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ORIGINAL ARTICLE

Neural mechanisms of oxytocin receptor gene mediating anxiety-related temperament

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Abstract A common variant (rs53576) of the *OXTR* gene has been implicated in a number of socio-emotional phenotypes, such as anxiety-related behavior. Previous studies have demonstrated that A-allele carriers have higher levels of physiological and dispositional stress reactivity and depressive symptomatology compared to those with the GG genotype, but the mediating neural mechanisms remain poorly understood. We combined voxel-based morphometry and resting-state functional connectivity analyses in a large cohort of healthy young Chinese Han individuals to test the hypothesis that the *OXTR* gene polymorphism influences an anxiety-related temperamental trait, as assessed by the harm avoidance subscale from the Tridimensional Personality Questionnaire via modulating the gray matter volume and resting-state functional connectivity of the brain, especially the limbic system. We revealed that female subjects with the AA genotype showed increased harm avoidance scores relative to G-carrier females. We also found that, compared to female individuals with the GG/GA genotype, female individuals with the AA genotype exhibited significantly smaller amygdala volumes bilaterally (especially the centromedial subregion), with a trend of allele-load-dependence. Compared to female individuals with the GG/GA genotype, female subjects with the AA genotype demonstrated reduced resting-state functional coupling between the prefrontal cortex and amygdala bilaterally, also with an allele-load-dependent trend. Furthermore, the magnitude of prefrontal-amygdala coupling in the left hemisphere was positively correlated with harm avoidance scores in female subjects. Our findings highlight a possible neural pathway by which a naturally occurring variation of the *OXTR* gene may affect an anxiety-related temperamental trait in female subjects by modulating prefrontal-amygdala functional connectivity.

Keywords Amygdala · Anxiety · Imaging genetics · Oxytocin receptor · Single nucleotide polymorphism

Drs. J. Wang and W. Qin contributed equally to this work.

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Introduction

As a neurotransmitter, oxytocin (OXT) has been shown to modulate socio-emotional behaviors, such as sexual behavior and pair bonding in rodents (Carter 1992; Hammock and Young 2006) and parental care and social recognition in humans (Kirsch et al. 2005; Feldman et al.

2012), and to exert anxiolytic and antidepressant effects in humans (Heinrichs et al. 2003; Scantamburlo et al. 2007). In humans, intranasal administration of OXT can affect the brain to increase trust, generosity, and mental inference, to modulate social memory, and to suppress responses to stress (Heinrichs et al. 2003; Kosfeld et al. 2005; Domes et al. 2007; Zak et al. 2007; Rimmele et al. 2009). The physiological effects of OXT are mediated via binding to the OXT receptor (OXTR) (Inoue et al. 2010), and numerous studies have implicated the OXTR in the regulation of social and emotional behaviors (Takayanagi et al. 2005; Bakermans-Kranenburg and Van IJzendoorn 2008; Lucht et al. 2009; Rodrigues et al. 2009; Yoshida et al. 2009; Kim et al. 2010; Tost et al. 2010; Saphire-Bernstein et al. 2011; Krueger et al. 2012). The expression of the OXTR is modulated by the *OXTR* gene, and variations of the *OXTR* gene may regulate these behaviors.

The human *OXTR* gene is located on chromosome 3p25.3 spanning approximately 19 kbp and contains three introns and four exons (Inoue et al. 1994). A single nucleotide polymorphism (SNP) within intron 3 involving an adenine (A)/guanine (G) transition (rs53576) has been associated with different socio-emotional phenotypes. Specifically, *OXTR* rs53576A promotes deficits in socio-emotional domains such as maternal sensitivity (Bakermans-Kranenburg and Van IJzendoorn 2008), empathy (Rodrigues et al. 2009), positive affect (Lucht et al. 2009), optimism, mastery, self-esteem (Saphire-Bernstein et al. 2011), emotional support seeking (Kim et al. 2010), prosocial temperament (Tost et al. 2010) and trust behavior (Krueger et al. 2012). Recently, genetic variation of *OXTR* rs53576 has been associated with anxiety-related behaviors. Specifically, compared to individuals with the GG genotype, A-allele carriers demonstrate higher levels of stress reactivity and depressive symptomatology (Rodrigues et al. 2009; Saphire-Bernstein et al. 2011).

The temperamental trait of harm avoidance (HA) is a heritable tendency to respond intensely to signals of aversive stimuli (Cloninger 1987a; Cloninger et al. 1993). A person with a higher HA score is characterized by excessive worrying, cautiousness, pessimism, shyness, and fearfulness (Cloninger 1987a; Cloninger et al. 1993). HA is thought to be associated with depression and anxiety-related disorders (Svrakic et al. 2002) and is an anxiety-related temperamental trait (Sen et al. 2004; Westlye et al. 2011). It is reasonable to predict that variation in *OXTR* rs53576 genotype will affect individual HA scores because this gene variation affects anxiety-related behaviors. If an association between specific genetic variants of *OXTR* rs53576 and HA scores is present, it would be interesting to know which brain networks and circuitry are involved in mediating this association. However, one should be noted that it remains unclear whether the OXTR or OXT itself is present in the human brain outside the hypothalamus, such as the amygdala.

Imaging genetics refers to the use of anatomical or physiological imaging technologies as phenotypic assays to evaluate genetic variation. It provides a unique tool to explore and evaluate the functional impact of brain-relevant genetic polymorphisms with the potential to understand their impact on behavior (Bigos and Weinberger 2010). This method has been used to study the neural mechanisms of the modulation of the *OXTR* rs53576 on the prosocial temperament (Tost et al. 2010). The authors found that *OXTR* rs53576 affects prosocial temperament via modulation of the structure and functional connectivity of the limbic system, especially the hypothalamus and amygdala, in Caucasian men (Tost et al. 2010).

In the present study, we used a stepwise imaging genetics approach to identify the neural mechanisms underlying the association between *OXTR* rs53576 and HA score in 290 healthy young Chinese Han subjects of both sexes. After we identified differences in HA score related to *OXTR* rs53576 genotype, we explored volumetric differences between genotypic groups throughout the whole brain, paying special attention to limbic structures, using a statistical threshold of $P < 0.001$ without correction for multiple comparisons. After finding group differences in the amygdala bilaterally, we further tested which amygdala subdivisions were more affected by the genetic variation of *OXTR* rs53576; this was prompted by previous reports that amygdala subdivisions exhibit distinct levels of OXTR binding in the rat brain (Bale et al. 2001; Huber et al. 2005), have distinct connection patterns (Roy et al. 2009), and are implicated in different functions (Li et al. 2012). Then, we examined whether amygdala regions with a volumetric difference also show differences in resting-state functional connectivity (rsFC) across genotypes. Finally, we assessed whether measurements of amygdala volume or rsFC that differed significantly between genotypic groups were correlated with HA scores. Gender differences were considered throughout the analyses because gender dimorphism has been frequently reported in anxiety-related personality traits (Cloninger et al. 1993) and related brain anatomy and function (Good et al. 2001; Luders et al. 2004).

Materials and methods

Participants

A total of 324 healthy young adults were recruited by local advertisements. Careful screening was performed to ensure that participants were free of any lifetime history of neurological and/or major mental illness and/or known or

suspected history of alcoholism or drug dependency and abuse. Because the screening procedure was not aimed at differential diagnostics or characterization of the incidence of different diagnoses in a population but rather at exclusion based on any prior or current psychiatric illness, we did not use a standardized psychiatric interview, such as Structured Clinical Interview for DSM-IV diagnostics (Westlye et al. 2011). Only Chinese Han populations were included to purify the sample because the allele frequencies of *OXTR* rs53576 are different across ethnics (Kim et al. 2010). All subjects were strongly right-handed, as judged according to the Chinese edition of the Edinburgh Handedness Inventory (Oldfield 1971), and had no MR contraindications. After a complete description of the study, all subjects gave written informed consent approved by the Ethics Committee of Tianjin Medical University.

OXTR rs53576 genotyping

Standard protocols were used to genotype *OXTR* rs53576 (for more detailed methods, see "Supplemental methods"). The distribution of *OXTR* rs53576 genotype (AA = 93, AG = 175, and GG = 22) was in line with previous reports of the variation in this gene in healthy Chinese Han populations (Wu et al. 2005). However, the frequency of the A-allele in our sample was much higher than that found in Caucasian populations. Neither the allele nor genotype frequencies differed between males and females. In the present study, the number of subjects with the GG genotype ($n = 22$) was much smaller than the number of subjects with the AA ($n = 93$) or AG ($n = 175$) genotypes. Therefore, subjects who were homozygous or heterozygous for the G-allele were merged into a single group of G-allele carriers (22 GG and 175 AG carriers) and compared with homozygotes for the A-allele (93 AA). This method may improve statistical power and help to avoid errors of statistical inference due to the sample size of one group being much smaller than the sample size of the other two groups. A similar method has been used in previous *OXTR*-related studies (Lucht et al. 2009; Kim et al. 2010).

Behavioral assessments

Harm avoidance scores, which quantify anxiety-related personality traits, were assessed using a Chinese version of the Tridimensional Personality Questionnaire (TPQ) (Cloninger 1987b). Full-scale Intelligence Quotient (FIQ) scores were measured using the Chinese Revised Wechsler Adult Intelligence Scale (Gong 1982). The Beck Depression Inventory-II (BDI-II) was used as a behavioral measurement of self-reported depressive symptoms (Beck and Steer 1990). Only one subject's BDI score was greater than 16, suggesting mild depression (Westlye et al. 2011). However, this subject was excluded due to the genotyping failure. Thus, the remaining 323 subjects had BDI scores within the normal range. Considering the correlation between HA and BDI scores (Grucza et al. 2003), we tried to reduce the influence of the BDI scores in the HA-related analyses by controlling for BDI scores.

Statistical analyses of the demographic, genetic and behavioral data

Statistical analyses of the demographic, genetic and behavioral data were performed using the Statistical Package for the Social Sciences version 18.0 (SPSS Inc, Chicago, Illinois) for Windows. Significance was set at $P < 0.05$. A Chi square test was used to compare group differences in categorical variables. Analysis of variance (ANOVA) with genotype and gender as factors was used to examine group differences in continuous demographic variables. ANOVA was also used to identify main effects of genotype and gender, and genotype-by-gender interactions in HA score with BDI score as a covariate. We performed partial correlation analysis between the HA and BDI scores while controlling for the sex because sex has shown a relationship to both HA (Miettunen et al. 2007; Hakamata et al. 2009) and BDI (Adewuya et al. 2007; Pietras et al. 2012).

Image acquisition

MR images were acquired using a Signa HDx 3.0 Tesla MR scanner (General Electric, Milwaukee, WI, USA). Tight but comfortable foam padding was used to minimize head motion, and earplugs were used to reduce scanner noise. Resting-state fMRI data were obtained using a Single-Shot Echo-Planar Imaging sequence (SS-EPI) with the following imaging parameters: repetition time (TR)/echo time (TE) = 2,000/30 ms; field of view (FOV) = 240 mm × 240 mm; matrix = 64 × 64; flip angle (FA) = 90°; slice thickness = 4 mm; no gap; 40 transverse slices; 180 volumes. During fMRI scans, all subjects were instructed to keep their eyes closed, to stay as motionless as possible, to think of nothing in particular and to not fall asleep. It took 6 min and 10 s for each subject. Sagittal 3D T1-weighted images were acquired by a brain volume (BRAVO) sequence (TR/TE = 8.1/3.1 ms; inversion time = 450 ms; FA = 13°; FOV = 256 mm × 256 mm; matrix = 256 × 256; slice thickness = 1 mm, no gap; 176 slices).

Voxel-based morphometry (VBM) analysis

Adopting a state of the art registration technique, the diffeomorphic anatomical registration through exponentiated

lie algebra (DARTEL) (Ashburner 2007), the VBM analysis was performed using Statistical Parametric Mapping software (SPM8) (See "Supplemental methods" for further details). After segmentation, registration, normalization, modulation, and smoothing, gray matter volume (GMV) maps were used for statistical analysis. We first performed voxel-based comparisons of whole-brain GMV between individuals with the AA genotype and G-allele carriers to identify brain regions with genotype-related GMV differences using a significance threshold of $P < 0.001$ without correction for multiple comparisons and a cluster size of >50 voxels. After identifying GMV differences between the genotypic groups in the amygdala bilaterally, we extracted the bilateral amygdala as a mask using Anatomy v1.7 software (Eickhoff et al. 2006). ANOVA with a fully factorial (genotype-by-gender) design with age, years of education and FIQ as nuisance variables was applied to compare the GMV differences between the two genotypic groups within the amygdala mask. The False Discovery Rate (FDR) was used to correct for multiple comparisons with a significance threshold of $P < 0.05$ and a cluster size of >50 voxels. We were interested in both the main effect of genotype and any interactions between genotype and gender. If the main effect or interaction was significant, the significant clusters were extracted as regions of interest (ROIs) and the GMV of these ROIs was compared across genotypic groups in male and female subjects, respectively.

Further, we wanted to investigate the differential involvement of amygdala subregions in the genotype-related GMV differences. Therefore, we extracted the three amygdala subregions (laterobasal, centromedial and superficial) using Anatomy v1.7 software (Eickhoff et al. 2006), which can provide the maximum probabilistic maps (MPM) of different cytoarchitectonic subregions of the amygdala. We then overlaid the clusters exhibiting significant GMV differences with the three amygdala subregions in MNI standard space using the software package MRIcron (http://www.mccauslandcenter.sc.edu/mricro/mricron/index.html) to identify which amygdala subdivisions were more involved.

rsFC analysis

Standard steps were used to preprocess the resting-state fMRI data (see "Supplemental methods"for additional detail). The seed ROI of each amygdala was defined by creating a binary mask that included voxels with significant differences in GMV between the genotypic groups (FDR corrected, $P < 0.05$). For each subject, the correlation coefficient between the mean time series of each seed ROI and that of each voxel in the whole brain was computed and converted to z-values to improve normality. Subsequently, each individual's z-values were entered into a random-effect one-sample t test to identify brain regions that were significantly correlated with the seed ROIs. Significant rsFC maps were corrected for multiple comparisons using the Family Wise Error rate (FWE, $P < 0.05$). Then, four masks, referring to the positive and negative rsFC maps of the two seed ROIs, were generated for the following analysis. ANOVA was used to test the rsFC differences of each seed ROI within each mask using genotype and gender as factors and age, years of education and FIQ as covariates of no interest ($P < 0.05$, FDR corrected; cluster size >50 voxels). If the main effect or interaction was significant, the significant rsFC was extracted and compared across genotypic groups in male and female subjects, respectively.

Correlation analysis between volumes and rsFCs of amygdala and HA scores

Besides the amygdala regions with significant genotypic differences, the region of each amygdala subregion with significant genotypic difference was also extracted as ROIs. To test the hypothesis that volumes of these amygdala ROIs and the rsFCs of amygdala would mediate the relationship between the *OXTR* rs53576 gene polymorphism and individual differences in HA, we calculated partial correlation coefficients (PCC) between these parameters and HA scores ($P < 0.05$) with age, years of education, FIQ and BDI scores as nuisance covariates (these factors may also modulate amygdala volume or rsFCs) in the gender group that showed significant genotypic differences.

Results

Demographic, genetic, and behavioral characteristics

A total of 324 healthy young adults were recruited in this study. After a careful evaluation of the exclusion criteria, 34 subjects were excluded due to genotyping failure ($n = 20$), visible structural abnormality ($n = 2$), poor image quality ($n = 2$), and great head motion ($n = 10$). Finally, 290 subjects (136 males and 154 females, mean age = 23.7 ± 2.5 years) were included in the final data analysis. The demographic, genetic, and behavioral data are presented in Table 1. There were no significant differences between the two genotypic groups in gender, age, years of education, FIQ, or BDI scores. With a Cronbach's alpha coefficient of 0.87, the HA subscale showed adequate internal consistency in our sample. As expected, there was a main effect of gender on HA scores ($t = 3.26$, $df = 288$, $P = 0.001$), with higher HA scores in women (mean ± SD, 15.86 ± 6.23) than in men (13.49 ± 6.15).

Table 1 Demographics and behavioral data

	AA genotype	GG/GA genotype[a]
Number of subjects	$n = 93$	$n = 197$
Gender (males/females)	39/54	97/100
Age (years)	24.0 ± 2.2	23.5 ± 2.6
Years of education	16.4 ± 2.0	16.1 ± 2.4
FIQ	118.6 ± 7.4	116.6 ± 9.5
BDI	8.1 ± 6.8	7.7 ± 7.0

Data are presented as mean ± SD and based on 290 participants

BDI beck depression inventory, *FIQ* full-scale intelligence quotient

[a] No significant differences in any measures between individuals with AA genotype and those with GG/GA genotype

BDI scores had a relatively weak correlation with HA scores (PCC = 0.37, $df = 289$, $P = 0.0008$), indicating an association between subclinical depression and increased HA. Notably, the HA scores showed significant genotypic differences (A-allele homozygotes > G-allele carriers) in females but not in males. The HA scores were as follows: 17.3 ± 6.6 (A-allele homozygotes) and 15.1 ± 6.0 (G-allele carriers) in females ($t = 2.16$, $df = 152$, $P = 0.03$) and 12.9 ± 6.4 (A-allele homozygotes) and 13.7 ± 6.0 (G-allele carriers) in males ($t = -0.74$, $df = 134$, $P = 0.46$) (Fig. 1).

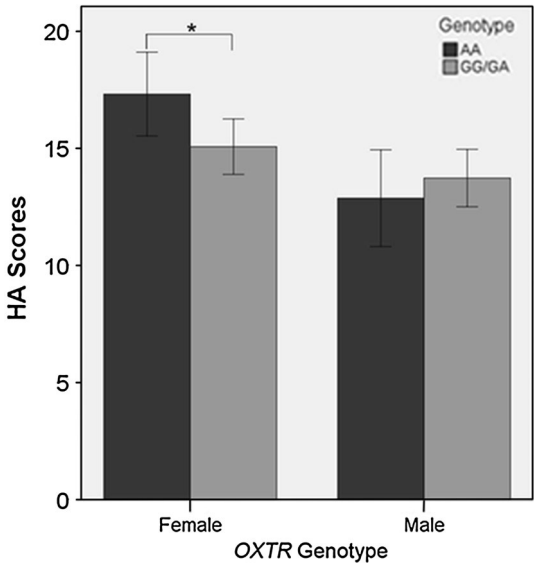

Fig. 1 Allelic variation in *OXTR* predicts differences in harm avoidance (HA). The exploratory plot of HA data in gender-stratified subsamples shows a significant difference between the two genotypic groups in female participants (A-allele homozygotes > G-allele carriers), but not in male subjects

Differences in amygdala volume between genotypes

The whole-brain VBM analysis demonstrated that, compared to G-allele carriers, the *OXTR* rs53576 A-allele homozygotes had decreased GMVs in the amygdala bilaterally as well as in several other brain regions ($P < 0.001$, without correction for multiple comparisons). In addition, no regions showed increased GMVs in A-allele homozygotes (Supplemental Table 1). Moreover, voxel-based comparisons between the two genotypes within the bilateral amygdala mask revealed a main effect ($P < 0.05$, FDR correction for multiple comparisons) of genotype in the left (peak $F_{1,286} = 10.09$) and right (peak $F_{1,286} = 11.06$) amygdala. Voxel-based post hoc comparisons between the two genotypes were then performed and revealed that A-allele homozygotes exhibited significantly smaller ($P < 0.05$, FDR correction within the bilateral-amygdala-mask) GMVs than G-allele carriers in both the left (peak $t = 3.47$; cluster size = 432 voxels; peak MNI coordinates: $x = -24$, $y = -10.5$, $z = -12$) and right amygdala (peak $t = 3.92$; cluster size = 233 voxels; peak MNI coordinates: $x = 24$, $y = -12$, $z = -12$) (Fig. 2a). These two amygdala regions exhibiting significant group differences were extracted as ROIs; and then ROI-based comparisons between the two genotypes were performed separately in male and female subjects. Only female A-allele homozygotes exhibited significantly smaller GMV in the left ($t = -3.60$, $df = 152$, $P < 0.001$) and right amygdala ($t = -3.15$, $df = 152$, $P = 0.002$) than female G-allele carriers (Fig. 2b). However, there were no significant genotypic differences in GMVs in the left ($t = -0.67$, $df = 134$, $P = 0.51$) and right amygdala ($t = -1.23$, $df = 134$, $P = 0.22$) in male subjects (Fig. 2b). The mean GMVs of the two amygdala regions in the three female genotypic groups are shown in Fig. 2c. We found an allele-load-dependent effect in the mean GMVs of the two amygdala regions (AA < AG < GG). Group comparisons revealed a significant difference between the AA and AG genotypic groups (left amygdala: $t = -3.23$, $df = 136$, $P = 0.002$; right amygdala: $t = -2.71$, $df = 136$, $P = 0.008$) but not between the AG and GG genotypic groups (left amygdala: $t = -0.70$, $df = 98$, $P = 0.486$; right amygdala: $t = -1.13$, $df = 98$, $P = 0.263$). Finally, we tested which amygdala subdivisions were more affected by genetic variation in *OXTR* rs53576. We found that the significant regions involved 80.7 % of the centromedial, 43.6 % of the superficial, and 18.4 % of the laterobasal subregion in the left amygdala, and involved 75.9 % of the centromedial, 43.3 % of the superficial, and 15.5 % of the laterobasal subregion in the right amygdala (Fig. 3, but also see Supplemental Table 2).

rsFC differences between genotypes

We calculated the rsFCs between the amygdala regions exhibiting significant differences in GMV and other brain areas in a voxel-wise manner and compared them

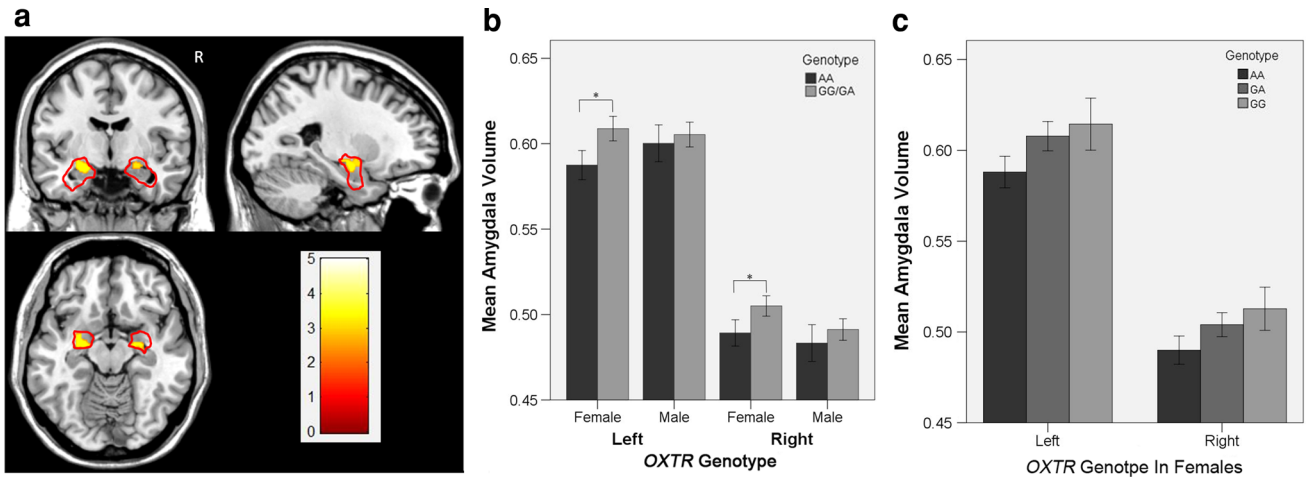

Fig. 2 Differences in amygdala volume between *OXTR* rs53576 genotypes. **a** *Yellow regions* represent the bilateral amagdala regions that show significant genotypic differences in GMV. The boundaries of the bilateral amygdala are marked using *red curves*. **b** and **c** *Bar plots* depict the group differences in mean amygdala volume and demonstrate that a significant effect occurs only in females (**b**) and has a trend of allele-load-dependence (**c**)

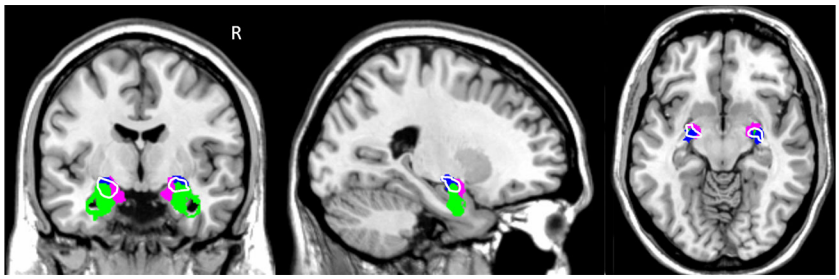

Fig. 3 Overlap between clusters (*white curves*) with significant genotypic differences in GMV and the centromedial (*blue*), laterobasal (*green*), and superficial (*pink*) subregions of the amygdala, showing that the centromedial subdivison accounts for the majority of the reduction of GMV

between the two genotypic groups. Significant interactions ($P < 0.05$, FDR correction for multiple comparisons) between genotype and gender were found in the rsFCs between the left amygdala and the left dorsolateral prefrontal cortex (DLPFC) (peak $F_{1,286} = 11.06$) and between the right amygdala and the right frontal pole (FP) (peak $F_{1,286} = 11.53$). No significant main effect of genotype was found on the rsFC of either the left or the right amygdala. Voxel-based post hoc comparisons indicated that, compared to female G-allele carriers, female A-allele homozygotes showed significantly ($P < 0.05$, FDR correction for multiple comparisons) reduced negative rsFC between the left amygdala and the left DLPFC (peak $t = 4.43$; cluster size = 186 voxels; peak MNI coordinates: $x = -28$, $y = 22$, $z = 40$) (Fig. 4a) and between the right amygdala and the right FP (peak $t = 4.79$; cluster size = 100 voxels; peak MNI coordinates: $x = 34$, $y = 58$, $z = -4$) (Fig. 5a). However, we did not find any significant group differences in rsFC in male subjects. We then extracted the rsFCs of the seed ROIs exhibiting significant differences and compared them between the two female genotypic groups. The ROI-based analysis confirmed the results derived from the voxel-based post hoc comparisons in that, compared to female G-allele carriers, female A-allele homozygotes showed significantly reduced negative rsFC between the left amygdala and the left DLPFC ($t = 4.68$, $df = 152$, $P < 0.001$) and between the right amygdala and the right FP ($t = 4.84$, $df = 152$, $P < 0.001$). Moreover, the reduced rsFCs exhibited an allele-load-dependent trend (AA < AG < GG) (Figs. 4b, 5b). Group comparisons showed a significant difference between the AA and AG genotypic groups (the rsFC between the left amygdala and the left DLPFC: $t = 2.52$, $df = 136$, $P = 0.014$; the rsFC between the right amygdala and the right FP: $t = 2.05$, $df = 136$, $P = 0.044$) but not between the AG and GG genotypic groups (the rsFC between the left amygdala and the left DLPFC: $t = 0.50$, $df = 98$, $P = 0.619$; the rsFC between the right amygdala and the right FP: $t = 1.34$, $df = 98$, $P = 0.184$).

Relationship between bilateral amygdala volumes and rsFCs

We tested the dependency of these significant rsFCs of prefrontal-amygdala circuitry on amygdala volumes that

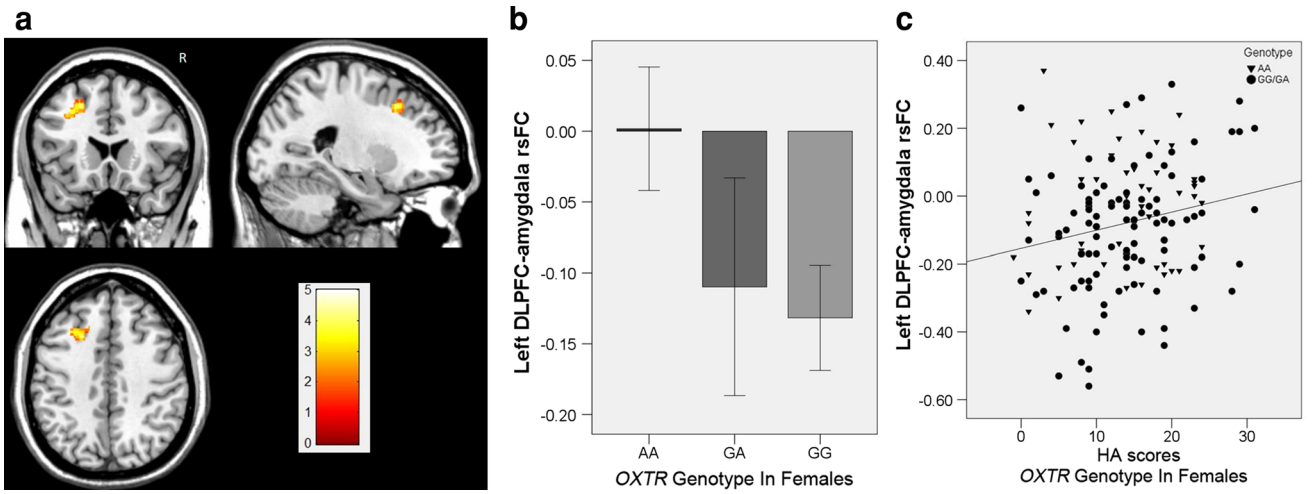

Fig. 4 Significant genotype × gender interaction in the rsFCs of the left amygdala seed region. The *color region* (left DLPFC) represents brain region whose rsFC with the left amygdala shows significant genotype × gender interaction (**a**). This genotypic difference is restricted to females and exhibits an allele-load dependent (**b**). Moreover, the rsFC between the left amygdala and the left DLPFC is positively correlated with HA score in female subjects (**c**)

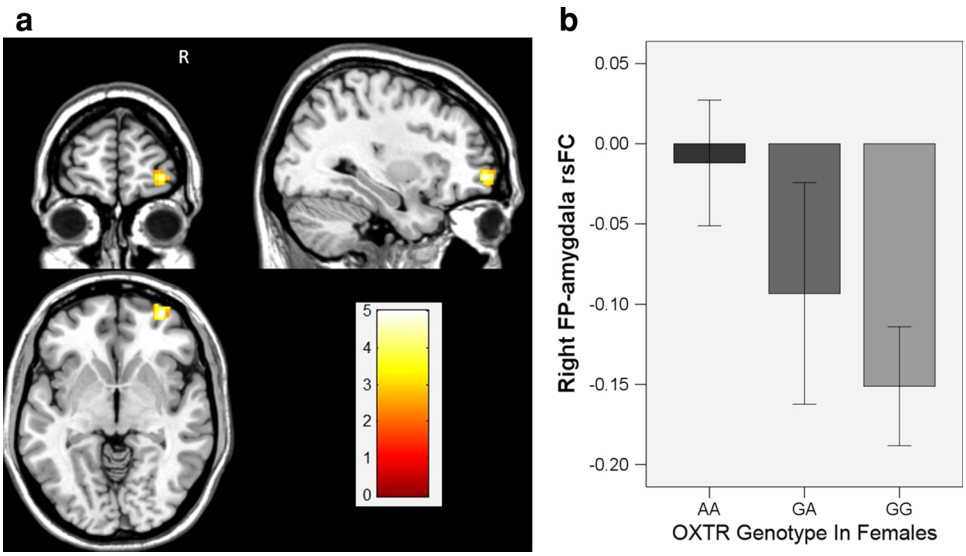

Fig. 5 Significant genotype × gender interaction in the rsFCs of the right amygdala seed region. The color region (right FP) represents brain region whose rsFC with the right amygdala shows significant genotype × gender interaction (**a**). This genotypic difference is restricted to females and exhibits an allele-load dependent (**b**)

showed significant differences between genotype groups. We did not observe significant correlations (*left* $r = 0.038$, $n = 290$, $P = 0.513$; *right* $r = 0.064$, $n = 290$, $P = 0.267$) between amygdala volume and prefrontal-amygdala rsFCs in female subjects, suggesting that genotypic differences in prefrontal-amygdala rsFCs are not driven by local structural alterations.

Correlation of amygdala characteristics with HA score

Partial correlation analyses of HA scores were restricted to female subjects because only this gender group showed significant genotypic differences in GMV or rsFCs of amygdala. There were no significant ($P > 0.05$) correlations between HA scores and the volumes of amygdala regions with significant genotypic differences in GMV. These female subjects did not show significant ($P > 0.05$) correlations between HA scores and the volume of the region of each amygdala subregion with significant genotypic differences in GMV, either. The rsFC between the left amygdala and the left DLPFC showed a weakly positive correlation with HA scores (PCC $= 0.224, df = 148, P = 0.005$) (Fig. 4c); however, the rsFC between the right FP and the amygdala did not correlate with HA scores in female subjects ($P > 0.05$).

Discussion

We followed a stepwise neuroimaging approach to investigate the neural mechanisms through which the *OXTR* rs53576 gene polymorphism affects HA temperamental predisposition in a large sample of healthy Chinese Han subjects of both sexes. After finding significantly higher HA scores in female subjects with the AA genotype, we found that, compared to female G-allele carriers, this subgroup demonstrated significantly reduced GMVs in the amygdala bilaterally, although it has not yet been reported whether OXTR is present in human amygdala. Furthermore, compared to female G-allele carriers, females with the AA genotype showed significantly reduced rsFC between the right FP-amygdala and the left DLFPC-amygdala, the latter of which was positively correlated with HA scores, a measurement of the temperamental propensity for anxiety and depression disorders. Our findings provide a possible neural mechanism for the association between the *OXTR* gene polymorphism and anxiety-related temperament traits. That is, the *OXTR* gene polymorphism affects the HA personality trait through the modulation of the functional coupling of prefrontal-amygdala circuitry.

The HA temperament trait suggests that there is a genetically determined bias toward being cautious, apprehensive and overly pessimistic (Baeken et al. 2009). Individuals with anxiety-related traits, anxiety disorder and depression, as well as those with genetic vulnerability to depression, score high for HA (Starcevic et al. 1996; Smith et al. 2005). OXT has been shown to exert an inhibitory influence on stress-responsive neuro-hormonal systems and to be an important modulator of anxiety and fear responses (Heinrichs et al. 2003; Kirsch et al. 2005). In patients with major depression, plasma levels of OXT are negatively correlated with scored symptoms of depression and anxiety (Scantamburlo et al. 2007). Moreover, an animal study has shown that OXT may regulate serotonin release and exert its anxiolytic effect by directly activating the OXTR expressed in serotonergic neurons (Yoshida et al. 2009), which suggests that expression of the OXTR may affect anxiety and depression behaviors. To the best of our knowledge, this study provides the first evidence that the *OXTR* rs53576 gene polymorphism affects the temperament trait HA in healthy female subjects. Specifically, females homozygous for the A-allele had higher HA scores than female G-allele carriers, which is consistent with previous findings that AA homozygotes have higher levels of physiological and dispositional stress reactivity and depression symptomatology (Rodrigues et al. 2009; Saphire-Bernstein et al. 2011).

Two previous studies investigated differences in amygdala volume between *OXTR* genotypes. One study that focused on Caucasians reported that male A-allele carriers had increased right amygdala volume. In contrast, the authors also found reduced volume of the same region in female A-allele carriers, as shown in the supplementary materials of that study (Tost et al. 2010). This finding is partially consistent with our finding of reduced amygdala volumes in female AA homozygotes. Ethnic differences in the allelic distribution of *OXTR* rs53576 and differences in the grouping methods employed may account for the main discrepancy between the Caucasian study and ours. The proportion of the *OXTR* A-allele is much higher in Asians than in Caucasians (Kim et al. 2010), and the GG homozygote is rare in the Chinese Han population (Wu et al. 2005). Thus, we merged the GG ($n = 22$) and AG ($n = 175$) genotypes into one group to improve statistical power when making comparisons with the AA ($n = 90$) genotype. In contrast, the AA and AG genotypes were combined into one group in the Tost et al. study. Another study of healthy Japanese adults did not find any significant difference in manually traced amygdala volume between the *OXTR* rs53576 genotypes (Inoue et al. 2010). The methodological differences in volume measurement may account for the discrepancy between the Japanese study and ours.

The amygdala is the critical structure involved in fear and anxiety (Thomas et al. 2001; Guyer et al. 2008), and reduced amygdala volume is commonly reported in healthy "anxiety-prone" individuals and patients with depression or anxiety-related disorders (Kronenberg et al. 2009; Spampinato et al. 2009). Thus, the finding that female A-allele homozygotes have smaller amygdala volumes is consistent with the findings of previous studies that they exhibit greater levels of depressive symptomatology (Saphire-Bernstein et al. 2011) and deficits in empathy (Rodrigues et al. 2009), affect (Lucht et al. 2009), emotional support seeking (Kim et al. 2010), and responses to stress (Rodrigues et al. 2009). Nonetheless, the lack of correlations between volumes of amygdala or its subregions and HA scores in female subjects suggests that amygdala size is not a critical factor in the relationship between the *OXTR* rs53576 gene polymorphism and the personality trait HA. In rat brain, the centromedial subregion of the amygdala contains the highest density of OXTR (Francis et al. 2000; Bale et al. 2001; Kalamatianos et al. 2010). Although this distribution has not been confirmed in human brains, our finding of an extensive reduction of the volume of the centromedial subregion of the amygdala in female A-allele homozygotes suggests that the human brain may have a similar OXTR distribution as the animal brain.

Compared to female G-allele carriers, female A-allele homozygotes showed reduced negative rsFC in prefrontal-amygdala circuitry bilaterally, including the left DLPFC-amygdala and the right FP-amygdala. A neural model

based on a physiological experiment in animals indicates that functional interactions between the prefrontal cortex and the amygdala play a role in fear conditioning and extinction (Sotres-Bayon et al. 2004). A large body of work has strongly suggested that a reduction of prefrontal-amygdala connectivity plays an essential role in subclinical anxiety and depressive disorders in humans (Anand et al. 2005; Johnstone et al. 2007). The observation of negative prefrontal-amygdala coupling suggests an inverse relationship between the activity of the prefrontal cortex and that of the amygdala, which is compatible with the idea that the prefrontal cortex exerts an inhibitory effect on amygdala activity and that this inhibitory circuitry is related to fear extinction and depression (Sotres-Bayon et al. 2004; Johnstone et al. 2007). The reduced functional coupling of the prefrontal-amygdala circuitry may reduce the regulatory inhibition of amygdala activity exerted by the prefrontal cortex, resulting in increased amygdala activity during rest and heightened responses to aversive stimuli. Indeed, such reductions of regulatory inhibition have been extensively reported in 'anxiety-prone' individuals and in depressed patients (Anand et al. 2005; Johnstone et al. 2007). Although the specific contributions of different regions of the prefrontal cortex to emotional control and/or regulation remain unclear, DLPFC dysfunction has been suggested to be a key feature of major depression (Phillips et al. 2003; Dannlowski et al. 2009). The function of the FP is less clear, although preliminary evidence suggests that it is involved in higher-level cognitive processing, anxiety-related personality traits and anxiety disorders (Milad and Rauch 2007; Yamasue et al. 2008).

More important, the strength of the left DLPFC-amygdala functional connectivity was positively correlated with individual HA scores in female subjects. This finding identifies a possible neural mechanism whereby the *OXTR* rs53576 gene polymorphism may affect the HA temperament trait by modulating the functional coupling of prefrontal-amygdala circuitry in healthy females. Our finding may explain why the *OXTR* rs53576 gene polymorphism affects stress reactivity and depressive symptoms. Specifically, we propose that the *OXTR* rs53576 gene polymorphism may affect the functional coupling of prefrontal-amygdala circuitry, which further affects amygdala activity and responses. This alteration of amygdala function may facilitate anxiety- and depressive-related symptoms. Although it is unclear whether the OXTR or OXT itself is present in the amygdala or prefrontal cortex of the human brain, much evidence from animal studies has revealed that the OXT system may affect the two brain regions. In central nervous system, the OXT is produced by the hypothalamus and can be transported to other brain regions via projections of the parvocellular neurons in the paraventricular nucleus or via the cycle of cerebral spinal fluid. The physiological effects of OXT are mediated via binding to the OXTR. In animals, the regional expression of OXTR is highly variable within and between species (Young and Wang 2004). In rodents, behaviorally relevant expression of OXTR in the central nervous system has been found in the amygdala (Bale et al. 2001; Huber et al. 2005). In rats, OXTR mRNA is detected in the frontal cortex (Gimpl and Fahrenholz 2001); moreover, female monogamous voles show high densities of OXTR binding in the prefrontal cortex (Smeltzer et al. 2006). Based on these findings, we speculate that *OXTR* gene polymorphism may affect the OXTR system in the amygdala and/or prefrontal cortex, which further influences the physiological effects of OXT on these two brain regions. The differential physiological effects of the OXT system may result in genotypic differences in the anatomical or functional characteristics of the two brain regions, which may be associated with the genotypic difference in functional coupling of prefrontal-amygdala circuitry.

We observed that the impact of the *OXTR* rs53576 polymorphism on amygdala volume and rsFC, and their links to HA, was gender dependent; it was only present in females. Although the exact cellular mechanism of the observed gender-by-genotype interaction is unknown, it has been shown that the temperament trait HA and the volume and rsFC of the amygdala are sexually dimorphic in humans (Cloninger et al. 1993; Cahill 2006). In addition, women have a propensity for anxiety and a higher rate of anxiety- and depressive-related disorders than men, indicating that there may be biological mechanisms in females that lead to a vulnerability to stressful life events. Another possible explanation for the gender-by-genotype interaction is that estrogens influence the activity of OXT producing neurons and heighten the rate of transcription of the *OXTR* gene (Choleris et al. 2008). Nevertheless, the exact mechanism should be studied in the future.

Several limitations should be noted when interpreting our findings. First, although our sample size is relatively large, it was composed exclusively of young adults; we cannot generalize the results to other age groups. Second, the allelic distribution of *OXTR* rs53576 differs widely among ethnic populations; the Chinese exhibit a much lower frequency of the GG genotype compared to Caucasians. Thus, we combined the GG and AG genotype into a group of G-carriers rather than combining the AA and AG genotypes into a group of A-carriers. This difference in genotypic grouping should be kept in mind when comparing studies of different ethnic populations. Third, previous studies have reported that the *OXTR* gene may contain at least two polymorphisms (rs53576 and rs2254298) that affect social behaviors in humans (Zink and Meyer-Lindenberg 2012). Thus, the effects of other SNPs and their common haplotypes on the brain and

behavior need to be further studied. Fourth, the rsFC analysis is based on the notion that if the activity of two brain areas is correlated, they are likely to be functionally connected. However, this correlation is non-directional and signal amplitude is not taken into account (Ramnani et al. 2004). Effective connectivity analysis between multiple brain regions might allow a more precise disambiguation of direct versus indirect effects of the prefrontal cortex on amygdala activity (Buckholtz et al. 2008). Finally, it should be noted that the rsFC method focuses on changes at systems-level functional neuroimaging. Thus, cellular-level changes such as postsynaptic inhibition or excitation should also be studied and related to alterations that occur at the systems level.

In summary, using a stepwise imaging genetics approach, we propose a neural mechanism underlying differences in HA score observed in female subjects with the *OXTR* gene (rs53576) polymorphism: the *OXTR* gene polymorphism affects the temperament trait HA, which is related to one's vulnerability to anxiety and depression disorders, by modulating functional coupling of the prefrontal-amygdala circuitry implicated in fear conditioning and extinction. Further studies are needed to validate our results in other ethnic populations and to confirm the predicted association between this *OXTR* gene polymorphism and anxiety and depressive disorders.

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Conflict of interest The authors have declared that no competing interests exist.

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