WESTFÄLISCHE
WILHELMS-UNIVERSITÄT
MÜNSTER

FACHGEBIET PSYCHOLOGIE

NEUROGENETICS OF EMOTION PROCESSING IN MAJOR DEPRESSION

INAUGURAL-DISSERTATION

ZUR

ERLANGUNG DES DOKTORGRADES

DER

PHILOSOPHISCHEN FAKULTÄT

DER

WESTFÄLISCHEN WILHELMS-UNIVERSITÄT

ZU

MÜNSTER (WESTF.)

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MÄRZ 2010

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1 General introduction

"I suggest that comprehensive study of the psychological as well as biological correlates of depression can provide a new understanding of this debilitating disorder"
Aaron T. Beck, American Journal of Psychiatry, 2008

Major depression is one of the most debilitating diseases of our times. Depression represents a highly prevalent, costly and burdensome disease, recently considered to be among the worldwide leading causes of disability adjusted life years (Evans & Charney, 2003; World Health Organization, 2001). Aside from environmental factors, a strong genetic contribution to the etiology of (unipolar) depression is assumed with estimations of heritability ranging around 40-50% (Sullivan, Neale, & Kendler, 2000). However, during decades of genetic association studies, there has been limited success in identifying robust candidate genes. Although several genetic variations have been identified - many of them affecting serotonin signal transduction - that were significantly associated with major depression or depression related personality traits in various single studies, these encouraging findings have been contrasted with several null findings or even contradictive results.

Associations of single polymorphisms with clinically defined phenotypes have been criticized for shortcomings and methodological difficulties, particularly in psychiatric genetics (Malhotra & Goldman, 1999) . In the context of complex polygenetic inheritance, epistasis, and epigenetic phenomena, single polymorphisms are unlikely to produce more than weak statistical effects, necessitating large samples, often exceeding several hundred subjects. Furthermore, it seems likely that there are etiological subgroups within major depression that would obscure effects at the broader group level. More importantly, genetic effects on the symptom level are mediated by their molecular and cellular effects on information processing in distinct brain circuitries. To understand the phenotypic heterogeneity in the context of a genetically complex background, researchers have begun to focus on endophenotypes within major depression and on elucidating their underlying genes (Hasler, Drevets, Manji, & Charney, 2004). Specifically, examining genetic effects

on neurobiological correlates of emotion processing in depression might represent a next step in understanding of genetic contribution to variability in the clinically defined phenotype.

During the last decade, an increasing number of studies have investigated such functional correlates of major depression by means of functional magnetic resonance imaging (fMRI). A common finding of neurobiological abnormalities in major depression is *amygdala hyperactivity*. Amygdala hyperactivity has been demonstrated in acutely depressed patients compared with controls at rest (Drevets et al., 2002), in expectation of negative pictures (Abler, Erk, Herwig, & Walter, 2007), in response to negative verbal stimuli (Siegle, Steinhauer, Thase, Stenger, & Carter, 2002; Siegle, Thompson, Carter, Steinhauer, & Thase, 2007), and emotional faces (Sheline et al., 2001), which was shown to resolve after antidepressant therapy (Fu et al., 2004; Sheline et al., 2001). Amygdala hyper-responsiveness to negative stimuli might represent the neural correlate of a stronger or deeper processing (Beck, 2008). Therefore, an increased responsiveness of the amygdala has been implicated in the pathogenesis of major depression, probably by causing negatively biased emotion processing (Dannlowski et al., 2007; Phillips, Drevets, Rauch, & Lane, 2003; Whalen, Shin, Somerville, McLean, & Kim, 2002).

Increased amygdala responsiveness has already been successfully employed as functional endophenotype for genetic association studies. Since the landmark study of Hariri and colleagues (Hariri et al., 2002), several studies have shown that genetic variation particularly in the serotonergic system has strong impact on amygdala responsiveness (Munafò, Brown, & Hariri, 2008). These studies founded the emerging research field called "imaging genetics" (Hariri, Drabant, & Weinberger, 2006) and for the first time, bridged the gap between molecular genetics and cognitive neuroscience.

However, the majority of studies investigating amygdala functioning in depression or using the imaging genetics approach employed paradigms using *overt* stimulus presentation with conscious processing of emotional stimuli. Nevertheless, according to neurobiological theories of emotions, the amygdala is particularly implicated in the rapid and automatic processing of emotional significance preceding conscious awareness (Ledoux, 1996). In healthy subjects, several studies have confirmed that the amygdala is

engaged during processing of emotional stimuli, even if presented briefly (< 40 ms) and backward-masked, and thus without conscious awareness (Whalen et al., 1998). Amygdala reactivity to covertly but not to overtly presented negative faces has been associated with individual differences in trait anxiety (Etkin et al., 2004). Thus, using *covert* stimulus presentation might be a more appropriate approach in order to investigate the role of the amygdala in dispositional emotional reactivity.

In the following experiments, we therefore have investigated *automatic* amygdala activity in response to emotional facial expressions presented briefly and backward-masked. The goals of the present thesis were:

1. To show that in major depression emotion processing is already biased on an automatic level of processing in the amygdala. We hypothesized that depressed patients show stronger amygdala responsiveness to negative facial expressions, also if the stimuli were not processed consciously.

2. To use this neurobiological trait as potential endophenotype. In two independent studies we investigated two recently discovered genetic variations, which have already been associated with depression and negatively biased emotion processing. We speculated that carriers of genetic risk variations in the investigated genes would show stronger automatic amygdala responsiveness to masked negative facial expressions.

In experiment 1 (chapter 2), we investigated automatic amygdala responsiveness to masked negative and positive faces in depressed patients and healthy controls using the subliminal affective priming paradigm of (Murphy & Zajonc, 1993)). In experiment 2 (chapter 3), we employed the same paradigm in a larger sample of healthy subjects. These subjects were genotyped for a prominent functional polymorphism in the serotonin transporter gene (5-HTTLPR). We investigated automatic amygdala responsiveness dependent on 5-HTTLPR genotype. Finally, in experiment 3 (chapter 4), we used a combined pharmacogenetic and imaging genetics approach to investigate the clinical and neurobiological impact of genetic variation in the neuropeptide Y (NPY) gene, which has been implicated in the pathophysiology of major depression.

2 Experiment 1: Automatic amygdala response bias in major depression[1]

"Odi et amo. Quare id faciam fortasse requiris. Nescio. Sed fieri *sentio et excrucior."*

Gaius Valerius Catullus, Carmen 85

2.1 Summary

Background: Cognitive theories of depression predict mood-congruent negative biases already at automatic stages of processing, although several behavioral studies seem to contradict this notion. That is, depression should potentiate emotional reactivity to negative emotional cues whereas it should reduce reactivity in response to positive stimuli. Assessing neurobiological substrates of automatic emotion processing might be a more sensitive challenge for automatic negative bias in depression than behavioral measures. Methods: In 30 acutely depressed inpatients and 26 healthy controls, automatic amygdala responses to happy and sad facial expressions were assessed by means of functional magnetic resonance imaging (fMRI) at 3 Tesla. In order to examine automatic responses, a presentation paradigm using subliminal, backward-masked stimuli was employed. A detection task was administered to assess participants' awareness of the masked emotional faces presented in the fMRI experiment. Results: Detection performance was at chance level for both patients and healthy controls, suggesting that the neurobiological reactions took place in absence of conscious awareness of the emotional stimuli. A robust emotion by group interaction was observed in the right amygdala. Whereas healthy controls demonstrated stronger responses to happy faces, depressed patients showed the opposite. Furthermore, amygdala responsiveness to happy facial expression was negatively correlated with current depression severity. Conclusions: Depressed patients exhibit potentiated amygdala reactivity to masked negative stimuli along with a reduced responsiveness to masked positive stimuli compared to healthy individuals. Thus, depression is characterized

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by mood congruent processing of emotional stimuli in the amygdala already at an automatic level of processing.

2.2 Introduction

Early cognitive theories of depression highlight the importance of negative biases affecting several if not all cognitive processes. Beck, for instance, suggested that depressogenic schemas operate in all aspects and stages of cognition, favoring or facilitating the processing of negative (mood-congruent) stimuli in major depression (Beck, 1967). Similar predictions can be derived from Bower's network model (Bower, 1981). According to these theories, mood congruency effects should be observable already at early, automatic processing stages. Pervasive negative mood states in depressed patients might contribute to an enhanced emotional reactivity to negative emotional cues (i.e. negative potentiation) and reduce reactivity in response to positive emotional stimuli (i.e. positive attenuation) (Beck, 2008; Depue & Iacono, 1989; Scher, Ingram, & Segal, 2005). Other theories propose that depression is rather characterized by cognitive biases in late or controlled stages of information processing (Mathews & MacLeod, 2005; Williams, Watts, MacLeod, & Mathews, 1997). Indeed, findings of negative biases in late stages of attention or explicit memory appear quite consistently in the literature, whereas findings of automatic emotion processing biases in major depression are rare (Dannlowski et al., 2006). However, most studies investigating cognitive bias in depression made use of reaction-time paradigms with behavioral measures as dependent variable. Neurobiological analyses might provide a more sensitive assessment of automatic emotion processing in depression, compared to behavioral testing.

The amygdala is a central structure in the limbic emotion processing circuit (Davis & Whalen, 2001). In addition to a slower, cortical route, the amygdala receives direct projections from the thalamus, allowing a rapid response to emotionally salient stimuli, even before conscious cortical representations have been formed (Ledoux, 1996). Amygdala hyperactivity has been implicated in the pathogenesis of major depression, probably by causing negatively biased emotion- processing (Phillips, Drevets, Rauch, & Lane, 2003; Whalen, Shin, Somerville, McLean, & Kim, 2002). Therefore, the amygdala was selected as region of interest in the present study. Functional magnetic resonance imaging was used to examine differences between depressed patients and healthy controls in automatic amygdala reactivity to facial emotions. Emotional faces were presented briefly

and masked backward by neutral faces to prevent conscious emotion processing. Facial expression serves as an important social signal that modulates interpersonal interactions (Ekman, 1984). The amygdala has been shown to be activated reliably by facial emotions, even when facial expressions were presented below the threshold of conscious awareness (Etkin et al., 2004; Killgore & Yurgelun-Todd, 2004; Liddell et al., 2005; Nomura et al., 2004). According to a recent meta-analysis of neuroimaging studies on amygdala activation, there is evidence for hemispheric lateralization during the processing of emotional stimuli. The right amygdala may subserve a high-speed detection role for unconscious stimuli (Costafreda, Brammer, David, & Fu, 2008).

We hypothesized that relative to healthy controls, acutely depressed patients show stronger automatic (right) amygdala activation in response to mood congruent (sad) facial expressions and less automatic activation to mood-incongruent (happy) facial expressions.

2.3 Methods and Materials

2.3.1 Subjects

Datasets of 30 right-handed inpatients with an acute major depressive episode according to DSM-IV criteria (American Psychiatric Association, 1994), diagnosed with the SCID-I interview (Wittchen, Wunderlich, Gruschwitz, & Zaudig, 1997) and 26 healthy control subjects were analyzed (see Table 1). Exclusion criteria were any neurological abnormalities, substance abuse, former electroconvulsive therapy, mood stabilizers, neuroleptic or benzodiazepine treatment. All patients were under antidepressant treatment (see Table 2 for details) which was coded in terms of dose and treatment duration into medication levels from 1 to 4, according to the suggestions of Sackeim (Sackeim, 2001). The study was approved by the Ethics Committee of the University of Münster. After complete description of the study to the subjects, written informed consent was obtained. Only patients with primary major depression were included (indicated by earlier onset and predominant symptoms). Secondary life-time diagnoses were undifferentiated somatoform disorder (n=4), social phobia (n=4), dysthymia (n=3), OCD (n=2), panic disorder (n=1),

specific phobia (n=1), generalized anxiety disorder (n=1), and pain disorder (n=1). Two patients had two and one patient had three comorbid disorders.

Table 1   Characteristics of study participants in experiment 1

	Patients (n = 30)	Controls (n = 26)	p-value, according to χ^2- or t-tests (2-tailed)
Age	38.8 (11.4)	36.2 (13.4)	0.44
Sex (m/f)	17/13	12/14	0.59
HAMD[1]	24.8 (4.9)	0.6 (0.8)	<0.001
HAMA[2]	21.1 (5.8)	1.5 (1.7)	<0.001
Total education time	13.6 (1.7)	14.2 (1.8)	0.24
Antidepressant potency[3]	2.6 (1.2)		
Number of episodes	2.7 (2.0)		
Life-time hospitalization (weeks)	7.6 (8.7)		
Duration of illness (months)	72.2 (75.0)		
Evaluation sad prime	-0.13 (0.23)	-0.01 (0.33)	0.12
Evaluation happy prime	-0.08 (0.33)	-0.04 (0.27)	0.60
Evaluation neutral prime	-0.10 (0.25)	-0.03 (0.24)	0.29
Evaluation no-face prime	-0.07 (0.29)	-0.03 (0.28)	0.58
Latency sad prime	1403.0 (537.7)	1319.6 (317.6)	0.51
Latency happy prime	1398.0 (575.7)	1315.6 (345.2)	0.54
Latency neutral prime	1400.3 (543.4)	1286.1 (338.2)	0.38
Latency no-face prime	1395.3 (511.6)	1303.3 (322.1)	0.45

Evaluation values reflect mean evaluative responses to the neutral face mask during the fMRI experiment dependent on prime condition (mean (s.d.)). [1]HAMD, Hamilton Rating Scale for Depression (Hamilton, 1960), [2]HAMA, Hamilton Rating Scale for Anxiety (Hamilton, 1959), [3]Antidepressant potency, coded in terms of dose and treatment duration into medication levels from 1 to 4 (Sackeim, 2001).

Table 2   List of antidepressant medication in the patient sample

Medication	Number of patients*
Mirtazapine	10
Venlafaxine	9
Escitalopram	4
Citalopram	3
Reboxetine	3
Duloxetine	2
Fluoxetine	2
Sertraline	2
Clomipramin	1
Buproprion	1
Doxepine	1

Values reflect number of patients treated with the respective medication; * Eight patients have been treated with a combination of 2 antidepressants, therefore the total number exceeds n=30

2.3.2 fMRI methods

Facial stimuli in the fMRI experiment consisted of grey-scale normalized sad, happy, and neutral expressions of 10 individuals (Ekman & Friesen, 1976). Emotional and neutral faces were presented for 33 msec and masked by neutral faces of the same individuals. To avoid identity of prime and mask in the neutral face condition, vertically mirrored faces were used as neutral primes. That is, neutral prime faces were produced by mirror-inversion (left to right) of neutral mask faces. Eighty trials were shown: 20 with sad, 20 with happy, and 20 with neutral prime faces; in 20 trials no prime-faces were presented. Faces were shown in two fixed pseudo-random sequences with the restriction of no repetition of an

individual and no more than one repetition of a prime condition on consecutive trials. Each trial lasted 9 sec. A fixation cross presented for 800 msec preceded a prime face shown for 33 msec which was followed by the corresponding neutral face mask, presented for 467 msec. A blank screen followed for 7.700 msec. During this time-period subjects had to evaluate whether the neutral (mask) face expressed rather negative or positive feelings, by pressing one of four buttons (−1.5, −0.5, +0.5, and +1.5). In each hand, participants held a fiber-optic response pad with two buttons (the positive or the negative response keys). One half of the sample gave positive responses with the left hand; the other with the right hand. Judgments and reaction times were registered. Images were projected to the rear end of the scanner (Sharp XG-PC10XE with additional HF shielding). T2* functional data were acquired at a 3 T scanner (Gyroscan Intera 3T, Philips Medical Systems, Best, NL), using a single shot echoplanar sequence with parameters selected to minimize distortion in the region of central interest, while retaining adequate signal to noise ratio (S/N) and T2* sensitivity (Robinson, Windischberger, Rauscher, & Moser, 2004). Volumes consisting of 40 axial slices were acquired (matrix 64^2, resolution 3.5×3.5×3.5mm; TR=3sec, TE=30msec, FA=90°). Functional imaging data were motion-corrected, spatially normalized to standard MNI space (Montreal Neurological Institute) and smoothed (Gaussian kernel, 8mm FWHM) using Statistical Parametric Mapping (SPM5, http://www.fil.ion.ucl.ac.uk/spm). An event-related analysis design was used. For each subject, trials were averaged separately for each prime condition, reducing the data to four average trials per subject. Brain responses to the prime stimulus categories were isolated by convolving a vector of onset times of the emotional and neutral primes and the no-face condition with a canonical hemodynamic response function. Since the two baseline conditions (neutral face and no face) did not differ with respect to amygdala responses elicited across both groups ($p>.05$, uncorrected), only the crucial contrasts of happy vs. neutral and sad vs. neutral expressions are reported. These two individual 1st level contrasts were entered into an ANOVA using the flexible factorial model implemented in SPM5, with emotion as within-subjects factor and group as between-subjects factor. A third factor "subjects" was also included in the model to account for the individual constants. In order to test whether the masked presentation of sad and happy expressions resulted in activation

of the amygdala, the main effect of each emotion condition (vs. the neutral prime baseline) was assessed separately within the whole sample. The model was used to calculate the main effects of group (patients vs. controls), emotion (happy vs. sad), and the crucial group x emotion interaction at a threshold of $p<.05$, family wise error (FWE) corrected for the amygdalae. The amygdala was defined according to (Tzourio-Mazoyer et al., 2002) and the amygdala mask was created by means of the WFU pickatlas (Maldjian, Laurienti, Kraft, & Burdette, 2003). To explore the nature of the group x emotion interaction, t-tests were used to investigate the effect of emotion within each group separately (paired t-tests) and to compare the activation due to masked sad and happy faces between groups at $p<.05$, uncorrected. At the location of maximal group x emotion interaction, the contrast values of happy vs. neutral and sad vs. neutral faces were extracted and used for exploratory analyses, controlling for potential effects of gender, detection task performance, medication level, number of episodes, comorbidity status, and duration of illness. These variables were included either as factor (gender) or covariate (detection task performance) in the group x emotion ANOVA, or (within the patient group) correlated with the extracted contrast values.

For exploratory reasons, a supplementary whole-brain analysis was conducted at $p<0.001$, uncorrected, with a cluster threshold of k=25 voxels. Finally, amygdala responsiveness to happy and sad faces was correlated with depression severity and anxiety level as measured with the Hamilton Depression Scale (HAMD; (Hamilton, 1960)) and the Hamilton Anxiety Scale (HAMA; (Hamilton, 1959)) within the patient sample.

2.3.3 Detection task

After the fMRI experiment, all subjects were asked whether they had noticed anything extraordinary during the scanner experiment. Then, subjects took part in a forced-choice detection task outside the scanner. The detection task consisted of 40 trials of the same stimulus presentation conditions and the same stimuli as in the fMRI experiment. Subjects were told that immediately before the face with the neutral expression another face was shown so briefly that it was very difficult to perceive. They should recognize the expression of the face presented before the neutral face. Subjects were informed that the covert face

could have a happy, a sad, or a neutral expression and that in some cases no covert face was shown. Subjects should indicate via button press which prime condition (happy, sad, neutral, no face) was presented before the neutral mask. *A'* was calculated as non-parametric measure of sensitivity, taking into account hit rate and false alarm rate, with *A'*=0.5 indicating chance level (Grier, 1971).

2.4 Results

2.4.1 Detection task

After the fMRI experiment, all subjects reported that they had not recognized any briefly presented emotional faces, even after being informed about their presence. In the detection task, average sensitivity of healthy controls and patients did not differ significantly from chance level, either for happy (controls: *A'*=0.49; patients: *A'*=0.51), sad (controls: *A'*=0.54; patients: *A'*=0.51), or neutral prime faces (controls: *A'*=0.48; patients: *A'*=0.43), according to t-tests (all *p*s > .2). Importantly, both groups did not differ concerning their sensitivity indexes for emotional or neutral faces (all *p*s > .55).

2.4.2 Behavioral results

Patients and controls did not differ in their evaluative ratings of the neutral mask faces or their reaction times, irrespective of prime condition (see Table 1).

2.4.3 fMRI results

Across both groups, the paradigm successfully elicited automatic amygdala responses to both masked happy and masked sad faces, compared with masked neutral faces, despite subjective unawareness of the emotional primes (see Fig. 1).

Figure 1   Amygdala responses to subliminal sad and happy faces

Coronal slices (y = 4) depicting amygdala responsiveness to subliminal sad (left) and happy (right) faces (vs. neutral faces) across both study groups ($p < 0.05$, FWE corrected). Both emotion conditions yielded strong bilateral amygdala responses.
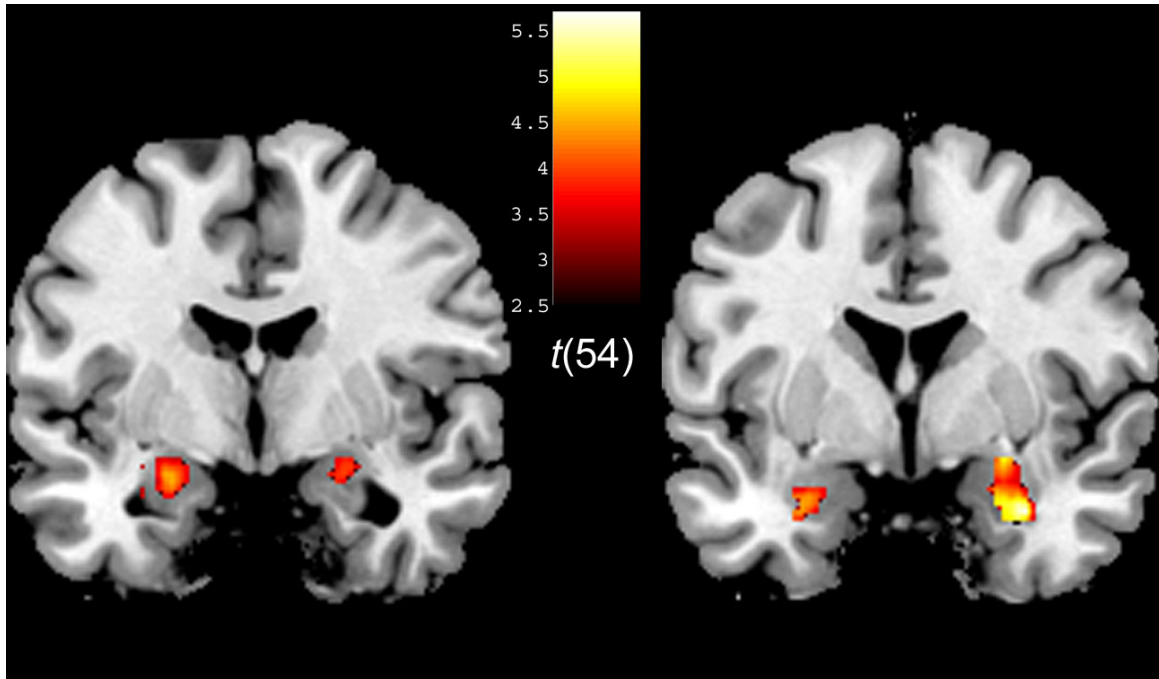
The ANOVA based on the contrasts of happy vs. neutral and sad vs. neutral expressions yielded no significant main effect of emotion or group within the amygdala. However, the hypothesized emotion x group interaction was observed in a cluster within the right amygdala, x = 30, y = 4, z = -20; $t(54)=3.56$, $d = 0.97$; $p_{uncorrected} = .00039$; $p_{FWE-corrected} = .016$; cluster size k = 7 (Figure 2). To determine the amygdalar subregion where differential processing of emotion faces occurred, the SPM Anatomy toolbox Version 1.5 (Eickhoff et al., 2005) was administered. The emotion x group interaction was located in the lateral and basal nuclei of the amygdala. Depressed patients showed higher amygdala responses to sad faces, than to happy faces, x = 28, y = 4, z = -20, $t(29) = 2.91$, $p = .003$, $d = 1.08$, k = 20, and compared with amygdala responses to sad facial expressions in healthy controls, x = 30, y = 2, z = -22, $t(54) = 2.34$, $p = .011$, $d = 0.64$, k = 29. Conversely, healthy controls showed the opposite pattern, with stronger amygdala responses to happy compared with sad

faces, x = 32, y = 0, z = -26, $t(25)$ = 2.74, p = .006, d = 1.10, k = 94, and with the depressed patients' amygdala responses to happy faces, x = 34, y = 4, z = -20, $t(54)$ = 2.26, p = .016, d = 0.62, k = 5.

Figure 2   Group x emotion condition interaction in the amygdala

Left: coronal view (y=4), depicting significant group x emotion interaction in the right amygdala, thresholded at p<0.05, FWE corrected for the amygdala volume.
Right: bar graphs depicting the mean contrast value for happy-neutral and sad-neutral extracted from x=30, y=4, z=-20, dependent on emotion and study group.
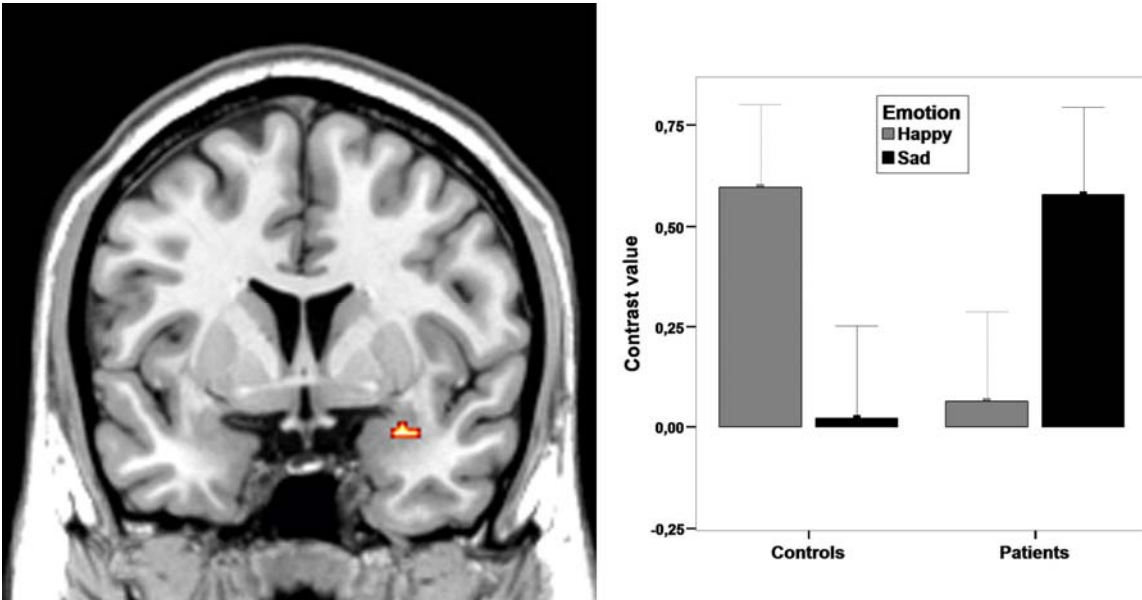
Introducing gender as additional factor or detection performance (A') as covariate did not alter the emotion x group interaction. Furthermore, there were no associations of medication level, number of episodes, comorbidity status or duration of illness on amygdala responsiveness to happy or sad faces in the patient group (all ps> .17).

The results of the supplementary whole-brain analysis of group x emotion interaction are reported in Table 3.

Table 3   Whole brain results of group x emotion interaction.

Results of a whole brain analysis of group x emotion interaction effects at p < 0.001, uncorrected, k = 25 voxels.

Anatomical region	Side	Cluster size	x	y	z	Z-score	p-value
IFG (orbital part) extending to STP, Insula, and PHG	L	57	-28	14	-24	4.26	0.00001
Fusiform Gyrus	L	25	-38	-56	-14	4.14	0.00002
Gyrus Rectus	L+R	69	0	42	-24	4.02	0.00003
MFG	L	41	-38	26	40	3.77	0.00008
MTG	R	48	64	-52	0	3.74	0.00009
ITG, extending to MTG	L	28	-54	2	-34	3.67	0.00012
MTG, extending to ITG	L	27	-60	-16	-24	3.67	0.00012
STP, extending to Amygdala*, PHG, IFG (orbital part), and Insula	R	51	28	12	-24	3.53	0.00021

Coordinates are given in MNI space. IFG, inferior frontal gyrus; MTG, middle temporal gyrus; ITG, inferior temporal gyrus; PHG, parahippocampal gyrus; STP, superior temporal pole. * 4 voxels of the cluster fall inside the right amygdala at p < 0.001, with peak coordinates at x=30, y=4, z=-20, Z=3.36, p= 0.00039

A correlation analysis in the patient sample yielded no significant correlation of HAMD-scores and amygdala responsiveness to sad faces. However, a negative correlation of HAMD-scores and amygdala responsiveness to happy faces was observed, again confined to the right amygdala, x = 26, y = -8, z = -12 (coordinates of the voxel with the highest correlation ($r = -.565$)); $t(28) = 3.62$; $p_{uncorrected} = .00058$; $p_{FWE-corrected} = .025$; k = 98. Thus, patients with weaker automatic amygdala responsiveness to happy facial expressions suffered from higher depression levels. The correlation of HAMA-scores and amygdala responsiveness to happy or sad faces yielded no significant results but showed a trend in the same direction.

2.5 Discussion

The present neuroimaging data provide evidence for a differential response pattern of the amygdala to subliminal emotion stimuli in depressed patients, as compared to healthy individuals. Our findings are consistent with the idea of automatic mood-congruent cognitive biases in major depression - as well as in mentally healthy subjects. As hypothesized, amygdalar reactivity in depressed patients was increased to masked negative emotional stimuli and decreased to masked positive emotional stimuli - in comparison with healthy controls. Thus, it appears that depression is characterized by a dysregulation of automatic neurobiological responsivity, showing negative potentiation and positive attenuation. In line with previous research on amygdala responsiveness, the backward-masked emotional faces successfully elicited robust amygdala responses. In accordance with previous research, differences in amygdala activation between groups were found in the right amygdala, which seems to be specifically important for the processing of unconscious stimuli (Costafreda, Brammer, David, & Fu, 2008).

The results of the detection task and the subjective experience reported by our participants indicate that the neurobiological reactions took place in absence of conscious awareness of the emotional stimuli. Healthy controls showed a stronger amygdala response to happy facial expression than to sad expression. This asymmetry is consistent with fMRI data reported by Killgore and Yurgelun-Todd (2004) and previous research from our laboratory (Dannlowski, Ohrmann, Bauer, Kugel, Arolt, Heindel, & Suslow, 2007). In a broader methodological context, the present data parallel several psychophysiological studies that demonstrated a positive or protective processing bias in healthy subjects (Deldin, Keller, Gergen, & Miller, 2001; Rottenberg, Gross, & Gotlib, 2005). Also in line with previous neuroimaging research, compared with healthy controls, depressed patients manifested stronger amygdala responses to negative stimuli (Abler, Erk, Herwig, & Walter, 2007; Sheline et al., 2001; Siegle, Steinhauer, Thase, Stenger, & Carter, 2002; Siegle, Thompson, Carter, Steinhauer, & Thase, 2007; Surguladze et al., 2005).

The present data suggesting a dysfunctional reactivity of the amygdala to emotion stimuli at an automatic processing level in major depression complement behavioral findings that depression is characterized by processing biases in late or controlled stages of

information processing (Mathews & MacLeod, 2005; Williams, Watts, MacLeod, & Mathews, 1997). It appears that neurobiological methods such as fMRI may represent a more sensitive way of assessing automatic emotion processing in depression than behavioral testing. Future studies on emotion processing in depression should combine different methods of response assessment and evaluate directly the relationship between response levels for different types of processing (automatic vs. controlled). In contrast with results from previous psychological studies based on the affective priming paradigm (Murphy & Zajonc, 1993; Murphy, Monahan, & Zajonc, 1995), no evaluative shifts due to masked facial emotions (compared to the neutral or the no prime condition) were observed in our experiment. The absence of priming effects could be due at least in part to the fact that we applied sad instead of angry facial expression in the negative prime condition. However, the pattern of our findings can also be interpreted in the sense that functional neuroimaging could represent a more sensitive tool to detect and measure subtle emotion processing compared to behavioral tests.

Numerous researchers have argued that the amygdala plays a central role in generating negative emotional experience (Abercrombie et al., 1998; Nomura et al., 2004; Schaefer et al., 2002). From this perspective, greater amygdala responsivity to negative faces in depressed patients could directly affect their mood state in a negative way. However, in the present study, no correlation was observed between severity of current depression and amygdala response to sad faces. Instead, we found an inverse relationship between intensity of depressive symptoms and amygdala response to happy faces. It is known that the amygdala modulates vigilance in order to exponentiate subsequent information processing throughout the brain (Davis & Whalen, 2001). A low automatic reactivity of the amygdala to positive stimuli could implicate less engagement in the encoding of positively valenced stimuli, or reduced recruitment of attentional resources that can bring (peripheral) emotional stimuli to conscious awareness (Adolphs et al., 1999; Amaral, 2002). Conversely, high amygdala responsivity to negative stimuli in depression was shown to be associated with automatic evaluative biases (Dannlowski, Ohrmann, Bauer, Kugel, Arolt, Heindel, Kersting, et al., 2007) and could bias negatively attention and higher cognitive processes. According to our results the between-group difference in

amygdalar activation in response to emotion faces was located in the basolateral nuclei. Findings from previous research indicate that basolateral amygdala activation in response to masked fearful faces is positively associated with trait anxiety (Etkin et al., 2004). The basolateral complex could represent an integral part of an amygdalar-cortical network for unconscious emotional vigilance. Recruitment of visual and prefrontal areas by the basolateral amygdala could enhance the allocation of attentional resources for processing of sad stimuli and reduce the processing of positive or happy stimuli in depression. The basolateral amygdala is also known to regulate the consolidation of memory through its projections to many other brain regions involved in storing new information (Chavez, McGaugh, & Weinberger, 2009; McGaugh, 2004). Depressed patients' differential responsivity of the basolateral amygdala to emotion stimuli may contribute to a preferential encoding of mood-congruent stimuli.

The present findings may also shed light on the problematic interpersonal functioning of depressed patients (Gotlib & Hammen, 2002). Our results show that automatic, subcortical reactions of depressed individuals are strong to negative but only weak to positive socio-emotional signals. Individuals manifesting a selective bias favoring the processing of negative facial responses may tend to experience interpersonal failures. Reduced responsiveness to positive facial expressions, which has already been shown in depressed patients on a behavioral level (Suslow et al., 2004), could lead to disturbed relationships, in the sense of less attunement and mutual involvement (Bouhuys, Geerts, & Mersch, 1997; Surguladze et al., 2004).

Some limitations must be acknowledged. All patients were medicated, which might represent a confounding factor. However, previous research has repeatedly shown that antidepressant agents reduce amygdala responses to negative stimuli (Fu et al., 2004; Harmer, Mackay, Reid, Cowen, & Goodwin, 2006; Norbury, Mackay, Cowen, Goodwin, & Harmer, 2007; Sheline et al., 2001), but enhance amygdala or subcortical responses to positive faces (Fu et al., 2007; Schaefer, Putnam, Benca, & Davidson, 2006). Given this consistent pattern, the medication in our sample would rather counteract the effects observed in our study (which might therefore be even stronger in unmedicated patients). Furthermore, our subjects were severely depressed inpatients. Thus, our results might not

generalize to more moderately depressed non-hospitalized patients, who constitute the usually recruited study population for behavioral studies of cognitive bias in depression. A larger sample size, with a broader spectrum of illness severity, would be necessary to investigate whether the mood congruency effects observed in our sample are restricted to severely affected patients or not. To control the effect of hospitalization on amygdala reactivity it would be necessary to include hospitalized, non-depressed subjects as second control group.

In sum, our study provides compelling support for the notion that depression is characterized by mood-congruent emotion processing already at early and automatic processing stages. Future studies should investigate potential effects of psychopharmacological and psychotherapeutic treatment on automatic mood congruent amygdala activation and its relation to clinical features or treatment response.

3 Experiment 2: 5-HTTLPR genotype biases automatic amygdala responsiveness[2]

3.1 Summary

A functional polymorphism in the serotonin transporter gene (5-HTTLPR) has been reported to modulate amygdala responsiveness to negative environmental cues. However, it remains unclear whether 5-HTTLPR modulates amygdala responses specifically to negative stimuli or rather to emotionally salient stimuli in general. In 44 healthy subjects, amygdala responses to subliminally presented happy and sad facial expressions were assessed by means of fMRI at 3 Tesla. All subjects were genotyped for 5-HTTLPR and the recently discovered 5-HTT rs25531. We observed a robust emotion by genotype group interaction in the right amygdala. Risk allele carriers (S or L_G) showed similar amygdala responses to happy faces compared to homozygous $L_A L_A$ carriers but increased amygdala responses to sad faces. The right amygdala was the only anatomical region across the whole brain demonstrating this interaction at a reasonable threshold. It appears that whereas 5-HTT gene variation modulates automatic amygdala responsiveness to sad faces, no such association was found for happy faces. We conclude that 5-HTTLPR genotype predominantly impacts the central processing predominantly of negative environmental cues but not of emotionally salient stimuli in general.

3.2 Introduction

Serotonin (5-HT) plays a major role in limbic neural transmission. During the last decade, genetic variants in the serotonin system have been described that modulate cognitive and neurobiological aspects of emotion processing. The most widely studied genetic variation is a common functional promoter polymorphism in the serotonin transporter gene (5-HTTLPR). A variety of studies has shown that the 5-HTTLPR low expressing risk allele (S) is associated with increased neurobiological, physiological, and cognitive responses particularly to negative stimuli (Beevers, Gibb, McGeary, & Miller, 2007; Brocke et al., 2006; Osinsky et al., 2008). Presumably the best established finding is that 5-HTTLPR biases the responsiveness to negative emotional stimuli of the amygdala, a core structure for early and rapid emotion processing. Since the seminal demonstration by Hariri et al. (Hariri et al., 2002), several studies have replicated these findings with different types of stimuli and tasks (Munafò, Brown, & Hariri, 2008). Furthermore, also the structure and connectivity of the amygdala regulatory areas appears to be modulated by 5-HTTLPR genotype (Pezawas et al., 2005; Pezawas et al., 2008). A common interpretation argues that the S-allele is associated with enhanced neural processing particularly of negative, aversive environmental cues, which could thereby increase the risk for emotional disorders in the context of aversive or stressful experiences. This notion corresponds well with the repeated finding that the 5-HTTLPR S-allele increases the risk for depression in the context of stressful life-events (Caspi et al., 2003). However, in the majority of imaging genetics studies, only negative stimuli have been used. Among the few studies also employing positive stimuli, results are inconclusive (Canli et al., 2005; Dannlowski et al., 2008; Heinz et al., 2005).

To the best of our knowledge, no study ever investigated valence-specific effects of 5-HTTLPR by directly comparing the 5-HTTLPR effect on amygdala responsiveness towards negative and positive stimuli. If the neurobiological effect of 5-HTTLPR indeed constitutes a risk factor for emotional disorders, it should be absent or at least less pronounced for limbic responses to positive cues. In the present study we sought to address this question directly with an established paradigm that assesses automatic aspects of amygdala responsiveness towards negative and positive facial expressions (Kugel et al.,

2008; Suslow et al., 2009; Suslow et al., 2010). A backward-masking procedure was selected to prevent conscious awareness of the emotional faces, therefore preventing voluntary emotion regulation processes. We predicted an interaction of genotype and emotional valence in the amygdala. Specifically, we predicted a significant modulation of amygdala responsiveness towards negative stimuli by 5-HTTLPR genotype, but a significantly weaker or even absent genotype effect for positive facial cues.

3.3 Materials and Methods

3.3.1 Subjects

Forty-four right-handed healthy German subjects participated (Table 4). Exclusion criteria were any previous or present psychiatric condition, neurological abnormalities, any psychotropic medication or drug use, and the usual MRI-contraindications. The study was approved by the Ethics Committee of the University of Münster. After complete description of the study to the subjects, written informed consent was obtained.

3.3.2 Genotyping

All subjects were genotyped for the 5-HTTLPR polymorphism, including SNP rs25531 (A/G), according to published protocols (Deckert et al., 1997; Wendland, Martin, Kruse, Lesch, & Murphy, 2006) with minor variation. Primers 5'-GGCGTTGCCGCTCTGAATGC-3' and 5'- GAGGGACTGAGCTGGACAACCAC-3' (10pM each) were used for 20µl PCR containing 60 ng DNA, 200 µM dNTPs, H_2O and 0.5 U HotStar Taq Polymerase with 1.5 mM $MgCl_2$, 1xQ-Solution and 1xBuffer (Qiagen), with an initial 15-min denaturation step at 95°C followed by 35 PCR cycles of 94°C (60s), 64°C (60s) and 72°C (120s) and a final extension step of 10 min at 72°C. For RFLP analysis PCR products were digested with HpaII at 37°C overnight, separated in 15% polyacrylamide gels (1xTBE, 230 V/cm) for 3.5 h and visualized by silver staining, which resulted in fragments between 62 and 340 bp length allowing differentiation and assignment of all 5-HTT-LPR and -rs25531 genotypes. The genotype distribution of 5-HTTLPR (20 LL, 15 LS, 9 SS) and rs25531 (38 AA, 6 AG, 0 GG) did not differ

significantly from the expected numbers calculated according to the Hardy-Weinberg equilibrium (both $p > 0.05$). Following the majority of previous studies, subjects were grouped into risk allele carriers (S or L_G) and homozygous non-risk (L_A) allele carriers.

Table 4   Characteristics of study participants in experiment 2

Sociodemographic and affective characteristics of study participants, and mean evaluative responses to the neutral face mask during the fMRI experiment dependent on prime condition (mean (s.d.))

	$L_A L_A$ (n=15)	S or L_G carrier (n=29)	p-value, according to χ^2- or t-tests (2-tailed)
Age	29.7 (9.2)	30.9 (10.8)	0.72
Sex (m/f)	7/8	18/11	0.33
BDI	3.4 (4.3)	3.8 (3.5)	0.75
STAI-trait	35.4 (7.0)	35.5 (9.6)	0.98
Verbal IQ (MWT-B)	121.3 (12.9)	116.3 (13.8)	0.26
Total education time	13.8 (1.6)	14.0 (1.8)	0.71
Evaluation sad prime	0.06 (0.31)	-0.09 (0.29)	0.13
Evaluation happy prime	0.11 (0.26)	-0.04 (0.37)	0.16
Evaluation neutral prime	0.09 (0.25)	-0.08 (0.29)	0.07
Evaluation no-face prime	0.07 (0.32)	-0.09 (0.28)	0.11

3.3.3 fMRI methods

The fMRI methods have been published previously (Suslow et al., 2009; Suslow et al., 2010). In short, facial stimuli with sad, happy, and neutral expressions were briefly (33 ms)

presented as prime stimuli (Ekman & Friesen, 1976) . Neutral faces of the same individuals served as masking stimuli. To avoid complete identity of prime and mask in the neutral face condition, mirrored faces were used as neutral primes. 80 trials were shown: 20 with sad, 20 with happy and 20 with neutral prime faces. In 20 trials no-face primes were presented. The no-face prime condition consisted of neutral faces in which central facial features (i.e., eyes, nose, and mouth) had been replaced by a surface without contours. Each trial had duration of 9 s. A fixation cross presented for 800 ms preceded a prime face shown for 33 ms which was followed by the corresponding neutral face mask for 467 ms. A blank screen followed for 7.7 s. Subjects were instructed to evaluate whether neutral (mask) faces expressed rather negative or rather positive feelings, by pressing one of four buttons (-1.5, -0.5, $+0.5$, and $+1.5$). Judgments and reaction times were registered.

T2* functional data were acquired at a 3 T scanner (Gyroscan Intera 3T, Philips Medical Systems, Best, NL) using a single shot echoplanar sequence. Volumes consisting of 40 axial slices were acquired (matrix 64^2, resolution 3.5×3.5×3.5mm; TR=3s, TE=30ms, FA=90°). Functional imaging data were motion-corrected, spatially normalized to standard MNI space (Montreal Neurological Institute) and smoothed (Gaussian kernel, 8 mm FWHM) using Statistical Parametric Mapping (SPM5, http://www.fil.ion.ucl.ac.uk/spm). For each subject, trials were averaged for each prime condition. Brain responses to the prime stimulus categories were isolated by convolving a vector of onset times of the emotional, neutral, and no-face conditions with a canonical hemodynamic response function. As described previously, two individual 1st level contrasts images (happy-neutral, sad-neutral) were entered into a factorial model, with emotion (happy vs. sad) as within-subjects factor and genotype group (S or L_G carriers vs. $L_A L_A$) as between-subjects factor. The model was used

a) to test for the expected group x emotion interaction in the whole brain at an uncorrected threshold of p<0.001, cluster threshold k=10.

b) for the amygdala, a region of interest analysis with a family-wise error (FWE) correction was performed using the anatomical definition of (Tzourio-Mazoyer et al., 2002).

c) to explore the nature and robustness of the group x emotion interaction. Therefore, contrast values in the significantly interacting area were extracted for each subject and emotion quality and processed further using the SPSS 17.0 software package. As additional analyses, gender was added as additional between-subjects factor, and age, verbal intelligence (MWT-B, (Lehrl, 1995)), trait anxiety (STAI-T, (Laux, Glanzmann, Schaffner, & Spielberger, 1981)), depression level (BDI, (Beck & Steer, 1987)), detection task performance for masked angry, sad, and neutral faces, and evaluative ratings were entered as covariates. T-tests were used on the extracted contrast values in order to compare the activation due to masked sad and happy faces between genotype-groups.

Given previous reports of 5-HTTLPR biasing amygdala-prefrontal connectivity in overt emotion processing tasks (Friedel et al., 2009; Heinz et al., 2005; Pezawas et al., 2005), we further conducted an exploratory functional connectivity analysis of the amygdala. The methods have already been described in Dannlowski et al. (Dannlowski et al., 2009). Briefly, the time course of bilateral amygdala activity was extracted for each participant and then entered as a regressor ('seed') in a subsequent fixed-effects first (individual) level analysis of the same subject. The presentation conditions and movement parameters were also modeled as nuisance variables to control movement and co-activation by the task. The resulting contrast images containing individual brain-wide connectivity of the amygdala were then entered into random effects group comparisons. The WFU PickAtlas Toolbox (Maldjian, Laurienti, Kraft, & Burdette, 2003) was used to create a mask for the prefrontal cortex (including all parts of the inferior, middle, and superior frontal gyrus, anterior cingulate gyrus, gyrus rectus, encompassing all parts of Brodmann's area 9, 10, 11, 24, 25, 32, and 44-47). The genotype groups were compared at $p < 0.001$, cluster threshold k=10.

3.3.4 Prime Detection task

After the fMRI experiment, subjects took part in a forced-choice prime detection task outside the scanner. The prime detection task consisted of 40 trials of the same stimulus presentation conditions and the same stimuli as in the fMRI experiment (33 ms prime presentation, followed by a neutral face mask of the same actor). However, in the prime

detection task, the subjects were asked to indicate the prime condition that was presented before the neutral mask via button press. *A'* was calculated as non-parametric measure of sensitivity, taking into account hit rate and false alarm rate, with *A'*=0.5 indicating chance level (Grier, 1971).

3.4 Results

3.4.1 Detection task

During debriefing, no subject reported having seen any briefly presented emotional faces during the fMRI task, even when informed about their presence. Data from the prime detection task confirmed this: The sensitivity indices in the whole sample did not differ from chance level neither for happy (*A'*=0.58, t(41)=1.02, p=0.32), sad (*A'*=0.53, t(41)=1.22, p=0.23) or neutral primes (*A'*=0.51, t(41)=0.31, p=0.76; two datasets were lost due to technical difficulties). Importantly, the two genotype-groups did not differ concerning their sensitivity indexes for any prime condition (all *p*s > 0.25).

3.4.2 Behavioral results

The genotype-groups did not differ significantly concerning their evaluative ratings of the neutral mask faces, irrespective of prime condition, albeit there was a general trend for more negative evaluative ratings in all prime conditions in the S/L$_G$-group (Table 4).

3.4.3 fMRI results

a) The whole-brain analysis yielded the hypothesized emotion x genotype-group interaction in one single cluster, x=28, y=6, z=-16; Z=4.02; p$_{uncorrected}$=0.00003; k=18, located in the right amygdala, labeled by the AAL-toolbox (Tzourio-Mazoyer et al., 2002); see Figure 3 and Table 5.

b) FWE-correction for the bilateral amygdala volume confirmed the detected cluster also at a corrected p-value (p$_{FWE-corrected}$=0.01, k=6).

Figure 3   Whole brain emotion x genotype interaction

Panel a) Glass brain depicing whole-brain emotion by genotype interaction, thresholded at p<0.001, k=10. Panel b): Bar graphs depicting the mean contrast value extracted from the right amygdala for the happy-neutral and sad-neutral contrast dependent on genotype group. Error bars, s.e.m.
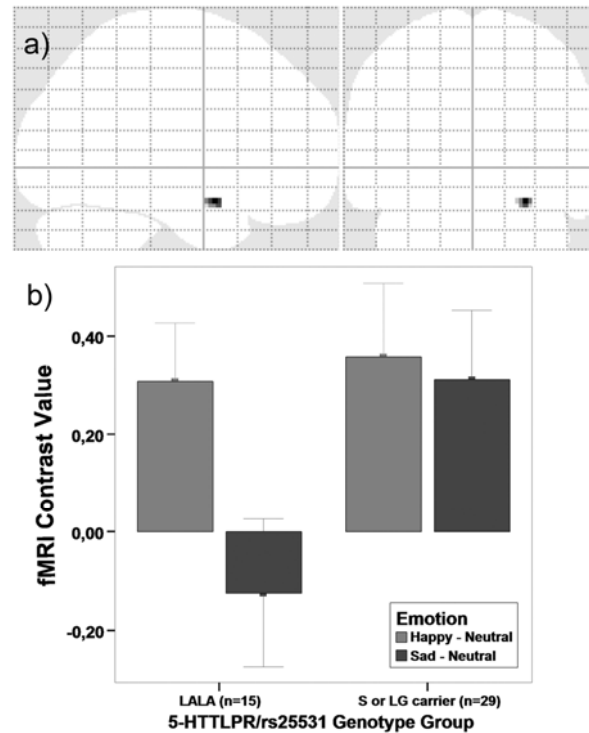
Table 5   Amygdala activation dependent on condition and baseline

Contrast values extracted from the right amygdala for happy and sad face condition vs. the neutral face or no face baseline, respectively.

Contrast	L_AL_A (n=15)	S or L_G carrier (n=29)
Sad – neutral	-0.124 (0.584)	0.312 (0.758)
Happy – neutral	0.309 (0.458)	0.359 (0.806)
Sad – no face	0.254 (0.715)	0.490 (0.647)
Happy – no face	0.687 (0.560)	0.538 (0.827)

c) In the subsequent analyses using the extracted contrast values from the significant cluster in a), the emotion x genotype group interaction remained highly significant if gender (as additional factor) or age, verbal intelligence, trait anxiety, depression level, evaluative responses, or detection task performance (as covariates) were added to the design (see Table 6 for details). None of the covariates had a significant effect on amygdala responsiveness to sad or happy expressions, including the questionnaire measures.

Table 6   Effects of covariates on group x genotype interaction in the amygdala

Results of the genotype group (LALA vs S/LG carrier) x emotion condition (happy vs. sad) ANOVA depending on the inclusion of various covariates. All covariates had no influence on the group x emotion interaction term and had no significant effect on amygdala responsiveness.

Covariate	Main effect of covariate	Group x emotion interaction term in the presence of covariate
None	---	$F(1,42)=21.1$, $p<.001$
Gender*	$F(1,40)=0.43$, $p=.52$	$F(1,40)=20.2$, $p<.001$
Age	$F(1,41)=0.35$, $p=.56$	$F(1,41)=20.6$, $p<.001$
Verbal intelligence	$F(1,41)=0.19$, $p=.66$	$F(1,41)=19.3$, $p<.001$
BDI-score	$F(1,41)=1.26$, $p=.27$	$F(1,41)=21.4$, $p<.001$
STAI-T score	$F(1,41)=0.10$, $p=.75$	$F(1,41)=21.8$, $p<.001$
Overall detection task performance	$F(1,41)=0.15$, $p=.70$	$F(1,41)=17.6$, $p<.001$

*Gender was added as additional factor.

While there were no differences between genotype-groups concerning amygdala responses to happy faces, $t(42)=0.5$, $p=0.62$, risk-allele carriers showed a significantly increased amygdala responsiveness to sad faces, compared with homozygous L_A carriers, $t(42)=2.93$, $p=0.006$.

It should be noted that the genotype group x emotion interaction term is not affected by the choice of the baseline condition (neutral faces) and the statistics remain identical, if the no face condition was used as baseline. However, since there have been reports of 5-HTTLPR biasing amygdala responsiveness to a neutral baseline in overtly presented emotion processing tasks (Canli et al., 2005; Heinz et al., 2007), we additionally extracted the contrast values for sad, happy, and neutral faces (vs. no face condition) for each subject separately. Also using the no face baseline, S carriers showed stronger amygdala responsiveness to sad faces compared to the LL genotype, $t(42)=2.62$, $p=0.012$. No differences were found for happy, $t(42)=0.77$, $p=0.46$, or neutral faces, $t(42)=0.32$, $p=0.75$. See Table 5 for the contrast values using the no-face baseline.

The functional connectivity analysis revealed no significant prefrontal area in which amygdala connectivity differed among the genotype groups.

3.5 Discussion

As a main result, our study yields strong evidence that, at an automatic level of processing, 5-HTTLPR genotype differentially modulates amygdala responses for negative and positive emotional content. In replication of previous studies, risk allele carriers demonstrated increased amygdala responsiveness to negative facial expressions (Bertolino et al., 2005; Canli et al., 2005; Dannlowski et al., 2007; Dannlowski et al., 2008; Hariri et al., 2002; Hariri et al., 2005; Heinz et al., 2005; Smolka et al., 2007). However, no such modulatory effect was found for positive faces, as evident from an emotion by valence interaction. Strikingly, the right amygdala was the only anatomical area across the whole brain exhibiting this interaction at a reasonable statistical threshold. Due to the masking procedure, participants were unaware of the presence of any emotional stimuli. Therefore, conscious and strategic aspects of processing, particularly voluntary emotion regulation processes, should have been sufficiently avoided. Hence, we had no hypotheses regarding genotype-group by valence interaction in cortical areas, particularly in parts of the prefrontal cortex, which is involved rather in conscious aspects of emotion processing.

To the best of our knowledge, this is the first report of a genotype-group by stimulus-valence interaction in brain activation. The few previous imaging genetics studies

using both positive and negative stimuli showed heterogeneous results. While two studies in medicated depressed patients (Dannlowski et al., 2007; Dannlowski et al., 2008) reported similar 5-HTTLPR effects on amygdala responsiveness to positive and negative stimuli, and one study reported even stronger 5-HTTLPR effects for positive stimuli in panic disorder (Domschke et al., 2006), other research indicated somewhat stronger genetic effects on amygdala responsiveness to negative stimuli in healthy subjects (Canli et al., 2005; Heinz et al., 2005; Smolka et al., 2007). However, no study has ever directly tested potential valence effects for significance. Furthermore, none of these studies has focused on the automatic stage of amygdala responsiveness, e.g. by using a masking procedure, which would prevent voluntary emotion regulation processes.

Our results parallel a recent pharmaco-fMRI study that studied automatic amygdala responses to subliminally presented fearful and happy faces before and after pharmacological challenge with a selective serotonin reuptake inhibitor (Harmer, Mackay, Reid, Cowen, & Goodwin, 2006). The authors reported that automatic amygdala responses to masked negative faces were attenuated by serotonergic pharmacological intervention, while no effect on amygdala responsiveness to positive faces was observed due to serotonergic challenge.

An alternative interpretation of our results could be that the L_AL_A carries show diminished amygdala activation by sad facial expressions, rather than the S carriers having increased responsiveness. This notion fits well with a recent neuropsychological study that reported a similar 5-HTTLPR genotype by emotion interaction regarding visual attention to positive and negative pictures in healthy subjects. In keeping with the notion of a "protective bias", LL carriers preferentially attended to positive compared to neutral pictures and to neutral, compared to negative pictures, whereas carriers of the S-allele showed an even-handed attention allocation (Fox, Ridgewell, & Ashwin, 2009).

It might be surprising that in our sample apparently more amygdala responsiveness was elicited by masked happy faces compared with negative facial expressions. However, most previous imaging studies used anxiety-relevant, particularly fearful faces, whereas in our study, depression-relevant stimuli (sad faces) were employed. A recent meta-analysis found no differences between happy and sad facial expressions regarding amygdala

responsiveness (Costafreda, Brammer, David, & Fu, 2008). Moreover, the only three independent studies that compared subliminally presented happy and sad faces reported stronger amygdala responsiveness to happy compared with sad faces (Dannlowski et al., 2007; Juruena et al., 2009; Killgore & Yurgelun-Todd, 2004), which is fully in line with our present data.

Unlike previous studies (Friedel et al., 2009; Heinz et al., 2005; Pezawas et al., 2005), we have detected no effect of 5-HTTLPR on amygdala-prefrontal functional connectivity. It is interesting to note that reduced amygdala-prefrontal connectivity has been interpreted in terms of a prefrontal – limbic emotion regulation deficit (Dannlowski et al., 2009; Friedel et al., 2009), which represents a controlled stage of emotion processing. Furthermore, all of the above mentioned studies used emotional stimuli that were overtly presented. In contrast, the present study used subliminally presented emotional faces, explicitly to target automatic emotion processing and to avoid controlled emotion regulation processes, which involve prefrontal areas.

The present findings provide further insight into how 5-HTTLPR could increase the risk for emotional disorders. Stronger processing of negative emotional content is one of the crucial findings in depression (Beck, 2008). A preference for enhanced processing of negative stimuli in depression has been described already for early, pre-attentive stages of cognition (Dannlowski et al., 2006), corresponding well with repeated findings of increased amygdala responsiveness to negative stimuli in major depression (Abler, Erk, Herwig, & Walter, 2007; Fu et al., 2004; Sheline et al., 2001; Siegle, Steinhauer, Thase, Stenger, & Carter, 2002; Siegle, Thompson, Carter, Steinhauer, & Thase, 2007; Suslow et al., 2010). Stronger amygdala responsiveness to subliminally presented negative, opposed to positive facial cues, has also been associated with automatic negative cognitive biases (Dannlowski et al., 2007; Dannlowski et al., 2007) and elevated trait anxiety (Etkin et al., 2004), which in turn, increases the risk for depression. The present study provides further evidence that at least part of these neurobiological abnormalities have a genetic background within the serotonergic signal transduction.

The evidence of a direct effect of 5-HTTLPR genotype on the clinical phenotype of major depression remains under debate, as reflected by the recent meta-analysis by (Risch

et al., 2009). However, particularly in the absence of clear genotype – phenotype associations and in the context of heterogeneous, purely clinically defined phenotypes, the endophenotype approach becomes a valuable approach. This strategy bears the potential to reveal distinct neurogenetic pathways, which might be able to outline different dimensions of depression-related psychopathology, and ultimately, could thereby lead to neurobiologically defined, more homogeneous subgroups.

Some limitations should be acknowledged. The sample size was limited, albeit in the range of several previous imaging genetics studies. While the recommended sample size for detecting 5-HTTLPR effects on amygdala responsiveness has been estimated $N>70$ (Munafò, Brown, & Hariri, 2008), and therefore, our study would be underpowered, our goal was to detect a genotype x emotion interaction, for which no previously published effects sizes were available for a power-analysis. Furthermore, we were not able to establish an association of amygdala responsiveness and affective characteristics, particularly trait anxiety, potentially due to the lack of statistical power, limited variance in our healthy control group regarding questionnaire measures, and the employment of sad rather than fearful or angry faces. While the subjects were screened for psychiatric disorders, including drug and alcohol abuse, smoking habits were not assessed in the present sample. Since the participants in our study were genotyped after their participation, the groups were not perfectly matched for socio-demographic variables such as gender. However, since there were no significant differences among the groups regarding all assessed variables, and inclusion of these variables as additional covariates did not alter the results, it seems very unlikely that our results are confounded by such factors.

In sum, the present study extends previous findings of a strong genetic influence on individual variation in early stages of limbic emotion processing. Future studies would strongly benefit from larger sample sizes, gene-gene interaction analyses and multiple paradigms to target different aspects of emotion processing, e.g. emotion regulation or reward processing.

4 Experiment 3: Neuropeptide Y gene biases amygdala responsiveness in depression[3]

4.1 Summary

Anxious features of depression have been suggested to particularly complicate the course of antidepressant treatment and to possibly constitute a separate nosological entity. Since neuropeptide Y (NPY) has been found to play a pivotal role in the pathomechanism of both anxiety and depression, NPY is a promising candidate in the investigation of the clinical phenotype of "anxious depression".

In the present study, NPY gene tagging variants were investigated for an influence on antidepressant treatment response in a sample of 256 patients with DSM-IV diagnosed major depression, with particular emphasis on the subgroup of 91 patients with anxious depression. Additionally, NPY gene impact on amygdala activation during facial emotion processing was analyzed in a subsample of 35 depressed patients applying an imaging genetics approach.

In anxious depression, the functional NPY rs16147 -399C/T variant was associated with treatment response, with the less active C allele conferring slow response after 2 weeks and failure to achieve remission after four weeks of treatment. The rs16147 C allele was further associated with stronger bilateral amygdala activation in response to threatening faces in an allele-dose fashion.

In conclusion, the present results point towards a possible influence of functional NPY gene variation on antidepressant treatment response in anxious depression, potentially conveyed by altered emotional processing.

4.2 Introduction

Depression and anxiety disorders occur highly comorbidly with increased morbidity, poorer acute and long-term outcome as well as increased suicide risk (Lydiard & Brawman-Mintzer, 1998). The clinical phenotype of anxious depression has been suggested to possibly constitute a diagnostic entity of its own requiring specific diagnostic and therapeutic attention (Silverstone & von Studnitz, 2003). Indeed, accumulating evidence points to anxious features of depression complicating the course of antidepressant treatment (Bagby, Ryder, & Cristi, 2002; Fava et al., 2008; Nelson, 2008). A recent study from our lab reported significantly decreased response rates after four (26.3% vs 54.2%, p=0.0005) and six (65.3% vs 78.1%, p=0.014) weeks of treatment for anxious depression as defined by a HAM-D anxiety/somatization factor score ≥ 7 in 340 Caucasian inpatients with a DSM-IV major depressive episode (MDE), particularly in the subsample of major depression (MDD) (N=256) (Domschke, Deckert, Arolt, & Baune, 2008).

Neuropeptide Y (NPY) (MIM *162640) is widely expressed in the central nervous system including the amygdala (Marcos et al., 1999) and has repeatedly been suggested to play a pivotal role in the pathophysiology of anxiety and depression as well as the mediation of treatment response in both disorders (Heilig et al., 2004; Obuchowicz, Krysiak, & Herman, 2004), which renders NPY a promising candidate in the investigation of the clinical phenotype of anxious depression.

NPY as well as NPY Y1 receptor knock-out mice and rats treated with NPY Y1 receptor antagonists exhibit significantly higher anxiety levels as compared to wild-type animals (Bannon et al., 2000; Karl, Burne, & Herzog, 2006; Wahlestedt, Pich, Koob, Yee, & Heilig, 1993). Reciprocally, in a wide range of animal models anxiolytic-like effects of NPY have been observed (Bannon et al., 2000; Broqua, Wettstein, Rocher, Gauthier-Martin, & Junien, 1995; Heilig et al., 1993; Heilig, Söderpalm, Engel, & Widerlöv, 1989; Karl, Burne, & Herzog, 2006; Karlsson, Holmes, Heilig, & Crawley, 2005; Sajdyk, Vandergriff, & Gehlert, 1999; Tovote et al., 2004; Wahlestedt, Pich, Koob, Yee, & Heilig, 1993). In animal models of depression, suppressed central NPY levels have been reported (Caberlotto et al., 1999; Caberlotto, Fuxe, Overstreet, Gerrard, & Hurd, 1998), while anti-depressant treatment led to an up-regulation of central NPY synthesis (Husum, Mikkelsen,

Hogg, Mathé, & Mørk, 2000; Mathé et al., 2007; Stenfors, Mathé, & Theodorsson, 1994; Wahlestedt et al., 1990). Accordingly, central NPY administration resulted in antidepressant-like effects (Redrobe, Dumont, Fournier, & Quirion, 2002; Stogner & Holmes, 2000). In depressed patients as well as in post-mortem tissue of suicide victims, a robust suppression of cerebrospinal fluid (CSF) NPY levels has been found (Heilig et al., 2004; Widerlöv, Lindström, Wahlestedt, & Ekman, 1988). Long-term treatment with the selective serotonin reuptake inhibitor (SSRI) citalopram led to a significant increase in NPY CSF concentrations (Nikisch et al., 2005). Anti-anxiety as well as anti-stress effects of NPY have been suggested to be in part mediated by the amygdala, particularly the lateral/basolateral complex (Primeaux, Wilson, Cusick, York, & Wilson, 2005; Sajdyk, Schober, & Gehlert, 2002), where NPY and GABA are co-localized (McDonald & Pearson, 1989).

In anxiety and anxiety disorders, particular evidence for a risk locus on chromosome 4q31-34 encompassing the NPY gene (chromosome 4q31.3-q32) has been reported (Kaabi et al., 2006). Neuropeptide Y system polymorphisms have been found to be possibly involved in the pathogenesis of panic disorder (Domschke, Hohoff, et al., 2008) and unipolar depression with a significantly elevated frequency of the NPY rs16147 -399C allele in depressed patients (Heilig et al., 2004), while another study failed to detect an influence of NPY gene variation on panic disorder or major depression (Lindberg et al., 2006). NPY rs16147 located in the promoter region of the gene is of particular functional relevance, since the -399C allele has been shown to alter NPY expression in vitro by accounting for 30% decrease in mRNA expression (Zhou et al., 2008). Furthermore, a low-NPY-expression diplotype containing the NPY -399C allele was reported to be associated with increased amygdala activity in response to threat-related facial expressions in healthy probands (Zhou et al., 2008).

Thus, given the converging lines of support for a pivotal role of NPY in both anxiety and depression as well as growing evidence for the combined clinical phenotype of anxious depression to be associated with impaired treatment response, in the present study the influence of NPY gene tagging variants on antidepressant treatment response was investigated in a sample of patients with major depression, particularly the subtype of

anxious depression. In order to identify potentially mediating neurobiological mechanisms, we further investigated the impact of NPY gene variants that were significantly associated with poor treatment response in our sample on amygdala activity by means of functional magnetic resonance imaging (fMRI). The amygdala is a core structure in limbic emotion processing circuitries (Davis & Whalen, 2001). Several studies have shown that depression (Phillips, Drevets, Rauch, & Lane, 2003a) as well as trait anxiety and anxiety disorders (Etkin & Wager, 2007) are associated with increased amygdala responsiveness particularly to negative facial expressions. Consequently, amygdala responsiveness to negative facial cues is regarded having endophenotype character (Hariri, Drabant, & Weinberger, 2006; Hasler, Drevets, Manji, & Charney, 2004), a notion which has stimulated several studies in the emerging research field of imaging genetics, including the study by Zhou et al. (2008) as mentioned above. Therefore, we have analyzed NPY rs16147 and rs9785023 impact on amygdala activation during facial emotion processing in a subsample of 35 depressed patients. We hypothesized that the low expressing variants, particularly rs16147 C alleles, are associated with increased amygdala responsiveness to negative facial expressions.

4.3 Materials and Methods

4.3.1 Samples

Samples of 268 unrelated Caucasian patients with current major depression (MDD) (mean age: 49.7 ± 15.4; f=154, m=114) and 72 patients with bipolar disorder, major depressive episode (mean age: 45.9 ± 14.5; f=40, m=32), admitted for inpatient treatment were consecutively recruited at the Department of Psychiatry, University of Muenster, Germany, between 2004 and 2006. For pharmacogenetic analyses, only patients with an HAM-D admission score >10 and a treatment cycle of at least 6 weeks from baseline were considered leaving a sample of N=256 patients with MDD (mean age: 50.4 ± 14.9; f=145, m=111). Patients with Schizoaffective Disorders or comorbid Substance Abuse Disorders, mental retardation, pregnancy and neurological, neurodegenerative disorders or other clinically unstable medical illnesses impairing psychiatric evaluation were not included in

this analysis. In order to minimize the risk of ethnic stratification, Caucasian descent was ascertained by Caucasian background of both parents.

From the overall sample, a subsample of N=35 patients (mean age: 37.3$\pm$12.6; f=24, m=11) with complete rs16147 genotype and fMRI data was drawn for the imaging genetics analysis. Besides the usual MRI contraindications, additional exclusion criteria were any neurological abnormalities, substance abuse, former electroconvulsive therapy, age of 60 and above and benzodiazepine treatment. Patients were scanned shortly after admission (mean HAM-D score 22.6$\pm$3.5). The genotype distribution of rs16147 in the imaging sample was TT: N=13, CT: N=14 and CC: N=8. Since the genotype distribution of rs9785023 was in complete linkage disequilibrium with rs16147 in our imaging sample, only imaging genetics data of rs16147 are reported. According to t-tests or χ^2-tests, TT homozygotes and C allele carriers did not differ significantly concerning age, gender, 5-HTTLPR genotype, presence of anxious depression, HAM-D score, number of episodes, duration of illness, or education years (all p>0.2).

Clinical data were obtained and analyzed in the context of a genetic study as approved by the local ethics committee of the University of Muenster, Muenster, Germany. After complete description of the study to the subjects, written informed consent was obtained. The present sample has previously been analysed for association between other candidate genes (e.g. catechol-O-methyltransferase, monoamine oxidase A) and antidepressant treatment response in published studies (Baune et al., 2008; Domschke et al., 2008).

4.3.2 Assessment

Patients' diagnoses were obtained by the use of a structured clinical interview (SCID-I) according to the criteria of DSM-IV (Wittchen, Wunderlich, Gruschwitz, & Zaudig, 1997). Clinical course of depression was assessed with the Hamilton Depression scale (HAM-D-21) on a weekly basis. Anxious depression (n=110, 32.3%; f=31.7%, m=32.6%; n.s.) was defined as published by Fava et al. (2008) and as applied in our previous study on pharmacoresponse in anxious vs. non-anxious depression (Domschke, Deckert, Arolt, & Baune, 2008). More specifically, a HAM-D anxiety/somatization factor score $\geq$7 was

regarded as high levels of anxiety. The anxiety/somatization factor, derived from Cleary and Guy's (1977) factor analysis of the HAM-D scale, includes six items from the original 17-item version: the items for psychic anxiety, somatic anxiety, gastrointestinal somatic symptoms, general somatic symptoms, hypochondriasis and insight.

4.3.3 Response

Treatment response (HAM-D reduction ≥50%) and remission (HAM-D ≤7) after 4 and 6 weeks of antidepressant treatment were applied as response parameters as defined by Fava et al. (2008) and based on our previous study on pharmacoresponse in anxious vs. non-anxious depression (Domschke, Deckert, Arolt, & Baune, 2008). In addition, the outcome measure of slow vs. fast response after 2 weeks (cutoff 50% HAM-D score reduction after 2 weeks) of treatment was applied. Side effects were not systematically assessed in detail.

4.3.4 Medication

Patients with MDD (N=256) were treated in a naturalistic setting with a variety of antidepressant medication (mirtazapine: N=28 (10.9%), citalopram/escitalopram: N=38 (14.8%), venlafaxine: N=45 (17.6%), mirtazapine plus citalopram/escitalopram: N=38 (14.8%); mirtazapine plus venlafaxine: N=63 (n=24.6%), other (TCA, MAO inhibitors, lithium): N=44 (17.2%)). As co-medication atypical neuroleptics (quetiapine, olanzapine, risperidone; N=121, 47.3%) as well as mood stabilizer (lithium, valproate acid; N=60, 23.4%) were used in addition to antidepressant treatment. Benzodiazepines were used in 3 cases only. None of the included patients had received electroconvulsive therapy within six months before the present investigation.

4.3.5 SNP selection and genotyping

Tagging SNPs covering the NPY gene region were selected by in silico analyses (UCSC human genome browser, HapMap). Further SNPs were included in the present study based on previous association findings in non-mental diseases as well as known functional relevance: SNP1 (rs16157), SNP2 (rs16147), -485T/C (Itokawa et al., 2003) analogous to -

399C/T (Lindberg et al., 2006; Mottagui-Tabar et al., 2005; Zhou et al., 2008), SNP3 (rs16139, 1128T/C, Leu-7-Pro (Karvonen et al., 1998)), SNP4 (rs9785023, 1258G/A (Skibola et al., 2005)), SNP5 (rs16474). In the present study, SNPs were named using 'rs' numbers and the respective alleles were called according to NCBI single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Genotypes of rs16147 were grouped according to functionality with the -399C allele conferring decreased mRNA expression (Zhou et al., 2008).

DNA isolated from EDTA anticoagulated venous blood samples was genotyped for the above mentioned polymorphisms by TaqMan 5'-exonuclease assays blind to disease status (ABI Prism 7900 Sequence Detection System, SDS software version 2.1, Applied Biosystems, Darmstadt, Germany).

4.3.6 Statistical analysis

Continuous variables were compared between two categories using student t-test and proportions between categorical variables were analysed using Chi-square test. Since in our previous study anxious depression had the strongest effects on pharmacoresponse in the subsample of MDD (Domschke, Deckert, Arolt, & Baune, 2008), the subsequent pharmacogenetic analyses were restricted to patients with MDD (see results section).

For pharmacogenetic analyses, the variables of treatment response and remission at weeks 4 and 6 of antidepressant treatment were entered as dependent variables, while NPY SNPs were considered as independent variables using single logistic regression models. Variables such as age, gender, polypharmacy, treatment with antidepressants plus neuroleptics, duration of depressive illness, lifetime number of depressive episodes, number of hospitalizations due to depression, class of antidepressant, i.e. SSRIs, SNRA, NaSSRA, TCA, MAO-inhibitors, antidepressants plus mood stabilizer or antipsychotic and family history of psychiatric disorders were included as covariates in logistic regression models, if a significant effect on treatment response or remission in anxious depression was observed in univariate analyses.

False Discovery Rate (FDR) (Benjamini & Hochberg, 1995) was applied to control for multiple testing and prevent from Type I error. FDR was calculated for the number of

hypotheses tested in the pharmacoresponse analyses of anxious depression vs. non-anxious depression as reported earlier plus the hypotheses tested in pharmacogenetic analyses as presented in this manuscript. The resulting FDR has a corrected p-value of $p \leq 0.014$.

Quanto (Gauderman, 2002; Gauderman, 2002) was used to approximate statistical power given the following assumptions: two-tailed a=0.05, 91 cases with anxious depression, failure of remission of 0.86 and a log additive genetic model. For statistical power of 0.80 (beta=0.20), the minimum detectable genotypic relative risk is 3.7 for high risk C-allele frequency of 0.43 (rs16147).

Effect sizes in previous imaging genetics studies have generally been large, often exceeding r=0.5. Our imaging genetics sample size had sufficient power to detect effect sizes of r=0.41 with sufficient power (1-ß=0.8), calculated with the G*power 3.0.4 software (Faul, Erdfelder, Lang, & Buchner, 2007). Hardy-Weinberg equilibrium was examined using the program Finetti provided as an online source (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl; Wienker TF and Strom TM).

4.3.7 fMRI Methods

All technical details of fMRI data acquisition and processing have been reported (Dannlowski et al., 2007; Dannlowski et al., 2007; Dannlowski et al., 2008). Briefly, subjects viewed alternating 30 s blocks of masked happy, sad, angry, and neutral facial stimuli (Ekman & Friesen, 1976) interleaved with a 30 s resting state (a gray rectangle). Emotional faces were presented twice per second for 33 ms directly followed by a 467 ms mask depicting a neutral face, resulting in subjective unawareness regarding the presence of emotional stimuli. This backward-masking procedure is widely used in the imaging literature to investigate automatic responsiveness patterns of the amygdala (Etkin et al., 2004; Morris et al., 1996; Sheline et al., 2001; Whalen et al., 1998) and has the advantage that it should be unconfounded by higher processes such as elaboration or rumination.

Functional imaging data were preprocessed (motion corrected, normalized to standard MNI space, and smoothed) with a published protocol using SPM2, (Wellcome Department of Cognitive Neurology, London, UK). In the first (individual) level analysis, activity during masked happy, sad, and angry face blocks was contrasted with the neutral

face baseline condition. Voxel values of each amygdala, as defined by automated anatomical labeling (Tzourio-Mazoyer et al., 2002) were extracted, summarized by mean and tested among the different conditions using the MarsBaR toolbox (Brett, Anton, Valabregue, & Poline, 2002). This procedure resulted in one average fMRI contrast value for each emotion condition for each amygdala. For comparability and display reasons, we further computed voxel-wise statistics by entering the first level contrast images into a second level random-effects group analysis with a statistical threshold set at $p<0.05$, using FDR correction for each amygdala. Outside the amygdalae, a threshold of $p<0.05$, corrected for the entire brain was employed.

4.4 Results

4.4.1 Sample characteristics

The distribution of rs16157, rs16147, rs16139, rs9785023 and rs16474 genotypes did not significantly differ from the expected numbers calculated on the basis of observed allele frequencies according to the Hardy-Weinberg equilibrium for the overall patient sample (rs16157: $p=0.82$; rs16147: $p=0.92$; rs16139: $p=0.91$; rs9785023: $p=0.90$; rs16474: $p=0.92$) and the sample of patients with MDD (rs16157: $p=0.98$; rs16147: $p=0.90$; rs16139: $p=0.74$; rs9785023: $p=0.91$; rs16474: $p=0.95$).

In the sample of 340 patients with MDE, the subsamples stratified for gender (f=194; m=146) and anxious (N=110, 32.4%; f=31.7%; m=32.6%; n.s.) vs. non-anxious depression (N=230, 67.7%; f=68.3%; m=67.4%; n.s.) did not significantly differ for education, marital status or age, except that patients with anxious depression were older than non-anxious depressed patients (52.4 vs. 47.8 y, $p=0.016$). There were no differences between anxious or non-anxious depression regarding lifetime number of depressive episodes, hospitalizations or duration of illness.

In the overall sample, mean HAM-D score at admission was 22.2 ± 8.3 and at discharge it was 5.8 ± 5.2, without showing any differences between genders. While patients with anxious depression had a higher HAM-D score at admission as compared to non-anxious depression (28.5 ± 7.5 vs. 19.2 ± 6.9; $p=0.001$), symptoms of depression were similar

at discharge among both groups (HAM-D: 6.3±5.8 vs. 5.5±4.9; p=0.2). Thus, all further pharmacogenetic analyses were additionally adjusted for HAM-D score at admission.

The type of antidepressant (SSRI vs others), antidepressant treatment alone (monotherapy vs. polypharmacy) or in combination with mood stabilizers (antidepressant alone vs. antidepressant plus mood stabilizer) or with neuroleptics (antidepressant alone vs antidepressant plus atypical neuroleptics) as well as the historical number of depressive episodes (continuous variable), duration of illness, number of hospitalizations (both continuous variables) and HAM-D score at baseline were not related to NPY genotypes.

4.4.2 Pharmacogenetic analyses

Since the strongest effects of anxious depression (N=91) on response and remission were found in patients with MDD (N=256), the following pharmacogenetic analyses focus primarily on the subsample of patients with MDD. Table 1 presents the results on the effects of the NPY SNPs on response (cut-off 50% HAM-D score reduction) and remission after 4 and 6 weeks of antidepressant treatment in patients with anxious depression. Failure to achieve remission after 4 weeks was related to both rs16147 (CC/CT (N=61) vs TT (N=30): OR=3.7, 95%CI 1.02-13.7; p=0.04) and rs9785023 (AA/AG (N=56) vs GG (N=35): OR=3.8, 95%CI 1.01-13.8; p=0.05). In contrast, 50% response at 4 or 6 weeks was not related to either of both SNPs. All other NPY SNPs showed no significant associations with pharmacoresponse (see Table 7).

In a next step, we calculated if rs16147 and rs9785023 were associated with slow vs fast response after 2 weeks (cut-off 50% HAM-D score reduction) of treatment. The grouped rs16147 CC/CT genotype was also associated with slow response after 2 weeks of treatment (CC/CT vs TT: OR=3.9, 95%CI 1.4-10.7, p=0.009), whereas rs9785023 showed no significant (p=0.5) association with outcome after 2 weeks. After applying FDR (cut-off p≤0.014) to all pharmacogenetic results, the association between NPY rs16147 CC/CT grouped genotype and slow response after 2 weeks of treatment remained significant. In the sample of patients with non-anxious MDD (N=165) as well as in the overall sample of patients with MDD (N=256), we did not discern any impact of NPY gene variants on antidepressant treatment response.

Table 7 Pharmacogenetic data

Pharmacoresponse dependent on NPY SNPs in patients with anxious depression (N=91) in the subsample of MDD (N=256)

NPY SNPs	Poor Response		No Remission	
	OR, 95% CI	p-value	OR, 95% CI	p-value
rs16157				
At week 4	1.2; 0.5-3.1	0.70	0.99; 0.3-3.5	0.99
At week 6	1.3; 0.5-3.4	0.55	1.2; 0.4-3.3	0.43
rs16147				
At week 4	2.1, 0.8-5.8	0.14	3.7, 1.02-13.7	*0.04*
At week 6	1.9, 0.7-5.1	0.18	1.2, 0.4-3.5	0.8
rs16139				
At week 4	0.27; 0.03-2.8	0.27	0.39; 0.08-1.9	0.17
At week 6	1.1; 0.1-8.1	0.95	0.9; 0.1-9.7	0.92
rs9785023				
At week 4	2.2, 0.8-5.9	0.14	3.8, 1.01-13.8	*0.05*
At week 6	2.0, 0.8-5.1	0.17	1.1, 0.3-3.5	0.8
rs16474				
At week 4	1.4, 0.4-4.4	0.6	0.3; 0.04-2.9	0.3
At week 6	1.3; 0.4-4.0	0.7	0.7; 0.2-2.9	0.66

OR denotes odds ratio and CI denotes confidence interval; response defined as HAM-D reduction ≥50%; remission defined as HAM-D ≤7 according to Fava *et al*, (2008); OR is adjusted for age, gender, baseline HAM-D score, number of hospitalizations, treatment with antidepressants plus atypical antipsychotics; genotypes were grouped by combining the less frequent homozygous plus the heterozygous genotype vs the more frequent homozygous genotype; details of the groups and N's of genotypes are reported in the results section; bold / italic: significant p=values.

4.4.3   Imaging genetics

In line with our hypotheses and data previously reported in healthy subjects (Zhou et al., 2008), we observed a strong association of NPY rs16147 with automatic amygdala responses to angry faces (see Figure 4).

Figure 4   Amygdala responsiveness dependent on NPY rs16147 genotype
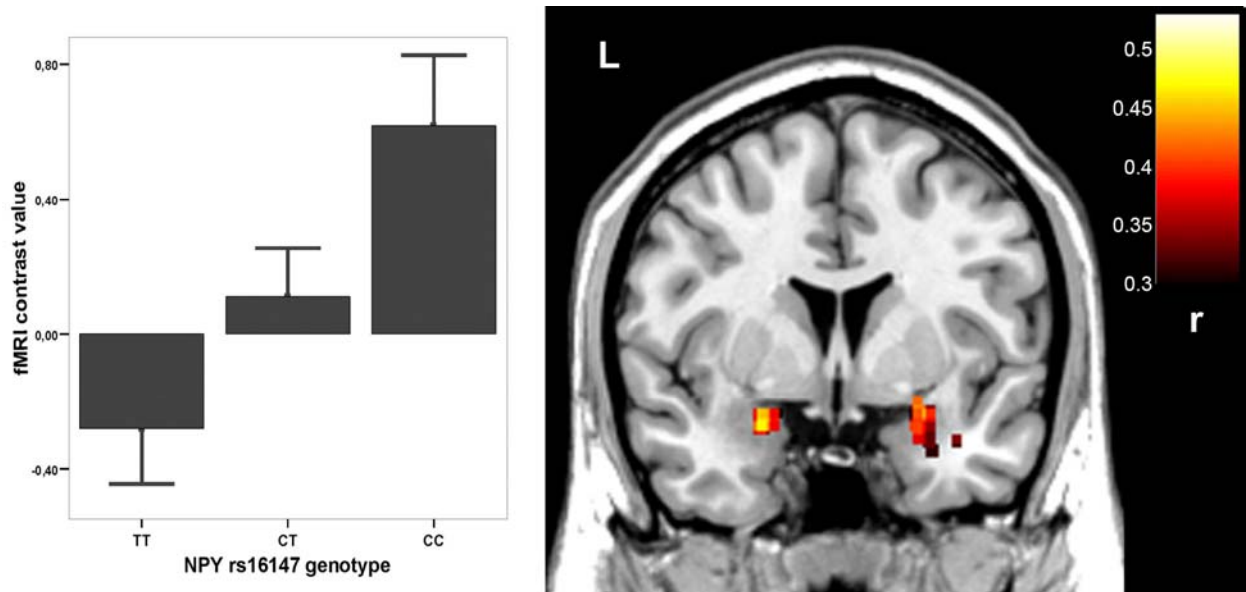
Right panel: Coronal view (y=4) of a voxel-wise regression of C alleles (0, 1, or 2) on the angry vs neutral face contrast in N=35 depressed patients showing a linear increase of amygdala responsiveness with increasing numbers of low-expressing C alleles, x=-24, y=4, z=-18, r=0.53, $p_{uncorrected}$=0.0005, $p_{corrected}$=0.023. The color bar represents effect size r. For display reasons, the image was thresholded at p<0.05, uncorrected. L, left.

Left panel: fMRI angry – neutral contrast values extracted from location x=-24, y=4, z=-18, dependent on genotype. Error bars, SEM.

As expected, carriers of the C allele showed stronger bilateral amygdala activation (averaged contrast value amygdala left: 0.19±0.60; right: 0.19±0.56) compared with TT homozygotes (left: -0.26±0.52, t(33)=2.23, p=0.032; right: -0.17±0.36, t(33)=2.06,

p=0.047). A similar effect was observed for amygdala responses to sad faces, although activation differences failed to reach significance (left: $t(33)=1.96$, $p=0.059$; right: $t(33)=1.86$, $p=0.072$). There was no significant impact of rs16147 on amygdala responsiveness to masked happy faces (both $p>0.2$).

As can be seen in figure 4, the effect of rs16147 apparently follows an allele-dose fashion with a maximal correlation of C alleles and amygdala responsiveness at MNI-coordinates $x=-24$, $y=4$, $z=-18$, $r=0.53$, $Z=3.27$, $p_{uncorrected}=0.0005$, $p_{FDRcorrected}=0.023$, cluster size $k=35$ voxels. No areas outside the amygdalae were associated with NPY rs16147 genotype at the corrected threshold.

Since our imaging subsample comprised only N=10 patients with anxious depression, it was not possible to calculate rs16147 effects on amygdala responsiveness only in this subgroup without violating statistical pre-requirements. However, on an exploratory level, also in this small subgroup, an almost significantly larger left amygdala responsiveness to masked angry faces was detected in TT homozygotes (N=5) compared with C allele carriers (N=5), $t(8)=2.27$, $p=0.053$, indicating that the anxious depressed patients were among the effect carriers. Furthermore, no differences between anxious and non-anxious depression with respect to amygdala responsiveness towards any emotion category was observed in the present imaging sample.

4.5 Discussion

The present pharmacogenetic analysis of NPY gene variants supports a potential role of the functional -399C/T polymorphism (rs16147) in the mediation of antidepressant treatment response in the clinical phenotype of anxious depression. The -399C allele of NPY SNP rs16147 was associated with impaired treatment response with a slower initial response after 2 and lower rates of remission after 4 weeks of treatment.

This finding is in line with and extends a report of the -399C allele being associated with the categorical diagnosis of unipolar depression (Heilig et al., 2004). Given an about 30% decrease in mRNA expression conferred by the -399C allele (Zhou et al., 2008), decreased NPY levels might underlie the presently observed effect in anxious depression. This is consistent with previous findings of NPY knock-out animals exhibiting significantly

elevated anxiety levels (Bannon et al., 2000; Karl, Burne, & Herzog, 2006; Wahlestedt, Pich, Koob, Yee, & Heilig, 1993) as well as suppressed NPY levels in animal models of depression or depressed patients (Caberlotto et al., 1999; Caberlotto, Fuxe, Overstreet, Gerrard, & Hurd, 1998; Heilig et al., 2004; Widerlöv, Lindström, Wahlestedt, & Ekman, 1988).

Reciprocally, the present observation further supports a potentially beneficial effect of NPY agonists in the treatment of depression as well as anxiety disorders or particularly the clinical phenotype of anxious depression, respectively, as suggested by a wide range of animal models reporting anxiolytic- as well as antidepressant-like effects of NPY (Broqua, Wettstein, Rocher, Gauthier-Martin, & Junien, 1995; Heilig et al., 1993; Heilig, Söderpalm, Engel, & Widerlöv, 1989; Karlsson, Holmes, Heilig, & Crawley, 2005; Redrobe, Dumont, Fournier, & Quirion, 2002; Sajdyk, Vandergriff, & Gehlert, 1999; Stogner & Holmes, 2000; Tovote et al., 2004).

Furthermore, the present study suggestes NPY gene variation as a potential neurobiological pathomechanism underlying the clinical phenomenon of anxious features complicating the course of antidepressant treatment in depression (Bagby, Ryder, & Cristi, 2002; Domschke, Deckert, Arolt, & Baune, 2008; Fava et al., 2008; Nelson, 2008). It therefore further supports the notion of anxious depression or an intermediate clinical phenotype common to major depression and anxiety disorders to possibly constitute a diagnostic entity of its own requiring specific diagnostic and therapeutic attention (Lydiard & Brawman-Mintzer, 1998; Silverstone & von Studnitz, 2003).

Our imaging genetics data in patients with depression provide in-vivo evidence of increased amygdala responsiveness in low-NPY-expression -399C allele carriers, thereby replicating and extending previous in-vitro and in-vivo findings of a low-NPY-expression diplotype containing the NPY -399C allele reported to be associated with increased amygdala activity in response to threat-related facial expressions in healthy probands (Zhou et al., 2008). Increased amygdala sensitivity to aversive, particularly threatening stimuli as a potential pathomechanism of depression or anxious depression in particular fits well with the amygdala's pivotal role as a core structure of the fear circuit, strongly implicated in aversive conditioning, and the modulation of stress responses. Furthermore, increased

automatic amygdala responsiveness to negative faces has been associated with negative cognitive biases in major depression (Dannlowski, Ohrmann, Bauer, Kugel, Arolt, Heindel, Kersting, et al., 2007), which were in turn associated with a chronic course of disease and poor treatment response (Dannlowski et al., 2006). It should be stressed, that NPY modulation of amygdala responsiveness was confined to negative, particularly threat relevant facial expressions, whereas no significant effect on the processing of happy facial cues was observed. This finding further underscores a particular role of NPY expression level on processing of anxiety-relevant information, and not emotion processing in general. Finally, the present imaging genetics finding of low-NPY-expression genotypes being associated with increased amygdala sensitivity to aversive stimuli provides an argumentum e contrario for previously reported anti-anxiety as well as anti-stress effects of NPY to be partly mediated by the amygdala, particularly the lateral/basolateral complex (Primeaux, Wilson, Cusick, York, & Wilson, 2005; Sajdyk, Schober, & Gehlert, 2002), where NPY and GABA are co-localized (McDonald & Pearson, 1989).

The following limitations have to be considered while interpreting the present results: Patients were recruited in a naturalistic setting allowing for a large sample size, however, implying treatment with a variety of antidepressants, no standardized dosage regime and no standardized control for plasma drug levels. Thus, treatment compliance could be only controlled for by routine nurse observations lacking objective measures of compliance, which has to be considered a possible major confounding factor. Furthermore, none of the patients was drug naïve with respect to antidepressant medication, with, however, no detailed data on the type of antidepressant pre-medication being available. Antidepressant treatment prior to the present investigation might have influenced the presently evaluated treatment response, which could not be controlled for in detail. In addition, since a-priori only patients with a treatment cycle of at least 6 weeks from baseline were included in the present study, no drop-outs due to non-response could be accounted for. Also, comorbidity with personality disorders could not be controlled for, which might have confounded the present pharmacogenetic finding. The imaging subsample was relatively small and underpowered for more subtle effects, albeit in the range of several previous imaging genetics studies.

In conclusion, the present results point towards a possible influence of NPY gene variation on antidepressant treatment response in anxious depression, potentially conveyed by altered emotional processing. The remarkable convergence of neurobiological, pharmacogenetic and imaging genetics data provides further support for a role of the NPY system in depression, anxiety or the clinical phenotype of anxious depression, respectively, and potentially aids in the future evaluation of pharmacological treatment options involving the NPY system in those disorders.

5 General Discussion

"I have reason to hope that future research will perhaps provide a new paradigm which for the first time can integrate findings from psychological and biological studies to build a new understanding of depression."

Aaron T. Beck, American Journal of Psychiatry, 2008

The present series of studies sought to integrate neurocognitive and genetic research to investigate a neurogenetic path of depression psychopathology (automatic emotion processing in the amygdala). In the first study (chapter 2), a neurobiological characteristic of depressed patients was demonstrated. It was shown that limbic responsiveness to negative (mood-congruent) facial expressions is exaggerated in depression, whereas limbic responsiveness to positive facial expressions is blunted. These findings are well in line with and were discussed in the context of classical cognitive theories of depression. Importantly, the participants of the study were unaware of the presence of emotional stimuli, since they have been presented in a backward-masked fashion. Therefore, it was concluded that the mood-congruent processing bias observed in the patient's amygdala occurs at an early, automatic stage of processing.

In experiment 2 (chapter 3), the genetic underpinnings of this neurobiological emotion processing bias were explored. Among the most prominent genetic variations discussed in the context of depression and emotion processing is the 5-HTTLPR polymorphism. A variety of studies has already investigated the neurobiological and cognitive effects of 5-HTTLPR genotype, showing that the 5-HTTLPR low expressing risk alleles (S or L_G) are associated with increased neurobiological, physiological, and cognitive responses particularly to negative stimuli (Beevers, Gibb, McGeary, & Miller, 2007; Brocke et al., 2006; Osinsky et al., 2008). Therefore, a sample of healthy subjects (n=44) was genotyped for 5-HTTLPR and underwent the same affective priming task as in experiment 1 during fMRI scanning. As expected, a robust emotion by genotype group interaction was observed in the right amygdala. Risk allele carriers showed similar amygdala responses to happy faces compared to homozygous $L_A L_A$ carriers but increased amygdala responses to sad faces. Interestingly, the right amygdala was the only anatomical

region across the whole brain demonstrating this interaction at a reasonable threshold. It appears that whereas 5-HTT gene variation modulates automatic amygdala responsiveness to sad faces, no such modulatory effect is evident for the processing of happy faces. Therefore, it could be concluded that 5-HTTLPR genotype predominantly impacts the central processing of negative environmental cues but not of emotionally salient stimuli in general.

Finally, in experiment 3, we investigated a recently discovered variation in the NPY gene, a neuropeptide which has been reported having antidepressant and anxiolytic properties. It was demonstrated that the risk allele (-399C) in this well characterized polymorphism resulting in reduced NPY transcription, is associated with reduced pharmacoresponse in a sample of depressed patients. Furthermore, the same risk allele is associated with increased amygdala responsiveness to subliminally presented aversive facial expressions. Thus, the data reveil a remarkable convergence of pharmacogenetic and imaging genetics data. It was concluded that NPY genotype influences the maintenance of depression potentially by biasing limbic responsiveness towards an increased processing of negative stimuli.

The present three experiments yield strong evidence for a genetic underpinning of the commonly observed emotion processing biases in major depression, in this case, particularly for the automatic aspects of emotion processing. It appears that the neurobiological response pattern observed in depressed patients in experiment 1 (automatic amygdala-hyperresponsiveness to negative stimuli) has an endophenotype character and is associated with genetic variation in at least two independent molecular systems (serotonergic system and neuropeptides). Albeit this conclusion cannot be drawn directly from the present data, it appears that potential susceptibility genes for depression exert their influence on the clinically defined phenotype indirectly, mediated by their effect on neurobiological correlates of emotion processing, particularly limbic activity (Figure 5).

Figure 5   Indirect genetic effects on depression mediated by limbic activity

| Susceptibility genes: | Neural processing bias: | Depression / |
| 5-HTTLPR, NPY | amygdala hyperresponsiveness | Pharmacoresponse |
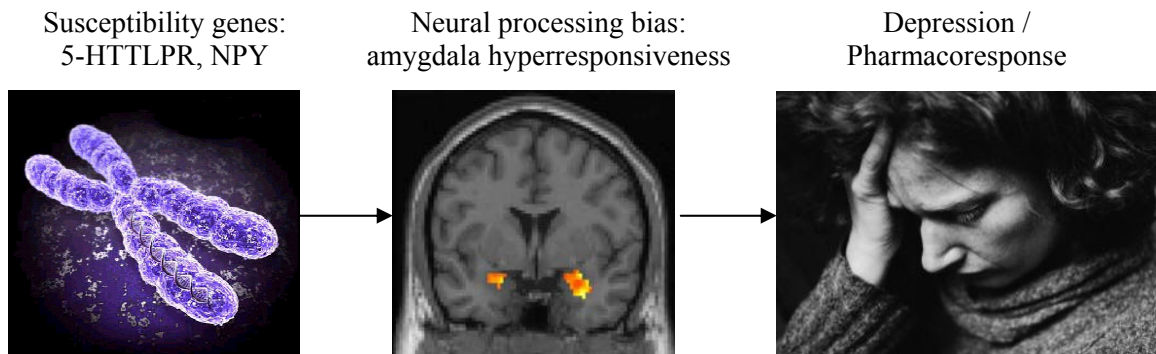
The present experiments have several limitations that should be acknowledged. First, the sample sizes were low and the experiments could be regarded underpowered, even for imaging genetics studies. E.g., a recent meta-analysis estimated the effect size of 5-HTTLPR on amygdala responsiveness as d=0.56, which would require a sample size of N>70 to detect such effects with sufficient statistical power. Furthermore, just two genetic polymorphisms have been selected for this thesis, although several more genetic variations have been discovered which might also contribute to the amygdala responsiveness patterns reported here.

The depressed patients in experiment 1 and experiment 3 were all under antidepressant medication, which could have biased the imaging data. However, using medication level as regressor did not change the pattern of results. It will be a demanding challenge for future studies to gather samples of depressed patients free from psychotropic medicine large enough for imaging genetics research.

Since no longitudinal fMRI data were acquired, no conclusions regarding the effect of treatment on amygdala responsiveness can be drawn. Furthermore, it is unknown, whether the amygdala hyper-responsiveness observed in depressed patients is a predisposing factor for - or a consequence of the disease.

However, despite all limitations, we observed a remarkable convergence of neurobiological, pharmacogenetic and imaging genetics data that potentially aids in the future evaluation of pharmacological treatment options involving the NPY and 5-HTTLPR system in depression. The strength and consistency of imaging genetics studies are hardly

rivaled by other research field involving genetic association studies or functional imaging of higher cognitive or emotional processes. This particular endophenotype approach bridges a wide gap between the limited effects of single genetic variations and complex heterogeneous pathological entities like major depression. Further studies should investigate the effect of haplotypes, gene-gene, and gene-environment interaction in longitudinal investigations.

6 Deutsche Zusammenfassung

Die depressive Störung ist in der Bevölkerung hoch prävalent und zählt unter allen Erkrankungen weltweit zu einem der Hauptverursacher verminderter Lebensqualität und ist damit ein bedeutender sozioökonomischer Faktor. Neben Umwelteinflüssen, verschiedenen körperlichen sowie psychischen Faktoren konnte ein starker genetischer Einfluss auf die Pathogenese der Depression nachgewiesen werden, mit einer geschätzten Heretabilität von etwa 40-50%. Dem beachtlichen genetischen Einfluss bei depressiven Erkrankungen steht eine sehr heterogene Befundlage hinsichtlich positiver Assoziationsbefunde mit möglichen Kandidatengenen gegenüber. Auch nach Jahrzehnten der Forschung mittels genetischer Assoziationsstudien konnte noch keine Genvariante widerspruchsfrei als Risikomarker für Depression identifiziert werden.

Genetische Assoziationsstudien bei psychiatrischen Erkrankungen wurden vielfältig wegen ihrer begrenzten Aussagekraft und methodischer Probleme im Kontext polygenetischer Erbgänge, Gen-Gen-, sowie Gen-Umwelt-Interaktionen und der rein klinisch definierten Phänotypen kritisiert. Neurobiologisch definierte Endophänotypen sollten hingegen stärker und direkter den Einfluss relevanter genetischer Polymorphismen abbilden können. Als mögliche Endophänotypen für die depressive Störung wurden Veränderungen neurobiologischer Aktivierungsmuster in limbischen Arealen vorgeschlagen, welche bei Depressiven oft beobachtet wurden. Einer der häufigsten Befunde bei Depressiven ist eine Hyperaktivität der Amygdala, einer zentralen Struktur in einem kortiko-limbischen Emotionsverarbeitungsnetzwerks. Derartige Befunde einer hyperresponsiven Amygdala auf eine Vielzahl negativer Reize, insbesondere auf negative Gesichter könnte das neurobiologische Substrat einer tieferen und anhaltenderen Verarbeitung darstellen. Daher ist das Ziel des vorliegenden Dissertationsprojektes:

1. Die Charakterisierung der Amygdalaresponsivität depressiver Patienten
2. Die Untersuchung der Effekte zweier bekannter genetischer Polymorphismen auf die Amygdalaresponsivität

Da die Amygdala vor allem im Kontext rascher, automatischer emotionaler Prozesse diskutiert wird, kamen in den vorliegenden Experimenten sogenanntes backward-masking zum Einsatz, eine Technik, in der Stimuli sehr kurz und maskiert präsentiert werden, dass die Probanden sie nicht mehr bewusst wahrnehmen können.

In Experiment 1 (Kapitel 2) wurde die Amygdalaresponsivität auf negative und positive emotionale Gesichter in einer Stichprobe depressiver Patienten (n=30) und soziodemografisch vergleichbaren gesunden Kontrollen (n=27) untersucht. Hierbei kam das sublimiale affektive Priming Experiment von Murphy und Zajonc (1993) zum Einsatz. Obwohl die Probanden nicht in der Lage waren, die maskiert präsentierten emotionalen Gesichter bewusst wahrzunehmen, zeigte sich über beide Gruppen hinweg eine robuste Amygdalaaktivierung durch traurige und fröhliche Gesichter im Vergleich zu neutralen Gesichtern. Wie vermutet zeigte sich als Hauptbefund eine starke Gruppe x Emotion Interaktion in der rechten Amygdala: Während Depressive eine signifikant stärkere Amygdalaaktivierung auf negative Gesichter zeigten, fand sich bei Gesunden das genau umgekehrte Muster einer stärkeren Aktivierbarkeit auf maskiert präsentierte fröhliche Gesichter. Das Ausmaß der Aktivierung durch fröhliche Gesichter sagte eine geringere Depressivität in der Patientengruppe vorher. Depressive Patienten scheinen also eine verzerrte Verarbeitung emotionaler Stimuli in der zentralen Schaltstelle des limbischen Systems aufzuweisen. Eine derart negativ verzerrte, automatische Verarbeitung emotionaler Reize könnte somit zur Entstehung und Aufrechterhaltung der Depression beitragen.

In Experiment 2 (Kapitel 3) wurde eine mögliche genetische Disposition für eine derartige Amygdalahyperreaktivität auf negative Gesichter untersucht. Einer größere (n=44) Gruppe gesunder Probanden wurde daher für einen bekannten Polymorphismus im Serotonintransportergen (5-HTTLPR) typisiert, dessen Risikoallel (S-Allel) bereits mit einer tieferen Verarbeitung negativer Reize, inklusive einer erhöhten Amygdalaresponsivität assoziiert wurde. In Experiment 2 kam das Selbe experimentelle Paradigma, wie in Experiment 1 zum Tragen. Es zeigte sich, dass die Nicht-Risikoalleleträger eine starke wiederum rechte Amygdalaresponsivität auf positive Gesichter aufweisen, während Träger eines oder zweier Risikoallele eine ebenso starke Amygdalaresponsivität auf negative Gesichter zeigen. Dieser Befund qualifizierte sich in

einer robusten Genotyp x Emotion Interaktion, die sich selbst in einer whole-brain Analyse ausschließlich in der rechten Amygdala manifestierte, und somit einen Amygdala-spezifischen Befund darstellt. Interessanterweise war lediglich die Amygdalareaktivität auf negativen Gesichtsausdruck durch den Genotyp beeinflusst, nicht jedoch Amygdalareaktivität auf positive Gesichter. Es scheint also, dass der in Experiment 1 gewonnene Befund einer automatischen Amygdalaresponsivität auf negative Stimuli Endophänotyp-Charakter besitzt und eine genetische Grundlage im serotonergen System zu haben scheint. Im foglenden Experiment wurde eine weitere genetische Variante untersucht, die bereits mit Depression und aberranter Emotionsverarbeitung assoziiert wurde.

In Experiment 3 (Kapitel 4) kam schließlich ein kombinierter Pharmakogenetik und Imaging Genetics Ansatz zum Tragen, um die klinischen und neurobiologischen Effekte genetischer Varianten im Neuropeptid Y (NPY) zu untersuchen. NPY, das in Interneuronen mit GABA kolokalisiert ist, hat eine nachgewiesene anxiolytische Wirkung und depressive Patienten scheinen eine Minderexprimierung dieses Neuropeptids aufzuweisen. Im Pharmakogenetikteil wurde ein bekannter Promotorpolymorphismus im NPY-Gen (-399C/T; rs16147) hinsichtlich seines Effektes auf die Ansprechensrate auf antidepressive Behandlung untersucht. Es zeigte sich, dass die Risiko-Variante (C-Allel), die mit einer verminderten NPY-Expression einhergeht, auch mit einem verminderten Ansprechen auf Pharmakologische Behandlung assoziiert war. Dieser Befund zeigte sich vor allem in der Substichprobe, die als „anxious depression" eingestuft wurde.

Im Imaging Genetics Teil des Experimentes wurden die neurobiologischen Auswirkungen des -399C-Allels erforscht. Hierfür wurde eine Teilstichprobe (n=35) der Patientenstichprobe mittels fMRT untersucht. Auch hierbei kamen emotionale Gesichter zum Einsatz, die den Patienten erneut maskiert, also nicht bewusst wahrnehmbar präsentiert wurden. Wie vermutet war das Risikoallel assoziiert mit einer verstärkten Amygdalaaktivierung durch negative, besonders wütende Gesichter. Patienten mit einer Genvariante, die mit verminderter NPY Expression einhergeht, zeigen also sowohl vermehrtes Therapieversagen, als auch eine Amygdalahyperreaktivität auf negative Stimuli. Es scheint also auch im System der Neuropeptide genetische Varianten zu geben, welche

die neurobiologischen Korrelate der Emotionsverarbeitung beeinflussen und damit (vermutlich indirekt) Einfluss auf den klinisch definierten Phänotyp aufweisen.

Der hier vertretene Forschungsansatz des „Imaging Genetics" schlägt eine Brücke über die Kluft zwischen kleinen Effekten einzelner genetischer Polymorphismen und den komplexen klinisch-psychiatrischen Phänotypen. Aus diesem Verständnis sind erhebliche Impulse für neue und individualisierbare Therapiestrategien denkbar. Die Identifizierung verschiedener neurogenetischer Pfade, bei denen unterschiedliche Transmittersysteme zu differenzierbaren Aktivitätsmustern führen, welche sich wiederum in unterscheidbaren psychopathologischen Phänomenen äußern, könnte schließlich zu einer neuen Klassifikation depressiver Störungen mit neurobiologisch abgrenzbaren nosologischen Entitäten führen. Zwar ist aus dieser Forschung bisher noch keine eine Aussage über den individuellen Patienten möglich, jedoch lässt dieser multidisziplinäre Ansatz jetzt schon großes Potential hinsichtlich einer profunden Weiterentwicklung unseres Grundlagenverständnisses der Depression erkennen.

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8 Danksagung

Ich möchte mich bei all denjenigen bedanken, die mich bei der Fertigstellung dieser Arbeit unterstützt und damit diese Arbeit erst ermöglicht haben. Diese Liste ist erfreulich lang. Mein erster Dank gilt Prof. Dr. Thomas Suslow. Er hat mich für die Wissenschaft begeistert und meine Forschungsinteressen geprägt. Ein herzlicher Dank gilt dem Direktor der Klinik für Psychiatrie, Prof. Dr. Volker Arolt, auf dessen Wohlwollen, Förderung und Wort ich mich jederzeit verlassen konnte. Weiterhin bedanke ich mich bei der Arbeitsgruppe Kognitive Neuropsychiatrie unter der Leitung von Prof. Suslow und Dr. Patricia Ohrmann, hier insbesondere bei Jochen Bauer für die vielen, kurzweiligen Stunden am Scanner, sowie bei unserem wichtigsten Kooperationspartner im Institut für Klinische Radiologie, Dr. Harald Kugel.

Bei Dr. Carsten Konrad, dem ehemaligen Leiter meiner Arbeitsgruppe, bedanke ich mich für die wertvolle, vertrauensvolle Kooperation. Ebenfalls bedanke ich mich bei allen Arbeitsgruppenmitgliedern, vor allem Dr. Sonja Schöning, Dr. Martin Pyka und Dr. Christina Sehlmeyer. Ein herzlicher Dank geht an Prof. Dr. Bernhard Baune, Priv.-Doz. Dr. Katharina Domschke und Dr. Christa Hohoff für die allseits hervorragende Kooperation in den genetischen Fragestellungen. Vielen Dank allen Mitarbeitern sowohl in der Uniklinik, wie auch in der LWL Klinik Münster für die Unterstützung der Rekrutierung.

Ein besonderer Dank gilt der Betreuerin dieser Arbeit, Prof. Dr. Pienie Zwitzerlood, die auch schon meine Diplomarbeit betreute und für mich jederzeit eine offene Tür hatte.

Ich bedanke mich bei dem IMF, der Rolf-Dierichs-Stiftung, dem IZKF und der DFG für die finanzielle Unterstützung meiner Studien, sowie bei allen Patienten und Probanden, ohne die diese Studien nicht möglich gewesen wären.

Mein ganz besonderer Dank gilt meiner Frau Gesine, meiner Tochter Lily und meinem Vater, auf dessen liebevolle Begleitung ich bis in seine letzten Tage vertrauen durfte.