Epigenetic Mechanisms in the Pathogenesis and Therapy of Anxiety Disorders

Epigenetische Mechanismen in der Pathogenese und Therapie von Angsterkrankungen

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AFFIDAVIT

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Ort, Datum Unterschrift

ABBREVIATIONS

µ	micro
5hmC	5-hydroxymethylcytosine
5-HTT	5-hydroxytryptamine (serotonin) transporter, encoded by SLC6A4
5mC	5-methylcytosine
AD	anxiety disorders
ANOVA	analysis of variance
Bdnf/BDNF	brain derived neurotrophic factor
bp	basepairs
C	cytosine
CBT	cognitive behavioural therapy
CG/CpG	cytosine-guanine dinucleotide
CH_3	methyl group
Chr	chromosome
COMT	catechol-O-methyltransferase
Dnmt/DNMT	DNA methyltransferase
FDA	Food and Drug Administration
FKBP5	FK506 binding protein 5
G	guanine
g	gram(s)
GAD1	glutamate decarboxylase 1
GR	glucocorticoid receptor
h	hour(s)
H1/2/3/4/5	histone 1/2/3/4/5
HAM-D-21	Hamilton rating scale for depression (21 items)
HAT	histone acetyltransferase
HDAC	histone deacetylase
HPA	hypothalamus-pituitary-adrenal

K	lysine
k	kilo
kb	kilobasepairs
l	liter
m	milli
MAOA	monoamine oxidase A
MBD	methyl-CpG-binding domain
MDD	major depressive disorder
MECP2	methyl CpG binding protein 2
min	minute(s)
miRNA	microRNA
mRNA	messenger RNA
N	number
NCBI	National Center for Biotechnology Information
ncRNA	non-coding RNA
Nr3c1/NR3C1	nuclear receptor subfamily 3, group C, member 1 (encodes GR)
OCD	obsessive compulsive disorder
OXT	oxytocin
OXTR	oxytocin receptor
PD	panic disorder
PCR	polymerase chain reaction
r	correlation coefficient
rpm	rounds per minute
RT	room temperature
s	second(s)
SAD	social anxiety disorder
SLC6A4	solute carrier family 6, member 4 (encodes 5-HTT)
SNP(s)	single nucleotide polymorphism(s)

SNRI(s)	serotonin and norepinephrine reuptake inhibitor(s)
SSRI(s)	selective serotonin reuptake inhibitor(s)
T	thymidine
TCA	tricyclic antidepressants
Tet	ten–eleven translocation
U	uracil
VNTR	variable number tandem repeat
(x) g	(multiplied by) earth’s gravitational force

SUMMARY

Anxiety disorders (AD) are common, disabling mental disorders, which constitute the most prevalent mental health condition conveying a high individual and socioeconomic burden. Social anxiety disorder (SAD), i.e. fear in social situations particularly when subjectively scrutinized by others, is the second most common anxiety disorder with a life time prevalence of 10%. Panic disorder (PD) has a life time prevalence of 2-5% and is characterized by recurrent and abrupt surges of intense fear and anticipatory anxiety, i.e. panic attacks, occurring suddenly and unexpected without an apparent cue.

In recent years, psychiatric research increasingly focused on epigenetic mechanisms such as DNA methylation as a possible solution for the problem of the so-called “hidden heritability”, which conceptualizes the fact that the genetic risk variants identified so far only explain a small part of the estimated heritability of mental disorders.

In the first part of this thesis, oxytocin receptor (*OXTR*) gene methylation was investigated regarding its role in the pathogenesis of social anxiety disorder. In summary, *OXTR* methylation patterns were implicated in different phenotypes of social anxiety disorder on a categorical, neuropsychological, neuroendocrinological as well as on a neural network level. The results point towards a multilevel role of *OXTR* gene hypomethylation particularly at one CpG site (CpG3, Chr3: 8 809 437) within the protein coding region of the gene in SAD.

The second part of the thesis investigated monoamine oxidase A (*MAOA*) gene methylation regarding its role in the pathogenesis of panic disorder as well as – applying a psychotherapy-epigenetic approach – its dynamic regulation during the course of cognitive behavioural therapy (CBT) in PD patients. First, *MAOA* hypomethylation was shown to be associated with panic disorder as well as with panic disorder severity. Second, in patients responding to treatment *MAOA* hypomethylation was shown to be reversible up to the level of methylation in healthy controls after the course of CBT. This increase in *MAOA* methylation along with successful psychotherapeutic treatment was furthermore shown to be associated with symptom improvement regarding agoraphobic avoidance in an independent replication sample of non-medicated patients with PD.

Taken together, in the future the presently identified epigenetic patterns might contribute to establishing targeted preventive interventions and personalized treatment options for social anxiety disorder or panic disorder, respectively.

ZUSAMMENFASSUNG

Angsterkrankungen sind die häufigsten psychischen Erkrankungen, welche in hohem Maße den Alltag der Betroffenen beeinträchtigen und eine große sozioökonomische Belastung darstellen. Eine der häufigsten Formen von Angsterkrankungen bildet die soziale Phobie, d.h. die Angst vor sozialen Situationen, in denen man im Mittelpunkt der Aufmerksamkeit steht, mit einer Lebenszeit-Prävalenz von circa 10%. Die Panikstörung, charakterisiert durch das wiederholte und unerwartete Auftreten von Panikattacken, ist eine weitere Form der Angsterkrankungen mit einer Lebenszeit-Prävalenz von circa 2-5%.

Epigenetische Mechanismen, wie zum Beispiel die DNA Methylierung, rücken in den letzten Jahren immer weiter in den Fokus der psychiatrischen Forschung. Hier werden sie als eine mögliche Lösung für das Problem der „*hidden heritability*" (versteckte Heritabilität) angesehen.

Im ersten Teil dieser Arbeit wurde die DNA Methylierung des Oxytozinrezeptorgens (*OXTR*) hinsichtlich ihrer Rolle in der Pathogenese der sozialen Phobie untersucht. Hierbei konnte eine verringerte Methylierung des Gens, speziell an einem CpG-Dinukleotid (CpG3, Chr3: 8 809 437) innerhalb der protein-kodierenden Genregion, auf verschiedenen Ebenen mit der Erkrankung an sozialer Phobie, dimensionalen Maßen der Erkrankungsschwere sowie der Stressverarbeitung auf neuro-endokrinologischer und neuronaler Ebene in Verbindung gebracht werden.

Der zweite Teil dieser Arbeit beschäftigt sich mit der Rolle von DNA Methylierungsmustern des Monoaminooxidase A (*MAOA*) Gens in der Pathogenese und der Therapie der Panikstörung. Zum einen konnte gezeigt werden, dass eine verringerte *MAOA* Methylierung mit dem Auftreten von Panikstörung sowie mit einer erhöhten Symptomschwere assoziiert ist. Zum anderen zeigten Patienten, welche auf eine kognitive Verhaltenstherapie (KVT) ansprachen, eine signifikante Erhöhung der *MAOA* Methylierung nach der Therapie, welche zusätzlich in einer unabhängigen Stichprobe mit einer Verringerung der Symptomschwere assoziiert war. Diese Veränderung zeigte sich jedoch nicht in Patienten, welche nicht auf die KVT ansprachen.

Zusammenfassend können beide im Rahmen dieser Arbeit untersuchten epigenetischen Muster und deren Rolle in der Pathogenese der sozialen Phobie sowie der Panikstörung zur Etablierung personalisierter Therapiemöglichkeiten wie auch targetierter präventiver Interventionen beitragen.

I. INTRODUCTION

1.1. Anxiety disorders

Anxiety disorders (AD) are common, disabling mental disorders, which are suggested to be the most prevalent mental health condition (Kessler et al. 2010). In 2013, one out of nine people worldwide suffered from an anxiety disorder in the past year and the global prevalence of anxiety disorders was estimated at 7.3% (Baxter et al. 2013). According to the "Global Burden of Disease Study" (Baxter et al. 2014) anxiety disorders are the sixth leading course of disability and even subclinical forms are associated with distress and impairment (Goodwin et al. 2005; Skapinakis et al. 2011). Recent evidence towards an increasing validation of anxiety symptoms is suggested to be due to increased exposure to threat-related information as well as refined methods of symptom detection (Twenge et al. 2010). Different forms of AD are known, such as generalised anxiety disorder, social anxiety disorder, specific phobias, panic disorder, agoraphobia, separation anxiety disorder, and selective mutism (American Psychiatric Association 2013). These specified diagnoses vary in their life time prevalence, but share main symptoms and their clinical presentation is described as excessively fearful, anxious or avoidant behaviour out of proportion following perceived threats in the environment. The displayed fear or anxiety has to be described as marked, persistent and associated with impairments in social, work-related or other central areas of functioning (American Psychiatric Association 2013).

Anxiety disorders frequently occur comorbidly with depression and other anxiety disorders (Kessler et al. 2005). The strong relation between depression and anxiety disorders is estimated to be present in 57% of anxiety disorder patients (Zimmerman et al. 2000) and further qualified by differential diagnoses of so-called anxious depression, or depression with anxious features (American Psychiatric Association 2013). Further described comorbidities of AD are alcohol and other substance use disorders (Moreno-Peral et al. 2014), as well as personality disorders (Welander-Vatn et al. 2016). An association between AD and obsessive-compulsive as well as stress-related disorders like post-traumatic stress disorder (PTSD) is underlined by having been placed close together the DSM-IV (American Psychiatric Association 1994), despite in the DSM-5 this concept has been left in favour of separate categories (American Psychiatric Association 2013).

Recent literature reports childhood maltreatment (Vachon et al. 2015), including physical punishment (Clauss and Blackford 2012), an overprotective or very harsh parenting style (Beesdo-Baum and Knappe 2012), parental history of mental disorders,

as well as a low socioeconomic status (Moreno-Peral et al. 2014) as common risk factors for developing AD. Nevertheless, it has to be mentioned that these factors are non-specific for AD, but represent risk factors for mental disorders in general. Additionally, women are twice as likely as men to have an anxiety disorder (Baxter et al. 2013), and for that matter reported distress and impairment caused by AD is greater for women than men (McLean et al. 2011). As anxiety disorders develop during childhood, adolescence and early adulthood, cause the highest burden between age 15 to 34 (Baxter et al. 2014), and due to the fact that people older than 55 are 20% less likely to have an anxiety disorder (Baxter et al. 2013), age is considered as another risk factor. Furthermore, the genetic constitution has to be stated as risk factor for AD, underlined by the observation of a moderate familial aggregation for anxiety disorders and an estimated heritability of 30 to 50% (Hettema et al. 2001).

1.1.1. Social anxiety disorder (SAD)

Social anxiety disorder (SAD) is characterized by marked fear, anxiety or avoidance of social situation or interactions. These involve being inspected while being in the focus of attention for example during performing, speaking or eating in front of other people. Furthermore, SAD comprises the fear of negative judgement, as well as being embarrassed, humiliated, or rejected (American Psychiatric Association 2013). For a diagnosis of SAD the reported fear or anxiety has to be out of portion to the actual situation in its frequency and/or duration. Furthermore, symptoms have to persistent and last for 6 months or longer resulting in significant distress or impairment of the routine in the patients' social settings (Fact Sheet Social Anxiety Disorder, DSM, American Psychiatric Association 2013). In addition to these characteristics described in the Diagnostic and Statistical Manual of Mental Disorders, DSM-5 (American Psychiatric Association 2013), physical symptoms and blushing symptoms like fear of vomiting or urgency, and fear of micturition or defecation are mentioned in the International Classification of Diseases (ICD-10, World Health Organization 1992). Some affected people report social anxiety only in performance situations. In general, SAD is characterized by a diverse clinical presentation, and different forms of SAD are discussed controversially (Bögels et al. 2010) such as generalized SAD, performance anxiety, interaction anxiety, fear of showing anxiety symptoms, and test anxiety. This underpins the need of subtypes or specifiers in the diagnosis of SAD to refine research into different branches of SAD, which may be hampered by the broad and thus imprecise diagnosis of this disorder (Bögels et al. 2010).

SAD is the second most common anxiety disorder with a life time prevalence of 10% (Baxter et al. 2013) affecting 15 million adults worldwide, or 6.8% of the US

population, and confers a high individual and socioeconomic burden (Stein and Stein 2008). Epidemiological data suggest that 19.5–35% of individuals with primary SAD meet diagnostic criteria for currently comorbid or lifetime major depression, respectively (Ohayon and Schatzberg 2010; Stein et al. 1990). SAD is characterized by an early onset in childhood or early adolescence (Chavira and Stein 2005) and, as described generally for anxiety disorders, has a higher prevalence in females (Kessler et al. 2005; Ruscio et al. 2008). Family studies revealed a higher risk for first degree relatives of SAD patients to develop social phobia as compared to first degree relatives of healthy controls (Reich and Yates 1988), suggesting an underlying role of genetic risk factors. This is further corroborated by twin studies that indicate a moderate heritability of 51% (Hettema et al. 2001; Stein et al. 2002; Stein and Stein 2008). In addition to and in interaction with this genetic aspect, different environmental factors are suggested to contribute to the susceptibility for SAD as well (Bienvenu et al. 2007). As a heritable trait, behavioural inhibition in early childhood is shown to be highly predictive for the development of social anxiety (Clauss and Blackford 2012; Hirshfeld-Becker et al. 2007), as well as an overprotective and hypercritical parenting environment (Rapee and Spence 2004). Various plausible candidate genes such as *COMT* (Stein et al. 2005), *GAD1* (Hettema et al. 2006) or *5-HTT* (Arbelle et al. 2003; Battaglia et al. 2005) and involved neurotransmitter systems like the serotonin (Furmark et al. 2005; Hariri et al. 2006; Argyropoulos et al. 2004) or dopamine system (Schneier et al. 2000; Tiihonen et al. 1997) are identified so far that are hoped to contribute to further therapy options and to a deeper understanding of the pathogenesis of SAD (reviewed in Stein and Stein 2008). Common therapy options for social anxiety disorder are cognitive behavioural therapy (CBT), which addresses the vicious cycle of anticipatory negative thoughts that lead to increased situational anxiety and avoidance behaviour, and pharmacotherapy with selective serotonin re-uptake inhibitors (SSRIs) and serotonin- und norepinephrine re-uptake inhibitors (SNRIs) as first line treatment options (Bandelow et al. 2013).

1.1.2. Panic disorder

Panic disorder (PD) is an anxiety disorder with a life time prevalence of 2-5% (Baxter et al. 2013). In the DSM-5 (American Psychiatric Association 2013), PD is characterized by recurrent situations of an abrupt surge of intense fear and anticipatory anxiety, so-called panic attacks occurring suddenly and unexpected without an apparent cue (American Psychiatric Association 2013). These attacks reach a peak within minutes and include four or more physiological or cognitive symptoms such as sweating, shaking, chest pair, or the fear of losing control. Patients often show intense

worries about possible further attacks and may avoid places or situations where panic attacks have occurred in the past. This change in behaviour related to the attacks leads to a significant hindrance in life style, possible socioeconomic problems, and to a higher risk for depression, alcohol abuse and suicidality (Fleet et al. 1996). Further, panic disorder is often comorbid with agoraphobia and other mood disorders, while different mood symptoms often follow the onset of panic attacks. As described for the whole group of anxiety disorders, life time prevalence rates of comorbid major depression in panic disorder are about 30 to 40% (Kessler et al. 2006). Further comorbid conditions are obsessive-compulsive disorder (OCD), schizophrenia, or additional anxiety disorders as specific phobias or social anxiety disorder (Buckley et al. 2009). Similar to the described epidemiology of anxiety disorders in general, women are affected two to three times more often than men (Kessler et al. 2006). PD develops during a range of 18 to 45 years of age, with an average age of onset of 24 years (Kessler et al. 2005). Besides sex and age, further important risk factors have been shown to be of relevance including environmental influences such as life events in childhood (e.g. abuse experience, loss, and separation) and adulthood (e.g. loss, threatening, adjustment life events, or separation, interpersonal conflicts) (extensively reviewed in Klauke et al. 2010) and a family history of PD. The influence of genetic factors on increased susceptibility for PD is supported by an estimated heritability of 48% (Hettema et al. 2001) as well as genetic association studies proposing a significant heritability in a polygenic manner suggesting a relation between common genetic variants and common diseases such as PD (Domschke and Reif 2012).

Both cognitive behavioural therapy (CBT) and pharmacotherapy (SSRIs, SNRIs) are highly effective therapy options for panic disorder, similar to anxiety disorders in general (see Bandelow et al. 2013).

1.2. Epigenetic Mechanisms

The term “epigenetics” literally translates to “above or in addition to genetics”. It was first introduced by Conrad Waddington in the early 1940s (Waddington 1942). Broadly, he described the field of epigenetics as “*the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being*” (Waddington 1968). Today, the term is applied more narrowly and refers to the reversible regulation of various genomic functions that appear largely unrelated to changes in DNA sequence and are mediated principally through changes in DNA methylation and chromatin structure. In other words, epigenetics defines chemical reactions that can activate or deactivate parts of the genome. These modifications can be heritable, and modified by environmental influences. In 1957, Conrad Waddington

coined the term "*epigenetic landscape*" as a metaphor for how gene regulation modulates development (Waddington 1957). Several major discoveries were made starting already in the late 1940s with the discovery of 5-methylcytosine as part of the mammalian genome (Hotchkiss 1948), followed by the detection of different histone modifications (Allfrey et al. 1964) in the 1960s. Thirty years after that, the discovery of the histone acetyltransferases (Brownell et al. 1996), and the histone deacetylases (Taunton et al. 1996) marked further milestones in epigenetic research, followed by the discovery of DNA methyltransferases (Yoder et al. 1997) as well as histone methyltransferases in the late nineties (Rea et al. 2000). Only a few years later, the first FDA approved drugs targeting epigenetic mechanisms such as Vidaza® (DNA methylation inhibitor, active compound: azacitidine, FDA approved in 2004) and Zolinza® (histone deacetylase inhibitor, active compound: vorinostat, FDA approved in 2006) were developed. Today, epigenetic mechanisms comprise mainly three types of regulation: DNA methylation, histone modifications, and the posttranscriptional regulation via non-coding RNAs (ncRNAs). These modifications will be described in detail in the following sections.

1.2.1. DNA methylation

DNA methylation describes a process by which a methyl group (CH_3) is added to cytosines in the DNA sequence. The occurrence of a CG in the DNA sequence is called a CpG site, and serves as an epigenetic signal in that a methyl group can be transferred to the cytosine in this particular sequence. This reaction is catalyzed by DNA methyltransferases (DNMTs). It needs S-adenosyl-L-methionine (SAM) as a substrate and leads to the addition of a methyl group to the C5 carbon of the cytosine pyrimidine ring (Moore et al. 2013). DNMTs are grouped into *maintenance* and *de novo* methyltransferases. DNMT1, a *maintenance* methyltransferase catalyzes the methylation of hemimethylated DNA, which occurs during DNA replication, and is highly expressed ubiquitously in all human tissues (Robertson et al. 1999). DNMT1 localizes to the replication fork where the newly synthesized hemimethylated DNA is formed, binds to the DNA strand and adds methyl groups to mimic the original distribution of methyl groups present before DNA replication. Additionally, DNMT1 has the ability to repair DNA methylation and thereby maintains the original DNA methylation pattern across multiple cell cycles. Its crucial role in cellular differentiation and in dividing cells is underlined by the fact that *Dnmt1* knockout in mice leads to embryonic mortality in early developmental stages. *De novo* methyltransferases, such as DNMT3a and DNMT3b, introduce methylation into unmethylated native or synthetic DNA, but show no preference for hemimethylated DNA. Both DNMT3a and DNMT3b

are expressed relatively ubiquitously in human tissues, but to a much lesser extent than DNMT1 on mRNA level (Robertson et al. 1999). How *de novo* methylation is targeted to specific gene regions is not exactly known, but different approaches suggest two possible mechanisms: DNMT3a and DNMT3b can be targeted to gene promoters by specific transcription factors or they randomly methylate all CpG sites across the genome, which are not protected by a transcription factor (reviewed in Moore et al. 2013). Similar to the *maintenance* methyltransferase DNMT1, *de novo* methyltransferases such as DNMT3a and DNMT3b possess a crucial role during early development and cellular differentiation, as shown in further studies where knockout of *Dnmt3b* in mice causes embryonic lethality (Okano et al. 1999), while *Dnmt3a* knockout mice survive up to four weeks after birth (Okano et al. 1999).

Furthermore, CpG sites can exist in several distinct chemical states. Aside from unmethylated and methylated forms, cytosines can be hydroxymethylated (5hmC). While the methylation of a cytosine is catalyzed by DNA methyltransferases as introduced earlier, the oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) is mediated by ten-eleven-translocation (TET) proteins (Tahiliani et al. 2009). Besides its supposed regulatory function on gene expression level, hydroxymethylation has been associated with cellular differentiation, neuronal development, and aging (reviewed in Pfeifer et al. 2013). In addition to these various roles, 5hmC is suggested as an intermediary step in the process of DNA demethylation (Moore et al. 2013). DNA demethylation or the erasure of DNA methylation is supposed to be either active or passive. In dividing cells, an inhibition or dysfunction of DNMT1 can lead to passive demethylation of DNA by allowing newly synthesized DNA strands to remain unmethylated. The process of active demethylation requires an enzymatic processing of methylated cytosines in order to degenerate it back to its unmethylated state (e.g. Zhang et al. 2007). Up to now, there is no distinct mechanism known which is able to cleave the covalent carbon-to-carbon bond connecting cytosines and methyl groups. As an alternative, demethylation is achieved through chemical degradation of 5mC, e.g. by oxidation into 5hmC via TET proteins, followed by recognition and replacement of the modified base with an unmethylated cytosine by base extinction repair (BER) (Bhutani et al. 2011; Moore et al. 2013).

Irrespective of the chemical state, CpG sites aggregate in some parts of the genome in so-called CpG islands. Because many human gene promoters (72%) are associated with these islands (Saxonov et al. 2006), their basal state must be unmethylated in order to allow transcription. Different circumstances can cause a CpG island to be methylated; in that case, methylation of CpG islands may interfere with the

binding of transcription factors that activate gene expression and thereby stably silence gene expression. An additional mechanism of transcriptional repression involves proteins that are attracted to methylated CpG sequences, so-called readers of DNA methylation. Such proteins are members of three protein families, namely methyl-CpG-binding domain (MBD) proteins (Nan et al. 1993), UHRF (ubiquitin-like with PHD and RING finger domain) proteins (Avvakumov et al. 2008), and zinc-finger proteins (Filion et al. 2006). For example, binding of MeCP2 a member of the MBD protein family leads to recruitment of a corepressor complex linking DNA methylation and histone modifications to repress gene transcription. This interaction between DNA methylation and histone modifications, i.e. chromatin remodeling, is detailed below (see 1.2.2).

Naturally, CpG sites also occur in the gene body which is considered the region of the gene downstream of the first exon. This distinct segmentation is caused by the fact that DNA methylation of the first exon, similar to methylation of promoter regions, leads to repression of gene transcription (Brenet et al. 2011), while methylation of the gene body does not repress, but rather initiates gene expression in dividing cells (Hellman and Chess 2007). Interestingly, in slowly and non-dividing cells gene body methylation is no longer associated with higher gene expression (Aran et al. 2011) indicating that the functional consequences of gene body methylation on gene expression regulation are not completely known.

1.2.2. Histone modifications

Besides DNA methylation, a second important epigenetic mechanism is the posttranslational modification of histone proteins that alters their interaction with DNA and nuclear proteins, i.e. the chromatin structure. By building up this chromatin structure, histone proteins mediate packing of the DNA molecule in the cell nucleus. Due to their distinct function, histones can be grouped into core histones (H2A, H2B, H3, and H4), which build the core of the nucleosome, and linker histones (H1 and H5), which lock the DNA into place at the nucleosome and allow for a higher order formation. One nucleosome as the unit of chromatin is comprised of an octamer of two copies of each histone H3, H4, H2A, and H2B and the DNA double helix wrapped around it 1.65 times (Kornberg 1974). All core histones share a distinct structure consisting of three alpha-helices which are separated by two loops. This structure allows for heterodimerization of H2A with H2B and H3 with H4 what consequently results in the dimeric structure of the nucleosome (Luger et al. 1997). Furthermore, core histones have long N-terminal amino terminal tails protruding from the nucleosome, which can be covalently modified at several amino acids. These modifications play a crucial role in the regulation of chromatin dynamics and determine

transcriptional availability of the DNA (Kouzarides 2007). Modifications of the tail include - among others - methylation, acetylation or phosphorylation (reviewed in Bannister and Kouzarides 2011). Combinations of modifications are thought to constitute a code, the so-called "histone code" which determines whether chromatin is present in an "open" (activated, euchromatin) or "closed" (inactivated, heterochromatin) state (Jenuwein and Allis 2001). Additionally, this hypothesis conceptualizes that modifications of the histone tails act as marks that can be read by other proteins to control the expression or replication of chromosomal regions. Broadly, there are two known mechanisms of action for the function of N-terminal modifications (Kouzarides 2007). First, chromatin structure is modulated by altered DNA-nucleosome or nucleosome-nucleosome interactions caused by changed histone charges or added physical units. Of all described modifications, one prominent example for the described mechanism is the acetylation of lysines (first described in Allfrey et al. 1964). In this highly dynamic process, histones are acetylated and deacetylated on lysine residues in the N-terminal tail. These reactions are catalyzed by histone acetyltransferases (HATs), or histone deacetylases (HDACs), respectively. HATs catalyze the addition of an acetyl group to lysine side chains by using acetyl-CoA as cofactor. By that, neutralization of the positive charge of lysines, and consequently a weakened DNA-histone interaction is achieved (Shogren-Knaak et al. 2006). Accordingly, histone acetylation is associated with transcriptionally active genes in an "open" chromatin structure. HDACs reverse this effect by catalyzing the deacetylation of lysines that is restoring positive charges and stabilizing chromatin confirmation. Therefore, deacetylation by HDACs is associated with inactive genes in a "closed" chromatin structure (Kouzarides 2007). Second, histone modifications are docking stations for specific binding proteins which recognize and interact with modified histones, thus influencing chromatin dynamics and function (Zeng and Zhou 2002).

As another important mechanism, chromatin activation status and DNA methylation often work in concert to control gene expression. For example, to form transcriptionally inactive heterochromatin, DNA methylation and histone deacetylation interact with each other. As already indicated above, methylated DNA is preferentially bound by different protein families (detailed in 1.2.1) including MeCP2, a member of the MBD protein family. This protein recruits a repressor complex of different proteins among others HDACs. The consecutive deacetylation of the histones leads to a stronger binding of DNA to the histone core and therefore causes a condensation of chromatin, the formation of heterochromatin and a repressed gene transcription (Jones et al. 1998; Nan et al. 1998). In addition, MeCP2 recruits histone methyltransferases which are potent repressors of the active chromatin state as well (Fuks et al. 2003).

1.2.3. Non-coding RNAs

In the past 10 years, non-coding RNAs (ncRNAs) have emerged as another important epigenetic mechanism. Broadly, ncRNAs are defined as RNAs which do not code for any protein, but indeed carry information and have many important functions in normal development, physiology and disease (reviewed in Esteller 2011). Recent literature describes the following subclasses of ncRNAs: transcribed ultraconserved regions (T-UCRs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs), long non-coding RNAs (lncRNAs), and micro RNAs (miRNAs). Among them, miRNAs are widely studied and their functional relevance is most evident (He and Hannon 2004; Mendell 2005). MicroRNAs are 18 to 24 nucleotides long, non-protein coding RNA molecules that function as posttranscriptional regulators. A single mRNA may be regulated by multiple miRNAs and in turn one particular microRNA has the potential to target hundreds of mRNAs and regulate their stability and translational efficiency. MicroRNAs are initially transcribed as long, capped and polyadenylated precursor transcripts, known as primary microRNAs (pri-miRNAs) by RNA polymerase II (Cai et al. 2004; Lee et al. 2002; Lee et al. 2004). Transcribed pri-miRNAs contain one or more hairpin structures in which the mature miRNA can be found. These hairpins are excised from the pri-miRNA in the cell nucleus and are now referred to as precursor microRNAs (pre-miRNAs) (Denli et al. 2004) which are recognized by the nuclear export factor exportin5 and transported to the cytoplasm (Lund et al. 2004). In the cytoplasm, pre-miRNAs are unwound and cleaved by Dicer in order to generate 18 to 24 nucleotides long double-stranded RNA molecules (Hutvágner et al. 2001). One strand of each molecule is incorporated into RISC (RNA induced silencing complex) and guides it to target mRNAs (Kim 2005). The regulation of target mRNAs is achieved by complementary binding to miRISC (miRNA containing RNA induced silencing complex). Two different mechanisms of post-transcriptional regulation of mRNAs via miRNAs are known and supposed to be determined by the degree of complementarity between mRNA and miRNA. If there is perfect complementarity, the target mRNA will be cleaved and further degraded, while if there is imperfect complementarity, translation will be prevented due to steric hindrance of the protein synthesis machinery (Zeng et al. 2003). Due to this epigenetic regulation no functional protein will be translated from transcribed mRNAs.

1.3. Epigenetic mechanisms in neuropsychiatric disorders

1.3.1. General importance of epigenetic mechanisms

The important role of gene expression regulation by all described epigenetic mechanisms becomes evident when studying monozygotic twins (MZ) who share 100% of their genetic information and are almost always similar in appearance, but they are often discordant for important phenotypes including complex diseases. Because epigenetic modification is a dynamic process and only partially stable, there is a great potential for epigenetic variation within monozygotic twin pairs causing this discordance (Wong et al. 2005). Such variation among organisms with identical DNA sequences has mainly been attributed to the effect of the environment influencing epigenetic mechanisms. The most important and prominent environmental influence is stress strongly influencing the psychological state of an organism. Several studies were able to link maternal behavior and stress-induced depressive-like behaviors in rodents. For example, glucocorticoid receptors play a major role in the negative feedback loop of the hypothalamic–pituitary–adrenal (HPA) axis and thus in the stress response. Consequently, increased DNA methylation of the glucocorticoid receptor gene *Nr3c1* after maternal separation is coupled to a decreased glucocorticoid receptor transcription in mice (Kember et al. 2012). The lowered glucocorticoid receptor levels resulting from these DNA methylation changes are thought to potentially cause an impaired negative feedback of the stress hormone system and thus result in long-lasting alteration of the HPA axis (Kember et al. 2012). Even in humans, stress in early developmental phases - as for example exposure to childhood maltreatment - has been shown to correlate with lower allele-specific methylation of the FK506 Binding Protein 5 (*FKBP5*) gene, an important player in the stress hormone system, i.e. the HPA axis. In fact, in risk allele carriers (T-allele carriers) of the single nucleotide polymorphism (SNP) rs1360780 (C/T) located in an intronic region of *FKBP5*, exposure to childhood abuse was shown to be associated with lower DNA methylation of *FKBP5* intron 7. Interestingly, this association was missing in individuals with the homozygous protective genotype (CC genotype carriers) (Klengel et al. 2013). This altered DNA methylation pattern leads to stronger *FKBP5* induction and to glucocorticoid receptor resistance followed by a dysregulation of the HPA axis, selectively in risk allele carriers (Klengel et al. 2013). This study adds to the growing body of evidence that gene by environment (GxE) interactions are able to influence epigenetic marks like DNA methylation and with that - among others - the stress response. A second important environmental factor influencing epigenetic mechanisms is the diet an organism is confronted with. An example of how epigenetic patterns are influenced by food intake is in the study of the viable yellow agouti (A^{vy}) mouse model. Because fur colour of this

mice is correlated to epigenetic patterns that are established during early development, this model has been widely used to study the influence of nutrition and environment on the fetal epigenome (Dolinoy 2008). In brief, pregnant A^{vy} mice are exposed to a diet that provides a high or a low amount of methyl (CH_3) groups, respectively. In the offspring of mice eating more methyl groups, the *Agouti* gene is repressed and switched off by means of DNA methylation, which becomes visible by yellowish fur color and obesity in the offspring. In addition, physical exercise has been shown to contribute to a positive therapeutic outcome in diverse diseases, potentially by altering important epigenetic marks. For example, Gomez-Pinilla and colleagues investigated how physical exercise can affect DNA methylation patterns in the brain-derived neurotrophic factor (*Bdnf*) gene which encodes a molecule deeply involved in neuronal excitability, learning and memory. In animals exposed to 1 week of voluntary exercise, one particular CpG site in a region crucial for *Bdnf* expression regulation was significantly less methylated. Interestingly, this CpG site is associated with binding of methyl-CpG binding protein 2 (MeCP2), the link between DNA methylation and chromatin condensation (please refer to 1.2.2). Consequently, physical exercise causing DNA hypomethylation on this particular CpG site lead to an increased *Bdnf* gene expression and to a decreased binding of MeCP2, followed by heightened synaptic plasticity and cognitive abilities (Gomez-Pinilla et al. 2011).

Neurodevelopmental deficits associated with mutations in the methyl-CpG binding protein 2 (*MeCP2*) gene in Rett syndrome (RTT, MIM 312750) further underline the importance of DNA methylation patterns and their interaction with chromatin structure for normal brain function and development (Amir et al. 1999). Rett syndrome was originally described by the Austrian pediatrician Andreas Rett in 1966 (Rett 1966). It is a neurodevelopmental disorder that occurs almost exclusively in females and is characterized by arrested development between 6 and 18 months of age, regression of acquired skills, loss of speech, stereotypic movements, microcephaly, seizures, and mental retardation with a life expectancy of about 40 years.

1.3.2. Importance in specific neuropsychiatric disorders

In recent years, epigenetic mechanisms came into focus of psychiatric research to serve as a possible solution for the problem of “hidden heritability”, which conceptualizes the fact that different identified risk genes and genetic variants only explain a small part of the estimated heritability of different psychiatric diseases (Manolio et al. 2009). Epigenetic mechanisms are supposed to possibly serve as the missing link to explain this phenomenon (Petronis 2010). In this section, some

examples for the impact of epigenetic patterns on different neuropsychiatric disorders are described.

First, DNA methylation of different genes is involved in vulnerability for depression as well as the pathogenesis of major depressive disorder (MDD) in particular. As one hypothesized model, adversity in early life is able to change epigenetic patterns in genes involved in two important systems: the HPA axis and the serotonergic system (extensively review in Booij et al. 2013). In brief, different studies reported altered DNA methylation patterns in genes involved in the HPA axis, as for example hypermethylation of the *NR3C1* gene coding for a glucocorticoid receptor in brains of suicide victims exposed to childhood maltreatment (McGowan et al. 2009). Within the serotonergic system, the serotonin transporter (*5-HTT*) gene has been observed to be hypermethylated in probands displaying more unresolved responses to loss or trauma (van IJzendoorn et al. 2010). Therefore, vulnerability for depression appears to be modulated by interaction of alterations in these two systems. With regard to *5-HTT* as a prime candidate gene for the pathogenesis of MDD, increased *5-HTT* DNA methylation has been reported to be related to the diagnosis of MDD (Philibert et al. 2008), and to severity of depressive symptoms (Kang et al. 2013). Furthermore, DNA methylation patterns of the *5-HTT* gene seem to modulate therapy outcome in depressed patients, with patients displaying a low *5-HTT* promoter methylation show a decreased reduction in HAM-D-21 score after six weeks of pharmacotherapy as compared to patients with high *5-HTT* promoter methylation (Domschke et al. 2014).

In addition to MDD, the serotonin transporter gene has been prominently studied in post-traumatic stress disorder (PTSD). For instance, the number of experienced traumatic events and DNA methylation of the serotonin transporter gene have been shown to interact in modulating vulnerability for PTSD. In detail, exposure to a greater number of experienced traumatic events was found to be associated with an increased predicted probability of PTSD diagnosis in presence of *5-HTT* hypomethylation, but in turn, if *5-HTT* methylation was high, exposure to a greater number of traumatic events has been observed to be associated with resilience to developing PTSD (Koenen et al. 2011).

a) Oxytocin receptor (*OXTR*) gene as a candidate gene for social anxiety disorder

Oxytocin (OXT) is a neurohypophysial hormone named after the Greek words for "quick birth". This neuropeptide consisting of nine amino acids has originally been described and investigated because of its uterotonic activity and its role in milk-ejection during lactation. Today, oxytocin is known to exert a wide range of central and peripheral effects, like the modulation of neuroendocrine effects, and the establishment of complex social behaviours in reproduction and care of offspring (Cochran et al. 2013). Oxytocin is synthesized in magnocellular and parvocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus. Magnocellular neurons release oxytocin either via the axonal terminals, where it is stored in secretory vesicles, into the posterior pituitary or via the dendrites into the extracellular space. Therefore, oxytocin can act locally (axonal release) and can diffuse through the brain to reach distant target regions (dendritic release), respectively. In addition, parvocellular neurons producing oxytocin project directly to different brain regions, for example amygdala, hippocampus, or brainstem (Meyer-Lindenberg et al. 2011).

With regard to social functioning, oxytocin promotes social affiliative and approach behaviours, even in the context of social threat, increases positive social interaction, and facilitates pair bonding, trust, empathy, and attachment in humans and animals. Due to its function in various studies, oxytocin has been suggested as a promising anxiolytic pharmacotherapeutic agent in disorders related to impaired social functioning (for review see Bakermans-Kranenburg and van IJzendoorn 2013; Meyer-Lindenberg et al. 2011). Specifically, in social anxiety disorder, oxytocin has been widely studied regarding its potential function as "prosocial" peptide. For example, decreased baseline oxytocin plasma levels have been observed in SAD patients (Hoge et al. 2012), and intranasal application of oxytocin in the course of behavioural exposure therapy sessions significantly improved self-reported speech performance compared to placebo (Guastella et al. 2009). On the central level, oxytocin is shown to depress amygdala activation in response to socially relevant or fear-conditioned emotional stimuli (e.g. Kirsch et al. 2005), a characteristic activation pattern for social anxiety (Phan et al. 2006). The various functions of oxytocin on central and peripheral level are mediated trough binding to a G protein coupled receptor (oxytocin receptor, OXTR). This class A receptor is encoded by the oxytocin receptor (*OXTR*) gene mapped to the gene locus 3p25-3p26.2 (Simmons et al. 1995). The gene spans 17 kb (kilobasepairs) and consists of three introns and four exons with exon 3 and exon 4

encoding the OXTR protein, a 389 amino acid polypeptide with 7 transmembrane domains.

Especially the oxytocin receptor gene has been implicated in social cognition and behaviour on the genetic level. The extensively researched single nucleotide polymorphism (SNP) rs53576 located in the third intron of the *OXTR* gene is associated with different social anxiety-related conditions. For example, rs53576 has been reported to play an important role in regulation of prosocial behaviour, sensitive parenting, shaping social traits, and modulating neuronal circuits (extensively reviewed in Kumsta and Heinrichs 2013). Homozygous carriers of the “protective” G-allele are characterized by beneficial social traits such as increased prosociality (Kogan et al. 2011), higher trust (Krueger et al. 2012), and a lowered negative association between threat and prosocial behaviours (Poulin et al. 2012). In contrast, A allele (“risk” allele) carriers show reduced sensitive parenting (Bakermans-Kranenburg and van IJzendoorn 2008; Riem et al. 2011), lower empathy (Rodrigues et al. 2009), decreased positive affect, as well as lower levels of optimism, mastery and self-esteem (Saphire-Bernstein et al. 2011), general social traits that are supposed to be important physiological resources and predictors of long term health. A neuroimaging study by Tost and colleagues reported an increased functional connectivity between hypothalamus and amygdala in A allele carriers during processing of social cues (Tost et al. 2010), which supports the notion that this genetic effect on social cognition and behaviour is achieved by modifying neuronal circuits for social information and negative affect processing (Meyer-Lindenberg and Tost 2012).

On the epigenetic level, the *OXTR* gene has been well studied and characterized especially with regard to one CpG island spanning exons 1 to 3 (Chr3:8 808 962–8 811 280, GRCh37/hg19) (reviewed in Kumsta et al. 2013). The part of the CpG site located upstream of the translation start site in exon 3 has been shown to regulate gene expression of *OXTR* with hypermethylation of this region decreasing *OXTR* mRNA levels by 70% (Kusui et al. 2001) and additionally to be associated with lower oxytocin plasma levels (Dadds et al. 2014). Recently, *OXTR* methylation of this CpG island has been studied regarding diverse phenotypes related to social cognition and functioning. For example, increased *OXTR* methylation has been associated with callous-unemotional traits in male adolescents with oppositional-defiant or conduct disorder (Dadds et al. 2014). Furthermore, increased *OXTR* methylation was observed in peripheral blood mononuclear cells and temporal cortex tissue in the context of autism spectrum disorder (Gregory et al. 2009). Even on a neuronal level, *OXTR* methylation has been shown to impact social function when applying an ‘imaging

epigenetic' approach in healthy subjects, where increased *OXTR* methylation has been associated with neural processes of social interpretation of ambiguous stimuli (Jack et al. 2012). Applying the Trier Social Stress Test (TSST) to healthy subjects, it has additionally been shown that *OXTR* methylation increased in a region located within the protein-coding part of exon 3 after exposure to social stress, but decreased at follow-up (90 min) (Unternaehrer et al. 2012).

b) Monoamine oxidase A gene (*MAOA*) as a candidate gene for panic disorder

The monoamine oxidase A isoform (MAOA) is a key catabolic enzyme in the degradation of biogenic amines such as serotonin and dopamine by catalysing the oxidative deamination of those neurotransmitters and therefore constitutes a promising candidate for psychiatric disorders. MAOA is localized in the outer mitochondrial membrane in the presynaptic terminal of monoaminergic neurons (Arai et al. 2002). MAOA is encoded by the monoamine oxidase A (*MAOA*) gene located on chromosome Xp11.4–p11.3 and is implicated in panic disorder on several levels.

First, MAO inhibitors have been shown to be highly effective in the treatment of anxiety disorders, and panic disorder in particular (Tiller et al. 1997; Tyrer and Shawcross 1988).

Second, on a genetic level, a functionally relevant 30bp variable number tandem repeat (VNTR) within the *MAOA* gene promoter has been extensively studied. Five different variants of the VNTR are known which can be grouped in to 'long' alleles (3.5, four and five repeats) and 'short' alleles (two and three repeats). *In vitro* studies demonstrated the functional relevance of this variable region, with 'long' alleles increasing gene expression as compared to 'short' alleles (Deckert et al. 1999; Sabol, et al. 1998). Interestingly, this VNTR is known to be associated to different diseases in a sex-specific manner. Diverse studies reported short alleles to be associated with impulsive and aggressive behaviour in males (e.g. Caspi et al. 2002; Reif et al. 2007), while others demonstrated long alleles to be related to panic disorder in females (Deckert et al. 1999; Maron et al. 2005) as well as to anxiety symptoms (Voltas et al. 2015). In detail, it was shown that the more active longer alleles of the *MAOA* VNTR (more than three repeats) occur more frequently in the female subgroup of panic disorder patients compared to controls (Deckert et al. 1999; Reif et al. 2014), and, interestingly, the more active longer *MAOA* alleles predicted impaired response to cognitive behavioural therapy in female patients with panic disorder (Reif et al. 2014).

Third, on an epigenetic level, DNA methylation of the *MAOA* gene has been shown to be altered in female panic disorder patients, with significant *MAOA* promoter hypomethylation (Domschke et al. 2012). In an epigenome by environment approach this methylation pattern was shown to be influenced by life events. Negative life events were associated with lower *MAOA* gene methylation, while positive life events correlated with increased methylation (Domschke et al. 2012). This points towards an important role of *MAOA* gene methylation as a dynamic and possibly crucial pathogenetic link between environment influences and the biological underpinnings of panic disorder. A negative environment might lead to DNA hypomethylation of a particular risk gene such as *MAOA* and consequently to its heightened expression, while positive influences might result in hypermethylation, silencing this risk gene and thereby possibly conferring resilience. In addition to panic disorder, *MAOA* hypomethylation has repeatedly been shown to be associated with the phenotype of depression in females, but not in males (Melas et al. 2013; Melas and Forsell 2015).

1.4. Aim of the thesis

In the first part of this thesis, *OXTR* DNA methylation was investigated regarding its role in the pathogenesis of social anxiety disorder, the second most common anxiety disorder. As the oxytocin system itself plays an important role in social anxiety disorder on several levels, we hypothesized the *OXTR* gene to be differentially methylated in social anxiety patients compared to healthy controls as well as to be associated with dimensional as well as intermediate biological, i.e. neuroendocrinological and neural network phenotypes of social anxiety.

In the second part of the thesis, we investigated the role of *MAOA* DNA methylation in the pathogenesis and treatment of panic disorder. Treatment resistance in panic disorder is a frequent problem with a high individual burden, and there is an urgent need to better understand biological mechanisms underlying such therapeutic interventions. Since DNA methylation of the *MAOA* gene has been found to be altered in female panic disorder patients and is suggested to possibly mediate antidepressant treatment success, this study aimed at replicating a former study suggesting *MAOA* hypomethylation as risk pattern for panic disorder in an independent case-control sample. Furthermore, we applied a proof-of-concept psychotherapy-epigenetic approach and analysed *MAOA* methylation changes during the course of a six-week CBT. Based on previous findings of *MAOA* hypomethylation as risk pattern for PD, we hypothesized a successful psychological treatment to reverse this epigenetic risk pattern, in that panic disorder patients responding to treatment would increase in their *MAOA* methylation during the course of CBT.

II. MATERIALS AND METHODS

2.1. Materials

2.1.1. Used kits

EpiTect® 96 Bisulfite Kit	Qiagen, Hilden, Germany
EZ-DNA Methylation-Gold^{TM} Kit	Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany
FlexiGene DNA Kit	Qiagen, Hilden, Germany
HotStarTaq Plus Master Mix Kit	Qiagen, Hilden, Germany
Human Methylated & Non-methylated DNA Set	Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany

2.1.2. Media, buffers and stock solutions

Agarose peqGold	peqLab, Erlangen, Germany
Loading buffer for gel electrophoresis	25 mg Bromphenol blue-Xylene Cyanol (Sigma-Aldrich, Munich, Germany)
	1.5 ml Glycerol (anhydrous, Merck, Darmstadt, Germany)
	Add ddH_2O to 10 ml
GeneRuler 100bp Plus DNA Ladder	Thermo Scientific, Munich, Germany
TAE-Buffer (1 l)	121 g Tris base
	28.5 ml Acetic acid (glacial)
	50 ml EDTA (0.5 M/pH 8)
	Add ddH_2O to 1 l

2.1.3. Chemicals

2-propanol for molecular biology	AppliChem, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	AppliChem, Darmstadt, Germany
Ethanol (absolute)	AppliChem, Darmstadt, Germany
Ethidium bromide solution (0.07 %)	Sigma-Aldrich, Taufkirchen, Germany
ddH_2O for chromatography (LiChrosolv®)	Merck, Darmstadt, Germany

2.1.4. Special consumable supplies

Eppendorf reaction tubes (1.5 ml/2 ml)	Eppendorf, Hamburg, Germany
Erlenmeyer flask (10-100 ml)	Schott-Duran, Wertheim, Germany
Falcon sample tubes (15 ml/50 ml)	Greiner Bio-One, Frickenhausen, Germany
PCR reaction tube (300 µl)	Sarstedt, Nümbrecht, Germany
Pipette tips (sterile) (10/100/1000 µl)	Sarstedt, Nümbrecht, Germany
Serological pipettes (5/10/25/50 ml)	Sarstedt, Nümbrecht, Germany

2.1.5. Equipment and devices

Description	Producer	Classification
Centrifuge	Eppendorf, Hamburg, Germany	5430
Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany	MIKRO 200 R
Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany	Rotana 460 R
Gel chamber	peqLab, Erlangen, Germany	B2
Incubator	Heraeus, Hanau, Germany	B 5042 E
Microwave	Sharp, Hamburg, Germany	Express
Photometer	peqLab, Erlangen, Germany	NanoDrop ND1000
Thermocycler	Biometra, Göttingen, Germany	T Gradient
Thermocycler	Biometra, Göttingen, Germany	T Professional Thermocycler
Thermomixer	Eppendorf, Hamburg, Germany	Thermomixer comfort
UV-Photochamber	BioRad Laboratories, Munich, Germany	ChemieDoc UV Shield
Vacuum manifold	Qiagen, Hilden, Germany	QIAVac 96
Vacuum pump	Welch, Alton, Hampshire, UK	Standard Lab-Duty oil free pump
Voltage device	Consort, Turnhout, Belgium	E431
Vortex-Mixer	Eppendorf, Hamburg, Germany	Microspin FV2400
Water bath	Great Instruments, Cambridgeshire, UK	JB Aqua 12

2.1.6. Software

Software	Source
Bisearch Primer Design and Search Tool	http://bisearch.enzim.hu/ Tusnady et al. 2005 Aranyi et al. 2006
DeFinetti program	http://ihg.gsf.de/cgi-bin/hw/hwa1.pl Wienker and Strom
MethPrimer	www.urogene.org/methprimer/index.html Li LC and Dahiya R, 2002
Epigenetic Sequencing Methylation analysis software (ESME)	Lewin et al. 2004
Sequence Scanner software	Applied Biosystems by Life Technologies, Darmstadt, Germany
SPSS statistical software (version 23.0)	SPSS Inc., Chicago, IL, USA

2.2. Methods

2.2.1. STUDY I

a) Overall sample

The sample consisted of 111 social anxiety disorder patients recruited between 2005 and 2008 under supervision of Prof. Dr. A. Gerlach and Prof. Dr. Dr. K. Domschke at the Institute of Psychology, University of Muenster, Germany (f=77, m=34; age=30.1±9.9 years±SD). The diagnosis of SAD was ascertained by experienced psychiatrists and/or clinical psychologists on the basis of medical records and structured clinical interviews (SCID-I) according to the criteria of DSM-IV (Wittchen 1997). Patients were evaluated regarding possible comorbid diagnoses. Furthermore, patients were scanned for the following exclusion criteria: psychotic disorders including schizoaffective disorder, bipolar disorder, mental retardation, or neurological and neurodegenerative disorders impairing psychiatric evaluation, current or past drug or alcohol abuse or dependence and any severe somatic disorder or pregnancy in females. All patients meeting one of the described criteria were excluded from the study. All patients were of Caucasian origin (for detail see Stevens et al. 2008).

The related control group comprised 111 healthy probands matched to the patient group by sex (f=77, m=34; p=0.56) and age (30.9±10.5 years±SD; p=0.59). Healthy subjects were recruited from 2005 through 2008 in the same context as SAD patients at the Institute of Psychology, University of Muenster, Germany. Absence of DSM-IV axis I disorders was evaluated by experienced psychologists on the basis of a structured clinical interview (Mini International Neuropsychiatric Interview (MINI); SCID-I) according to the criteria of DSM-IV (Wittchen 1997). Similar to the patient cohort, all healthy probands were scanned for several exclusion criteria as current or past drug or alcohol abuse or dependence, any severe somatic disorder and pregnancy. Probands meeting one of the described criteria were excluded from the study. All healthy probands were of Caucasian origin (for detail see Stevens et al. 2008).

Dimensional measures of social anxiety severity were assessed in patients (N=110, data missing for 1 patient) and controls (N=108, data missing for 3 controls) using the German versions of the following psychometric instruments: Social Phobia Scale (SPS; Stangier et al. 1999) and Social Interaction Anxiety Scale (SIAS; Stangier et al. 1999). The SPS was originally designed to measure social phobia in the context of activities in the presence of other people, whereas the SIAS measures social phobia in situations of meeting, talking, or interacting with other people (Mattick and Clarke 1998). Thereby, the two measures were used to differentiate between scrutiny fears and worries about interaction (Peters 2000).

b) Trier Social Stress Test (TSST) sample

This sample was recruited by Prof. Dr. J. Hoyer and Dr. D. Bräuer at the Institute of Psychology, University of Dresden, Germany as an independent sample of healthy subjects (N=20, f=9, m=11; age=26.7±9.0 (years±SD)) for analysis of a possible association of *OXTR* methylation and salivary cortisol response to a standardized psychosocial stress situation the Trier Social Stress Test (TSST; Kirschbaum et al. 1993).

Subjects were included under absence of any lifetime diagnosis of psychiatric disorders indicated by the stem questions of the Composite International Diagnostic Interview (DIA-X/M-CIDI, Wittchen and Pfister 1997). Further inclusion criteria were: no participation in a study applying the Trier Social Stress Test before, and smoking of less than ten cigarettes per day (Kirschbaum et al. 1992). Regarding the TSST procedure, all female participants were tested in the luteal phase of their menstrual cycle (Kirschbaum et al. 1999) and all participants were requested to abstain from alcohol the evening before and on the day of testing, they were asked to have a regular meal on the testing day, and to avoid smoking, drinking, and eating 2 h before arriving at the laboratory of the Institute of Psychology, University of Dresden, Germany.

The stress paradigm was conducted by Dr. D. Bräuer at the Institute of Psychology, University of Dresden, Germany and is described in detail in the thesis-related publication (Ziegler et al. 2015).

c) fMRI sample

Twenty-five female patients (age=28.8±8.3 years±SD) diagnosed with current social anxiety disorder according to DSM-IV criteria (SCID-I) represented a subsample of the overall sample of SAD patients described above and additionally underwent an fMRI experiment assessing amygdala responsiveness to social phobia-relevant verbal stimuli. All patients were recruited according to the inclusion and/or exclusion criteria as described for the overall sample and did not receive any psychotropic medication including benzodiazepines. Further inclusion criteria were: absence of comorbid diagnosis of a current major depressive episode or generalized anxiety disorder, as well as no neurological illnesses or history of seizures or head trauma, and fulfilment of the general MRI restrictions. All participants were German native speakers and had normal or corrected-to-normal vision. This patient subsample and the fMRI task have been detailed elsewhere (Laeger et al. 2014), with patients showing greater amygdala responsiveness to social phobia- related words than to generally negative words when compared with healthy controls. The fMRI task was conducted by Dr. I. Laeger under

supervision of Prof. Dr. Dr. U. Dannlowski and Prof. Dr. P. Zwanzger at the University of Münster, Germany, and is described in detail in the thesis-related published manuscript (Ziegler et al. 2015).

d) Ethics

Written informed consent was obtained from all participants prior to inclusion in the study, which was approved by the ethics committees of the Universities of Muenster and Dresden, Germany, and conducted according to the ethical principles of the Helsinki Declaration.

2.2.2. STUDY II

a) Discovery sample

For the discovery sample, 28 female patients with PD (age (mean±SD): 34.57±8.51 years) with (N=14; 50%) or without agoraphobia were recruited at the Department of Psychiatry, University of Würzburg, Germany, within the Collaborative Research Centre SFB-TRR-58 'Fear, Anxiety, Anxiety Disorders', subproject C02 explicitly for this study by Dipl.-Psych. M. Mahr and Dr. A. Gajewska under supervision of Prof. Dr. Dr. K. Domschke. Based on the female-specific associations of *MAOA* variation (VNTR, Reif et al. 2014) and *MAOA* methylation patterns with panic disorder (Domschke et al. 2012), as well as the X-chromosomal location of the *MAOA* gene entailing hemizygosity in men, all analyses were restricted to all-female samples of panic disorders patients (Ziegler et al. 2016).

Diagnosis of panic disorder with or without agoraphobia was ascertained by experienced psychiatrists and/or clinical psychologists on the basis of a structured clinical interview (SCID-I). The occurrence of comorbid axis I diagnoses except bipolar disorder, psychotic disorders, current alcohol dependence, current abuse or dependence on benzodiazepines and other psychoactive substances were allowed if panic disorder was the primary diagnosis. Medicated patients (N=19) were only included in the study, if received medication was stable for at least 2 weeks and furthermore if pharmacological treatment remained unmodified during the course of the therapy.

Patients were excluded from the study if they met at least one of the following exclusion criteria: current or previous internal or neurological somatic illnesses, any somatic medication, consumption of illegal drugs including cannabis (assessed by urine toxicology), pregnancy and excessive alcohol (more than 15 glasses of alcohol per week) or nicotine (more than 20 cigarettes per day) use, and non-Caucasian

background (for at least two proceeding generations). Smokers included in the study where instructed to keep smoking behaviour (number of smoked cigarettes per day) constant during the time course of therapy. Because smoking behaviour has been shown to influence *MAOA* methylation (Philibert et al. 2010), smoking status was documented in detail with the total number of smoked cigarettes per day during the last 4 weeks. Nineteen patients were classified as smokers, the mean number of smoked cigarettes per day in the discovery sample was 4.64±7.26 (mean±SD).

Patients underwent a standardized cognitive behavioural psychotherapy (CBT) over six-weeks (see below; 2.2.2 (c)) and were evaluated at T0 and T1 (post-treatment).

The corresponding control group consisted of healthy female subjects (N=28) recruited at the Department of Psychiatry, University of Würzburg, Germany, within the Collaborative Research Centre SFB-TRR-58 'Fear, Anxiety, Anxiety Disorders', subprojects C02 and Z02 explicitly for the present study by Dipl.-Psych. M. Mahr and Dipl.-Psych. M. A. Schiele, under supervision of Prof. Dr. J. Deckert, Prof. Dr. A. Reif, Prof. Dr. P. Pauli and Prof. Dr. Dr. K. Domschke. All healthy controls were matched to the discovery patient sample by age and smoking status according to the number of smoked cigarettes. Healthy probands were included based on the absence of mental axis 1 disorders (assessed by experienced psychologists on the basis of a SCID (Mini International Neuropsychiatric Interview (MINI)) according to the criteria of DSM-IV). The same exclusion criteria as listed for the patient sample were used for the control sample. Healthy volunteers were evaluated at T0 and - in parallel to the course of CBT in the discovery sample - after a 6-week waiting period (T1).

b) Replication sample

The replication sample comprised 20 female patients as part of a total sample of 154 patients recruited within the second multicenter (Greifswald, Münster, Würzburg, Bremen, Marburg) clinical trial of the MAC study within the BMBF network 'Improving the Treatment of Panic Disorder' (Gloster et al. 2011). For these 20 female patients (age (mean±SD): 33.55±11.15 years) with a primary diagnosis of PD with (N=14; 70%) or without agoraphobia, DNA samples were available for two time points (T0=pre- and T1=post-therapy). These 20 patients were thus used as an independent replication sample for the above mentioned discovery sample. Diagnoses were established using a standardized computer-administered face-to-face interview (CAPI-WHO-CIDI). CIDI was conducted by trained expert interviewers who were certificated and supervised by certified CIDI assessors of the clinical coordination centre (Bremen).

Inclusion criteria were: Caucasian descent, a current primary diagnosis of panic disorder with or without agoraphobia, age between 18–65 years, ability and availability to regularly attend treatment sessions, a score of more than four on the Clinical Global Impression scale (CGI, Busner and Targum 2007).

A comorbid axis I diagnosis (except bipolar disorder, psychotic disorders, current alcohol dependence, current abuse or dependence on benzodiazepines and other psychoactive substances) was allowed, if PD with or without agoraphobia was the primary diagnosis. Patients were excluded if they displayed current suicidal intent, were diagnosed with borderline personality disorder, or received ongoing psychotherapeutic or psychopharmacological treatment for any mental disorder. Furthermore, patients with severe cardiovascular, renal and neurological diseases as important limitations for exposure-based cognitive behavioural therapy were excluded from the study. Similar to the discovery sample, smoking status was ascertained in detail with the total number of smoked cigarettes per day during the last 4 weeks. Ten patients were classified as smokers (50%); the mean number of smoked cigarettes per day in the overall sample was 6.10±7.82 (mean±SD). Given the exclusion criteria, none of the patients received any kind of drugs including psychiatric medication.

c) Cognitive behavioural therapy (CBT)

The used psychotherapy setup for panic disorder patients of the discovery sample in a regular outpatient clinical setting comprised six semi-standardized sessions over approximately six weeks and was designed as to a shortened version of the exposure-based CBT manual as applied in the ‘Mechanisms of Action for CBT’ (MAC) study within the BMBF network Improving the Treatment of Panic Disorder’ (Gloster et al. 2011). The therapists (Dipl.-Psych. M. Mahr and Dr. A. Gajewska) were experienced graduate or clinical psychologists having participated in a training workshop on this manual and were additionally involved in weekly supervision to maintain therapy integrity during the course of this study. The therapy settings are described in detail in the thesis-related publication (Ziegler et al. 2016). In brief, the first three therapy sessions covered psychoeducational information followed by a second three-session block comprising interoceptive exercises for all patients. Furthermore, these sessions were accompanied by intensive homework adapted to the individual´s particular fears of situations.

Within the replication sample, all patients followed a 12-session written manualized treatment protocol focusing on *in situ* exposure to target avoidance

behaviour. The content, structure and doses of therapy were identical to the published MAC study (Gloster et al. 2011).

d) Outcome criteria

With regard to the interoceptive exposure in the short-term, proof-of-principle treatment design (six sessions in six weeks) for patients of the discovery sample, the reduction of panic attacks per se rather than the reduction of avoidance behaviour was supposed to be the primary indicator of disease severity and treatment response. Therefore, all patients were grouped into “responders” and “non-responders” according to the change in the number of panic attacks per week assessed before (T0) and after (T1) therapy, respectively. Patients reporting a decrease in the number of experienced attacks at T1 compared with T0 (T1–T0 < 0) were defined as responders (N=11), while patients characterized by no change in the number of panic attacks or even more attacks after CBT (T1–T0⩾0) were defined as non-responders (N=17). In addition, the Mobility Inventory (MI) was ascertained as a complementary psychometric index. The MI is a self-report questionnaire measuring agoraphobic avoidance in specific situations with (MI-Accompanied subscale) or without (MI-Alone subscale) company of a trusted person (Chambless et al. 1985).

Similar to the discovery sample, responders (N=8) and non- responders (N=8) to CBT were defined according to the difference in number of panic attacks at T1 (post therapy) compared with T0 (baseline). In addition and with regard to the intensified exposure-based CBT mainly targeting avoidance behaviour, the MI score - as particularly suitable to measure changes of pathological avoidance behaviour in patients with panic disorder and comorbid agoraphobia (Chambless et al. 1985) - was chosen as the primary indicator of disease severity and treatment response for the replication sample.

e) Ethics

For the discovery sample, the study was approved by the ethics committee of the University of Würzburg, Germany, and was conducted according to the ethical principles of the Helsinki Declaration. Written informed consent was obtained from all patients and controls prior to inclusion in the study. With regard to the replication sample, all patients gave written informed consent after receiving a detailed description of the study program. This study was approved by the Ethics Committee of the German Psychological Society and was conducted according to the ethical principles of the Helsinki Declaration.

2.3. Blood collection

2.3.1. STUDY I

Venous blood was collected using EDTA (Ethylenediaminetetraacetic acid)-coated tubes (Sarstedt, Nümbrecht, Germany), which were stored at -20°C immediately after blood collection until DNA isolation at the Department of Psychiatry, University of Muenster, Germany, or the Department of Psychology, Technical University of Dresden, respectively (see 2.2.1, Trier Social Stress Test (TSST) sample).

2.3.2. STUDY II

Venous blood of all patients and controls included in the discovery sample was collected at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, before (T0, baseline) and after CBT (T1, day of last CBT session) using EDTA-coated tubes (Sarstedt, Nümbrecht, Germany), which were stored at -80°C immediately after blood collection to prevent degradation of DNA itself as well as of distinct DNA methylation patterns until DNA isolation (see 2.4). In the control sample, blood was taken at T0 and - in parallel to the course of CBT in the patients of the discovery sample - after a 6-week waiting period (T1).

2.4. DNA Extraction from frozen whole blood samples

DNA was isolated from EDTA-blood samples using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) in batches of 24-48 samples, and following the protocol "Isolation of DNA from 4-14 ml Whole Blood" as part of the manufacturer's instruction (FlexiGene-DNA-Handbook, Qiagen, Hilden, Germany) with minor modifications. The buffer volumes were adapted for DNA isolation from blood samples of 8 ml.

All frozen blood samples were thawed quickly at 37°C in a water bath (JB Aqua 12, Great Instruments, Cambridgeshire, UK) prior to the DNA isolation procedure. Furthermore, lyophilized QIAGEN Protease was resuspended in 1.4 ml FG3 hydration buffer and mixed with FG2 Buffer to a Buffer FG2/QIAGEN Protease mixture.

After all blood samples were thawed completely, 20 ml Buffer FG1 were pipetted into labelled 50 ml centrifugation tubes (Greiner Bio-one, Frickenhausen, Germany) and mixed with 8 ml whole blood by inverting the tube five times. The tubes were centrifuged at 3000 rpm in a swing-out rotor (Hettich, Tuttlingen, Germany) for 15 min. The supernatant was discarded and the tube was left on a clean sheet of absorbent paper for 2 min making sure that the pellet remained in the tube. This

handling minimizes the backflow of supernatant which may inhibit the following protein lysis from the rim and sides of the tube onto the pellet. Now, 4 ml freshly prepared Buffer FG2/QIAGEN Protease mixture was pipetted onto the pellet and vortexed immediately until the pellet was completely homogenized. After all tubes were vortexed, they were inverted another three times and placed into a water bath for protein digestion at 65°C for at least 10 min, but 15 min maximum. The samples changed colour from red to green which indicated successful protein digestion. After that, 4 ml isopropanol (2-propanol for molecular biology, AppliChem, Darmstadt, Germany) were added to the sample, and the DNA precipitated by inverting the tubes at least 20 times or until the precipitate became visible as threads or a clump. The pellet the precipitated DNA samples were centrifuged for 15 min at 3000 rpm in a swing-out rotor. The supernatant was discarded slowly and the tubes were inverted on a clean sheet of absorbent paper to remove remaining quantities of isopropanol. Afterwards, 4 ml 70% ethanol (for analysis, AppliChem, Darmstadt, Germany)) were added, and samples were vortexed for 5 s. To pellet the precipitated DNA again, samples were centrifuged at 3000 rpm for 15 min. The supernatant was discarded slowly and tubes were inverted on a clean sheet of paper for at least 5 min to remove all remaining ethanol quantities. All pellets were air-dried for additional 5 min followed by addition of 800 µl Buffer FG3. This buffer served as storage buffer for DNA samples, which were completely dissolved in this buffer by heating the sample to 65°C for 1 h in a water bath. After complete dissolution DNA sample were transferred to clean labelled 1.5ml reaction tubes (Eppendorf, Hamburg, Germany) and stored at -80°C until further use (e.g. for bisulfite conversion, see 2.5.1. ,or PCR reaction, see 2.6 and 2.7). DNA isolation for all samples of this STUDY I were conducted in the Laboratory of “Clinical Genetics”, Department of Psychiatry, University of Muenster, Germany, except for blood samples of the sub-study conducted at the Technical University of Dresden (see 2.2.1). The samples were stored at -80°C until shipment to the laboratory of “Functional Genomics”, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany. All DNA samples from STUDY II (see 2.2.1., Trier Social Stress Test (TSST) sample) were isolated and stored in the laboratory of “Functional Genomics”, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany.

2.5. DNA methylation analysis

2.5.1. Bisulfite conversion of human genomic DNA

a) STUDY I

The EZ-DNA Methylation-Gold^{TM} Kit (Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany) is based on a three-step reaction process which converts un-methylated cytosine into uracil using sodium bisulfite in a single spin-column setup. The procedure was performed according to manufacturer's instruction with minor modifications.

All genomic DNA samples (isolated from human whole blood samples, see 2.4) were diluted to a concentration of 22.5 ng/µl with ddH_2O (Merck, Darmstadt, Germany) in a total volume of 20 µl (450 ng) prior to bisulfite conversion. All components of the EZ-DNA Methylation-Gold^{TM} Kit (Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany) were prepared according to the manufacturer's instructions prior to use. In brief, CT Conversion reagent was dissolved in 900 µl ddH2O, 300 µl M-Dilution buffer and 50 µl M-Dissolving buffer by shaking it at 1400 rpm and 37°C for 10 min on a horizontal shaker (Eppendorf, Hamburg, Germany). Furthermore, M-wash buffer concentrate was diluted with 96 ml Ethanol (absolute, Sigma-Aldrich, Taufkirchen, Germany).

Each DNA sample (450 ng) was mixed with 130 µl freshly prepared CT conversion reagent in single PCR reaction tubes (Sarstedt, Nümbrecht, Germany) for small sample sizes (e.g. optimization purpose or conversion of control DNA) or in 8-strip format PCR tubes (Sarstedt. Nümbrecht, Germany) for larger sample sizes (e.g. conversion of all samples of one study in one batch). Bisulfite conversion was performed in a standard thermal cycler (T Professional Thermocycler. Biometra, Göttingen, Germany) by heating the samples for 10 min to 98°C, followed by incubation for 4 h at 53°C. After completing bisulfite conversion, samples were cooled down to 4°C and stored at this temperature until further processing.

Bisulfite converted DNA samples were cleaned up following the recommended protocol using a spin-column technology and a microcentrifuge (Hettich, Tuttlingen, Germany). In detail, 600 µl M-Binding buffer were pipetted into each spin column followed by whole reaction volume from bisulfite conversion reaction (approx. 150 µl). Both contents were mixed well by inverting the spin column several times (min. 5). Spin columns were centrifuged full speed (14,000 rpm for the described centrifuge) for 30 s and the flow trough was discarded. The membrane of the spin column containing bisulfite converted DNA was washed with 100 µl M-Wash Buffer by centrifugation at full

speed for 30 s. In a next step, bisulfite converted DNA was desulphonated to eliminate DNA precipitates by adding 200µl M-Desulphonation buffer to the spin column followed by 15 to 20 min incubation at room temperature (RT). During this incubation all spin columns were put into a dark place (e.g. a cupboard) to prevent the reaction from light. After this, 200 µl M-Wash Buffer were added to the spin column and the flow trough was discarded after centrifugation at full speed for 30 s. This step was repeated, the flow through discarded and the membrane of the spin column was dried by centrifugation at full speed for 2 additional min. This drying process minimizes ethanol and salt carry over. Finally, cleaned-up bisulfite converted DNA was eluted into a 1.5 ml reaction tube (Eppendorf, Hamburg, Germany) by pipetting 20 µl ddH2O directly on the membrane, incubating the columns for 10 min at RT and centrifuging the samples at full speed for 1 min. The obtained bisulfite converted DNA samples were directly separated into 1.5 µl aliquots and stored at -20°C until further use (e.g. bisulfite-PCR, see 2.5.3).

b) STUDY II

Due to refinement of the procedure of bisulfite conversion during the course of this thesis the used bisulfite conversion kit was changed to EpiTect® 96 Bisulfite Kit (Qiagen, Hilden, Germany). This kit allowed for complete bisulfite conversion and clean-up of genomic DNA for methylation analysis in 96-well format. Furthermore, using the EpiTect® 96 Bisulfite Kit (Qiagen, Hilden Germany) yielded higher concentrations of bisulfite converted DNA, which additionally was stable at -20°C for a longer time as compared to samples obtained from EZ-DNA Methylation-Gold™ Kit (Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany). The procedure was performed according to the protocol for “Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Using a Vacuum Manifold” as part of the manufacturer’s instruction (EpiTect-96-Bisulfite-Handbook, Qiagen, Hilden, Germany).

All genomic DNA samples (isolated from human whole blood samples, see 2.3.) were diluted to a concentration of 25 ng/µl with ddH_2O (Merck, Darmstadt, Germany) in a total volume of 20 µl (500 ng) prior to bisulfite conversion. All components of EpiTect® 96 Bisulfite Kit were prepared according to the manufacturer’s instructions prior to use. In brief, Buffer BW concentrate was diluted with 120 ml ethanol (absolute, 99 %, Sigma-Aldrich, Taufkirchen, Germany), Buffer BD concentrate was diluted with 27 ml ethanol (absolute, Sigma-Aldrich, Taufkirchen, Germany), lyophilized carrier RNA was dissolved in 1350 µl RNase-free water and split into two 675 µl aliquots, Buffer BL was heated to approx. 55°C to dissolve precipitates, and 600 µl of the

dissolved carrier RNA was added to this buffer. All provided components as well as DNA samples were equilibrated to room temperature.

The protocol is divided into two parts: bisulfite DNA conversion and clean-up of the bisulfite converted DNA. For bisulfite DNA conversion, the provided Bisulfite Mix was prepared by dissolving it in 9 ml RNase-free water and vortexing the mixture for at least 5 min. To guarantee complete dissolution, the mixture was heated to 60°C and vortexed again. The bisulfite reaction mixture was assembled in the provided EpiTect Conversion Plate as follows: 20 µl DNA sample (500 ng), followed by 85 µl dissolved Bisulfite Mix and 35 µl DNA Protect Buffer (consequently changed colour from green to blue). All components were mixed well by pipetting. The EpiTect Conversion Plate was sealed securely using the EpiTect Cover Foil and centrifuged briefly at 650 xg in a plate centrifuge (Centrifuge 5430, Eppendorf, Hamburg, Germany) to collect the reactions in the bottom of the wells. Subsequently, bisulfite conversion was performed using a standard thermal cycler (T Professional Thermocycler, Biometra, Göttingen, Germany) programmed according to Table 1.

Table 1: Thermal cycler conditions for bisulfite conversion using the EpiTect® 96 Bisulfite Kit (Qiagen, Hilden, Germany).

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min	60°C
Denaturation	5 min	95°C
Incubation	175 min	60°C
Hold	Indefinite (up to overnight)	20°C

After that, bisulfite converted DNA was cleaned up according to manufacturer's protocol using a QIAvac 96 vacuum manifold (Qiagen, Hilden, Germany) assembled with a vacuum pump (Welch, Alton, Hampshire, UK). First, the EpiTect Conversion Plate containing the bisulfite reactions was briefly centrifuged at 650 xg. The vacuum manifold was prepared according to manufactures instruction (QIAvac 96 vacuum manifold handbook, Qiagen, Hilden, Germany) and connected to a vacuum pump (Welch, Alton, Hampshire/UK). An EpiTect 96 Plate was placed securely onto the vacuum manifold. Now, 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA were dispensed into the wells of the EpiTect 96 Plate followed by transferring of complete bisulfite reactions to the EpiTect 96 Plate and mixing with the Buffer BL by pipetting up and down four times. After that, vacuum source was switched on until all

liquid had passed through the membrane. Vacuum sourced was switched off and 500 µl Buffer BW were added carefully to each well. Again, vacuum source was switched on until all liquid had passed and switched off afterwards. For desulphonation of the samples, 250 µl Buffer BD were added to each well followed by incubation for 15 min at room temperature. After that time, vacuum source was switched on until all liquid had passed through and switched off again. Now, the membranes were washed twice with 500 µl Buffer BW by adding the required volume to each well, switching the vacuum source on until all liquid has passed and switching it off again. This step was repeated once. To remove remaining quantities of Buffer BW all wells were washed with 250 µl ethanol (absolute, Sigma-Aldrich, Taufkirchen, Germany). After ethanol in all wells had passed through the membrane, maximum vacuum was applied for additional 10 min to dry the membrane and remove residual ethanol. Now, the vacuum manifold was ventilated slowly and the top plate of the manifold together with the EpiTect 96 plate was lifted and latter was tapped vigorously on clean absorbent paper to remove residual ethanol from the nozzles of the plate. To elute the bisulfite converted DNA into the provided elution plate, the waste tray was removed from the vacuum manifold and replaced by the elution plate together with a vacuum manifold adapter for elution plates. After placing the top plate together with the EpiTect 96 plate back onto the manifold, 70 µl of Buffer EB and 10 µl Top Elute Fluid were dispensed directly to the centre of the membrane of each well. The vacuum source was switched on for a maximum of 1 min. After that, the vacuum source was switched off and the manifold was ventilated slowly. The elution plate was sealed for storage using the provided Tape Pads and stored at -20°C until further use.

2.5.2. Design of DNA methylation assays

a) Oxytocin receptor (*OXTR*) gene

The *OXTR* gene is located on chromosome 3p25-3p26.2 and consists of four exons with the translation start site (ATG) in exon 3 and the stop codon (TGA) in exon 4. As target sequence for DNA methylation analysis an amplicon localized in exon 3 of the *OXTR* gene was chosen. An analogous amplicon has been described in a previous study on *OXTR* gene methylation dynamics within a social stress test setting (Unternaehrer et al. 2012). The chosen amplicon spans the genomic region Chr3: 8 809 281–8 809 534 (UCSC Human Genome Browser; February 2009; GRCh37/hg19) and contains 22 CpG sites. Due to technical difficulties particularly at the 5’ and 3’ ends of the sequence, only 12 of 22 CpG sites were robustly readable regarding their DNA methylation status. Location and detailed sequence information are summarized in

Figure 1; genomic positions for all 12 analysed CpG sites as well as analogous CpG sites from Unternaehrer et al. (2012) are given in Table 2.

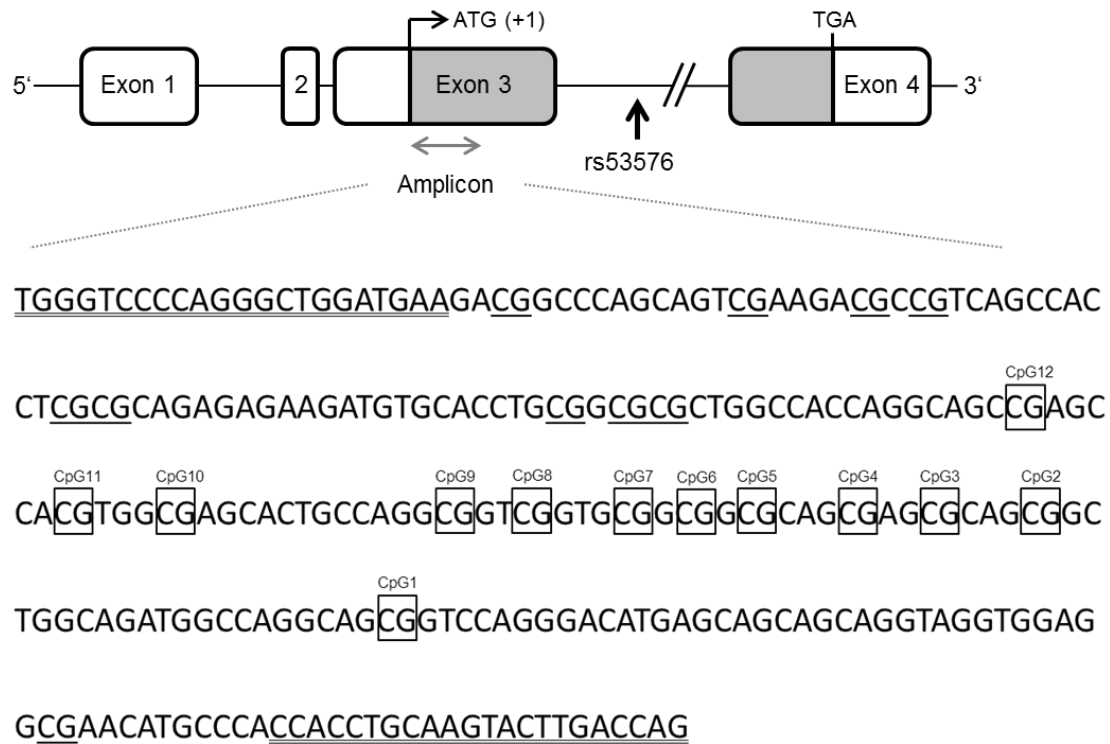

Figure 1: Schematic illustration of the *OXTR* gene. The gene consists of four exons with the translation start site (ATG (+1)) in exon 3 and the stop codon (TGA) in exon 4. The analysed SNP *OXTR* rs53576 is localized in the third intronic region (not to scale). The amplicon for methylation analysis (not to scale) is located within the protein coding region of *OXTR* exon 3. The sequence of the amplicon is displayed as per GRCh37 build, National Center for Biotechnology Information (NCBI) reference sequence NC_000017.10 (Chr3:8 809 281–8 809 534), with double underlined primer pair binding sites. CpG sites 1–12 analysed in STUDY I are marked with boxes, CpG sites not analysed due to technical difficulties are underlined.

Table 2: Analysed CpG sites within *OXTR* exon 3. CpG sites are numbered according to their position within the amplicon; analogous CpG sites from previous publication (Unternaehrer et al. 2012) as well as their genomic position are shown.

CpG site in the present study (Ziegler et al. 2015)	Analogous CpG site as published in (Unternaehrer et al. 2012)	Genomic position (UCSC Human Genome Browser; February 2009; GRCh37/hg19)
CpG1	CpG2	Chr3: 8 809 464
CpG2	CpG3	Chr3: 8 809 442
CpG3	CpG4	Chr3: 8 809 437
CpG4	CpG5	Chr3: 8 809 433
CpG5	CpG6	Chr3: 8 809 428
CpG6	CpG7	Chr3: 8 809 425
CpG7	CpG8	Chr3: 8 809 422
CpG8	CpG9	Chr3: 8 809 417
CpG9	CpG10	Chr3: 8 809 413
CpG10	CpG11	Chr3: 8 809 399
CpG11	CpG12	Chr3: 8 809 394
CpG12	CpG13	Chr3: 8 809 387

b) Monoamine oxidase A (*MAOA*) gene

The human *MAOA* gene is located on chromosome Xp11.4-p11.3 and its promoter region as well as exon 1 and intron 1 have been studied previously regarding their DNA methylation pattern (Domschke et al. 2012; Domschke et al. 2015; Philibert et al. 2008). For this study, an amplicon comprising *MAOA* exon 1 and parts of intron 1 (chromosome X, GRCh38.p2 Primary Assembly, NCBI Reference Sequence: NC_000023.11, 43656260–43656613) was chosen for DNA methylation analyses in analogy to previous studies on *MAOA* methylation (Domschke et al. 2012; Domschke et al. 2015) covering the CpG sites most significantly associated with panic disorder (amplicon C in Domschke et al. 2012). DNA methylation of 13 CpG sites in the described amplicon C were analysed in patients of the discovery sample as well as in the healthy control sample matched to the discovery sample (CpGs 1–13). Due to technical difficulties, only 12 of these CpG sites could be analysed in the replication sample (CpGs 2–13). CpG sites were numbered according to their position in the amplicon as well as in analogy to a previous study on *MAOA* methylation in panic disorder patients (Domschke et al. 2012): CpG1=43,656,316; CpG2= 43,656,327; CpG3=43,656,362; CpG4=43,656,368; CpG5=43,656,370; CpG6=43,656,383; CpG7=43,656,386; CpG8=43,656,392; CpG9= 43,656,398; CpG10=43,656,427;

CpG11=43,656,432; CpG12=43,656,514; CpG13=43,656,553. All genomic positions refer to UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) assembly.

2.5.3. Polymerase chain reaction (PCR)

a) Oxytocin receptor (*OXTR*) gene

The described amplicon (see 2.5.2 (a)) was amplified from bisulfite-converted DNA (see 2.5.1) using polymerase chain reaction (PCR) with oligonucleotide primers (Metabion, Steinkirchen, Germany) specifically designed for bisulfite-converted DNA (forward primer: 5’-TTGGTTAAGTATTTGTAGGTGGTG-3’ and reverse primer: 5’-TAAATCCCCAAAACTAAATAAAAAC-3’). All required components were assembled to generate a PCR-premix (Table 3). Eighteen µl PCR-premix were dispensed in each well of a 96-well MTP. Afterwards, 2 µl bisulfite converted DNA were added to each well. All DNA samples were amplified in duplicates to account for run variability. PCR reaction was conducted in a thermal cycler (Biometra, Göttingen, Germany) programmed according to the following protocol: initial denaturation at 94°C for 3 min, 40 cycles of 1 min at 94°C, 1 min at 59.5°C (primer annealing), 2 min at 72°C, and final elongation at 72°C for 5 min. All PCR products were stored at 4°C. All generated PCR products were proven to have the predicted length of 253 bp as well as sufficient quality for sequencing using gel electrophoresis (see 2.5.4). Furthermore, non-template controls (ddH_2O) were used to account for possible cross-contaminations. Additionally, non-methylated and fully methylated commercially available DNAs (Human Methylated & Non-methylated DNA Set, Zymo Research, HiSS Diagnostics GmbH, Freiburg im Breisgau, Germany) were used to control for complete bisulfite conversion.

Table 3: Components of PCR mix used for amplification of bisulfite converted DNA.

Component	Amount in µl for one reaction
$MgCl_2$ (25 mM)	1
Forward primer (10 µM)	0.8
Reverse primer (10 µM)	0.8
HotStarTaq Plus Master Mix	10
ddH_2O	5.4
Bisulfite-converted DNA	2
Total volume	20

b) Monoamine oxidase A (*MAOA*) gene

The chosen amplicon (see 2.5.2 (b)) was amplified from bisulfite-converted DNA (see 2.5.1) using polymerase chain reaction (PCR) with oligonucleotide primers (Metabion; Steinkirchen, Germany) specifically designed for bisulfite-converted DNA (forward primer: 5'- GGGGAGTTGATAGAAGGGTTTTTTTTAT-3' and reverse primer: 5'-TATATCTACCTCCCCCAATCACACC-3'). All required components were assembled to generate a PCR-premix (Table 3). Eighteen µl PCR-premix were dispensed in each well of a 96-well MTP. Afterwards, 2 µl bisulfite converted DNA were added to each well. All DNA samples were amplified in duplicates to account for run variability. PCR reaction was conducted in a thermal cycler (Biometra, Göttingen, Germany) programmed according to the following protocol: initial denaturation at 94°C for 3 min, 40 cycles of 1 min at 94°C, 1 min at 64°C (primer annealing), 2 min at 72°C, and final elongation at 72°C for 5 min. All PCR products were stored at 4°C. All PCR products were verified to have the predicted length of 353 bp as well as sufficient quality for sequencing using gel electrophoresis (see 2.5.4). Furthermore, non-template controls (ddH_2O) were used to account for possible cross-contaminations. Additionally, non-methylated and fully methylated commercially available DNAs (Human Methylated & Non-methylated DNA Set, Zymo Research, HiSS Diagnostics, Freiburg im Breisgau, Germany) were used to control for complete bisulfite conversion.

2.5.4. Gel electrophoresis

For quality control of PCR products, 2% agarose gels were prepared by dissolving 2 g agarose (peqGold, Peqlab, Erlangen, Germany) in 100 ml Tris-Acetat-EDTA (TAE) buffer by heating the solution in a microwave (Sharp, Hamburg, Germany). After the agarose-TAE solution was cooled down to approximately 60°C, 3 µl ethidium bromide solution (EtBr, Sigma-Aldrich, Germany) - serving as a DNA intercalating dye to visualize DNA fragments using ultraviolet (UV) light. - were added. Afterwards, the agarose solution was poured into the gel tray, which had been prepared by placing a gel chamber and a suitable comb into a casting system. After the agarose mixture had polymerized, the comb was removed carefully and the gel was placed in an electrophoresis chamber and covered with TAE-buffer. Loading buffer needed to be added to the samples before loading them to the gel. In addition to the samples, a standard marker (100 bp Plus Gene Ruler, Thermo Scientific, Munich, Germany) was loaded on the gel. Electrophoresis was performed at 120 V for approximately 45 min, and the fragments were visualized as bands on the gel using an UV light imaging system (ChemiDoc, BioRad Laboratories, Munich, Germany). The length of the fragments was estimated by means of the used DNA marker.

2.5.5. Direct sequencing

All amplified PCR products were purified by EXO/SAP (Exonuclease I/Shrimp Alkaline Phosphatase) clean-up and sequenced by LCG Genomics (Berlin, Germany). The sequencing services were delivered on ABI 3730 XL platforms for traditional Sanger sequencing. The provided electropherograms were analysed regarding quality and reliability using Sequence Scanner software (Applied Biosystems by Life Technologies, Darmstadt, Germany). After passing quality control, sequences were analysed using the Epigenetic Sequencing Methylation analysis tool ESME (see 2.5.6).

2.5.6. Epigenetic Sequencing Methylation analysis tool – ESME

The obtained sequence files (.ab1 format) were quantitatively analysed by determining relative peak heights (C/C+T) from the normalized sequence trace files using Epigenetic Sequencing Methylation analysis software (ESME). This freely available software was specifically designed and evaluated for artificially generated single nucleotide polymorphisms (C/T and G/A). It automatically performs quality control, normalizes signals, corrects for incomplete bisulfite conversion and aligns the generated bisulfite sequence and a genomic reference sequence to compare C to T peak heights at CpG sites displaying the respective methylation status of each CpG site (Lewin et al. 2004). This software has proven to generate reliable results for the analysis of methylation profiles in other psychiatric disorders (Alasaari et al. 2012; Domschke et al. 2013; Domschke et al. 2014; Domschke et al. 2015; Tadic et al. 2013). To account for run variability, all samples were tested in duplicate, yielding a mean individual methylation score for each CpG, as well as an individual standard deviation (SD) for each duplicate. The SD of each duplicate was used as a first step of quality control with methylation values of duplicates with SD>0.1 set as missing values. In a second step, outliers (⩾3 SD from mean methylation of the respective CpG site) were defined as missing data. A cut-off of >20% of missing data was defined as an exclusion criterion for the whole sample.

2.6. Genotyping of *OXTR* rs53576

The single nucleotide polymorphism (SNP) rs53576 is localized in the third intronic region of the gene and was investigated in addition to *OXTR* DNA methylation. To determine rs53576 genotypes in STUDY I, genomic DNA isolated from whole blood was amplified by PCR. The PCR premix was assembled as indicated in Table 4 using the following oligonucleotide primers (Metabion, Steinkirchen, Germany): forward primer: 5’-ATTTGTACCCAGAAGGGCCG-3’ and reverse primer: 5’-ACAGAACTGGCAACCTGGAA-3’. Twenty-four µl premix were dispensed into each

well of a 96-well MTP. Afterwards, 1 µl genomic DNA was added and PCR reaction was conducted in a thermal cycler (T Professional Thermocycler, Biometra, Göttingen, Germany) programmed according to the following protocol: initial denaturation at 95°C for 3 min, 45 cycles of 45 s at 95°C, 45 s at 62.5°C (primer annealing), 45 s at 72°C, and final elongation at 72°C for 5 min. All PCR products were digested for 3 h at 37°C using the restriction endonuclease BamHI (New England Biolabs, Frankfurt am Main, Germany). Digested PCR products were separated on a 3% agarose gel and visualized by ethidium bromide staining using ultraviolet light (see 2.5.4). The digest resulted in distinct DNA fragments which differed in size according to the respective genotype of each sample. Homozygous G-allele carriers were characterized by a 372 bp fragment, heterozygous A/G carriers by three fragments with 372 bp, 196 bp and 172 bp, while homozygous A-allele carriers were represented by two fragments with 196 bp and 172 bp length.

Table 4: Components of PCR mix used for amplification of genomic DNA. Used oligonucleotide primers as well as PCR buffer conditions optimized for genotyping of *OXTR* rs53576.

Component	Amount in µl for one reaction
PCR buffer (15mM $MgCl_2$)	2.5
Nucleotides (2.5mM each)	1
Forward primer (10µM)	1
Reverse primer (10µM)	1
HouseTaq polymerase	0.3
ddH_2O	18.2
Genomic DNA	1
Total volume	25

2.7. Genotyping of *MAOA* VNTR

Isolated genomic DNA from all participants of STUDY II was additionally genotyped for the *MAOA* VNTR (variable number tandem repeat). DNA isolated from whole blood was amplified by PCR with the following oligonucleotide primers (Metabion, Steinkirchen, Germany): forward primer: 5'-AGCCTGACCGTGGAGAAGG-3' and reverse primer: 5'-GGACCTGGGCAGTTGTGC-3', which flank the polymorphic region covering all variants of the VNTR. Twenty-four µl premix (for details please see Table 5) were dispensed into each well of a 96-well MTP. Afterwards, 1 µl genomic DNA was added, and PCR reaction was conducted in a thermal cycler (Biometra, Göttingen, Germany) programmed according to a special touchdown PCR protocol as follows: initial denaturation at 95°C for 3 min, 6 cycles with 94°C for 40 s, 70°C for 40 s and 72°C for 40 s (annealing temperature decreased about 1°C each cycle), 35 cycles

of 40 s at 94°C, 40 s at 63°C, and 40 s at 72 °C for 35 cycles, finalized by elongation at 72°C for 5 min. All PCR products were stored at 4°C. PCR products were run on a 3% agarose gel and visualized by EtBr staining using ultraviolet light (see 2.5.4). Different variants of the investigated VNTR result in different DNA fragments characterized by their specific length: 2-repeat = 231 bp, 3-repeat = 261 bp, 3.5-repeat = 279 bp, 4-repeat = 291 bp, 5-repeat = 321 bp, and 6-repeat = 351 bp.

Table 5: Components of PCR mix used for amplification of genomic DNA. Used oligonucleotide primers as well as PCR buffer conditions optimized for genotyping of *MAOA* VNTR.

Component	Amount in µl for one reaction
PCR buffer (Goldstar)	2.5
$MgCl_2$ (25mM)	1.5
Nucleotides (2.5mM each)	1
Forward primer (10µM)	1
Reverse primer (10µM)	1
HouseTaq polymerase	0.3
ddH_2O	16.7
Genomic DNA	1
Total volume	25

2.8. Statistics

2.8.1. STUDY I

In general, categorical data were analysed using the Chi squared test or Fisher's exact test, respectively. Furthermore, differences in continuous variables were evaluated by Student's t-test or multivariate ANOVA corrected for age and *OXTR* rs53576 genotype. Correlations between dimensional variables (as for example SPS and SIAS scores) and *OXTR* DNA methylation were calculated by correlation or regression analyses, respectively. *Post hoc* Bonferroni correction for multiple comparisons with regard to *OXTR* methylation in SAD patients vs. healthy controls set the significance level to $p<0.004$ ($p<0.05$ divided by 13 tests, as DNA methylation across the entire *OXTR* amplicon plus DNA methylation at 12 individual CpG sites were tested). All tests were carried out two-sided with an alpha-level of <0.05 considered significant. Statistical analysis was performed using SPSS statistical software (version 22.0; SPSS Inc., Chicago, IL, USA). Fulfilment of Hardy–Weinberg criteria for *OXTR* rs53576 genotype distribution was tested by the online program DeFinetti (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl; TF Wienker and TM Strom).

2.8.2. STUDY II

In general, differences in baseline *MAOA* methylation between PD patients of the discovery sample and matched healthy controls were statistically evaluated using mixed linear models for repeated measures; (Schuster et al. 2016; Unternaehrer et al. 2015), with *MAOA* methylation as within factor and group (PD patients vs. healthy controls) as between factor and the number of smoked cigarettes as covariate (Philibert et al. 2010). To test for differences on the level of single CpG sites, mixed linear models were followed-up by univariate analysis of variance (ANOVA), again controlled for the number of smoked cigarettes per day. Associations between *MAOA* methylation and number of panic attacks and MI score at baseline (T0) were tested by Pearson's correlation. Furthermore, repeated measures ANOVAs with "time" (discovery sample: T0 vs. T1; replication sample: T0 vs. T1 vs. T2) as within-subject variable were conducted to test for potential dynamics in amplicon-wide *MAOA* methylation during CBT irrespective of treatment response. Similarly, possible dynamics in *MAOA* methylation within the healthy control sample were evaluated using repeated measures ANOVAs with two time points (T0 vs. T1) as within-subject variables were calculated. Differences in percentage methylation change (T1–T0 in percent of T0: [T1−T0]/T0×100) between responders and non-responders were statistically evaluated using univariate ANOVA with baseline *MAOA* methylation as covariate for average *MAOA* methylation change, as well as for CpG site specific changes. Baseline *MAOA* methylation differed significantly between therapy responders and non-responders at CpG site 6 ($p=0.044$) and 12 ($p=0.026$) only and was thus not included as a covariate in further analyses for analysis of methylation dynamics at these two CpG sites. Again, associations between percentage *MAOA* methylation change and MI score change (T1–T0) during CBT were evaluated by Pearson's correlations. For the discovery sample, *post hoc* Bonferroni correction for multiple comparisons regarding percentage methylation change at individual CpG sites (N=13) resulted in a corrected significance level of $p \leqslant 0.004$. Given the confirmatory nature of the analysis in smaller replication sample, here, no Bonferroni correction was applied. All data tested were normally distributed and all tests were carried out two-sided with an alpha-level of <0.05 considered significant. Statistical analysis was performed using SPSS statistical software (version 23.0; SPSS Inc., Chicago, IL, USA).

III. RESULTS

3.1. STUDY I

3.1.1. Oxytocin receptor gene hypomethylation is associated with the categorical phenotype of SAD

To investigate the role of *OXTR* DNA methylation in the pathology of social anxiety disorder 111 patients with social anxiety disorder and 111 controls matched for sex and age were analysed (see 2.2.1), but due to failure of meeting quality criteria for sequencing data the final sample size was reduced to N=220 (f=153, m=67). Within this reduced sample, *OXTR* methylation was available for 110 healthy control subjects and 110 social anxiety disorder patients. Characteristics of this final sample regarding age, sex, *OXTR* rs53576 genotype, comorbidities, medication, Social Phobia Scale (SPS) scores, and Social Interaction Anxiety Scale (SIAS) scores are summarized in Table 6 for controls and patients, respectively.

Table 6: Sample characteristics for the overall sample comprising 110 healthy controls and 110 SAD patients: [a] Hardy–Weinberg criteria were fulfilled for *OXTR* rs53576 genotype distribution (p=1.00) [b] Genotypes were grouped according to previous studies for further analysis (Rodrigues et al. 2009; Saphire-Bernstein et al. 2011) [c] data missing for 2 control subjects.

	Controls (N=110)	**SAD Patients (N=110)**	**Statistics**
Age (mean±SD, years)	30.9±10.5	30.1±9.9	Mann–Whitney U-test, p=0.46
Sex			
Females	77	76	X^2 test: X^2=0.02, p=0.88
Males	33	34	
***OXTR* rs53576 genotype**[a]			
A/A	12	9	X^2 test: X^2=0.72, p=0.70
A/G	48	53	
G/G	50	48	
Grouped genotype[b]			
(A/A+A/G vs. GG)	60 vs. 50	62 vs. 48	X^2 test: X^2=0.07, p=0.79
Comorbidities			
Depression	-	N=21	
Specific phobia	-	N=5	
Panic disorder/agoraphobia	-	N=1	
Medication	-	N=1 (15 mg citalopram)	
SPS score[c]	3.6±0.4	36.6±1.3	Mann–Whitney U-test, p<0.001
SIAS score[c]	9.1±0.6	45.5±1.3	Mann–Whitney U-test, p<0.001

Only minor average *OXTR* DNA methylation across all 12 CpG sites of the investigated amplicon was observed in the combined sample of controls and SAD patients. Correlation analyses revealed moderate to high correlations between *OXTR* methylation levels of single CpG sites ranging between 0.53 and 0.95 and reaching significance for correlations between all single CpG sites (all p<0.05). This result suggested a high functional connectivity between the CpG sites of the investigated amplicon. Furthermore, possible confounding variables such as age, sex, or *OXTR* rs53576 genotype were tested regarding their influence on *OXTR* methylation status in the overall sample. Here, age was found to be positively correlated with average methylation status across all 12 CpG site as well as at individual CpG sites 2 and 5–12 (r=0.14–0.22, all p<0.05). Consequently, age was included as a covariate in further analyses. Regarding other potential confounders, neither sex showed a significant association with overall *OXTR* methylation or single CpG site methylation (all ps>0.26) nor had comorbid major depression (all ps>0.24) an influence on *OXTR* methylation levels. However, *OXTR* rs53576 genotype seemed to affect average *OXTR* DNA methylation (p=0.007) and methylation at CpG sites 1, 2, 5–10, and 12 (p=0.03–0.001) with A-allele carriers showing decreased methylation levels. Interestingly, when patients and controls were analysed separately, this latter association was particularly true for the patient group. This effect of *OXTR* rs53576 genotype on DNA methylation is summarized in Table 7 as well as in Figure 2 for the overall sample.

Table 7: Influence of *OXTR* rs53576 genotype on *OXTR* methylation at single CpG sites and overall *OXTR* methylation for controls, SAD patients and the overall sample. N=sample size, F- and p-values from MANOVA with grouped *OXTR* rs53576 genotype (AA/AG vs. GG) as independent variable, age as covariate. Bold= significant results; *=significant at p≤0.05; **=significant at p≤0.01.

Position	Controls (N=110)		SAD Patients (N=110)		Overall sample (N=220)	
	F	p-value	F	p-value	F	p-value
Average methylation	0.802	0.451	4.861	**0.010***	5.045	**0.007****
CpG1	0.506	0.604	4.236	**0.017***	3.702	**0.026***
CpG2	1.798	0.171	3.221	**0.044***	4.883	**0.008****
CpG3	0.055	0.946	3.126	**0.048***	1.336	0.265
CpG4	0.933	0.397	3.102	**0.049***	1.730	0.180
CpG5	0.383	0.683	4.953	**0.009****	3.452	**0.033***
CpG6	0.407	0.667	6.023	**0.003****	4.613	**0.011***
CpG7	0.655	0.522	5.650	**0.005****	5.289	**0.006****
CpG8	1.799	0.170	5.198	**0.007****	6.596	**0.002****
CpG9	2.360	0.099	5.270	**0.007****	7.473	**0.001****
CpG10	0.600	0.551	3.613	**0.030***	3.643	**0.028***
CpG11	0.336	0.715	4.149	**0.018***	2.787	0.064
CpG12	2.072	0.131	3.919	**0.023***	5.757	**0.004****

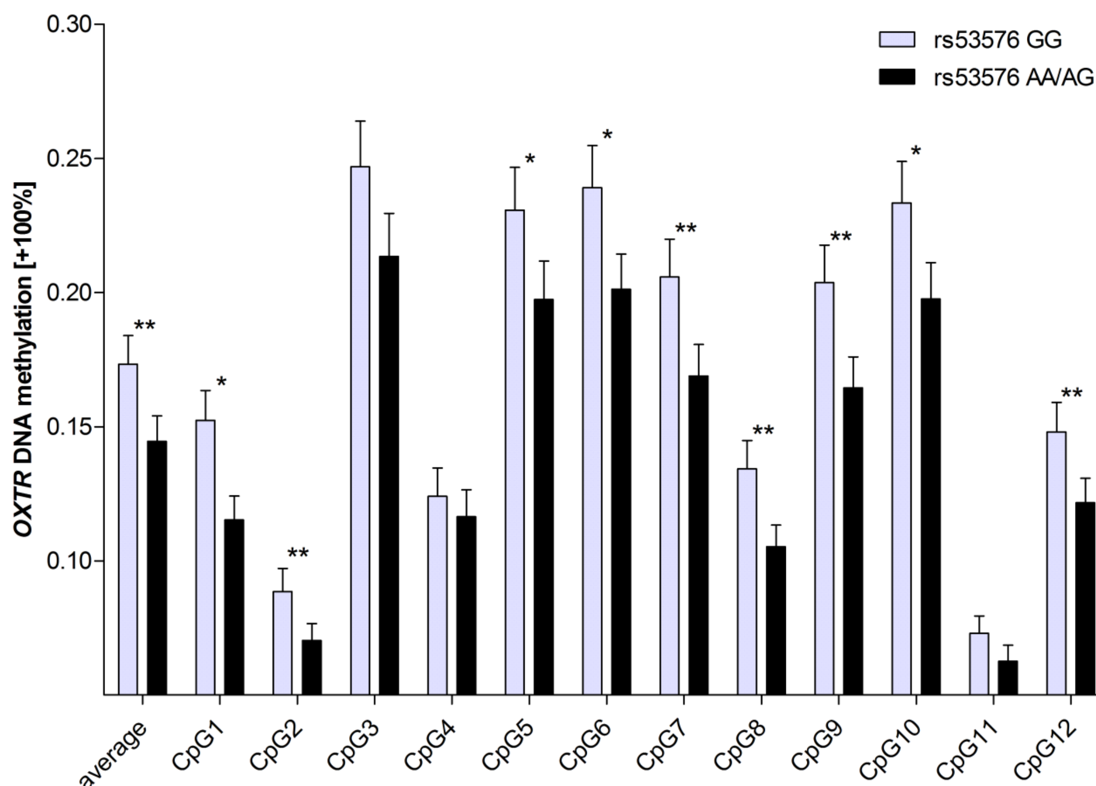

Figure 2: Genotype dependent *OXTR* DNA methylation in the overall sample of healthy controls and SAD patients. Bars represent mean methylation for average *OXTR* methylation across all 12 CpG sites as well as for single CpG sites in *OXTR* rs53576 GG carriers (N=98, grey bars) and *OXTR* rs53576 A-allele carriers (AA+AG, N=122, black bars). Error bars: ±SE. ** significant at p<0.01; * significant at p<0.05.

Furthermore, *OXTR* methylation was compared between healthy controls and social anxiety disorder patients (see Table 8 and Figure 3). Here, a significantly lower average methylation across all 12 *OXTR* CpG sites was found in patients with SAD as compared with healthy controls (see Table 8). In detail, multivariate analysis of variance (MAOVA) for single CpG sites corrected for age showed a significant effect of group (SAD patients vs. controls; p<0.001), with subsequent univariate tests revealing significant hypomethylation in SAD patients compared to healthy controls at single CpG sites 2, 3, 6, 7, 8, and 9, with p-values ranging from 0.03 to <0.001. Interestingly, CpG site 12 was significantly higher methylated in patients than in controls. Association of lower methylation at CpG3 (Chr3:8 809 437, UCSC Human Genome Browser; February 2009; GRCh37/hg19) with social anxiety disorder remained significant when applying Bonferroni correction for multiple testing (corrected p-value of <0.004). Based on this result all further analyses (for results see 3.1.2, 3.1.3, and 3.1.4) focused on overall *OXTR* methylation as well as on methylation level at CpG3 in particular.

Given the *OXTR* genotype effect on methylation as described above, genotype was added as second fixed factor to the MANCOVA in complementary analyses.

Significance of results remained stable ($p<0.001$), univariate tests still revealed significant hypomethylation in SAD patients compared to healthy controls across all 12 *OXTR* CpG sites ($p=0.02$) and at individual CpG sites 2, 3, 6, 7, 8, 9, and 12 ($p=0.05$–0.001).

Table 8: *OXTR* DNA methylation levels for healthy controls and SAD patients. N=sample size, p-value from MANOVA corrected for age. Bold=significant results. *=significant at p≤0.05; **=significant at p≤0.01; ***=significant at p≤0.001.

Position	**Controls**		**SAD Patients**		**p-value**
	Mean	SE	Mean	SE	
Average methylation	0.164	0.009	0.151	0.011	**0.03***
CpG1	0.123	0.010	0.141	0.010	0.29
CpG2	0.079	0.008	0.078	0.007	**0.03***
CpG3	0.273	0.018	0.183	0.014	**<0.001*****
CpG4	0.131	0.011	0.109	0.009	0.07
CpG5	0.223	0.015	0.202	0.015	0.06
CpG6	0.232	0.014	0.204	0.015	**0.02***
CpG7	0.192	0.012	0.179	0.014	**0.03***
CpG8	0.120	0.009	0.116	0.009	**0.01****
CpG9	0.186	0.012	0.178	0.013	**0.01****
CpG10	0.214	0.013	0.213	0.016	0.10
CpG11	0.064	0.006	0.071	0.006	0.07
CpG12	0.128	0.009	0.139	0.011	**0.01****

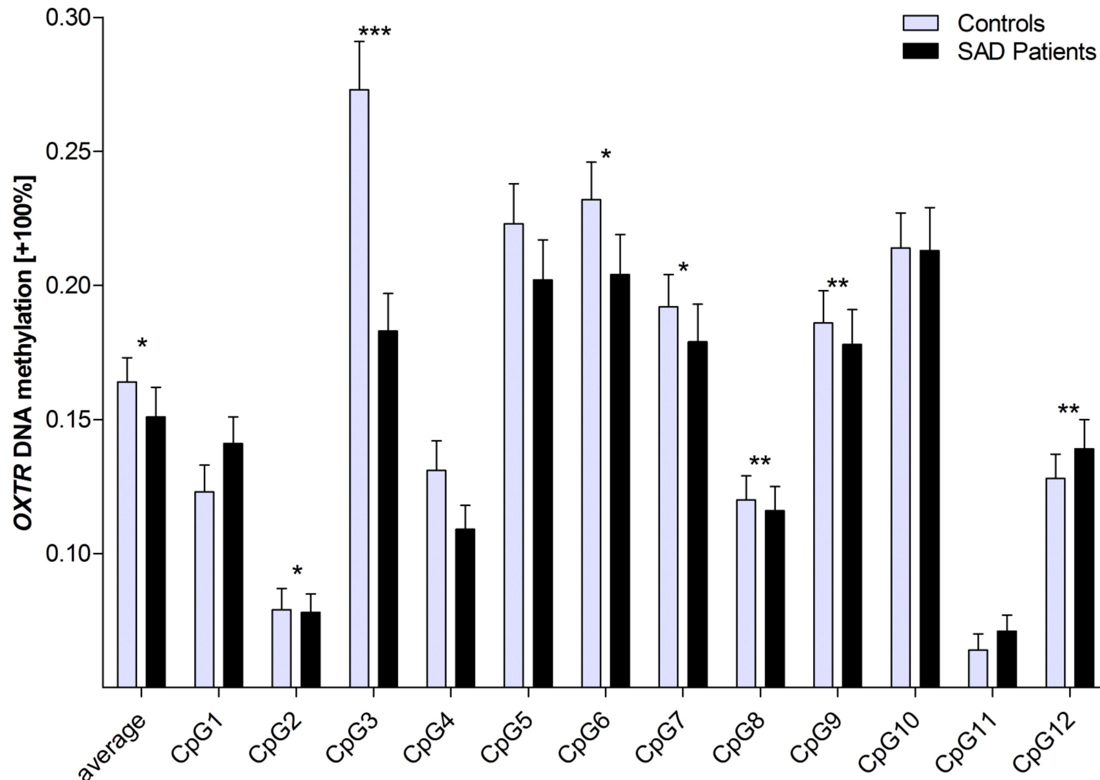

Figure 3: *OXTR* DNA hypomethylation in SAD patients. Bars represent mean methylation for average *OXTR* methylation across all 12 CpG sites as well as for single CpG sites in healthy controls (N=110, grey bars) and patients with social anxiety disorder (N=110, black bars). Error bars: ±SE. *** significant at p<0.001; ** significant at p<0.01; * significant at p<0.05.

3.1.2. Oxytocin receptor gene hypomethylation is associated with dimensional traits of social anxiety

First, a partial correlation analysis controlled for age was conducted within the combined sample (N=218; patients=110, controls=108) to unravel a potential relation between *OXTR* methylation status and SPS and SIAS scores, respectively. Here, a significant negative correlations between overall *OXTR* methylation across all 12 CpG sites and scores on the SPS scale (r=-0.20, p=0.02) and the SIAS scale (r=-0.18, p=0.04) were detected. Second, testing for correlation between methylation levels at single CpG sites and SPS/SIAS scores revealed a significant negative correlation of *OXTR* CpG3 methylation with SPS scores (r=-0.39, p<0.001) and SIAS scores (r=-0.40, p<0.001).

3.1.3. Oxytocin receptor gene hypomethylation is associated with increased cortisol response to social stress (TSST)

With regard to the results described in this section, please note that the Trier Social Stress Test (TSST) procedure, analysis of cortisol levels and blood collection were conducted at the Institute of Psychology, University of Dresden, Germany, by Dr. D. Bräuer and Prof. Dr. J. Hoyer. Analysis of *OXTR* methylation levels was conducted in the context of this thesis (C. Ziegler). Statistical analysis regarding salivary cortisol data was accomplished by Dr. D. Bräuer.

To test for relation between *OXTR* methylation levels and response to a psychosocial stressor (TSST), salivary cortisol levels were measured 45 (t1) and 1 min (t2) before, and 1 (t3), 10 (t4), 20 (t5), 30 (t6), 45 (t7), and 60 (t8) min after the TSST (Ziegler et al. 2015). The maximum salivary cortisol response as well as delta measures of salivary cortisol were calculated (for details please see Ziegler et al. 2015). In the investigated healthy subjects (N=16), pairwise correlation controlled for age revealed a significant negative correlation between *OXTR* methylation and maximum salivary cortisol response to the TSST, both for average *OXTR* methylation across all 12 CpG sites (r=-0.56, p=0.03) and CpG3 (r=-0.59, p=0.02). The same pattern was detected for association of delta salivary cortisol response to the social stressor and *OXTR* methylation, again for both average overall *OXTR* methylation (r=-0.56, p=0.03) and CpG3 (r=-0.53, p=0.04).

3.1.4. Oxytocin receptor gene hypomethylation is associated with increased amygdala activity to social phobia related words

With regard to the results described in this section, it has to be noted that the entire fMRI procedure and all analyses of amygdala reactivity were conducted at the Department of Psychiatry and Psychotherapy, University of Münster, Germany, by Dr. I. Laeger supervised by Prof. Dr. Dr. U. Dannlowski and Prof. Dr. P. Zwanzger. Experimental analysis of *OXTR* methylation levels was conducted in the context of this thesis (C. Ziegler). Statistical analyses were accomplished in the group led by Prof. Dr. Dr. U. Dannlowski, Department of Psychiatry and Psychotherapy, University of Münster, Germany.

Amygdala responsiveness was tested to be predicted by *OXTR* methylation status in a multiple regression model with age as nuisance regressor. Mean methylation across all 12 CpG sites as well as methylation rate at CpG3 were used as predictors according to the results from the overall sample reported in section 3.1.1 Given the working hypothesis of particular relevance of the amygdala region to confer

emotional responses to social anxiety related verbal stimuli, a region of interest (ROI) analysis of the bilateral amygdalae was conducted (for details please refer to Ziegler et al. 2015).

Here, a strongly significant negative correlation of overall *OXTR* methylation across all 12 CpG sites and amygdala responsiveness to social phobia-related words in comparison to generally negative words was detected (corrected p-values: right: p=0.0002; left: p=0.0018). Similar patterns emerged for methylation at CpG3 (corrected p-values: right: p=0.0002; left: p=0.0054). Furthermore, when testing the contrast between social phobia-related words and neutral words again significant associations of amygdala responsiveness with mean overall *OXTR* methylation (corrected p-values: right: p=0.0014; left: p=0.017) and methylation at CpG3 (corrected p-values: right: p=0.0148; left: p=0.0962, trend) were discerned.

3.2. STUDY II

3.2.1. Monoamine oxidase A *(MAOA)* gene hypomethylation in female PD patients

Twenty-eight female patients with panic disorder and 28 female healthy controls matched for age and smoking behaviour (number of smoked cigarettes per day) were analysed regarding *MAOA* DNA methylation status. Detailed characteristics of the sample regarding age, number of smoked cigarettes per day, *MAOA* VNTR genotype, diagnosed comorbidities, and medication are given in Table 9 for female controls and PD patients, respectively, since only the female subgroups were relevant for subsequent analyses (see 2.2.2).

Table 9: Characteristics of the discovery sample for female healthy controls and female panic disorder patients. SSRIs = selective serotonin re-uptake inhibitors; SNRIs = selective serotonin and norepinephrine re-uptake inhibitors; NaSSA = noradrenaline and selective serotonin agonists; TCA = tricyclic antidepressants [a] genotype data missing for two controls and two patients.

	Controls (N=28)	PD Patients (N=28)	Statistics
Age (mean±SD)	34.96±9.02	34.57±8.51	t=0.17, p=0.867
Smoking status (yes vs. no)	8 vs. 20	9 vs. 19	
Number of smoked cigarettes per day (mean±SD)	5.59±8.82	4.64±7.26	t=0.44, p=0.663
***MAOA* VNTR**[a]			
3/3	3	3	
3/4	13	14	
3a(3.5)/4	1	1	
3/5	2	-	
4/4	7	8	
Grouped *MAOA* VNTR			
"low expression" (33/34/3a4/35)	19	18	X^2 test,
"high expression" (44/45)	7	8	p=1.000
Comorbidities			
Depression	-	12	
Social anxiety disorder	-	3	
Specific phobias	-	1	
Medication (yes vs. no)		19 vs. 9	
SSRIs	-	12	
SNRIs	-	2	
NaSSA	-	4	
TCA	-	3	
pregabaline	-	2	
quetiapine	-	2	
zopiclone	-	1	

First, the sample was analysed with regard to possible confounding variables influencing *MAOA* methylation status in controls as well as in PD patients. In the control sample, neither age nor *MAOA* VNTR genotype showed a significant association with *MAOA* DNA methylation status. But as expected (cf. Philibert et al. 2010), smoking behaviour (number of smoked cigarettes per day) correlated inversely with *MAOA* methylation level at CpG sites 3, 12 and 13 (r=−0.54 to −0.40, all p<0.05). In the patient sample, again age and *MAOA* VNTR genotype did not significantly impact *MAOA* methylation status or - particularly relevant for the patient sample - comorbidity with agoraphobia or depression and medication. However, similar to the control sample, the number of smoked cigarettes per day correlated inversely with

average *MAOA* methylation (r=−0.38, p=0.047) at baseline (T0). Thus, all analyses at baseline (T0) were corrected for the number of smoked cigarettes per day.

In a case–control approach, *MAOA* methylation at baseline (T0) was compared between female healthy controls and female panic disorder patients (see Table 10 and Figure 4). Here, mixed linear models for repeated measures showed that *MAOA* DNA methylation levels differed significantly between PD patients and healthy controls (p<0.001), in that panic disorder patients displayed decreased average methylation compared with healthy controls (p<0.001). In follow-up univariate tests, as compared to healthy controls lower methylation was detected in panic disorder patients at CpG sites 1– 4, 6–10, 12 and 13 (p-values ranging from 0.049 to <0.001; for details please see Table 10 and Figure 4). When applying Bonferroni correction for multiple testing, association of average *MAOA* hypomethylation, as well as hypomethylation at CpG sites 3, 6–9, 12 and 13 with the categorical phenotype of panic disorder remained significant.

Table 10: *MAOA* methylation levels in the discovery sample of healthy controls and patients with panic disorder at baseline (T0). p-value for average methylation from mixed linear model with number of smoked cigarettes as covariate; p-values for single CpG sites from univariate ANOVA controlled for number of smoked cigarettes. Bold= significant results. *Significant at p<0.05; **significant at p<0.01; ***significant at p≤0.001.

Position	**Controls (N=28)**		**PD Patients (N=28)**		**p-value**
	Mean	SE	Mean	SE	
Average methylation	0.435	0.007	0.404	0.005	**<0.001*****
CpG1	0.369	0.011	0.341	0.008	**0.033***
CpG2	0.358	0.010	0.326	0.006	**0.019***
CpG3	0.387	0.011	0.343	0.007	**<0.001*****
CpG4	0.420	0.007	0.395	0.008	**0.049***
CpG5	0.281	0.011	0.268	0.008	0.169
CpG6	0.364	0.008	0.321	0.008	**<0.001*****
CpG7	0.450	0.006	0.414	0.005	**<0.001*****
CpG8	0.330	0.010	0.288	0.007	**0.004****
CpG9	0.478	0.006	0.442	0.006	**0.001*****
CpG10	0.485	0.007	0.461	0.005	**0.013***
CpG11	0.293	0.010	0.317	0.013	0.233
CpG12	0.910	0.008	0.870	0.009	**<0.001*****
CpG13	0.553	0.015	0.473	0.010	**<0.001*****

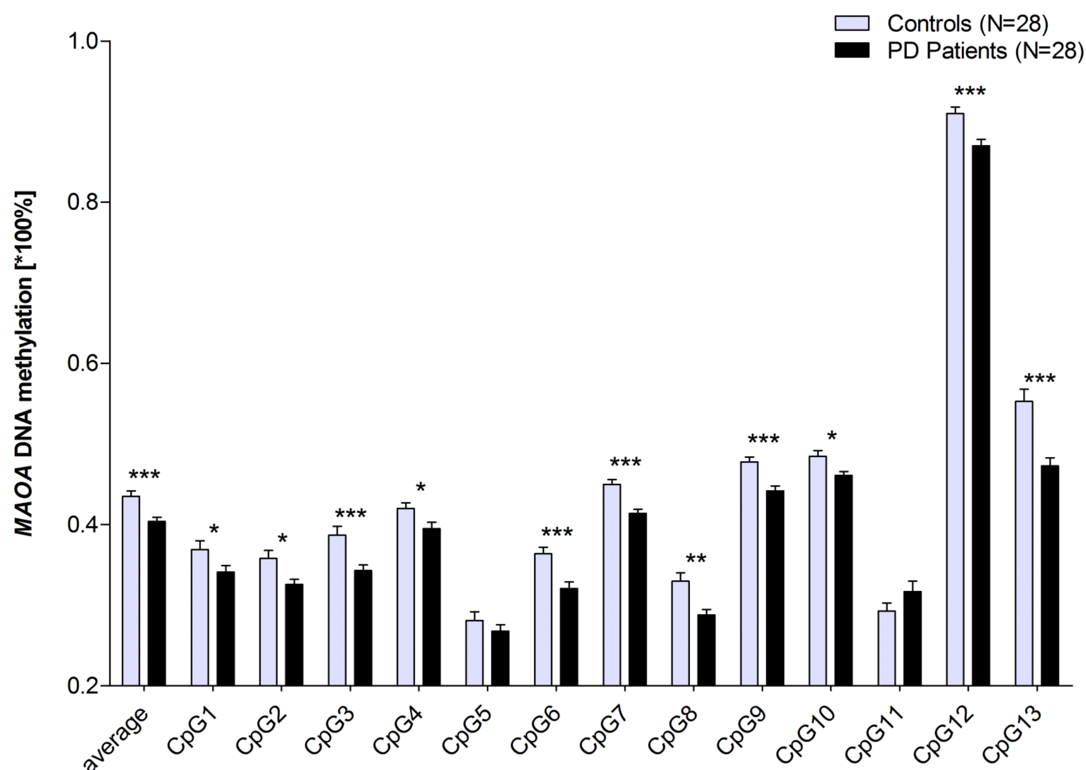

Figure 4: *MAOA* DNA hypomethylation in PD patients. Bars represent mean methylation for average *MAOA* methylation across all 13 CpG sites as well as for single CpG sites in healthy controls (N=28, grey bars) and patients with panic disorder (N=28, black bars) from the discovery sample. Error bars: ±SE. *** significant at $p \leq 0.001$; ** significant at $p<0.01$; * significant at $p<0.05$.

3.2.2. *MAOA* hypomethylation is associated with panic disorder severity in females

Furthermore, a potential association of *MAOA* methylation at baseline (T0) and disease severity reflected by the number of panic attacks as well as scores on the Mobility Inventory (MI) was investigated using partial correlations controlled for the number of smoked cigarettes. In the discovery sample, a negative correlation between *MAOA* methylation and the number of panic attacks was detected at CpG site 4 (r=−0.49, p=0.010). Regarding baseline MI-Accompanied scores, a significant inverse correlation with average *MAOA* methylation (r=−0.47, p=0.013) as well as with methylation at CpG sites 4 (r=−0.52, p=0.005), 6 (r=−0.39, p=0.046), 8 (r=−0.42, p=0.031), 12 (r=−0.41, p=0.036) and 13 (r=−0.45, p=0.018) was discerned. In addition, average *MAOA* methylation correlated negatively with the MI-Alone subscale score at baseline (r=−0.52, p=0.005). On the level of single CpG sites, this pattern was significant for methylation at CpG sites 3 (r=−0.41, p=0.035), 4 (r=−0.50, p=0.008), 6 (r=−0.49, p=0.009), 7 (r=−0.43, p=0.024) and 8 (r=−0.59, p=0.001).

In the replication sample, no significant associations between the number of panic attacks and *MAOA* methylation levels were detected. However, with regard to

panic disorder severity as reflected by scores on the Mobility Inventory, we discerned an inverse correlation of the MI-Accompanied subscale scores with *MAOA* methylation at CpG sites 4 ($r=-0.46$, $p=0.047$), 7 ($r=-0.51$, $p=0.026$), 8 ($r=-0.55$, $p=0.014$), 10 ($r=-0.52$, $p=0.023$) and 12 ($r=-0.51$, $p=0.027$). Analysing MI-Alone subscale scores, only a trend towards an association with decreased methylation at CpG8 ($r=-0.44$, $p=0.061$) was discerned.

3.2.3. *MAOA* methylation change during CBT treatment

First, *MAOA* methylation change during the course of treatment (CBT) was investigated irrespective of responder status in the patient groups of the discovery and the replication sample as well as in the healthy control group. Across all patients of the discovery and replication sample, respectively, *MAOA* methylation did not significantly change from baseline (T0) to post-treatment (T1). The same pattern was found in the healthy control group.

In a next step, female patients were stratified into therapy (CBT) responders and non-responders defined according to the number of panic attacks experienced before (baseline, T0) and after (T1) therapy (for details please see 2.2.2 (d), “Outcome criteria”). To test for potentially differential dynamics in *MAOA* methylation depending on responder/non-responder status, percentage *MAOA* methylation change (details regarding calculation in 2.2.2 (d), “Outcome criteria”) was compared between the respective groups. In the discovery sample, analyses revealed a significant increase in average *MAOA* methylation (mean percentage change±SE, 3.37±2.17%) in the responder group, while non-responders decreased in average *MAOA* methylation level (mean percentage change±SE, −2.00±1.28%; $p=0.001$). On the level of single CpG sites, this pattern was proven to be significant for CpG sites 1, 3-6, and 9-11 (for detailed data see Table 11), and remained significant for CpG sites 3, 4, 6 and 11 after Bonferroni correction for multiple testing (see Figure 5).

Table 11: Percentage *MAOA* methylation change in female patients with panic disorder stratified for responders and non-responders to CBT (discovery sample). p-values for average *MAOA* methylation and single CpG sites from univariate ANOVA with baseline *MAOA* methylation of the respective site as covariate (except CpG site 6 and 12; p-values for CpG sites 6 and 12 from univariate ANOVA). Bold=significant results. *significant at $p<0.05$; **significant at $p<0.01$; ***significant at $p \leq 0.001$.

Position	**Responders (N=11)**		**Non-responders (N=17)**		**p-value**
	Mean [%]	SE	Mean [%]	SE	
Average methylation	3.37	2.17	-2.00	1.28	**0.001*****
CpG1	0.51	5.28	-1.90	3.75	**0.005****
CpG2	4.15	3.69	-0.87	2.23	0.087
CpG3	7.22	3.53	-1.85	2.70	**0.001*****
CpG4	9.68	5.67	-3.40	2.23	**0.003****
CpG5	3.70	2.77	-4.08	3.82	**0.040***
CpG6	9.18	2.65	-2.42	2.33	**0.003****
CpG7	4.19	3.58	-1.85	2.54	0.098
CpG8	4.60	2.84	0.69	2.84	0.085
CpG9	2.68	2.04	-1.62	2.19	**0.009****
CpG10	2.57	1.91	-1.70	2.12	**0.027***
CpG11	0.01	7.34	-6.99	4.79	**0.002****
CpG12	0.50	1.77	0.82	1.62	0.898
CpG13	4.54	3.86	-2.06	2.21	0.276

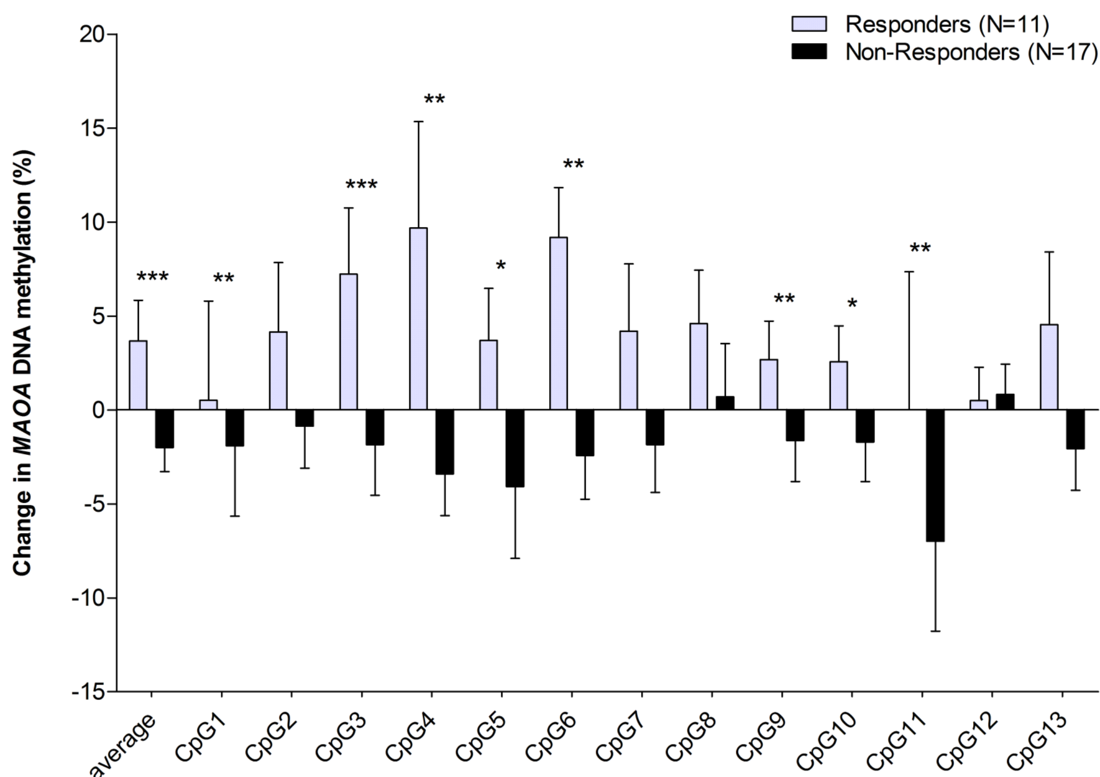

Figure 5: Differential dynamics of *MAOA* methylation during the course of CBT in therapy responders and non-responders in the discovery sample. Bars represent percentage *MAOA* methylation change for average *MAOA* methylation across all 13 CpG sites as well as for single CpG sites in therapy responders (N=11, grey bars) and non-responders (N=17, black bars). Error bars: ±SE. *** significant at p≤0.001; ** significant at p<0.01; * significant at p<0.05.

At baseline, average *MAOA* methylation differed significantly between healthy controls (mean±SE: 0.435±0.007) and responders (mean±SE: 0.405±0.005; p=0.015) as well as between healthy controls and non-responders (mean± SE, 0.404±0.008; p=0.001). After 6 weeks of waiting time for healthy controls or CBT for panic disorder patients, respectively, healthy controls (mean±SE: 0.432±0.005) and therapy responders (mean±SE: 0.418±0.007) did not differ anymore with regard to their average *MAOA* methylation levels (p=0.148). In contrast, differences in average *MAOA* methylation levels between healthy controls and non-responders (mean±SE: 0.395±0.007) remained significant (p<0.001). This “normalization” of *MAOA* hypomethylation detected in the discovery sample in contrast to healthy controls is illustrated in Figure 6.

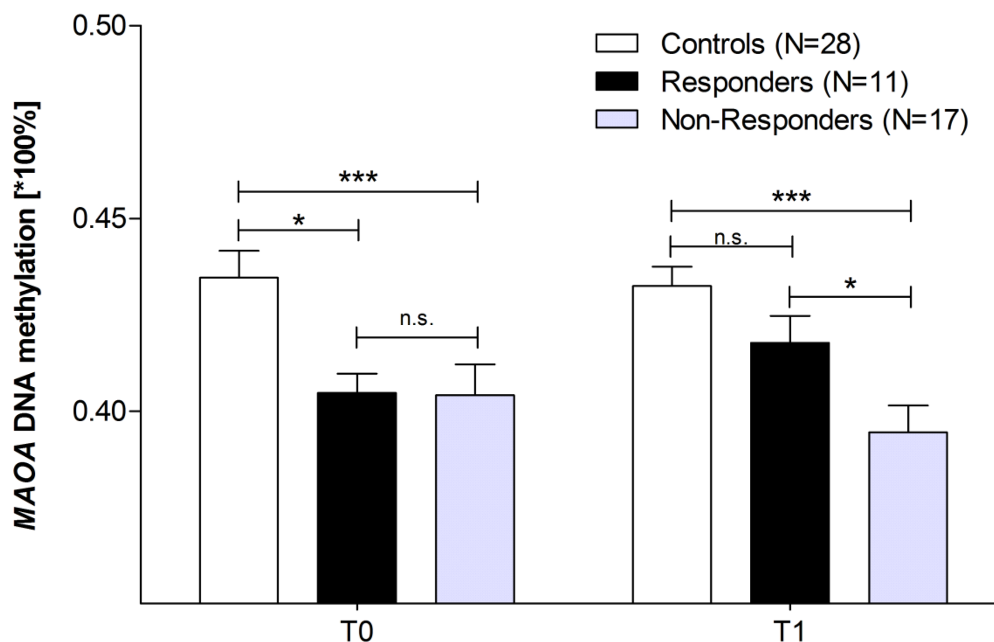

Figure 6: Average *MAOA* methylation levels at baseline (T0) and after six weeks of CBT for responders/non-responders or waiting time for healthy controls (T1) in the discovery sample. Bars represent average *MAOA* methylation in healthy controls (N=28, white bars), therapy responders (N=11, black bars) and non-responders (N=17, grey bars). Error bars: ±SE. *** significant at p≤0.001; ** significant at p<0.01; * significant at p<0.05.

In the replication sample, no significant differences in *MAOA* methylation dynamics between responder/non-responder groups defined according to the number of panic attacks were detected.

When focusing on the relation of percentage *MAOA* methylation change after treatment (CBT) with improvement of panic disorder symptoms reflected by a decrease in MI scores rather than the number of panic attacks, in the patient discovery sample revealed a significant association between symptom improvement (reduction in MI-Alone subscale score) and increase in *MAOA* methylation at CpG site 12 (r=−0.43, p=0.022) was discerned irrespective of responder status. No significant associations were detected for MI-Accompanied subscale scores. In the replication sample, however, a reduction in MI-Accompanied subscale scores after CBT was found to be significantly associated with an increase in *MAOA* methylation at CpG sites 4 (r=−0.56, p=0.025), 7 (r=−0.55, p=0.027), 8 (r=−0.55, p=0.029), 9 (r=−0.55, p=0.028), 10 (r=−0.54, p=0.030) and 12 (r=−0.57, p=0.020).No significant associations were detected for MI-Alone subscale scores.

IV. DISCUSSION

STUDY I

The first part of the thesis comprised a multilevel epigenetic approach investigating the role of oxytocin receptor (*OXTR*) gene methylation in the pathogenesis of social anxiety disorder (SAD), the second most prevalent anxiety disorder (Baxter et al. 2013). In summary, *OXTR* methylation patterns were implicated in different intermediate phenotypes of social anxiety disorder on a categorical, dimensional, neuroendocrinological as well as on a neural network level. The results point towards a multilevel role of *OXTR* gene hypomethylation particularly at one CpG site within the protein coding region of the gene (CpG3, Chr3: 8 809 437, UCSC Human Genome Browser; February 2009; GRCh37/hg19) in SAD. In detail, decreased DNA methylation in this region was associated with (i) the phenotype of social anxiety disorders, with SAD patients being significantly lower methylated than healthy controls; (ii) dimensional traits of social anxiety (SPS and SIAS scores), with *OXTR* hypomethylation being correlated with higher disorder severity; (iii) heightened stress response to a psychosocial stressor in healthy individuals, with *OXTR* hypomethylation being related to increased cortisol response to TSST; and (iv) neuronal processing of social contents in patients, with SAD with *OXTR* hypomethylation being associated with increased amygdala activity in response to social phobia-related word stimuli.

The investigated amplicon containing the strongly associated CpG site 3 is located within the gene body of the *OXTR* gene, namely in the protein coding region of exon 3. In contrast to the region upstream of the translation start site (promoter, exons 1 and 2), methylation patterns of the *OXTR* gene body including exon 3 have not been investigated so far regarding their functional consequences on gene expression and protein levels. The *OXTR* promoter region (including exons 1 and 2) is known to regulate gene expression in that hypermethylation leads to decreased *OXTR* mRNA levels by 70% (Kusui et al. 2001). Furthermore, hypermethylation in the intron 1 region has been shown to be associated with lower oxytocin plasma levels (Dadds et al. 2014) and to be associated with callous-unemotional traits in male adolescents with oppositional-defiant or conduct disorder (Dadds et al. 2014). *OXTR* exon 1 / intron 1 hypermethylation has been reported to be related to autism spectrum disorder (Gregory et al. 2009), and increased *OXTR* intron 1 methylation has been associated with neural processes of social interpretation of ambiguous stimuli (Jack et al. 2012).

According to the notion that gene promoter hypomethylation causes an upregulation of gene expression, while hypomethylation of the gene body rather

represses gene expression (Suzuki and Bird 2008), *OXTR* hypomethylation of exon 3 as presently identified to be associated with SAD is hypothesized to lead to decreased gene expression and thereby to lower levels of oxytocin receptor protein. Decreased oxytocin (OXT) transmission as possibly conferred by *OXTR* hypomethylation of exon 3 in social anxiety and related traits is entirely in line with previous studies reporting decreased OXT transmission in social anxiety related traits. For instance, decreased baseline oxytocin plasma levels were found to be associated with SAD (Hoge et al. 2012). Furthermore, several studies showed that oxytocin administration represses amygdala activation in response to socially relevant or fear-conditioned stimuli suggesting an attenuated OXT transmission in social phobia related traits in healthy individuals as well as in SAD patients (Domes et al. 2007; Kirsch et al. 2005; Labuschagne et al. 2010; Petrovic et al. 2008). Reciprocally, intranasal oxytocin has been shown to significantly improve self-reported speech performance and speech appearance in SAD patients (Guastella et al. 2009).

Adding to these results, we showed that in healthy individuals *OXTR* hypomethylation predicts higher cortisol response to social stress as applied via the Trier Social Stress Test (TSST) supporting the role of *OXTR* exon 3 hypomethylation as a risk factor for altered coping with social stressors and thus for developing social anxiety disorder. In accordance with this notion, high salivary cortisol levels during the TSST have previously been shown to correlate inversely with endogenous oxytocin levels (Pierrehumbert et al. 2010) as well as to be attenuable by intranasal oxytocin administration during the TSST in healthy subjects (Heinrichs et al. 2003). Interestingly, in healthy individuals Unternaehrer et al. reported a significant increase in *OXTR* exon 3 methylation in response to acute social stress applied in form of the TSST (Unternaehrer et al. 2012). This short-term upregulation of *OXTR* exon 3 methylation after acute social stress – resulting in temporarily higher levels of oxytocin receptor protein – might therefore be interpreted as an adaptive response to cope with social stress.

Furthermore, on a neuronal network level, we were able to show that in SAD patients *OXTR* exon 3 hypomethylation was associated with increased amygdala response to social phobia-related words. Here, decreased oxytocin receptor gene methylation levels may mediate amygdala hyper-responsiveness to social threat, especially to social phobia-related words as already shown to be characteristic for SAD (Schmidt et al. 2010) as well as positively associated with dimensional measures of anxiety (Laeger et al. 2012) and social anxiety (Phan et al. 2006). As the oxytocin receptor gene is strongly expressed in the amygdala (Bale et al. 2001), peripheral

OXTR methylation as presently observed might indeed reflect a central role of *OXTR* methylation in mediating emotion processing of social environmental signals.

Besides *OXTR* DNA methylation, *OXTR* rs53576 genotype has previously been shown to be implicated into regulation of prosocial behaviour, sensitive parenting, shaping social traits, and modulating neuronal circuits (extensively reviewed in Kumsta and Heinrichs 2013). Within the present study, no association of *OXTR* rs53576 genotype with the phenotype of social anxiety disorder was detected, but *OXTR* exon 3 methylation levels differed significantly between homozygous GG carriers and A-allele carriers, with lower methylation in A-allele carriers. This is in line with reports of the *OXTR* rs53576 A-allele to constitute an SAD "risk allele" by conferring lower levels of generally positive social traits that such as optimism, mastery and self-esteem (Saphire-Bernstein et al. 2011). This allele-specific methylation - as already reported in the context of other psychiatric phenotypes (e.g. Klengel et al. 2013) - was specifically found in the SAD patient sample but not in healthy controls, suggesting DNA methylation as a possible mediator of disease risk genotypes. This adds to the hypothesis that epigenetic mechanisms in general as well as allele-specific methylation in particular may serve as a mechanism partly explaining the extensively discussed problem of the so-called 'hidden heritability' in the context of complex genetic diseases (Meaburn et al. 2010). With regard to *OXTR* rs53576 in particular, allele-specific methylation patterns were recently reported to be present in autistic children, in that *OXTR* exon 3 hypermethylation was found to be associated with social problems only in *OXTR* rs53576 G-allele homozygous children, but not in A-allele carriers (Rijlaarsdam et al. 2016). Thus, allele-specific *OXTR* methylation patterns might be a disease-specific risk marker and might change in the course of development from childhood to adulthood. These results underline the importance of including 'epi-allelic' information into genetic studies. Also, previous apparently contradictory genetic association findings could be reconciled by additionally considering epigenetic information. In sum, gene by 'epi-gene' interaction studies might aid in further elucidating the role of the oxytocin system in the development and maintenance of SAD.

The reported results have to be interpreted in the light of some limitations. First, the present results need to be replicated in an independent and sufficiently powered sample of patients with SAD and the respective matched healthy controls. Second, effects of medication, comorbid diseases, gender or environmental influences have to be considered. In the present study, a confounding effect of antidepressant medication previously reported to impact the oxytocin system (Uvnäs-Moberg et al. 1999) can be

excluded, because all patients except one were not receiving any psychoactive medication. Furthermore, no differences in *OXTR* exon 3 methylation were detected in SAD patients with comorbid depression compared to those without depression suggesting no major confounding effect of comorbid depression. Regarding a potential gender specific effect, no methylation differences were discerned between males and females in the samples of the present study, although females are at higher risk to develop social anxiety disorder (Kessler et al. 2005; Ruscio et al. 2008). However, given an overrepresentation of females in the overall sample and an entirely female fMRI subsample, but equal distribution of gender in the TSST subsample, gender effects cannot be evaluated conclusively from the present study. Life events experienced prior to inclusion to the study as well as experienced childhood traumata are another important factor, as life events have been suggested to induce stable alterations in the epigenome (reviewed in Szyf et al. 2016), especially in the context of anxiety or stress-related disorders or traits (Domschke et al. 2012; Domschke et al. 2013; Kember et al. 2012; Klengel et al. 2013; Yehuda et al. 2013). Recent life events or childhood traumata were not considered within this study and could thus have constituted confounders of the reported results. Finally, the exact functional consequences of differential *OXTR* exon 3 methylation levels on *OXTR* mRNA or oxytocin receptor protein levels were not investigated in the context of this study and thus remain to be elucidated. This particularly, since the role of the oxytocin system in social anxiety appears not to be elucidated unequivocally yet. The simplistic view of heightened oxytocin levels to facilitate anxiolytic or prosocial behaviour is challenged by several findings. For example, high oxytocin levels were associated with increased SAD symptom severity (Hoge et al. 2008), and a positive correlation between oxytocin levels and anxiety symptoms was identified in the context of experiences in close relationships (Marazziti et al. 2006). Furthermore, oxytocin application failed to increase approach behaviour to threatening stimuli in severe social anxiety (Radke et al. 2013), and adult autistic patients have been shown to be characterized by increased basal OXT levels (Jansen et al. 2006). Finally, amygdala reactivity in response to social and non-social threatening scenes was positively associated with OXT levels in healthy females (Lischke et al. 2012). In summary, the role of oxytocin levels as well as *OXTR* methylation status in social anxiety remains unclear, and has to be clarified in detail in further, preferably longitudinal studies to elucidate whether low *OXTR* exon 3 methylation levels are the cause or rather a consequence of social anxiety.

Along these lines, a general problem with regard to epigenetic analyses in neuropsychiatric phenotypes has to be discussed at this point. Given the difficulty to obtain human brain tissue *in vivo* as well as the small number of post-mortem samples

available, DNA samples from whole blood or buccal cells, i.e. peripheral biomaterial, are widely used in DNA methylation studies in neuropsychiatric phenotypes (reviewed in Terry et al. 2011), for example in Alzheimer’s disease (Chouliaras et al. 2010), schizophrenia (Walton et al. 2015), or Parkinson’s disease (Masliah et al. 2013). However, it has to be noted that DNA methylation patterns measured in peripheral tissues do not readily allow for direct conclusions on DNA methylation patterns present in the brain. Therefore, it has to be clarified whether differences in *OXTR* methylation detected in the periphery – such as in whole blood like in the present study – can be translated to central *OXTR* methylation and oxytocin receptor levels in the brain, and whether altered *OXTR* levels in the periphery correlate with altered brain function. Some peripheral epigenetic patterns have indeed been suggested as viable sensors for central processes, as for example in major depressive disorder, where there is growing evidence for differentially methylated regions (DMRs) not to be restricted to the affected tissue, but to be detectable in other tissues as well (reviewed in Mill and Petronis 2007). Further epigenetic studies in rodents, rhesus monkeys and humans support the fact that peripheral DNA methylation patterns are comparable with those in central brain tissues. For example, a rodent study examined pairwise associations between DNA methylation values of *Fkbp5* in blood versus the hippocampus of mice. Data suggests that glucocorticoid-induced methylation changes observed in blood can serve as a proxy for both DNA methylation and expression changes in the brain (Ewald et al. 2014). In addition, overlapping DNA methylation changes in the prefrontal cortex (PFC) and T-cells in response to chronic pain were reported in rats, where 72% of all investigated promoter regions of genes affected in T-cells were differentially methylated in the prefrontal cortex as well (Massart et al. 2016). In a rhesus macaque model, promoter methylation differences between T-cells and PFC across all chromosomes genome were analyzed and - similar to what was found in mice - promoter methylation in peripheral blood cells overlapped with central methylation patterns in the prefrontal cortex (Provençal et al. 2012). In a cross-species, cross-tissues approach, Nieratschker et al. identified DNA methylation patterns of 30 genes to overlap between tissues (blood, PFC) and species (humans, rhesus monkeys, and rats). In addition, DNA methylation patterns in seven gene promoters were affected in the same way in response to early life stress in all analyzed tissues and species (Nieratschker et al. 2014). In humans, there is similar evidence for moderate to high correlation between peripheral and central DNA methylation patterns, e.g. of genes implicated in neurological and psychiatric conditions (e.g. *BDNF* (Stenz et al. 2015), or *C9ORF72* (Russ et al. 2015)). Furthermore, a genome-wide analysis revealed a significant correlation between differential blood and brain methylation levels in a gene set for

precursors of metabolites in schizophrenic patients compared to controls (Walton et al. 2015) as well as a strong correlation of differentially methylated regions in the brain (post-mortem frontal cortex) and leucocytes from the same individuals with Parkinson's disease and healthy controls (Masliah et al. 2013). In addition, Wang and colleagues showed a strong negative correlation between peripheral *5-HTT* (*SLC6A4*) DNA methylation and in vivo serotonin (5-HT) synthesis in the brain (Wang et al. 2012). With regard to oxytocin receptor gene methylation in particular, a recent study investigating *OXTR* methylation patterns in autistic patients revealed similar *OXTR* methylation levels in peripheral blood cells and temporal cortex cells from post mortem tissue of ASD patients (Gregory et al. 2009). In summary, DNA methylation analyses in peripheral materials such as blood or buccal cells may prove valuable as biomarkers of neuropsychiatric disorders possibly reflecting central processes. Additionally – given the possibility of repeated sample collection over the lifespan – studies on peripheral DNA methylation allow for confounder analyses considering e.g. medication, drugs, or stress particularly accumulating in the periphery and for evaluating the dynamics of epigenetic patterns throughout the life time course of a specific disorder regarding development, remission, and relapse (cf. Mill and Petronis 2007).

To further unravel the role of *OXTR* exon 3 methylation in social dysfunction and social anxiety disorder, respectively, it would be worthwhile to combine methylation analyses with interventional approaches. For example, analysis of *OXTR* methylation pre- and post-intranasal application of oxytocin might reveal potential methylation dynamics caused by alternating oxytocin levels. In addition, *OXTR* methylation - already shown to be highly temporally dynamic (Unternaehrer et al. 2012) - could be investigated in SAD patients during the course of a therapeutic intervention such as cognitive behavioural psychotherapy aiming at identifying a) epigenetic risk patterns of treatment response/resistance and b) epigenetic correlates/mechanisms of treatment response as reflected by possibly differential dynamics in *OXTR* methylation depending on treatment response, hypothesizing that therapy responders might increase in *OXTR* exon 3 methylation while non-responders would remain unaltered or rather decrease in methylation. With regard to further investigation of the adaptive role of *OXTR* methylation in response to social stress, SAD patients might differ from healthy controls in their response to a Trier Social Stress Test, failing to compensatorily increase in *OXTR* exon 3 methylation and thus to adapt to social stress as has been shown for healthy participants (Unternaehrer et al. 2012). Finally, it should be further elucidated whether *OXTR* exon 3 hypomethylation is a cause or a consequence of social anxiety disorder, which warrants future longitudinal studies preferably in a cohort of healthy children/adolescents followed-up well into adulthood. These longitudinal analyses of

methylation patterns require sample collections at multiple time points e.g. by blood sampling or buccal swabs to collect DNA from buccal cells, which have also been suggested as surrogate tissue for brain DNA methylation (Lowe et al. 2013).

In summary, the present results provide strong, multi-level evidence for *OXTR* exon 3 hypomethylation to be associated with SAD and social phobia-related traits. However, given the above mentioned limitations and the overall controversial role of oxytocin in social anxiety, there is a strong need for robust replication of the present findings, before *OXTR* methylation patterns will hopefully be useful as accessible biomarkers of SAD. These epigenetic patterns might furthermore contribute to establishing tailored preventive interventions and personalized treatment options which target especially the oxytocin system.

STUDY II

The second part of the thesis investigated monoamine oxidase A (*MAOA*) gene methylation regarding its role in the pathogenesis of panic disorder as well as – applying a psychotherapy-epigenetic approach – its dynamic regulation during the course of CBT in PD patients. First, *MAOA* hypomethylation was shown to be associated with panic disorder as well as with panic disorder severity. Second, in patients responding to treatment *MAOA* hypomethylation was shown to be reversible up to the level of methylation in healthy controls after the course of CBT. This increase in *MAOA* methylation by successful psychotherapeutic treatment was furthermore shown to be associated with symptom improvement regarding agoraphobic avoidance in an independent replication sample of non-medicated patients with PD.

The present results of decreased methylation of a distinct amplicon covering *MAOA* exon I and parts of intron I to be associated with panic disorder and higher disorder severity are in line with a previous study suggesting *MAOA* hypomethylation as risk pattern for PD (Domschke et al. 2012). Together, both studies provide converging evidence for a role of *MAOA* hypomethylation in female panic disorder patients. Previous studies have suggested *MAOA* hypomethylation to drive increased *MAOA* expression *in vitro* and *in vivo* (Checknita et al. 2015; Pinsonneault et al. 2006; Shumay and Fowler 2010). This notion could be corroborated by an own luciferase-based assay *in vitro* (Kollert 2016), where methylation of the investigated amplicon was observed to significantly silence luciferase activity. In further support, a study using a positron emission tomography (PET) approach with [(11)C]clorgyline demonstrated peripheral *MAOA* promoter hypomethylation to correlate with higher levels of MAOA protein in the brain (Shumay et al. 2012). Given that MAOA is a key catabolic enzyme

in the degradation of biogenic amines such as serotonin and dopamine with catalysing the oxidative deamination of those neurotransmitters, *MAOA* hypomethylation possibly mediates lower levels of those neurotransmitters in the synaptic cleft as known to be pathogenetically relevant for anxiety disorders (reviewed in Maron and Shlik 2005; Bell and Nutt 1998). This pathogenetic model is in line with previous genetic studies robustly showing association of the longer, more active alleles of the *MAOA* VNTR with panic disorder (Deckert et al. 1999; Maron et al. 2005; Reif et al. 2014; Samochowiec et al. 2004).

As main result of the present study, *MAOA* methylation was observed to be temporally dynamic under the influence of cognitive behavioural therapy. These dynamic changes in *MAOA* methylation were shown to be dependent on symptom improvement. Responders to CBT characterized by a reduction in the number of experienced panic attacks displayed an increase in *MAOA* methylation after CBT, while therapy non-responders experiencing no improvement or even more panic attacks after CBT remained unaltered or rather decreased in *MAOA* methylation. Furthermore, responders did not significantly differ from healthy controls any more regarding *MAOA* methylation levels after CBT, suggesting a 'normalization' of an epigenetic disease risk pattern by psychotherapy. This observation is in line with results from a previous study reporting *MAOA* methylation levels to be influenced by life events, with positive life events – assumed to strengthen resilience to anxiety disorders – correlating with increased *MAOA* methylation (Domschke et al. 2012). Given the functional consequences of *MAOA* methylation patterns as described above, a psychotherapy-mediated increase in *MAOA* methylation in therapy responders may lead to decreased MAOA protein levels by gene silencing and thus to higher availability of neurotransmitters such as serotonin, norepinephrine and dopamine. This admittedly highly speculative theoretical model (illustrated in Figure 7) suggests a biochemical mechanism of psychotherapy similar to the action of SSRIs or SNRIs also increasing catecholamines in the treatment of panic disorder.

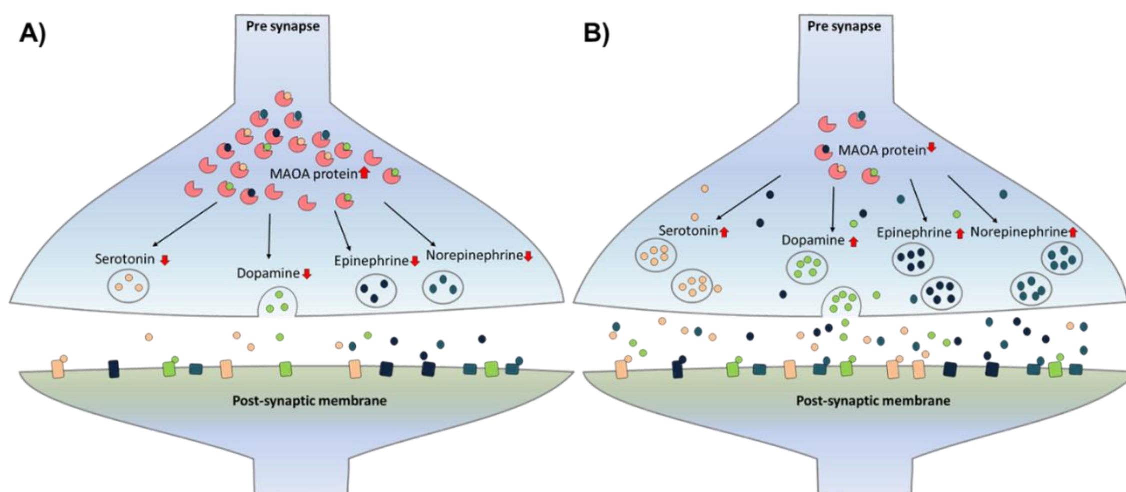

Figure 7: Schematic illustration of possible functional consequences of differential *MAOA* methylation in PD patients before (A) and after successful CBT (B). Low *MAOA* methylation conferring higher MAOA enzyme activity results in lower catecholamine availability (A). After successful CBT, increased *MAOA* methylation may "normalize", i.e. lower MAOA enzyme activity, thereby increasing catecholamine signalling (B).

DNA methylation has been shown to constitute both, stable, possibly even heritable markers (Yehuda et al. 2015) reflecting events from childhood (Heijmans et al. 2008; Klengel et al. 2013) on the one hand and rapidly changeable biological signals on the other hand (Ramchandani et al. 1999), which can be altered within less than 1 hour (Unternaehrer et al. 2012). With regard to differential DNA methylation dynamics along with therapy response, Roberts and colleagues reported increases in *5-HTT* promoter methylation in children with mixed primary anxiety diagnoses responding to CBT (Roberts et al. 2014). In the same sample, a nominally significant association between decrease in *FKBP5* methylation and treatment response (reduction in severity) has been shown (Roberts et al. 2015). Up to now and to the best of my knowledge, only a few other studies have focused on epigenetic dynamics as correlates of treatment outcome in psychiatric disorders or related animal models. For example, *P11* gene promoter hypermethylation was shown to be reversed to normal by antidepressant treatment with escitalopram in animals of the Flinders Sensitive Line, a rodent genetic depression model (Melas et al. 2012). In patients with MDD, histone 3 lysine 27 trimethylation (H3K27me3) levels of the *BDNF* gene (promoter IV) were found to be decreased in therapy responders (HAM-D score <9 in week 8) after eight weeks of citalopram treatment (Lopez et al. 2013). In addition, in patients with borderline personality disorder *BDNF* gene DNA hypermethylation (exon I and exon IV) decreased significantly in responders (>50% improvement) to intensive dialectical behaviour therapy (I-DBT) (Perroud et al. 2013). Furthermore, patients with PTSD responding to a 12-week psychotherapy were characterized by a significant decrease

in *FKBP5* gene promoter methylation (Yehuda et al. 2013). Various pharmacological drugs have already been shown to target epigenetic mechanisms on histone and DNA level (reviewed in Lötsch et al. 2013). For example, valproate, used in treatment of mania and bipolar disorder, inhibits histone deacetylase (HDAC) activity by binding to the catalytic center of the enzyme, leading to a higher overall transcriptional activity in the genome (Göttlicher et al. 2001). With regard to DNA methylation, selective serotonin reuptake inhibitors (SSRIs), which are used in the treatment of many psychiatric disorders such as anxiety disorders, depression and obsessive-compulsive disorder, indirectly influence DNA methylation levels, as fluoxetine induces expression of methyl-CpG binding proteins MeCP2 and MBD1 resulting in increased protein levels (Cassel et al. 2006). Also, escitalopram causes reduced mRNA expression of *maintenance* DNA methyltransferases and consequently decreases gene-specific methylation levels (Melas et al. 2012). Taken together, the present results – in synopsis with the literature as reviewed above – provide converging evidence that epigenetic patterns such as DNA methylation can be altered by a therapeutic intervention, i.e. epigenetic risk patterns can be reversed to 'normal' levels along with psychotherapy response and thus might constitute a dynamic epigenetic correlate of a therapeutic intervention. These findings add some evidence to theoretical discussions in the recent literature: "*It is consistent with the idea that the changes that define the phenotype are not caused only by inherited genetic polymorphisms but also by reversible epigenetic marks such as DNA methylation. The remaining challenge is to determine whether these conclusions could be translated to humans and whether behavioral interventions could substitute for pharmacological interventions […].*" (Szyf et al. 2016).

The reliability of the described results is underlined by several strengths of the study. The patient sample is characterized by low demographic and clinical heterogeneity as patients were recruited following strict inclusion and exclusion criteria. Furthermore, healthy controls were recruited to exactly match the patient sample regarding age, sex, and smoking behavior with the number of smoked cigarettes per day. All analyses were conducted considering potential confounding factors of epigenetic mechanisms such as age-, sex-, and smoking status. Also, the main results showing differential methylation dynamics depending on responder status survived Bonferroni-correction for multiple testing, which is to be considered overly conservative given the high intercorrelation between single CpG site methylation (cf. Tyrka et al. 2016). In addition, results were corroborated by analyses in an independent replication sample.

Still, the present results have to be interpreted in the light of some limitations, warranting replication in larger samples including males and additional follow-up assessments: (1) Sizes of subsamples, especially of the replication sample, which was not explicitly recruited for this study, were small, caused by the fact that only females were included. (2) CBT design regarding the number of sessions and overall focus was not exactly similar between the discovery and the replication sample. CBT in the discovery sample comprised six sessions within six weeks with focus on interoceptive exposure, while patients of the replication sample underwent 12 sessions within 6 weeks with focus on *in situ* exposure. (3) Clinical composition differed slightly between both patient samples: in the discovery sample, comorbidity with agoraphobia was present in 50% of the patients, while in the replication sample 70% of the patients suffered from comorbid agoraphobia. Association of *MAOA* methylation dynamics with different parameters of treatment response (discovery samples: number of panic attacks; replication sample: avoidance behavior) might reflect these differences in treatment focus, therapy intensity and comorbidity rates between the two samples. (4) Medication is suggested to strongly influence DNA methylation with different antidepressants targeting and altering the DNA methylation machinery as already described above (for review see Lötsch et al. 2013). Furthermore, panic disorder symptoms can be negatively influenced by psychoactive drugs at the beginning of pharmacotherapy (Sinclair et al. 2009). To minimize the confounding effect of antidepressant treatment in the discovery sample of this study, all medicated patients were only included if they were stable on the respective medication for at least two weeks prior to inclusion. In addition, medication (type and dose) was not changed during the course of CBT. Most importantly, average *MAOA* methylation did not differ between medicated and non-medicated patients, and *MAOA* methylation change was not impacted by medication status. In the replication sample, a potential confounding effect of medication can be excluded, since all patients were entirely medication-free. (5) One of the most important confounding factors for *MAOA* methylation analysis is smoking, repeatedly shown to significantly impact *MAOA* DNA methylation levels, e.g. (Domschke et al. 2012; Philibert et al. 2008; Philibert et al. 2010). Also, smoking is to be considered an independent risk factor of PD (Cosci et al. 2010). Indeed, smoking was significantly associated with *MAOA* methylation at baseline in the present study, necessitating control for this aspect on several levels: smoking behavior of patients as well as controls was assessed in detail with the number of smoked cigarettes per day and was not changed during course of CBT or waiting time, respectively. Furthermore, healthy controls were matched to patients according to the number of smoked cigarettes. To control for smoking statistically, all analyses regarding baseline

methylation were conducted with the number of smoked cigarettes as a covariate. (6) Finally, results were obtained using DNA from peripheral blood as study material, not allowing for direct translation of *MAOA* methylation patterns in the periphery to the respective *MAOA* methylation patterns in the brain. However, as already discussed in detail above, several studies suggest a moderate to high correlation between peripheral and central methylation patterns (please refer to 'Discussion', 'STUDY I'). Furthermore, a recent study using a positron emission tomography (PET) approach with [(11)C]clorgyline proved peripheral *MAOA* promoter methylation to be associated with central MAOA protein levels in that peripheral *MAOA* promoter hypomethylation correlated with higher levels of MAOA protein in the brain (Shumay et al. 2012). In sum, these results point towards peripheral *MAOA* methylation as well as peripheral methylation patterns in general to potentially function as sensors or surrogates of central brain action. Still, results obtained from peripheral study material have to be interpreted cautiously regarding their reflection of central brain functions.

Adding to the growing body of evidence of *MAOA* as a risk gene for PD, the presently reported results provide strong evidence for *MAOA* hypomethylation to be associated with PD as well as PD severity and therefore replicate previous studies. In addition, *MAOA* hypomethylation was shown to be reversible by CBT, and furthermore increasing *MAOA* methylation was associated with significant symptom improvement. Provided robust replication of the present findings, *MAOA* methylation patterns are hoped to be useful as accessible biomarkers of PD and as peripheral mechanistic correlates of therapy response, particularly successful fear extinction. These patterns might contribute to establishing tailored preventive interventions and personalized treatment options. As an exemplary clinical application of the present line of research, patients with extremely low *MAOA* methylation levels may benefit from CBT accompanied by pharmacotherapy with MAO inhibitors to counterbalance the high *MAOA* protein levels and thereby increase therapy response. Furthermore, healthy participants at a high epigenetic risk to develop PD as defined by *MAOA* hypomethylation might profit from individualized preventive interventions, while high *MAOA* methylation and the ability to maintain it could be discussed as a 'resilience' factor against developing anxiety disorders, especially panic disorder.

V. References

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Ziegler, C., J. Richter, M. Mahr, A. Gajewska, M. A. Schiele, A. Gehrmann, B. Schmidt, et al. 2016. “MAOA Gene Hypomethylation in Panic Disorder-Reversibility of an Epigenetic Risk Pattern by Psychotherapy.” *Translational Psychiatry* 6 (4): e773.

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6.3. Curriculum Vitae

PERSONAL DETAILS

Name	Christiane Ziegler, M. Sc.
Date of birth	18.04.1989
Place of birth	Suhl

ACADEMIC EDUCATION

since 01/2013	PhD student at the Graduate School of Life Sciences, Section Neuroscience, University of Wuerzburg, within the Collaborative research center SFB TRR 58, subproject C02 "Epigenetic profiling of anxiety: the role of DNA methylation in the pathogenesis and therapeutic mechanisms of anxiety disorders", funded by the German research foundation (DFG) under the supervision of Prof. Dr. Dr. Katharina Domschke, Prof. Dr. Klaus-Peter Lesch, Prof. Dr. Charlotte Förster
10/2010 – 12/2012	Master of Science (1.1) with a focus on neurobiology and infection biology, University of Wuerzburg
	Master thesis on "Dual-luciferase assay in human fibroblasts ex vivo" at the Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital Wuerzburg under the supervision of Prof. Dr. Andreas Reif, Prof. Dr. Charlotte Förster
10/2007 – 05/2010	Bachelor of Science (1.9), University of Wuerzburg
	Bachelor thesis on "Effekte von mutierten, humanen LMNA-Genen auf die Zebrafisch-Frühentwicklung (Danio rerio HAMILTON, 1822)" at the Imaging Core Facility, Department of Cell and Developmental Biology, University of Wuerzburg, under the supervision of Prof. Dr. Marie-Christine Dabauvalle
1999 – 2007	Heinrich-Ehrhardt-Gymnasium Zella-Mehlis
	Degree Abitur (1.7)

WORK EXPERIENCE

since 10/2015	Research scientist at the Department of Psychiatry, Psychosomatics and Psychotherapy (Prof. Dr. J. Deckert), University Hospital Wuerzburg within the research association PROTECT-AD subproject P5 “(Epi)Genetic Mechanisms of extinction learning and therapy response in anxiety disorders”
since 01/2013	Research scientist at the Department of Psychiatry, Psychosomatics and Psychotherapy (Prof. Dr. J. Deckert), University Hospital Wuerzburg within the SFB TRR 58 subproject C02 “Epigenetic profiling of anxiety: the role of DNA methylation in the pathogenesis and therapeutic mechanisms of anxiety disorders”
09/2011	Research assistant at the Department of Psychology I: Biological Psychology, Clinical Psychology, and Psychotherapy, University of Wuerzburg (Prof. Dr. P. Pauli, Dr. M. Andreatta)

PROFESSIONAL MEMBERSHIPS

Gesellschaft für Angstforschung (GAF)

FUNDING AND ACADEMIC AWARDS

2016 Travel Fellowship to attend the 29th ECNP Congress 2016 in Vienna, awarded by the Graduate School of Life Sciences

2015 Travel Fellowship to attend the 45th annual meeting of the Society for Neuroscience (SfN) 2015 in Chicago, awarded by the Graduate School of Life Sciences

2015 Travel Award to attend the 28th ECNP Congress 2015 in Amsterdam, awarded by the European College of Neuropsychopharmacology (ECNP)

2014 Travel Award to attend the Annual Meeting of the German Association of Biological Psychiatry 2014 in Aachen (Deutsche Gesellschaft für Biologische Psychiatrie, DGBP), awarded by the German Association of Biological Psychiatry (Deutsche Gesellschaft für Biologische Psychiatrie, DGBP)

RESEARCH AREAS

Genetics, Epigenetics, Anxiety Disorders

POSTER PRESENTATIONS

1) Ziegler C, Kollert L, Schiele MA, Mahr M, Gajewska A, Lesch KP, Deckert J, Domschke K (2016, September) MAOA methylation change as a marker of treatment response: Epigenetic signature of CBT in panic disorder. Annual Meeting of German Association of Biological Psychiatry, Würzburg

2) Ziegler C, Richter J, Mahr M, Gajewska A, Schiele MA, Lang T, Pauli P, Reif A, Rief W, Kircher T, Arolt V, Hamm AO, Deckert J, Domschke K (2016, September) Monoamine oxidase A (MAOA) gene hypomethylation in panic disorder - an epigenetic risk pattern reversed by psychotherapy? 29th European College of Neuropsychopharmacology (ECNP) Congress, Wien

3) Ziegler C, Richter J, Mahr M, Gajewska A, Lang T, Pauli P, Rief W, Kircher T, Arolt V, Hamm AO, Deckert J, Domschke K (2016, March) Reversibility of MAOA gene hypomethylation by psychotherapy in panic disorder patients. WPA Section on Epidemiology and Public Health 2016 Meeting, Munich

4) Ziegler C, Laeger I, Stevens S, Lesch KP, Arolt V, Gerlach A, Deckert J, Zwanzger P, Domschke K (2015, October) Epigenetics of social anxiety disorder: Oxytocin receptor gene (OXTR) hypomethylation as a risk marker? Society for Neuroscience Meeting 2015, Chicago (Travel Fellowship, Graduate School of Life Sciences)

5) Ziegler C, Dannlowski U, Bräuer D, Stevens S, Laeger I, Kugel H, Reif A, Lesch KP, Heindel W, Kirschbaum C, Arolt V, Gerlach AL, Hoyer J, Deckert J, Zwanzger P, Domschke K (2015, September) Oxytocin Receptor Gene Methylation: Epigenetic Modulator of Social Anxiety and its Endophenotypes? SFB-TRR58 4th International Symposium “Fear, Anxiety, Anxiety Disorders”, Münster, Germany

6) Ziegler C, Laeger I, Stevens S, Lesch KP, Arolt V, Gerlach A, Deckert J, Zwanzger P, Domschke K (2015, August) Oxytocin receptor gene (OXTR) hypomethylation as a potential risk factor in social anxiety? 28th European College of Neuropsychopharmacology (ECNP) Congress, Amsterdam (ECNP Travel Award 2015)

7) Ziegler C, Laeger I, Stevens S, Mahr M, Lesch KP, Reif A, Arolt V, Deckert J, Gerlach A, Zwanzger P, Domschke K (2014, November) Oxytocinrezeptor (OXTR) Methylierung – Epigenetischer Marker der Sozialen Phobie? Annual Meeting of the German Association for Psychiatry, Psychotherapy and Psychosomatics (DGPPN), Berlin

8) Ziegler C, Laeger I, Stevens S, Lesch KP, Reif A, Arolt V, Deckert J, Gerlach A, Zwanzger P, Domschke K (2014, September) Epigenetics of social anxiety disorder – A role of Oxytocin receptor gene (OXTR) methylation. Annual Meeting of German Association of Biological Psychiatry, Aachen (Travel award from the German Association of Biological Psychiatry (Deutsche Gesellschaft für Biologische Psychiatrie, DGBP))

9) Ziegler C, Mahr M, Gajewska A, Gehrmann A, Reif A, Pauli P, Lesch KP, Deckert J, Domschke K (2013, November) Epigenetic Mechanisms in the Pathogenesis and Therapy of Anxiety Disorders. Annual Meeting of the Gesellschaft für Angstforschung (GAF), Göttingen

10) Mahr M*, Ziegler C*, Gajewska A, Gehrmann A, Reif A, Pauli P, Lesch KP, Deckert J, Domschke K (2013, September) Epigenetic Profiling of Anxiety: The Role of DNA Methylation in the Pathogenesis and Therapeutic Mechanisms of Anxiety Disorders. Symposium of the collaborative research center SFB-TRR 58, Hamburg (*authors contribute equally)

CONGRESS TALKS

1) Ziegler C (2015, November) Monoaminoxidase A (MAOA) Methylierung – Epigenetische Signatur der Pathogenese und Therapie von Panikstörung?. Annual meeting of the Gesellschaft für Angstforschung (GAF), Wasserburg am Inn

2) Ziegler C (2014, November) Oxytozin Rezeptor Hypomethylierung – Risikofaktor der Sozialen Phobie? Annual meeting of the Gesellschaft für Angstforschung (GAF), Berlin

INVITED TALKS

1) Ziegler C, Domschke K. (2016, September) Epigenetik. Workshop „NetBio-mics – ein Querschnittsprojekt im BMBF-Netz zu psychischen Erkrankungen: Einführung in „omics“ und Biobanking“, Annual Meeting of German Association of Biological Psychiatry, Würzburg

2) Ziegler C, Domschke K. (2016, March) Psychiatric Epigenetics. Intersectional Educational Track, WPA Section on Epidemiology and Public Health 2016 Meeting, Munich

Wuerzburg, November 01, 2016

Signature (Christiane Ziegler)

6.4. Publications

1) **Ziegler, C.**, J. Richter, M. Mahr, A. Gajewska, M. A. Schiele, A. Gehrmann, B. Schmidt, et al. 2016. “MAOA Gene Hypomethylation in Panic Disorder-Reversibility of an Epigenetic Risk Pattern by Psychotherapy.” *Translational Psychiatry* 6 (4): e773.

2) **Ziegler, C.**, U. Dannlowski, D. Bräuer, S. Stevens, I. Laeger, H. Wittmann, H. Kugel, et al. 2015. “Oxytocin Receptor Gene Methylation: Converging Multilevel Evidence for a Role in Social Anxiety.” *Neuropsychopharmacology* 40 (6): 1528–38.

3) Kulenovic A. D., Agani F., Avdibegovic E., Jakovljevic M., Babic D., Kucukalic A., Kucukalic S.,[…], **Ziegler C.**, Wolf C., Warrings B., Domschke K., and Deckert J. 2016. “Molecular Mechanisms of Posttraumatic Stress Disorder (PTSD) as a Basis for Individualized and Personalized Therapy: Rationale, Design and Methods of the South Eastern Europe (SEE)-PTSD study”. *Psychiatria Danubina* 28(2):154-63.

4) Schiele M. A., **Ziegler C.**, Holitschke K., Schartner C., Schmidt B., Weber H., Reif A., Romanos M., Pauli P., Zwanzger P., Deckert J., and Domschke K. 2016. „Influence of 5-HTT variation, childhood trauma and self-efficacy on anxiety traits: a gene-environment-coping interaction study.” *Journal of Neural Transmission (Vienna)* 123(8):895-904.

5) Notzon S., Domschke K., Holitschke K., **Ziegler C.**, Arolt V., Pauli P., Reif A., Deckert J., and Zwanzger P. 2016. „Attachment style and oxytocin receptor gene variation interact in influencing social anxiety.” *World Journal of Biological Psychiatry* 17(1):76-83.

6) Kittel-Schneider S., Schreck S., **Ziegler C.**, Weißflog L., Hilscher M., Schwarz R., Schnetzler L., Neuner M., and Reif A. 2015. „Lithium-induced Clock Gene Expression in Lymphoblastoid Cells of Bipolar Affective Patients”. *Pharmacopsychiatry*, 48(4-5):145-9.

7) Domschke K., Tidow N., Schwarte K., **Ziegler C.**, Deckert J., Arolt V., Zwanzger P., and Baune B.T. 2014. „Pharmacoepigenetics of depression - no major influence of MAO-A DNA methylation on treatment response.” *Journal of Neural Transmission (Vienna)* 122(1):99-108.

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