CASL), as a correlate of tonic activity (*22*). We extracted perfusion data, calculated by pair-wise subtraction of the label/control image pairs, from the cluster of amygdala activation found with conventional fMRI by creating regions of interest (ROIs) for each individual participant. The results showed an increase in perfusion in the amygdala during the stress condition as compared to the neutral condition ($F(1,34) = 6.17$, $p = .018$), but no significant ADRA2B genotype x stress interaction ($F(1,34) = 0.57$, $p = .46$). Thus, these results indicate that while acute stress leads to a shift in tonic amygdala activity, this shift appears not responsible for the differences in phasic responses as observed with

conventional fMRI. Both genotypes show increased amygdala perfusion under stress, with deletion carriers showing an increase in phasic responses on top of that (Fig. 3).

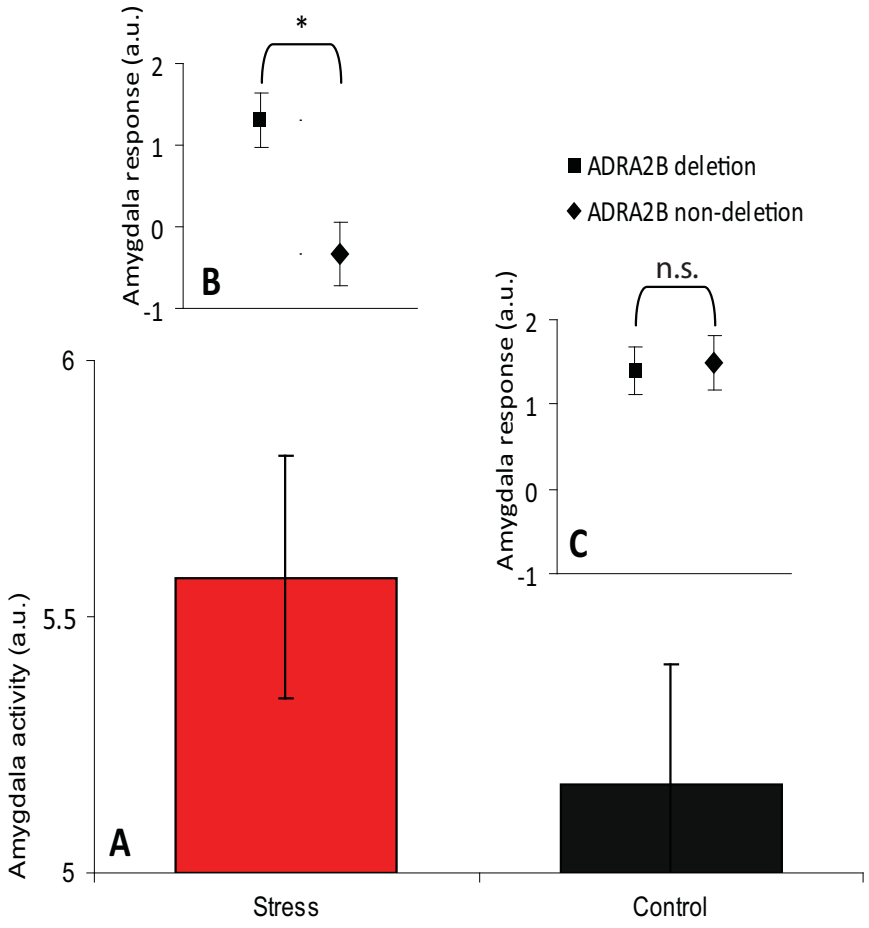

Fig. 3. The influence of acute stress and a common deletion in ADRA2B on amygdala perfusion and amygdala responses. For visualization purposes, the figure shows the combination of tonic activity and phasic responses that was observed in the amygdala cluster. (A) Tonic activity: amygdala perfusion was higher in the stress than control condition, and this effect was independent of ADRA2B genotype. (B) Phasic responses: during the stress condition, ADRA2B deletion carriers showed higher amygdala responses than non-carriers. (C) Phasic responses: during the control condition, no significant differences in amygdala response were observed between ADRA2B deletion and non-deletion carriers. (* $p < .05$)

To investigate the specificity of the observed interaction in the amygdala, we performed an exploratory whole brain analysis ($p = .001$ uncorrected, cluster ≥ 10 voxels). This analysis revealed an additional ADRA2B genotype x stress interaction in the left [4, -2, -8] and right [-4, -4, -8] hypothalamus, a crucial brain region within the stress system. As in the amygdala, deletion carriers displayed increased activity during the stress condition when compared to non-deletion carriers ($p < .001$ uncorrected), while there was no difference in the neutral condition ($p > .05$ uncorrected). Thus, we show that the gene x stress interaction is relatively specific for the amygdala and hypothalamus, which are two of the most important stress regions in the brain.

Discussion

In the present study, we examined for the first time whether genotype effects can be modulated by the current environmental context. The results demonstrate that the influence of ADRA2B genotype on brain processes is dependent on a state of acute stress. While under normal conditions no effect of ADRA2B on amygdala processing was revealed, deletion carriers showed increased amygdala activity compared to non-deletion carriers in a state of acute stress. Thus, our model of ADRA2B genotype by acute stress shows that the effects of genes on the brain can be state-dependent, such that they only exert their effects under specific, often environmentally controlled, conditions.

A similar pattern was found in the hypothalamus, which is a crucial brain region for the stress response because of its involvement in both cortisol and noradrenaline signaling (*23*). Situations of acute stress usually engage the hypothalamus and it has been shown previously that face processing tasks can lead to hypothalamus activation (*24*).

The finding that this activation pattern resembles that of the amygdala indicates that the state-dependent effects of ADRA2B are seen in multiple brain regions involved in the stress response.

The increased amygdala activity we observed in deletion carriers as compared to non-deletion carriers seems to result from changes in noradrenaline availability, since the deletion leads to perturbation of the relationship between incoming noradrenaline signals and receptor responsiveness. The α2b-adrenoceptor is a presynaptic receptor and has a negative feedback function. In vitro data suggest that the deletion exerts both agonistic and antagonistic effects, making it impossible to predict whether the deletion leads to increased or decreased noradrenergic signaling (*14*). However, behavioral data indicate that the deletion acts as either a loss-of-function or reduced-function polymorphism, thereby leading to increased noradrenaline availability (*13*). Our finding of a relative increase in amygdala activity for deletion carriers provides additional evidence for the idea that the ADRA2B deletion potentiates noradrenergic activity. In both groups, we found increased tonic activation in the amygdala during acute stress, a state associated with an increase in noradrenaline, but only the deletion group, with putatively increased noradrenaline availability, is still able to increase noradrenaline and thus show phasic responses to emotional stimuli.

In sum, in this study we uncovered the effect of ADRA2B genotype on amygdala processing by inducing acute stress. One may speculate whether the interaction revealed here may act as a vulnerability factor when it comes to stress-related mental disorders. Most importantly, the results demonstrate that the influence of genes on brain function can be dependent on the current context. This finding underlines the importance of

studying gene-environment interactions for the understanding of the mechanisms leading to interindividual differences in brain function. Future studies may reveal whether previously observed effects of other polymorphisms show the same state dependency.

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Materials and Methods

Participants

Forty-one Caucasian men (aged 18-35 years) with normal or corrected-to-normal vision participated in this study. Only men were included because hormonal fluctuations across the menstrual cycle and hormonal contraceptives influence the stress response (*1*). Participants reported no history of psychiatric, neurological, or endocrine diseases and no current use of psychoactive drugs or corticosteroids. All had participated in previous MRI experiments to ensure that no stress response would be evoked by unfamiliarity with the environment and procedures. After the experiment, ADRA2B genotype was determined for each participant and 56% were carriers of the deletion variant of three glutamic acids (residues 301–303) in the third intracellular loop, which has an allele frequency of 30% (*2*). All participants were college students and the groups did not differ significantly in age ($p > .1$), trait anxiety ($p > .6$), and baseline levels of cortisol ($p > .8$) and α-amylase ($p > .2$) as measured the day before the experiment (i.e. before dinner) at home. Written informed consent was obtained before the experiment and the study was carried out in accordance with the guidelines of the local ethical review board (CMO Region Arnhem-Nijmegen, The Netherlands) and in accordance with the declaration of Helsinki. Data of one participant were excluded due to technical failure and MRI data of one participant were excluded due to excessive head movement.

General Procedure

In the week preceding the experiment, participants were contacted by telephone and answered a set of questions that formed the exclusion criteria for this experiment. After

the phone call, participants were sent a salivette collection device (Sarstedt, Rommelsdorf, Germany) and were asked to take a baseline saliva sample in the late afternoon on the day before the experiment. The experiment took place in the afternoon or early evening to ensure low and relatively stable levels of endogenous cortisol. Upon arrival, participants completed the trait version of the State Trait Anxiety Inventory (*3*) and had the experiment explained to them. All individuals participated in both the stress and the control condition and this was done in counterbalanced order. One condition consisted of the amygdala activation task, which was interleaved with stressful or neutral movies. Immediately after the last movie, perfusion was measured with Continuous Arterial Spin Labeling (CASL). Before and after each condition, saliva samples and subjective affect ratings were collected and heart rate was measured throughout the experiment. The two conditions were separated by approximately 20 minutes in which a structural MRI scan was made for anatomical normalization purposes.

Stress Induction

In the stress condition, moderate psychological stress was induced by showing short movie clips within the MRI scanner containing scenes with strongly aversive content (extreme violence) selected from a commercially available movie (Irréversible, 2002 by Gaspar Noé). In contrast, during the control condition participants watched equally long movie clips from another movie (Comment j'ai tué mon père, 2001 by Anne Fontaine) which were equal in luminance and similar in language but contained only non-arousing scenes. After short introductory texts, participants were asked to watch the movies attentively and take an eye-witness perspective as to involve them maximally in the

movie clips. The present stress induction method closely corresponds to the determinants of the human stress response as described by Mason (*4*), i.e., unpredictability, novelty, and uncontrollability. Moreover, previous studies have shown that this method elicits a measurable physiological stress response (*5-7*).

Amygdala Activation Task

In between the movie clips, participants passively viewed blocks of faces morphing dynamically into happy or fearful expressions. The perceptual processing of emotional faces has been shown to reliably and robustly engage the amygdala (*8*) and even more so with dynamic rather than static presentation of facial expressions (*9*). Stimuli consisted of short 133 ms animation clips for each of ten different faces (taken from a standardized set and equalized in luminance and contrast (*10*)) showing a morphing sequence consisting of four frames (55%, 70%, 85%, and 100% emotional expression) repeated at 2Hz. The amygdala activation task lasted 7.45 minutes and consisted of six blocks of 25 seconds of each emotion and six blocks of 25 seconds of fixation cross. The whole task was divided into two sub-parts so that it was fully embedded into a stressful context. The first part of the task was carried out in between the first and second movie and the second part in between the third and fourth movie. Blocks were presented in a mirrored design avoiding covariation with linear drift, and adjacent blocks of the same emotion or fixation cross were avoided. The order of blocks was counterbalanced across participants. Participants were requested to press a button with their right index finger when they saw a fixation cross to ensure they were paying attention.

Subjective and Physiological Measurements

Subjective mood was assessed by obtaining scores on the positive and negative affect scale (PANAS) (*11*) before and after both conditions. Ten items for positive and ten for negative affect had to be rated on a five-point scale ranging from 1-not at all to 5-extremely. A mean score was calculated for subjective negative affect.

To assess the autonomic response and the HPA-axis response to the context manipulation, saliva was sampled with salivette collection devices to determine the levels of α-amylase and cortisol. Samples were taken the day before the experiment and before and after both conditions (five in total) and were stored at -20°C until analysis. The analysis was carried out at the Biopsychology department in Dresden, where samples were prepared for biochemical analysis by centrifuging at 3,000 rpm for 5 minutes, which resulted in a clear supernatant of low viscosity. Salivary-free cortisol concentrations were determined employing a chemi-luminescence-assay (CLIA) with high sensitivity of 0.16 ng/ml (IBL; Hamburg, Germany). 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 afterwards, 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 37°C 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). For one subject no data were acquired and for one subject the analysis did not succeed, while data of a third subject were not taken into account because he consumed caffeine shortly before the experiment.

To assess autonomic activity throughout the experiment, we continuously recorded heart rate with an infrared pulse oximeter (accompanying the MRI scanner, Siemens, Erlangen, Germany) placed on a finger of the left hand. Offline artifact correction and analysis of the heart rate frequency was done with in-house software. The heart rate frequency was averaged for the duration of each movie clip and the task. For one subject, data were not available.

fMRI Data Acquisition

During both conditions, whole brain T2*-weighted blood oxygenation level-dependent (BOLD) fMRI data were acquired using echo-planar imaging (EPI) with a Siemens TIM Trio 3.0 Tesla MR-scanner using an ascending slice acquisition sequence (37 axial slices, volume repetition time (TR) = 2.18 s, echo time (TE) = 25 ms, flip angle = 90°, slice

matrix size = 64 x 64, slice-thickness = 3.0 mm, slice gap = .3 mm, field of view (FOV) = 212 mm. Two hundred and five images were acquired during the amygdala activation task.

At the end of both conditions, resting-state CASL data were recorded with an ascending slice acquisition sequence (labeling time = 2 s, post label delay time = 1 s, label offset = 8.0 cm, TR = 3.69 s, TE = 11 ms, flip angle = 90°, matrix size = 64 x 64, slice thickness = 5 mm, slice gap = 1.5 mm, FOV = 224 mm, bandwidth = 2694 Hz per pixel). Eighty images were acquired for each participant in each condition. For three participants, no CASL data were acquired due to technical failure.

High-resolution structural images were acquired using a T1-weighted three dimensional magnetization-prepared rapid gradient echo (MP-RAGE) sequence (TR = 2.3 s, TE = 3.03 s, flip angle = 8°, 192 contiguous sagittal slices, slice matrix size = 256 x 256, FOV = 256 mm).

fMRI Data Analysis

Image preprocessing and statistical analysis of the BOLD fMRI data was preformed using SPM5 (Wellcome Department of Imaging Neuroscience, London, UK). The first five EPI volumes were discarded to allow for T1 equilibration. Remaining functional images were realigned with rigid body transformation and coregistered to the anatomical T1-weighted MR-image. Subsequently, images were transformed into a common stereotactic space (MNI152 T1-template) and resampled into $2 \times 2 \times 2$ mm^3 isotropic voxels. Spatial smoothing was performed with an isotropic 3D Gaussian kernel of 8 mm full-width at half-maximum.

Statistical analysis was performed within the framework of the general linear model. The presentation of emotional faces was modeled as boxcar regressor and convolved with the canonical hemodynamic response function of SPM5. Additionally, realignment parameters were included to model potential movement artifacts. Contrast parameter images generated at the single subject level (emotion vs. fixation) were submitted to second level group analysis. This group analysis was a 2 (genotype) x 2 (stress) mixed-model analysis of variance. Statistical tests were corrected for multiple comparisons across the entire brain or for the search volume for the amygdala using a small volume correction (*12*), which was anatomically defined using the WFU Pickatlas (*13*). Additionally, we carried out an exploratory whole-brain analysis ($p<.001$ uncorrected, cluster ≥ 10 voxels).

Preprocessing of the CASL-data was carried out with the SPM-based ASL perfusion fMRI data processing toolbox using standard settings (*14*). Images were realigned and spatial smoothing was applied with a 3D isotropic kernel with 9 mm full-width at half-maximum, followed by image coregistration between the raw EPI and structural images. Perfusion difference images were calculated by pair-wise subtraction of the label/control image pairs. The amygdala cluster observed in the conventional fMRI analysis at a threshold of $p < .05$ uncorrected was defined and transformed back into the anatomical space for each individual subject, thereby creating ROIs for all individual participants. Mean perfusion data was extracted for these ROIs and subjected to a 2 (genotype) x 2 (stress condition) ANOVA to determine differences in perfusion.

Genetic analysis

For the analysis of the insertion/deletion polymorphism in ADRA2B, we used fragment length analysis on a genetic analyzer. In short, amplification of a fragment containing the variant was performed in a total volume of 10 µl containing 50 ng of DNA and 1 x PCR buffer II (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), 2.5 mM MgCl2 (Applied Biosystems), 0.25 mM dNTPs (GE Healthcare, Zeist, The Netherlands), 0.5 µl (10 pmol/µl) of each primer (Applied Biosystems), i.e. a NED-labeled forward primer (NED-AGA AGG AGG GTG TTT GTG GGG) and a reverse primer carrying a 'PIG tail' (ACC TAT AGC ACC CAC GCC CCT-GTTTCTT), 0.5 M betaine (Sigma, Zwijndrecht, The Netherlands), 0.04 units AmpliTaq Gold (Applied Biosystems). The amplification protocol consisted of an initial 12 minutes at 95°C, followed by 32 cycles of 1 minute at 94°C, 1 minute at 66°C and 1 minute at 72°C, and finishing with a step of 7 minutes at 72°C. For the fragment length analysis on an ABI Prism 3730 Genetic Analyser, 1 µl of (diluted) PCR product was added to 8.7 µl formamide (Applied Biosystems) and 0.3 µl Liz600 standard (Applied Biosystems). The results were analyzed using GeneMapper® Software, version 4.0 (Applied Biosystems). Testing for Hardy-Weinberg equilibrium did not show deviations from the expected distribution ($X^2(1) = .65$, $p = .42$).

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