

The association between the 5-HTTLPR and neural correlates of fear conditioning and connectivity

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Strong evidence links the 5-HTTLPR genotype to the modulation of amygdala reactivity during fear conditioning, which is considered to convey the increased vulnerability for anxiety disorders in s-allele carriers. In addition to amygdala reactivity, the 5-HTTLPR has been shown to be related to alterations in structural and effective connectivity. The aim of this study was to investigate the effects of 5-HTTLPR genotype on amygdala reactivity and effective connectivity during fear conditioning, as well as structural connectivity [as measured by diffusion tensor imaging (DTI)]. To integrate different classification strategies, we used the bi-allelic (s-allele vs l/l-allele group) as well as the tri-allelic (low-functioning vs high-functioning) classification approach. S-allele carriers showed exaggerated amygdala reactivity and elevated amygdala-insula coupling during fear conditioning (CS+ > CS-) compared with the l/l-allele group. In addition, DTI analysis showed increased fractional anisotropy values in s-allele carriers within the uncinate fasciculus. Using the tri-allelic classification approach, increased amygdala reactivity and amygdala insula coupling were observed in the low-functioning compared with the high-functioning group. No significant differences between the two groups were found in structural connectivity. The present results add to the current debate on the influence of the 5-HTTLPR on brain functioning. These differences between s-allele and l/l-allele carriers may contribute to altered vulnerability for psychiatric disorders.

Keywords: classical conditioning; fear; 5-HTTLPR genotype; amygdala; emotion

INTRODUCTION

Fear conditioning is an established model for the development and maintenance of anxiety disorders (Hamm and Weike, 2005; Delgado *et al.*, 2006). In fear conditioning paradigms, a neutral stimulus (CS+) is paired with an aversive stimulus (unconditioned stimulus, UCS) while a second stimulus (CS-) predicts the absence of the UCS. After a few trials, the CS+ elicits conditioned responses (CRs) like increased skin conductance responses (SCRs), changes in preference ratings and altered neural activity (Delgado *et al.*, 2006; Olsson and Phelps, 2007; Tabbert *et al.*, 2011). Regarding the neural correlates of fear conditioning, studies have identified a network including the amygdala, the anterior cingulate cortex (ACC), the insula, the orbito-frontal cortex (OFC) and the occipital cortex (LeDoux, 2000; Olsson and Phelps, 2007). The amygdala is essentially involved in the formation of the conditioned stimulus (CS)/UCS association (LaBar and LeDoux, 1996; Büchel and Dolan, 2000). Conditioned blood oxygen level-dependent signal change (BOLD) responses within the OFC, the insula and the occipital cortex are often interpreted as neural correlates of conscious evaluation processes of bodily arousal, increased attention and the evaluation of the CS value (O'Doherty, 2007; Ochsner *et al.*, 2008; Craig, 2011; Klucken *et al.*, 2012).

Substantial effort has been made to investigate the association between specific genetic variations and fear conditioning because fear conditioning is considered to be a central mechanism in the development of psychiatric disorders (Mineka and Oehlberg, 2008; Lonsdorf *et al.*, 2009, 2015; Caspi *et al.*, 2010; Schweckendiek *et al.*, 2011). The functional genetic variation within the promoter region of the serotonin transporter gene (SLC6A4; serotonin

transporter-linked polymorphic region; 5-HTTLPR) is of special interest (Lesch *et al.*, 1996; Hariri *et al.*, 2002; Munafò *et al.*, 2008; Caspi *et al.*, 2010). A 43 base pair insertion/deletion located in the promoter region of the serotonin transporter gene results in two allelic variations: a long (l-) and a short (s-) allele. Initial *in vitro* studies point to a ('bi-allelic') dominant effect of the s-allele, with reduced 5-HTT availability and 5-HTT functioning in s-allele carriers compared with homozygote l-allele carriers (l/l-allele group) (Lesch *et al.*, 1996; Stoltenberg *et al.*, 2002 but see Heinz *et al.*, 2000). However, this classification strategy is currently under debate (Jonassen and Landro, 2014). More recent studies assume an alternative 'tri-allelic' classification owing to the observation that a single nucleotide polymorphism [A/G polymorphism (rs25531)] renders the L_G-allele functionally similar to the s-allele in terms of the reduced 5-HTT availability ('low-functioning group': S, L_G vs 'high-functioning group': L_A) (Nakamura *et al.*, 2000; Hu *et al.*, 2006; Praschak-Rieder *et al.*, 2007). S-allele carriers and subjects with the low-functioning allele are characterized by an increased vulnerability for psychiatric disorders, which could be explained by exaggerated fear processing (Hariri *et al.*, 2002; Caspi *et al.*, 2003; Heinz *et al.*, 2004; Munafò *et al.*, 2008, 2009a, b; Karg *et al.*, 2011; Jiang *et al.*, 2013; Northoff, 2013).

Although a prominent twin study has suggested that genetic factors explain a substantial proportion of the variance in CRs (Hettema *et al.*, 2003), so far only few studies have investigated the association between the 5-HTTLPR and fear conditioning. Thereby, the majority of studies reported increased CRs in s-allele carriers as compared with the l/l-allele group, as measured by startle responses and hemodynamic activity. However, no group differences were found in conditioned SCRs (Lonsdorf *et al.*, 2009; Agren *et al.*, 2012; Klucken *et al.*, 2013a, b; for an exception see Garpenstrand *et al.*, 2001). Regarding neural activity, increased BOLD responses to the CS+ were observed in the low-functioning group as compared with the high-functioning group, respectively, in s-allele carriers as compared with l/l-allele carriers in different brain structures like the amygdala, the insula, the

Received 18 February 2014; Revised 7 July 2014; Accepted 13 August 2014

Advance Access publication 19 August 2014

This work was supported by a research grant from the DFG German Research Foundation (KL 2500-1). We thank Luise Blochberger, Paul R. Bretschneider, and Aisha J. Munk for data acquisition.

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ACC and the occipital cortex (Hermann *et al.*, 2012; Klucken *et al.*, 2013a). Increased amygdala, ACC and occipital responses were often associated with increased attention to salient stimuli and/or an exaggerated stress response (Bradley *et al.*, 2003; Delgado *et al.*, 2006; Alexander *et al.*, 2009; Homberg and Lesch, 2011; Klucken *et al.*, 2013b). The enhanced insula activity to the CS+ in s-allele carriers was interpreted to mirror an increase of anticipatory anxiety and/or an increased sensitivity to bodily cues (Crişan *et al.*, 2009; Hermann *et al.*, 2012).

In addition to enhanced BOLD responses, alterations of effective connectivity and structural connectivity (e.g. white matter microstructure integrity) have been hypothesized to contribute to the development of psychiatric disorders (Cremers *et al.*, 2010; Meyer-Lindenberg, 2010; Ayling *et al.*, 2012). In detail, amygdala-insula and amygdala-ventromedial prefrontal cortex (vmPFC) connectivity have been related to (dysfunctional) emotion regulation as well as state and trait anxiety (e.g. Baur *et al.*, 2013; Hilbert *et al.*, 2013). Regarding structural connectivity, the delineation of the fiber architecture of tissue using diffusion tensor imaging (DTI) has recently gained increased attention (White *et al.*, 2008; Ayling *et al.*, 2012; Jones *et al.*, 2013). Although a number of different tracts have recently been associated with psychiatric disorders (White *et al.*, 2008; Ayling *et al.*, 2012; Baur *et al.*, 2013), we focused on the uncinate fasciculus (UF) due to the following reasons: first, the UF connects the limbic system with the prefrontal cortex and has repeatedly been linked to (dysfunctional) emotion processing, psychiatric disorders and subclinical (e.g. trait) anxiety (White *et al.*, 2008; Thomason and Thompson, 2011; Ayling *et al.*, 2012; Montag *et al.*, 2012; Baur *et al.*, 2013). Second, the 5-HTTLPR has been associated with altered white matter microstructure in the UF (Pacheco *et al.*, 2009; Jonassen *et al.*, 2012). However, DTI results are not always consistent. For instance, Montag and colleagues (2012) found a positive correlation between the UF and trait anxiety in males but not in females (and also further positive correlations with other tracts), while other studies showed negative correlations with trait anxiety and/or anxiety disorders (Kim and Whalen, 2009; Phan *et al.*, 2009). Nevertheless, it has been speculated that serotonergic transmission and hence related polymorphisms like the 5-HTTLPR might be critical for neuroplasticity, which could influence the development and strength of connectivity of the PFC and other areas (like the amygdala) involved in the serotonergic circuitry (e.g. Homberg *et al.*, 2011; Jonassen and Landro, 2014 for a comprehensive review). For instance, studies suggest that postnatal serotonergic transmission has long-term effects on the neuroplasticity within and around the amygdala (Homberg *et al.*, 2011) and thus may alter its connectivity. In addition, serotonin is involved in early differentiation and maturation of nerve cells not only in the amygdala but also in many other cortical and subcortical brain areas (Jonassen and Landro, 2014).

To date, associations of the 5-HTTLPR genotype with fear conditioning as well as with structural and functional connectivity alterations have been investigated only separately. The integration of these measurements constitutes an essential step toward a more detailed understanding of the impact of 5-HTTLPR genotype on brain mechanisms. Therefore, the first aim of the study was to analyze the association between 5-HTTLPR genotype and fear conditioning. The second aim was to examine effective as well as structural connectivity of the amygdala. We expected increased amygdala responses to the CS+ during fear conditioning as well as increased connectivity in s-allele carriers because fear conditioning may provoke (state) anxiety and probably emotion regulation (e.g. by trying to down-regulate) when confronted with the CS+ (e.g. Delgado *et al.*, 2008; Hermann *et al.*, 2014). We analyzed the data with respect to the bi-allelic and the

tri-allelic classification approach to add to the ongoing classification debate.

METHODS

Participants

For the present study, 107 participants (mean age: 24.3; s.d.: 4.4, 58 males) were recruited. To avoid potential confounds due to stratification strategy, we included only Caucasian participants with European background, who were native German speakers. Current or past mental, sexual or chronic health problems as well as consumption of psychotropic drugs were defined as exclusion criteria. All participants were right-handed, had normal or corrected-to-normal vision and received 40 Euro for their participation. Participants signed an informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. Seven participants (five males) were excluded owing to neurological problems ($n = 1$; arachnoidal cyst) or owing to excessive (>6 mm) head motion during scanning ($n = 6$), leaving 100 participants in the final sample. The genotype frequencies were as follows: 9 s/s (4 males; mean age: 23.3; s.d.: 2.6), 42 s/l (24 males; mean age: 23.2; s.d.: 3.0) and 49 l/l (25 males; mean age: 24.7; s.d.: 5.5). There was no significant deviation from Hardy–Weinberg Equilibrium ($\chi^2_{(1)} < .1$; $P > 0.9$).

Genotyping

DNA was extracted from buccal cells using a standard commercial extraction kit (High Pure PCR Template Preparation Kit; Roche, Mannheim, Germany) in a MagNA Pure1 LC System (Roche). Participants were genotyped for the 5-HTTLPR genotype (and rs25531) by means of polymerase chain reaction and gel electrophoresis. A detailed protocol is provided elsewhere (Alexander *et al.*, 2009). The bi-allelic results (s carrier vs l/l homozygote) are presented in the result section. As a supplement, additional results are provided using a tri-allelic dichotomized model ('SS, SL_G, L_GL_G, SL_A, L_AL_G' vs 'L_AL_A'). We decided to use this tri-allelic dichotomized model because no group differences occurred between the two 'low-functioning' groups ('SS, SL_G, L_GL_G' and 'SL_A, L_AL_G').

Conditioning procedure

A differential fear conditioning procedure (each CS: 16 trials) was conducted using colored squares as reinforced conditioned (CS+) or as non-reinforced stimuli (CS−). Electrical stimulation was used as UCS (50% reinforcement). Each CS was presented for 8 s. The UCS duration was 100 ms. The UCS was delivered 7.9 s after the CS+ onset and co-terminated with the CS+ offset. The intertrial interval ranged from 4.5 to 7 s. Electrodes were fixed to the middle of the left shin and stimulus intensity was set individually using a gradually increasing procedure to achieve an 'unpleasant but not painful' level of sensation. A custom-made impulse generator (833 Hz) provided transcutaneous electrical stimulation (UCS) for 100 ms through two Ag/AgCl electrodes (1 mm² surface). Two different colored CS+ were used (Wittmann *et al.*, 2007). The two CS+ differed significantly neither in valence, arousal and UCS-expectancy ratings nor in SCRs (all $P > 0.700$) or hemodynamic responses, and are summarized in the analyses. The stimuli were projected onto a screen at the end of the scanner (visual field = 18°) using a liquid-crystal display (LCD) projector and were viewed through a mirror mounted on the head coil. A magnetic resonance imaging (MRI)-compatible video camera was used to check whether participants watched the stimuli. Throughout the experiment, SCRs were sampled simultaneously. Immediately after the conditioning procedure, preference and expectancy ratings of the

CS were collected. An extinction phase was further assessed but will not be reported in the present manuscript.

Subjective ratings

Valence and arousal ratings of the CS were collected using nine-point Likert scales that ranged from 1, very pleasant/not arousing at all, to 9, very unpleasant/very arousing. In addition, UCS-expectancy was rated from 0 (no shock) to 100 (certain shock) using a Likert scale. For the CS ratings (arousal, valence, UCS-expectancy), statistical analyses were performed via analysis of variance (ANOVA) in a 2 (stimulus: CS+ vs CS-) × 2 (genotype: s-allele vs l/l-allele group, respective low-functioning vs high-functioning group) design followed by *post hoc* tests in SPSS 21 (IBM Corporation, Armonk, USA) for each rating. Appropriate Bonferroni-corrected *post hoc t*-tests were conducted to further analyze significant effects.

Skin conductance measures

SCRs were sampled simultaneously with MR scans using Ag/AgCl electrodes filled with isotonic (0.05 M NaCl) electrolyte medium placed hypothenar at the non-dominant (left) hand. SCRs were defined in two analysis windows: the maximum response within the time window 1–5 s after the CS (CS+ or CS-) onset was counted as the first interval response (FIR) and within the time windows 5.1–9 s as the second interval responses (SIR). Statistical analyses were performed via ANOVAs in a 2 (stimulus: CS+ vs CS-) × 2 (group: s-allele vs l/l-allele group; or low-functioning vs high-functioning group) design followed by Bonferroni-corrected *post hoc* tests in SPSS 21.

Magnetic resonance imaging

Hemodynamic activity

All images were acquired with a 1.5 Tesla whole-body tomograph (Siemens Symphony with a quantum gradient system) with a CP head coil. Structural image acquisition consisted of 160 T1-weighted sagittal images (MPRage, 1 mm slice thickness; TR = 1.9 s; TE = 4.16 ms; field of view 250 mm × 250 mm). For functional images, 292 images were registered using a T2*-weighted gradient echo-planar imaging (EPI) sequence with 25 slices covering the whole brain (slice thickness = 5 mm; 1 mm gap; descending slice order; time of repetition (TR) = 2.5 s; echo time (TE) = 55 ms; flip angle = 90°; field of view 192 mm × 192 mm; matrix size = 64 × 64). The first two volumes were discarded owing to the incomplete state of magnetization. The orientation of the axial slices was paralleled to the OFC tissue–bone transition. Data were analyzed using Statistical Parametric Mapping (SPM8, Wellcome Department of Cognitive Neurology, London UK; 2008) implemented in MATLAB 7.5 (Mathworks Inc., Sherbourn, MA). Before all statistical analyses, data were preprocessed as described before (Klucken et al., 2012). The experimental conditions were the CS+, the CS-, the UCS and the non-UCS modelled as events. Regressors were convolved with the hemodynamic response function. The six movement parameters of the rigid body transformation obtained by the realignment procedure were introduced as covariates in the model. The voxel-based time series were filtered with a high pass filter (time constant = 128 s). On the first level of analysis, the contrast CS+ > CS- was analyzed for each participant and introduced as dependent variable into the group analyses to investigate the potential effects of 5-HTTLPR genotype.

On the second level, a full-factorial model was used to avoid potentially biased type I errors due to the use of pooled errors (Boik, 1981; Barcikowski and Robey, 1984). The full-factorial model included the group factor 5-HTTLPR genotype implemented in SPM8 and was analyzed for main effects of task (CS+ > CS- and CS- > CS+). Whole brain analyses were conducted with $P < 0.05$ [family-wise

error (FWE) corrected] and $k > 10$ voxels. Region of interest (ROI) analyses were performed using the small volume correction in SPM8 $P < 0.05$ (FWE-corrected; $k > 5$ voxels). ACC, amygdala, insula, OFC and thalamus masks were taken from the ‘Harvard-Oxford cortical and subcortical structural atlases’ provided by the Harvard Center for Morphometric Analysis. The occipital cortex mask was created with MARINA (Walter et al., 2003). Hermann and colleagues (2012) kindly provided the vmPFC mask.

Effective Connectivity Analyses

To assess connectivity, we conducted a psychophysiological interaction (PPI) analysis, which explores the effective connectivity between a seed region and other brain areas in interaction with an experimental task (Friston et al., 1997; Gitelman et al., 2003; O’Reilly et al., 2012). We used the amygdala as seed region (volume of interest; VOI) and extracted the first eigenvariate as implemented in SPM8. Then, the interaction term was created by multiplying the extracted signal with the contrast of interest (CS+ > CS-) for each participant. Three vectors were created containing (i) the psychological variable (main effect of the contrasts of interest), (ii) the physiological variable (VOI time-course) and (iii) the interaction term. First-level analysis was conducted for each participant and included the three regressors (psychological variable, physiological variable, interaction term) in the design matrix. At the second level, we analyzed genotype-dependent differences in the effective connectivity in the insula and the vmPFC.

Diffusion Tensor Imaging

Diffusion-weighted images were acquired using a single shot, pulsed gradient, EPI protocol (slice thickness = 3 mm; interleaved slice procedure; TR = 9.8 s; TE = 111 ms; field of view 192 mm × 192 mm; matrix size = 128 × 128, 12 directions, b-values = 0 and 1000 s/mm², 3 averages). Skeletonization was carried out using the Tract-Based Spatial Statistics module implemented in FSL. First, data were preprocessed (eddy current and head motion correction, brain mask for the DTI data, tensor calculation). Anisotropy was expressed as fractional anisotropy (FA). The fiber tract of interest (UF) was taken from the John Hopkins University (JHU) DTI white matter atlas provided by the FMRIB software library (FSL) software package (Wakana et al., 2007; Hua et al., 2008). We were interested in group differences in (the frontal part of) the UF tract, which is provided in the JHU DTI white matter atlas because Jonassen et al. (2012) found differences in the frontal but not in the temporal part. To analyze further if potential differences occur exclusively in the hypothesized UF-tract or are found generally in white matter microstructure integrity, we analyzed the posterior corona radiata as a ‘control tract’. This tract is assumed to be uninfluenced by the 5-HTTLPR. In the first-level analyses, FA-values were computed for each participant and introduced as dependent variable for the group analyses. In the group analyses, main effects as well as differences between the genotype groups were calculated using the permutation program ‘randomise’ (FSL software package). In addition, we analyzed both groups separately to test whether group means are significantly increased against chance. A threshold of $P < 0.05$ (FWE-corrected) was used.

RESULTS

Subjective ratings

ANOVAs showed strong main effects of stimulus-type for valence [$F_{(1,98)} = 275.93$, $P < 0.001$], arousal [$F_{(1,98)} = 271.63$, $P < 0.001$] and UCS-expectancy ratings [$F_{(1,98)} = 469.46$, $P < 0.001$], but no interaction effects with 5-HTTLPR genotype as compared with previous studies. *Post hoc* tests showed that the CS+ was rated as significantly

Table 1 Mean subjective ratings (s.d.) and SCRs (FIR and SIR) for the CS+ and the CS–

Stimulus	Valence	Arousal	UCS expectancy	FIR	SIR
CS+	6.6 (1.3)	6.7 (1.2)	6.5 (1.5)	0.14 (0.13)	0.16 (0.16)
CS–	2.3 (1.8)	2.4 (1.9)	1.6 (1.4)	0.09 (0.09)	0.10 (0.12)

more unpleasant, more arousing and with a higher UCS-expectancy rating as compared with the CS– (all $P < 0.001$; see Table 1).

Skin conductance responses

ANOVAs revealed a significant main effect of stimulus-type regarding the FIR [$F_{(1,98)} = 43.54$, $P < 0.001$] and the SIR [$F_{(1,98)} = 37.10$, $P < 0.001$], showing increased SCRs to the CS+ as compared with the CS– (all $P < 0.05$). Neither a main effect of 5-HTTLPR genotype nor a stimulus \times genotype interaction effect was found for SCRs (all $P > 0.1$). We also analyzed group differences regarding the first half (trial 1–10) and the second half (trial 11–20) of the experiment because time might be an important variable in fear conditioning. Again, the results remain stable: No group differences were found, neither in the first nor in the second half (all $P > 0.3$) of the experiment.

MRI results

Fear conditioning

Main effect of stimulus (CS+ > CS–). Whole brain results showed significant differences between the CS+ and the CS– within many different brain structures. We found increased BOLD responses to the CS+ in the occipital cortex ($x/y/z = 15/ -79/10$, $z_{\max} = 7.82$, $P < 0.0001$), the left OFC ($x/y/z = -3/35/ -14$, $z_{\max} = 6.280$, $P < 0.0001$), the supplemental motor area ($x/y/z = -6/11/43$, $z_{\max} = 5.73$, $P < 0.0001$), the middle temporal gyrus ($x/y/z = 54/ -7/52$, $z_{\max} = 5.45$, $P = 0.0001$), the rolandic operculum ($x/y/z = -51/2/13$, $z_{\max} = 5.55$, $P < 0.001$), the supramarginal gyrus ($x/y/z = -51/ -28/28$, $z_{\max} = 5.17$, $P < 0.001$) and the postcentral gyrus ($x/y/z = 18/ -43/61$, $z_{\max} = 5.34$, $P < 0.001$). Further, ROI analyses revealed significant results in the contrast CS+ > CS– (see Table 2 for detailed P -values and localization). Finally, no main effect of stimulus was found in the contrast CS– > CS+.

Group differences in the contrast CS+ > CS–

Whole brain results showed increased BOLD responses in the contrast CS+ > CS– in s-allele carriers as compared with the l/l-allele group in the left insula ($x/y/z = -36/ -19/22$, $z_{\max} = 4.47$, $P < 0.001$). ROI analyses further displayed higher amygdala reactivity in s-allele carriers as compared with the l/l-allele group for the contrast CS+ > CS– ($x/y/z = 24/ -1/ -29$, $z_{\max} = 3.19$, $P < 0.05$; see Figure 1a). In addition, we found increased insula ($x/y/z = -36/ -19/19$, $z_{\max} = 3.55$, $P < 0.05$) and occipital ($x/y/z = -27/ -1/ -44$, $z_{\max} = 4.21$, $P < 0.05$) activity in s-allele carriers as compared with the l/l-allele group (see Table 2). In addition, no group differences occurred for the contrast CS– > CS+. Finally, whole brain and ROI analyses showed no significantly elevated activity in l/l-allele carriers as compared with the s-allele group.

Effective connectivity

In addition to amygdala reactivity, we used PPI to explore effective connectivity between the amygdala and cortical brain structures. Increased effective connectivity in s-allele carriers as compared with the l/l-allele group was found between the amygdala and the left insula

($x/y/z = -36/ -22/16$; $z = 3.48$; $P < 0.05$; effect size = 0.33; see Figure 1b), but not between the amygdala and the right insula or between amygdala and the vmPFC. In addition, no significant results occurred in further areas in the whole brain analysis.

White matter microstructure integrity

Next, we analyzed the DTI data to investigate white matter microstructure integrity. An effect of genotype was found in the UF but not in the control tract. S-allele carriers showed significantly higher FA-values (max. peak: $x/y/z = 24/ -1/ -29$; $z = 3.19$; $P = 0.027$) as compared with the l/l-allele group (see Figure 1c). Notably, the peak FA-values differentiating s-allele carriers from the l/l-allele group were located in the limbic part of the UF. As an additional finding, we also analyzed the s-allele group separately and found increased FA-values bilaterally in both UF-tracts. Peak voxels were located in the limbic part of the UF-tract (see Figure 1c).

Finally, to investigate if age and gender influenced the effects of the 5-HTTLPR, we entered age and gender as (co)variates into an AN(C)OVA (cf. Jonassen *et al.*, 2012). We did not find any interaction effects of age and gender on FA-values or on other measurements. One reason might be that participants were mostly students, which may have led to restricted variance at least in age. Jonassen and colleagues (2012) investigated a sample with a broader age range.

DISCUSSION

The aim of the present study was to investigate the relationship between 5-HTTLPR genotype and fear conditioning, effective coupling and white matter microstructure integrity. As the main result, s-allele carriers exhibited enhanced amygdala reactivity, increased amygdala-insula connectivity during fear learning and increased white matter integrity in the UF tract.

Fear conditioning

In detail, we found elevated amygdala responses in s-allele carriers as compared with the l/l-allele group during fear learning. Enhanced BOLD responses in response to fear stimuli in s-allele carriers is a frequently observed finding, which has been reported by many studies using various stimuli and designs (Hariri *et al.*, 2002, 2006; Munafò *et al.*, 2008; Alexander *et al.*, 2012; Klucken *et al.*, 2013a). The substantial influence of the serotonergic system on amygdala functioning and fear learning is also mirrored in pharmacological studies showing alterations by direct manipulation of serotonergic neurotransmission (Inoue *et al.*, 2004; Burghardt *et al.*, 2007; Almada *et al.*, 2009; Homberg, 2012). The increased activity might constitute an important process for the stabilization of the fear learning signal, the production of conditioned fear responses and the maintenance of anxiety disorders (Delgado *et al.*, 2006; Schwackendiek *et al.*, 2011).

Importantly, the increased amygdala reactivity during fear conditioning in the s-allele group offers an explanation for the observed dissociation of the 5-HTTLPR on different response levels: As mentioned in the introduction, in contrast to startle responses (Lonsdorf *et al.*, 2009) and hemodynamic activity (Klucken *et al.*, 2013a), most studies have not found a genotype-dependent effect on SCRs. While amygdala reactivity is crucially involved in conditioned startle responses, conditioned SCRs have been reported to be (mostly) dissociated from amygdala responses (Davis and Whalen, 2001; Hamm and Weike, 2005; Weike *et al.*, 2005; Tabbert *et al.*, 2006; Klucken *et al.*, 2009). We assume that the 5-HTTLPR may predominantly alter amygdala-dependent CRs (like startle response) rather than CRs (e.g. SCRs) modulated by other brain areas (Critchley *et al.*, 2002).

Table 2 Significant ROI activations (localization and statistics of the peak voxels) for the contrast CS+ > CS− and for group differences (s-allele group vs l/l-allele group)

Group analysis	Contrast	Structure	Side	k	x	y	z	z _{max}	p _{corr}	Effect size
Main effect of stimulus	CS+ > CS−	ACC	L	420	−3	35	−8	5.27	<0.001	0.50
		Amygdala	L	10	−12	−7	−17	3.23	0.022	0.32
		Amygdala	R	23	24	−1	−29	4.33	0.001	0.42
		Insula	L	172	−33	20	7	3.99	0.012	0.37
		Insula	R	97	−30	23	7	3.76	0.015	0.37
		Occipital cortex	L	3257	−6	−82	10	7.80	<0.001	0.68
		Occipital cortex	R	2920	15	−79	10	7.82	<0.001	0.68
		OFC	L	96	−21	32	−17	5.02	<0.001	0.48
		OFC	R	23	15	14	−14	3.60	0.025	0.35
		Thalamus	L	208	−12	−16	7	3.49	0.029	0.34
		Thalamus	R	165	21	−28	4	3.52	0.026	0.34
		vmPFC	L	70	−3	35	−14	6.28	<0.001	0.58
		vmPFC	R	189	3	38	−14	5.66	<0.001	0.53
		s-allele group > l/l-allele group	CS+ > CS−	Amygdala	R	11	24	−1	−29	3.19
Insula	L			35	−36	−19	19	3.55	0.029	0.35
Occipital cortex	L			54	−27	−1	−44	4.21	0.030	0.41

The threshold was $P < 0.05$ (FWE-corrected; small volume correction according to SPM8). L: left hemisphere, R: right hemisphere. k: cluster size. Effect sizes are given in point biserial correlation of the respective peak voxels. All coordinates are given in MNI space.

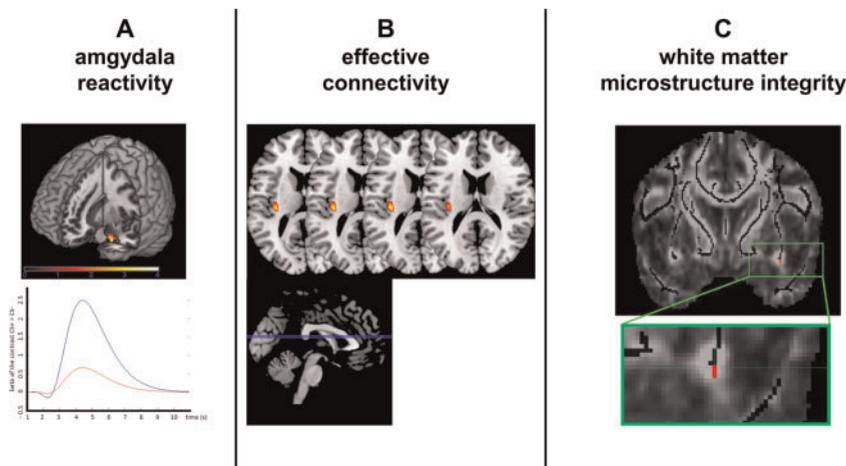


Fig. 1 (A) Neural activations for the main effect of 5-HTTLPR genotype (s-allele carriers vs l/l-allele group) in the right amygdala (above) and a standard modeled canonical hemodynamic response function (HRF) depicting the neural activation from the s-allele (blue line) and the l/l-allele group (red line) for the peak voxel within the amygdala for contrast CS+ > CS− (below). (B) Neural activations for the main effect of 5-HTTLPR genotype in the left insula (above) for the effective connectivity results. (C) Coronal view of the higher FA values in s-allele carriers as compared with the l/l-allele group observed in the limbic part of the UF (red; $x/y/z = 34/3 - 12$). The displaying threshold was $P < 0.05$. Results are displayed on the mean FA skeleton overlaid on the mean FA image.

Effective connectivity

We observed increased amygdala-insula coupling dependent on 5-HTTLPR genotype. Altered amygdala-insula coupling during (dysfunctional) emotion processing is a robust finding (Stein et al., 2007) and has been reported to be modulated by 5-HTTLPR genotype in the context of emotion regulation (Schardt et al., 2010; Lemogne et al., 2011). In detail, s-allele carriers showed increased (anterior) insula responses and altered insula coupling processes as compared with l/l-allele carriers (Schardt et al., 2010; Lemogne et al., 2011). The enhanced coupling in s-allele carriers possibly reflects the increased effort necessary for emotion regulation during the anticipation of the unconditioned fear stimulus (Hermann et al., 2014). However, because in the present study subjects were not explicitly instructed to (actively) regulate their emotions, this interpretation should be treated with caution. Alternatively, because amygdala and insula responses are involved in interoceptive processing, the enhanced connectivity could be caused by increased interoceptive processing in s-allele carriers (Paulus and Stein, 2006; Domschke et al., 2010; Drabant, 2012; Klucken et al., 2013b). However, this is a *post hoc* explanation and future studies have to examine this in more detail.

Structural connectivity

In addition to effective connectivity, the DTI data provides evidence that the 5-HTTLPR genotype is linked to alterations of structural connectivity within the UF. The UF constitutes the white matter tract that connects structures of the limbic system with prefrontal cortex areas (Ebeling and Cramon, 1992; Pacheco et al., 2009; Ayling et al., 2012). Alterations of UF white matter microstructure integrity have been speculated to influence bottom-up processing of fear-relevant signals from the limbic system to cortical structures (Montag et al., 2012), which are responsible for CS evaluation (Milad and Rauch, 2007; O'Doherty, 2007). This view is supported by recent reports of positive correlations between anxiety-related traits and white matter microstructure integrity in the UF (Montag et al., 2012; Modi et al., 2013). Notably, previous studies have also reported negative associations between the UF-tract and anxiety-related traits. Thus, the functionality of white matter microstructure integrity is to date unclear (Phan et al., 2009; Baur et al., 2011, 2013).

The present results allow speculations about larger scale network functioning: The observed increased amygdala reactivity could be a

correlate of a facilitated acquisition process, which renders formerly neutral stimuli into salient stimuli more easily in s-allele carriers (Klucken *et al.*, 2013b). Moreover, the fear signal could be further augmented by increased bottom-up processing enabled by altered white matter microstructure integrity of the UF (Montag *et al.*, 2012). Consequently, s-allele carriers may be prone to encounter a larger number of fear-provoking stimuli (Lonsdorf *et al.*, 2009), which may lead to the experience of negative affect more often in s-allele carriers. This in turn might require enhanced emotion regulation efforts, which could be reflected in increased coupling processes (Volman *et al.*, 2013). It is then conceivable that additional stressors such as stressful life events hamper successful emotion regulation and therefore increase the risk for psychiatric disorders (Karg *et al.*, 2011; Alexander *et al.*, 2012; Miller *et al.*, 2013).

Limitations and overall conclusion

When evaluating the findings of the present study, some limitations have to be considered. First, although we showed that the 5-HTTLPR genotype is associated with white matter microstructure integrity, the biological mechanisms that link altered serotonergic transmission to structural connectivity are undetermined so far. The understanding of the functional consequences of altered white matter microstructure integrity is to date far from comprehensive and thus the current results should be interpreted with caution until replication is available. It is possible that other white matter tracts, which were not investigated in this study, are also associated with the 5-HTTLPR. Only two other studies investigated the 5-HTTLPR genotype and white matter microstructure integrity and observed enhanced white matter microstructure integrity in high-functioning allele carriers in the UF or no clear relationship, which is in contrast to our findings (Pacheco *et al.*, 2009; Jonassen *et al.*, 2012). These findings could have been caused by small sample sizes (37 participants in both studies) and the investigation of female subjects only. Further, the present study only included individuals of European descent. Results based on other (e.g. Asian) backgrounds may not be comparable with the present findings. In addition, we investigated potential variance in the group differences with respect to classification strategies (bi-allelic vs tri-allelic). The low-functioning group displayed increased amygdala activation and amygdala-insula coupling, but no group differences regarding the UF-tract were found. These discrepancies might be explained by the increase of error variance in the tri-allelic dichotomized model (see Jonassen and Landro, 2014, for a comprehensive overview), which decreases the power of the analysis. Finally, in contrast to previous studies, we found increased activations in the vmPFC in the contrast CS+ > CS. Previous results have linked vmPFC activity to fear inhibition (e.g. during fear extinction) and/or to emotion regulation (Milad *et al.*, 2007; Goldin *et al.*, 2008; Hermann *et al.*, 2009; Merz *et al.*, 2012; Lissek *et al.*, 2014; Klucken *et al.*, 2013c). The vmPFC is a brain area that has been associated with many different functions. Thus, the effects observed in this study could be caused by functionally heterogeneous subdivisions. However, this is a *post hoc* interpretation. Further studies should investigate the vmPFC during fear acquisition to clarify its role.

In conclusion, in search of the determinants of the present results, it has been suggested that the association between the 5-HTTLPR and altered emotional processing are determined by early neural developmental rather than differences in 5-HT transmission in adulthood (Jonassen and Landro, 2014). If such early differences in neural plasticity occur, it seems plausible that s-allele carriers show dysfunctional connectivity in the serotonergic circuitry, which is associated with

increased stress and fear (e.g. Alexander *et al.*, 2009, 2012; Belsky *et al.*, 2009; Kuepper *et al.*, 2012).

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