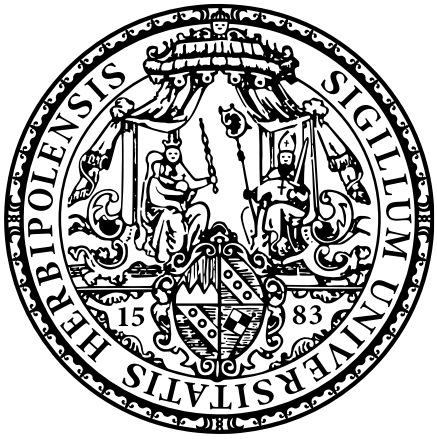

Epigenetics of anxiety and depression – a differential role of TGFB-Inducible Early Growth Response Protein 2 gene promoter methylation

Epigenetik von Angst und Depression – Die differentielle Rolle von TGFB-Inducible Early Growth Response Protein 2 Gen Promotor Methylierung

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Hiermit erkläre ich an Eides statt, die Dissertation „Epigenetik von Angst und Depression – Die differentielle Rolle von TGFB-Inducible Early Growth Response Protein 2 Gen Promotor Methylierung“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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ABBREVIATIONS

ρ	Spearman's rank correlation coefficient
(x)g	(multiplied by) earth's gravitational force
5-HTT	5-hydroxytryptamine (serotonin) transporter, encoded by SLC6A4
A	Adenine
ACQ	Agoraphobic Cognitions Questionnaire
AD	Anxiety disorders
ASI	Anxiety Sensitivity Index
ATP	Adenosine triphosphate
BAI	Beck Anxiety Inventory
BDI-II	Beck Depression Inventory II
BDNF/Bdnf	Brain derived neurotrophic factor
bp	Base pairs
C	Cytosine
CBT	Cognitive behavioral therapy
CCDs	Charged coupled devices
CG/CpG	Cytosine-guanine dinucleotide
CH_3	Methyl group
Chr	Chromosome
CRC-TRR58	Collaborative Research Center - Transregio 58
DAT	Dopamine transporter
ddH_2O	High purified water
DEPC	Diethyl dicarbonate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DSM-IV/5	Diagnostic and Statistical Manual of mental disorders (fourth/fifth edition)
EDTA	Ethylenediaminetetraacetic acid
ENCODE	Encyclopedia of DNA Element

ESME	Epigenetic Sequencing Methylation analysis tool
f	Female
FKBP5	FK506 binding protein 5
g	Gram(s)
G	Guanine
GAD1	Glutamate decarboxylase 1
GR	Glucocorticoid receptor
h	Hour(s)
HAMD	Hamilton Depression Rating Scale
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferases
HPA	Hypothalamus-pituitary-adrenal
HPTM	Post-translational histone modifications
IGF2	Insulin-like growth factor II
kb	Kilobase pairs
KLF11	Krüppel-like factor 11 (encodes TIEG2)
l	Liter(s)
LSAS	Liebowitz Social Anxiety Scale
m	Male
M.I.N.I.	Mini International Neuropsychiatric Interview
MAOA	Monoamine oxidase A
MB-COMT	Membrane binding catechol-O-methyltransferase
MDD	Major depressive disorder
MECP2	Methyl CpG binding protein 2
mg	Milligram(s)
$MgCl_2$	Magnesium chloride
min	Minute(s)
miRNA	Micro RNA
ml	Milliliter(s)
mM	Millimolar

mRNA	Messenger RNA
N	Number
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding RNA
NCS(-R)	National Comorbidity Survey (replicated)
ng	Nanogram(s)
nm	Nanometer(s)
NR3C1/Nr3c1	Nuclear receptor subfamily 3, group C, member 1
nt	Nucleotide
ºC	Celsius
PCR	Polymerase chain reaction
PD	Panic disorder
piRNA	Piwi-interacting RNAs
PPi	Pyrophosphate
PSWQ	Penn State Worry Questionnaire
PTSD	Post-traumatic stress disorder
r	Pearson correlation coefficient
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpm	Rounds per minute
RT	Room temperature
s	Second(s)
SAM	Substrate S-adenosyl-L-methionine
SCID	Structured Clinical Interview for DSM disorders
SCL6A4	Solute carrier family 6, member 4 (encodes 5-HTT)
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Short interfering RNA
sncRNA	Small non-coding RNA

SNP	Single nucleotide polymorphism
SNRI	Serotonin and norepinephrine reuptake inhibitor
SPAI	Social Phobia and Anxiety Inventory
SSRI	Selective serotonin reuptake inhibitor
STAI-T	State Trait Anxiety Inventory – Trait version
T	Thymine
TIEG2	TGFB-inducible early growth response protein 2 (encodes KLF11)
TSS	Transcriptional start site
UCSC	University of California, Santa Cruz
VNTR	Variable number tandem repeat
µl	Microliter(s)

SUMMARY

Among mental disorders, panic disorder (PD) is one of the most common anxiety disorders characterized by recurring and unexpected episodes of extreme fear i.e. panic attacks. PD displays lifetime prevalence rates in the general population between 2.1-4.7 % and in about 30 to 40 % occurs comorbid with major depressive disorder (MDD). Differential methylation levels of the *monoamine oxidase A* (*MAOA*) gene have previously been associated with the etiology of both PD and MDD. The TGFB-Inducible Early Growth Response Protein 2 (TIEG2*;* alias KLF11), an activating transcription factor of the *MAOA* gene, has been reported to be increased in MDD, but has not yet been investigated in PD on any level.

Therefore, in an attempt to further define the role of an impaired TIEG2-MAOA pathway in anxiety and affective disorders, in the present thesis *TIEG2* promoter DNA methylation was analyzed in two independent samples of I) PD patients with or without comorbid MDD in a case/control design and II) MDD patients with and without anxious depression. Additionally, in PD patients of sample I), *TIEG2* methylation was correlated with Beck Depression Inventory (BDI-II) scores. Finally, in a third independent healthy control sample, correlation of *TIEG2* promoter methylation levels with Anxiety Sensitivity Index (ASI) scores as a PD-related measure was analyzed.

No overall association of *TIEG2* promoter methylation with PD was detected. However, PD patients with comorbid MDD showed significant *TIEG2* hypomethylation compared to PD patients without comorbid MDD (p=.008) as well as to healthy controls (p=.010). In addition, MDD patients without anxious features displayed a statistical trend in decreased *TIEG2* methylation in comparison to MDD patients with anxious depression (p=.052). Furthermore, *TIEG2* methylation was negatively correlated with BDI-II scores in PD patients (p=.013) and positively correlated with ASI scores in the healthy control sample (p=.043).

In sum, the current study suggests *TIEG2* promoter hypomethylation as a potential epigenetic marker of MDD comorbidity in PD or of non-anxious depression, respectively. If replicated and verified in future studies, altered *TIEG2* methylation might therefore represent a differential pathomechanism of anxiety and mood disorders.

ZUSAMMENFASSUNG

Die Panikstörung (PD) ist eine der häufigsten Angststörungen, die durch wiederkehrende und unerwartete Episoden extremer Angst gekennzeichnet ist. Die PD tritt in der Allgemeinbevölkerung mit Lebenszeitprävalenzraten zwischen 2,1 und 4,7 % und in etwa 30 bis 40 % der Fälle komorbid mit einer schweren Depression (MDD) auf. Unterschiedliche Methylierungs-Niveaus des *Monoaminoxidase A* (*MAOA*) Gens wurden bereits mit der Ätiologie von PD und MDD assoziiert. Das TGFB-Inducible Early Growth Response Protein 2 (TIEG2; alias KLF11) fungiert als ein aktivierender Transkriptionsfaktor des *MAOA* Gens und wurde bei Patienten mit MDD in seiner Expression erhöht gefunden. Bei der PD wurde TIEG2 bis heute jedoch noch nicht untersucht.

Um die Rolle eines gestörten TIEG2-MAOA Signalwegs bei Angst- und affektiven Störungen genauer zu definieren, wurde in der vorliegenden Studie die Methylierung des *TIEG2* Promotors in zwei unabhängigen Stichproben bestehend aus I) PD Patienten mit bzw. ohne komorbider MDD, sowie II) MDD Patienten mit bzw. ohne erhöhte Angstsymptomen untersucht. Zusätzlich wurde in der PD Stichprobe die *TIEG2* Methylierung mit dem Beck Depression Inventar II (BDI-II) korreliert. Schließlich wurde in einer dritten unabhängigen Stichprobe gesunder Probanden die Korrelation der *TIEG2* Methylierung mit den Punktwerten des Angstsensitivitätsindex (ASI) analysiert.

Es wurde keine Assoziation von *TIEG2* Promotor-Methylierung mit PD beobachtet. Allerdings waren PD Patienten mit komorbider MDD im Vergleich zu PD Patienten ohne komorbide MDD (p=,008) sowie zu gesunden Kontrollprobanden (p=,010) signifikant niedriger methyliert. MDD Patienten ohne ängstliche Symptome zeigten einen statistischen Trend von verringerte *TIEG2* Methylierung im Vergleich zu MDD Patienten mit ängstlicher Depression (p=,052). Zusätzlich korrelierte die *TIEG2* Methylierung negativ mit den BDI-II Werten bei PD Patienten (p=,013) und positiv mit den ASI Werten in der gesunden Probandenstichprobe (p=,043).

Die aktuelle Studie legt nahe, dass eine Hypomethylierung des *TIEG2* Promotors einen epigenetischen Marker für die MDD-Komorbidität bei PD bzw. für die nicht-ängstliche Depression darstellt und damit eine differentielle Rolle im Pathomechanismus von Angst- bzw. affektiven Störungen einnehmen könnte. Diese Hypothese muss allerdings in weiterführenden Studien repliziert und verifiziert werden.

I INTRODUCTION

1.1 Mental disorders

1.1.1 Anxiety disorders

Each year about 38.2 % of the European population suffer from mental disorder. The most frequent mental disorders are depressive disorders, substance use disorders and anxiety disorders (AD) with 14.0 % of the EU population affected by an anxiety disorder (Wittchen et al. 2011). ADs are the most predominant mental health complaint (Kessler et al. 2010), and the global prevalence of anxiety disorders was estimated at 7.3 % (Baxter et al. 2013).

The onset of anxiety disorders occurs during childhood, adolescence or early adulthood, with a median age of onset of 11 years and a peak occurring in middle age (Jalnapurkar and Allen 2018). In the 50- to 64-year age group, prevalence rates decline and were lowest in the elderly group (65 to 79 years; Bandelow and Michaelis 2015). Therefore, age can be considered as a potential risk factor for the etiology of anxiety disorders. Furthermore, genetic predisposition has been suggested to influence the development of any AD, emphasized by heritability estimates of 30 to 50 % (Hettema et al. 2001). Additionally, women are affected twice as often as men (Baxter et al. 2013), and related distress and impairment caused by AD is greater for women than men (McLean et al. 2011). Accordingly, sex can also be considered as a potential risk factor for developing any AD.

Different types of AD are social anxiety disorder, specific phobias, agoraphobia, selective mutism, generalized anxiety disorder, separation anxiety disorder, and panic disorder (American Psychiatric Association 2013). Although these specified forms of AD differ in their lifetime prevalence, they share principal symptoms, with mainly disproportionately fearful, anxious or avoidant behavior, mostly resulting of perceived threats in the environment. These persistent symptoms lead to severe impairments in social life, such as in the work environment and other situations, in which the extreme fear or anxiety makes normal functioning impossible (American Psychiatric Association 2013). If not treated properly, anxiety disorders can lead to the development of several adverse consequences including reduced educational and job-related opportunities, greater functional impairment and overall increase in morbidity and mortality rates (Jalnapurkar and Allen 2018).

The comorbidity of anxiety disorders with other mental disorders or other subtypes of anxiety disorders is common (Kessler et al. 2005). In the epidemiological program National Comorbidity Survey (NCS), replicated in the years 2001 to 2003 (NCS-R), the highest tetrachoric correlations among the anxiety disorders were found between social anxiety disorder and agoraphobia, between panic disorder and agoraphobia, and between specific phobia and agoraphobia (Bandelow and Michaelis 2015). Depression and anxiety disorders are estimated to occur comorbidly in 57 % of patients with an anxiety disorder (Zimmerman et al. 2000). Apart from true comorbidity, depression can be specified as anxious depression, or depression with anxious features (American Psychiatric Association 2013).

1.1.1.1 Panic Disorder

Panic disorder (PD) is one of the most common anxiety disorders, with lifetime prevalence rates in the general population between 2.1-4.7 % (Baxter et al. 2014) and a 12-month prevalence of 1.8 % (Goodwin et al. 2005). PD is characterized by sudden, unexpected panic attacks, i.e. episodes of extreme fear (DSM-5; American Psychiatric Association 2013). These unexpected attacks imply at least four physiological or cognitive symptoms and reach a peak within minutes. Patients with PD often complain about the feeling of going crazy, losing control or even dying (Bandelow et al. 2013). Additionally, they mostly develop anticipatory anxiety with the fear of new, unexpected panic attacks happening at any time and repeatedly. Physical symptoms of panic attacks are various and include stomach discomfort, chills, nausea, sweating, chest pain, palpitations, and shortness of breath or hyperventilation. Further physical characteristics are dizziness, derealization or depersonalization (Yates 2009). Similar to anxiety disorders in general, panic attacks can result in a significant impairment in social life, in socioeconomic problems and a higher risk for developing comorbid depression, alcohol abuse and suicidality (Fleet et al. 1996).

PD is a complex heterogeneous, multifactorial and polygenic disorder (Kim 2019). This heterogeneity not only complicates panic disorder diagnosis but also the treatment outcomes and prognosis. The onset of PD falls between 18 and 45 years (average age of onset: 24 years; Kessler et al. 2005). Besides, women suffer from anxiety disorders two to three times more often than men, comparable to the epidemiology of anxiety disorders in general (Kessler et al. 2006). In addition, a moderate genetic component with a heritability of up to 48 % is involved in the etiology

of PD, indicated by twin studies (e.g. Hettema et al. 2001). Additionally, genetic association studies suggest an association between several candidate gene variants and anxiety diseases such as PD (Gottschalk and Domschke 2016). Environmental factors are also implicated as a possible risk factor for developing PD. Specific as well as cumulative negative life events, such as threat, child abuse or loss/separation experiences in childhood as well as critical interpersonal and health-related events in adulthood might have a meaningful influence on the pathogenesis of PD. Also, personality and behavioral characteristics, such as anxiety sensitivity, neuroticism and cognitive appraisal might impact the development of PD (Klauke et al. 2010). Additionally, studies indicated that head injuries (Koponen et al. 2002), alcohol or nicotine dependence (Philibert et al. 2008a, 2010) can also result in a higher risk of developing PD.

The course of PD is often accompanied by other comorbid anxiety disorders such as agoraphobia (Noyes et al. 2006). Furthermore, depression and disorders of abuse also frequently occur as comorbidities of PD (Gorman and Coplan 1996; Smith and Book 2008). The lifetime prevalence rates of comorbid major depression in PD are about 30 to 40 %, similar to the lifetime prevalence rates of comorbid MDD in the whole group of anxiety disorders (Kessler et al. 2006).

According to the current S3-guidelines (Bandelow and Wiltink 2014), cognitive behavioral therapy (CBT) accompanied by pharmacotherapy are highly effective treatment possibilities for PD, analogous to anxiety disorders in general (Bandelow et al. 2013). Highly effective drugs in the treatment of PD are selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). Benzodiazepines are also effective drugs but are to be used with extreme caution, because of the high potential of abuse. Besides pharmacological treatment, cognitive behavioral therapy in PD including psychoeducation and exposure exercises (Lang 2012) is one of the primary treatment options. Exposure exercises comprise confrontation and habituating to fearful situations in which the patient feels extremely uncomfortable but realizes that the fear is unfounded and can be overcome with the right treatment techniques. CBT can be supplemented by endurance sports, relaxation techniques, breathing techniques and confidence training (Barlow et al. 2000). In addition, participation in self-help groups can be recommended to patients and their families. The combination of pharmacotherapy and cognitive behavioral therapy are highly effective in the treatment of PD, but still 20-40 % of all patients do not respond

to the initial treatment. These patients suffer from a poor quality of life as well as socio-economic difficulties and a heightened rate of suicide attempts (Bystritsky 2006).

1.1.2 Major Depressive Disorder

Major depressive disorder (MDD) is a serious, recurring disorder associated with decreased quality of life, medical morbidity and mortality. Affected patients suffer from irregularities of mood, neurovegetative functions (e.g. appetite and sleep disorders) and cognition (e.g. inappropriate feelings of guilt and feelings of worthlessness) as well as disturbed psychomotor activity (e.g. arousal or disability). According to the DSM-5, the average life prevalence and 12-month prevalence rates are 14.6 % and 5.5 %, respectively, in countries with high incomes as compared to 11.1 % and 5.9 % in countries with low to medium incomes. Similar to PD, women are two times more often affected than men (Bromet et al. 2011). The average age of onset is 25.7 years in countries with high income and 24.0 years in countries with low to middle income (Bromet et al. 2011). However, MDD is not a disorder that is limited to adults and the elderly. There is strong evidence that adverse conditions in childhood are significant risk factors for the occurrence of depressive disorders (Fava and Kendler 2000; Gilman et al. 2002) and, in contrast, sufficient parenting alleviates the effects of negative life events (Armsden et al. 1990; Dekovíc and Meeus 1997). For most people, MDD is a lifelong, repetitive episodic disease, with approximately 20 to 25 % of MDD patients experiencing a chronic, relentless progression. The chronic and recurrent course of MDD is an important clinical problem that often requires long-term prophylactic treatment (Fava and Kendler 2000). Depression heritability is moderate at 31-42 % (Sullivan et al. 2000), and it is supposed that genetic factors influence the risk of depression also by affecting sensitivity to stressful life events (Kendler et al. 1995).

1.2 Epigenetic mechanisms

This section is based on a part of the introduction of the master thesis "Functional characterization of DNA methylation patterns in candidate genes for panic disorder", which was submitted by Leonie Kollert to the University of Würzburg in 2016 (Kollert 2016).

The term epigenetics describes the study of regulation of gene expression, which is not directly caused by a change in the nucleotide sequence (Bird 2007). The

discovery of epigenetic mechanisms started in the late 1940s with the discovery of 5-methylcytosine as part of the mammalian genome (Hotchkiss 1948).

Epigenetic mechanisms affect gene expression levels by chemical reactions that can turn parts of the genome on or off, thereby epigenetic mechanisms are heritable via cell mitosis or cell meiosis (Berger et al. 2009). These epigenetic modifications can influence the development of various diseases like for example cancer, by either regulating cell fate or even causing cell damage (Egger et al. 2004). Several factors like age, environment, nutrition, activity or disease can influence epigenetic changes in the genome. Epidemiological studies have publicized that prenatal as well as early postnatal environmental influences may also effect the risk of developing various chronic diseases and behavioral disorders (Jirtle and Skinner 2007). For instance, if a pregnant woman was exposed to famine during the period of the so called “Dutch Famine” (1944-1945), their children have a higher risk of developing coronary heart disease and obesity. Furthermore, hypomethylation of the *insulin-like growth factor II* (*IGF2*) gene has been indicated as a molecular basis of this phenomenon (Heijmans et al. 2008).

At present, three main molecular mechanisms of epigenetic gene regulation are known: the posttranscriptional regulation via DNA methylation, histone modifications and non-coding RNAs (Hagood et al. 2014; Schuebel et al. 2016).

1.2.1 DNA methylation

Among the three main epigenetic processes (DNA methylation, histone modifications and micro-RNAs) DNA methylation is probably the first discovered epigenetic mechanism, dating back to reports by Griffith and Mahler in 1969 (Griffith and Mahler 1969). DNA methylation can have a significant effect on the formation of specific cell identities by critically influencing their gene expression. For example, DNA methylation can regulate X-chromosome inactivation in women and therefore has an important influence on the formation and maintenance of genomic imprinting. Since DNA methylation patterns can be transmitted from one cell to another, this epigenetic mechanism is proposed to be stable during cell division (Jaenisch and Bird 2003).

During the process of DNA methylation a methyl-(CH3) group is added to the cytosine base, which occurs when cytosine is coupled with guanine (5'-CpG-3') as a CpG-dinucleotide (Robertson 2005). Thereby, the substrate S-adenosyl-L-methionine (SAM) acts as a methyl donor converting cytosine into 5-methyl-cytosine. This reaction

is catalyzed by DNA methyltransferases (DNMT). The three DNA methyltransferases DNMT1, DNMT3A and DNMT3B form a highly conserved protein family and catalyze either “de novo” or “maintenance” methylation (Yoder and Bestor 1998; Okano et al. 1999; Bestor 2000; Cheng et al. 2008). DNMT1 is usually expressed in somatic cells, primarily during the S-phase and preferentially affects hemi-methylated DNA. Therefore, DNMT1 is responsible for maintenance methylation. This mechanism is necessary to reserve important DNA methylation patterns after every DNA replication cycle. The loss of maintaining DNA methylation would lead to passive de-methylation of the template strand (Bestor et al. 1988). In contrast, DNMT3A and DNMT3B are associated with non-methylated CpG-dinucleotides and expressed during embryonic development and catalyze de novo methylation (Kafri et al. 1992; Okano et al. 1999). Therefore, this methylation mechanism is important for the establishment of new methylation patterns. Both, maintenance and de novo methylation seem to be essential for normal neuronal development. This statement is supported by studies showing that knock-out of genes coding for DNMT1, 3A and 3B results in a prenatal or an early postnatal death in mice (Li et al. 1992, 1998).

In the human genome, 65 % of all genes include short CpG-rich regions (CpG islands) that are at least 200 base pairs (bp) long and comprise more than 55 % of CpG-dinucleotides (Bestor et al. 1988; Takai and Jones 2002). In somatic cells, the majority of these CpG islands are non-methylated, while methylated CpG islands are mostly associated with tissue-specific and imprinting genes (Ehrlich et al. 1982; Bird 1986). The position of CpG methylation in the transcriptional unit makes an important difference in gene control. A CpG hypermethylation in the promoter region of the gene impairs the initiation of gene transcription, whereas DNA methylation in the gene body mostly results in an activation of gene transcription (Suzuki and Bird 2008; Ball et al. 2009).

The regulation of gene expression through CpG methylation is achieved in two ways: Either by the recruitment of repressor complexes which can cause a change of the chromatin structure or by a direct blockade of the binding of transcription factors in the promoter of the gene (Nan et al. 1997; Bird et al. 1999; Jones 2012).

1.2.2 Histone modifications

Histones are alkaline proteins of the cell nucleus of eukaryotes that create a structure known as heterochromatin by interacting – due to their positive charges –

with the negatively charged nucleic acid of chromosomes. There are two major classes of histones containing five different histone protein families: core histones (H2A, H2B, H3 and H4) and linker histones (H1 and H5). Nucleosomes are structural units of the nucleic acid strand. A nucleosome consists of a histone-octamer comprising eight histones, two copies of each core histone. Each histone-octamer is surrounded by 1.75 spiral turns of the DNA strand. The linker histone H1 induce the next higher packing unit of DNA by binding further nucleic acids appearing close to the nucleosomes (Allis et al. 2007).

Post-translational histone modifications (HPTMs) produce many variants in chromatin structures and can be divided into two groups: the first group includes acetylation, phosphorylation and methylation as minor chemical modification of histone proteins. The second group comprises peptides, which are the basis of larger modifications like ubiquitylation and sumoylation. Histone modifications can affect gene expression by changing the structure of chromatin (cis-modification), by disturbing the binding of negative-acting proteins or by providing altered binding surfaces that appeal to certain effector proteins (trans-modifications; Allis et al. 2007).

Activation of gene transcription is induced by histone acetylation occurring only at lysine bases of the N-terminal tail of histone proteins. In detail, histone acetyltransferases (HAT), and acetyl coenzyme A as a co-factor, catalyze the transfer of an acetyl group to the amino group of lysine sidechains. The neutralization of the positive charge of lysine residues by the addition of an acetyl group leads to a reduction of the electrostatic interaction between lysine and the negative charged DNA molecule. This process allows binding of transcription factors of the transcription machinery to specific DNA regions by opening of the chromatin structure (cis-effect) and therefore induces gene transcription. Histone acetylation can be actively undone by histone deacetylases (e.g. HDAC4; Allfrey et al. 1964).

Additionally, gene transcription can be activated by a second histone modification. The histone phosphorylation occurs at common amino acids like serines, threonines and tyrosines, which contain a hydroxyl group. A negative charge is added by the transfer of a phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of target amino acid side-chains (Oki et al. 2007).

The third histone modification, the histone methylation seems to modulate gene transcription both positively and negatively, depending on which residue (lysine or

arginine) is modified. This modification does not result in any alteration of charge and occurs on lysine and arginine residues. To the lysine residues, up to three methyl groups can be added, while arginine residues can be transformed with up to two methyl groups. These dissimilar methylation conditions are often disproportionately allocated in the genome and thus may fulfill diverse biological functions. Histone methylation is caused by histone methyltransferases (HMT) and removed by histone demethylases (HDM; Lan and Shi 2009).

1.2.3 Non-coding RNAs

The last epigenetic mechanism is characterized by transcriptional regulation via so-called non-coding RNAs (ncRNAs). After DNA transcription, NcRNA molecules are synthesized by RNA polymerase II or RNA polymerase III and remain untranslated into a protein structure (Bartel et al. 2004). A nowadays well investigated mechanism, known as RNA interference (RNAi; Fire et al. 1998), was the first evidence of an epigenetic regulation via ncRNAs. Fire and colleagues showed that in the common model organism *Caenorhabditis elegans*, an artificially induced double-stranded RNA results in an effective and specific gene knockdown. In this process, the messenger RNA (mRNA) is cleaved into multiple fragments and therefore the information is destroyed and the translation into a protein is stopped (Fire et al. 1998).

Approximately 98 % of all transcriptional outputs in humans result from ncRNAs (Mattick 2001), which play an important role in the formation of heterochromatin, histone modifications, DNA methylation targeting and as a consequence in gene silencing. NcRNAs can be divided into two groups: short (<30nt) and long (>200nt) non-coding RNAs. Predominantly short ncRNAs (sncRNAs), comprising micro-RNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs), play an important role in the epigenetic regulation of gene expression (Zhou et al. 2010). The long non-coding RNAs rather have influence on chromatin remodeling, transcriptional regulation, post-transcriptional regulation and act as precursor for siRNAs (Kaikkonen et al. 2011).

In the group of sncRNAs, MicroRNAs (miRNAs) control the expression of homologous target gene transcripts and are highly conserved, non-protein coding RNAs. Mature miRNA forms a so called RNA-induced silencing complex (RISC; Hutvágner and Zamore 2002), which target specific mRNA. Blocking of the post-transcriptional regulation of the target mRNA via RNA cleavage or degradation is

induced by binding of this specific complex to target mRNA (Collins et al. 2011). In detail, miRNAs bind to the 3'-untranslated region (3'-UTR) of the target mRNA and translation of these mRNAs will be prevented or the mRNAs will be destroyed by cutting. Which mechanism is induced, depends on the complementarity of the binding sequence and the proteins involved in translation. Partial complementarity results in inhibition of translation, while perfect base pairing end in the degradation of the target mRNA (Wienholds and Plasterk 2005). This RISC mechanism was initially developed as a defense mechanism but has gained an additional function in the progress of evolution and plays now an important role in the complex network of gene regulation, predominantly in gene silencing.

Another mechanism that induces mRNA degradation involves SiRNAs function similar to previous described miRNAs function. SiRNA functions induces also post-transcriptional silencing of gene expression. In addition, they stimulate formation of heterochromatin via a RISC mechanism, which supports the histone methylation and chromatin condensation (Carthew and Sontheimer 2009).

As a last member of sncRNAs, PiRNAs interact with proteins of the piwi-family. They stimulate chromatin regulation trough suppression of transposon activity in germline and somatic cells (O'Donnell and Boeke 2007). PiRNAs bind to expressed transposons in an antisense orientation and cleave the transposons by forming complexes with proteins of the piwi-family. Additional piRNAs are generated due to cleavage of transposons and that again leads to cleavage of further transposons. This cycle produces an abundance of piRNAs and ultimately results in silencing of the transposon (O'Donnell and Boeke 2007).

1.3 Epigenetic mechanisms at the interface of environment-gene interactions

Many different environmental factors (e.g. nutrients, pollutants, chemicals, physical activity, lifestyle, physical and mental stress) can modulate epigenetic mechanisms during lifespan. Epigenetic mechanisms, in turn, can be associated with the pathology of several mental diseases in humans.

In the rodent model, Kember et al. showed that deficiency in the stress hormone system and therefore in the stress response can be caused by methylation of genes, involved in the negative feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis. After maternal separation of mice, an increased methylation of the glucocorticoid receptor gene *nuclear receptor subfamily 3 group C member 1* (*Nr3c1*) was discerned,

which implicated a reduced transcription of *Nr3c1* and subsequently a deficiency in the HPA axis and the stress system (Kember et al. 2012). This phenomenon was already demonstrated in 1999, investigating glucocorticoid receptor *Nr3c1* methylation levels in the hippocampus of rats (Francis et al. 1999). Another study reports that increased pup licking and grooming by rat mothers altered activity of the *Nr3c1* gene in the offspring's hippocampus, leading to a reduction of the stress hormone cortisol. Here, maternal care led to decreased *Nr3c1* methylation levels and therefore to a higher expression of the glucocorticoid receptor gene in the hippocampus and an increased negative feedback sensitivity (Weaver et al. 2004).

Similarly, an influence of stress in early developmental phases (e.g. childhood maltreatment) on decreased DNA methylation of the *FK506 binding protein 5* gene (*FKBP5*), a member of the stress hormone system, was demonstrated in humans. This hypomethylation causes an increase in *FKBP5* expression and consequently a receptor resistance of glucocorticoid, followed by dysregulation of the hormone stress system. Interestingly, this *FKBP5* methylation was found to be allele-specific. This means that only risk allele carriers (T allele carriers) of the single nucleotide polymorphism (C/T) exhibited this reduced methylation. Carriers of the homozygous protective genotype (CC genotype carriers) did not show a lower *FKBP5* methylation in association with childhood maltreatment (Klengel et al. 2013).

Another important factor that can influence the mental health of an organism is the diet. The "Dutch famine of 1944", already described in Introduction section 1.2, serves as an example of the connection between epigenetics and nutrition, particularly in humans. Studies have shown that children of pregnant women who had been exposed to this famine, suffered from diseases such as diabetes, obesity or cardiovascular diseases (Stein et al. 2004). This went along with a lower methylation of the gene controlling the production of the growth factor IGF-2 (insulin like growth factor-2). These observations suggest that environmental influences could have altered the epigenetic make-up of the fetus, already during pregnancy (Heijmans et al. 2008). Furthermore, in the yellow Agouti (Avy) mouse model, in which the coat color is determined by epigenetic patterns in an early developmental phase, it was shown that a diet with a high amount of methyl groups (CH3) led to an increased DNA methylation and thus to an inactivation of the *agouti* gene along with a yellowish coat color and obesity of the offspring (Dolinoy 2008).

In addition to the factors mentioned above, it has also been shown that physical exercise can lead to an epigenetic change potentially relevant for various mental diseases. For example, exercise has been shown to be associated with decreased methylation of the *brain derived neurotrophic factor* (*Bdnf*) in rats. This factor encodes a molecule that is involved in hippocampal learning and memory, and an epigenetically conferred increased *Bdnf* expression would therefore lead to an increased cognitive performance and heightened synaptic plasticity (Gomez-Pinilla et al. 2011).

1.4 Epigenetic mechanisms in mental disorders

Epigenetic mechanisms such as DNA methylation may also play a crucial role at the interface of genetic and environmental influences in the conferral of mental disorder pathogenesis (for review see Schiele and Domschke 2018). This interplay of genetic predisposition and epigenetic influence might be able to solve the "hidden heritability" problem described by Manolio and colleagues. Epigenetic mechanisms like DNA methylation could therefore serve as the “missing link” between already identified genetic variants of risk genes and further risk factors (Manolio et al. 2009; Petronis 2010).

For example, promoter hypomethylation of the *membrane binding catechol-O-methyltransferase* (*MB-COMT*) gene was linked to schizophrenia and bipolar disorder (Abdolmaleky et al. 2006). Additionally, hypermethylation of the *dopamine transporter* (*DAT*) gene promoter was associated with lower rates of alcohol addiction (Hillemacher et al. 2009). Concerning the *serotonin transporter gene* (*SLC6A4*, 5-*HTT*) as a prime candidate gene in the pathogenesis of major depressive disorder, hypermethylation of the *5-HTT* gene promoter was associated with major depression (Philibert et al. 2008b) and higher severity of depressive symptoms (Kang et al. 2013). In a pharmacoepigenetic study, depressive patients with lower *5-HTT* promoter methylation at baseline showed a decreased reduction in HAMD (Hamilton 1960) scores after six weeks of pharmacotherapy as related to patients with increased 5-HTT promoter methylation at baseline and therefore an impaired antidepressant treatment response (Domschke et al. 2014). In addition to MDD, the *serotonin transporter* gene seems to play an important role in post-traumatic stress disorder (PTSD). In short, individuals with lower *5-HTT* methylation levels and having experienced a greater number of experienced traumatic events had a higher risk of PTSD diagnosis.

However, probands with a higher *5-HTT* methylation level displayed resilience against developing PTSD despite increased traumatic events (Koenen et al. 2011).

Another very interesting candidate gene in the pathology of mental disorders, the *monoamine oxidase A* (*MAOA*) gene, is located on the X chromosome (Xp11.4-p11.3). The corresponding protein is concentrated on the outer membrane of the mitochondria and is highly expressed in neuronal and cardiac cells. In general, MAOA plays an important role in the degradation of biogenic amines including serotonin, norepinephrine and epinephrine, by oxidative deamination (Shih and Chen 1999). In anxiety disorders and panic disorder in particular, *MAOA* methylation seems to have an important influence on the categorical phenotype as well as on treatment response on both a genetic and epigenetic level. On a genetic level, the *MAOA* gene carries a functionally relevant 30 base pair long, variable number tandem repeat (VNTR) in its promoter region (Sabol et al. 1998). This polymorphic region has been linked to panic disorder in a sex-specific manner. The long variants of the VNTR were associated with the pathology of panic disorder and impaired treatment response in different independent female cohorts (Reif et al. 2014). In contrast, short variants were related to aggressive behavior in males. This phenomenon is entitled “warrior vs. worrier gene dichotomy” (Gibbons 2004). On an epigenetic level, female patients displayed lower DNA methylation levels in the exonI/intronI region of the *MAOA* gene compared to healthy controls (Domschke et al. 2012; Ziegler et al. 2016). In addition, DNA methylation status seems to be dynamic and influenced by the environment: negative life events correlated with a decreased, positive life events with an increased DNA methylation (Domschke et al. 2012). Moreover, *MAOA* hypomethylation has been observed to be predictive of response to pharmacological treatment in MDD (Domschke et al. 2012) and to be modifiable by psychotherapeutic interventions in anxiety disorders (Ziegler et al. 2016; Schiele et al. 2018; for review see Schiele et al. 2020). For instance, PD patients displayed differential dynamics in their methylation status dependent on their treatment response: responders displayed an increase in DNA methylation, while non-responders showed an unaltered or even decreasing *MAOA* methylation after therapy (Ziegler et al. 2016). In general, *MAOA* seems to be a key player in anxiety and other mood or stress-related disorders (for review see Ziegler and Domschke 2018), as further studies have implicated differential *MAOA* methylation in post-traumatic stress disorder (Ziegler et al. 2018), acrophobia (Schiele et al. 2018) and MDD (Melas et al. 2013; Melas and Forsell 2015).

1.5 Relationship between TIEG2 and MAOA

Recent reports have underlined the TGFB-Inducible Early Growth Response Protein 2 (TIEG2*;* alias Krüppel-like factor 11, KLF11) as a novel transcriptional activator of *MAOA* gene expression (for review see Duncan et al. 2012). *TIEG2* gene is located on chromosome 2 (chr2:10,182,976-10,194,963; GRCh37/hg19 Assembly, UCSC Genome Browser), and the protein encoded by this gene is a zinc finger transcription factor. *TIEG2* regulates various cellular processes e.g. apoptosis, cell growth, differentiation etc. (Buck et al. 2006; Cook et al. 1998; Fernandez-Zapico et al. 2003; Tachibana et al. 1997) through a Pol II mediated initiation of transcription within the promoter region of neuronal genes (Cook et al. 1998; Tachibana et al. 1997). Ou and colleagues have proposed that TIEG2 is a robust transcription factor of the *MAOB* gene by binding to GC-rich sites near the transcription start sites in the *MAOB* gene and therefore increasing gene transcription (Ou et al. 2004). Similarly, the *MAOA* gene promoter also contains four Sp1-binding sites, which have recently been described to be a target of TIEG2 (Grunewald et al. 2012). TIEG2 as an activating transcription factor of *MAOA* would therefore stimulate the transcriptional activity as well as increase mRNA levels of this gene. This would result in an increased MAOA protein level and higher enzymatic activity (see Fig. 1).

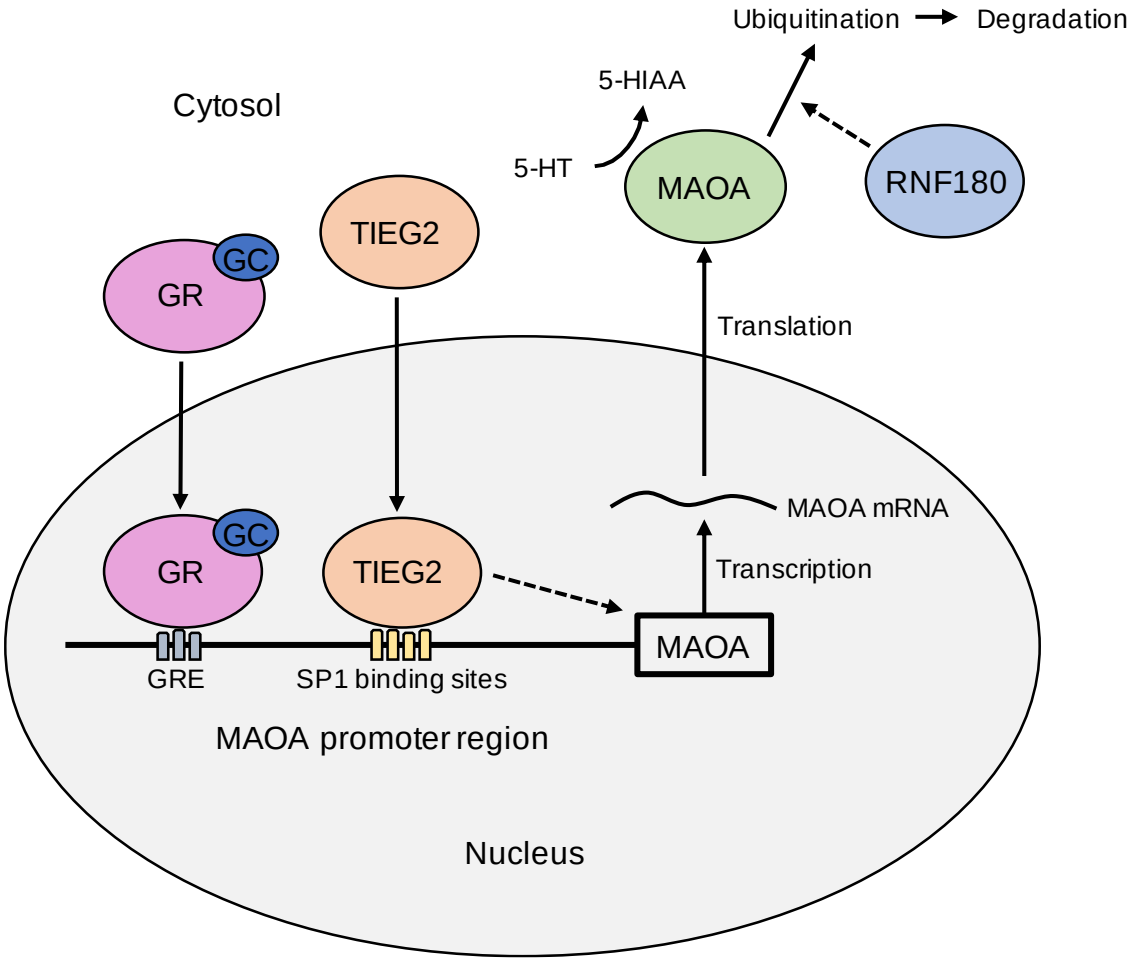

Fig. 1 Pathway of TGFB-Inducible Early Growth Response Protein 2 (TIEG2) and *MAOA* gene

TIEG2 binds as an activating transcription factor to Sp1 binding sites within the *MAOA* gene promoter. This leads to an activation of *MAOA* gene transcription and therefore to an increase in MAOA mRNA and protein levels, followed by an increase of MAOA enzymatic activity. This pathway results in higher degradation of monoamines via ubiquitination. [Figure modified from Higuchi et al. 2017]

This hypothesis was reinforced by a study showing that both, TIEG2 mRNA levels and protein levels were increased in cultured neuronal cells by administration of glucocorticoids. In addition, MAOA mRNA levels and enzymatic activity were 2-fold increased after overexpression of *TIEG2*. An siRNA-mediated *TIEG2* knockdown, however, reduced *MAOA* expression in neuronal cells. Furthermore, *Tieg2* knockout in mice induced decreased MAOA mRNA levels and catalytic activity in the cortex compared to wild-type mice. Additionally, exposure of rats to chronic social defeat stress test led to an activation of the TIEG2 pathway in the brain and thus to increased MAOA mRNA levels and enzymatic activity (Grunewald et al. 2012).

Further support for a TIEG2-MAOA pathway, especially in the pathophysiology of MDD, emerges from a study showing elevated TIEG2 protein levels correlating with

increased MAOA levels in post mortem brain samples of patients with MDD (Harris et al. 2015).

Given these close links between *MAOA* expression and neurobiological processes involved in the pathogenesis of anxiety disorders like PD and the association of increased TIEG2 and MAOA protein levels in patients with MDD, *TIEG2* as an activating transcription factor of *MAOA* may therefore constitute a prime candidate gene in the pathogenesis of panic disorder and MDD as well.

1.6 Aim of the thesis

Extending previous studies demonstrating differential *MAOA* methylation in the pathology of mental disorders such as panic disorder and MDD (Domschke et al. 2012; Melas et al. 2013; Melas and Forsell 2015; Ziegler et al. 2016) and building on previous evidence for a role of TIEG2 in depression closely linked to MAOA function (Harris et al. 2015), the aim of this thesis was to investigate the role of *TIEG2* for the first time in panic disorder and major depressive disorder. For this purpose, a promoter sequence of the human *TIEG2* gene was explored in silico, followed by analysis of *TIEG2* methylation and its role in panic disorder by pyrosequencing of a 72 bp long DNA sequence in a case/control sample (sample I). To account for the high rate of comorbid depression in panic disorder, association of *TIEG2* methylation with a dimensional phenotype of depression (Beck Depression Inventory II, BDI-II; Hautziger et al. 2009) was investigated in the PD patient group (sample I). Reciprocally, in a sample of patients with major depression *TIEG2* methylation was analyzed for association with the phenotype of anxious vs. non-anxious depression (anxiety/somatization factor; Hamilton Depression Rating Scale, HAMD; Hamilton 1960; sample II). Finally, the role of *TIEG2* methylation in depression/panic disorder-related traits was investigated in a large sample of healthy probands with respect to various dimensional phenotypes of anxiety (e.g. Anxiety Sensitivity Index, ASI; Reiss et al. 1986) and depression (e.g. Beck Depression Inventory II, BDI-II; Hautziger et al. 2009; sample III). This three-step approach was expected to aid in further delineating the epigenetic effects of the TIEG2-MAOA pathway in anxiety and affective disorders.

II MATERIALS AND METHODS

2.1 Materials

2.1.1 Kits

Tab. 1: Kits

Name	Producer
EpiTect® 96 Bisulfite Kit	Qiagen, Hilden, Germany
EpiTect® PCR Control DNA Set	Qiagen, Hilden, Germany
FlexiGene® DNA Kit	Qiagen, Hilden, Germany
HotStarTaq® Plus Polymerase Mastermix	Qiagen, Hilden, Germany
PyroMark® Gold Q96 Reagents	Qiagen, Hilden, Germany

2.1.2 Media, buffers and stock solutions

Tab. 2: Media, buffers and stock solutions

Solution	Composition
Blue Loading Buffer	25 mg bromophenol blue-xylene cyanole 1.5 ml glycerol Add ddH_2O to 10 ml
DEPC-treated ddH2O	0.1 % v/v diethyl dicarbonate (DEPC) ddH2O incubated for 1 h and autoclaved
Freezing Lysis Buffer	2.5 ml Nonident P40 22.5 g NaCl Add ddH_2O to 2500 ml
Lysis Buffer	155 ml 1M NH_4Cl 10 ml 1M $KHCO_3$ 200 µl 0.5 M EDTA-solution pH 8.0 Add ddH_2O to 1000 ml
PCR Buffer	500 µl 1 M KCl 100 µl 1 M tris-HCl pH 8.3 25 µl 10 % Polysorbate 20 (Tween 20) 100 µl 0.1 M MgCl2 200 µl ddH2O

SE Buffer	4.383 g NaCl (75 mM) 50 ml EDTA-solution 0.5 M pH 8.0 Add ddH_2O to 1000 ml
TAE-Buffer (1l)	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, fluids and organic solvents

Tab. 3: Chemicals

Chemicals	**Producer**
Agarose peqGold	peqLab, Erlangen, Germany
Annealing Buffer	Qiagen, Hilden, Germany
Binding Buffer	Qiagen, Hilden, Germany
CoralLoad Concentrate	Qiagen, Hilden, Germany
ddH_2O for Chromatography (LiChrosolv®)	Merck, Darmstadt, Germany
Deoxyribonucleotide Triphosphate	Promega, Mannheim, Germany
Ethanol (absolute, 99.8 %)	ChemSolute, Renningen, Germany
Ethidium Bromide Solution (0.07 %)	Sigma-Aldrich, Taufkirchen, Germany
GeneRuler 100bp plus DNA-ladder	Thermo Scientific, Munich, Germany
$MgCl_2$	Sigma-Aldrich, Taufkirchen, Germany
NaOH	AppliChem, Darmstadt, Germany
Streptavidin Sepharose	GEHealthcare, Munich, Germany
Wash Buffer	Qiagen, Hilden, Germany

2.1.4 Enzymes

Tab. 4: Enzymes

Enzymes	**Producer**
Pronase E	Applichem, Darmstadt, Germany

2.1.5 Consumables

Tab. 5: Consumables

Consumable	Producer
96-well PCR Plate	4titude Ltd, Berlin, Germany
Cryo Sample Tubes 1.5 ml/2 ml	Sarstedt, Nümbrecht, Germany
Eppendorf Combitips	Eppendorf, Hamburg, Germany
Eppendorf Reaction Tube 1.5 ml/2 ml	Eppendorf, Hamburg, Germany
Falcon Sample Tube 15 ml/50 ml	Greiner Bio-One, Frickenhausen, Germany
PCR Cover Foil	Thermo Scientific, Munich, Germany
PCR Reaction Tube 300 µl	Sarstedt, Nümbrecht, Germany
Pipette (sterile) 2/5/10/25/50 ml	Sarstedt, Nümbrecht, Germany
Pipette Tips (sterile) 10/100/1000 µl	Sarstedt, Nümbrecht, Germany
Q96 ID Cartridge	Qiagen, Hilden, Germany

2.1.6 Equipment and devices

Tab. 6: Equipment and devices

Equipment	Producer
Centrifuge 5430	Eppendorf, Hamburg, Germany
Centrifuge MIKRO 220 R	Hettich, Tuttlingen, Germany
Centrifuge Rotana 460 R	Hettich, Tuttlingen, Germany
Freezer (-20 °C)	Liebherr, Biberach, Germany
Freezer (-80 °C)	Fisher Scientific, Schwerte, Germany
Gel Chamber B2	BioRad, Munich, Germany
Incubator B 5042 E	Heraeus, Hanau, Germany
KNF Lapoport Vacuum Pump	Sigma-Aldrich, Taufkirchen, Germany
Magnetic Stirrer	Heidolph, Schwabach, Germany
Microwave Express	Sharp, Hamburg, Germany
NanoDrop ND1000	pegLab, Erlangen, Germany
PCR Plates PP	Nb Nerbe plus, Winsen, Germany
Photochamber ChemieDoc UV Shield	BioRad, Munich, Germany
PyroMark® Q96 ID instrument	Qiagen, Hilden, Germany
PyroMark® Q96 Plate low	Qiagen, Hilden, Germany

PyroMark® Q96 Workstation	Qiagen, Hilden, Germany
Shaker Bioshake iQ	BioShake, Jena, Germany
Thermocycler T Gradient	Biometra, Göttingen, Germany
Thermocycler T Professional	Biometra, Göttingen, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Vacuum manifold QIAVac 96	Qiagen, Hilden, Germany
Vacuum pump standard lab-duty oil free	Welch, Alton, Hampshire, UK
Voltage device E431	Consort, Turnhout, Belgium
Vortex mixer Microspin FV-2400	Eppendorf, Hamburg, Germany
Water bath JB Aqua 12	Great Instruments, Cambridgeshire, UK

2.1.7 Software

Tab. 7: Software

Software	**Company**
Bisearch - Primer Design and Search Tool	http://bisearch.enzim.hu/; Tusnady et al. 2005
ESME	Lewin et al. 2004
Prism 6 - GraphPad	GraphPad Software, San Diego, USA
MethPrimer	www.urogene.org/methprimer/index.html; Li LC and Dahiya R, 2002
Microsoft® Excel 365	Microsoft, Redmond, USA
Microsoft® Office 365	Microsoft, Redmond, USA
PyroMark® Assay Design 2.0	Qiagen, Hilden, Germany
PyroMark® Q96 Software	Qiagen, Hilden, Germany
Sequence Scanner Software	Applied Biosystems by Life Technologies, Darmstadt, Germany
IBM SPSS Statistics for Windows, Version 25.0	IBM Corporation, Armonk, USA

2.2 Methods

2.2.1 Studies

All studies were approved by the ethics committees of the University of Würzburg/Hamburg/Münster, Germany with ethic votes of sample I: 79/12, sample II: 104/12 and sample III: 304/15. All participants gave their written informed consent and the studies were conducted according to the ethical principles of the Helsinki Declaration.

2.2.1.1 Sample I – PD case/control sample

Sample I comprised 60 Caucasian patients (f=47; age [mean±SD]: 34.28±9.49 years) with PD as well as 60 healthy subjects, which were recruited and psychophysiologically characterized by Dipl.-Psych. M. Mahr and Dr. A. Gajewska under supervision of Prof. Dr. Dr. K. Domschke at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, from 2012 to 2016 within the Collaborative Research Centre SFB-TRR-58 'Fear, Anxiety, Anxiety Disorders', 2nd funding period, project C02. PD diagnosis was determined by experienced psychiatrists based on medical records and a Structured Clinical Interview according to the criteria of DSM-IV (SCID; Wittchen et al. 1997). Within the patient group, twenty-eight PD patients suffered from comorbid agoraphobia (N=28, 46.6 %). As long as PD was the primary diagnosis, other comorbid axis I diagnoses were tolerated, except bipolar disorder, psychotic disorders, current alcohol dependence, current abuse of or dependence on benzodiazepines and other psychoactive substances (depression: N=28; social anxiety disorder: N=3; specific phobias: N=2; cf. Ziegler et al. 2016, 2019; Schiele et al. 2019; Kollert et al. 2020). Further exclusion criteria comprised pregnancy, current or previous internal or neurological somatic illnesses and any somatic medication. Additionally, probands taking illegal drugs e.g. cannabis (assessed by urine toxicology) and reporting excessive alcohol (>15 glasses of alcohol per week) or nicotine (>20 cigarettes per day) use were excluded from the study (cf. Ziegler et al. 2016, 2019; Schiele et al. 2019; Kollert et al. 2020). Furthermore, psychiatric medication was recorded. Thirty-four patients received medication: selective serotonin reuptake inhibitors (SSRIs) N=18; selective serotonin and norepinephrine re-uptake inhibitors (SNRIs) N=5; noradrenaline and selective serotonin agonist (NaSSA) N=7; tricyclic antidepressants (TCA) N=7; pregabaline N=2; quetiapine: N=2; zopiclone: N=1; no other medication was prescribed (cf. Schiele

et al. 2019; Kollert et al. 2020). The control group was matched to the patient group by age (mean±SD: 34.30±9.25 years; t=.010, df=118, p=.992) and sex (f=47, X^2=0.0, df=1, p=1.0). Absence of current and lifetime mental axis I disorders in the control group was ascertained according to the criteria of DSM-IV (Mini International Neuropsychiatric Interview, M.I.N.I; Sheehan et al. 1998). Smoking status was evaluated in the patient group (smokers: N=19, 31.7 %) as well as in the control group (N=18, 30 %; X^2=0.0, df=1, p=.843; cf. Schiele et al. 2019; Kollert et al. 2020). The severity of depressive symptoms in the patient group was assessed using the 21-item Beck Depression Inventory II (BDI-II). The BDI-II contains 21 multiple-choice self-report inventory questions and is one of the most widely used psychometric tests for measuring the severity of depression. Each answer is scored on a scale from 0 to 3. Higher total scores indicate more severe depressive symptoms (Hautziger et al. 2009).

2.2.1.2 Sample II – MDD sample

Sample II comprised 170 Caucasian patients with major depressive disorder (MDD; f=98; age [mean±SD] 44.61±14.86 years). Recruitment, blood sampling and psychophysiological characterization was conducted by Dr. S. Stonawski under the supervision of Prof. Dr. Dr. K. Domschke and PD Dr. A. Menke at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany within the GEParD (Genetics and Epigenetics of Pharmaco-/Psychotherapy in Acute or Recurrent Depression) project. MDD diagnosis was determined by experienced psychiatrists based on medical records and a Structured Clinical Interview according to DSM-IV criteria (SCID, Wittchen et al. 1997). Exclusion criteria were current obsessive-compulsive disorder, schizoaffective disorder, psychosis or dementia, and/or presence of substance abuse disorder or eating disorder currently or within the last ten years (cf. Kollert et al. 2020). Smoking status was assessed in the overall sample (smokers: N=52, 30.59 %). Anxious depression was defined as an anxiety/somatization factor ≥7 (cf. Fava et al. 2008; Domschke et al. 2010) on the Hamilton Depression Rating Scale (HAMD; Hamilton 1960).

2.2.1.3 Sample III – Healthy subjects sample

Sample III constitutes an independent sample of healthy probands including 1,057 subjects (f: N=755, m: N=302; age: 25.05±5.762 years±SD) of Caucasian descent. The sample was recruited by experienced psychologists (K. Herzog, Dr. L. Leehr, K. Rosenkranz) under supervision of Prof. Dr. P. Pauli, Prof Dr. Dr. K.

Domschke, Prof. Dr. J. Deckert and Dr. M.A. Schiele at the Department of Psychology and the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, under supervision of Prof. Dr. Dr. U. Dannlowski at the Department of Psychiatry and Psychotherapy, University of Münster, Germany, and under supervision of Dr. T.B. Lonsdorf at the Institute for Systems Neuroscience, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, respectively, within the Collaborative Research Center (CRC) TRR58, subproject Z02, 3rd funding period (2017-2020). Absence of current and lifetime mental axis I disorders was established by experienced psychologists on the basis of the M.I.N.I according to DSM-IV criteria (Sheehan et al. 1998). Further exclusion criteria were severe medical conditions, intake of centrally active medication and excessive consumption of alcohol (>15 units/week), nicotine (>15 cigarettes/day) or caffeine (>4 cups/day). Dimensional measures of anxiety were assessed using the Agoraphobic Cognitions Questionnaire (ACQ; Chambless et al. 1984), the Anxiety Sensitivity Index (ASI; Reiss et al. 1986), the Beck Anxiety Inventory (BAI; McKee et al. 2011), the Liebowitz Social Anxiety Scale (LSAS; Liebowitz 1987), the Social Phobia and Anxiety Inventory (SPAI; Beidel et al. 1989) and the State Trait Anxiety Inventory - Trait Version (STAI-T; Spielberger 2010). Dimensional depression was evaluated by the Beck Depression Inventory II (BDI-II; Hautziger et al. 2009), and pathological worries by the Penn State Worry Questionnaire (PSWQ; Meyer et al. 1990).

2.2.2 Blood collection

Venous blood samples of all participants (samples I – III) were collected at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, as well as for sample III at the Department of Psychiatry and Psychotherapy, University of Münster, Germany and at the Institute for Systems Neuroscience, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, using EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). All blood samples were stored immediately after blood collection at -80 °C to prevent degradation of DNA itself as well as of distinct DNA methylation patterns until further processing.

2.2.3 DNA isolation from frozen whole blood samples

2.2.3.1 DNA purification

DNA isolation was accomplished using either the FlexiGene® DNA Kit (sample I; Qiagen, Hilden, Germany) or a standardized salting out procedure (samples II and III; Miller et al. 1988). DNA isolated from frozen whole blood was stored at -80 °C in the Laboratory of Functional Genomics, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany.

DNA isolation by FlexiGene® DNA Kit was carried out according to the manufacturer's instruction, following the protocol "Isolation of DNA from 4-14 ml whole blood" (*method description derived from FlexiGene® DNA Handbook, Qiagen, Hilden, Germany*). First, frozen blood samples were thawed quickly at 37 °C using a prewarmed water bath (JB Aqua 12, Great Instruments, Cambridgeshire, UK). For the following DNA isolation procedure, 50 ml centrifugation tubes (Greiner Bio-one, Frickenhausen, Germany) were labelled and filled with 20 ml Buffer FG1. The buffer was mixed with 8 ml whole blood by inverting the tube five times. Then, the mixture was centrifuged in a swing-out rotor (Hettich, Tuttlingen, Germany) for 15 min at 3,000 rpm. While making sure that the pellet persisted in the tube by carefully discarding the supernatant, the tubes were put upside down on a clean sheet of absorbent paper for 2 min. For homogenization of the pellet, 4 ml freshly prepared FG2/QIAGEN Protease mixture was pipetted onto the pellet and the tubes were vortexed straightaway. Then, the tubes were inverted another three times and placed into a prewarmed water bath. To ensure a successful protein digestion, the mixture was incubated at 65 °C for 10 min. Successful protein digestion designates a color change of the samples from red to green. For precipitation of the DNA, 4 ml isopropanol (2-propanol for molecular biology, AppliChem, Darmstadt, Germany) were pipetted to the sample, and the tubes were inverted until the precipitate became detectable as white fibers or a small pellet. The pellet of the precipitated DNA samples was then centrifuged in a swing-out rotor for 15 min at 3,000 rpm. The supernatant was carefully discarded. To eliminate the residual quantities of isopropanol, the tubes were again put upside down on a clean sheet of absorbent paper. For washing the pellet, 4 ml 70 % ethanol (for analysis, AppliChem, Darmstadt, Germany) were added, and tubes were vortexed for 5 s to homogenize the pellet again. Subsequently, samples were centrifuged again at 3,000 rpm for 15 min, and the supernatant was carefully discarded. The tubes were put upside down on a clean sheet of paper for at least 5 min to remove all residual ethanol.

The pellets were air-dried for further 5 min until they became white. Afterwards, 800 µl storage Buffer FG3 were added and samples were heated to 65 °C for 1 h in a water bath for complete dissolution of the pellets in the storage Buffer FG3. After complete homogenization, DNA samples were transferred to clean labelled 1.5 ml reaction tubes (Eppendorf, Hamburg, Germany) and stored at -80 °C until further usage.

Standardized salting out blood isolation was performed according to Miller et al. (Miller et al. 1988). 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. Probes of 9 ml EDTA-blood were gently mixed with 30 ml 4 °C cold Lysis Buffer to accomplish cell lysis of erythrocytes in labelled 50 ml centrifugation tubes (Greiner Bio-one, Frickenhausen, Germany). After 30 min incubation on ice, the lysis mix was inverted four times and centrifuged in a swing-out rotor (Hettich, Tuttlingen, Germany) for 15 min at 4 °C and 2,500 rpm. The supernatant was decanted, and the pellet of leucocytes was resolved with 5 ml Lysis Buffer. For removing the Lysis Buffer completely, the samples were centrifuged again at 2,500 rpm and at 4 °C and the pellet was resuspended in 5 ml SE Buffer. For preventing protein contamination, 250 µl of 20 mg/ml pronase E (AppliChem, Darmstadt, Germany) and 250 µl of 20 % SDS were added. The solution was incubated in a water bath (Great Instruments, Cambridgeshire, UK) at 37 °C overnight. On the next day, 2.5 ml SE Buffer were added, and cell debris precipitated by mixing 6 M NaCl for 15 s and a centrifugation for 20 min at 4,000 rpm. Supernatant was mixed with 7.5 ml 2-propanol and centrifuged for 5 min at 3,000 rpm to precipitate the DNA. After washing the DNA pellet with 70 % ethanol, DNA was dried at RT and resolved in 400 µl TE-Buffer by overnight incubation at 37 °C. DNA was stored at -80 °C until further use.

2.2.3.2 Photometric determination

To define the DNA and RNA concentrations and purity, samples were quantified using the photometer NanoDrop ND100 (pegLab, Erlangen, Germany) and the corresponding software. As advocated in the instructions of the device, the instrument was initialized and blanked with 1.2 µl RNase-free water. For quantification and qualification, 1.2 µl of DNA or RNA, respectively, were applied to the lower optical surface of the device. The DNA or RNA concentration was subsequently determined based on the extinction at 260 nm. DNA and RNA purity were determined by extinction between 230 nm and 280 nm. The absorbance at 260 and 280 nm is used to assess

the purity of DNA and RNA. In general, a ratio of ~1.8 is proposed as pure for DNA, a ratio of ~2.0 is commonly accepted as pure for RNA. The 260/230 absorbance-ratio is used as a secondary measure of nucleic acid purity. Accepted 260/230 values are generally in the range of 1.8-2.2.

2.2.4 DNA methylation analysis

All DNA methylation analyses were conducted at the Laboratory of Functional Genomics, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, under supervision of Dr. C. Thiel and Prof. Dr. Dr. K. Domschke.

2.2.4.1 Bisulfite conversion

For DNA methylation analyses, the EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany) was used for bisulfite conversion of human DNA. This kit enables a successful bisulfite conversion and clean-up of bisulfite converted DNA for methylation analysis in 96-well format. The procedure was executed according to the manufacturer's instructions and to the protocol for "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Using a Vacuum Manifold" (*method description derived from EpiTect® 96 Bisulfite Handbook, Qiagen, Hilden, Germany*). To avoid batch effects, case and control samples were bisulfite converted in the same process. Prior to bisulfite conversion, genomic DNA samples were diluted to a concentration of 25 ng/µl with ddH2O (Merck, Darmstadt, Germany) in a total volume of 20 µl (500 ng). All components of the EpiTect® 96 Bisulfite Kit were prepared according to the manufacturer's instructions prior to use: Buffer BW concentrate was homogenized in 120 ml ethanol (absolute, 99.8 %, Sigma-Aldrich, Taufkirchen, Germany), Buffer BD concentrate was diluted with 27 ml ethanol (absolute, Sigma-Aldrich, Taufkirchen, Germany), and Buffer BL was heated to approx. 55 °C to dissolve precipitates. All provided components as well as DNA samples were equilibrated to room temperature.

First, the powdery Bisulfite Mix was dissolved in 9 ml RNase-free water and the mixture was heated to 60 °C and vortexed for at least 5 min to assure complete homogenization. Then, 85 µl dissolved bisulfite reaction mixture was combined with 35 µl DNA Protect Buffer and 20 µl DNA sample (500 ng) in the provided EpiTect® Conversion Plate. Protect Buffer subsequently should change colour from green to blue. All components were mixed well by carefully pipetting the mixture up and down

and the EpiTect® Cover Foil was carefully used to seal the EpiTect® Conversion Plate before centrifugation briefly at 650 (x)g in a plate centrifuge (Centrifuge 5430, Eppendorf, Hamburg, Germany) to accumulate the reaction mixture in the bottom of the wells. Bisulfite reactions were performed in a thermal cycler according to following protocol (see Tab. 8).

Tab. 8 Bisulfite conversion conditions

Step	Temperature	Time
Denaturation	95 °C	5 min
Incubation	60 °C	25 min
Denaturation	95 °C	5 min
Incubation	60 °C	85 min
Denaturation	95 °C	5 min
Incubation	60 °C	175 min

The reaction was terminated by incubation at 20 °C up to 12 h (overnight). After that, a QIAvac 96 vacuum manifold (Qiagen, Hilden, Germany) connected with a vacuum pump (Welch, Alton, Hampshire, UK) was used to clean up the bisulfite converted DNA according to the manufacturer's introductions. For that, the vacuum manifold was prepared according to manufactures instruction (QIAvac 96 Vacuum Manifold Handbook, Qiagen, Hilden, Germany) and the EpiTect® Conversion Plate containing the bisulfite reactions was briefly centrifuged at 650 (x)g. Then, the EpiTect® 96 Plate was firmly placed onto the holding position of the vacuum manifold. Now, the complete bisulfite reaction was added to 560 µl freshly prepared Buffer BL previously dispensed into the wells of the EpiTect® 96 Plate and mixed with the Buffer BL by pipetting up and down until a homogeneously blue mixture was acquired. After that, all liquid was passed through the membranes in each well of the EpiTect® 96 Plate by switching on the vacuum. Then, 500 µl Buffer BW were added carefully to each well and vacuum source was switched on again until all liquid had passed. Afterwards, 250 µl Buffer BD were added to each well and the mixture was incubated for 15 min at room temperature to ensure successful desulphonation of the samples. After incubation time, the vacuum pump was switched on again until all liquid had passed through. Now, the membranes attaching the genomic DNA were washed by pipetting 500 µl Buffer BW into each well and switching the vacuum pump on until all liquid had

passed. This step was repeated once. As a last cleaning step, wells were washed with 250 µl ethanol (absolute, 99.8 %; Sigma-Aldrich, Taufkirchen, Germany) to remove residual Buffer BW. The bisulfite converted DNA, attached to the membranes, was air-dried for additional 10 min by maximal vacuum, to ensure a complete removing of remaining quantities of ethanol. Now, the top plate of the manifold together with the EpiTect® 96 plate was carefully lifted by slowly ventilating the vacuum manifold. Then, the EpiTect® 96 plate was repeatedly tapped on clean absorbent paper to remove remaining quantities of ethanol from the nozzles of the 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. To dissolve the dried bisulfite converted DNA again, the EpiTect® 96 plate was placed back onto the manifold and 70 µl of Buffer EB and 10 µl Top Elute Fluid were dispensed directly to the midpoint of the membrane of each well. The vacuum pump was switched on for a maximum of 1 min. After switching off the vacuum pump again, the manifold was slowly and carefully ventilated. The elution plate containing the dissolved genomic DNA was sealed using the provided Tape Pads and stored at -20 °C until further use.

2.2.4.2 Design of *TIEG2* DNA methylation assays

For this study, a 313 bp long region of interest comprising parts of the *TIEG2* promoter region (chr2:10,182,891-10,183,204; GRCh37/hg19 Assembly, UCSC Genome Browser) was chosen for DNA methylation pre-analyses using direct Sanger sequencing. For this purpose, DNA methylation of this region containing 14 CpG was first analyzed in the healthy control sample (sample III, see Fig. 2).

These pre-analyses by Sanger sequencing revealed that only five of these CpG sites displayed variance in their methylation status (see Results section 3.1) and were thus further analyzed in the PD and matched control sample (sample I), in the MDD sample (sample II) as well as in the healthy subjects sample (sample III) by pyrosequencing of a 72 bp long amplicon (10,183,094-10,183,166; GRCh37/hg19 Assembly, UCSC Genome Browser). CpG sites were numbered according to their position in the amplicon CpG1=chr2:10,183,094; CpG2=chr2:10,183,106; CpG3=chr2:10,183,115; CpG4=chr2:10,183,125; CpG5=chr2:10,183,140 (see Fig. 2, all genomic locations according to GRCh37/hg19 Assembly, UCSC Genome Browser).

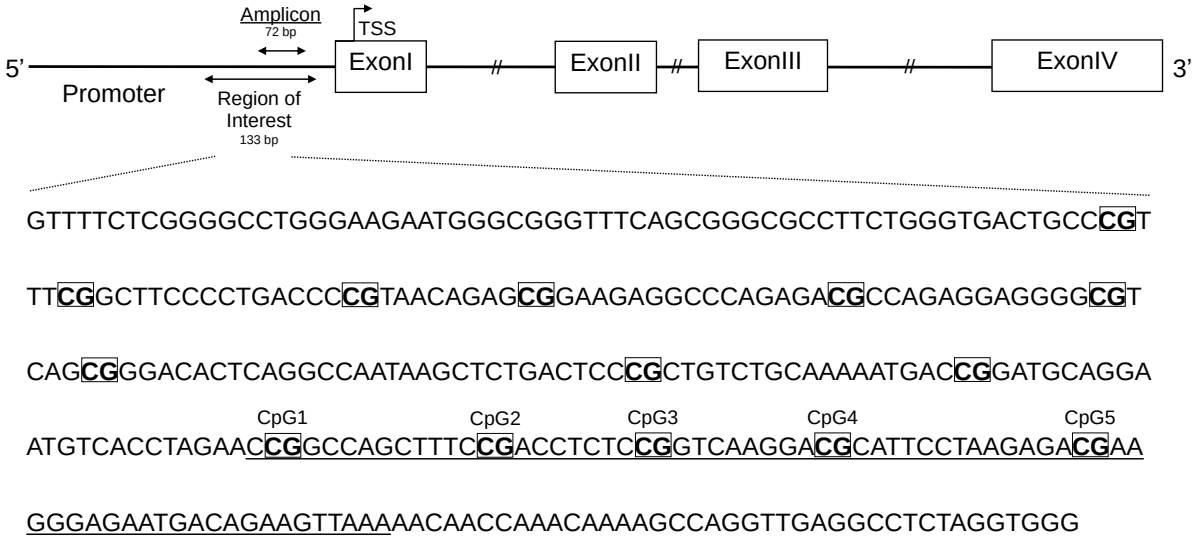

Fig. 2 Schematic illustration of the *TIEG2* gene

The *TIEG2* gene (not to scale) consists of four exons. The region of interest (133 bp) presently chosen for pre-analyses via Sanger sequencing is located within the promoter region, approximately 500 bp upstream of the *TIEG2* transcriptional start site (TSS; not to scale). The DNA sequence of the region of interest is given below and contains fourteen CpG sites (framed; first nine CpGs are unnumbered). Five of the fourteen CpGs were further analyzed via pyrosequencing and are numbered according to their order (CpGs 1-5) within the 72 bp long pyrosequencing amplicon (underlined).

Primers for direct Sanger sequencing were designed using the MethPrimer database provided by the Peking Union Medical College Hospital, Chinese Academy of Medical Sciences (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; Long Cheng and Rajvir 2002). Position and sequences of *TIEG2* CpG island were obtained from UCSC Genome Browser (https://genome.ucsc.edu; Kent et al. 2002) and NCBI Refseq database (Pruitt et al. 2014). The MethPrimer online tool selects primers in the CpG island sequence considering possible masking effects of DNA methylation at all CpG sites in the sequence (Long Cheng and Rajvir 2002). Hence, the obtained oligonucleotide primers bind to bisulfite-converted DNA derived from methylated as well as non-methylated DNA. Oligonucleotide primers were validated using primer design and search tool BiSearch (http://bisearch.enzim.hu/?m=genompsearch; Tusnády 2005). Oligonucleotide primers (see Tab. 9) were purchased at Sigma-Aldrich (Munich, Germany).

Tab. 9 *TIEG2* forward and reverse primer sequence for Sanger sequencing

Primer name	Sequence (5' – 3' direction)
*TIEG2*_Forward primer	TTTTTTAGGGGTTTGGGAAGAATGGG
*TIEG2*_Reverse primer	CCCACCTAAAAACCTCAACCTAACTTT

Primers for bisulfite pyrosequencing were designed using the PyroMark® Assay Design 2.0 Software (Qiagen, Hilden, Germany). This software creates forward, reverse and sequencing primers, according to the region of interest set up by the costumer. Given the results of the above-mentioned pre-analyses of the *TIEG2* promoter region via Sanger sequencing, just a short fragment of the promoter region was analyzed using the PyroMark® Primer Software. Position and sequences of this specific *TIEG2* region - containing five CpGs - were obtained from UCSC Genome Browser (https://genome.ucsc.edu; Kent et al. 2002) and NCBI Refseq database (Pruitt et al. 2014). Forward and reverse primer settings were as follows: Primer length varied between 18 and 30 bp, amplicon length varied between 100 and 400 bp, primer concentration was 0,2 µM and the optimal melting temperature was between 50-70 °C. Sequencing primer settings differed from the other two primer settings in primer length between 15-25 bp and optimal melting temperature between 29-59 °C. All oligonucleotides (see Tab. 10) were purchased at Sigma-Aldrich (Munich, Germany).

Tab. 10 *TIEG2* forward, reverse and sequencing primer sequence for pyrosequencing

Primer name	Sequence (5' – 3' direction)
*TIEG2*_pyro_forward primer	GGATGTAGGAATGTTATTTAGAA
*TIEG2*_pyro_reverse primer	AAAACCTCAACCTAACTTTTATTTAATT
*TIEG2*_pyro_sequencing primer	ATGTAGGAATGTTATTTAGAAT

2.2.4.3 Bisulfite polymerase chain reaction

2.2.4.3.1 Bisulfite polymerase chain reaction for Sanger sequencing

For the direct Sanger sequencing bisulfite polymerase chain reaction, the region of interest was analyzed with PCR using the HotStarTaq® Plus Master Mix according to the standard protocol (Domschke et al. 2012). In brief, 2 µl bisulfite converted DNA were mixed with 10 µl HotStarTaq® Plus Master Mix, 0.8 µl of 10 µM forward and

reverse primer individually, 1 µl of 25 mM $MgCl_2$ and subsequently filled with water to a reaction volume of 20 µl. 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 protocol given in Tab. 11.

Tab. 11 Sanger sequencing PCR conditions

Step	Temperature	Time
Denaturation	94 °C	5 min
Annealing (40 cycles)	94 °C	1 min
	65 °C	1 min
	72 °C	2 min
Elongation	72 °C	3 min

PCR products were stored at 4 °C and were proven to have the predicted length as well as sufficient quality for sequencing using gel electrophoresis (see Methods section 2.2.4.4). Furthermore, non-template controls (ddH_2O) were used to account for possible cross-contaminations. Additionally, non-methylated, semi-methylated and methylated DNA samples were used as controls for quality of bisulfite conversion (EpiTect® PCR Control DNA Set; Qiagen, Hilden, Germany).

2.2.4.3.2 Bisulfite polymerase chain reaction for pyrosequencing

For the pyrosequencing bisulfite polymerase chain reaction, the target amplicon was analyzed with PCR using the HotStarTaq® Plus Master Mix according to the standard protocol (Qiagen, Hilden, Germany). In brief, 2 µl bisulfite converted DNA were mixed with 12.5 µl HotStarTaq® Plus Master Mix, 0.8 µl of 10 µM forward and reverse primer, respectively, 1 µl of 25 mM MgCl2, 2.5 µl Coral Load and subsequently filled with water to a reaction volume of 25 µl. 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 protocol given in Tab. 12. PCR products were stored at 4 °C for further use.

Tab. 12 Pyrosequencing PCR conditions

Step	Temperature	Time
Denaturation	95 °C	15 min
Annealing (45 cycles)	95 °C	30 sec
	54.4 °C	30 sec
	72 °C	30 sec
Elongation	72 °C	10 min

All generated PCR products were proven to have the predicted length of as well as sufficient quality for sequencing using gel electrophoresis (see Methods section 2.2.4.4). Furthermore, non-template controls (ddH_2O) were used to account for possible cross-contaminations. Additionally, non-methylated, half-methylated and fully methylated commercially available DNA controls (EpiTect® PCR Control DNA Set; Qiagen, Hilden Germany) were used to control for complete bisulfite conversion. All amplified PCR products were sequenced by using the Q96 ID instrument for pyrosequencing analyses (Qiagen, Hilden, Germany; see Methods section 2.2.4.7).

2.2.4.4 Agarose gel electrophoreses

For quality control of PCR products, gel electrophoresis was performed. Gel electrophoresis is executed in an electric field, in which the DNA molecules move towards the anode, due to their negative net charge. In general, larger DNA fragments migrate more slowly through the agarose than smaller fragments, due to their different friction. For preparation of a 2 % agarose gel, 2 g agarose (peqGold, Peqlab, Erlangen, Germany) were dissolved 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 55 °C, 3 µl Ethidium Bromide Solution (Sigma-Aldrich, Germany) were added. This chemical intercalated to DNA fragments, resulting in visibility using ultraviolet light. The agarose solution was transferred into the gel tray, which had been prepared before by placing a gel chamber and a suitable comb into a casting system. After the agarose mixture had cooled down to room temperature and polymerized, the comb was removed carefully. Then, the gel was placed in an electrophoresis chamber and covered with TAE-Buffer. The samples were mixed with Loading Buffer before loading them to the gel. Additionally, a standard marker (100 bp

Plus Gene Ruler, Thermo Scientific, Munich, Germany) was loaded on the gel. Electrophoresis was executed at 120 V for approximately 30 min. 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 GeneRuler 100 bp plus DNA-ladder (Thermo Scientific, Munich, Germany), which consists of distinctly sized DNA fragments with a size range between 100 to 1000 bp as defined by the manufacturer.

2.2.4.5 Direct Sanger sequencing

Sequencing of the PCR products was conducted by LGC Genomics (Berlin, Germany) on ABI 3730 XL platforms. The provided electropherograms were evaluated considering quality and reliability using the Sequence Scanner software (Applied Biosystems by Life Technologies, Darmstadt, Germany). To avoid batch effects, case and control samples were sequenced in the same run and in randomized order (cf. Domschke et al. 2012; Ziegler et al. 2016).

2.2.4.6 Epigenetic sequencing methylation analysis tool

Sequencing data were quantitatively analyzed using the Epigenetic Sequencing Methylation analysis tool (ESME; Lewin et al. 2004). This freely available software was explicitly designed for artificially generated single nucleotide polymorphisms (C/T and G/A) and analyzes sequencing results by aligning the bisulfite converted DNA sequence with the original sequence obtained from the NCBI reference sequence database (http://www.ncbi.nlm.nih.gov/refseq/; Pruitt et al. 2014). By determining relative peak heights (C/C+T) using the normalized sequence trace files and comparing C to T peak heights at individual CpG sites the program estimates the respective methylation status of each CpG site. This software automatically executes quality control, normalizes signals and corrects for incomplete bisulfite conversion (Lewin et al. 2004). ESME has already been proven to generate reliable results for the analysis of methylation profiles in other mental disorders (Frieling et al. 2007; Domschke et al. 2012, 2014, 2015; Tadić et al. 2014; Ziegler et al. 2016, 2018; Schiele et al. 2018, 2019). To take care of run variability, all samples were tested in duplicate, resulting in a mean methylation score for each CpG site, as well as an individual standard deviation (SD) for each duplicate. For quality control, the SD of each duplicate with SD>0.1 were defined as missing values. Outliers (≥3 SD from mean methylation of the individual CpG site) were also defined as missing data. For the total sample, a

cut-off of >20 % of missing data was defined as an exclusion criterion (cf. Domschke et al. 2012; Ziegler et al. 2016).

2.2.4.7 Pyrosequencing

For pyrosequencing analysis of the investigated *TIEG2* amplicon, the PyroMark® Q96 ID system (Qiagen, Hilden, Germany) was used, following the manufacturer's instructions (*method description derived from PyroMark® Q96 ID User Manual and PyroMark® Gold Q96 Reagents Handbook, Qiagen, Hilden, Germany*).

Quantitative DNA methylation analysis by pyrosequencing is a "sequencing-by-synthesis" principle. In short: a sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template. One of four nucleotides is added to the reaction, and if the nucleotide is complementary to the base in the template strand, it will be incorporated into the DNA strand by the DNA polymerase. Each incorporation is supplemented by release of pyrophosphate (PPi) in an equimolar amount to the number of incorporated nucleotides. ATP sulfurylase quantitatively converts PPi to ATP and drives the conversion of luciferin to oxyluciferin by luciferase, generating visible light in amounts proportional to the quantity of ATP. This light is detected using charged coupled devices (CCDs) and seen as a peak in the pyrogram. Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added. As the process continues, the complementary sequence is built up and the nucleotide sequence is determined from the peak in the pyrogram (PyroMark® Q96 ID User Manual, Qiagen, Germany).

The pyrosequencing procedure was performed according to the standard protocol for pyrosequencing instruction, and all components were prepared according to the manufacturer's instructions prior to use (PyroMark® Q96 ID User Manual, Qiagen, Hilden, Germany). In brief, 10xWashing Buffer Concentrate (Qiagen, Hilden, Germany) was diluted with ddH2O to a concentration of 1xWashing Buffer; Denaturation Buffer was assembled by dissolving 8 g NaOH (AppliChem, Darmstadt, Germany) in 1 l ddH2O and 70 % ethanol was produced by mixing 700 ml ethanol absolute (99.8 %, ChemSolute, Renningen, Germany) with 300 ml ddH2O. All provided components as well as DNA samples were equilibrated to room temperature.

2.2.4.7.1 Immobilization of biotinylated PCR products

The first step of pyrosequencing procedure was the immobilization of the biotinylated PCR products (see Methods section 2.2.4.3.2) to the Streptavidin Sepharose High Performance Beads (GE Healthcare, Munich, Germany). The bottle containing the streptavidin-coated Sepharose High Performance Beads (GE Healthcare, Munich, Germany) was gently shaken from side to side until a homogenous solution was acquired. A mixture of Streptavidin Sepharose High Performance Beads (GE Healthcare, Munich, Germany), Binding Buffer (Qiagen, Hilden, Germany) and high-purity water was prepared conferring to the reaction setup showed in the table below (see Table Tab. 13).

Tab. 13 Reaction mixture for immobilization of the biotinylated PCR products

Component	Volume per sample
Biotinylated PCR Product	10 µl
Streptavidin Sepharose High Performance Beads	1.5 µl
Binding Buffer	40 µl
H2O	28.5 µl
Total volume	80 µl

For each sample, 80 µl of the immobilization mixture was added in each well of a 96-well PCR plate (Nb Nerbe plus, Winsen, Germany), which was carefully sealed by using a PCR cover foil (Thermo Scientific, Munich, Germany) and shortly centrifuged at 2,000 rpm (Centrifuge 5430, Eppendorf, Hamburg, Germany). Then the PCR plate (Nb Nerbe plus, Winsen, Germany), was constantly agitated at 1,900 rpm for at least 10 min using a horizontal mixer (Shaker-Bioshaker iQ, Bioshake, Jena, Germany).

During immobilization, the PyroMark® Q96 Vacuum Workstation (Qiagen, Hilden, Germany; see Fig. 3) was prepared by filling up the five separate troughs (see Tab. 14).

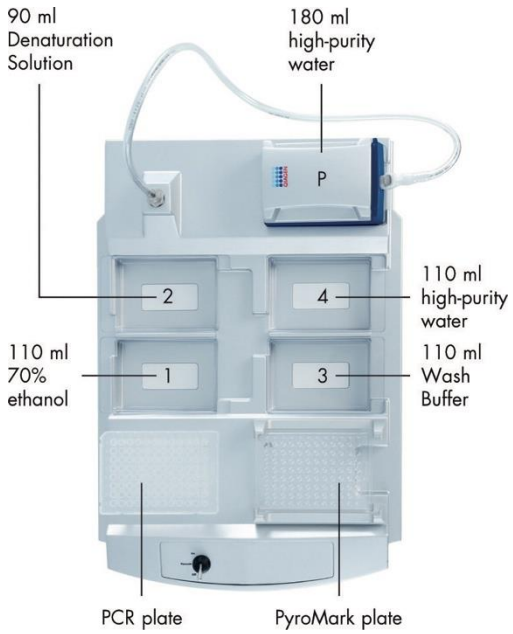

Fig. 3 PyroMark® Q96 Vacuum Workstation

PyroMark® Q96 Vacuum Workstation with indicated five separate troughs for 70 % Ethanol, Denaturation Solution, Wash Buffer and high purity water. Additionally, settings for PCR plate and PyroMark® Plate Low are shown. [Figure adapted from PyroMark® Q96 ID User Manual, version 5, page 43, © QIAGEN, all rights reserved]

Tab. 14 Components for preparation of PyroMark® Q96 Vacuum Workstation

Component	Volume per trough
70 % Ethanol	110 ml
Denaturation Solution	90 ml
Wash Buffer	110 ml
High-purity water	110 ml
High-purity water (Parking Position)	180 ml

2.2.4.7.2 Strand separation

The second step comprised the strand separation, therefore a second 96-well plate, the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany), was prepared by adding 0.4 µM sequencing primer in 40 µl Annealing Buffer (Qiagen, Hilden, Germany) to each well that was used. After the 10 min shaking of the 96-well PCR plate (Nb Nerbe plus, Winsen, Germany; see Methods section 2.2.4.7.1), the PCR plate as well as the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany) were placed on the worktable of the PyroMark® Q96 Vacuum Workstation (Qiagen, Hilden, Germany; see Fig. 3).

After switching on the vacuum, the Vacuum Prep Tool (Qiagen, Hilden, Germany) containing the filter probes was carefully lowered into the PCR plate (Nb Nerbe plus, Winsen, Germany) to capture the beads containing the immobilized template and was hold in place for 15 s. Then, the tool was transferred to the first trough containing 70 % ethanol and the filter probes were flushed for 5 s. Afterwards, the tool was transferred to the trough containing Denaturation Solution (Qiagen, Hilden, Germany) for 5 s and to the trough containing Wash Buffer (Qiagen, Hilden, Germany) for 10 s. Subsequently, the Vacuum Prep Tool was raised to beyond 90° vertical for 5 s to drain liquid from the filter probes. While holding the tool over the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany), the vacuum was switched off. Carefully transferring of the filter probs of the Vacuum Prep Tool (Qiagen, Hilden, Germany) in the wells of the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany), led to the release of the beads into the PyroMark® Q96 Plate Low, containing 0.4 µM sequencing primer in 40 µl Annealing Buffer (Qiagen, Hilden, Germany) by shaking the tool gently from side to side in the plate.

For cleaning up the Vacuum Prep Tool (Qiagen, Hilden, Germany), it was first transferred to the trough containing high-purity water and agitated for 10 s applying vacuum, then raised to beyond 90° vertical for 5 s, to drain liquid from the filter probes. After that, the vacuum switch on the worktable was closed, and the Vacuum Prep Tool parked in the parking position (see Fig. 3).

2.2.4.7.3 Annealing of sequencing primer to samples

The third step of the pyrosequencing procedure included annealing of sequencing primer to PCR samples by placing the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany) in the prewarmed PyroMark® Q96 Sample Prep Thermoplate Low (Qiagen, Hilden, Germany) and heating the samples on a heating block (Heidolph, Schwabach, Germany) at 80 °C for 2 min. Afterwards, the PyroMark® Q96 Plate Low (Qiagen, Hilden, Germany) was removed from the PyroMark® Q96 Sample Prep Thermoplate Low (Qiagen, Hilden, Germany), and the samples were cooled down to room temperature (15-25 °C) for at least 8 min. Finally, the plate was processed in the PyroMark® Q96 ID instrument (Qiagen, Hilden, Germany).

2.2.4.7.4 Reagent preparation

The fourth step contained preparation of the reagents according to the manufacturer's instructions included in the PyroMark® Gold Q96 Reagents Kit (Qiagen, Hilden, Germany). The reagent cartridge (Qiagen, Hilden, Germany), used for reagent

preparation, had to be handled with care in order to maintain accuracy. First, the recommended volume of substrate solution was pipetted into the S compartment of the reagent cartridge. Second, the recommended volume of enzyme solution was pipetted into the E compartment of the cartridge. Then, the recommended volume of each dNTP solution was pipetted into the A, C, G, and T compartments according to their positions (see Fig. 4). Finally, also the cartridge containing all six reagents was processed in the PyroMark® Q96 ID instrument (Qiagen, Hilden, Germany).

Fig. 4 Schematic illustration of PyroMark® Q96 Cartridge

PyroMark® Q96 Cartridge with indicated compartments for enzymes, substrate, and nucleotides from above. Label of PyroMark® Q96 Cartridge should face the front. [Figure adapted from PyroMark® Q96 ID User Manual, version 5, page 47, © QIAGEN, all rights reserved]

As a last step, die actual run was started within the PyroMark® Q96 software (Qiagen, Hilden, Germany) displaying the current state of a run (e.g. preparing for run, adding reagents, run, end of run, connection lost) and the time remaining to complete the run.

To adjust for run variability, all samples were tested in duplicate, resulting in a mean individual methylation score for each CpG, as well as an individual standard deviation (SD) for each duplicate. The first step of quality control was checking whether the standard deviation of each duplicate did not exceed the upper limit of SD>0.1. In a second step, outliers (≥3SD 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.

2.2.4.8 Statistics

Statistical analyses were conducted at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, under supervision of Dr. C. Thiel and Dr. M.A. Schiele. Independent samples t-test were used to analyze differences in dimensional sample characteristics, differences in categorical variables were tested by means of Chi-square tests. In sample I, differences in *TIEG2* methylation (mean methylation, single CpG sites) between PD patients and controls were explored by means of non-parametric Kruskal-Wallis test. Due to non-nominal distribution, correlations of *TIEG2* methylation and BDI-II (Hautziger et al. 2009) scores were assessed by means of Spearman correlation. In sample II, non-parametric Kruskal-Wallis test was used to analyze differences between anxious depression patients and non-anxious depression patients. In sample III, correlations of *TIEG2* methylation and dimensional phenotypes of anxiety or depression were calculated by means of Spearman correlation. No significant associations between average *TIEG2* methylation and sex (sample I: t_{112}=1.056, p=.293; sample II: t_{162}=-0.137, p=.891; sample III: t_{1055}=0.989, p=.323), age (sample I: r=0.156; p=.097; sample II: r=0.054; p=.495; sample III: r=0.012, p=.703) or smoking behavior (sample I: t_{112}=0.745, p=.458; sample II: t_{161}=1.213; p=.227; sample III: r=-0.27; p=.387) were found in either sample. Thus, these parameters were not included as covariates. The significance level was set at p<.05. Given the present proof-of-concept approach and high correlation between single CpG sites (see Results sections 3.2, 3.3 and 3.4), no correction for multiple testing was applied when analyzing single CpG sites and average *TIEG2* methylation (cf. Schiele et al. 2018, Kollert et al. 2020). Statistical analysis was performed using SPSS statistical software (version 22.0; SPSS Inc., Chicago, IL, USA).

III RESULTS

Parts of the results are published online in the Journal of Neural Transmission (see Kollert et al. 2020). Copyright remained to the author after publication and references are represented under the corresponding figures.

3.1 Sanger sequencing methylation analyses

In search of regulatory regions relevant for DNA methylation in the *TIEG2* promoter region, the Genome Browser of the University of California in Santa Cruz (UCSC; W. J. Kent et al. 2002) was used. Further analyses led to the discovery of a 2,067 bp long CpG Island, spanning the transcriptional start side of *TIEG2* (chr2:10,182,636-10,184,702; GRCh37/hg19 Assembly, UCSC Genome Browser). In total, this CpG island contained 183 individual CpG sites. No other CpG island across the *TIEG2* gene region could be detected. Within this CpG island, a target region of 313 bp (chr2:10,182,891-10,183,204 based on GRCh37/hg19 Assembly, UCSC Genome Browser) located approximately 500 bp upstream of the *TIEG2* transcriptional start site (TSS) and partly covering the 5' part of a CpG island was chosen for DNA methylation analyses (see Fig. 2; cf. Methods section 2.2.4.2). Additionally, this target region was selected because it was located in a promoter region associated with higher activation of transcription as indicated by ENCODE Enhancer- and Promoter-associated H3K27Ac histone mark track (an active enhancer marker, often found near active regulatory elements; cf. Encyclopedia of DNA Elements ENCODE; available on GRCh37/hg19 Assembly, UCSC Genome Browser; see Fig. 5).

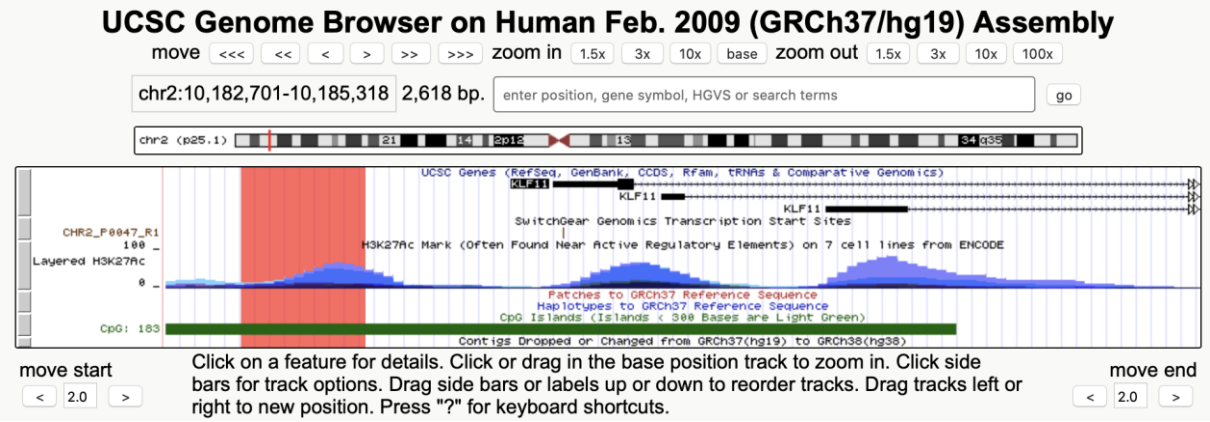

Fig. 5 Illustration of UCSC Genome Browser output of *TIEG2 (*alias *KLF11)*

Screenshot of UCSC Genome Browser output of *TIEG2* (alias *KLF11*) (https://genome.ucsc.edu; 04-07-20, 15:25). First exon (black box) and first intron (black line) of *TIEG2* (alias *KLF11*) are shown. Highlighted in red: Target region of interest chosen for DNA Sanger sequencing analyses located in the promoter region and partly covering the 5' part of a CpG island (shown in green). Three active regions indicated by ENCODE Enhancer- and Promoter- associated H3K27Ac histone mark track (an active enhancer marker; cf. Encyclopedia of DNA Elements; ENCODE) are shown in blue. The *TIEG2* (alias *KLF11)* target region of interest is located in the first H3K27Ac histone mark track associated with higher activation of transcription.

The region of interest contained 18 CpG sites; however, due to technical difficulties particularly at the 3′ and 5' ends of the region of interest, only 14 CpG sites were robustly readable using Sequence Scanner software (Applied Biosystems by Life Technologies) and commercially available Sanger sequencing (LGC Genomics, Berlin, Germany; see Fig. 6).

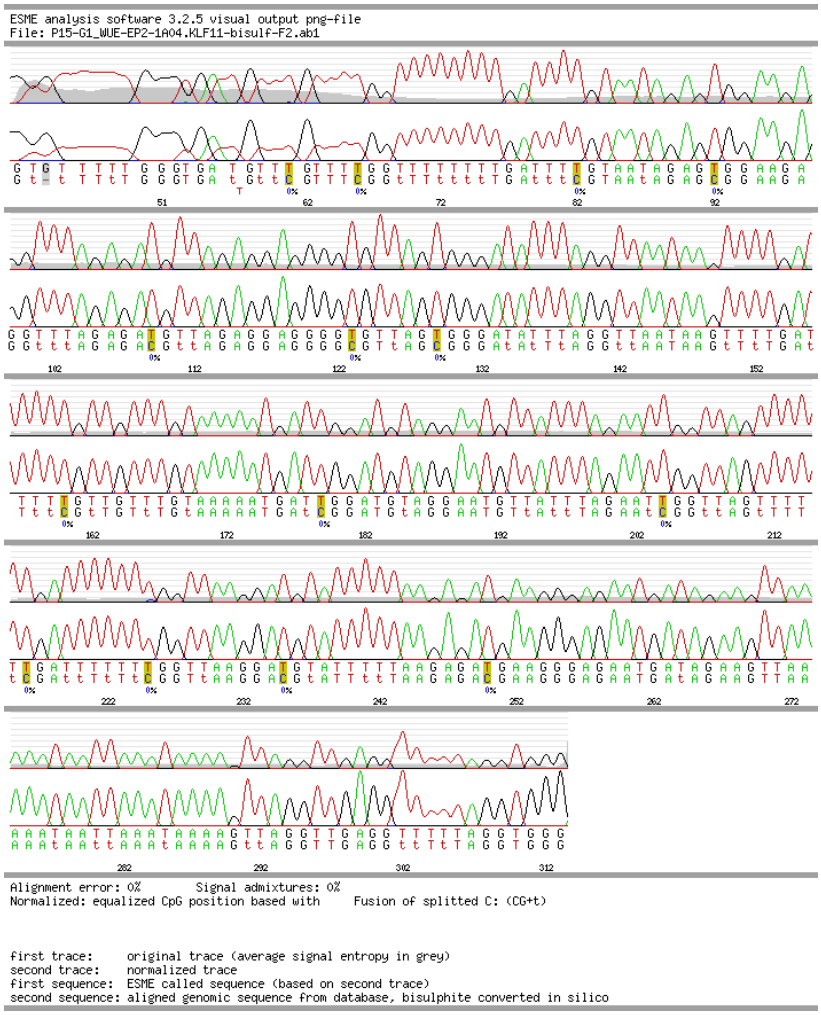

Fig. 6 Example of an electropherogram of the analyzed *TIEG2* region of interest

A 313 bp long region of interest (chr2:10,182,891-10,183,204 based on GRCh37/hg19 Assembly, UCSC Genome Browser) located approximately 500 bp upstream of the *TIEG2* transcription start and partly covering the 5'-part of a CpG island was chosen for DNA methylation analyses. The region of interest contained 14 CpG sites (highlighted in yellow) readable using commercially available Sanger sequencing (LGC Genomics, Berlin, Germany).

Across the 14 readable CpG sites in the analyzed region of interest, only at CpG sites 10-14 a sufficient variance in mean methylation in a test sample consisting of N=53 (f=38, m=15) healthy control subjects of Caucasian decent and without any current and lifetime mental axis I disorder was detected. Consequently, all subsequent epigenetic analyses were carried out for a 72 bp long pyrosequencing amplicon containing those five CpG sites only (cf. Methods sections 2.2.4.2, 2.2.4.3.2, 2.2.4.7, 2.2.4.8).

3.2 Sample I

3.2.1 *TIEG2* hypomethylation in PD patients with comorbid MDD

In order to examine the role of *TIEG2* DNA methylation as a marker of panic disorder, a sample of 60 patients with PD and 60 controls matched for sex and age was analyzed (see Methods section 2.2.1.1). The final sample size was reduced to N=114 (f=88, m=26), since sequencing data of six samples failed quality control. Within this reduced sample, *TIEG2* methylation was available for 57 healthy control subjects and 57 patients with panic disorder. In the combined sample, *TIEG2* DNA methylation across all five CpG sites (mean±SE:11.58±4.12) as well as of all single CpG sites (CpG1: mean±SE: 2.69±1.32; CpG2: mean±SE: 11.80±4.51; CpG3: mean±SE: 24.70±8.44; CpG4: mean±SE: 8.97±3.16 and CpG5: mean±SE: 9.37±2.77) was observed to be modest. Correlation analyses revealed statistically significant moderate to high correlations between *TIEG2* methylation levels of single CpG sites ranging between ρ=.834 and ρ=.990 (all ps<.01). This points towards a high functional connectivity between the singe CpG sites in the investigated amplicon. Furthermore, possible confounding variables such as age, sex, or smoking behavior were tested regarding their impact on *TIEG2* methylation status in the overall sample. Sex did not show a significant association with overall *TIEG2* methylation or single CpG site methylation (all ps>.13), nor did age (all ps>.37) or smoking behavior (all ps>.32).

In the main analysis, *TIEG2* methylation was compared in a case/control design in 57 healthy subjects and 57 panic disorder patients. Here, no significant differences in average methylation or in DNA methylation at single CpG sites emerged (see Tab. 15, Fig. 7).

Tab. 15: *TIEG2* DNA methylation levels in patients with panic disorder (PD) and matched healthy controls

CpG	PD patients (mean±SE) N=57	Controls (mean±SE) N=57	Statistics[a]
Average	11.29±0.45	11.88±0.64	z=-.249; p=.803
1	2.55±0.14	2.83±0.21	z=-.598; p=.552
2	11.67±0.55	11.93±0.65	z=-.102; p=.919
3	24.01±0.91	25.38±1.30	z=-.332; p=.740
4	8.90±0.38	9.04±0.46	z=-.003; p=.998
5	9.29±0.35	9.46±0.39	z=-.055; p=.956

Legend to Tab. 15: PD: panic disorder; [a] p-values from non-parametric Mann-Whitney-U test are reported for average DNA methylation or methylation at the respective single CpG sites 1-5 as dependent variables and group (PD patients vs. healthy controls) as independent variable. Mean methylation is given in %; SE: standard error of the mean.

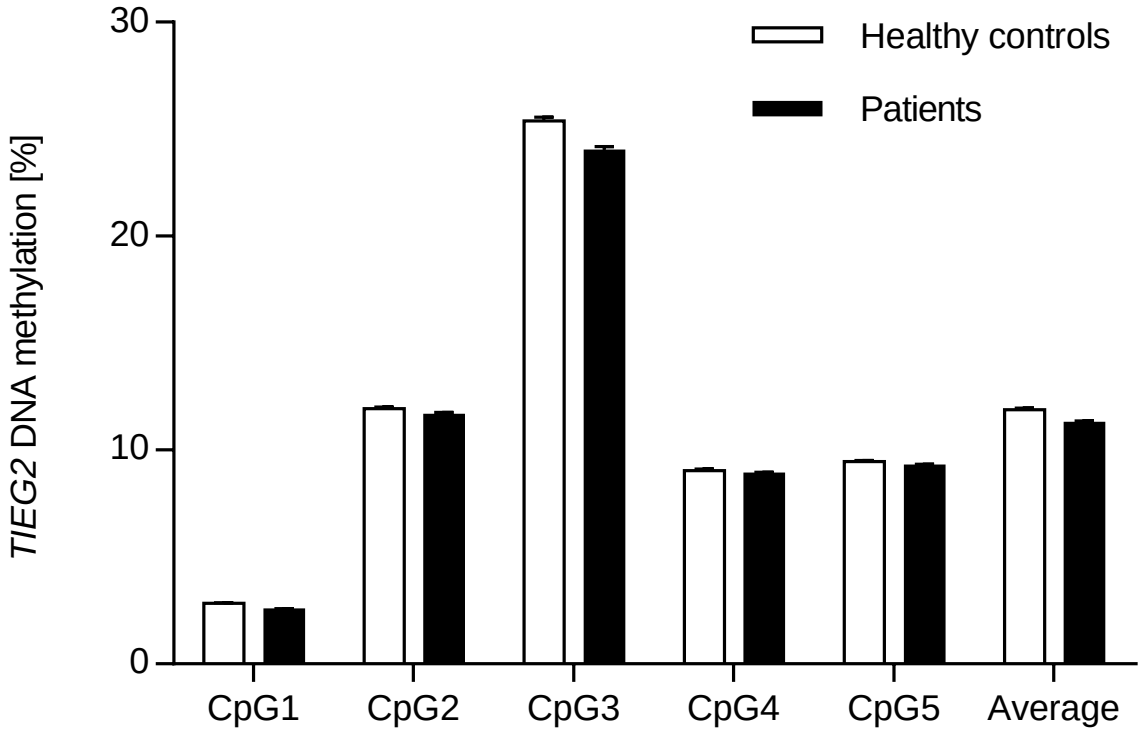

Fig. 7 *TIEG2* DNA methylation levels in patients with panic disorder (PD) and matched healthy controls

Controls: healthy controls without any anxiety disorder (N=57), Patients: panic disorder patients (N=57); no significant difference in *TIEG2* DNA methylation (given in %) between healthy controls and panic disorder patients is shown.

In order to investigate the strong comorbidity between panic disorder and depression, further analyses were carried out with regard to *TIEG2* methylation and its effect on the comorbidity rate with MDD. These analyses indicated nominally significant group differences for average methylation (p=.049) and methylation at CpG3 (p=.041) between PD patients with and without comorbid MDD. Follow-up tests revealed a significantly decreased methylation in PD patients with comorbid MDD (PD+MDD; N=27) concerning average *TIEG2* methylation (p=.008) and CpG site 3 methylation (p=.006; see Tab. 16, Fig. 8) compared to PD patients without comorbid MDD as well as compared to healthy controls, again regarding average methylation (p=.010) and methylation at CpG site 3 (p=.006; see Tab. 16, Fig. 8). Conversely, PD patients without comorbid MDD diagnosis (PDonly; N=30) did not differ from healthy controls with regard to *TIEG2* DNA average methylation or methylation at single CpG sites (all p>.05; see Tab. 16).

Tab. 16: *TIEG2* DNA methylation levels in patients with panic disorder (PD) with and without comorbid major depression (MDD) and matched healthy controls

CpG	PDonly (mean±SE) N=30	PD+MDD (mean±SE) N=27	Controls (mean±SE) N=57	Statistics[a]	PDonly vs. Controls[b]	PD+MDD vs. Controls	PDonly vs. PD+MDD
Average	12.22±0.61	10.25±0.62	11.88±0.63	H=6.04; **p=.049***	z=-1.94; p=.053	z=-2.73 **p=.010****	z=-2.65; **p=.008****
1	2.79±0.20	2.29±0.18	2.84±0.21	H=4.35; p=.113	-	-	-
2	12.72±0.76	10.51±0.73	11.93±0.65	H=5.06; p=.080	-	-	-
3	25.99±1.22	21.81±1.25	25.38±1.30	H=6.39; **p=.041***	z=-1.93; p=.054	z=-2.56; **p=.006****	z=-2.73; **p=.006****
4	9.63±0.51	8.09±0.54	9.04±0.46	H=5.26; p=.072	-	-	-
5	9.95±0.47	8.56±0.49	9.46±0.39	H=5.59; p=.061	-	-	-

Legend to Tab. 16: PD: panic disorder; MDD: major depressive disorder; [a] p-values from non-parametric Kruskal-Wallis test are reported with average DNA methylation or methylation at the respective single CpG sites 1-5 as dependent variable and group (healthy controls vs. PDonly patients vs. PD+MDD patients) as independent variable. [b] z- and p-values from post-hoc tests (Mann-Whitney-U test) are reported. Bold: (nominally) significant results: *= nominally significant at p≤.05; **=significant at p≤.01; Mean methylation is given in %; SE: standard error of the mean.

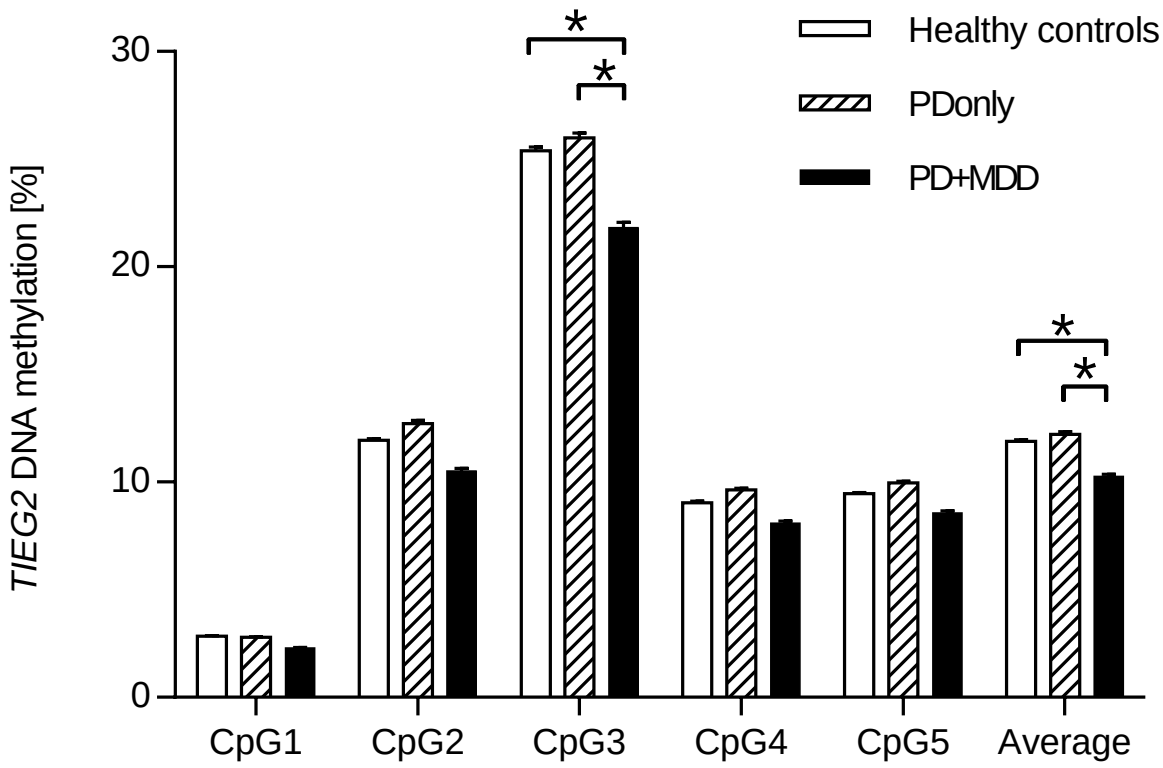

Fig. 8 *TIEG2* DNA methylation in panic disorder patients with or without comorbid major depressive disorder and matched healthy controls

PD: panic disorder; MDD: major depressive disorder; Controls: healthy controls without any anxiety disorder; PDonly: PD patients without comorbid MDD; PD+MDD: PD patients with comorbid MDD; Significant differences of average *TIEG2* methylation (given in %) as well as *TIEG2* methylation at CpG3 between healthy controls and PD patients with comorbid MDD (Controls vs. PD+MDD) as well as between patients without and with MDD (PDonly vs. PD+MDD) are marked with an asterisk (*=p≤.05). [Figure adapted from Kollert et al. 2020]

3.2.2 *TIEG2* DNA methylation and dimensional depression in PD

In addition to the categorical association between *TIEG2* methylation and comorbid MDD, a bivariate correlation analysis within the patient group (available for N=54, f=41, m=13) was conducted to detect a potential relation between *TIEG2* methylation status and the severity of depressive symptoms as evaluated by the Beck Depression Inventory II (BDI-II) questionnaire (Hautziger et al. 2009). Here, a nominally significant negative correlation between average *TIEG2* methylation and scores of the BDI-II questionnaire was discovered (ρ=-.336, p=.013; see Fig. 9).

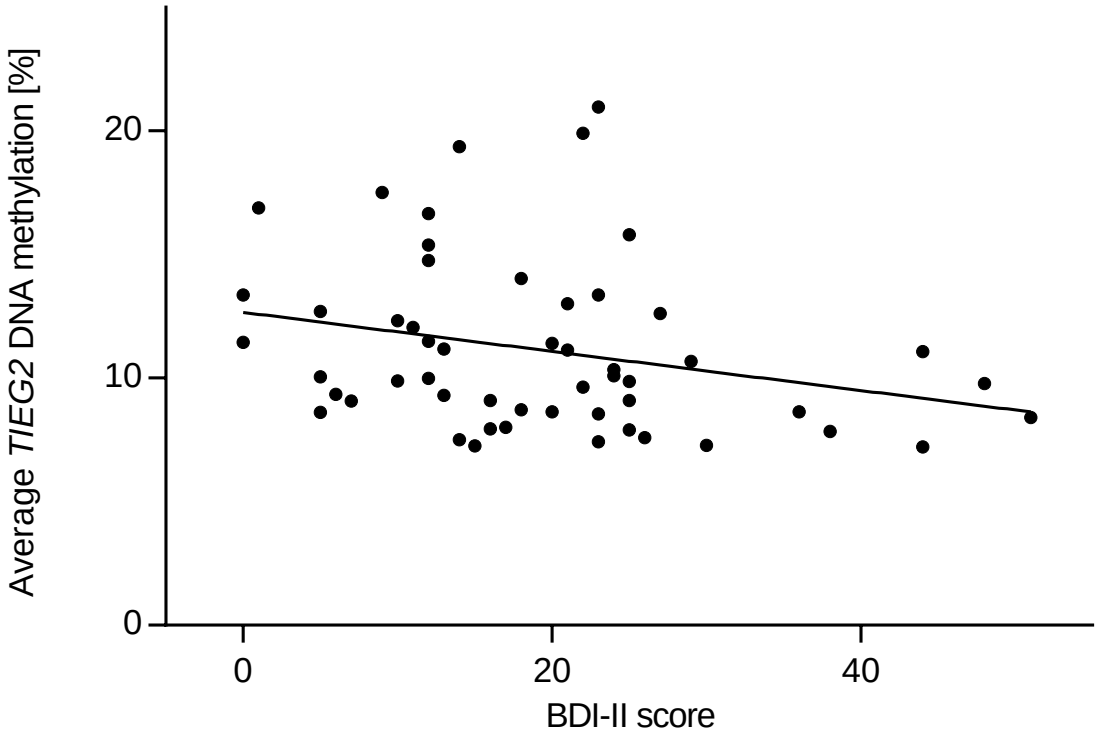

Fig. 9 Average *TIEG2* DNA methylation and dimensional depression in panic disorder

Negative correlation between average *TIEG2* DNA methylation and Beck Depression Inventory II (BDI-II) scores by means of Spearman correlation (ρ=-.336, p=.013) in 54 patients with panic disorder. [Figure adapted from Kollert et al. 2020]

3.3 Sample II

To further elucidate the previously discerned association of *TIEG2* hypomethylation in PD patients with comorbid depression (see Results section 3.2.1), methylation analyses were carried out in an independent sample of patients with major depression (initial sample size N=170; cf. Methods section 2.2.1.2). The final sample size was reduced to N=164 (f=96, m=68), as six samples had to be excluded due to failing quality check of the sequencing data. In this final MDD sample, patients were grouped into anxious (N=98, HAMD-score≥7) or non-anxious (N=66, HAMD-score<7) MDD patients (cf. Fava et al. 2008; Domschke et al. 2010; see Methods section 2.2.1.2). Again, in the overall sample only minor average *TIEG2* methylation was detected across all investigated CpG sites (average: mean±SE: 11.86±3.52) or at single CpG sites (CpG1: mean±SE: 2.92±1.139; CpG2: mean±SE: 11.81±3.96; CpG3: mean±SE: 24.63±6.82; CpG4: mean±SE: 9.75±3.32 and CpG5: mean±SE: 10.16±2.93). Correlations between single CpG methylation levels ranged between

ρ=.935 and ρ=.982; all ps<.01). Moreover, possible cofounders such as age (all ps>.15), sex (all ps>.38) or smoking behavior (all ps>.09) showed no significant association with average *TIEG2* methylation or methylation at single CpG sites.

A nominally significant difference in *TIEG2* DNA methylation was observed between patients with and without anxious depression at CpG site 1 (p=.041). Given a statistical trend, also average *TIEG2* DNA methylation (p=.052) as well as *TIEG2* methylation at CpG sites 2-4 (ps=.053 - .081; see Tab. 17, Fig. 10) differed between anxiously and non-anxiously depression patients, in that patients with non-anxious depression displayed decreased *TIEG2* methylation in comparison to MDD patients with anxious features.

Tab. 17: *TIEG2* DNA methylation levels in MDD patients with or without anxious depression

CpG	Anxious depression (mean±SE) N=98	Non-anxious depression (mean±SE) N=66	Statistics[a]
Average	12.28±0.37	11.23±0.41	z=-1.95; p=.052[#]
1	3.07±0.12	2.70±0.13	z=-2.04; **p=.041***
2	12.23±0.41	11.20±0.46	z=-1.75; p=.081[#]
3	25.46±0.70	23.41±0.80	z=-1.75; p=.079[#]
4	10.13±0.35	9.18±0.38	z=-1.77; p=.077[#]
5	10.51±0.31	9.64±0.33	z=-1.94; p=.053[#]

Legend to Tab. 17: MDD: major depressive disorder; Anxious depression: 98 patients with anxious depression (anxiety/somatization factor of the Hamilton Depression Rating Scale ≥7); Non-anxious depression: 66 patients without anxious depression (anxiety/somatization factor of the Hamilton Depression Rating Scale <7). [a] p-values from non-parametric Mann-Whitney U test are reported with average DNA methylation or methylation at the respective single CpG sites as dependent variable and group (anxious vs. non-anxious MDD patients) as independent variable. Bold: nominally significant results: *=nominally significant at p≤.05; [#]=statistical trend at p<.1; Mean methylation given in %; SE: standard error of the mean.

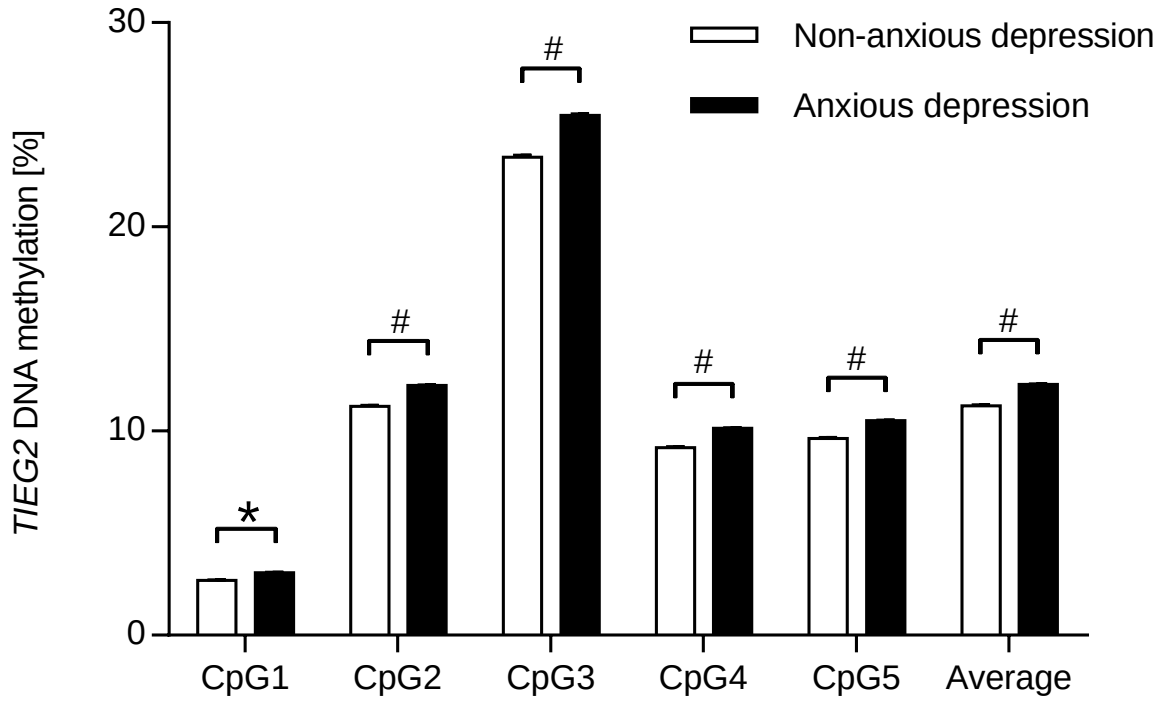

Fig. 10 *TIEG2* DNA methylation in MDD patients with or without anxious depression

MDD: major depressive disorder; Anxious depression: 98 patients with anxious depression (anxiety/somatization factor of the Hamilton Depression Rating Scale ≥7); Non-anxious depression: 66 patients without anxious depression (anxiety/somatization factor of the Hamilton Depression Rating Scale <7). Statistical trends in difference of *TIEG2* methylation (given in %) between MDD patients with or without anxious depression are marked with #=p≤.1; nominally significant results are marked with *=p≤.05. [Figure adapted from Kollert et al. 2020]

3.4 Sample III

Further methylation analyses regarding *TIEG2* methylation patterns and their effects on dimensional anxiety or depression-related behavior were carried out in a large, independent sample of healthy subjects (N=1,057, f=755, m=302; see Methods section 2.2.1.3). The final sample size was reduced to N=1,050 (f=749, m=301), given that sequencing data of seven samples failed outlier analysis. Again, *TIEG2* methylation was modest across all investigated CpG sites (mean±SE: 11.25±3.12) as well as at every single CpG site (CpG1: mean±SE: 2.48±1.25; CpG2: mean±SE: 11.52±3.57; CpG3: mean±SE: 23.43±6.00; CpG4: mean±SE: 9.31±2.88 and CpG5: mean±SE: 9.51±2.70). Comparable to the two previous samples (sample I and II), moderate to high correlations across all five CpG sites and average *TIEG2* methylation were discerned (ρ=.834-.976) reaching significance for all investigated CpG sites (all ps<.01). Also, the confounding factors sex (all ps>.25), age (all ps>.18) and smoking

behavior (all ps>.18) did not impact *TIEG2* average methylation or methylation at single CpG sites.

For each questionnaire on anxiety- and depression-related measures (for details see Methods section 2.2.1.3), a correlation analysis with average *TIEG2* methylation was conducted. Only one nominally significant positive correlation between average *TIEG2* methylation and the Anxiety Sensitivity Index score (ASI; available for N=1,050; Reiss et al. 1986) was detected (ρ=.062, p=.043; see Tab. 18, Fig. 11), which, however, would not withstand correction for multiple testing.

Tab. 18: Correlations between *TIEG2* average methylation and scores of anxiety/depression-related questionnaires in 1,050 healthy probands

Questionnaire	Statistics	
Agoraphobic Cognitions Questionnaire (ACQ)	ρ	0.037
	p-value	.229
Anxiety Sensitivity Index (ASI)	ρ	0.062
	p-value	**.043***
Beck Anxiety Inventory (BAI)	ρ	-0.006
	p-value	.854
Beck Depression Inventory II (BDI-II)	ρ	0.058
	p-value	.060
Liebowitz Social Anxiety Scale (LSAS)	ρ	0.048
	p-value	.124
Penn State Worry Questionnaire (PSWQ)	ρ	0.051
	p-value	.096
Social Phobia and Anxiety Inventory (SPAI)	ρ	0.013
	p-value	.674
State Trait Anxiety Inventory – Trait Version (STAI-T)	ρ	0.017
	p-value	.574

Legend to Tab. 18: Statistics are based on bivariate correlations; N: number of included subjects; correlation coefficients (ρ) and p-values of Spearman correlation are reported. *=nominally significant at p≤.05 (bold).

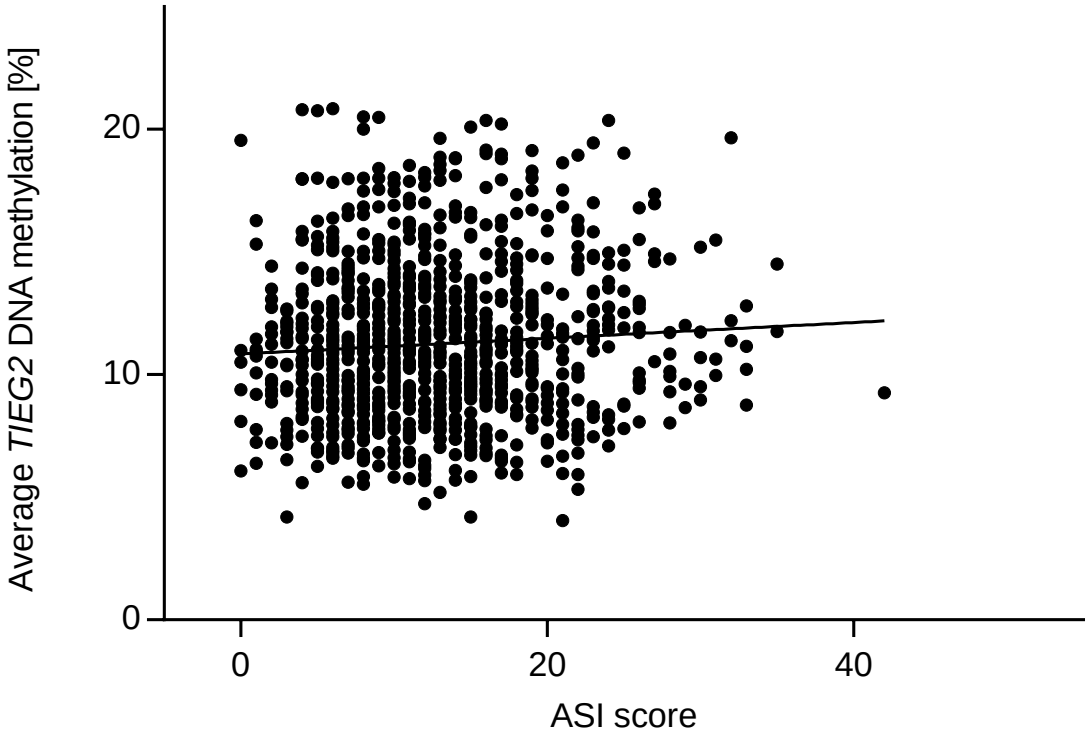

Fig. 11 Average *TIEG2* DNA methylation and dimensional anxiety in healthy subjects

Positive correlation between average *TIEG2* DNA methylation and Anxiety Sensitivity Index (ASI) score by means of Spearman correlation (ρ=.062, p=.043) in 1,050 healthy subjects.

IV DISCUSSION

In the current study, DNA methylation patterns in the promoter region of the *TGFB-inducible Early Growth Factor 2* (*TIEG2*) gene were examined with regard to a potential role of *TIEG2* as a differential epigenetic marker of comorbid depression in panic disorder or non-anxious depression, respectively, and anxiety in three independent samples. *TIEG2* hypomethylation was found to be associated with the phenotype of comorbid MDD in PD, were PD patients with comorbid MDD showed significantly decreased methylation levels compared to patients without comorbid depression as well as compared to healthy controls. Additionally, in PD patients *TIEG2* hypomethylation correlated negatively with Beck Depression Inventory II scores (BDI-II; Hautziger et al. 2009), indicating *TIEG2* hypomethylation to be associated with an increased severity of depression in PD patients. Additionally, in an independent sample of patients with MDD *TIEG2* hypomethylation was nominally associated with MDD without anxious traits as compared to MDD patients with anxious depression. In another independent sample of healthy controls decreased *TIEG2* methylation was found to be associated with lower Anxiety Sensitivity Index (ASI; Reiss et al. 1986) scores. These findings suggest that *TIEG2* hypomethylation might constitute an epigenetic marker of depression rather than of anxiety-related phenotypes. This result might aid in disentangling the differential pathogenesis of MDD and PD within the TIEG2-MAOA pathway as detailed below.

A model of the TIEG2-MAOA pathway in the differential MDD/PD pathogenesis

The presently investigated amplicon containing five CpG sites is located within the promoter region of the *TIEG2* gene and covers a highly active transcriptional region. Methylation patterns of this *TIEG2* gene region have not been investigated so far regarding their functional consequences on gene expression and protein levels. Nevertheless, epigenetic modifications of the promoter region of a gene are known to regulate gene expression in that hypomethylation leads to increased mRNA levels and consequently increased protein expression, while hypermethylation of the gene promoter rather represses gene expression (see Fig. 12; Suzuki and Bird 2008). Accordingly, hypermethylation of *TIEG2* promoter has already been reported to result in decreased gene expression in ovarian cancer (Wang et al. 2015).

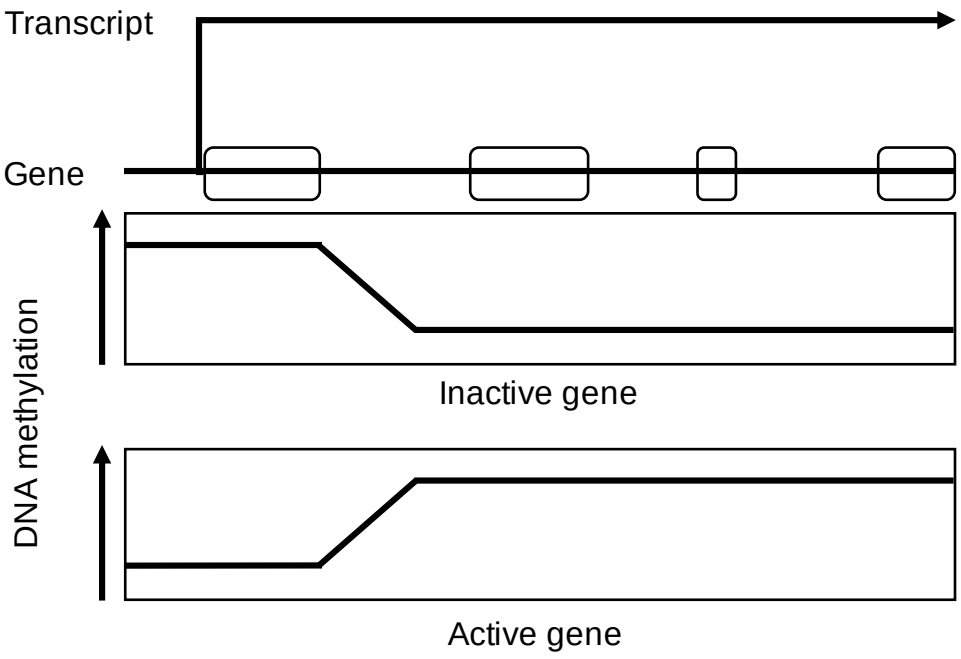

Fig. 12 Influence of differentially located DNA methylation on gene expression

The upper part of this figure illustrates that DNA hypermethylation in the promoter region of a gene can lead to inactivation of the gene. The lower part of the figure depicts hypermethylation in the gene body, which results in activation of the gene. [Figure modified from Suzuki and Bird 2008]

In accordance with the notion that gene promoter hypomethylation is associated with an upregulation of gene expression, *TIEG2* hypomethylation in the investigated amplicon – as presently identified to be associated with comorbid MDD in PD, depression severity in PD as well as non-anxious depression – is hypothesized to lead to higher TIEG2 protein levels and thus higher expression and consequently enzymatic activity of the TIEG2 target monoamine oxidase A (MAOA; Grunewald et al. 2012; see Introduction section Fig. 1). Biochemically, MAOA overexpression followed by increased MAOA protein levels results in an enhanced degradation of catecholamines such as serotonin, norepinephrine and dopamine and subsequently a reduced availability of these neurotransmitters in the synaptic cleft (e.g. Shih and Chen 1999; Ou et al. 2006; Duncan et al. 2012; Naoi et al. 2018; see Fig. 13).

TIEG2 hypomethylation and thus increased TIEG2 expression in PD patients with comorbid MDD as well as in non-anxious MDD patients is in line with a study reporting TIEG2 protein expression to be elevated by 44 % in the frontal cortex of *Tieg2* wild-type mice, when mice were exposed to chronic social defeat stress, which is considered a rodent model of depression. In contrast, locomotor activities, central box duration and sucrose preference were significantly reduced in the stressed wildtype

mice, suggesting that wildtype mice are more harshly affected by the stress model compared to *Tieg2* knock out mice (Harris et al. 2015). Accordingly, TIEG2 immunoreactivity in the frontal cortex, in the medial prefrontal cortex and in the CA1 of the hippocampus was increased by chronic social defeat stress in rats (Duncan et al. 2015). The present hypothesis that *TIEG2* hypomethylation resulting in increased TIEG2 expression would lead to enhanced MAOA activity is in line with a previous study showing that exposure of rats to chronic social defeat stress test led to an activation of the TIEG2 pathway in the brain and consequently to increased MAOA mRNA levels and enzymatic activity (Grunewald et al. 2012). On a human level, the present results are in accordance with increased TIEG2 and MAOA protein levels in the postmortem prefrontal cortex of MDD patients as compared to healthy controls (Harris et al. 2015) and previous epigenetic studies having observed *MAOA* hypomethylation in the first exon region – presumably leading to increased MAOA expression – in female patients with MDD as compared to healthy controls (Melas et al. 2013; Melas and Forsell 2015). Increased MAOA activity is known to contribute to a catecholamine depletion as proposed in one of the most widely accepted pathogenetic models of MDD (e.g. Meyer et al. 2006, 2009; Barton et al. 2008; Johnson et al. 2011; Chiuccariello et al. 2014; Harris et al. 2015; Homan et al. 2015; Zhen et al. 2017) and, reciprocally, the monoamine oxidase inhibitors such as moclobemide, tranylcypromine or phenelzine are highly efficacious pharmacological treatment options in MDD (e.g. Tiller et al. 1997; Youdim et al. 2006; Lopez-Munoz and Alamo 2009; Wimbiscus et al. 2010; Shulman et al. 2013).

In sum, *TIEG2* hypomethylation – potentially resulting in increased MAOA activity – as presently observed in depression-related phenotypes might provide further insight into the pathomechanism of the so-called “catecholamine depletion”, which has been proposed as a major factor in MDD pathogenesis. By further understanding the molecular processes that affect *MAOA* regulation in the brain, new drug targets to control MAOA activity – such as TIEG2 – could be identified.

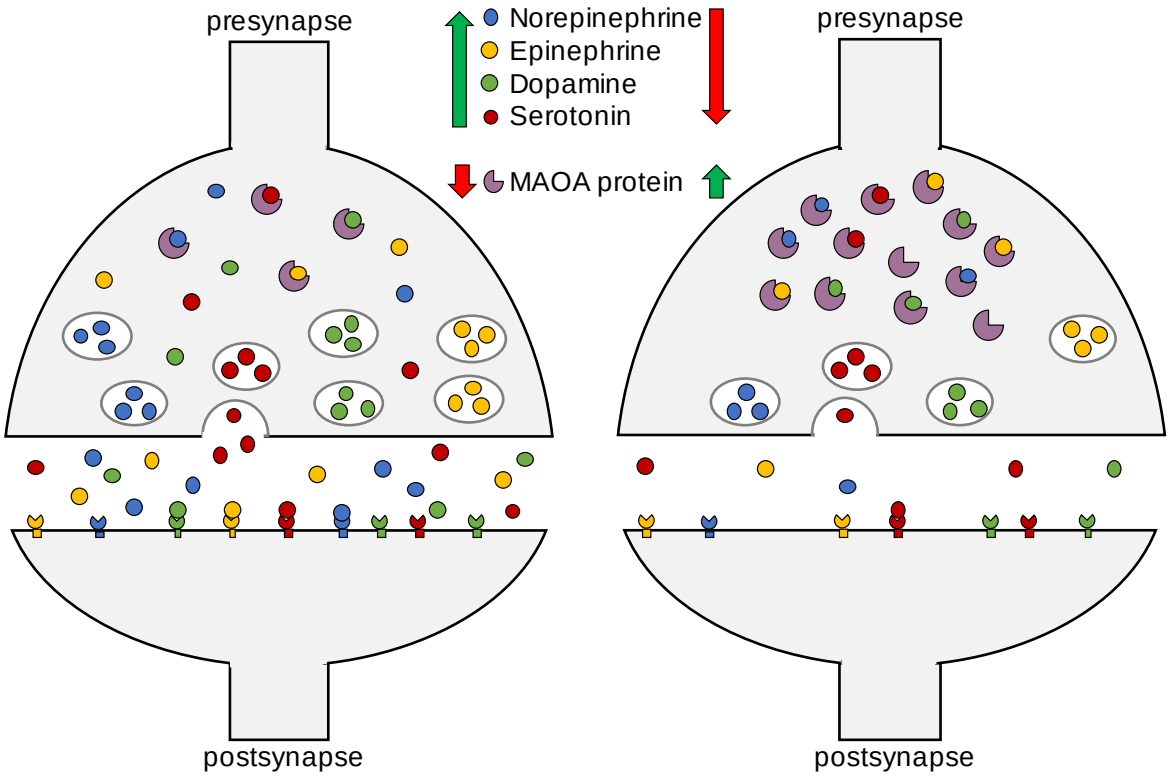

Fig. 13 Schematic illustration of possible functional consequences of differential *MAOA* methylation levels

On the left, less MAOA protein activity due to high *MAOA* methylation is shown to confer higher catecholamine availability. On the right, higher MAOA protein activity due to low *MAOA* methylation is depicted to confer lower catecholamine availability. [Figure modified from Ziegler et al. 2016]

Interestingly, *TIEG2* DNA methylation was not associated with panic disorder per se or with anxious depression. Rather, a positive correlation between an anxiety-related trait – as captured by ASI score – and *TIEG2* DNA methylation was observed in the healthy proband sample indicating lower *TIEG2* methylation to go along with decreased anxiety. Therefore, *TIEG2* DNA hypomethylation seems to constitute a depression- rather than anxiety-related marker. This is at first sight surprising given 1) the assumed TIEG2-MAOA pathway as detailed above and 2) a number of previous studies reporting increased MAOA activity in anxiety disorders (e.g. Zhen et al. 2017; Schiele et al. 2018; for review see Ziegler and Domschke 2018), association of the more active alleles of the *MAOA* VNTR with anxiety phenotypes (e.g. Deckert et al. 1999; Reif et al. 2012, 2014) and recent reports of *MAOA* hypomethylation – presumably leading to increased *MAOA* activity – in anxiety disorders (Domschke et al. 2012; Ziegler et al. 2016; Schiele et al. 2018). However, it should be noted that the influence of regulatory factors such as TIEG2 on *MAOA* gene activity can vary across different brain regions. For example, the knockout of another regulatory factor of the

Maoa gene in mice, known as ring finger protein 180 (RNF180; see Fig. 1), leads to an increase of MAOA mRNA and protein levels in the Locus coeruleus, but not in other brain regions. This could indicate that this regulatory factor only controls the regulation of *Maoa* expression in a specific brain region (Kabayama et al. 2013) pointing towards brain region specificity of different regulatory factors of *Maoa*. Thus, TIEG2 might drive *Maoa* expression in brain regions relevant for the pathogenesis of depression rather than anxiety-related phenotypes. This hypothesis, however, needs to be tested in future animal studies analyzing TIEG2-driven MAOA activity in a comparative design of anxiety- and depression-related behavioral phenotypes.

Strengths and limitations

Strengths of the present study include: 1) patients in samples I and II were recruited according to strict inclusion and exclusion criteria, implicating low demographic and clinical heterogeneity, 2) healthy controls of sample I were recruited to exactly match the patient sample regarding age, sex, and smoking status, and 3) potential confounding factors of epigenetic mechanisms such as age, sex, and smoking status were considered.

However, the current findings should be interpreted in the light of some limitations:

First, it is worth mentioning, that the main results showing differential methylation dynamics depending on patient's status (PD+MDD vs. PD and PD+MDD vs. controls) did not survive Bonferroni-correction for multiple testing ($p<.01$), which, however, is to be considered overly conservative given the high intercorrelation between single CpG site methylation (cf. Schiele et al. 2018).

Second, replication of the results in further independent and larger samples is needed to validate *TIEG2* hypomethylation as a potential differential diagnostic marker of MDD comorbidity in PD or non-anxious MDD, respectively.

Additionally, it should be noted that MDD can occur comorbidly not only with PD but also with other anxiety and mental disorders. Therefore, the role of *TIEG2* methylation levels as a diagnostic marker of MDD should be investigated in different comorbidity profiles. This would involve a direct comparison of an MDD sample with a PD sample, as well as with samples with other anxiety or mental disorders. In addition,

upcoming studies should examine the association of *TIEG2* methylation with symptom subtypes and with disorder-specific intermediate phenotypes.

Another limitation is the missing control group for the MDD sample II. Thus, it cannot be conclusively confirmed that the observed differences in *TIEG2* methylation in MDD patients with or without anxiety depression can be attributed to the presence of anxiety symptoms.

Importantly, it has to be noted that DNA methylation patterns measured in peripheral tissues do not allow for direct conclusions on DNA methylation patterns existing in the brain. However, DNA samples extracted from whole blood are commonly used in DNA methylation studies for neuropsychiatric disorders (for review see Terry et al. 2011), given the difficulty to obtain human brain tissue *in vivo* as well as the small number of post-mortem samples available. Furthermore, animal studies have postulated some evidence for DNA methylation levels measured in blood as a surrogate for central processes by intraindividually comparing peripheral and central methylation levels. Moreover, a genome-wide analysis study revealed a significant correlation between differential methylation levels in blood or in the brain in a gene pool of precursors of metabolites in patients with schizophrenia (Walton et al. 2016). Additionally, in patients with Parkinson disease, a strong correlation of differentially methylated regions in the brain (post-mortem frontal cortex) and leucocytes was revealed (Masliah et al. 2013). Finally, human studies comparing peripheral methylation levels with central activity of the respective metabolite by means of positron emission tomography (PET) identified a functional correlation (e.g. Murphy et al. 2005; Nohesara et al. 2011; Shumay et al. 2012). For instance, a robust negative correlation between peripheral *5-HTT* (*SLC6A4*) DNA methylation level and *in vivo* serotonin (5-HT) synthesis in the brain was identified (Wang et al. 2012). These different lines of evidence could suggest that differentially methylated regions are not limited to the affected tissue but are detectable in other tissues as well (for review see Mill and Petronis 2007). However, it remains to be experimentally determined whether differences in *TIEG2* methylation detected in peripheral material such as blood can be generalized to central *TIEG2* methylation and TIEG2 levels in the brain, and whether altered TIEG2 levels in the periphery correlate with altered brain function.

Outlook

Longitudinal studies, preferably in a cohort of healthy children/adolescents, should be considered in order to disentangle trait and state characteristics of *TIEG2* methylation in interaction with environmental factors. This longitudinal analysis of *TIEG2* methylation patterns would imply regular collections of blood samples at several times, reaching into adulthood, as well as a detailed documentation of psychometric scores and life events. This latter aspect is of particular importance, since previous studies indicated a strong influence of environmental factors such as stress on *TIEG2* gene expression (Grunewald et al. 2012; Harris et al. 2015). Therefore, the impact of positive or negative life events (cf. Domschke et al. 2012) on *TIEG2* methylation status should be explored in future studies applying an (epi)gene-environment approach.

Further studies are furthermore warranted to combine methylation analyses with interventional approaches thereby providing an improved understanding of the role of *TIEG2* methylation in mental disorders such as PD or MDD and their treatment. In analogy to previous studies showing reversibility of for instance *MAOA* hypomethylation along with successful cognitive behavioral psychotherapy in panic disorder (Ziegler et al. 2016) as well as in acrophobia (Schiele et al. 2018) and obsessive-compulsive disorder (Schiele et al. 2020), *TIEG2* methylation patterns could be investigated for possible alterations along with successful therapy in MDD and for whether these changes would constitute a direct mechanism of an effective treatment. Given the present finding of *TIEG2* hypomethylation in PD patients with comorbid MDD and non-anxious depression, an increase in *TIEG2* methylation upon successful therapy in responders would be assumed, whereas *TIEG2* methylation levels of non-responders should not change or rather decrease.

In general, the functional consequences of *TIEG2* methylation status remain highly speculative and need to be confirmed by future studies regarding TIEG2 mRNA and protein levels. One possible method to quantify mRNA levels would be real-time quantitative PCR (qPCR). The qPCR is a variation of the polymerase chain reaction (PCR). Through directly added non-specific fluorescent dyes or sequence-specific DNA probes, the concentration of the products can be followed in real-time during PCR. The change of the concentrations permits evaluation of the initial concentration of the nucleic acid. Thereby, mRNA levels from patients suffering from a mental disorder could be compared with mRNA levels from healthy controls. This method has

already been carried out with cDNA samples of a case/control approach regarding TIEG2 mRNA. However, due to poor quality of the mRNA, data normalization by including an invariant endogenous control (reference gene) to correct for variations in qPCR efficiency and errors in sample quantification could not confirm the reliability of the qPCR experiment (Bustin et al. 2009). A second way to functionally characterize DNA methylation patterns is a dual luciferase assay, which allows for analyzing the functional consequences of DNA methylation patterns regarding gene expression as represented by luciferase activity. This technique has successfully been applied by the author of this thesis in previous studies (Schiele, Kollert et al. 2018; Schiele, Ziegler, Kollert, et al. 2019).

Furthermore, it would be interesting to investigate how altered methylation status of certain transcription factor binding sites, like SP1 binding sites for *TIEG2*, within the promoter region of certain target genes affects the binding of transcription factors to these specific binding sites. To answer this question, a chromatin immunoprecipitation (ChIP) would be suitable. ChIP is an experimentally powerful technique for determining possible DNA-protein interactions in the cell. This method helps to define whether specific genomic regions are associated with certain proteins, such as TIEG2 transcription factor with *MAOA* promoter or in more detail with SP1 binding sites. However, the execution of this technique bears some challenges: it is not easy to find an appropriate antibody because the target protein needs to be correctly predicted, DNA-protein interactions with signals of low-affinity might not be detected, and there might be a further protein in a certain DNA-protein interaction, which was not considered.

As the results of this study reflect a recently published report of *serotonin transporter gene* (*SLC6A4*) hypermethylation in PD patients with comorbid MDD compared to healthy controls, but not in PD *per se* (Schiele et al. 2019), future studies should examine the possible biochemical association between *TIEG2* and *SLC6A4* function in the context of MDD, especially in light of the fact that the *SLC6A4* promoter region has been shown to contain Sp1 binding sites (Bengel et al. 1997; Heils et al. 1998), which have been identified as a target of TIEG2 (Grunewald et al. 2012).

Additionally, other members of the Krüppel-like family (KLF) of transcription factors – to which TIEG2 belongs – should be investigated to clarify their role in the pathophysiology of mental disorders. Interestingly, recent literature reports that

another member of the KLF family was associated with anxious behavior. The Krüppel-like factor 9 (KLF9), a zinc finger transcription factor, has been shown to interact directly with the scaffold corepressor protein Sin3A and therefore activate transcription as well as repress expression of different genes (Zhang et al. 2001). KLF9 plays a role in various biological processes involving stem cell maintenance (Jiang et al. 2008) and differentiation of T- and B-lymphocytes (Good and Tangye 2007). Furthermore, KLF9 expression seems to be perceptive to changes in serotonin signaling (Khawaja et al. 2004). Based on behavioral experiments, Scobie and colleagues showed that *Klf9* null mice exhibited a subtle increase in anxious behavior. This was demonstrated with the light/dark choice test, a well-established model for assessing anxiety-like behavior. *Klf9* null mice spent less time and traveled less distances in the light compartment, which indicates an increased anxiety-like behavior (Scobie et al. 2009). Additionally, *Klf9* null mice and control wild-type mice were tested in a single trial contextual fear conditioning paradigm, to determine the influence of *Klf9* deficiency on hippocampal dependent learning (Scobie et al. 2009). Both groups showed no significant difference in contextual fear conditioning performance. Moreover, authors tested the performance of both groups (*Klf9* null and control mice) in a contextual fear discrimination learning paradigm. In contrast to the control group, *Klf9* null mice showed no change in freezing behavior comparable to the fear conditioning paradigm. These results imply that *Klf9* dependent neuronal maturation could be essential for normal contextual fear discrimination learning. Therefore, also in humans, *KLF9* appears to be a suitable candidate to be investigated for association with anxious behavior particularly with regard to methylation patterns.

Finally, beyond candidate gene approaches such as the present one focusing on the *TIEG2* gene, epigenome-wide association studies (EWAS) should be carried out to investigate differential DNA methylation markers for panic disorder and MDD on an epigenome-wide level (cf. Shimada-Sugimoto et al. 2017; Iurato et al. 2017; Shimada et al. 2018; Ziegler et al. 2019).

In conclusion, the present data suggest *TIEG2* promoter hypomethylation as a potential epigenetic marker of MDD comorbidity in PD or non-anxious depression, respectively, possibly contributing to a differential pathomechanism of anxiety and mood disorders. In line with previous findings implicating TIEG2 in depression-related phenotypes (Harris et al. 2015), the results of this study are of mechanistic relevance and might promote research efforts into exploring innovative therapeutic methods targeting this pathway in the treatment of major depression. In general, the identification of epigenetic mechanisms contributing to the development of mental disorders does not only aid in a deepened understanding of disease pathomechanisms, but might also allow for the development of innovative pharmacological agents and their individualized application according to the patient's genetic/epigenetic risk factor constellation, leading to a more effective treatment of mental diseases such as anxiety disorders or major depression.

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