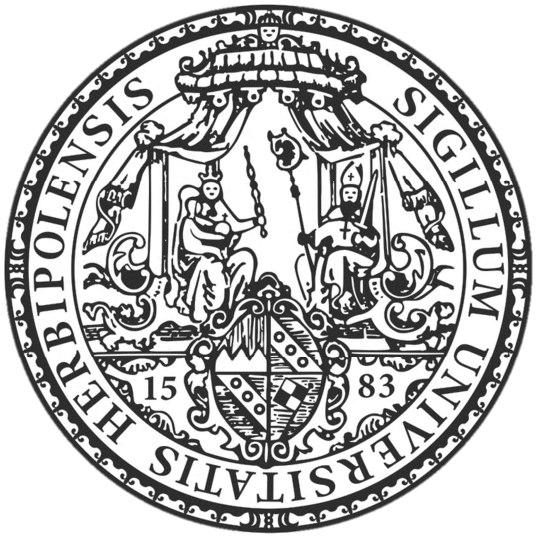

Interaction of *5-HTT/NPSR1* variants with distal and acute stress on dimensional and neuroendocrine anxiety endophenotypes – A multi-dimensional model of anxiety risk

[Interaktion von *5-HTT/NPSR1* Varianten mit distalem und akutem Stress bei dimensionalen und neuroendokrinen Endophänotypen von Angst – Ein multidimensionales Modell der Angstentstehung]

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SUMMARY

The etiology of anxiety disorders is multifactorial with contributions from both genetic and environmental factors. Several susceptibility genes of anxiety disorders or anxiety-related intermediate phenotypes have been identified, including the serotonin transporter gene (*5-HTT*) and the neuropeptide S receptor gene (*NPSR1*), which have been shown to modulate responses to distal and acute stress experiences. For instance, gene-environment interaction (GxE) studies have provided evidence that both *5-HTT* and *NPSR1* interact with environmental stress, particularly traumatic experiences during childhood, in the moderation of anxiety traits, and both *5-HTT* and *NPSR1* have been implicated in hypothalamic-pituitary-adrenal (HPA) axis reactivity – an intermediate phenotype of mental disorders – in response to acute stress exposure. The first part of this thesis aimed to address the interplay of variations in both *5-HTT* and *NPSR1* genes and distal stress experiences, i.e. childhood trauma, in the moderation of anxiety-related traits, extended by investigation of the potentially protective effect of positive influences, i.e. elements of successful coping such as general self-efficacy (GSE), on a GxE risk constellation by introducing GSE as an indicator of coping ability ("C") as an additional dimension in a GxExC approach conferring – or buffering – vulnerability to anxiety. Increased anxiety was observed in *5-HTTLPR*/rs25531 L_AL_A genotype and *NSPR1* rs324981 AA genotype carriers, respectively, with a history of childhood maltreatment but only in the absence of a person's ability to cope with adversity, whereas a dose-dependent effect on anxiety traits as a function of maltreatment experiences irrespective of coping characteristics was observed in the presence of at least one *5-HTT* S/L_G or *NSPR1* T allele, respectively. The second part of this thesis addressed the respective impact of *5-HTT* and *NPSR1* variants on the neuroendocrine, i.e. salivary cortisol response to acute psychosocial stress by applying the Maastricht Acute Stress Test (MAST). A direct effect of *NPSR1* – but not *5-HTT* – on the modulation of acute stress reactivity could be discerned, with carriers of the more active *NPSR1* T allele

displaying significantly higher overall salivary cortisol levels in response to the MAST compared to AA genotype carriers.

In summary, study 1 observed a moderating effect of GSE in interaction with childhood maltreatment and *5-HTT* and *NPSR1*, respectively, in an extended GxExC model of anxiety risk, which may serve to inform targeted preventive interventions mitigating GxE risk constellations and to improve therapeutic interventions by strengthening coping ability as a protective mechanism to promote resilient functioning. In study 2, a modulation of HPA axis function, considered to be an endophenotype of stress-related mental disorders, by *NPSR1* gene variation could be discerned, suggesting neuroendocrine stress reactivity as an important potential intermediate phenotype of anxiety given findings linking *NPSR1* to dimensional and categorical anxiety. Results from both studies may converge within the framework of a multi-level model of anxiety risk, integrating neurobiological, neuroendocrine, environmental, and psychological factors that act together in a highly complex manner towards increasing or decreasing anxiety risk.

ZUSAMMENFASSUNG

Die Entstehung von Angsterkrankungen ist multifaktoriell bedingt durch sowohl genetische als auch umweltbezogene Faktoren. Verschiedene Suszeptibilitätsgene von Angsterkrankungen und angstbezogenen Phänotypen konnten identifiziert werden, darunter das Serotonintransportergen (*5-HTT*) und das Neuropeptid S Rezeptorgen (*NPSR1*). Für beide Gene konnte gezeigt werden, dass sie die Reaktion auf sowohl distale als auch akute Stresserlebnisse beeinflussen können. Unter anderem legen Befunde aus Gen-Umwelt-Interaktionsstudien (GxE) nahe, dass sowohl *5-HTT* also auch *NPSR1* mit Umwelteinflüssen interagieren, insbesondere mit traumatischen Kindheitserlebnissen, und somit unterschiedliche Ausprägungen der Angst mitbedingen. Weiterhin konnten sowohl *5-HTT* als auch *NPSR1* in Bezug zu veränderter Reaktivität der Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) auf psychosozialen Stress hin gebracht werden, deren Funktion einen intermediären Phänotyp von psychischen Erkrankungen darstellt. Im ersten Teil dieser Arbeit wurde das Zusammenwirken von Varianten in sowohl dem *5-HTT* als auch dem *NPSR1* Gen mit distalen Stresserlebnissen, d.h. Kindheitstraumata, unter Einbezug der möglicherweise protektiven Funktion von positiven Einflussfaktoren im Sinne von erfolgreichen Bewältigungsstrategien (engl. Coping) wie der generellen Selbstwirksamkeitserwartung (GSE) untersucht. Dazu wurde GSE als Indikator für Coping-Eigenschaften („C“) als zusätzliche Ebene in einem erweiterten GxExC-Ansatz eingeführt, welche je nach Ausprägung die Vulnerabilität für Angst zusätzlich mitbedingen oder aber abschwächen kann. Es zeigten sich jeweils erhöhte Angstwerte in Trägern des *5-HTTLPR*/rs25531 L_AL_A Genotyps sowie des *NPSR1* rs324981 AA Genotyps, welche traumatische Ereignisse während der Kindheit erlebt hatten, aber nur bei gleichzeitig vorliegender niedriger Coping-Fähigkeit. Das Vorliegen von mindestens einem *5-HTT* S/L_G-Allel beziehungsweise einem *NSPR1* T-Allel war hingegen mit einem Anstieg der Angstmaße mit steigender Zahl erlebter

Kindheitstraumata assoziiert unabhängig von der Ausprägung von Bewältigungsmöglichkeiten. Der zweite Teil dieser Arbeit behandelte den jeweiligen Einfluss von *5-HTT* beziehungsweise *NPSR1* Varianten bezüglich der neuroendokrinen, d.h. Speichelkortisol-Stressantwort auf einen akuten psychosozialen Stressor im Rahmen des Maastricht Acute Stress Tests (MAST). Es konnte ein direkter Einfluss von *NPSR1*, aber nicht von *5-HTT*, auf die Veränderung der akuten Stressreaktivität gezeigt werden. Träger des höher aktiven *NPSR1* T-Allels waren gekennzeichnet durch höhere Speichelkortisollevel in Reaktion auf den MAST im Vergleich zu Trägern des AA Genotyps.

Zusammenfassend konnte in der ersten Studie ein moderierender Einfluss von GSE in Interaktion mit Kindheitstrauma und *5-HTT* beziehungsweise *NPSR1* im Sinne eines erweiterten GxExC-Modells des Angstrisikos gezeigt werden. Dies kann zum einen zur Entwicklung gezielter präventiver Maßnahmen und zum anderen zur Verbesserung therapeutischer Interventionen beitragen, durch welche jeweils Bewältigungsfähigkeiten im Sinne eines protektiven, resilienzfördernden Mechanismus gestärkt werden. In der zweiten Studie zeigte sich eine veränderte Funktion der HPA-Achse, welche einen Endophänotyp von stressbezogenen psychischen Erkrankungen darstellt, in Abhängigkeit von einer *NPSR1* Genvariante, was die neuroendokrine Stressreaktivität als möglichen intermediären Angstphänotyp im Zusammenhang von *NPSR1* Variation und dimensionaler bzw. kategorialer Angst nahelegt. Ausblickend können die Ergebnisse aus beiden Studien im Rahmen eines Mehrebenenmodells des Angstrisikos zusammenfließen, welches neurobiologische, neuroendokrine, umweltbezogene und psychologische Faktoren integriert, die auf hochkomplexe Art zusammenwirken und somit das Angstrisiko erhöhen oder herabsetzen können.

1. INTRODUCTION

Fear and anxiety arise in response to threat or danger, enabling an organism to react in an appropriate manner, e.g. by activating the sympathetic nervous system ("fight or flight" response; Cannon, 1915). Anxiety disorders, however, are characterized by excessive or inappropriate fears that emerge in the face of situations that do not constitute real threat, with profoundly disabling consequences. The category of anxiety disorders according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association, 2013) subsumes specific phobias, social anxiety disorder, agoraphobia, panic disorder, generalized anxiety disorder, separation anxiety disorder, and selective mutism. Although formerly grouped together, DSM-5 no longer lists post-traumatic stress disorder and obsessive-compulsive disorder under anxiety disorders. Rather, they are now described in separate chapters ('Obsessive-Compulsive and Related Disorders', and 'Trauma- and Stressor-Related Disorders', respectively). The etiology of anxiety disorders is thought to be multifactorial, involving the complex interplay of biological, psychological, and environmental factors. The following section will provide an overview of the epidemiology of anxiety disorders and the complex-genetic constructs, environmental aspects, as well as their interactions contributing to pathological anxiety.

1.1 EPIDEMIOLOGY OF ANXIETY DISORDERS

Anxiety disorders as a group constitute the most commonly occurring psychiatric disorders in Europe. Annual prevalence rates for any anxiety disorder are estimated at 14%, affecting approximately 61.5 million persons over the age of 14 within the European Union. Within the collective of anxiety disorders, specific phobias are the most common (12-month prevalence rate estimate of 6.4%), followed by social anxiety disorder (2.3%), agoraphobia (2.0%), panic disorder (1.8%), and

generalized anxiety disorder (1.7%) (Wittchen et al., 2011). Past-year prevalence of separation anxiety disorder is estimated at 1.0% (Silove et al., 2015). Selective mutism is rare, with point prevalence estimates between 0.03 and 0.2% (Sharp et al., 2007). Anxiety disorders are highly comorbid, both with each other and other mental disorders, with 45% of patients diagnosed with an anxiety disorder also meeting criteria for a comorbid psychiatric diagnosis across the lifespan (Kessler et al., 2005b). They often co-occur with mood disorders, e.g. depression (Mergl et al., 2007) and bipolar disorder (Freeman et al., 2002), as well as substance use and dependence (Grant et al., 2004), or personality disorders (Welander-Vatn et al., 2016).

Anxiety disorders confer a high socioeconomic burden, and present with high chronicity. They are one of the leading causes of disability worldwide, ranking sixth among all disorders – including somatic disorders – with regard to years lived with disability (YLDs), i.e. the number of years of life lived in less than full health, and are responsible for a substantial number of disability adjusted life years (DALYs) at a rate 389.7 DALYs per 100,000 people (Baxter et al., 2014). In 2010, annual costs of anxiety disorders were estimated at 74.38 billion Euros, ranking fourth within the group of psychiatric and neurologic disorders after mood disorders, dementia, and psychotic disorders, and accounting for approximately 9.3% of the total costs generated by these disorders combined (Olesen et al., 2012).

Prevalence rates are roughly twice as high in women compared to men (Baxter et al., 2013), though there do not appear to be any differences with regard to age of onset, chronicity, or the constellation of genetic and environmental risk factors between the sexes (Hettema et al., 2005; McLean et al., 2011). However, the frequency of comorbidities appears to differ between men and women, with comorbid other anxiety disorders, depressive disorders, or bulimia nervosa occurring more often in women, whereas comorbid diagnoses of attention-deficit hyperactivity disorder, intermittent explosive disorder, or substance abuse are more frequent in men

(McLean et al., 2011). Additionally, the burden of anxiety disorders is higher in women than in men, with women accounting for 65% of total DALYs credited to anxiety disorders as a group (Baxter et al., 2014).

Anxiety disorders manifest early in life and are characterized by a considerably earlier age of onset than other groups of psychiatric disorders, the median age of onset estimated at age 11. Within the group of anxiety disorders, specific phobias and separation anxiety disorders present with the earliest median age of onset during childhood (age 7), followed by social phobia during adolescence (age 13). Panic disorder and agoraphobia typically first manifest in early adulthood (ages 20-24), whereas generalized anxiety disorder displays a later age of onset with a median age of 31 years (Kessler et al., 2005a). Furthermore, childhood anxiety disorders tend to persist or progress towards other anxiety disorders or other mental disorders, e.g. depression, across the lifespan (Beesdo-Baum and Knappe, 2012).

1.2 CLINICAL GENETICS

Clinical genetic studies assess the contribution of genetics to the etiology of a given disorder by means of family studies, twin/adoption studies, and segregation studies. Anxiety disorders exhibit substantial familial aggregation – i.e. the observation of higher prevalence rates among first-degree relatives of patients compared to the prevalence rates among first-degree relatives of unaffected control subjects – with a four- to sixfold increased risk of anxiety disorders in first-degree relatives of patients with panic disorder, phobic disorders, generalized anxiety disorder, and obsessive-compulsive disorder (Hettema, Neale, & Kendler, 2001). This observed familiality points to the involvement of genetic factors and/or common environmental influences in the development of anxiety disorders. Evidence for genetic risk factors stems from twin studies, which infer the proportion of genetic contributions to the pathogenesis of a disease by comparing concordance rates of a

given disorder between monozygotic versus dizygotic twins. Heritability estimates from twin studies are moderate at a rate of 43% for anxiety disorders as a group, with estimates for individual anxiety disorders ranging from 32% for generalized anxiety disorder, over 48% for panic disorder and 51% for social phobia, up to 67% for agoraphobia (Hettema et al., 2001; Kendler, Karkowski, & Prescott, 1999). Segregation analyses point to a complex-genetic model of inheritance featuring the interaction of several different genes of small individual effect (i.e. an oligo- or polygenic model) in addition to environmental influences rather than a Mendelian pattern of inheritance of anxiety traits (Vieland et al., 1996).

1.3 MOLECULAR GENETICS

Once heritability is established by means of clinical genetics studies, molecular genetic methods can be applied to identify specific susceptibility genes that contribute to the overall risk of disease. The following section summarizes evidence stemming from the two main approaches in molecular genetics: linkage studies, which asses the co-inheritance of a genetic marker with the disorder of interest within affected families, and association studies, which assess the contribution of individual candidate genes to phenotypic manifestation by comparing the allelic frequencies between patient and control groups.

1.3.1 LINKAGE STUDIES

Linkage studies have yielded evidence for several potential chromosomal risk loci that co-segregate with anxiety disorders in families. Linkage analyses in panic disorder have implicated regions on chromosomes 1q (Gelernter et al., 2001), 2q (Fyer et al., 2006), 4q (Kaabi et al., 2006), 5q (Kaabi et al., 2006), 7p (Crowe et al., 2001; Knowles et al., 1998), 9q (Thorgeirsson et al., 2003), 11p (Gelernter et al., 2001), 12q (Smoller et al., 2001), and 15q (Fyer et al., 2006). Chromosome 13q has

been suggested as a risk locus for a "social or specific phobia" phenotype in families with a familial aggregation of panic disorder (Fyer et al., 2012), as well as for a broader "panic syndrome" in which panic disorder is accompanied by several medical conditions (i.e. bladder/renal dysfunction, mitral valve prolapse, headache, or thyroid conditions) (Hamilton et al., 2003). In panic disorder with comorbid bipolar disorder, joint risk loci were identified on chromosomes 2, 12, and 18 (Logue et al., 2009; MacKinnon et al., 1998). Linkage on chromosome 1q has also been associated with early-onset susceptibility to anxiety disorders in panic disorder pedigrees (Smoller et al., 2001). For a comprehensive review of linkage studies in panic disorder, see also Maron, Hettema, & Shlik (2010). Regarding other anxiety disorders, risk loci have been described on chromosome 3q for agoraphobia (Gelernter et al., 2001), chromosome 14q for specific phobia (Gelernter et al., 2003), and 16q for social phobia (Gelernter, Page, Stein, & Woods, 2004). Linkage on chromosome 14 has also been reported for trait anxiety as measured via the State-Trait Anxiety Inventory (Middeldorp et al., 2008; Spielberger et al., 1970). However, replication findings regarding the susceptibility loci described so far are scarce, and the loci in question often span large chromosomal regions containing a multitude of potential vulnerability genes. Still, the cumulative evidence from linkage studies highlights the involvement of various regions, and thus, multiple genes, in support of a polygenic etiology of anxiety disorders.

1.3.2 CANDIDATE GENE ASSOCIATION STUDIES

Since anxiety disorders are considered to be complex-genetic disorders, with contributions from several different genes, association studies have focused on the identification of risk variants in *a priori* defined candidate genes. Accordingly, a wide range of polymorphisms in genes of interest has been investigated in the context of genetic contributors underlying anxiety-related phenotypes and anxiety disorders.

Specific candidate genes can be selected on the basis of chromosomal location from linkage studies (see 1.3.1), or prior observations, e.g. derived from animal models, pharmacological studies, or genome-wide approaches.

Efforts have prominently focused on candidate genes related to monoaminergic function, neuropeptides, or hypothalamic-pituitary-adrenal axis-related systems. For instance, significant evidence has been provided for associations between variants in the catechol-O-methyltransferase (*COMT*) gene with panic disorder in a sex- and ethnicity-specific manner (Domschke et al., 2004; Hamilton et al., 2002; Rothe et al., 2006; Woo et al., 2004; Woo, Yoon, & Yu, 2002; for meta-analyses, see Domschke, Deckert, O'Donovan, & Glatt, 2007; Howe et al., 2016; Zintzaras & Sakelaridis, 2007) and specific phobias (McGrath et al., 2004), the monoamine oxidase A (*MAOA*) gene in panic disorder (Deckert et al., 1999; Maron et al., 2005a; Samochowiec et al., 2004; for meta-analyses, see Howe et al., 2016; Reif et al., 2012) and generalized anxiety disorder (Tadic et al., 2003), as well as the serotonin transporter gene (*5-HTT*) with panic disorder (Gyawali et al., 2010; Maron et al., 2005a, 2005b; Ohara et al., 1998; Strug et al., 2010) and social anxiety disorder (Reinelt et al., 2013), although several studies have failed to establish a direct link between *5-HTT* and panic disorder (Deckert et al., 1997; Hamilton et al., 1999), and a meta-analysis was unable to discern an overall effect (Blaya et al., 2007).

Recent research has also spotlighted neuropeptides in the mediation of anxiety disorders, particularly panic disorder, such as genes coding for the receptors of neuropeptide S (*NPSR1*; Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007), neuropeptide Y (*NPY Y5*; Domschke et al., 2008), or oxytocin (*OXTR*; Onodera et al., 2015). Association of genes related to hypothalamic-pituitary-adrenal (HPA) axis function, e.g. encoding the corticotropin releasing hormone 1 receptor (*CRHR1*), has also been reported for panic disorder (Keck et al., 2008; Weber et al., 2016). Comprehensive overviews of candidate genes in genetic association studies in

anxiety disorders are given in Bandelow et al. (2016), Domschke and Maron (2013), Domschke and Reif (2012), and Gottschalk and Domschke (2016).

The genetic dissection of anxiety disorders may furthermore be aided by the consideration of so-called "intermediate phenotypes", or "endophenotypes". Endophenotypes constitute heritable neurobiological or neuropsychological markers that are linked to a disorder. Importantly, while psychiatric disorders are defined categorically, endophenotypes are narrowly described dimensional constructs, and therefore assumed to be closer to the underlying genetic architecture than the clinical phenotype itself (Gottesman and Gould, 2003). In relation to anxiety disorders, several such intermediate phenotypes have been described, such as behavioral inhibition (Rosenbaum et al., 1991; Smoller and Tsuang, 1998), trait anxiety (Legrand et al., 1999), and anxiety sensitivity (Stein et al., 1999), and have furthermore been linked to genetic variation. For instance, behavioral inhibition was linked to the gene coding for the corticotropin releasing hormone (*CRH*) (Smoller et al., 2005, 2003). On a neurobiological level, several intermediate phenotypes have been connected to genetic variants, for example alterations of the startle response (e.g. *5-HTT*: Brocke et al., 2006; Klumpers et al., 2012; *COMT*: Montag et al., 2008; *NPSR1*: Domschke et al., 2012), carbon dioxide (CO_2) sensitivity (e.g. *5-HTT*: Schmidt et al., 2000; Schruers et al., 2011), cholecystokinin tetrapeptide (CCK-4) challenge response (e.g. *5-HTT*: Maron et al., 2004; *MAOA*: Maron et al., 2004), and sympathetic activation such as heart rate (e.g. *NPSR1*: Domschke et al., 2011). Neuronal activation correlates of emotional processing constitute another intermediate phenotype for anxiety disorders, and in so-called 'imaging genetics' approaches, several polymorphisms have been linked to altered prefrontal (e.g. *NPSR1*: Domschke et al., 2011) and amygdala activation (e.g. *5-HTT*: Domschke et al., 2006; Furmark et al., 2004; *COMT*: Domschke et al., 2008b; *NPSR1*: Dannlowski et al., 2011).

Although strong evidence has accumulated for several susceptibility genes of anxiety disorders or anxiety-related intermediate phenotypes, negative results/non-replications, or discrepancies concerning the allelic direction of association have also been reported. High comorbidity rates, etiological heterogeneity, and unclear distinction between clinical and non-clinical anxiety impede the search for candidate genes (McGrath, Weill, Robinson, Macrae, & Smoller, 2012), necessitating re-evaluation in well-defined, sufficiently powered samples.

Nonetheless, despite the heterogeneity of findings, interest in the nature of the association between *5-HTT* in particular and anxiety disorders is unwavering in the attempt to reconcile conflicting findings. In addition, given the accumulating evidence linking *NPSR1* to the pathogenesis of anxiety disorders, as well as findings regarding the interplay of each of these genes with environmental factors as outlined below in section 1.5, both genes as well as their relation to categorical and dimensional anxiety will be described in greater detail below.

1.3.2.1 SEROTONIN TRANSPORTER GENE (*5-HTT*)

The serotonin transporter (5-HTT) is a protein in the cell membrane and involved in the regulation of serotonergic function in the brain by mediating synaptic serotonin re-uptake. The gene encoding the serotonin transporter (*5-HTT*; *SLC6A4*) is located on chromosome 17q11. A 44-base pair functional insertion/deletion polymorphism in the promoter region of the *5-HTT* gene – the serotonin transporter gene linked polymorphic region (*5-HTTLPR*) – comprises a low-expressing short allele (S) and a high-expressing long allele (L) (Lesch et al., 1996). Additionally, a single nucleotide polymorphism within *5-HTTLPR* (rs25531 A>G) influencing expression of the L allele has been identified, with the presence of the G allele (L_G) rendering it functionally equivalent to the S allele. In turn, the L_A variant leads to increased 5-HTT expression (Hu et al., 2006; Wendland et al., 2006).

Although several association studies argue against a major role of *5-HTTLPR* in anxiety disorders (Blaya et al., 2010; Deckert et al., 1997; Hamilton et al., 1999; Strug et al., 2010), there is some evidence for significant associations between the LL genotype and panic disorder (Maron et al., 2005a). Similarly – although no overall effect could be discerned – Hamilton et al. (1999) observed a higher frequency of the LL genotype in a subset of female panic disorder patients. The LL genotype has also been linked to cholecystokinin tetrapeptide (CCK-4)-induced panic attacks (Maron et al., 2004; but: Maron et al., 2008) and more pronounced responses to carbon dioxide (CO_2) challenge experiments in healthy controls (Schmidt et al., 2000; Schruers et al., 2011), but not in patients with panic disorder (Perna et al., 2004). A higher frequency of the combined *5-HTTLPR*/rs25531 L_AL_A genotype was also observed in patients with social anxiety disorder (Reinelt et al., 2013). By contrast, associations of the S allele have been reported with symptom severity in social anxiety disorder, e.g. increased trait anxiety and symptoms of depression (Furmark et al., 2004) or blushing propensity (Domschke et al., 2009). In animal models of 5-HTT function in anxiety, inactivation of the *5-HTT* gene has been observed to lead to enhanced anxiety-like behavior and decreased locomotor activity in mice (Holmes et al., 2003; Lesch et al., 2003). Relatedly, in healthy participants, the S allele has been found to moderate anxiety-related traits (e.g. Greenberg et al., 2000; Lesch et al., 1996), responses to fear conditioning (e.g. Lonsdorf et al., 2009; Wendt et al., 2015), increased startle response (Brocke et al., 2006; Klumpers et al., 2012), or HPA axis reactivity (Gotlib et al., 2008; Way and Taylor, 2010; for meta-analysis, see Miller et al., 2013).

Imaging genetic studies investigating the relationship between *5-HTT* variation and neuronal activation have suggested an overall effect of the *5-HTTLPR* and the S allele in particular on amygdala activation (for meta-analyses, see Munafò et al., 2008; Murphy et al., 2013), although the most recent meta-analysis failed to discern a significant association (Bastiaansen et al., 2014), suggesting that the previously

reported effects may have been overestimated due to publication bias (Bastiaansen et al., 2014; Murphy et al., 2013). With regard to anxiety disorders, increased amygdala activity has been reported in *5-HTTLPR* S allele carriers in patients with social anxiety disorder in response to a public speaking task (Furmark et al., 2004), and in panic disorder to the presentation of happy faces (Domschke et al., 2006). Conversely, the L_AL_A genotype was linked to increased amygdala and anterior insula activity in generalized anxiety disorder during anticipation of and in response to aversive pictures (Oathes et al., 2015). Lau et al. (2009) also reported an association between the L_AL_A genotype and heightened amygdala responses to fearful and happy faces in adolescents with anxiety and depressive disorders. A moderating effect of the *5-HTTLPR* effect on amygdala activation to anxiety-relevant stimuli has also been observed in healthy controls (for review, see Domschke and Dannlowski, 2010).

Furthermore, the LL genotype has been found to be related to better selective serotonin reuptake inhibitor (SSRI) treatment response in social anxiety disorder (Stein et al., 2006) and panic disorder (female patients only; Perna et al., 2005), and serotonin-norepinephrine reuptake inhibitor (SNRI) response in generalized anxiety disorder (for combined *5-HTTLPR*/rs25531 genotype L_AL_A, Lohoff et al., 2013). Associations of *5-HTTLPR* genotype and response to psychotherapy have also been investigated (cf. Lueken et al., 2016). One study linked the S allele (including L_G) to better response to exposure therapy in agoraphobia (Knuts et al., 2014), and the S allele has also been found to increase psychological flexibility after cognitive behavioral therapy (Gloster et al., 2015). The SS genotype was furthermore reported to facilitate treatment response in childhood anxiety disorders (Eley et al., 2012), although this finding could not be confirmed in a replication study (Lester et al., 2016). This latter finding is corroborated by several other studies that did not observe an effect of *5-HTTLPR* genotype on primary psychotherapy outcome in social anxiety disorder (Andersson et al., 2013; Hedman et al., 2012), or panic disorder (Lonsdorf et al., 2010; Lueken et al., 2015). However, while no direct influence of

5-HTTLPR genotype could be discerned in a study assessing therapy outcome in panic disorder with agoraphobia after exposure-based cognitive behavioral therapy, pre-treatment negative connectivity of anterior cingulate cortex and amygdala during fear conditioning was found to predict treatment response in L_AL_A genotype carriers (Lueken et al., 2015).

Taken together, these conflicting findings regarding allelic direction and especially concerning negative findings may be reconciled by the view that *5-HTTLPR* is highly sensitive to environmental influences (see Caspi et al., 2010), and thus acts in a complex, interactive manner with the environment rather than by modulating outcome parameters directly, which will be addressed in more detail in section 1.5.

1.3.2.2 NEUROPEPTIDE S RECEPTOR GENE (*NPSR1*)

Neuropeptide S (NPS) is a 20-amino acid peptide that acts as an agonist at its cognate G-protein coupled receptor (NPSR), which results in an increase in intracellular calcium and cyclic adenosine monophosphate (cAMP) concentrations (Reinscheid and Xu, 2005; Xu et al., 2004). In rodent models, NPS administration has been found to induce arousal, i.e. by increasing locomotor activity and wakefulness (Reinscheid and Xu, 2005; Rizzi et al., 2008; Xu et al., 2004; Zhao et al., 2012), and elicit anxiolytic-like effects (Leonard et al., 2008; Pulga et al., 2012; Vitale et al., 2008; Wegener et al., 2012; Xu et al., 2004). It has furthermore been linked to activation of the HPA axis (Smith et al., 2006) and decreased food intake (Beck et al., 2005; Peng et al., 2010; Smith et al., 2006). In humans, the gene coding for NPSR (*NPSR1*) is located on chromosome 7p14. Several single nucleotide polymorphisms in this gene have been identified and linked to susceptibility to asthma (Hersh et al., 2007; Kormann et al., 2005; Laitinen et al., 2004; Melén et al., 2005), rheumatoid arthritis

(D'Amato et al., 2010), inflammatory bowel disease (D'Amato et al., 2007), and panic disorder (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007).

The single nucleotide polymorphism rs324981 codes for an amino acid exchange, changing asparagine (Asn) to isoleucine (Ile) at position 107, the functional consequence being an about tenfold increase in NPS potency at NPSRIle^{107} (T) relative to NPSRAsn^{107} (A) (Reinscheid et al., 2005). The more active T allele has been linked to panic disorder (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007). The association of the T allele initially appears to conflict with findings from animal models reporting an anxiolytic effect of NPS administration. This apparent inconsistency may, however, be reconciled by the notion that panic disorder is largely driven by heightened arousal (Bouton et al., 2001; cf. Domschke et al., 2011), and, thus, an overactive NPS system, which is in line with the literature on the arousal-inducing effect of NPS administration in rodents (Reinscheid and Xu, 2005; Rizzi et al., 2008; Xu et al., 2004; Zhao et al., 2012).

Furthermore, elevated levels of anxiety sensitivity (Beste et al., 2013; Domschke et al., 2011), enhanced response inhibition and increased error monitoring (Beste et al., 2013), increased heart rate and higher symptom reports during a behavioral avoidance test (Domschke et al., 2011), higher impulsivity (Laas et al., 2015, 2014b), and increased neuroendocrine and subjective responses to acute stress (Kumsta et al., 2013) have been demonstrated in carriers of at least one T allele. TT homozygosity has furthermore been shown to influence affect-modulated startle magnitude to neutral and negative pictures following caffeine administration in healthy controls (Domschke et al., 2012).

On a neural level, the T allele appears to be related to altered cortico-limbic function in panic disorder, with attenuated activation of the dorsolateral prefrontal, lateral orbitofrontal, and anterior cingulate cortex during the processing of fearful faces (Domschke et al., 2011). Interestingly, negative correlations were observed

between anxiety sensitivity – constituting an intermediate phenotype/risk factor for panic disorder (Stein et al., 1999) – and dorsolateral and medial prefrontal cortex activity in response to negative pictures in an emotional n-back task in healthy T allele carriers (Guhn et al., 2015). Furthermore, in healthy probands, T allele carriers exhibited increased amygdala activation in response to fearful and angry faces (Dannlowski et al., 2011). The suggested dysfunctional cortico-limbic interaction might result from a delayed maturation of connectivity between the amygdala and the medial frontal cortex across development in carriers of the TT genotype, with otherwise increasing connectivity strength from early to late adolescence facilitated by the A allele (Domschke et al., 2015). Presence of at least one T allele has also been linked to altered neural responses during fear conditioning, with increased activity to threat cues in the rostral dorsomedial prefrontal cortex, which was paralleled by enhanced subjective fear ratings (Raczka et al., 2010). In an emotional n-back task, T allele carriers showed higher activation of the dorsolateral and medial prefrontal cortex in response to negative pictures (Guhn et al., 2015). TT homozygosity has furthermore been linked to attentional functions, with observations of enhanced engagement of the right prefrontal cortex and locus coeruleus in relation to alertness, and increased fronto-parietal activity during an executive control task (Neufang et al., 2015). Across studies, the observed increases in prefrontal activity in healthy T allele carriers may constitute a compensatory top-down mechanism offsetting heightened subcortical activation driven by an overactive NPS system, that, if disrupted, can predispose to pathological anxiety (cf. Neufang et al., 2015).

Taken together, *NPSR1* appears to play a significant role in anxiety and anxiety disorders, particularly panic disorder, and is involved in the modulation of a variety of intermediate anxiety phenotypes on neural and neurophysiological levels. Recent research has also focused on the interplay of *NPSR1* with the environment. The relationship between *NPSR1* rs324981 and environmental risk factors regarding anxiety disorders and anxiety-related phenotypes is discussed in section 1.5.

1.4 ENVIRONMENTAL STRESS

While heritability estimates obtained from family studies provide evidence for a significant genetic component in the etiology of anxiety disorders, they also point to the involvement of shared environmental factors that account for the remaining variability not already explained by genetic contribution (Hettema et al., 2005). Following the summary of genetic risk factors in chapter 1.3, the current section will highlight the role of environmental influences – focusing on stressful life events – in the moderation of anxiety risk.

The occurrence of environmental adversity, e.g. traumatic and stressful life events, has repeatedly been implicated in the vulnerability to the development of mental disorders (Carr et al., 2013). Stressful life events such as health problems, interpersonal conflicts, bereavement, or experiences of threat, loss, or separation, have been found to directly precede the onset of anxiety disorders. For instance, an occurrence of one or more life events, particularly negative ones (e.g. loss, threat, severe illness, conflict) in the year prior to disease onset has been reported for panic disorder with or without agoraphobia (e.g. Batinic et al., 2009; Faravelli and Pallanti, 1989; Faravelli, 1985; Scocco et al., 2006). Higher overall frequency of traumatic experiences has been linked to generalized anxiety disorder (Roemer et al., 1997), with a threefold increased risk for subsequent disorder onset following the experience of at least one negative, major life event (Blazer et al., 1987), especially pertaining to experiences of loss, or danger of future loss or trauma (Kendler et al., 2003). A higher frequency of negative life events has also been found to be predictive of relapse in patients with generalized anxiety disorder, particularly concerning experiences of death, health-related issues, and stressors related to social contacts in the month prior to relapse (Francis et al., 2012). While stressful life events have been considered extensively either cumulatively across the lifespan or more narrowly focusing on recent life events during adulthood (e.g. in the year prior to assessment) with respect

to manifestation of disease (cf. Klauke et al., 2010), a particular focus in the dissection of environmental risk factors has been on adverse events occurring during the sensitive period of childhood. Indeed, a recent meta-analysis reported a significant link between the experience of childhood trauma and anxiety disorders, with an almost two- to fourfold increased risk for the development of panic disorder, social anxiety disorder, or generalized anxiety disorder in individuals with a history of early trauma (Fernandes and Osório, 2015). For example, for panic disorder, negative experiences during childhood such as sexual abuse (Bandelow et al., 2002; Cougle et al., 2010; Goodwin et al., 2005; Stein et al., 1996), physical abuse (Goodwin et al., 2005; Sareen et al., 2013; Stein et al., 1996), domestic violence (Bandelow et al., 2002; Sareen et al., 2013), parents' marital problems (Bandelow et al., 2002), separation experiences (Bandelow et al., 2002), or bereavement (Keyes et al., 2014) were identified as risk factors. Similarly, higher rates of adverse experiences during childhood, including separation experiences (Bandelow et al., 2004), physical abuse (Bandelow et al., 2004; Bishop et al., 2014; Sareen et al., 2013), emotional abuse (Bishop et al., 2014; Kuo et al., 2011; Reinelt et al., 2013), sexual abuse (Bandelow et al., 2004; Bishop et al., 2014; Cougle et al., 2010), parents' marital quality (Bandelow et al., 2004), domestic violence (Bandelow et al., 2004; Sareen et al., 2013), or dysfunctional parental rearing behavior (Bandelow et al., 2004) were reported in relation to onset of social anxiety disorder. Death of a parent (Torgersen, 1986) as well as childhood sexual abuse (Cougle et al., 2010) have also been linked to increased generalized anxiety disorder risk. Furthermore, physical abuse has been implicated as a risk factor for specific phobia (Cougle et al., 2010). Comparing the incidence of childhood adversity between different anxiety disorders, there is some evidence that history of sexual and physical abuse is significantly more frequent among patients with panic disorder relative to patients with social phobia, while rates among patients with generalized anxiety disorder do not appear to differ considerably from either group (Safren et al., 2002). The detrimental effect of childhood adversity on mental

health outcomes is further corroborated by higher rates of suicide ideation and suicide attempts observed in individuals reporting childhood experiences of physical abuse, sexual abuse, or domestic violence (Afifi et al., 2008).

Taken together, there is converging evidence for the role of adverse experiences in the pathogenesis of anxiety disorders, and disease onset is often preceded by stressful life events. Traumatic experiences during childhood appear to exert a particularly harmful effect on mental health outcomes. However, it should be noted that despite the higher frequency of stressful life events reported in patient groups, a considerable portion of patients do not report any history of traumatic experiences (e.g. Bandelow et al., 2002). Therefore, it seems unlikely that stressful life events constitute a sole etiological factor in anxiety disorders, but rather act by increasing vulnerability to disease in an interactive manner with other, e.g. genetic, risk factors. Life events occurring shortly before disease onset may therefore have a triggering function that, if coinciding with dormant risk factors, can lead to pathological anxiety.

On a neurobiological level, the detrimental effect of stressful life events may be conferred by permanently altered HPA axis function. The HPA axis represents an organism's major stress response system involving the interplay of the hypothalamus, the pituitary gland, and the adrenal glands. The HPA axis can be activated by stress by releasing corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which is then transported via the hypophyseal portal system to the pituitary gland, in turn triggering the release of adrenocorticotropin hormone (ACTH). ACTH binds to its receptors on the adrenal cortex, which prompts synthesis of glucocorticoids (i.e. cortisol). Termination of the stress response is executed via negative feedback loops (Smith and Vale, 2006). Prolonged stress, however, can lead to dysfunction of the HPA axis and, consequently, repeated elevation of glucocorticoids, with long-term detrimental effects on brain structures

and function and the development of pathologies (McEwen, 2000). In rodents, exposure to early life stress such as maternal separation has been linked to abnormal HPA axis function and expression of CRH (cf. de Kloet et al., 2005). Likewise, HPA axis disturbances following stress experiences have been observed in humans, although both increased and decreased cortisol secretion has been reported (Heim and Nemeroff, 2001; Miller et al., 2007). This discrepancy may, however, be reconciled by taking into account the nature of the stressor, person characteristics, or the temporal relationship between stress onset and time of assessment, with cortisol output changing adaptively over time (Miller et al., 2007). Disrupted HPA axis function has been described in anxiety disorders (e.g. Abelson et al., 2007; Erhardt et al., 2006; Mantella et al., 2008; Vreeburg et al., 2010). Cortisol responses to an acute stressor can be elicited in a laboratory setting by standardized stress protocols, such as the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), the (socially evaluated) cold pressor test (CPT; e.g. Lovallo, 1975; SECPT; Schwabe et al., 2008), or the Maastricht Acute Stress Test (MAST; Smeets et al., 2012). For instance, blunted cortisol reactivity in response to the TSST has been observed in healthy adolescents or adults with a history of childhood maltreatment (Carpenter et al., 2007; Elzinga et al., 2008; Sumner et al., 2014), particularly for experiences of sexual abuse, physical abuse, and emotional neglect (Carpenter et al., 2007), and if trauma was unresolved (Pierrehumbert et al., 2009). The former finding could be partially replicated in a larger sample, with decreased cortisol responses in relation to childhood physical abuse (Carpenter et al., 2011). By contrast, childhood abuse has been linked to increased cortisol reactivity in clinical populations. Heim et al. (2000) reported heightened cortisol levels after exposure to psychosocial stress in clinically depressed – but not healthy – women with a history of maltreatment. Increased cortisol levels were also observed in patients with social anxiety disorder with a history of childhood abuse in response to the TSST compared to both patients without maltreatment and a healthy control group (Elzinga et al., 2010), although no

differences in salivary cortisol to acute stress between social anxiety disorder patients and controls have also been reported (Klumbies et al., 2014).

1.5 GENE-ENVIRONMENT INTERACTIONS

As outlined above, the etiology of anxiety disorders is complex, with contributions from both genetic and environmental factors which are assumed to not only act independently, but interactively with each other to increase the risk towards manifestation of disease as proposed by the "diathesis-stress" model (Zubin and Spring, 1977). The investigation of the interplay of genetic markers with environmental influences in so-called gene-environment (GxE) interaction approaches thus constitutes a crucial step in the dissection of the pathogenesis of anxiety disorders. Accordingly, the combined effect of a variety of candidate genes (see 1.3.2) and environmental variation (see 1.4) on anxiety traits has been the topic of a range of studies in clinical and non-clinical populations.

With regard to GxE effects on anxiety disorders as categorical nosological entities, the *5-HTTLPR*/rs25531 L_AL_A genotype in the presence of family adversity has been linked to increased risk for any DSM-IV anxiety disorder diagnosis (Laucht et al., 2009). The L_AL_A genotype has furthermore been observed to increase risk towards social anxiety disorder in combination with lack of social support (Reinelt et al., 2014). Conversely, the *5-HTTLPR* SS genotype (in combination with rs25531 L_GL_G and SL_G genotypes) has been shown to increase panic disorder risk in the presence of at least two separation life events (Choe et al., 2013). By contrast, Blaya et al. (2010) failed to discern an interactive effect of *5-HTTLPR* genotype and childhood maltreatment in the moderation of panic disorder risk. Concerning generalized anxiety disorder, a GxE interaction of *NPY* rs16147 and hurricane exposure on disorder risk has been observed (Amstadter et al., 2010). GxE interactions have also been studied in relation to dimensional anxiety traits. For instance, childhood

maltreatment has been shown to increase anxiety sensitivity in an interactive manner with *5-HTT* variation, although findings conflict regarding the direction of this association. While Klauke et al. (2011) reported enhanced anxiety sensitivity in *5-HTTLPR*/rs25531 L_AL_A carriers with a history of maltreatment, a study by Stein et al. (2008) implicated the *5-HTTLPR* SS genotype. Presence of at least one S or L_G allele was furthermore shown to confer higher levels of anxiety in response to the experience of daily stressors (Gunthert et al., 2007). By contrast, some studies were unable to discern an interactive effect of *5-HTTLPR* and adverse life events in the moderation of anxiety-related phenotypes (Cividanes et al., 2014; Zavos et al., 2012). Furthermore, both the *NPSR1* rs324981 TT genotype and the *COMT* rs4680 AA genotype have been linked to increased anxiety sensitivity in concert with experiences of childhood maltreatment (Baumann et al., 2013; Klauke et al., 2014). However, in contrast to the findings by Klauke et al. (2014), Laas et al. (2014a) reported a female-specific interaction of the *NPSR1* rs324981 AA genotype with a history of stressful life events leading to increased trait anxiety, as well as with family adversity on higher risk of affective and anxiety disorders, and on the frequency of attempted suicides. A GxE effect conferring increased symptoms of social anxiety has also been observed in carriers of the *OXTR* rs53576 A risk allele with an insecure attachment style (Notzon et al., 2016).

Additionally, the interplay of genetic and environmental influences has not only been assessed with regard to dimensional or categorical anxiety, but also in the context of research on biomarkers or intermediate phenotypes (e.g. 'imaging genetics' approaches) of vulnerability or resilience to anxiety. For example, urban upbringing was shown to further moderate the link between amygdala reactivity and *NPSR1* variation (see 1.3.2.2) by conferring enhanced amygdala activation during exposure to stress in *NPSR1* rs324981 T allele carriers (Streit et al., 2014). Increased affect-modulated startle magnitude in response to unpleasant pictures was observed in *COMT* rs4680 GG genotype carriers with a history of childhood maltreatment

(Klauke et al., 2012). Furthermore, stress reactivity as measured by salivary cortisol levels has also been found to be subject to GxE influences: a polymorphism (rs1360780) in the FK506 binding protein 5 (*FKBP5*) gene, which encodes a co-chaperone protein influencing glucocorticoid receptor sensitivity, was shown to interact with childhood maltreatment, resulting in blunted cortisol response to the TSST in CC genotype carriers with a history of abuse (Buchmann et al., 2014). Similarly, blunted cortisol reactivity to the TSST was observed in *NPY* rs16147 TT carriers with a history of early life adversity (Witt et al., 2011). A GxE effect of *COMT* variation and stressful life events was observed in response to the TSST in children, with higher cortisol levels in *COMT* A allele carriers who had experienced adversity (Armbruster et al., 2012). *5-HTTLPR* SS genotype carriers with a history of life events also showed elevated cortisol reactivity in response to the TSST (Alexander et al., 2009).

Taken together, findings from GxE interaction approaches highlight the complex underpinnings of anxiety traits and anxiety disorders, with contributions from multiple domains that, depending on their individual constellation, can increase – or suspend – vulnerability to manifestation of disease.

1.6 PROTECTIVE FACTORS

While much attention has been paid to the detrimental effect of environmental adversity as outlined above (1.4), positive environmental influences, i.e. elements of successful coping with adversity that may exert a protective or buffering effect on anxiety risk, have been studied to a lesser extent.

Availability of social support has been linked inversely to anxiety, with high social support buffering symptoms of anxiety (Hart and Hittner, 1991; Reinelt et al., 2014; Roohafza et al., 2014; Sangalang and Gee, 2012; Yasin and Dzulkifli, 2010), possibly via moderating fear-relevant neuronal activation patterns (Hyde et al.,

2011). Furthermore, high social support has also been shown to mediate psychotherapy outcome in patients with anxiety and mood disorders (Dour et al., 2014; Lindfors et al., 2014). Similarly, peer relationships, friendship quality and romantic relationships (Festa and Ginsburg, 2011; La Greca and Harrison, 2005), and a secure attachment style have been linked to lower symptoms of anxiety (Muris and Meesters, 2002; Notzon et al., 2016). Self-efficacy constitutes another promising construct related to coping that may be contributing to resilient functioning. The concept of self-efficacy constitutes one of the pillars of Bandura's social-cognitive theory: It refers to a person's belief in his or her own ability to successfully cope with adversity, and consequently plays a role in whether coping behavior will be initiated and sustained depending on outcome expectations (Bandura 1977). Self-efficacy can be measured as a general construct – termed general self-efficacy (GSE) – or with regard to more narrowly defined, specific situations, such as emotional, social, or academic self-efficacy (e.g. Cassidy, 2015; Choi et al., 2013; Smith and Betz, 2000). High GSE has been linked to lower levels of trait anxiety in healthy adolescents (Muris, 2002) and adults (Endler et al., 2001), decreased risk for symptoms of social anxiety in childhood (Rudy et al., 2012), and diminished general psychological distress and posttraumatic stress symptoms in trauma survivors (Luszczynska et al., 2009). GSE has further been shown to alleviate symptoms of anxiety and depression in healthy adolescents (Muris, 2002) and in patients with cancer during treatment (Mystakidou et al., 2013). Importantly, GSE was found to exert a protective effect against the deleterious effects of daily stressors (Schönfeld et al., 2016), thus highlighting its function as an important link between environmental adversity and mental health outcomes. Moreover, self-efficacy constitutes a modifiable quality than can be targeted by therapeutic interventions: it has been linked to therapy outcome in panic disorder and social anxiety disorder (Bouchard et al., 2007; Gallagher et al., 2013; Gaudiano and Herbert, 2007), and has been shown to increase following stress management training (Molla Jafar et al., 2016). However, the potential links between

GSE and genetic aspects, as well as its role in the search for protective mechanisms in GxE models of anxiety-related traits, have yet to be elucidated.

1.7 AIMS AND OBJECTIVES

The aims of this dissertation are twofold: first, to address the moderating influence of positive factors in an extension of GxE risk constellation models by introducing coping factors as an additional dimension in the context of anxiety-related traits, and second, to further elucidate potential genetic mechanisms affecting acute stress reactivity.

1.7.1 STUDY 1: GENE-ENVIRONMENT-COPING INTERACTIONS

Although protective factors have not gone unnoticed, little attention has been paid to the interplay of positive and negative environmental aspects against the background of an individual's genetic make-up, and – if at all – efforts have mostly focused on the availability of social support. For instance, high social support has been observed to exert a protective effect on risk for social anxiety disorder, and, applying a GxE approach, this effect was further qualified by an interaction with *5-HTTLPR*/rs25531 genotype, with high social support buffering social anxiety disorder risk conferred by L_AL_A genotype (Reinelt et al., 2014). However, the interplay of negative environmental influences such as childhood maltreatment in synopsis with beneficial conditions has scarcely been addressed in the framework of GxE research. In one study assessing the effect of social support on the interaction of childhood maltreatment and *5-HTTLPR* genotype, social support was shown to buffer depression risk in children with the SS genotype and a history of maltreatment (Kaufman et al., 2004), highlighting the necessity of simultaneously considering both beneficial and detrimental factors in the moderation of genetic susceptibility to disease towards the definition of "plasticity" rather than "risk" genes of mental

disorders (Belsky et al., 2009). Hence, the additional consideration of protective next to deleterious environmental aspects in an extension of classic GxE models based on a "diathesis-stress" approach may further elucidate the complex underpinnings of vulnerability to anxiety. Given findings outlined above implicating variations in both *5-HTT* and *NPSR1* genes as moderators of anxiety-related traits, particularly in synopsis with childhood maltreatment, the first part of this thesis aims to expand GxE interaction models regarding each of these genes by proposing GSE as an indicator of coping ability ("C") as an additional dimension (GxExC) conferring – or buffering – anxiety risk in an attempt to reconcile the heterogeneity of previous findings in GxE research.

1.7.2 STUDY 2: GENETIC DETERMINANTS OF STRESS REACTIVITY

HPA axis reactivity is moderately heritable (Federenko et al., 2004), and neuroendocrine stress reactivity has been discussed as an intermediate phenotype of mental disorders (e.g. Hasler et al., 2004; Mehta and Binder, 2012). Variation in genes such as *5-HTT* and *NPSR1* has been assessed with regard to HPA axis activation. *5-HTTLPR* genotype has repeatedly been shown to moderate cortisol reactivity in response to acute stress, although, paralleling the conflicting findings on the direction of allelic association with (chronic) environmental stress, both the S (Gotlib et al., 2008; Way and Taylor, 2010; for meta-analysis, see Miller et al., 2013) and the L allele (Mueller et al., 2011) have been linked to increased cortisol responses, while negative findings have also been reported (Alexander et al., 2014, 2009; Verschoor and Markus, 2011; Wüst et al., 2009). NPS administration has been shown to activate the HPA axis in rats (Smith et al., 2006), and, in humans, first concrete evidence for the involvement of the NPS system in stress regulation stems from a recent study addressing stress reactivity as a function of *NPSR1* rs324981 genotype. Acute stress responses were elicited by means of the group version of the Trier Social

Stress Test in a sample of male healthy probands. T-allele carriers displayed larger salivary cortisol levels in response to acute stress, which was paralleled by enhanced anticipatory subjective stress ratings (Kumsta et al., 2013).

In light of these findings on genetic determinants of acute stress responding, the second part of this thesis addresses possible genotype-dependent differences in the neuroendocrine response to acute psychosocial stress. Specifically, this study aims to address the respective impact of variants in the *5-HTT* and *NPSR1* genes on acute stress reactivity in a mixed sample of both male and female volunteers (the sample reported by Kumsta et al. (2013) was exclusively male), and to increase generalizability by implementing a stress paradigm different from the commonly used TSST.

2. METHODS

2.1 STUDY 1: GENE-ENVIRONMENT-COPING INTERACTIONS

Parts of this study have been published in Schiele et al. (2016) on the interactive effect of *5-HTT* variation, childhood trauma, and general self-efficacy.

2.1.1 SAMPLE

A total of 695 participants (427 female, 268 male, mean age±SD=25.12±5.28 years) was recruited in the context of project Z02 within the Collaborative Research Center SFB-TRR58 "Fear, Anxiety, Anxiety Disorders" during the project's second funding period at the Universities of Würzburg and Münster, Germany, between 2013 and 2015. Participant recruitment at the Würzburg site (N=324) was carried out by the experimenter (M. Schiele), probands at the Münster site were recruited by K. Holitschke under supervision of Prof. Dr. P. Zwanzger. Inclusion criteria were predefined as follows: Caucasian descent (self-report up to third generation), age at participation between 18 and 50 years, right-handedness, and fluency in German. Exclusion criteria comprised past or present diagnosis of any DSM-IV axis I disorder, history or presence of severe neurological or internal diseases, intake of centrally active medication, excessive consumption of alcohol (more than 15 units per week), nicotine (more than 20 cigarettes per day), and caffeine (more than 4 cups per day), consumption of illegal drugs, and pregnancy. Absence of mental axis I disorder was ascertained using the German version of the Mini International Psychiatric Interview (M.I.N.I.; Sheehan et al., 1998). All participants received 50€ remuneration upon participation. Written informed consent was obtained from all participants. The study was reviewed and approved by the ethical committees of the Universities of Würzburg and Münster, and conducted in compliance with the declaration of Helsinki.

2.1.2 EXPERIMENTAL SETUP

Participants were screened for general inclusion and exclusion criteria in a telephone interview before being invited to participate in the study. Participants were asked to refrain from consuming alcohol the day before the experiment and to be well rested on the day of. All participants were tested individually in a single session. A venous blood sample (2x9ml) was taken for genetic analyses at the beginning of the experimental session (see 2.1.4 and 2.1.5). Participants then filled in a set of questionnaires (for a selection as relevant for this dissertation see 2.1.3). The experimenter was present in the same room with the participant for the duration of the experiment. Comparable general experimental set-up, including verbatim consent forms, study information and instructions, and surrounding conditions between both study sites were ascertained by adherence to a detailed standard operating procedure.

2.1.3 SELECTED SELF-REPORT MEASURES

CHILDHOOD TRAUMA QUESTIONNAIRE

The short form of the Childhood Trauma Questionnaire (CTQ; Bernstein et al., 2003; Wingenfeld et al., 2010), comprising 28 items, retrospectively assesses the frequency and kind of childhood maltreatment. It contains five subscales addressing different kinds of abuse (physical, emotional, sexual) and neglect (physical, emotional). Answers are scored on a five-point scale (1=never true, 2=rarely true, 3=sometimes true, 4=often true, 5=very often true). Seven items are inversely formulated. After re-coding of the respective items, a total score is obtained by calculating the sum of all items, resulting in possible sum scores between 25 and 128. For each subscale, addition of the respective item scores returns sum scores ranging from five to 25.

GENERAL SELF-EFFICACY SCALE

The General Self-Efficacy Scale (GSE; Schwarzer & Jerusalem, 1995) consists of 10 items addressing perceived self-efficacy, referring to a person's belief in their own ability to cope with difficulties. The GSE is scored on a four-point scale (1=not at all true, 2=hardly true, 3=moderately true, 4=exactly true) and evaluated by summing up all items, resulting in total scores between 10 and 40 points.

AGORAPHOBIC COGNITIONS QUESTIONNAIRE

The Agoraphobic Cognitions Questionnaire (ACQ; Chambless et al., 1984; Ehlers et al., 1993) comprises 14 items that are scored on a five-point scale (1=thought never occurs, 2=thought rarely occurs, 3=thought occurs during half of the times, 4=thought usually occurs, 5=thought always occurs) and address the frequency of anxiety-related cognitions. The total score is calculated as the overall mean of the individual item scores.

LIEBOWITZ SOCIAL ANXIETY SCALE

The Liebowitz Social Anxiety Scale (LSAS; Liebowitz, 1987; Stangier and Heidenreich, 2004) assesses fear and avoidance in social situations. It contains 24 items that are rated on a four-point scale with regard to the degree of fear of a specific situation (0=none, 1=mild, 2=moderate, 3=severe), and how often the situation is avoided (0=never, 1=occasionally, 2=often, 3=usually). An overall sum score across all items can be obtained, resulting in possible scores between zero and 144 points.

STATE-TRAIT ANXIETY INVENTORY – TRAIT VERSION

The State-Trait Anxiety Inventory (STAI; Laux et al., 1981; Spielberger et al., 1970) comprises two scales, capturing anxiety as a transitory state (state anxiety, STAI-S) as well as a stable, transsituational disposition (trait anxiety, STAI-T). Both scales can be used together or independently of each other. Since the focus of the present study was on anxiety as a trait rather than a state marker, only the STAI-T form was applied and is thus described further in the following. The STAI-T consists of 20 items that are scored on a four-point scale (1=almost never, 2=sometimes, 3=often, 4=almost always). Seven items are reverse-coded to minimize errors due to arbitrary responding. After recoding of the respective items, the total STAI-T score is calculated as the sum of all items, resulting in possible scores between 20 and 80.

2.1.4 BLOOD SAMPLING

Venous blood samples (2x9ml) were drawn (at Würzburg site by the experimenter) using S-Monovettes® (Sarstedt, Nümbrecht, Germany) from each participant at the beginning of the experimental session. Samples were stored at 4°C upon collection. DNA was extracted following the salting-out procedure described by Miller et al. (1988) with minor modifications in the Laboratory of Functional Genomics (Head: Prof. Dr. Dr. K. Domschke), Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg. Briefly, erythrocytes were lysed using hypotonic NH_4Cl buffer. Lysis of leukocytes returned after centrifugation was carried out using sodium dodecyl sulfate (SDS) lysis buffer and proteinase. Following treatment with sodium chloride (NaCl) and centrifugation, DNA was precipitated with isopropanol. Aliquots of isolated DNA were stored at 4°C until further processing.

2.1.5 GENOTYPING

Isolated DNA was genotyped for *5-HTTLPR* and the functionally related single nucleotide polymorphism rs25531 (Hu et al., 2006) as well as for *NPSR1* rs324981 in the Laboratory of Functional Genomics (Head: Prof. Dr. Dr. K. Domschke), Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

5-HTTLPR and rs25531 were genotyped according to published protocols with minor modifications (Wendland et al., 2006). DNA was amplified by polymerase chain reaction (PCR) (60 s at 94°C, 60 s at 64°C, 120 s at 72°C for 35 cycles) using the following oligonucleotide primers F: 5'-TGCCGCTCTGAATGCCAGCAC-3' and R: 5'-GGGATTCTGGTGCCACCTAGACG-3'. PCR products were digested with MspI at 37°C overnight, separated on 3% agarose gel containing ethidium bromide, and visualized by ultraviolet light (ChemiDoc UV chamber, BioRad, Munich, Germany).

For genotyping of *NPSR1* rs324981, DNA was amplified by PCR (45 s at 95°C, 45 s at 58°C, 45 s at 72°C for 35 cycles) applying the oligonucleotide primers F: 5'-TGCTTTGCATTTCCTCAGTG-3' and R: 5'-TTGTCTCATCACATTTGGAAGG-3'. PCR products were digested with AseI at 37°C overnight, separated on 3% agarose gel containing ethidium bromide, and visualized by ultraviolet light (ChemiDoc UV chamber, BioRad, Munich, Germany).

Genotypes were determined by two independent investigators blinded for phenotypes. *5-HTTLPR*/rs25531 genotype information was unavailable for 17 participants, resulting in a reduced sample size of N=678 for all analyses with respect to *5-HTT* variation. Hardy-Weinberg criteria as calculated by the online program DeFinetti (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) were fulfilled for *5-HTTLPR* genotype distribution (SS=110, SL=325, LL=243; p=.939) and the triallelic model (L_AL_A=197, L_GL_A/SL_A=318, L_GL_G/SL_G/SS=163; p=.121; Hu et al., 2006), as well as for the distribution of *NPSR1* rs324981 genotypes (AA=200, AT=341, TT=154; p=.704).

2.2 STUDY 2: GENETIC DETERMINANTS OF ACUTE STRESS REACTIVITY

2.2.1 SAMPLE

A subsample of 104 participants (62 female, 42 male; mean age±SD=28.34±7.61 years) drawn from the overall sample recruited by the experimenter via the SFBTRR-58 project Z02 in Würzburg between 2014 and 2016 took part in a standardized laboratory stress paradigm with minor modifications (Maastricht Acute Stress Test, MAST, see 2.2.2; Smeets et al., 2012) probing the time course of salivary cortisol in response to an acute stress situation. Inclusion and exclusion criteria corresponded to those of the overall Z02 sample as described above (see 2.1.1). Cold sensitivity of the hands was defined as an additional exclusion criterion for participation in the MAST. All participants received 15€ remuneration and gave written informed consent. The study protocol was approved by the ethical committee of the University of Würzburg and complied with the Declaration of Helsinki.

2.2.2 EXPERIMENTAL SETUP

EQUIPMENT AND PROCEDURES

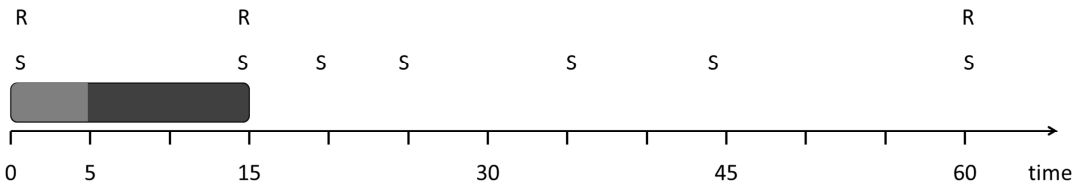
Experimental sessions were conducted between 1 pm and 4:30 pm in order to reduce variability due to circadian cortisol levels. Participants were asked to refrain from consumption of alcohol the day before, and from eating or drinking, chewing gum, or smoking at least 30 minutes prior to the experimental session. Intake of hormonal contraceptives and menstrual cycle phase at the time of testing were documented in female participants. Participants were asked to remove jewelry etc. worn on the right hand or wrist and take off their watch. Instructions were displayed using Presentation software (Version 17.2; Neurobehavioral Systems, Inc., Albany, CA, USA) on a 19" LCD monitor at a distance of approximately 80 cm from the participant. A high definition webcam (Logitech C270) attached centrally at the top

of the monitor was used for videotaping during the experimental session. A green light next to the camera lens signaled active recording to the participant. Participants were instructed to face the camera during the experiment for subsequent analysis of their facial expression. Participants were seated to the left of a water bath containing ice-cold water (2°C) (see below). Water temperature was kept constant (±0.03°C) and controlled with a circulation pump (JULABO ED-19). A towel was provided for in between hand immersion trials. All experimental sessions were conducted by a female researcher (M. Schiele) who was present in the room with the participant throughout the experimental session.

THE MAASTRICHT ACUTE STRESS TEST

The MAST was conducted according to the authors' protocol (Smeets et al. 2012) with minor modifications for practicability. For a schematic overview of the time course of the MAST as applied in the present study, see figure 2.1.

Figure 2.1. Schematic overview of the time course of the Maastricht Acute Stress Test



Light grey=anticipation phase; dark grey=stress phase; S=saliva sample; R=rating; time is given in minutes

The MAST is composed of a five-minute preparation/anticipation phase, and a ten-minute acute stress phase. The acute stress phase is characterized by alternating hand immersion trials, during which participants were instructed to place the right hand into ice-cold water (2°C) (physical stressor), and mental arithmetic trials (mental stressor), during which the participants had to count backwards from 2043

in steps of 17. Participants were asked to count as fast and correctly as possible. They received negative feedback from the experimenter in case of a counting error and had to recommence counting at 2043. During the mental arithmetic trials, participants placed their hand on a towel next to the water bath. In addition to an instruction being displayed on the screen, an acoustic computer signal indicated the start of the next trial. Participants were told that the overall number of trials and duration of individual trials was randomized, the only limitation being that no trial would be longer than 90 s, that the time between hand immersion trials would be at least 45 s, and that the overall duration of the experimental paradigm (including the anticipation phase) would be 15 minutes. Additionally, they were told that there would be a break, also randomly selected by a computer algorithm. For the duration of the experiment, participants were watched by the experimenter and recorded on video for subsequent facial expression analysis (social stressor).

In reality, the order and duration of trials was fixed and identical for all participants, consisting of a total of five hand immersion trials alternating with four mental arithmetic trials in the following order: hand immersion (90 s), mental arithmetic (45 s), hand immersion (60 s), mental arithmetic (60 s), hand immersion (60 s), mental arithmetic (90 s), hand immersion (90 s), mental arithmetic (45 s), hand immersion (60 s). The last hand immersion trial was followed by an instruction indicating a short break during which participants filled in a set of questionnaires and provided a saliva sample. Upon completion, it was explained to the participants that the announced break constituted in fact the end of the experiment, and that no further hand immersion trials or mental arithmetic would follow. For the remainder of the experimental session, participants were allowed to engage in non-stressful activities, e.g. reading. Participants provided saliva samples at seven time points during the experiment in order to track the course of cortisol reactivity to the acute stress paradigm: prior to (t_0; baseline), immediately after the acute stress phase while participants were still under the assumption that the stress phase would continue (t_1),

and 5 (t_2), 10 (t_3), 20 (t_4), 30 (t_5), and 45 (t_6) minutes after t_1, respectively. Ratings of subjective stress were obtained at three time points (t_0, t_1, t_6), with participants indicating their perceived level of stress on a visual analogue scale (VAS) ranging from 1-10 with 1="not stressed" and 10="very stressed".

2.2.3 BLOOD AND SALIVA SAMPLING

Blood sampling and genotyping for *5-HTTLPR*/rs25531 and *NPSR1* rs324981 for all MAST participants was performed within the context of project Z02 of the SFBTRR-58 (see 2.1.3).

Saliva samples were collected by the experimenter at seven time points (t_0-t_6) throughout the experiment using Salivette® Cortisol swabs (Sarstedt, Nümbrecht, Germany). Participants were instructed to place a synthetic fiber swab in their mouth and chew on it for approximately 60 s to stimulate saliva flow before transferring it to a plastic container and sealing it according to the manufacturer's instructions. Saliva samples were stored at room temperature for the duration of the experimental session and then refrigerated at 4°C. Within seven days of collection, samples were centrifuged at 3,500 rpm for 10 minutes. The obtained saliva was pipetted into 1 ml Eppendorf tubes and stored at -80°C until further processing. Samples were then thawed and centrifuged at 3,000 rpm for three minutes. Salivary-free cortisol concentrations were determined by commercially available chemiluminescence-immunoassays (CLIA; IBL, Hamburg, Germany) in cooperation with the Department of Biopsychology, Technical University of Dresden (Prof. Dr. C. Kirschbaum).

2.3 STATISTICAL ANALYSES

All statistical tests were performed by the experimenter using SPSS (Version 23; SPSS Inc., Chicago, Illinois, USA). Alpha level was set at .05. Greenhouse-Geisser corrections were applied where appropriate.

2.3.1 STUDY 1: GENE-ENVIRONMENT-COPING INTERACTIONS

DESCRIPTIVE STATISTICS

5-HTTLPR and rs25531 were grouped into a high expression group containing the combined genotype L_AL_A, and a low expression group comprising the remaining genotypes SS, SL_G, SL_A, L_GL_A and L_GL_G (cf. Baffa et al., 2010; Baune et al., 2008; Wendland et al., 2006). *NPSR1* rs324981 was grouped into T allele (AT/TT) versus AA genotype carriers (cf. Domschke et al., 2011). Genotype group differences regarding continuous variables – i.e. age, CTQ, GSE, ACQ, LSAS, STAI-T – were assessed by means of one-way ANOVAs. Differences with respect to sex were analyzed via Chi square (χ^2) tests. Possibly confounding gene-environment correlations (rGE) were evaluated by Pearson's correlations.

MAIN ANALYSIS

The influence of *5-HTT* and *NPSR1* genotype, respectively, CTQ, and GSE, as well as their interactions on anxiety phenotypes ACQ, LSAS, and STAI-T was tested via hierarchical multiple regression analyses.

According to recommendations by Kraemer and Blasey (2004), grouped genotype variables were centered in order to minimize statistical interference errors. *5-HTT* genotypes were thus coded as .5 (L_AL_A) and -.5 (SS, SL_G, SL_A, L_GL_A, L_GL_G). In the same vein, *NPSR1* genotype was coded as .5 (AA) and -.5 (AT, TT). CTQ and GSE sum scores were centered (mean=0, SD=1). The variance inflation factor (VIF)

was assessed as a measure of multicollinearity. A VIF equal to one indicates no collinearity, whereas values greater than 10 indicate high correlations between predictor variables and are thus cause for concern (Belsley et al., 1980). Independence of errors was tested by means of the Durbin-Watson test statistic, with values close to two indicating that the residuals are uncorrelated, whereas values greater than three or less than one are considered to be problematic (Field, 2013).

Regression analyses were performed in three steps. In the first step, main effects were entered into the model, i.e. grouped genotype, centered CTQ sum score, and centered GSE sum score. In a second step, all two-way interaction terms – genotype x CTQ, genotype x GSE, and CTQ x GSE – were included, and lastly, in a third step, the three-way interaction term comprising genotype x CTQ x GSE was added.

Participants with psychometric scores ≥3 SD (Osborne and Overbay, 2004) were identified as outliers separately for each questionnaire and excluded from analysis of the respective psychometric measure. The False Discovery Rate (FDR) (Benjamini and Hochberg, 1995) was applied as a correction for multiple testing. However, since all tests remained significant at an alpha level of $p<.05$, uncorrected p-values are reported in the following for clarity.

EXPLORATORY ANALYSIS OF SEX DIFFERENCES

Although the sample was not pre-stratified for sex and the distribution was skewed, with females representing a larger portion of the sample than males, and the addition of a fourth main factor and the resulting interaction terms leading to a considerable decrease in statistical power, regression analyses for *5-HTT* and *NPSR1* were repeated to include sex as an additional factor to account for potentially confounding sex-specific effects (cf. Domschke et al., 2011). All potential interactions terms with sex were created, resulting in a fourth step containing the four-way interaction term of grouped genotype x CTQ x GSE x sex. Sex was coded as .5 for

females and -.5 for males. Due to their exploratory nature, no correction for multiple testing was applied for the analyses including sex.

EXPLORATORY ANALYSIS OF TRAUMA SUBTYPES

In order to test for GxExC effects of specific types of trauma as assessed via the five CTQ subscales, the main analyses were repeated with centered sum scores of each of the subscales (physical abuse, emotional abuse, sexual abuse, physical neglect, emotional neglect) in place of the CTQ overall sum score. For the same reason as stated above, no correction for multiple testing was applied, and sex was not included as an additional variable.

2.3.2 STUDY 2: GENETIC DETERMINANTS OF ACUTE STRESS REACTIVITY

DESCRIPTIVE STATISTICS

As described above, *5-HTTLPR*/rs25531 were grouped into L_AL_A versus SS, SL_G, SL_A, L_GL_A and L_GL_G genotypes, and *NPSR1* genotype was grouped as described above into AA versus combined AT/TT genotypes. Differences between the respective genotype groups and sex, oral contraceptive users (users vs. non-users), menstrual cycle phase (luteal phase vs. follicular phase vs. ovulation), and smoking status (smokers vs. non-smokers) were evaluated by means of χ^2 tests, as well as via one-way ANOVAs for age and counting errors.

Since cortisol reactivity may be influenced by several factors including sex, age, smoking status, or intake of oral contraceptives (Kudielka et al., 2009), associations between cortisol reactivity at single time points (t_0-t_6) and sex, oral contraceptive use (users vs. non-users), menstrual cycle phase (luteal phase vs. follicular phase vs. ovulation phase), and smoking status (smokers vs. non-smokers) were tested via one-

way ANOVAs. Associations between cortisol reactivity and age were tested via bivariate correlation analysis. In the overall sample, cortisol levels were associated with sex at t_1-t_3 (males>females; p=.006–.022) and intake of oral contraceptives at t_0-t_5 (no>yes; p≤.001–.010) and thus entered as covariates in all subsequent analyses. No associations were found between cortisol reactivity and menstrual cycle phase, smoking status, or age (all ps>.05).

MAIN ANALYSIS

Time course of cortisol reactivity was evaluated by means of ANCOVA with repeated-measures, with measurement time points (t_0-t_6) entered as within-subject factor, *5-HTT* and *NPSR1* genotype groups as between-subject factor, respectively, and sex and oral contraceptive intake as covariates. For analysis of subjective stress ratings, measurements at three time points were entered as within-subject factors, with genotype-groups as between-subject factor. Greenhouse-Geisser (GG-ε) corrections were applied where indicated, although, for clarity, uncorrected degrees of freedom are reported.

Concerning the overall sample, subjective ratings were unavailable for N=2 participants. No sufficient amount of saliva could be extracted from one sample for N=1 participant. *NPSR1* genotype information was missing for N=3 participants; similarly, no *5-HTT* genotype was available for N=5 participants. Therefore, all analyses reported in the following concerning salivary time course are restricted to a total sample size of N=100, and with respect to subjective measurements to N=98 participants for the analysis of *NPSR1*. For *5-HTT*, analysis of cortisol measurements is restricted to N=98 and for evaluation of subjective stress ratings to N=97 participants.

EXPLORATORY ANALYSIS OF GENERAL SELF-EFFICACY ON CORTISOL RESPONSES AND GENOTYPE EFFECTS

Given the results obtained from study 1 on the buffering effect of GSE on the interplay of genetic risk variants and distal stress experiences (CTQ), in an exploratory approach the potentially moderating role of GSE on cortisol stress reactivity was analyzed as an extension of the main analyses of *5-HTT* and *NPSR1* genotype effects, respectively, on the acute stress response. Therefore, the main analyses as described above were repeated to include GSE (see 2.1.3) as an additional between-factor, dichotomized by median split into a high (scoring on level of the median or above) and low (below the median) GSE group. For the whole sample, the median GSE score was 30. Sex and use of oral contraceptives were entered as covariates as described above.

3. RESULTS

3.1 STUDY 1: GENE-ENVIRONMENT-COPING INTERACTIONS

3.1.1 DESCRIPTIVE STATISTICS

Descriptive characteristics of the whole sample (N=695) and respective *5-HTT* and *NPSR1* genotype groups are given in table 3.1.

Table 3.1. Sample characteristics of study 1

		Overall sample	*5-HTTLPR*/rs25531		*NPSR1* rs324981	
			L_AL_A	$SS/SL_G/SL_A/L_GL_A/L_GL_G$	AA	TT/AT
N		695	197	481	200	495
Sex	(f:m)	427:268	117:80	301:180	122:78	305:190
Age	M	25.12	25.09	25.05	25.51	24.97
	(SD)	(5.28)	(4.86)	(5.40)	(6.00)	(4.95)
CTQ	M	43.48	42.99	43.64	43.48	43.49
	(SD)	(6.11)	(5.60)	(6.32)	(6.07)	(6.14)
GSE	M	29.78	30.16	29.59	30.16	29.62
	(SD)	(3.68)	(3.67)	(3.72)	(3.51)	(3.73)
ACQ	M	1.33	1.31	1.33	1.32	1.33
	(SD)	(0.23)	(0.22)	(0.23)	(0.21)	(0.24)
LSAS	M	20.86	19.88	21.26	21.27	20.70
	(SD)	(14.86)	(15.43)	(14.80)	(15.01)	(14.81)
STAI-T	M	34.58	34.34	34.72	34.59	34.57
	(SD)	(8.08)	(8.58)	(7.92)	(7.70)	(8.24)

f=female, m=male, M=mean, SD=standard deviation, CTQ=Childhood Trauma Questionnaire, GSE=General Self-Efficacy Scale, ACQ=Agoraphobic Cognitions Questionnaire, LSAS=Liebowitz Social Anxiety Scale, STAI-T=Trait Scale of the State-Trait Anxiety Inventory

No differences were observed between *5-HTT* genotype groups regarding age ($F(1,677)=.01$, $p=.929$), sex ($\chi^2(1)=.60$, $p=.438$), CTQ ($F(1,677)=1.59$, $p=.207$), GSE ($F(1,677)=3.31$, $p=.070$), ACQ ($F(1,677)=.2.09$, $p=.149$), STAI-T ($F(1,693)=.305$, $p=.581$), or LSAS ($F(1,693)=1.18$, $p=.279$). Likewise, *NPSR1* genotype groups did not differ with regard to age ($F(1,694)=1.52$, $p=.219$), sex ($\chi^2(1)=.02$, $p=.880$), CTQ

(F(1,694)<.001, p=.989), GSE (F(1,694)=3.00, p=.084), ACQ (F(1,694)=.67, p=.412), STAI-T (F(1,694)<.001, p=.984), or LSAS (F(1,694)=.21, p=.651). No significant rGEs between the respective genotypes with either of the environmental predictors were observed (all ps≥.07).

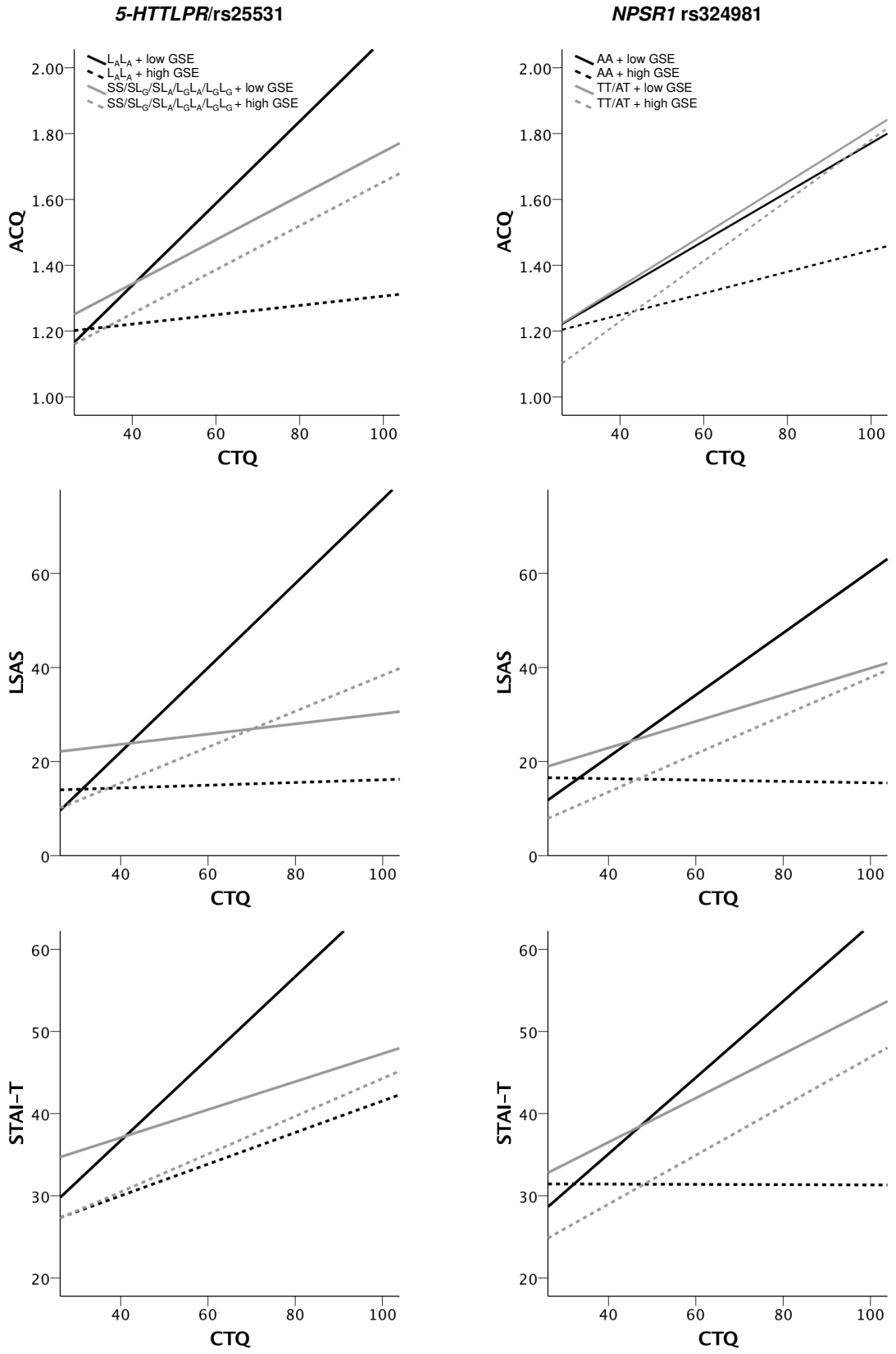
After exclusion of outliers regarding psychometric scores applying the criteria described above, and taking into account minimal discrepancies in total N for *5-HTT* and *NPSR1* due to unavailability of the respective genotype information for a fraction of the whole sample, interaction analysis for *5-HTT* genotype, CTQ and GSE were restricted to N=669 participants for ACQ and LSAS, and to N=674 for STAI-T. For analysis of *NPSR1* interaction effects, outlier exclusion resulted in adjusted sample sizes of N=686 for ACQ and LSAS, and N=691 for STAI-T.

3.1.2 MAIN ANALYSES

The relationships between *5-HTTLPR*/rs25531 genotype and *NPSR1* rs324981, respectively, CTQ, GSE and outcome variables (ACQ, LSAS, and STAI-T) are depicted in figure 3.1.

The VIF statistic as a test of possible inter-correlations between the predictor variables yielded values between 1.004 and 1.625 across all models, thus indicating no significant multicollinearity between any of the predictor variables. The assumption of independence of errors was tested by means of the Durbin-Watson statistic, which returned values between 1.595 and 2.109, indicating no first-order autocorrelations.

Figure 3.1. Effect of childhood trauma on anxiety scores as a function of 5-*HTT* and *NPSR1* genotypes and general self-efficacy (GSE)



CTQ=Childhood Trauma Questionnaire, GSE=General Self-Efficacy Scale, ACQ=Agoraphobic Cognitions Questionnaire, LSAS=Liebowitz Social Anxiety Scale, STAI-T=Trait Scale of the State-Trait Anxiety Inventory

5-HTT X E X C

ACQ. Significant main effects of CTQ (β=.184, t=5.130, p<.001) and GSE (β=−.322, t=−8.948, p<.001) were observed in step 1. Step 2 yielded significant main effects of CTQ (β=.209, t=4.918, p<.001) and GSE (β=−.370, t=−9.223, p<.001), and a significant interaction of CTQ × GSE (β=.079, t=2.031, p=.043), accounting for a significant increase in explained variance (R^2=.157, ΔR^2=.012, ΔF=3.174, p=.024). In step 3, significant main effects of CTQ (β=.216, t=5.079, p<.001) and GSE (β=−.372, t=−9.302, p<.001), as well as a significant interaction of *5-HTT*× CTQ × GSE (β=−.135, t=−2.191, p=.029) were observed. The addition of the three-way interaction term in step 3 accounted for a significant increment in explained ACQ variance (R^2=.170, ΔR^2=.006, ΔF=4.801, p=.029).

LSAS. Significant main effects of CTQ (β=.105, t=2.931, p=.003) and GSE (β=−.378, t=−10.576, p<.001) emerged in step 1. In step 2, significant main effects of CTQ (β=.158, t=3.776, p<0.001) and GSE (β=−.415, t=−10.490, p<.001) were observed as well as a significant interaction of CTQ × GSE (β=.087, t=2.268, p=.024), explaining a significant portion of LSAS variance (R^2=.176, ΔR^2=.015, ΔF=4.060, p=.007). Step 3 returned significant main effects of CTQ (β=.170, t=4.080, p<.001) and GSE (β=−.421, t=−10.717, p<.001) and a significant interaction term of *5-HTT*× CTQ × GSE (β=−.210, t = −3.437, p=.001). The addition of the three-way interaction term in step 3 accounted for a significant increment in explained LSAS variance (R^2=.191, ΔR^2=.014, ΔF=11.814, p=.001).

STAI-T. CTQ (β=.153, t=4.754, p<.001) and GSE (β=−.528, t=−16.426, p<.001) main effects reached significance in step 1. In step 2, significant main effects of CTQ (β=.213, t=5.696, p<.001) and GSE (β=−.564, t=−15.909, p<.001) emerged as well as a significant interaction of CTQ × GSE (β=.121, t=3.523, p<.001), explaining a significant portion of STAI-T variance (R^2=.336, ΔR^2=.020, ΔF=6.766, p=.006). Step 3 yielded significant main effects of CTQ (β=.222, t=5.950, p<.001) and GSE (β=−.569,

t=−16.136, p<.001) in addition to a significant interaction term of *5-HTT* × CTQ × GSE (β=-.165, t=−3.051, p=.002). The addition of the three-way interaction term in step 3 accounted for a significant increment in explained variance (R^2=.345, ΔR^2=.009, ΔF=9.308, p<.001).

NPSR1 X E X C

ACQ. Step 1 of the analysis yielded significant main effects of CTQ (β=.188, t=5.305, p<.001) and GSE (β=−.320, t=−8.984, p<.001), as did step 2 (CTQ: β=.190, t=4.823, p<.001; GSE: β=-.325, t=-8.032, p<.001) in addition to a significant interaction of CTQ x GSE (β=.122, t=2.967, p=.003) and a marginally significant interaction of *NPSR1* x CTQ (β=-.076, t=-1.772, p=.077), resulting in an overall increase in ACQ variance (R^2=.155, ΔR^2=.012, ΔF=3.204, p=.023). However, inclusion of the three-way interaction term of *NPSR1* x CTQ x GSE did not result in an additional change in explained variance (R^2=.163, ΔR^2<.001, ΔF=.280, p=.597).

LSAS. Significant main effects of CTQ (β=.108, t=3.060, p=.002) and GSE (β=−.380, t=−10.786, p<.001) were observed in step 1. Inclusion of the two-way interaction terms in step 2 led to a significant increase of LSAS variance (R^2=.174, ΔR^2=.011, ΔF=2.971, p=.031), with significant main effects of CTQ (β=.125, t=3.197, p=.001) and GSE (β=−.389, t=−9.692, p<.001) and a significant CTQ x GSE interaction term (β=.118, t=2.907, p=.004). Step 3 returned significant main effects of CTQ (β=.172, t=3.984, p<.001) and GSE (β=−.375, t=−9.288, p<.001), and a significant three-way interaction of *NPSR1* x CTQ x GSE (β=-.127, t=-2.527, p=.012). Addition of the three-way interaction term explained a significant portion in LSAS variance (R^2=.182, ΔR^2=.008, ΔF=6.386, p=.012).

STAI-T. Main effects of CTQ (β=.163, t=5.175, p<.001) as well as GSE (β=-.529, t=-16.646, p<.001) reached significance in step 1. A significant increase in explained variance was observed in step 2 (R^2=.338, ΔR^2=.014, ΔF=6.112, p=.001), with

significant main effects of CTQ (β=.180, t=5.155, p<.001) and GSE (β=-.534, t=-14.660, p<.001), and a significant CTQ x GSE interaction (β=.153, t=4.218, p<.001). Step 3 again returned significant main effects of CTQ (β=.252, t=6.427, p<.001) and GSE (β=-.525, t=-14.555, p<.001), and a significant interaction of *NPSR1* x CTQ x GSE (β=-.185, t=-3.877, p<.001). The addition of the three-way interaction term in step 3 accounted for a significant increment in explained variance (R^2=.353, ΔR^2=.014, ΔF=15.033, p<.001).

3.1.3 EXPLORATORY ANALYSIS OF SEX DIFFERENCES

For both *5-HTT* and *NPSR1*, the GxExC effects reported above (3.1.2) could be confirmed when sex was introduced as an additional component. Step 4 of the regression model, resulting from the inclusion of the four-way interaction term containing genotype, CTQ sum score, GSE sum score, and sex, did not reach significance for any of the outcome measures, for either genotypic model, as detailed below.

5-HTT X E X C AND SEX EFFECTS

ACQ. Inclusion of sex as an additional moderator in the interaction model did not lead to an increase in explained ACQ variance in the resulting fourth step of the model (R^2=.187, ΔR^2<.001, ΔF=.169, p=.681). Step 3, as described above, returned a significant three-way interaction of *5-HTT* x CTQ x GSE (β=-.135, t=-2.039, p=.042) and an overall increase in explained variance (R^2=.187, ΔR^2=.013, ΔF=2.604, p=.035).

LSAS. Likewise, the three-way interaction term in step 3 of the regression on LSAS sum score remained significant after the inclusion of sex (β=-.215, t=-3.231, p=.001; R^2=.197, ΔR^2=.019, ΔF=3.882, p=.004). No significant effect on explained

LSAS variance was observed regarding the four-way interaction term in step 4 (R^2=.199, ΔR^2=.002, ΔF=1.288, p=.257).

STAI-T. A G x E x C interaction with sex did not account for an increase in explained STAI-T variance (step 4: R^2=.359, ΔR^2=.001, ΔF=.733, p=.392). Analysis confirmed the above observed significance of step 3 (R^2=.358, ΔR^2=.022, ΔF=5.567, p<.001) and the contained three-way interaction of *5-HTT* x CTQ x GSE (β=-.124, t=-2.131, p=.033).

NPSR1 X E X C AND SEX EFFECTS

ACQ. For ACQ, neither step 3, containing the three-way interaction terms (R^2=.181, ΔR^2=.009, ΔF=1.910, p=.107), nor step 4, comprising the four-way interaction term including sex (R^2=.185, ΔR^2=.003, ΔF=2.580, p=.109), returned significant results.

LSAS. Inclusion of sex did not result in a significant increase in explained LSAS variance (R^2=.188, ΔR^2=.001, ΔF=.851, p=.356). The above reported significant increment in total LSAS variance after inclusion of the three-way interaction terms emerged with borderline significance (R^2=.187, ΔR^2=.011, ΔF=2.358, p=.052), confirming the significant interaction of NPSR1 x CTQ x GSE (β=-.118, t=-2.101, p=.036).

STAI-T. Sex did not moderate the relationship between *NPSR1*, CTQ, and GSE on STAI-T (R^2=.363, ΔR^2=.002, ΔF=2.191, p=.139). Step 3 confirmed the increase in explained STAI-T variance (R^2=.361, ΔR^2=.022, ΔF=5.739, p<.001) and significance of the three-way interaction term of *NPSR1* x CTQ x GSE (β=-.118, t=-2.095, p=.037).

3.1.4 EXPLORATORY ANALYSIS OF TRAUMA SUBTYPES

Descriptive statistics of the CTQ subscales and severity of childhood maltreatment (cf. Bernstein and Fink, 1998) of the whole sample are summarized in table 3.2.

Table 3.2. Descriptive statistics of Childhood Trauma Questionnaire subscales and severity of maltreatment

	M	SD	Severity of maltreatment N (%)			
			none/ minimal	low to moderate	moderate to severe	severe to extreme
CTQ subscale						
Emotional abuse	6.82	2.35	571 (82.6)	103 (14.8)	12 (1.7)	6 (0.9)
Physical abuse	5.30	1.15	673 (96.8)	13 (1.9)	7 (1.0)	2 (0.3)
Sexual abuse	5.29	1.33	621 (89.4)	54 (7.8)	16 (2.3)	4 (0.6)
Emotional neglect	8.03	3.22	510 (73.4)	144 (20.7)	28 (4.0)	13 (1.9)
Physical neglect	6.23	2.03	560 (80.6)	90 (12.9)	34 (4.9)	11 (1.6)

M=mean, SD=standard deviation, CTQ=Childhood Trauma Questionnaire

5-HTT X E X C AND TRAUMA SUBTYPES

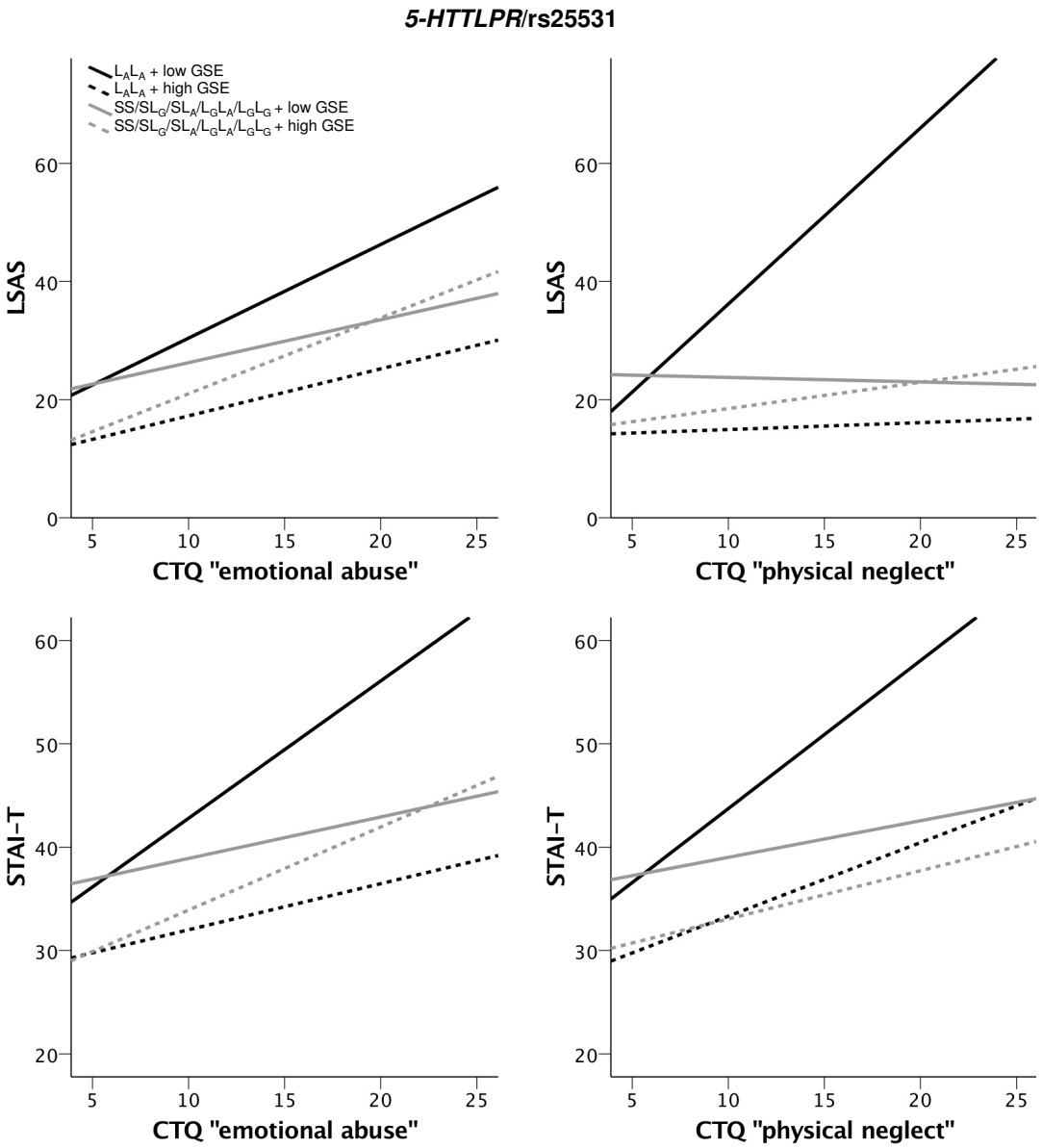
ACQ. No significant interaction terms of *5-HTT* x E x C on ACQ were observed for the subscales "emotional abuse" (R^2=.173, ΔR^2=.002, ΔF=1.989, p=.159), "physical abuse" (R^2=.136, ΔR^2=.001, ΔF=.688, p=.407), and "emotional neglect" (R^2=.148, ΔR^2=.001, ΔF=.781, p=.377). A trend could be observed for the three-way interaction term regarding "sexual abuse" (R^2=.141, ΔR^2=.004, ΔF=3.392, p=.066; β=-.134, t=-1.842, p=.066) and "physical neglect" (R^2=.135, ΔR^2=.005, ΔF=3.735, p=.054; β=-.121, t=-1.933, p=.054).

LSAS. Significant increases in explained LSAS variance after addition of the three-way interaction term could be observed with regard to the subscales "emotional abuse" (R^2=.194, ΔR^2=.008, ΔF=6.879, p=.009; β=-.117, t=-2.623, p=.009) and "physical neglect" (R^2=.187, ΔR^2=.020, ΔF=16.397, p<.001; β=-.252, t=-4.049, p<.001), but not "physical abuse" (R^2=.169, ΔR^2=.001, ΔF=1.188, p=.276), "sexual abuse" (R^2=.161, ΔR^2=.001, ΔF=.869, p=.352), or "emotional neglect" (R^2=.181, ΔR^2=.002, ΔF=1.472, p=.226).

STAI-T. Inclusion of the *5-HTT* x E x C interaction term in step 3 yielded significant increases in explained variance for the subscales "emotional abuse" (R^2=.355, ΔR^2=.004, ΔF=4.177, p=.041; β=-.148, t=-3.716, p<.001), "physical neglect" (R^2=.327, ΔR^2=.013, ΔF=13.806, p<.001; β=-.110, t=-2.044, p=.041), and on a trend level, for sexual abuse (R^2=.302, ΔR^2=.003, ΔF=3.133, p=.077; β=-.115, t=-1.770, p=.077). No significant results were obtained for "physical abuse" (R^2=.315, ΔR^2<.001, ΔF=.238, p=.626) and "emotional neglect" (R^2=.358, ΔR^2=.001, ΔF=1.307, p=.253).

The relationship between the CTQ subscales "emotional abuse" and "physical neglect", *5-HTT* variation, and GSE on LSAS and STAI-T is depicted in figure 3.2.

Figure 3.2. Effect of CTQ subscales on anxiety scores as a function of *5-HTT* genotype and general self-efficacy (GSE)



CTQ=Childhood Trauma Questionnaire, GSE=General Self-Efficacy Scale, ACQ=Agoraphobic Cognitions Questionnaire, LSAS=Liebowitz Social Anxiety Scale, STAI-T=Trait Scale of the State-Trait Anxiety Inventory

NPSR1 X E X C AND TRAUMA SUBTYPES

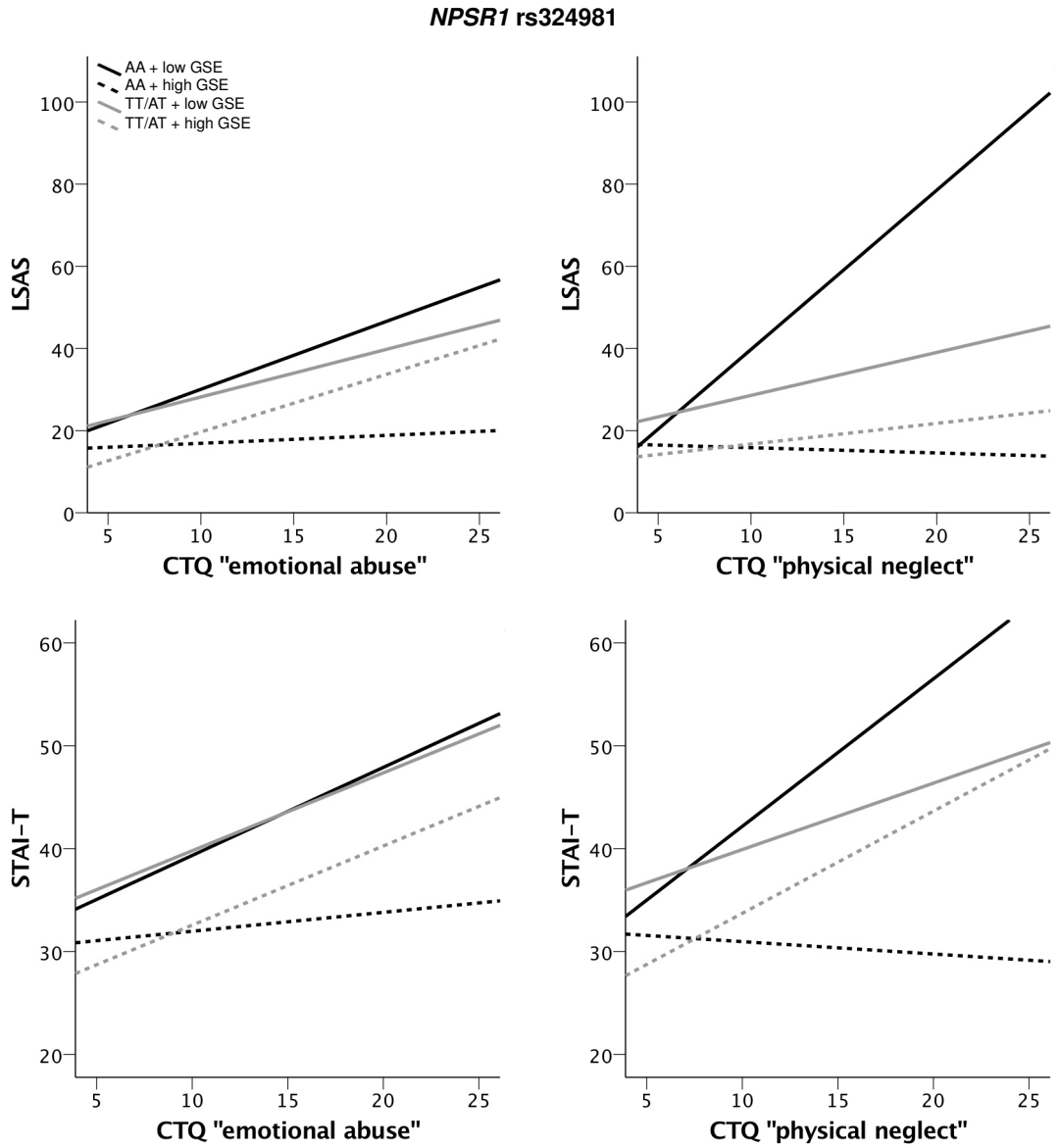
ACQ. Similar to the main analysis reported above, no three-way interaction effects emerged regarding the subscales "emotional abuse" (R^2=.161, ΔR^2<.001, ΔF=.002, p=.964), "physical abuse" (R^2=.129, ΔR^2=.001, ΔF=.772, p=.380), "sexual abuse" (R^2=.126, ΔR^2=.003, ΔF=2.169, p=.141), "emotional neglect" (R^2=.143, ΔR^2<.001, ΔF=.266, p=.606), or "physical neglect" (R^2=.133, ΔR^2<.001, ΔF=.053, p=.818).

LSAS. A significant increase in explained LSAS variance was observed for the subscale "physical neglect" after addition of the three-way interaction term (R^2=.167, ΔR^2=.008, ΔF=6.500, p=.011; β=-.112, t=-2.549, p=.011). No effects emerged regarding the subscales "emotional abuse" (R^2=.188, ΔR^2=.002, ΔF=1.965, p=.161), "physical abuse" (R^2=.163, ΔR^2<.001, ΔF=.146, p=.703), "sexual abuse" (R^2=.159, ΔR^2<.001, ΔF=.172, p=.679), and "emotional neglect" (R^2=.180, ΔR^2<.001, ΔF=.044, p=.834).

STAI-T. Inclusion of the *NPSR1* x E x C interaction term led to significant increases in STAI-T variance for "emotional abuse" (R^2=.350, ΔR^2=.006, ΔF=6.648, p=.010; β=-.105, t=2.578, p=.010) and "physical neglect" (R^2=.337, ΔR^2=.015, ΔF=15.373, p<.001; β=-.160, t=-3.921, p<.001), on a trend level for "emotional neglect" (R^2=.366, ΔR^2=.003, ΔF=3.243, p=.072; β=-.065, t=-1.801, p=.072), but not "physical abuse" (R^2=.314, ΔR^2<.001, ΔF=.155, p=.694), or "sexual abuse" (R^2=.296, ΔR^2<.001, ΔF=.120, p=.729).

The relationships between *NPSR1* genotype, GSE, and the CTQ subscales "emotional abuse" and "physical neglect", respectively, on LSAS and STAI-T are depicted in figure 3.3.

Figure 3.3. Effect of CTQ subscales on anxiety scores as a function of *NPSR1* genotype and general self-efficacy (GSE)



CTQ=Childhood Trauma Questionnaire, GSE=General Self-Efficacy Scale, ACQ=Agoraphobic Cognitions Questionnaire, LSAS=Liebowitz Social Anxiety Scale, STAI-T=Trait Scale of the State-Trait Anxiety Inventory

3.2 STUDY 2: GENETIC DETERMINANTS OF ACUTE STRESS REACTIVITY

3.2.1 DESCRIPTIVE STATISTICS

Descriptive statistics are summarized in table 3.3. For *5-HTT* genotype groups, no differences were observed for sex ($\chi^2(1)=1.244$, p=.265), age ($F(1,98)=.04$, p=.836), or, in female participants, for intake of oral contraceptives ($\chi^2(1)=3.84$, p=.05) and menstrual cycle phase ($\chi^2(1)=1.065$, p=.302). *NPSR1* genotype groups did not differ with regard to sex ($\chi^2(1)=2.02$, p=.156). In female participants, no genotype group differences emerged with regard to contraceptive use ($\chi^2(1)=.07$, p=.792), or menstrual cycle phase ($\chi^2(1)=1.51$, p=.220). A statistically significant difference in age was observed ($F(1,100)=4.34$, p=.040), with AA genotype carriers being slightly older (mean age±SD=30.93±8.12 years) relative to T allele carriers (mean age±SD=27.47±7.31 years), which might constitute a confounding factor. However, since there was no relationship between age and any of the outcome measures, this seems unlikely. Specifically, age was not associated with cortisol levels (all ps≥.533) or subjective stress levels (all ps≥.546) at any time point.

Table 3.3. Sample characteristics of study 2

		Overall sample	*5-HTTLPR/* rs25531		*NPSR1* rs324981	
			L_AL_A	SS/SL_G/ SL_A/L_GL_A /L_GL_G	AA	TT/AT
N		104	31	68	29	72
Sex	f:m	62:42	21:10	38:30	20:9	39:33
Age	M (SD)	28.34 (7.61)	28.10 (7.81)	28.44 (7.74)	30.93 (8.12)	27.47 (7.31)
Smoking status	yes:no	17:27	5:26	12:56	3:26	13:59
Intake of contraceptives[1]	yes:no	37:25	16:5	19:19	12:8	22:17
Menstrual cycle phase[1,2]	luteal:follicular: ovulation	40:16:0	12:7:0	26:8:0	11:7:0	27:8:0
Counting errors[3]	M (SD)	3.71 (2.25)	3.64 (2.15)	3.75 (2.38)	3.07 (2.27)	3.99 (2.25)

f=female, m=male, M=mean, SD=standard deviation, [1]female participants only, [2]information about menstrual cycle phase was unavailable for N=6 women, [3]number of counting errors averaged across all mental arithmetic trials of the MAST

3.2.2 EFFECTS OF GENOTYPE ON STRESS REACTIVITY

The time course of salivary cortisol depending on *5-HTT* and *NPSR1* genotypes, respectively, is depicted in figure 3.4. Subjective stress ratings separated by genotype at t_0, t_1, and t_6 are summarized graphically in figure 3.5.

5-HTT AND STRESS REACTIVITY

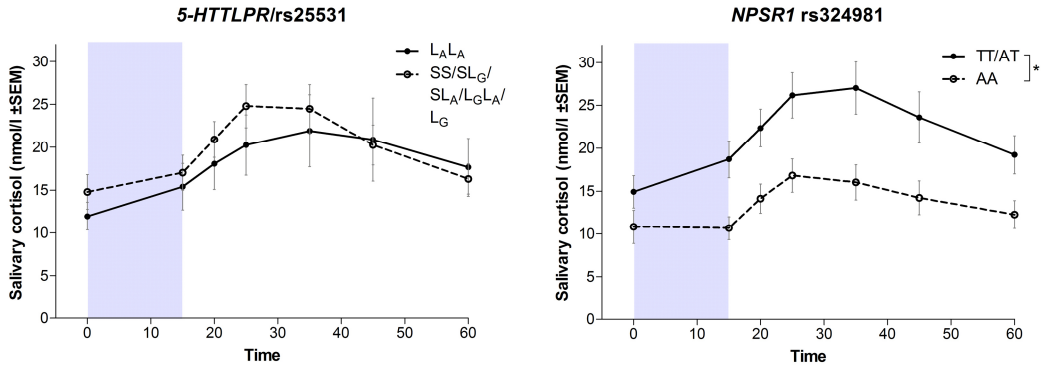
Analysis of the effect of *5-HTT* genotype on cortisol reactivity revealed a significant main effect of time ($F(6,564)=5.63$, GG-$\varepsilon=.42$, $p<.001$), but no time x genotype interaction ($F(6,564)=.709$, GG-$\varepsilon=.42$, $p=.524$) or main effect of genotype ($F(1,94)=.16$, $p=.688$) could be observed. The same pattern was true for stress ratings (main effect time: $F(2,190)=163.02$, GG-$\varepsilon=.81$, $p<.001$), while again, no interaction effect ($F(2,190)=1.36$, GG-$\varepsilon=.81$, $p=.259$) or main effect of genotype were discerned

(F(1,95)=.01, p=.925). No differences with regard to counting errors could be observed (F(1,98)=.04, p=.835).

NPSR1 AND STRESS REACTIVITY

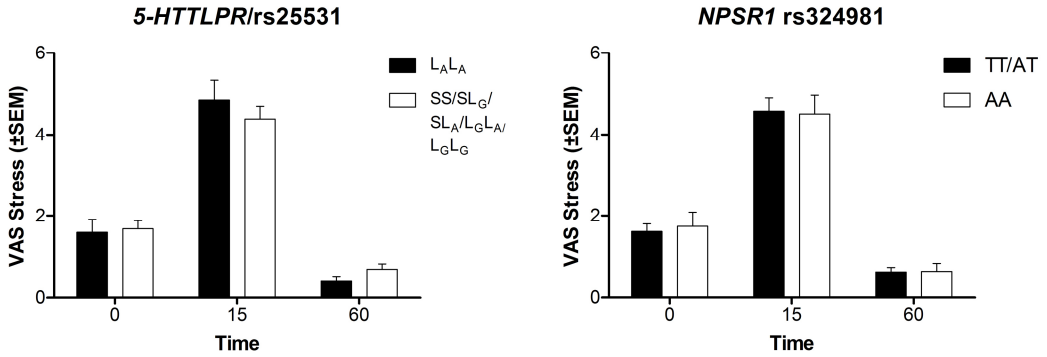
For *NPSR1*, there was a significant main effect of time (F(6,576)=6.01, GG-ε=.39, p=.002), indicating significant increases in salivary cortisol in reaction to the stress manipulation. While no significant time x genotype interaction was observed (F(6,576)=.91, GG-ε=.39, p=.417), however, a significant main effect of genotype emerged (F(1,96)=4.37, p=.039), with higher salivary cortisol levels in T allele carriers compared to carriers of the AA genotype. While subjective ratings of perceived stress followed the expected time course of increasing from t_0 to t_1, and decreasing from t_1 to t_2 (main effect time: F(2,194)=135.77, GG-ε=.79, p<.001), no interaction with genotype (F(2,194)=.08, GG-ε=.79, p=.885) or overall difference between genotype groups (all Fs(1,97)=.01, p=.938) could be observed. Interestingly, there was a trend towards more counting errors in *NPSR1* T-allele carriers (F(1,99)=3.374, p=.069).

Figure 3.4. Genotype effects on time course of salivary cortisol levels in the context of the Maastricht Acute Stress Test (MAST)



SEM = standard error mean. Time is given in minutes. Shaded area represents the duration of the MAST.
* p<.05

Figure 3.5. Genotype effects on subjective stress ratings in the context of the Maastricht Acute Stress Test (MAST)



VAS=visual analogue scale. SEM=standard error mean. Time is given in minutes.

3.2.3 EXPLORATORY ANALYSIS OF GENERAL SELF-EFFICACY ON CORTISOL RESPONSES AND GENOTYPE EFFECTS

5-HTT, GSE AND CORTISOL RESPONSES

Neither a significant main effect of GSE group (F(1,92)=1.01, p=.319) nor an interaction effect of GSE group and *5-HTT* genotype (F(1,92)=2.23, p=.139) were observed on salivary cortisol levels overall. Regarding the time course of cortisol responses, no differences between GSE groups were obtained, neither independently of *5-HTT* genotype (time x GSE: F(6,552)=.75, GG-ε=.42, p=.50) nor interactively (time x GSE x genotype: F(6,552)=1.31, GG-ε=.42, p=.272).

NPSR1, GSE AND CORTISOL RESPONSES

Again, no main effect of GSE (F(1,94)=1.16, p=.284) or interaction of GSE x *NPSR1* genotype (F(1,94)=.89, p=.348) on salivary cortisol were observed, and no two-way interaction of time x GSE (F(6,564)=.57, GG-ε=.39, p=.591) or three-way interaction of time x GSE x genotype (F(6,564)=.78, GG-ε=.39, p=.478) on the time course of salivary cortisol responses could be discerned.

4. DISCUSSION

Study 1 observed a moderating effect of general self-efficacy (GSE) in interaction with childhood maltreatment and *5-HTT* and *NPSR1*, respectively, in an extended GxExC model of anxiety risk. In study 2, a modulation of HPA axis function, considered to be an endophenotype for stress-related mental disorders, by *NPSR1* gene variation could be discerned. Results from both studies as well as their implications are discussed separately below, and finally within a converging framework comprising genetics, environmental adversity, and coping-related resources, as well as potential intermediate mechanistic links.

4.1 STUDY 1: GENE-ENVIRONMENT-COPING INTERACTIONS

In accordance with the "diathesis-stress" model, variants in both the *5-HTT* and *NPSR1* genes have been observed to interact with environmental factors in the moderation of vulnerability to anxiety and anxiety disorders. On the heels of a landmark study by Caspi et al. (2003) on its interaction with childhood maltreatment on depression, the *5-HTTLPR* variant has been a central focus in GxE research, although results have been mixed (cf. Munafò et al., 2009). With regard to anxiety, both the S (Choe et al., 2013; Gunthert et al., 2007; Stein et al., 2008) and the L allele (Klauke et al., 2011; Laucht et al., 2009; Reinelt et al., 2014) have been implicated, or no interactive effect with environmental factors in either direction has been discerned (Blaya et al., 2010; Cividanes et al., 2014; Zavos et al., 2012). Interest in the involvement of the NPS system in the moderation of anxiety risk has only recently emerged, and while first findings support its involvement in a GxE manner, results have differed regarding the allelic direction of this interaction (Klauke et al., 2014; Laas et al., 2014a). While GxE models constitute a crucial step in the disentanglement of putative risk factors, they are most likely subject to concurrent moderating influences that may increase or reduce disease risk. Therefore, one aim of this thesis

was to expand for the first time existing GxE models by an additional dimension, i.e. coping ("C") with adversity, in a GxExC approach of anxiety risk. To this end, the interactive effect of *5-HTT* and *NPSR1* variation, respectively, with history of childhood trauma, and the potentially buffering effect of general self-efficacy (GSE) on dimensional anxiety phenotypes, was examined in a large sample of healthy volunteers.

Results demonstrated a moderating influence of GSE on the deleterious effects of childhood maltreatment in a genotype-dependent fashion regarding a range of anxiety traits, including agoraphobic cognitions, social anxiety, and trait anxiety. Specifically, in carriers of the more active *5-HTTLPR*/rs25531 L_AL_A genotype with a history of childhood maltreatment and characterized by low GSE, the highest scores were observed on all considered measures of anxiety. However, this pattern was reversed when GSE was high, with the lowest anxiety scores observed in L_AL_A carriers despite the experience of childhood adversity. A similar effect was obtained for *NPSR1* rs324981, with high GSE buffering social and trait anxiety in AA homozygotes despite a history of maltreatment, whereas low GSE led to increased anxiety scores in individuals with an otherwise equal genetic and environmental risk constellation. Moreover, concerning specific types of childhood trauma, results suggest a particular role of both physical neglect and emotional abuse.

5-HTT X E X C

The findings regarding the interactive relationship of *5-HTTLPR*/rs25531 with childhood trauma and GSE are in line with GxE literature reporting the high-expressing L allele to confer vulnerability to anxiety and anxiety disorders (Klauke et al., 2011; Laucht et al., 2009; Reinelt et al., 2014), with association studies linking the L allele to panic disorder (Maron et al., 2005a) and social anxiety disorder (Reinelt et al., 2013) as categorical disease entities *per se*, impaired psychotherapy response in

patients with panic disorder and agoraphobia (Knuts et al. 2014), neuroimaging-based intermediate phenotypes such as heightened amygdala responsiveness in anxiety disorder patients (Lau et al., 2009; Oathes et al., 2015), as well as CCK-4-induced panic attacks (Maron et al., 2004) and increased anxiety responses to CO_2 in healthy volunteers (Schmidt et al., 2000; Schruers et al., 2011). Importantly, they do not contradict findings linking the S allele to increased anxiety traits and psychopathology (Choe et al., 2013; Gunthert et al., 2007; Stein et al., 2008): while differential GSE modulated anxiety levels in L_AL_A carriers in either an anxiety-buffering or enhancing manner, the detrimental effect of childhood trauma in the presence of at least one S or L_G allele was largely unaffected by GSE, resulting in heightened anxiety as a function of CTQ. Rather, the obtained results may aid in the reconciliation of incongruent findings regarding the allelic direction of association across a wealth of studies investigating the relationship between *5-HTT* gene variation and environmental factors in the moderation of anxiety-related traits and manifest anxiety disorders (Choe et al., 2013; Gunthert et al., 2007; Klauke et al., 2011; Laucht et al., 2009; Reinelt et al., 2014) by highlighting the importance of considering favorable next to adverse conditions. A similar interactive effect has previously been observed with regard to childhood depression, where availability of social support buffered depression risk in maltreated children as a function of *5-HTTLPR* genotype (Kaufman et al., 2004), arguing for a resilience-increasing impact of beneficial conditions on an otherwise vulnerable GxE risk profile.

NPSR1 X E X C

In a similar vein, the results on *NPSR1* rs324981 genotype, childhood trauma, and GSE interactively moderating anxiety traits broaden the current understanding of GxE interactions contributing to anxiety. While low or high GSE, respectively, increased or decreased trait and social anxiety in AA genotype carriers with a history

of maltreatment, increases in anxiety measures as a function of childhood maltreatment were observed in T allele carriers largely unaffected by GSE. Consequently, the present results do not contradict but rather correspond to the existing GxE literature linking the T allele to increased anxiety sensitivity (Klauke et al., 2014) or heightened amygdala activation (Streit et al., 2014) constituting endophenotypes of categorical anxiety via environmental variation. They additionally corroborate previous findings of the T allele to mediate panic disorder as a nosological entity (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007), psychophysiological and neurophysiological responding (Beste et al., 2013; Domschke et al., 2012a, 2011), altered neuronal activation patterns (Dannlowski et al., 2011; Domschke et al., 2011; Guhn et al., 2015; Neufang et al., 2015; Raczka et al., 2010), or neuroendocrine stress responsiveness (Kumsta et al., 2013), whereas the AA genotype was generally not found to predispose to panic disorder risk *per se*, or to influence anxiety traits on an intermediate level (however, see Laas et al., 2014a). The present findings corroborate the view that the T allele, particularly in combination with adversity, leads to increased anxiety irrespective of the influence of GSE, while the AA genotype does not – unless accompanied by low GSE, i.e. insufficient coping ability. This is important in light of the apparent discrepancy of the AA genotype having also been associated with a higher frequency of anxiety disorders observed in women reporting a negative family environment, and higher trait anxiety dependent upon the experience of past stressful life events (Laas et al., 2014a), in that coping characteristics might constitute an additional and previously unconsidered dimension able to buffer or, if maladaptive, further increase disease risk. It should be noted that, for *NPSR1*, GxExC interaction effects were restricted to social anxiety (LSAS) and trait anxiety (STAI-T), whereas with regard to ACQ, only a trend for a GxE effect was obtained, which – although visually suggested in the graphical representation of the interaction model in figure 3.1 – was not further moderated by GSE status (see annex, figure I.1). Descriptively, dependent on high CTQ scores,

ACQ scores were as high in T allele carriers as in AA homozygotes with low GSE. The ACQ addresses symptoms and cognitions that are related to arousal commonly occurring in panic disorder (e.g. autonomic hyperarousal, loss of control; Chambless et al., 1984). Given that a hyperfunction of the NPS system in animal models or as conferred by the *NPSR1* gain-of-function T allele in humans has been related to arousal (Reinscheid and Xu, 2005; Rizzi et al., 2008; Xu et al., 2004; Zhao et al., 2012), heightened sensitivity to arousal-related sensations or cognitions (Klauke et al., 2014), and panic disorder (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007), a relatively stronger genetic influence in this particular phenotype might have concealed subtle moderating effects by GSE.

SECONDARY ANALYSES OF TRAUMA SUBTYPES AND SEX

Exploratory analyses regarding different trauma subtypes yielded preliminary evidence for a distinct role of emotional abuse and physical neglect in interaction with GSE and genetic variation both in the *5-HTT* and *NPSR1* genes. This suggests that different kinds of trauma might not interact with a person's genetic makeup and coping ability in the same way, and that particular subtypes confer vulnerability to a greater degree than others. Accordingly, emotional abuse has previously been linked to the physical concerns domain of anxiety sensitivity in a GxE manner (Stein et al., 2008), and the influence of different traumatic occurrences during childhood has been addressed in the context of anxiety disorder risk, with some studies linking experiences of emotional abuse to increased risk for social anxiety disorder (Bishop et al., 2014; Kuo et al., 2011; Reinelt et al., 2013). Emotional abuse and physical neglect have furthermore been shown to influence cortisol responses to an acute stress situation (Carpenter et al., 2007).

Re-analyses of the GxExC interaction effects to include sex as an additional factor did not yield any evidence for a modulation of the interplay between genetic

variation, childhood trauma, and GSE in a sex-specific manner. This corresponds to the GxE effects of variants in *5-HTT* and childhood trauma (Klauke et al., 2011), and *NPSR1* and childhood trauma (Klauke et al., 2014) that were observed independently of sex.

BEYOND DIATHESIS-STRESS: "G" AS RISK VS PLASTICITY

The "diathesis-stress" framework proposes that due to inherent risk factors (e.g. genetic vulnerability conveyed by "risk" genes), these individuals are disproportionally more vulnerable to environmental stress, and, in turn, more likely to develop psychopathologies as a consequence. In this context, however, with the central focus being on the negative aspects of the environment, beneficial outcomes conferred by positive influences are often overlooked. Rather than the classical notion of "risk" genes, the present findings argue for a redefinition in line with the 'differential susceptibility hypothesis' (Belsky et al., 2009; Belsky and Pluess, 2009) in that genes do not confer risk for a disease *per se*, but are susceptible to environmental influences – both positive *and* negative – and hence towards a definition of "plasticity" rather than "risk" genes. That is, genes seem to drive differential sensitivity to environmental conditions as a whole, and depending on the nature of these environmental influences, their contribution can be beneficial or harmful and manifest on a phenotypic level (Schiele et al., 2016). Here, *5-HTTLPR*/rs25531 L_AL_A carriers and *NPSR1* rs324981 AA carriers, respectively, with a history of childhood maltreatment were characterized by higher anxiety traits when they were not equipped with sufficient coping abilities, but were able to counterbalance the detrimental impact of adversity in the presence of high self-efficacy. This illustrates that rather than representing "risk" genotypes, *5-HTT* L_AL_A and *NPSR1* AA genotypes appear to convey a greater sensitivity to both positive and negative environmental influences than those comprising at least one S/L_G or T allele,

respectively, and may thus constitute "plasticity" factors. Consequently, carriers of *5-HTT* SS, SL_G, SL_A, L_GL_A and L_GL_G and *NPSR1* TT/AT genotypes, respectively, were less susceptible to effects of the environment as reflected by a lack of modulation of the outcome variables by childhood trauma and the presence or absence of sufficient coping capabilities.

BEYOND ADVERSITY: "E" AND "C"

Across a wealth of studies, negative and positive poles of environmental circumstances have been addressed separately. Efforts have mostly been focused on the negative end of the spectrum, subsuming different concepts of adversity, although there is considerable variability in the definition, time of occurrence, and assessment of the environmental risk factors in question. Life events have been defined in a broader sense, e.g. cumulatively across the lifespan, or more narrowly, i.e. referring to specific types of trauma such as abuse, loss or separation experiences, stressful life events, war or combat experiences, or exposure to natural disasters. Moreover, environmental adversity has also been considered in light of a specific time frame, comprising recent life events (e.g. in the year prior to assessment), or more distant adverse experiences during childhood, adolescence, and adulthood. Consequently, GxE research and comparability between studies is further complicated by how environmental variables are conceptualized, and the instruments used to capture them, which, in turn, may further explain some of the discrepancies in the literature. For instance, in GxE models of anxiety regarding *5-HTT* variation, the environmental component has been defined as childhood maltreatment (Cividanes et al., 2014; Klauke et al., 2011; Stein et al., 2008), family adversity (Laucht et al., 2009), recent (past-year) stressful life events (Zavos et al., 2012), cumulative stressful life events across the lifespan (Choe et al., 2013), or daily stressors (Gunthert et al., 2007). Similarly, interactions of *NPSR1* with environmental aspects on anxiety-

related outcomes have been described for childhood maltreatment (Klauke et al., 2014), family adversity (Laas et al., 2014a), and urban upbringing (Streit et al., 2014). These examples illustrate the complexity of the environmental component in GxE models concerning the specificity, timing, and sequence of stressors: There are obvious qualitative differences in the definition of a stressor, e.g. major events such as experiences of severe abuse, or more minor stressors such as so-called daily hassles, underlining that while the overall term may be consistent across several studies, the underlying constructs are not. Furthermore, the timing of adversity may be crucial in the moderation of long-term effects (Nederhof and Schmidt, 2012). For instance, *5-HTTLPR* may be particularly sensitive to adversity occurring early in life but not at later stages (Caspi et al., 2010). Finally, the sequence of environmental conditions – positive *and* negative – can differentially modulate vulnerability to disease (Nederhof and Schmidt, 2012). Importantly, the absence of adverse experiences alone does not necessarily equate with advantageous environmental circumstances (Seery et al. 2010; Seery et al. 2013). Rather, definition of a positive environment beyond the mere absence of adversity by taking into account inter- and intrapersonal factors such as social support, coping strategies, or self-efficacious beliefs seems crucial. To date, such factors have sparsely been addressed in the literature with reference to GxE models though first results confirm a protective effect of beneficial conditions on anxiety risk (Reinelt et al., 2014). Consequently, there is a need for novel conceptual frameworks like the 'coping with challenge hypothesis' integrating developmental timing of beneficial and adverse events as well as their sequence in the context of GxE research (cf. Bodden et al., 2015). The present results constitute a first step towards reconciling a number of discrepancies in the literature by simultaneously focusing on traumatic experiences in the sensitive period of childhood, which impose an up to almost two- to fourfold increased risk for the development of anxiety disorders (Fernandes and Osório, 2015), and self-efficacy, which constitutes a promising coping-related concept able to cancel out the negative effects of stress (Schönfeld et

al., 2016), together with variation in two genes that have been shown to interact specifically with childhood adversity (Klauke et al., 2014, 2011; Stein et al., 2008).

LIMITATIONS

Some limitations should be taken into account in the interpretation of the present findings. While the results clearly highlight the necessity of including coping-related mechanisms as an additional dimension in GxE research, other factors are likely to contribute further to the complexity.

Haplotypic effects with other relevant polymorphisms within one gene, and epistatic effects, i.e. interactions between two or more genes representing a particular pathway or different neurotransmitter systems, should be taken into account, and, with regard to the *5-HTT* gene, have for example been shown to modulate panic disorder risk (Freitag et al., 2006; Strug et al., 2010). Gene-gene interactions have been reported in the context of GxE research, for instance between *5-HTTLPR* and the rs6265 (Val66Met) polymorphism of the brain derived neurotrophic factor (*BDNF*) gene, with the *BDNF* A allele decreasing depressiveness in *5-HTTLPR* SS genotype carriers in the presence of childhood abuse, and, in turn, *BDNF* GG genotype interacting with *5-HTTLPR* SS genotype and childhood abuse in a depression-enhancing manner (Grabe et al., 2012). Also, an interaction between *5-HTTLPR* and *NPSR1* has been found to modulate contextual fear conditioning, with increased startle potentiation being conferred by the simultaneous presence of at least one of each *5-HTTLPR* S and *NPSR1* T alleles (Glotzbach-Schoon et al., 2013).

In addition, given increasing evidence for the role of epigenetic processes in the modulation of gene function that furthermore constitute temporally dynamic mechanisms partly responsive to environmental influences in the context of anxiety disorders or anxiety-related phenotypes (Domschke et al., 2013, 2012b; Duman and

Canli, 2015; Kang et al., 2013; Tyrka et al., 2016; Ziegler et al., 2015), future GxExC models will benefit from the inclusion of epigenetic markers such as *5-HTT* DNA methylation (cf. Domschke et al., 2014; Roberts et al., 2014).

The assessed sample of healthy volunteers was relatively young with a mean age of 25.12 years, and was furthermore characterized by primarily high education status, which limits the generalizability of the present results to other populations. Therefore, future studies should include more heterogeneous samples of healthy volunteers, and – extending the proposed GxExC approach to a clinical context – patients with anxiety disorders. Moreover, longitudinal designs would provide promising insights into the developmental trajectories of anxiety and anxiety disorders as well as contribute to the understanding of resilient functioning. This is particularly of interest in light of a recent finding demonstrating a shift in the importance of environmental contributions from childhood and adolescence to adulthood, with the influence of environmental factors on the phenotypic stability of symptoms of anxiety and depression increasing with advancing age (Nivard et al., 2015).

In addition, childhood trauma was assessed retrospectively via a self-report questionnaire, which may be subject to recall bias (Hardt and Rutter, 2004). Overall, reported rates of experiences of childhood maltreatment were low as indicated by low CTQ sum scores, reflecting a limited range of childhood maltreatment experiences in the assessed sample. While exploratory analyses taking into account the different CTQ subscales rather than their cumulative effect (i.e. CTQ overall sum score) suggest that different types of neglect and abuse – particularly physical neglect and emotional abuse – may be more closely related to genetic and coping-influences than others, interpretation of these effects is highly speculative due the low frequency of traumatic experiences both overall and for specific subtypes in the present sample. Therefore, the relationship between specific, more narrowly defined types of trauma,

genetic susceptibility, and coping strategies constitutes a promising and necessary future direction. Sex-specific effects have repeatedly been described regarding *NPSR1* (Domschke et al., 2011; Laas et al., 2014b; Okamura et al., 2007), and sex has also been discussed to influence serotonin transporter binding in panic disorder (Cannon et al., 2012). No modulation by participant sex was observed regarding the presently reported GxExC effects, which is in line with both *5-HTT* and *NPSR1* GxE effects emerging irrespective of sex (Klauke et al., 2014, 2011). However, since the present sample was predominantly female, replication in samples with a more equal sex distribution would aid in the clarification of potential sex-specific effects in GxExC models.

SUMMARY AND OUTLOOK

The present results suggest that the availability of successful coping mechanisms exerts a protective effect compensating for the deleterious impact of environmental and genetic susceptibility in a resilience-enhancing way. In other words, history of childhood maltreatment may increase the risk for anxiety in *5-HTT* L_AL_A genotype and *NSPR1* AA genotype carriers, respectively, but only in the absence of a person's ability to cope with adversity, whereas a dose-dependent effect on anxiety traits as a function of maltreatment experiences irrespective of coping characteristics was observed in the presence of at least one *5-HTT* S/L_G or *NSPR1* T allele, respectively. These findings corroborate previous GxE studies regarding the interactive relationship between *5-HTT and NPSR1* genotypes and experiences of childhood adversity in the moderation of anxiety-related traits, and furthermore expand these findings by implicating coping-related qualities to function as an additional and important dimension buffering the effects of a GxE risk constellation. This extended GxExC approach carries great potential for clinical practice, particularly in early developmental stages: while genetic constellations and past

experiences of adversity are invariant, general self-efficacy by contrast constitutes a dynamic, modifiable quality, and could therefore constitute a target for psychotherapeutic interventions. Bandura (1997) himself postulated self-efficacy as a therapy target, and, indeed, increased self-efficacy has been observed following stress management training (Molla Jafar et al., 2016) and in the course of cognitive behavioral therapy (CBT) (Gallagher et al., 2013). It has furthermore been linked to symptom improvement in panic disorder (Gallagher et al., 2013) and social anxiety disorder (Bouchard et al., 2007; Gaudiano and Herbert, 2007). Therefore, the present results could inform targeted preventive interventions mitigating GxE risk constellations, for example in the form of trainings tailored to increase self-efficacy in at-risk populations by developing or improving positive coping strategies. Moreover, given that anxiety disorders typically manifest already early in childhood and are characterized by high chronicity and progression towards other anxiety disorders across the lifespan (e.g. Beesdo-Baum and Knappe, 2012), especially early targeted preventive interventions informed by complex-genetic susceptibility markers are particularly relevant within the crucial time window of childhood and adolescence (Schiele et al., 2016).

4.2 STUDY 2: GENETIC DETERMINANTS OF STRESS REACTIVITY

In recent years, there has been increasing interest in the definition and study of intermediate phenotypes of mental disorders and their relation to genetic markers. For instance, HPA axis reactivity to stress has been proposed as such an intermediate phenotype (e.g. Hasler et al., 2004; Mehta and Binder, 2012), and has consequently been addressed in the context of acute stress manipulation experiments. Unsurprisingly, variants in the *5-HTT* gene have been studied especially with regard to HPA axis function in depression and anxiety, although findings regarding if and

how *5-HTTLPR* genotype is related to markers of the stress response are inconclusive.

The present results in the context of the Maastricht Acute Stress Test (MAST) do not support a direct role of *5-HTTLPR* in the moderation of the neuroendocrine response to acute stress, as neither a main effect nor an interaction effect with genotype was observed. This is at odds with a number of studies reporting *5-HTTLPR* to influence cortisol reactivity to acute stress (Gotlib et al., 2008; Mueller et al., 2011; Way and Taylor, 2010), and a recent meta-analysis reporting an overall association, albeit small, of the S allele with cortisol responses elicited in response to a stress manipulation in a laboratory setting (Miller et al., 2013). However, several other studies have also not been able to discern a direct effect of *5-HTTLPR* genotype on stress reactivity (Alexander et al., 2014, 2009; Verschoor and Markus, 2011; Wüst et al., 2009), although a moderation of the relationship between *5-HTT* genotype and cortisol responding by stressful life events in a GxE manner has been suggested (Alexander et al., 2009; Mueller et al., 2011).

By contrast to the findings obtained regarding *5-HTT* variation, after undergoing the MAST carriers of the more active *NPSR1* T allele displayed significantly higher overall salivary cortisol levels compared to AA genotype carriers. This confirms observations from a previous study utilizing a different stress protocol (TSST for groups), which reported enhanced cortisol levels in healthy men carrying at least one T allele relative to AA homozygotes (Kumsta et al., 2013), and extends this previous study by investigating a more heterogeneous sample comprising both female and male volunteers. The present results are furthermore in line with HPA axis activation by NPS administration in animal models: Paraventricular NPS administration has been reported to increase plasma ACTH and corticosterone in rats, and, additionally, NPS was shown to facilitate the release of CRH and arginine vasopressin (AVP), a pituitary hormone, from hypothalamic explants *in vitro*,

suggesting that NPS affects HPA axis function via the hypothalamus through CRH and AVP release (Smith et al., 2006). Accordingly, enhanced reactivity of the HPA axis in humans is likely to be driven by higher NPS signaling in T allele carriers and is thus in line with studies observing increased physiological activation in T allele carriers across different paradigms (e.g. Domschke et al., 2012a, 2011). In the present study, the observed modulation of the neuroendocrine stress response by *NPSR1* genotype did not extend to subjective stress ratings, which were comparable between *NPSR1* genotype groups. This is in contrast to the study by Kumsta et al. (2013), who observed higher self-report anticipatory stress ratings in T allele carriers. One explanation may concern the operationalization of the subjective stress rating, which was different between the two studies. While presently, perceived momentary stress was explicitly inquired on a rating scale (VAS), in the study by Kumsta et al. (2013) "subjective stress" was obtained as the mean value of three rating scales addressing the participants' desire to leave the situation, anxiety levels, and emotional arousal, and may thus reflect slightly different aspects of stress perception. Interestingly, on a trend-level, T allele carriers exhibited more counting errors during the mental arithmetic trials of the MAST, which is somewhat at odds with a previous observation of enhanced response inhibition and increased error monitoring in T allele carriers in a Go/NoGo paradigm, with, on a behavioral level, lower rates of false alarms and increased post-error accuracy in T allele carriers (Beste et al., 2013). It could be speculated that this effect is reversed under high levels of acute stress as applied in the present context. The T allele has, however, also been linked to higher impulsivity (Laas et al., 2015, 2014b), which might constitute another explanation for the higher number of mistakes in T allele carriers observed in the present study. Further research is needed to address the mechanistic underpinnings of error-related processing depending on stress load.

SECONDARY ANALYSES OF GSE ON CORTISOL REACTIVITY

In an exploratory approach aiming to extend findings from study 1 on GSE buffering the interplay of *5-HTT/NPSR1* variation and childhood trauma (i.e. distal stress) by addressing GSE and genetic variation also in the context of acute stress as modeled by the MAST, no moderating effects of GSE on acute stress reactivity as indexed by salivary cortisol levels could be obtained, neither independently of nor in interaction with *5-HTT/NPSR1* genotypes. While it may be conceivable that GSE does not affect responses to acute stress in a similar manner as to distal or chronic stressors, these results are highly preliminary and should be interpreted cautiously given the presently investigated sample size of N=104. In fact, not taking into account potential covariates, a total sample size of at least N=148 would be required in order to detect an interactive effect with a small effect size of f=.1 of grouped GSE and grouped genotype, thus resulting in four groups, on cortisol levels (7 measurements), applying an alpha level of .05 and a power (1-β) of .8, under the assumption of moderate correlations among repeated measurements and a nonsphericity correction of 1 as calculated using G*Power (Version 3.1.9.2; Faul et al., 2007). Therefore, re-analysis in larger, sufficiently powered samples is needed in order to conclusively evaluate the potential impact of GSE on the relationship between genetic variation and stress reactivity.

LIMITATIONS

The present results need to be considered in light of some limitations. While they add to the literature on genetic determinants of acute stress reactivity by investigating the effects of *5-HTT* and *NPSR1* variation on cortisol reactivity by means of a recently developed stress paradigm, other mechanisms are likely involved, constituting a complex structure involving genetic and non-genetic factors.

While presently only salivary cortisol and subjective stress ratings were assessed, it would be interesting to compare genotype effects across a number of additional markers in the neuroendocrine system, e.g. ACTH or CRH. Additionally, it would be useful to additionally assess physiological correlates of the stress response, e.g. heart rate, blood pressure, or skin conductance level, in future studies. Furthermore, particularly pertaining to the null effect of *5-HTTLPR*, given the sample size of N=104 participants, the present study may have been underpowered to detect a possible main effect of *5-HTT* genotype since its estimated effect on HPA axis reactivity is assumed to be small (Miller et al., 2013), necessitating re-evaluation in larger samples. Similarly, while a main effect of grouped genotype on cortisol reactivity emerged with regard to *NPSR1*, no interaction effect was obtained on cortisol levels, as well as no main or interaction effects with regard to subjective stress ratings. This may also be owed to the relatively small sample size for a genetic study; given that the effect sizes reported by Kumsta et al. (2013) were small, the present study may have been underpowered to detect these effects.

Since multiple genes are involved in the regulation of HPA axis function, haplotypic and/or epistatic effects should be taken into account in future studies, for instance with the glucocorticoid receptor (*NR3C1*) gene (Kumsta et al., 2007), between *5-HTT* and *BDNF* variants (Dougherty et al., 2010), or between *5-HTT* and the D4 dopamine receptor gene (*DRD4*) (Armbruster et al., 2009b), which have been shown to affect HPA axis reactivity. Additionally, accumulating evidence for epigenetic mechanisms in the regulation of stress reactivity proposes that epigenetic markers such as DNA methylation should be considered in future studies on gene function modulating the response to acute stress, for instance DNA methylation in the *5-HTT* (Alexander et al., 2014; Duman and Canli, 2015; Ouellet-Morin et al., 2012), *OXTR* (Unternaehrer et al., 2012; Ziegler et al., 2015), *NR3C1* (Tyrka et al., 2016), or *FKBP5* (Höhne et al., 2015) genes. To illustrate, DNA methylation has been reported to impact the association between *5-HTTLPR* and cortisol responses to

psychosocial stress, with an effect of the S allele emerging only under conditions of low *SLC6A4* methylation (Alexander et al., 2014).

While inclusion of a mixed sample of female and male participants constitutes an advantage of the present study compared to previous studies on acute stress reactivity investigating only male cohorts (e.g. Duman and Canli, 2015; Kumsta et al., 2013), and although all analyses were controlled for sex, a specific investigation of differences in cortisol responding to the MAST as a function of *5-HTT* and *NPSR1* genotypes between men and women was limited by the overall small sample size and low genotype frequencies. Therefore, future studies in sufficiently powered samples designed to investigate sex-specific effects are needed. Furthermore, for a more sound interpretation of the observed differences with regard to counting errors, it would be useful to take general mathematical ability into account, which may constitute a confounding factor.

Given that *NPSR1* gene variation has been found to interact with environmental stress (study 1 of this thesis and Klauke et al., 2014), it seems pertinent to address this interaction in the context of HPA axis reactivity similar to research pertaining to e.g. *5-HTTLPR* (Alexander et al., 2009; Mueller et al., 2011), *FKBP5* (Buchmann et al., 2014; Höhne et al., 2015), *COMT* (Armbruster et al., 2012), *CRHR1* (Tyrka et al., 2009), or *NPY* (Witt et al., 2011) variation, and furthermore extend this approach to clinical populations. In light of the association between *NPSR1* variation and panic disorder (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007), and observations of HPA axis dysregulation in panic patients (Abelson et al., 2007; Erhardt et al., 2006), addressing stress reactivity in patients with panic disorder depending on *NPSR1* genotype constitutes a promising future study subject adding to the understanding of neurobiological disease mechanisms.

SUMMARY AND OUTLOOK

The present study adds support for the direct involvement of *NPSR1* – but not *5-HTTLPR* – in the modulation of acute stress reactivity, which has been discussed as an intermediate phenotype for mental disorders (e.g. Hasler et al., 2004; Mehta and Binder, 2012). In light of findings linking *NPSR1* to dimensional and categorical anxiety as well as endophenotypes of anxiety (Gottschalk and Domschke, 2016), the present findings suggest stress reactivity as an important potential intermediate phenotype of anxiety, which will have to be elucidated further in future studies, especially in the context of GxE, or, as proposed above in study 1, GxExC approaches. Acute stress responsiveness has previously been shown to be interactively influenced by genetic and adverse environmental factors (Alexander et al., 2009; Armbruster et al., 2012; Buchmann et al., 2014; Witt et al., 2011), and importantly, recent research points to an involvement of coping styles in the modulation of stress reactivity (Höhne et al., 2014; Villada et al., 2016), implicating that not only the effects of past stressful experiences, but also responses to an acute stress situation can be affected by beneficial influences pertaining to coping. While exploratory analyses in the present sample on the role of GSE in interaction with *5-HTT* and *NPSR1* genotype did not point to a moderating impact on acute stress responding, this was likely due to the limited sample size. Therefore, an extension in a GxExC manner in larger samples in order to ensure sufficient statistical power seems promising, and would add valuable insights into stress reactivity and its role in the mediation of vulnerability or resilience to psychopathology.

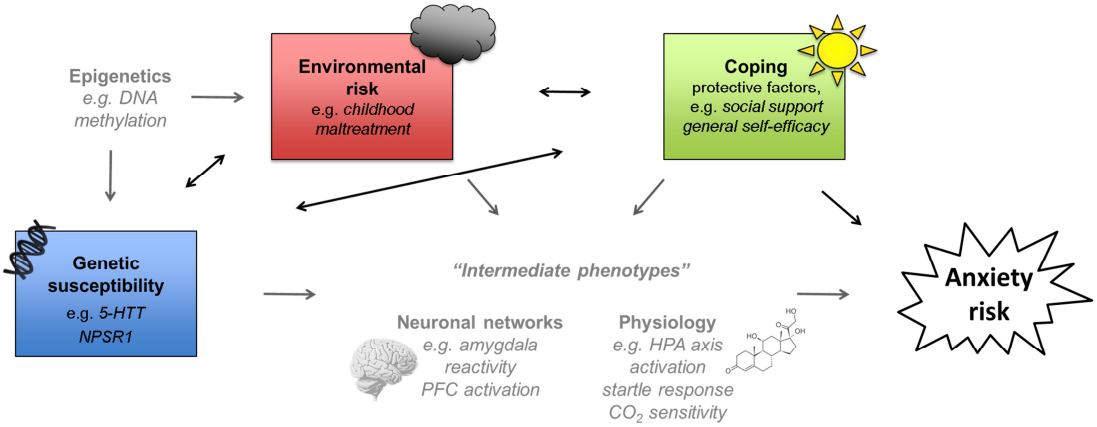
4.3 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The presently applied GxExC approach extends existing GxE models of anxiety risk by including an additional dimension able to buffer an otherwise disadvantageous profile of genetic and environmental circumstances, reconciling

previous findings that have addressed two, but rarely all three dimensions simultaneously. Therefore, the findings obtained in this thesis may inform future research directions and furthermore carry important implications for clinical practice.

The moderation of risk or resilience to anxiety is highly complex, comprising the interplay of a multitude of different factors, including genetics, environmental conditions, epigenetic mechanisms, neuronal networks, or physiological systems. In order to further elucidate the underlying mechanisms by which risk or resilience are conferred, future research would benefit greatly from embedding the proposed GxExC model into current risk factor models of anxiety, and address the mechanisms by which genetic variation, environmental stress, and protective factors shape anxiety risk and are reflected on an intermediate phenotype level (figure 4.1).

Figure 4.1. Extended gene x environment x coping (GxExC) model of anxiety risk



Two-dimensional gene-environment models postulate an interactive effect of genetic and environmental risk factors in the development of anxiety. Protective, i.e. coping ("C") factors, representing an additional dimension in an extended GxExC approach, may exert a buffering effect on an existing GxE risk factor constellation via epigenetic, neuronal, and physiological mechanisms and thus decrease anxiety risk or, in turn, increase resilience, respectively.

It is commonly understood that multiple genes are involved in the pathogenesis of anxiety and anxiety disorders (cf. Vieland et al., 1996), contributing in an individual or interactive manner ('***epistasis***'), and furthermore in interaction with

environmental risk factors and – as proposed in the present approach – protective factors. Interactions with negative environmental circumstances – e.g. childhood adversity or recent stressful experiences – have commonly been addressed with regard to variants in single candidate genes in the moderation of anxiety risk (e.g. Amstadter et al., 2010; Baumann et al., 2013; Choe et al., 2013; Klauke et al., 2014, 2011; Laucht et al., 2009), but interactive effects have also been described between genetic variation and protective factors, for instance *5-HTTLPR* and social support (Reinelt et al., 2014). Epistatic effects in GxE research on depression have been discerned between *5-HTT* and *BDNF* variation (Grabe et al., 2012; Kaufman et al., 2006), and in line with an extended GxExC model, this gene-gene interaction with childhood maltreatment has been observed to be further moderated by presence or absence of social support (Kaufman et al., 2006).

On an intermediate phenotype level, modulations by genetic variation as well as environmental and psychosocial factors, individually and interactively, have been described. Disruptions in ***neural network regulation*** have been discussed in the mediation of anxiety, particularly with regard to components of the 'fear circuit' such as amygdala, hippocampus, and prefrontal regions (Shin and Liberzon, 2010), and connected to variation in candidate genes such as *5-HTT*, *COMT*, or *NPSR1* (e.g. Dannlowski et al., 2011; Domschke et al., 2011, 2008; Furmark et al., 2004; see also Bandelow et al., 2016; Domschke and Dannlowski, 2010; Domschke and Deckert, 2009). Aside from genetic factors, environmental stress has also been linked to brain structures and function; for example, increased amygdala responsiveness to threat-related stimuli in a face-processing task has been observed as a function of childhood maltreatment, and, on a structural level, reductions in hippocampal and prefrontal volumes were related to history of maltreatment (Dannlowski et al., 2012). Concerning protective factors, high social support has been shown to buffer the link between increased amygdala reactivity in response to threat-related face stimuli and trait anxiety (Hyde et al., 2011). Moreover, interactive effects of genetic and

environmental factors have been described in imaging research, for example for *NPSR1* and urban upbringing on amygdala activation (Streit et al., 2014), or *5-HTTLPR* and childhood adversity on hippocampal volume in depression (Frodl et al., 2010). Therefore, the integration of a GxExC approach into an 'imaging genetics' framework could constitute a logical next step in anxiety research.

With regard to ***psychophysiological and neuroendocrine endophenotypes*** of anxiety disorders, increased startle magnitude to safe conditions has been found to be predictive of anxiety disorder onset (Craske et al., 2012). Genetic markers of the startle response have been described, e.g. for variants in the *5-HTT* (Brocke et al., 2006; Klumpers et al., 2012) and *COMT* genes (Montag et al., 2008), as well as moderating GxE effects on startle magnitude conferred by *5-HTT* and recent stressful life events (Armbruster et al., 2009a), and *COMT* and childhood maltreatment (Klauke et al., 2012), respectively. CO_2 reactivity has been proposed as another endophenotypic marker of panic disorder (Coryell, 1997). Studies in healthy volunteers suggest a moderating role of *5-HTTLPR* in CO_2 reactivity (Schmidt et al., 2000; Schruers et al., 2011), although no such effect was observed in patients with panic disorder (Perna et al., 2004). Additionally, history of stressful life events has been found to predict CO_2 sensitivity (Ogliari et al., 2010), presumably in a GxE manner as derived from a clinical genetic study comparing monozygotic and dizygotic twins (Spatola et al., 2011). In CCK-4 challenge experiments, which reliably provoke panic attacks in a dose-dependent fashion, variants in the *5-HTT* and *MAOA* genes for example have been described to differentially modulate panic responses (Maron et al., 2004; but: Maron et al., 2008; see Zwanzger et al., 2012). Combining a CCK-4 challenge test with an 'imaging genetics' approach, *NPSR1* rs324981 T allele carriers showed blunted anterior cingular glutamate-glutamine levels (Ruland et al., 2015). Furthermore, CCK-4-induced panic responses have been found to be associated with increased HPA axis activity (Zwanzger et al., 2013) although no moderation of this link was observed depending on *NPSR1* genotype

(Ruland et al., 2015). Altered HPA axis reactivity has also been implicated as a marker of clinical anxiety (e.g. Abelson et al., 2007; Erhardt et al., 2006; Mantella et al., 2008; Vreeburg et al., 2010). In addition, HPA axis activity is presumed to be partly determined by genetic factors, such as variants in *NPSR1* as reported in this thesis and by Kumsta et al. (2013), *CRHR1* (Mahon et al., 2013; Sumner et al., 2014), *FKBP5* (Ising et al., 2008; Mahon et al., 2013), *NR3C1* (Kumsta et al., 2007), *HTR1A* (Armbruster et al., 2011), or *5-HTT* (Miller et al., 2013), although several findings point to a moderation of the link between e.g. *5-HTT* and acute stress reactivity by environmental factors or other genes. For instance, interactions between genes, e.g. between *5-HTT* and *BDNF* (Dougherty et al., 2010) or between *5-HTT* and *DRD4* (Armbruster et al., 2009b) have been found to moderate reactivity of the HPA axis. Additionally, history of adversity may also impact stress reactivity, for example childhood maltreatment (Carpenter et al., 2011, 2007, Elzinga et al., 2010, 2008; Sumner et al., 2014), and joint effects of genetic variants and environmental stressors such as childhood adversity as addressed in GxE approaches have been identified to interactively affect HPA axis response, e.g. for *5-HTTLPR* (Alexander et al., 2009; Mueller et al., 2011), *FKBP5* (Buchmann et al., 2014; Höhne et al., 2015), *COMT* (Armbruster et al., 2012), *CRHR1* (Tyrka et al., 2009), or *NPY* (Witt et al., 2011). Moreover, positive factors have also been found to modulate the stress response. Social support, for instance, has been shown to influence HPA axis function and cortisol levels in response to an acute stressor (Hostinar et al., 2014), suggesting that positive contextual influences exert a buffering effect on stress reactivity and may therefore represent an important moderator in accordance with a GxExC framework. Furthermore, regulation of the HPA axis is linked to neural structure and function, particularly pertaining to limbic, hippocampal and prefrontal brain regions (Pruessner et al., 2010), which may additionally be influenced by early adverse experiences (Frodl and O'Keane, 2013). An integration of these multi-level findings,

then, within a cohesive GxExC framework would constitute an important future direction towards a better understanding of anxiety and anxiety phenotypes.

In recent years, evidence has accumulated proposing ***epigenetic effects*** such as DNA methylation as a mechanistic link between genetic determinants and environmental influences in conveying vulnerability to anxiety (cf. Schuebel et al., 2016). For instance, DNA hypomethylation of the *MAOA* gene (Domschke et al., 2012b; Ziegler et al., 2016) as well as the glutamate decarboxylase 1 (*GAD1*) gene (Domschke et al., 2013) has been reported in patients with panic disorder, and lower *OXTR* methylation has been observed in patients with social anxiety disorder (Ziegler et al., 2015). Epigenetic mechanisms are subject to environmental influences, and accordingly, similar to GxE approaches, DNA methylation patterns have also been addressed in the context of adverse experiences. For example, *MAOA* and *GAD1* hypomethylation has been shown to be related to negative recent life events in panic disorder (Domschke et al., 2013, 2012b), and history of childhood maltreatment has been linked to decreased *NR3C1* methylation in healthy volunteers (Tyrka et al., 2016). An association between *FKBP5* hypomethylation and history of childhood maltreatment has been observed in a genotype-dependent fashion (Klengel et al., 2013). DNA methylation patterns have also been connected to acute stress responsiveness, for instance a negative correlation has been reported between *OXTR* methylation and cortisol responses to the TSST (Ziegler et al., 2015), and *NR3C1* methylation was found to be positively related to cortisol reactivity to the dexamethasone/corticotropin-releasing hormone (Dex/CRH) test (Tyrka et al., 2016) and to cortisol response recovery following TSST exposure (van der Knaap et al., 2015). Furthermore, dynamic changes in *OXTR* methylation to psychosocial stress have been reported, with an initial increase relative to pre-stress methylation levels immediately after exposure to the TSST and a subsequent decrease in *OXTR* methylation after 90 minutes (Unternaehrer et al., 2012). Genotype-dependent effects of DNA methylation on HPA axis function have also been reported and may thus aid

in the reconciliation of observed discrepancies in the literature on if and how genetic variation is related to acute stress reactivity. Correspondingly, a moderating effect of the *5-HTTLPR* S allele on cortisol levels in response to an acute psychosocial stress manipulation has been observed only when *SLC6A4* promoter methylation was low (Alexander et al., 2014). Finally, on a neural level, a positive relationship between *SLC6A4* promoter methylation and hippocampal gray matter volume could be discerned (Dannlowski et al., 2014), and *OXTR* hypomethylation was found to be associated with increased amygdala activity during the processing of social anxiety related words (Ziegler et al., 2015), pointing to an involvement of epigenetic processes in the shaping of brain structures and functions.

Taken together, there is converging evidence for genetic, environmental and protective factors in accordance with the proposed GxExC model that act together in a highly complex manner via epigenetic mechanisms, neuronal structures and function, neuroendocrine systems, and psychophysiological indices towards increasing or decreasing anxiety risk. The present approach argues for a multi-level integration of neurobiological, physiological, environmental, and psychological factors towards an understanding of how multiple putative risk factors interact to increase the vulnerability towards disease, and under which circumstances a risk factor constellation actually leads to the progression from *risk* to *manifestation* of a disease, and under which circumstances it does not. Protective factors, then, including for example functional coping strategies and social integration/support, may mitigate, suspend, or even inverse the effects of existing vulnerability factors on multiple levels. This carries great potential for clinical practice in terms of prediction, prevention, and intervention towards effective personalized treatment options integrating individual genetic susceptibility, environmentally-shaped traits, and pre-existing resources. As a promising future direction, GxExC interactions should be addressed under consideration of epigenetic processes, in clinical populations, in the context of the study of intermediate phenotypes, or with relation to psycho- and

pharmacotherapy outcome in an attempt to unify findings from the literature addressing selective aspects of a GxExC model as discussed above. While adding the dimension of coping constitutes an important step forward in the dissection of putative risk factors and resilient functioning, it does not constitute an exhaustive model of anxiety risk. Further extension is necessary towards a comprehensive multi-dimensional explanatory model, for example regarding the role of microRNAs (Hommers et al., 2015) or copy number variation (Ono et al., 2015). Beyond the consideration of specific candidate genes as well as haplotypic or epistatic effects, future research might want to address GxE(xC) approaches in the context of genome-wide association studies (Thomas, 2010). Moreover, investigation of GxExC approaches in longitudinal designs would add valuable insights into the trajectory from susceptibility to manifestation, and aside from how GxExC interactions can influence disease risk, future attempts should also consider the combination and interplay of genes, environment, and coping factors in relation to their contribution to the maintenance of a disease after its onset.

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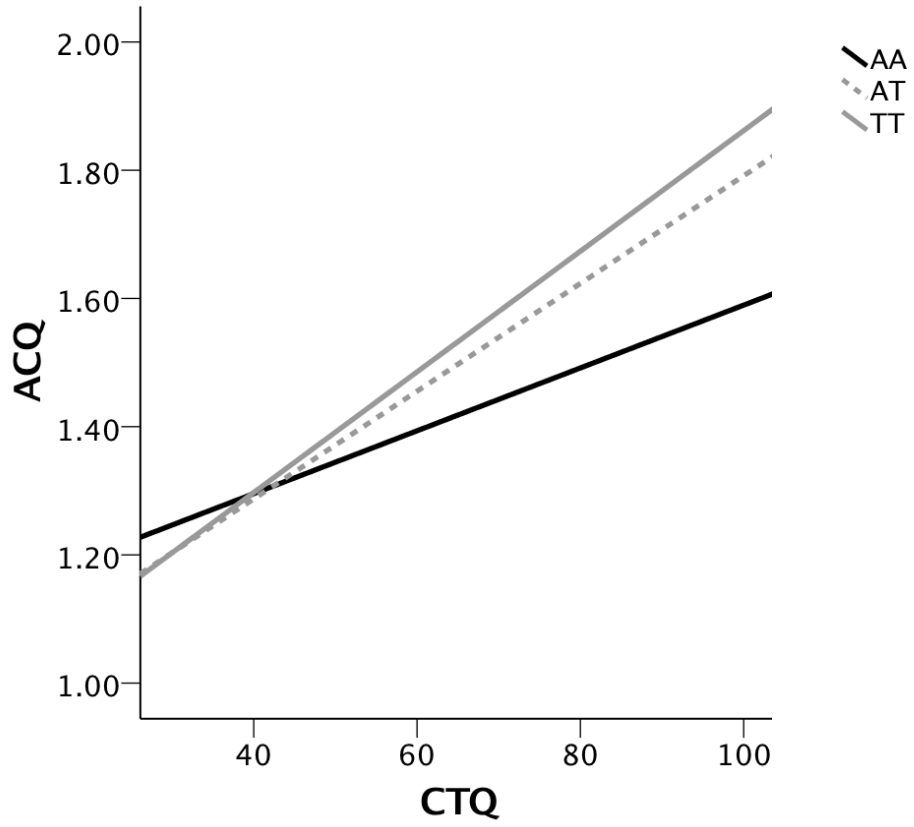
ANNEX

I ADDITIONAL FIGURES

Figure I.1. GxE effect of childhood trauma on ACQ as a function of *NPSR1* rs324981 genotype



CTQ=Childhood Trauma Questionnaire, ACQ=Agoraphobic Cognitions Questionnaire.

II LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter gene linked polymorphic region
ACTH	adrenocorticotropin hormone
ACQ	Agoraphobic Cognitions Questionnaire
Asn	asparagine
AVP	arginine vasopressin
CBT	cognitive behavioral therapy
CCK-4	cholecystokinin tetrapeptide
CLIA	chemiluminescence-immunoassay
CO_2	carbon dioxide
COMT	catechol-O-methyltransferase
CPT	Cold Pressor Test
CRH	corticotropin releasing hormone
CRHR1	corticotropin releasing hormone 1 receptor
CTQ	Childhood Trauma Questionnaire
DALYs	disability adjusted life years
Dex	dexamethasone
DNA	deoxyribonucleic acid
DRD4	D4 dopamine receptor
FDR	false discovery rate
FKBP5	FK506 binding protein 5
GxE	gene-environment
GxExC	gene-environment-coping
GSE	general self-efficacy
HPA	hypothalamic-pituitary-adrenal
HTR1A	5-hydroxytryptamine receptor 1A

Ile	isoleucine
LSAS	Liebowitz Social Anxiety Scale
MAOA	monoamine oxidase A
MAST	Maastricht Acute Stress Test
NaCL	sodium chloride
NH_4Cl	ammonium chloride
NPS	neuropeptide S
NPSR	neuropeptide S receptor
NPY	neuropeptide Y
NR3C1	nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor)
OXTR	oxytocin receptor gene
PCR	polymerase chain reaction
rGE	gene-environment correlation
rpm	revolutions per minute
s	seconds
SDS	sodium dodecyl sulfate
SECPT	Socially Evaluated Cold Pressor Test
SLC6A4	solute carrier family 6 member 4 (serotonin transporter)
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
STAI	State-Trait Anxiety Inventory
STAI-T	State-Trait Anxiety Inventory, Trait Version
TSST	Trier Social Stress Test
VAS	visual analogue scale
VIF	variance inflation factor
YLDs	years lived with disability

III LIST OF FIGURES

IV LIST OF TABLES

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