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

Abbreviated title: 5-HTTLPR and fear conditioning

Klucken, Tim¹; Schweckendiek, Jan¹; Blecker, Carlo²; Walter, Bertram²; Kuepper³; Yvonne; Hennig, Juergen³; & Stark, Rudolf¹,²

1 Department of Psychotherapy and Systems Neuroscience, Justus Liebig University Giessen
2 Bender Institute of Neuroimaging, Justus Liebig University Giessen
3 Department of Personality Psychology and Individual Differences, Justus Liebig University Giessen

Corresponding author
Dr. Tim Klucken
Department of Psychotherapy and Systems Neuroscience
Justus Liebig University Giessen
Otto-Behaghel-Str. 10 F
35394 Giessen, Germany
Phone: +49 641 9926088
Fax: +49 641 9926009
E-Mail: tim.klucken@psychol.uni-giessen.de

Number of words: 4839

Keywords: classical conditioning, fear, 5-HTTLPR genotype, amygdala, emotion
Abstract

Strong evidence links the 5-HTTLPR genotype to the modulation of amygdala reactivity, 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). In order 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 FA-values in s-allele carriers within the uncinate fasciculus. approach, increased amygdala reactivity and amygdala insula coupling were observed in the low-functioning compared 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.
Introduction

Fear conditioning is an established model for the development and maintenance of anxiety disorders (Delgado et al., 2006; Hamm, et al., 2005). In fear conditioning paradigms, a neutral stimulus (CS+) is paired with an aversive 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 orbitofrontal cortex (OFC), and the occipital cortex (LeDoux, 2000; Olsson and Phelps, 2007). The amygdala is essentially involved in the formation of the CS/UCS association (Büchel and Dolan, 2000; LaBar and LeDoux, 1996). 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 (Craig, 2011; Klucken et al., 2012; Ochsner et al., 2008; O'Doherty, 2007).

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 (Caspi et al., 2010; Lonsdorf et al., 2009; Mineka and Oehlberg, 2008; Schweckendiek et al., 2011). The functional genetic variation within the promoter region of the serotonin transporter gene (SLC6A4; serotonergic transporter-linked polymorphic region; 5-HTTLPR) is of special interest (Caspi et al., 2010; Hariri et al., 2002; Lesch et al., 1996; Munafò et al., 2008). 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 to 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. More recent studies assume an alternative “tri-allelic” classification due to the observation that a single nucleotide polymorphism (A/G polymorphism (rs25531)) renders the L_C-allele functionally similar to the s-allele in terms of the reduced 5-HTT availability (“low-functioning group”: S, L_C vs. “high-functioning group”: L_A) (Hu et al., 2006; Nakamura et al., 2000; 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 (Caspi et al., 2003; Hariri et al., 2002; Heinz et al., 2004; Jiang et al., 2013; Karg et al., 2011; Munafò et al., 2008; Munafò et al., 2009a; Munafò et al., 2009b; 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 to the l/l-allele group, as measured by startle responses and hemodynamic activity. However, no group differences were found in conditioned SCRs (Agren et al., 2012; Lonsdorf et al., 2009; 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 to the high-functioning group, respectively, in s-allele carriers as compared to l/l-allele carriers in different brain structures like the amygdala, the insula, the ACC,
and the occipital cortex (Hermann et al., 2012; Klucken et al., 2013a). The increased amygdala, ACC, and occipital responses were associated with increased attention to salient stimuli and/or an exaggerated stress response (Alexander et al., 2009; Bradley et al., 2003; Delgado et al., 2006; 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 (Ayling et al., 2012; Cremers et al., 2010; Meyer-Lindenberg, 2010). 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 (Ayling et al., 2012; Jones et al., 2013; White et al., 2008). Although a number of different tracts have recently been associated with psychiatric disorders (Ayling et al., 2012; Baur et al., 2013; White et al., 2008), 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 (Ayling et al., 2012; Baur et al., 2013; Montag et al., 2012; Thomason and Thompson, 2011; White et al., 2008). However, it should be noted that 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 (Phan et al., 2009; Kim and Whalen, 2009). Second, the 5-HTTLPR has been associated with altered white matter microstructure in the UF (Pacheco et al., 2009; Jonassen et al., 2012). Finally, 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 towards 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 in order to add to the ongoing classification debate.
Methods

Participants

For the present study, 107 participants (mean age: 24.3; SD: 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 due to neurological problems (n = 1; arachniodal cyst) or due 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; SD: 2.6), 42 s/l (24 males; mean age: 23.2; SD: 3.0), and 49 l/l (25 males; mean age: 24.7; SD: 5.5). There was no significant deviation from Hardy-Weinberg-Equilibrium ($x^2_{(1)} < .1; p > .9$).

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 (PCR) 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, SLG, LG, SL, LG", vs. "LA, LG"). We decided to use this tri-allelic dichotomized model, because no group differences occurred between the two "low-functioning" groups ("SS, SLG, LG", and "SL, LG").

**Conditioning procedure**

A differential fear conditioning procedure (each conditioned stimulus: 16 trials) was conducted using colored squares as reinforced conditioned (CS+) or as non-reinforced stimuli (CS-). Electrical stimulation was used as unconditioned stimulus (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 inter-trial-interval (ITI) 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+ did neither differ significantly in valence, arousal, and UCS-expectancy ratings nor in SCRs (all \(p > .700\)) 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 an LCD projector and were viewed through a mirror mounted on the head coil. An 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 (ANOVAs) in a 2 (stimulus: CS+ vs. CS-) x 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-) x 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 x 250 mm). For functional images, a total of 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; TR = 2.5 s; TE = 55 ms; flip angle = 90°; field of view 192 x 192 mm; matrix size = 64 x 64). The first two volumes were discarded due 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). Prior to 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 was 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 in order to investigate the potential effects of 5-HTTLPR genotype.

On the second level, a full-factorial model was used in order to avoid potentially biased type I errors due to the use of pooled errors (Barcikowski and Robey, 1984; Boik, 1981). 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 $p < .05$ (family-wise-error corrected (FWE)) and $k > 10$ voxels. Region of interest (ROI) analyses were performed using the small volume correction in SPM8 $p < .05$ (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**

In order to assess connectivity, we conducted a 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 (1) the psychological variable (main effect of the contrasts of interest), (2) the physiological variable (VOI time-course), and (3) 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, echo planar imaging protocol (slice thickness = 3 mm; interleaved slice procedure; TR = 9.8 s; TE = 111 ms; field of view 192 x 192 mm; matrix size = 128 x 128, 12 directions, b-values = 0 and 1000 s/mm$^2$, 3 averages). Skeletonization was carried out using the Tract-Based Spatial Statistics (TBSS) 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 (uncinate fasciculus; UF) was taken from the JHU DTI white matter atlas provided by the FSL software package (Hua et al., 2008; Wakana et al., 2007). 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 in order to test if group means are significantly increased against chance. A threshold of $p < .05$ (FWE-corrected) was used.
Results

Subjective Ratings

ANOVA\text{s} showed strong main effects of stimulus-type for valence ($F_{(1,98)}=275.93$, $p<.001$), arousal ($F_{(1,98)}=271.63$, $p<.001$), and UCS-expectancy ratings ($F_{(1,98)}=469.46$, $p<.001$), but no interaction effects with 5-HTTLPR genotype as compared to previous studies. Post-hoc tests showed that the CS+ was rated as significantly more unpleasant, more arousing, and with a higher UCS-expectancy rating as compared to the CS- (all $p<.001$; see table 1).

Skin Conductance Responses

ANOVA\text{s} revealed a significant main effect of stimulus-type regarding the FIR ($F_{(1,98)}=43.54$, $p<.001$) and the SIR ($F_{(1,98)}=37.10$, $p<.001$), showing increased SCRs to the CS+ as compared to the CS- (all $p<.05$). Neither a main effect of 5-HTTLPR genotype nor a stimulus x genotype interaction effect was found for SCRs (all $p>.1$). We also analyzed group differences regarding the first half (trial 1-10) and the second half (trial 11-20) of the experiment since 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 $p>.3$) of the experiment.

Table 1: Mean subjective ratings (SD) and skin conductance responses (first interval responses, FIR and second interval responses, SIR) for the CS+ and the CS-.

<table>
<thead>
<tr>
<th>stimulus</th>
<th>valence</th>
<th>arousal</th>
<th>UCS-expectancy</th>
<th>FIR</th>
<th>SIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS+</td>
<td>6.6 (1.3)</td>
<td>6.7 (1.2)</td>
<td>6.5 (1.5)</td>
<td>0.14 (0.13)</td>
<td>0.16 (0.16)</td>
</tr>
<tr>
<td>CS-</td>
<td>2.3 (1.8)</td>
<td>2.4 (1.9)</td>
<td>1.6 (1.4)</td>
<td>0.09 (0.09)</td>
<td>0.10 (0.12)</td>
</tr>
</tbody>
</table>
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 <.0001), the left orbitofrontal cortex (x/y/z= -3/35/-14, z_max= 6.280, p <.0001), the supplemental motor area (x/y/z= -6/11/43, z_max= 5.73, p <.0001), the middle temporal gyrus (x/y/z= 54/-7/52, z_max= 5.45, p <.001), the rolandic operculum (x/y/z= -51/2/13, z_max= 5.55, p <.001), the supramarginal gyrus (x/y/z= -51/-28/28, z_max= 5.17, p <.001), and the postcental gyrus (x/y/z= 18/-43/61, z_max= 5.34, p <.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 to the l/l-allele group in the left insula (x/y/z= -36/-19/22, z_max= 4.47, p <.001). ROI-analyses further displayed higher amygdala reactivity in s-allele carriers as compared to the l/l-allele group for the contrast CS+ > CS- (x/y/z= 24/-1/-29, z_max= 3.19, p <.05; see figure 1a). In addition, we found increased insula (x/y/z= -36/-19/19, z_max= 3.55, p <.05) and occipital (x/y/z= -27/-1/-44, z_max= 4.21, p <.05) activity in s-allele carriers as compared to 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 to the s-allele group.
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).

<table>
<thead>
<tr>
<th>group analysis</th>
<th>contrast</th>
<th>structure</th>
<th>side</th>
<th>k</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>z_max</th>
<th>p_corr</th>
<th>effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td>main effect of stimulus</td>
<td>CS+ &gt; CS-</td>
<td>ACC</td>
<td>L</td>
<td>420</td>
<td>-3</td>
<td>35</td>
<td>-8</td>
<td>5.27</td>
<td>&lt;.001</td>
<td>.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amygdala</td>
<td>L</td>
<td>10</td>
<td>-12</td>
<td>-7</td>
<td>-17</td>
<td>3.23</td>
<td>.022</td>
<td>.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amygdala</td>
<td>R</td>
<td>23</td>
<td>24</td>
<td>-1</td>
<td>-29</td>
<td>4.33</td>
<td>.001</td>
<td>.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insula</td>
<td>L</td>
<td>172</td>
<td>-33</td>
<td>20</td>
<td>7</td>
<td>3.99</td>
<td>.012</td>
<td>.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insula</td>
<td>R</td>
<td>97</td>
<td>-30</td>
<td>23</td>
<td>7</td>
<td>3.76</td>
<td>.015</td>
<td>.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occipital Cortex</td>
<td>L</td>
<td>3257</td>
<td>-6</td>
<td>-82</td>
<td>10</td>
<td>7.80</td>
<td>&lt;.001</td>
<td>.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occipital Cortex</td>
<td>R</td>
<td>2920</td>
<td>15</td>
<td>-79</td>
<td>10</td>
<td>7.82</td>
<td>&lt;.001</td>
<td>.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFC</td>
<td>L</td>
<td>96</td>
<td>-21</td>
<td>32</td>
<td>-17</td>
<td>5.02</td>
<td>&lt;.001</td>
<td>.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFC</td>
<td>R</td>
<td>23</td>
<td>15</td>
<td>14</td>
<td>-14</td>
<td>3.60</td>
<td>.025</td>
<td>.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thalamus</td>
<td>L</td>
<td>208</td>
<td>-12</td>
<td>16</td>
<td>7</td>
<td>3.49</td>
<td>.029</td>
<td>.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thalamus</td>
<td>R</td>
<td>165</td>
<td>21</td>
<td>-28</td>
<td>4</td>
<td>3.52</td>
<td>.026</td>
<td>.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vmPFC</td>
<td>L</td>
<td>70</td>
<td>-3</td>
<td>35</td>
<td>-14</td>
<td>6.28</td>
<td>&lt;.001</td>
<td>.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vmPFC</td>
<td>R</td>
<td>189</td>
<td>3</td>
<td>38</td>
<td>-14</td>
<td>5.66</td>
<td>&lt;.001</td>
<td>.53</td>
</tr>
<tr>
<td>s-allele group &gt;</td>
<td>CS+ &gt;</td>
<td>Amygdala</td>
<td>R</td>
<td>11</td>
<td>24</td>
<td>-1</td>
<td>-29</td>
<td>3.19</td>
<td>.026</td>
<td>.32</td>
</tr>
<tr>
<td>l/l-allele group</td>
<td>CS-</td>
<td>Insula</td>
<td>L</td>
<td>35</td>
<td>-36</td>
<td>-19</td>
<td>19</td>
<td>3.55</td>
<td>.029</td>
<td>.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occipital Cortex</td>
<td>L</td>
<td>54</td>
<td>-27</td>
<td>-1</td>
<td>-44</td>
<td>4.21</td>
<td>.030</td>
<td>.41</td>
</tr>
</tbody>
</table>

The threshold was $p < .05$ (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.

**Effective Connectivity**

In addition to amygdala reactivity, we used PPI in order to explore effective connectivity between the amygdala and cortical brain structures. Increased effective connectivity in s-allele carriers as compared to the l/l-allele group was found between the amygdala and the left insula ($x/y/z=-36/-22/16$; $z=3.48$; $p < .05$; effect size= .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 in order 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; \(p= .027\)) as compared to 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 lead to restricted variance at least in age. Jonassen and colleagues (2012) investigated a sample with a broader age range.

--please insert figure 1 about here--
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 to 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 (Alexander et al., 2012; Hariri et al., 2002; Hariri et al., 2006; Klucken et al., 2013a; Munafò et al., 2008). 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 (Almada et al., 2009; Burghardt et al., 2007; Homberg, 2012; Inoue et al., 2004). 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; Schweckendiek 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 et al., 2005; Klucken et al., 2009; Tabbert et al., 2006; Weike et al., 2005). 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).

**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 (Lemogne et al., 2011; Schardt et al., 2010). In detail, s-allele carriers showed increased (anterior) insula responses and altered insula coupling processes as compared to I/I-allele carriers (Lemogne et al., 2011; Schardt et al., 2010). 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 (Chritchley et al., 2004; Domschke et al., 2010; Drabant, 2012; Klucken et al., 2013b; Paulus and Stein, 2006). 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 (Ayling et al., 2012; Ebeling and Cramon, 1992; Pacheco et al., 2009). 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 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 (Modi et al., 2013; Montag et al., 2012). 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 (Baur et al., 2011; Baur et al., 2013; Phan et al., 2009).

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 (Alexander et al., 2012; Karg et al., 2011; Miller et al., 2012).

**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 decent. 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 (Lissek et al., 2013; Goldin et al., 2008; Hermann et al., 2009; Klucken et al., 2013; Merz et al., 2012; Milad et al. 2007). 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 in order 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).
Conflicts of interest: The authors declare no competing financial interests

Funding: This work was supported by a research grant from the DFG German Research Foundation (KL 2500-1).

Acknowledgements: We thank Luise Blochberger, Paul R. Bretschneider, and Aisha J. Munk for data acquisition.
REFERENCES


Figure Legends

Figure 1.

Figure 1a. Neural activations for the main effect of 5-HTTLPR genotype (s-allele carriers vs. I/I-allele group) in the right amygdala (above) and a standard modelled canonical haemodynamic response function (HRF) depicting the neural activation from the s-allele (blue line) and the I/I-allele group (red line) for the peak voxel within the amygdala for contrast CS+ > CS- (below).

Figure 1b. Neural activations for the main effect of 5-HTTLPR genotype in the left insula (above) for the effective connectivity results.

Figure 1c. Coronar view of the higher FA values in s-allele carriers as compared to the I/I-allele group observed in the limbic part of the uncinate fasciculus (red; x/y/z = 34/3-12). The displaying threshold was p < .05. Results are displayed on the mean FA skeleton overlaid on the mean FA image.