Biological and personality factors underlying Major Depression

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List of scientific articles included within this thesis:

This cumulative dissertation is based on four publications and one preprint. Two studies (Sanwald et al. (2019) and Sanwald et al. (2021)) were published open-access and distributed under the terms of the Creative Commons CC BY license (CC BY 4.0). Sanwald, Gahr et al. (2020) was reprinted by permission from Springer Nature Customer Service Centre GmbH. Sanwald, Widenhorn-Müller et al. (2020) was published in the Journal of Affective Disorders, which is the article's original source. I retained the right to include and reprint the article in my dissertation. Reprints of the four studies and a preprint version of a study published in BMC Psychiatry are included in the following sections of this dissertation:

Section “2.1. Study I” is a reprint of

Section “2.2. Study II” is a reprint of

Section “2.3. Study III” is a reprint of

Section “2.4. Study IV” is a reprint of

Section “2.5. Study V” is a preprint version. A later version of this manuscript is published in BMC Psychiatry:
Table of contents

List of abbreviations ................................................................. 6
List of Figures ........................................................................ 7
Summary ................................................................................... 8
Zusammenfassung ..................................................................... 9
Synopsis .................................................................................. 11
1. Introduction ........................................................................ 11
  1.1. Epidemiology and course of MDD ........................................... 12
  1.2. Etiology of MDD ................................................................. 13
  1.2.1. The genetics of MDD ......................................................... 13
  1.3. From biological to psychological to environmental factors implicated in MDD development ......................................................... 14
  1.3.1. Sex, MDD and the 2D:4D ratio .......................................... 14
  1.3.2. Serotonin and MDD ......................................................... 15
  1.3.3. Oxytocin and MDD ......................................................... 17
  1.3.4. Primary emotions associated with MDD .......................... 19
  1.3.5. Stressful life events and MDD .......................................... 21
  1.4. Epigenetics – bridging the gap ............................................ 23
  1.4.1. Definition and epigenetic mechanisms .............................. 23
  1.4.2. Methylation of SLC6A4 and OXT in stress and depression research ................................................................. 24
  1.5. A comprehensive model of depression development ............. 25
  1.6. Aims and outline of the present work .................................... 28
2. Original research articles .......................................................... 30
  2.1. Study I - Depression is associated with the absence of sex differences in the 2D:4D ratio of the right hand .......................... 30
  2.2. Study II - Relation of promoter methylation of the oxytocin gene to stressful life events and depression severity .......................... 40
  2.3. Studies III - Relation of promoter methylation of the structural oxytocin gene to critical life events in major depression: A case control study ......................................................... 52
  2.4. Study IV - Factors related to age at depression onset: The role of SLC6A4 methylation, sex, exposure to stressful life events and personality in a sample of inpatients suffering from major depression ........................................ 63
  2.5. Study V - When a playful personality counteracts fear: Predictors for fear of COVID-19 in former inpatients with major depressive disorder and healthy control participants ........ 78
3. General discussion and future directions ...................................... 107
  3.1. The 2D:4D ratio and MDD .................................................... 108
  3.2. SLEs and MDD .................................................................. 109
  3.3. Primary emotions associated with MDD .............................. 111
  3.4. Biological factors associated with MDD ................................ 112
3.4.1. SLC6A4 and MDD .............................................................................................................. 113
3.4.2. OXT and MDD .................................................................................................................. 114
3.5. Interrelations and interactions of environmental, psychological and biological factors ................................................................................................................................. 115
3.6. Implications of the current findings for research and clinical practice .................................. 118
3.7. Limitations and future directions ............................................................................................ 120
3.8. Conclusions ............................................................................................................................ 121

References ....................................................................................................................................... 123

List of abbreviations
2D:4D ratio .............................................................. ratio of the index finger (2D) relative to the ring finger (4D)
5-HT ................................................................. serotonin
5-HTT .................................................................................................................. serotonin transporter
5-HTTLPR ............................................................. 5-HTT-linked polymorphic region
A ............................................................................................................................... adenine
ACTH .................................................................................................................... adrenocorticotropic hormone
BDNF ................................................................................................................... brain-derived neurotrophic factor
C .............................................................................................................................. cytosine
CpG .......................................................................................................................... cytosine-guanine dinucleotides
CRF ......................................................................................................................... corticotropin-releasing factor
DNA ......................................................................................................................... Deoxyribonucleic acid
G ............................................................................................................................... guanine
GC ............................................................................................................................ glucocorticoid
GR ............................................................................................................................ glucocorticoid receptor
GWAS ..................................................................................................................... genome wide association studies
HPA .......................................................................................................................... hypothalamic-pituitary-adrenal
MDD ......................................................................................................................... Major Depressive Disorder
mRNA ....................................................................................................................... messenger RNA
NR3C1 ..................................................................................................................... glucocorticoid receptor gene
OXT ........................................................................................................................... oxytocin gene
OXTR ....................................................................................................................... oxytocin receptor gene
RNA .......................................................................................................................... ribonucleic acid
SLC6A4 ...................................................................................................................... serotonin transporter gene
SLEs ........................................................................................................................... stressful life events
SSRIs .................................................................................. selective serotonin reuptake inhibitors
T ........................................................................................................ thymine

List of Figures

Figure 1. A comprehensive model of depression development. .................................................. 27
Summary

Research in recent years has revealed a large amount of biological correlates of depression. It has become increasingly clear that the initial hope that progress in the field of genetics would be able to unravel the genetic basis of the disease will not be fulfilled in the near future. Rather, depression is characterized by a complex interplay of many genes and associated biological systems, making the current findings difficult to interpret. This could be remedied by integrating the findings from gene-environment interactions with personality constructs. The latter could facilitate the classification of current findings and might represent a step toward theory-based research into the biological basis of depression etiology. Primary emotional systems are considered the emotional basis of human personality. They have a neurobiological basis in subcortical brain regions experimentally identified using deep brain stimulation studies and psychopharmacological challenge tests in animal models. The neural substrate of primary emotions allows for theory-driven investigations of the emotional consequences of stressful life events (SLEs) and their associations with biological systems associated with depression like the serotonin and the oxytocin system.

This dissertation aims at collecting evidence for a comprehensive model of depression development comprising environmental, psychological and biological factors. Accordingly, the interplay between the 2D:4D (second digit to fourth digit) ratio a marker of prenatal androgen exposure, stressful life events, primary emotional systems, and methylation patterns of the serotonin and oxytocin systems was examined. This dissertation comprises five studies. In the first study, the associations between the 2D:4D ratio, sex and depression were investigated using a case-control design. The study showed no direct association between the 2D:4D ratio and depression. However, an absence of sex differences in the 2D:4D ratio of the right hands of patients as compared to controls was observed. The second and third study showed that SLEs were negatively associated with methylation status of the structural oxytocin gene. In addition, patients exhibited a significantly lower methylation status of the oxytocin gene as compared to matched controls. The fourth study provided evidence for the predictive value of the primary emotions SEEKING and SADNESS for first depression onset. Furthermore, methylation status of the serotonin transporter gene was positively associated with depression severity in women. The fifth study showed that SLEs and primary emotions explained a significant amount of variance in fear of a future SLE.

The present dissertation provides evidence for associations between environmental, psychological as well as biological factors and depression. The consideration of primary emotions helps in the interpretation of associations between SLEs, epigenetic alterations and depression. Therefore, personality might bridge the gap between the environment and biological adaptions to the environment.
Zusammenfassung


Somit liefert die vorliegende Dissertation Belege für Zusammenhänge zwischen Umwelt-, psychologischen und biologischen Faktoren mit Depressionen. Die Berücksichtigung von Primäremotionen hilft, Assoziationen zwischen SLEs, epigenetischen Veränderungen und der Major Depression zu interpretieren. Daher könnte die Persönlichkeit die Kluft zwischen der Umwelt und der biologischen Anpassungen an die Umwelt überbrücken.
Synopsis

1. Introduction

“The trick to forgetting the big picture is to look at everything close-up.”
(Chuck Palahniuk, 2003, p. 34)

When we talk about depression, we all may have an idea what it feels like to be depressed, at least in a subclinical way. We may feel inconsolable sadness when a romantic relationship ends or when a beloved person dies. This kind of sadness, also known as separation distress (Watt & Panksepp, 2009), is one of the most intense feelings one can experience and may be accompanied by anhedonia, loss of interest and changes in sleep and appetite. It shows us how essential relationships with others, living together in groups and romantic love are for our wellbeing, survival and the continued existence of our species. Therefore, the experience of separation distress is common, makes evolutionary sense and initially has no pathological value. The symptoms, however, resemble the criteria of Major Depressive Disorder (MDD). MDD is a highly disabling mental illness characterized by at least two weeks with depressed mood, decreased interest or pleasure in most activities, changes in appetite or sleep, loss of energy, feelings of guilt or worthlessness, diminished ability to think or concentrate, indecisiveness and thoughts of death or suicide (American Psychiatric Association, 2018). The similarities between separation distress and MDD are one of the reasons why Watt and Panksepp (2009) argued that depression was conserved throughout the evolution. They suggest that depression might be an emotional shutdown to end chronically prolonged separation distress. From this point of view, it becomes clear that a search for the reason for a depressive phenotype investigating exclusively individual factors is too simplistic. Accordingly, current theories of depression development postulate an interaction of individual vulnerability and environmental stressors to result in the pathogenesis of depression (Colodro-Conde et al., 2018). Thus, it seems promising to investigate stressors in combination with systems involved in the bodily stress response. In addition, an individual’s perceptions, interpretations and reactions with respect to its environment and their neurobiological underpinnings could shed light on the link between environment and the experience of an event as stressful. The aim of this dissertation is therefore to integrate factors associated with depression development from different branches of research, i.e., biology, psychiatry and psychology, taking a first step to a more thorough understanding of the disorder. We investigated the biological sex, a marker for sexual hormone exposure, a potential genetic risk factor, stressors, Deoxyribonucleic acid (DNA)-methylation of two gene loci and primary emotions. However, first I want to give a short description of MDD epidemiology and etiology.
1.1. Epidemiology and course of MDD

After anxiety disorders, MDD is the most widespread mental disorder and among mental disorders the number one cause of years lived with disability (James et al., 2018). In Germany, the prevalence of administrative MDD diagnoses increased from 12.5 % in 2009 to 15.7 % in 2017 with women being twice as likely as men to receive a diagnosis (Steffen, Thom, et al., 2020). However, studies based on standardized MDD diagnosis indicate a stable 12-month prevalence for MDD of 7.4 % in Germany (Bretschneider et al., 2018). It is worth noting that rising depression risk within specific subgroups, e.g., an increased lifetime depression prevalence and chronicity in younger cohorts has been documented (Bretschneider et al., 2018; Kessler et al., 2005; Murphy et al., 2000). Mood and anxiety disorders are highly comorbid: Of individuals diagnosed with a depressive disorder, 67 % have a current and 75 % a lifetime comorbid anxiety disorder (Lamers et al., 2011). These comorbidity rates are comparable to a study using ambulatory claims data of the German population which reported the prevalence of neurotic disorders in individuals diagnosed with a depressive disorder to range from 52.4 % to 65.5 % depending on the severity of the depressive disorder (Steffen, Nübel, et al., 2020). A recent study reported that if a mood disorder was diagnosed, 10 years later 27.2 % of the diagnosed men and 28.9 % of diagnosed women additionally had received a diagnosis of a neurotic disorder with even higher risks in case of an early onset of the mood disorder (Plana-Ripoll et al., 2019). Therefore, it is hardly surprising that depression and anxiety disorders are thought to share a common etiological background (Garber & Weersing, 2010).

There are at least four possible courses of MDD: a single brief episode with complete recovery that does not recur, multiple acute episodes interspersed with periods of complete recovery, acute episodes followed by periods of residual symptoms and chronic episodes with fluctuating symptom severity (Otte et al., 2016). In population-based samples, mean episode duration varies between 13 and 30 weeks. Up to 90% of patients recover within one year (Keller et al., 1992; Otte et al., 2016; Spijker et al., 2002; Üstün et al., 2004). In outpatient care settings, relative remittance is only about 25% within six months. After two years, more than 50% of patients still show above threshold MDD symptom severity (Boschloo et al., 2014; Penninx et al., 2011; Wells et al., 1992). About 50% of individuals recover from their first depressive episode with no future episodes, 15% of cases experience an unremitting and chronic course and 35% suffer from recurrent episodes (Eaton et al., 2008). Psychiatric comorbidity, older age and a younger age of onset are associated with chronicity (Murphy & Byrne, 2012). Female sex is associated with a more chronic depression course and a longer duration of episodes. In addition, a lower age of onset predicts a worse course of MDD in females (Eaton et al., 2008; Essau et al., 2010; Üstün et al., 2004).
1.2. Etiology of MDD

The literature on depression development impressively shows that there is a plethora of neurobiological correlates of MDD but no coherent, depression-specific etiological integration of these findings (Watt & Panksepp, 2009). There are reports of monoamine deficiency (Heninger et al., 1996), hypothalamic-pituitary-adrenal (HPA) axis alterations (Heim et al., 2000; Heim & Binder, 2012; Pariente & Lightman, 2008; Weaver et al., 2004) and associated atrophic changes in the hippocampus (Lee et al., 2002), alterations in neuronal growth factors (Duman & Monteggia, 2006), alterations in opioid systems (Filliol et al., 2000; Yoo et al., 2004), oxytocin (Cyranowski et al., 2008), glutamate (Sanacora et al., 2012) and γ-aminobutyric acid (GABA) activity (Möhler, 2012). In addition, there are reports of chronic inflammation (Miller et al., 2009) and alterations in mitochondrial activity (Gumpp et al., 2020; Karabatsiakis et al., 2014). Furthermore, the role of alterations in prefrontal activity (Wang et al., 2008) and changes in large-scale emotional brain networks is discussed (Hare & Duman, 2020; Menon, 2011; Watt & Panksepp, 2009). The integration of all these disparate candidate mechanisms into one theoretical framework may help contextualizing new findings and derive specific falsifiable hypotheses regarding their contribution to the development and maintenance of depression. The following sections give a short explanation of some of the candidate mechanisms investigated in the current work, which are further integrated in the aims and outlines section. Before doing so, I would like to give a brief overview of the field of research on which great hopes have been pinned with regard to the early detection and prevention of depressive disorders: genetics.

1.2.1. The genetics of MDD

Since depression is considered having a moderate heritability of about 37% (Sullivan et al., 2000), the investigation of the genetic basis of MDD seemed to bring the possibility of reliable biomarkers of the disorder within reach. As permanent biomarkers, genes could indicate the diagnostic probability that an individual has or will have depression (Gururajan et al., 2016). The so sparked fascination led to a rapid increase in the number of publications with respect to genetic association studies. These studies mainly used two different approaches: genome wide association studies (GWAS) and candidate gene studies. GWAS studies examine the associations of a large number of polymorphisms with a phenotype of interest, i.e., depressive symptoms or a diagnosis of MDD. This approach reveals correlative associations and is exploratory in nature. The investigation of candidate genes is theory driven or subsequent to GWAS studies but investigates only a small number of genes and polymorphisms. Logical candidate genes for depression are genes repeatedly found in GWAS studies or genes theoretically associated with vulnerability and resilience to depression (Montag et al., 2020). For instance, the glucocorticoid receptor gene (NR3C1), the serotonin transporter gene (SLC6A4) or genes of the oxytocin system are valid candidates according to the
aforementioned findings on neurobiological correlates. However, GWAS as well as candidate gene studies had up to now limited success in identifying reliable biomarkers (Milaneschi et al., 2016). Several reasons for difficulties in the identification and replication of depression associated genetic risk loci have been identified and discussed. For instance, depression is a polygenic disorder with single genes only explaining small amounts of variance (Montag et al., 2020; Mullins & Lewis, 2017). In addition, there is an ongoing debate as to whether depression is one syndrome or whether there are a number of subtypes of depression (Milaneschi et al., 2016). Striking evidence for depression being heterogeneous comes from twin studies indicating that 45 % of genetic liability to depression is not shared between sexes (Kendler et al., 2001, 2006a; Weissman et al., 1996). In addition, recurrent depressive episodes or early onset depression yielded higher heritability estimations (Power et al., 2012; Sullivan et al., 2000). Biological, genetic and psychological factors associated with the development of MDD are described below.

1.3. From biological to psychological to environmental factors implicated in MDD development
The identification of solely intraindividual factors, e.g., genes, implicated in MDD development was not fruitful with respect to the development of a theory able to explain the variety of symptoms that are grouped under the diagnosis of MDD. Since there is an ongoing debate about sex differences in the development and course of MDD as well as about sex specific effects of antidepressants, the investigation of sex differences is fundamental to promote our understanding of MDD (LeGates et al., 2019). Therefore, after a description of one possible source of sex differences in depression, two neurobiological systems, which are assumed to play an important role in the development of the disorder, are described. A candidate gene of the serotonin system was investigated since it has a longstanding tradition in research regarding depression development (Dell’Osso et al., 2016). The oxytocin gene was investigated since oxytocin has been shown to modulate the bodily stress response and is considered having alleviating properties regarding separation distress (Amico et al., 2004; Panksepp, 2004). Thereafter, primary emotions as the basis of personality and a possible endophenotype of depression are explained. Last, the role of stressful experiences and their association with MDD development are outlined.

1.3.1. Sex, MDD and the 2D:4D ratio
The stable sex difference in depression incidence and sex specific courses of the disorder encouraged the investigation of estradiol and testosterone with respect to the development of depressive disorders. An involvement of at least estradiol is especially plausible against the background of increasing prevalence of depression correlating with hormonal changes in women during puberty, prior to menstruation, following pregnancy and at perimenopause.
Introduction

Albert, 2015). However, testosterone is considered to be potentially effective in the treatment of MDD in men (Walther et al., 2019). Humans are prenatally exposed to estrogen and testosterone and these hormones have been shown to affect fear reactivity (Bergman et al., 2010). In addition, prenatal exposure to these sexual hormones has been reported to influence the stress system and stress responsivity later in life in animal studies (Wilson et al., 2020). Therefore, it has been suggested that the observed sex differences in the prevalence of MDD might be associated with the exposure of the embryo to testosterone relative to estrogen during development (Smedley et al., 2014). Androgen-to-estrogen signaling during digit development in an animal model has been found to be associated with the ratio of the index finger (2D) relative to the ring finger (4D). Inactivation of the androgen receptor decreased growth of 4D resulting in a higher 2D:4D ratio. In contrast, inactivation of estrogen receptor α increased growth of 4D, which led to a lower 2D:4D ratio (Zheng & Cohn, 2011). Studies investigating the associations of the 2D:4D ratio and neuroticism as well as depression report heterogeneous and inconclusive results (Austin et al., 2002; Bailey & Hurd, 2005; Martin et al., 1999; Smedley et al., 2014). Furthermore, the relation between 2D:4D ratio and depression has only been assessed in non-clinical samples, rendering a further investigation of the associations between 2D:4D ratio and depression necessary. The investigation of sex differences beyond prenatal exposure to gonadal hormones on the other hand is of utmost importance for understanding the biological basis of depression and for the development of new antidepressant treatments. The importance of examining sex differences is further substantiated by estrogen potentially influencing serotonin synthesis and serotonin receptor binding (LeGates et al., 2019; Staley et al., 2006). Accordingly, there are reports of sex differences in the clinical presentation and course of MDD (Eid et al., 2019). For instance, sex differences in selective serotonin reuptake inhibitors (SSRIs) with women responding better to SSRI treatment than men have been reported (LeGates et al., 2019).

1.3.2. Serotonin and MDD

Early theories of depression development postulated depression to result from a deficiency in the transmission within the monoamine systems, i.e., the noradrenaline and the serotonin (5-HT) system (Heninger et al., 1996). The 5-HT system is large and complex and 5-HT is involved in a plethora of psychological and behavioral processes, e.g., mood, cognition, sleep, appetite, activity and sexual behaviors. However, it is essential for none of them (Jacobs & Azmitia, 1992). Several alterations in the 5-HT system were found comparing MDD patients to healthy controls pointing towards diminished functioning of the 5-HT system in MDD patients (for a detailed review, see Jans et al., 2007). However, there is no conclusive evidence supporting a causal role of serotonin in the development of MDD (Lacasse & Leo, 2005). For instance, experiments with the aim of inducing depression by depleting serotonin levels reaped no consistent results (Heninger et al., 1996). Likewise, huge increases in brain
serotonin, induced by administering high-dose L-tryptophan, a precursor amino acid of 5-HT, were ineffective at relieving depression (Mendels et al., 1975). Still, 5-HT seems to play a role in the pathophysiology of depression as studies of tryptophan depletion showed. In tryptophan depletion studies, an acute dietary manipulation is employed that diminishes the availability of tryptophan, which in turn lowers brain serotonin activity. In healthy controls, there are no clinically significant changes in mood after tryptophan depletion. Recovered MDD patients free of medication, however, show a brief, clinically relevant, depressive symptomatology (Smith et al., 1997). In sum, diminished 5-HTergic function is involved in the pathophysiology of depression but may not have a causal role in depression development. Similarly, not all patients suffering from MDD present with 5-HT abnormalities. Likewise, not all patients benefit from drugs inducing an upregulation of 5-HT neurotransmission (Jans et al., 2007). A dimensional approach has been suggested which fits better with the picture of findings pointing towards an imbalance of many transmitter systems, nerve growth factors, and neuropeptides, characterizing depression as a systemic disease that can be triggered by different factors (Dell’Osso et al., 2016) which may comprise 5-HT abnormalities in some individuals (Jans et al., 2007). Thus, diminished functioning of the 5-HT system constitutes a risk factor for mood regulation disorders (Jans et al., 2007). Accordingly, the serotonin system is supposed to interact for example with environmental factors like stressful life events (SLEs) or the steroid hormones regulating HPA activity (Gotlib et al., 2008; Hammen et al., 2010; Lanfumey et al., 2008). For instance, a sustained increase in glucocorticoid secretion in response to stress can affect serotonergic neurotransmission (Lanfumey et al., 1999, 2008). This interaction is substantiated by the effects of corticosteroids on the activity and expression of several types of serotonin receptors (Lanfumey et al., 2008). Accordingly, chronically increased glucocorticoid production induced by stress was shown to desensitize 5-HT autoreceptors in the mice brain associated with a downregulation of serotonergic neurotransmission (Lanfumey et al., 1999).

The dimensional approach of 5-HTergic neurotransmission in depression is in line with results from candidate gene studies examining the 5-HT transporter (5-HTT) gene (SLC6A4) on chromosome 17 (Lesch et al., 1994). The 5-HTT is an integral membrane protein that transports 5-HT from the synaptic space into presynaptic neurons, thus playing a critical role in the termination of 5-HTergic neurotransmission (Kanner & Schuldiner, 1987). The 5-HTT-linked polymorphic region (5-HTTLPR) is composed of 16 repeat elements located approximately 1 kilobase upstream of the SLC6A4 gene transcription initiation side and consists of a 44 base pair insertion or deletion (Lesch et al., 1994). Thus, the 5-HTTLPR has a short (S) and a long (L) allele. The S allele has been associated with less transcription of the serotonin transporter compared with the L allele (Lesch et al., 1996). Initial enthusiasm for the hypothesis that the S allele of the 5-HTTLPR might be associated with depression risk
gradually veined in the face of inconsistent findings (Lasky-Su et al., 2005; Levinson, 2006). Inconsistencies were argued to be a result of an interaction of SLEs and the 5-HTTLPR (Caspi et al., 2003). A recent meta-analysis, however, did neither find a significant effect of the 5-HTTLPR nor of interactions of the 5-HTTLPR and a number of covariates considering depression severity or diagnosis (Culverhouse et al., 2018). Furthermore, previous meta-analyses yielded inconsistent results (Karg et al., 2011; Risch et al., 2009).

Single genes explain only a small amount of variance in a complex disorder like MDD. There are various interactions between the genes of the various systems implicated in depression development. As such, there is also evidence for an interaction between the 5-HT and the oxytocin system (McQuaid et al., 2014). Accordingly, an interaction between the 5-HTTLPR and the gene coding for the oxytocin receptor (OXTR) in the prediction of negative emotionality has been documented (Montag et al., 2011). In addition, repeated treatment with citalopram produced increased plasma levels of oxytocin in rats and it was suggested that the antidepressant effect of SSRIs is partly due to their actions on oxytocin (de Jong et al., 2007; Uvnäs-Moberg et al., 1999). Furthermore, 5-HT fibers have an influence on brain regions rich in oxytocin (Emiliano et al., 2007; Sawchenko et al., 1983) and peripheral markers of oxytocin have been postulated to interact with the 5-HTT (Marazziti et al., 2012).

1.3.3. Oxytocin and MDD

Central oxytocin is synthesized in the hypothalamus (Landgraf & Neumann, 2004) and is not only considered to be involved in local signal transmission from axon to dendrite via hard-wired neuronal connections but also diffuses to the extracellular fluid. This allows oxytocin to act broadly as a neuromodulator in the central nervous system (Landgraf & Neumann, 2004). Originally, oxytocin was known for its role in parturition (Russell et al., 2003) and lactation (for a review, see Richard, Moos, & Freund-Mercier, 1991). In addition, oxytocin soon became famous as the social hormone being implicated in trust (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005; for replication problems, see Nave, Camerer, & McCullough, 2015), empathy (Decety & Batson, 2009) and neural activation in brain areas associated with sociality (Jack et al., 2012). In accordance with the initially mentioned theory of depression development, oxytocin is a major modulator of separation distress (Panksepp, 2004) and has been shown to attenuate the stress response (Amico et al., 2004), presumably by modulation of cortisol levels. The pleotropic actions exerted by oxytocin led to an increase in studies investigating oxytocin in the context of depression development and treatment resulting in mounting evidence that the neuropeptide may have an important role in understanding mood disorders (Grinevich et al., 2016; Grinevich & Neumann, 2021; McQuaid et al., 2014).

Oxytocin is considered playing an important role in HPA stress reactivity especially for separation distress. For instance, oxytocin levels were higher and cortisol levels returned to baseline levels more swiftly subsequent to a social stressor in children who were able to see
or hear their mothers as compared to children in a no-contact condition \citep{Seltzer2010}. Further, oxytocin may exert an antidepressant-like effect in mice that were separated from their mothers mitigating separation-associated neuroinflammation \citep{Amini-Khoei2017}. Additionally, SLEs have been reported to exert long lasting effects on the oxytocin system \citep{Donadon2018, LondonoTobon2018}. For instance, women with a history of childhood abuse had reduced oxytocin concentrations in their cerebrospinal fluid \citep{Heim2009}. A similar association has been reported in men \citep{Opacka-Juffry2012}. It has even been suggested that oxytocin may have different effects on social behavior depending on the presence or absence of early life adversity \citep{Perry-Paldi2019}. Accordingly, oxytocin had a trust-lowering effect if childhood trauma was present \citep{Ebert2013}. Thus, lasting alterations in the oxytocin system might be entwined with HPA alterations due to early adversity. In line with this, oxytocin has been shown to modulate the stress response after acute stressors depending on the presence or absence of early adversity \citep{Grimm2014, Pierrehumbert2010}. This is in line with theories postulating that a combination of genes and early life experiences may program phenotypes with differential susceptibility to later-life challenges. If the programmed phenotype cannot cope with later-life SLEs, the individual is at risk of depression development \citep{Daskalakis2013}.

In light of the association between oxytocin and stress, it is hardly surprising that there are reports of dysregulated oxytocin pathways in depression \citep{Cyranowski2008, Thomas2020} and an ongoing debate about oxytocin being potentially effective in the treatment of depression \citep{Li2020}. However, there are also reports of oxytocin increasing stress-induced social avoidance pointing towards diverse, circuit-specific effects of oxytocin \citep{Duque-Wilckens2020}. Hence, even though a relationship between stress, oxytocin and depression is strongly supported by previous evidence, it is considered dynamic and context specific in nature and may depend on the environment in early developmental stages \citep{Brown2016, McQuaid2014}.

In addition to the interactions between oxytocin and the bodily stress system, oxytocin has been shown to affect other systems considered central for depression development, like monoamines and cytokines and there exist first indications for an interaction with neuronal growth factors \citep[for a review, see][]{McQuaid2014}. There is a considerable amount of evidence for the interaction of the oxytocin and the 5-HT system: Selective 5-HT agonists lead to an increase in the expression of oxytocin mRNA in the brain \citep{Jorgensen2003}. Moreover, the infusion of oxytocin exerted an anxiolytic effect via facilitation of 5-HT release within the median raphe nucleus \citep{Yoshida2009}.

The gene coding for both neurophysin I as well as oxytocin (OXT) is localized on human chromosome 20p13 \citep{Rao1992}. While the OXTR has received growing attention, investigations of other genes of the oxytocin system are sparse. This is a gap in knowledge,
especially against the backdrop of a potential gene by environment interaction modulating the risk for depression development that is currently discussed for two polymorphisms in the OXTR (Cataldo et al., 2018; Smearman et al., 2016). In fact, there are first indications for a role of OXT in psychopathology linking polymorphisms in OXT to lower emotional well-being in females (Love et al., 2012), social anxiety (Olofsdotter et al., 2018) and postpartum depression (Mileva-Seitz et al., 2013). Moreover, a diathesis-stress type of interaction between polymorphisms of OXT has been suggested (Olofsdotter et al., 2018).

However, candidate gene approaches in the investigation of MDD reaped inconsistent and non-replicable results for all candidate genes including OXT,OXTR and SLC6A4 (Border et al., 2019; Montag et al., 2020). Therefore, it has been suggested to investigate endophenotypes of complex disorders.

1.3.4. Primary emotions and MDD

Endophenotypes occupy an intermediate position on the pathway from genes to the full-blown disorder and are thus considered having a simpler genetic architecture (Goldstein & Klein, 2014). Neuroticism is a multifaceted personality dimension defined as the tendency to experience negative emotions (Costa & McCrae, 1992) and also an endophenotype of depression (Goldstein & Klein, 2014). Neuroticism is highly associated with depression as well as the onset of depressive episodes, has a huge trait component as well as a high genetic correlation with depression and co-segregates with MDD (Farmer et al., 2002; Goldstein & Klein, 2014; Kendler et al., 2006b). In addition personality is a predictor of the adaption to life challenges in all spheres of life (Ozer & Benet-Martínez, 2006) and neuroticism has been postulated to result from an interaction of genetic predispositions and experiences of stress or trauma much like depression (Barlow et al., 2014). Therefore, the emotional components of neuroticism are worth investigating in the context of diathesis-stress interaction in depression development.

Personality can be defined as stable differences between individuals considering cognitive, emotional and motivational aspects of mental states resulting in stable behavioral action tendencies (Montag & Panksepp, 2017). The personality dimension neuroticism is defined as stable tendency to respond with negative emotions to frustration, stress or loss (Lahey, 2009) and is strongly associated with MDD (Goldstein & Klein, 2014). Since depression is an affective disorder, the emotional aspect of Neuroticism is of utmost importance when examining the association of Neuroticism and depression and could promote a neuroscientific and evolution-based understanding of the associations between personality and MDD (Karterud et al., 2016; Montag & Panksepp, 2017). The theory of primary emotions is based on the notion that the human brain can be divided into at least three evolutionary layers (MacLean, 1990). Evolutionarily old brain areas, e.g., the periaqueductal gray, the basal ganglia, the amygdala or the hippocampus are responsible for emotional-affective and motivational aspects of
Introduction

personality. They have not only been preserved throughout the evolution but influence behavior in a bottom-up fashion. This notion is supported by recent studies showing primary emotions to moderate the association between the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism and executive functions as well as reaction times in a masked priming paradigm (Berger et al., 2021; Sanwald, Montag, et al., 2020). On the contrary, phylogenetically young brain areas like the neo-cortex can regulate emotional states top-down. Hence, human personality is a complex interaction of bottom-up emotional urges and top-down regulation abilities (Montag & Davis, 2018; Montag & Panksepp, 2017, 2020). Primary emotions are promising candidates in the research of depression development since they have a huge trait component and have their neural substrate in subcortical brain regions experimentally identified using deep brain stimulation studies and psychopharmacological challenge tests in animal models (Delgado et al., 1954; MacLean & Delgado, 1953; Montag & Panksepp, 2017; Olds & Milner, 1954; Panksepp, 1971, 2004). Until now, seven primary emotions have been identified: SEEKING, CARE, PLAY and LUST on the positive side and FEAR, SADNESS and ANGER as negative primary emotions. The SEEKING system is our drive to explore and investigate appetitive stimuli. It is essential for acquiring all the vital resources needed. The LUST and CARE systems are deeply entwined with the SEEKING circuitry. LUST promotes sexual desire and sexual engagements. CARE arouses the urge to nurture and protect the offspring, and may promote satisfying relationships among adult partners. The social PLAY system, also closely related to SEEKING urges, promotes abundant pro-social activities during development (Montag & Panksepp, 2017). A lack of these activities may lead to diminished social competence in adulthood (Pellegrini & Smith, 1998).

In order to escape life threatening and dangerous situations, the FEAR circuitry was preserved phylogenetically. ANGER urges us to defend our lives and resources. The ANGER circuitry is also active in situations of frustration, when access to expected reward is barred. Finally, the SADNESS system activates in situations of separation distress such as the loss of a child or being apart from a loved one. It goes along with feelings of loneliness and sadness eventually precipitating depression (Montag & Panksepp, 2017).

A theory of depression development basing on primary emotions has been described in short in the introduction. This theory suggests a stressor to cause an activation of the SADNESS circuitry and initially elevated efforts to terminate the stress response as well as to soothe separation distress. When termination fails, a behavioral shutdown is triggered protecting the individual against the potentially fatal consequences of wasting too much energy. However, the shutdown may also result in lassitude and despair (Panksepp & Watt, 2011). This mechanism does make sense from an evolutionary perspective since in young animals a protracted panic phase would increase chances for falling prey to predators or metabolic exhaustion due to prolonged distress (Watt & Panksepp, 2009). Accordingly, depression
should be strongly associated with two primary emotions: SEEKING and SADNESS (Watt & Panksepp, 2009). As a recent study showed, low SEEKING and high SADNESS are indeed associated with depression severity and patients suffering from depression showed lower SEEKING and higher SADNESS (but also lower PLAY and higher FEAR) than did healthy controls (Montag et al., 2017). In addition, the personality dimension Neuroticism was associated with FEAR, ANGER and SADNESS (Davis & Panksepp, 2011; Montag & Davis, 2018). These findings demonstrate that primary emotions might provide a more specific way of investigating the emotional basis of depression since neuroticism by definition comprises the whole range of negative emotions.

From an evolutionary perspective, the shutdown mechanism should be self-terminating which is reflected by depression having a phasic course in 85% of cases (Eaton et al., 2008). However, the shutdown mechanism may not be without flaws and become disinhibited, i.e., recruited pathologically even in the context of minimal stressors (Watt & Panksepp, 2009). It would also explain why depression is such a common mental disease: there may not be categorical differences between patients suffering from depression and healthy controls. Instead, depression severity might constitute a spectrum from functional forms of SADNESS increase and SEEKING reduction to disinhibited and pathologically prolonged states of depressed mood, anhedonia and loss of interest. The view of chronically prolonged separation distress also explains the variety of symptom combinations all subsumed under the diagnosis of depression: Episodes of separation distress early in life could later be recruited to cope with various adaptive challenges when sustained efforts to pursue difficult goals become dangerous to the individual. Thus, different combinations of symptoms constituting MDD may result from different responses depending on the nature of an individual’s adaptive challenge when there is a vulnerability formed by genetic predispositions and early life adversity (Nesse & Ellsworth, 2009; Watt & Panksepp, 2009). In short, the hypothesis of chronically prolonged separation distress and a subsequent emotional shutdown is in line with HPA axis dysfunction, which is currently considered the essential mechanism predisposing for depression (Heim & Binder, 2012; Heim & Nemeroff, 2001; Pariante & Lightman, 2008). In addition, this hypothesis provides a heuristic framework to coordinate findings of the various dysregulations associated with depression (Watt & Panksepp, 2009). Since HPA hyper(re-)activity is considered having the central role in depression development, the next section gives a brief overview covering current findings.

1.3.5. Stressful life events and MDD
A finding highly consistent across many studies is SLEs being a potent risk factor for MDD that often precipitates the onset of MDD episodes (Kendler et al., 1995, 1999). It has been shown that stressors, especially early in life, leave traces in the individual, sensitizing it for the depressive effects of subsequent stressors (Heim et al., 2008; Heim & Nemeroff, 2001). This
can be observed in the form of an altered stress response in stress-exposed individuals suffering from depressive symptoms (Burke et al., 2005; McLaughlin et al., 2015). The autonomic sympathetic stress response acts via the secretion of epinephrine by the adrenal glands, which activates a cascade of events known as HPA axis. First, the paraventricular nucleus releases the corticotropin-releasing factor (CRF) which stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) by the anterior pituitary. ACTH stimulates the synthesis and release of cortisol by the adrenal cortex. This in turn causes downregulation of CRF release and thus helps maintaining homeostasis in response to stress (Juruena, 2014).

Growing evidence supports the idea of impaired glucocorticoid (GC)-mediated feedback inhibition associated with reduced glucocorticoid receptor (GR) function and increased activity of the hypothalamic-pituitary-adrenal (HPA) axis in depressed patients (Heim et al., 2008; Pariante & Miller, 2001).

Accordingly, subsequent studies showed an impaired stress suppression after a stress induced GC release due to impaired GR binding and reduced expression of central GRs to be associated with early life stressors and depressive symptoms (Heim et al., 2000; Heim & Binder, 2012; Pariante & Lightman, 2008; Weaver et al., 2004). However, not all depressive episodes are associated with SLEs (accessible to active retrieval) and not all individuals experiencing SLEs develop a depressive episode (Heim et al., 2008; Kendler et al., 1999). This issue might in part be explained by different types of SLEs and other interacting variables: Recent meta-analyses showed that especially emotional abuse during childhood is an important risk factor for depression development in child or youth as well as in adult samples (Lemoult et al., 2020; Nelson et al., 2017). The authors argue that early life stress affecting the caregiving environment in childhood may pose the highest risk for depression development, while other stressors are less specific in terms of their consequences considering subsequent psychopathology (Lemoult et al., 2020). This explanation is in line with the above mentioned theory building upon separation distress (Watt & Panksepp, 2009) as well as with findings indicating that the influence of SLEs is moderated by attachment style in children (Suzuki & Tomoda, 2015). Attachment style itself is an important predictor for depression risk in children and in adults: while a secure attachment style is protective, an insecure (preoccupied) attachment is associated with greater risk for depression development (Dagan et al., 2018; Spruit et al., 2020). Further findings suggesting the hypothesis of a relationship between depression and experiences of separation/loss of important attachments come from a large epidemiological survey and show that loss and humiliation (unlike other stressors) were strongly linked to risk for depressive episodes (Kendler et al., 2003). In addition, there might be timing effects of SLEs with sensitive periods probably associated with the relative plasticity of different brain areas (for a review, see Heim & Binder, 2012). For an integration of these findings, a three-hit concept of vulnerability and resilience to depression has been suggested.
The authors postulate that the individual genome (hit-1) interacts with early-life (also prenatal) environmental factors (hit-2) programming a phenotype with specific susceptibilities to later-life challenges (hit-3) (Daskalakis et al., 2013). In summary, it is important to consider early as well as acute stressors and stress experiences across the lifespan, different types of stressors and individual characteristics, i.e. personality but also biological factors like sex, genes and epigenetics. After all, there seems to be an interaction of personality, sex, an individual’s genetic liability and the environment it finds itself in (or chooses) (Kendler et al., 2004, 2010). However, in order to create a conclusive link between a stressor that may have occurred a long time ago and persistent changes in the bodily stress system, a mechanism is needed that allows an individual with its (aside from mutations) hard-wired genome to adapt to different environmental conditions. The following section describes this link, which is now commonly known as epigenetics.

1.4. Epigenetics – bridging the gap

1.4.1. Definition and epigenetic mechanisms

The human body comprises a plethora of different cell types each with its specific functions. Each cell type has a different set of actively transcribed genes and therefore produces an individual set of proteins that is necessary to fulfill the cell type's role in the complex system of a multicellular organism. Yet nearly all cell types share the same genome. Therefore, other regulatory mechanisms in addition to the evolutionary processes of randomly arising genetic variants must exist (O'Donnell & Meaney, 2020).

The ‘recipe’ for all functions of all multicellular organisms is encoded by the four nitrogenous bases of the DNA. The four bases are adenine (A), cytosine (C), guanine (G) and thymine (T). The DNA is a two-stranded molecule that consists of nucleotides. A nucleotide is defined as the combination of a nitrogenous base, the sugar deoxyribose and a phosphate group. Various combinations of nucleotides store genetic information and a tremendous amount of information is needed as blueprint for complex organisms (about 6 billion base pairs of DNA for humans). Therefore, the genetic information needs to be tightly packed to fit into the nucleus. In humans, the DNA together with histones forms chromosomes, which serve as vehicles of the highly condensed genetic information. However, the packaging of DNA is not static but changes in response to cellular processes. For instance, DNA needs to be less tightly packed and the two nucleotide strands must unwind to expose the base sequence in order to be transferred to ribonucleic acid (RNA). RNA in turn is translated into an amino acid sequence that ultimately specifies the structure of a protein. Since the base sequence is the same for nearly all different cell types of an organism (aside from spontaneous mutations), the second regulatory layer stipulates which genes are transcribed or translated into proteins by changing for instance, the accessibility of genes for the transcription apparatus. This control over the operation of the genome without changes in the DNA sequence itself is referred to as epigenetics. Thus,
epigenetics can be defined as software that directs the activity of the genetic hardware (O’Donnell & Meaney, 2020). Epigenetic mechanisms allow for (in part reversible) adaption processes on an individual level and can be modified by environmental conditions throughout life thereby bridging the gap between nature and nurture, genotype and phenotype (Goldberg et al., 2007; O’Donnell & Meaney, 2020; Weaver et al., 2004; Zannas & Chrousos, 2017). Epigenetic mechanisms comprise histone modifications (Maze et al., 2015; Sun et al., 2013), polycomb complexes (Schuettengruber et al., 2017), noncoding RNAs (O’Donnell & Meaney, 2020) and DNA methylation (Bird, 2002). DNA-methylation is probably the most investigated epigenetic mechanism and denotes the addition of a methyl (CH$_3$) group to the 5’-position of cytosine (O’Donnell & Meaney, 2020). These methylated cytosine residues are most commonly found in cytosine-guanine dinucleotides (CpG sites; p represents phosphate) (Razin & Riggs, 1980). While DNA methylation in a gene’s regulatory regions is usually considered to repress gene transcription through multiple pathways (Klose & Bird, 2006), DNA methylation and transcription within the gene body is more complex and not yet fully understood (Shenker & Flanagan, 2012). However, methylated CpGs may attract methyl-CpG-binding proteins that recruit repressor complexes, which may ultimately result in histone modifications. These methylation induced histone-DNA interactions lead to a more condensed chromatin structure and thus diminished accessibility of the gene for transcription factors (Klose & Bird, 2006; Lim & Maher, 2010; O’Donnell & Meaney, 2020).

1.4.2. Methylation of SLC6A4 and OXT in stress and depression research
Methylation of the SLC6A4 promoter coding for the serotonin transporter has been associated with reduced transcription rate of SLC6A4 (D. Wang et al., 2012), messenger RNA (mRNA) concentrations (Philibert et al., 2007), SLEs, well-being and family history of depression (Kang et al., 2013) as well as post-stroke depression (Kim et al., 2013). A recent review summarized that depression has mostly been associated with hypermethylation of SLC6A4 (Li et al., 2019). There was, however, some heterogeneity considering the associations between SLC6A4 methylation and depression (Olsson et al., 2010; Zhao et al., 2013). This heterogeneity was suggested to result from the 5-HTTLPR moderating the association between stress and SLC6A4 methylation (Duman & Canli, 2015). This is in line with studies pointing towards gene by environment interactions explaining about 75% of the interindividual variation in variably methylated regions better than genes or environmental factors alone (Czamara et al., 2019; Teh et al., 2014).

In case of the oxytocin system, there are fewer studies investigating the associations between stress, methylation status of genes of the oxytocin system and depression. In fact, prior to the present work, methylation of the gene coding for oxytocin, OXT, has never been studied in the context of depression. Therefore, after a brief recapitulation of studies investigating the associations of stress, methylation of the gene coding for the oxytocin receptor, OXTR, and
depression, the – to the best of my knowledge – only two studies investigating OXT methylation are summarized. Studies on OXTR methylation present ambiguous results: One study reports a positive association between adult adversity (but not childhood adversity) and OXTR methylation but no direct association between OXTR methylation and depression. Instead, OXTR methylation mediated the association between SLEs and negative cognitive schemas (Simons et al., 2017). Another study reported no significant associations between OXTR methylation and child abuse as well as psychiatric symptoms (Smearman et al., 2016). Reiner and colleagues (2015) found a significantly lower OXTR methylation in women suffering from depression as compared to non-depressed controls and an interaction with a single nucleotide polymorphism (OXTR rs53576). Differences between women suffering from depression and non-depressed women were more pronounced in GG carriers than in women carrying an A allele (Reiner et al., 2015). Bell and colleagues (2015) on the other hand reported women homozygous for the G allele of the same polymorphism to have an increasing risk for developing postpartum depression with increasing OXTR methylation. With respect to the heterogeneity of findings, reviews on candidate gene methylation and depression conclude that there is no conclusive association between stress, OXTR methylation and depression. Therefore, further research is needed (Chen et al., 2017; Li et al., 2019; Park et al., 2019). The only two studies investigating the methylation of OXT in humans reported that OXT methylation was negatively associated with a secure attachment style, the ability to recognize emotional facial expressions, as well as greater superior temporal sulcus activity (Haas et al., 2016). In the second study, OXT methylation decreased from early to mid-pregnancy with no further change until late pregnancy. Further, intrusive as compared to nonintrusive mothers had 6% higher methylation of one CpG site in the promoter region of OXT in late pregnancy (Toepfer et al., 2019).

Adding to the complexity of the genetic and epigenetic basis of depression, DNA methylation may also be sex specific. In line with this, SLC6A4 has been reported to be differentially methylated as a function of sex with females exhibiting higher SLC6A4 methylation as compared to men (Palma-Gudiel et al., 2019). While sex differences in OXT methylation are not sufficiently investigated, sexually dimorphic methylation of OXTR has been reported (Gouin et al., 2017).

1.5. A comprehensive model of depression development
In summary, MDD is a psychiatric disorder characterized by possible dysregulations in all of the brain’s functional domains: cognition, emotion and homeostasis (American Psychiatric Association, 2018; Watt & Panksepp, 2009). Homeostasis refers to the principle of a finely regulated and balanced overall state of the body. For this purpose, there are feedback loops, which supply central nervous regulation areas with information regarding the actual state of the periphery (Schandry, 2011). Under certain circumstances, these areas have to exert a
regulating influence to match the actual state of peripheral systems to their target condition. Since depression associated dysregulations can be found in all functional domains, abnormalities in a variety of neurobiological systems have been documented in association with a diagnosis of MDD – from serotonin to oxytocin (Montag & Reuter, 2014). In accordance, it becomes apparent that the genetic architecture of depression is far more complex than initially hoped with single genes having a very small effect (Border et al., 2019; Montag et al., 2020). The endophenotype approach has been suggested to overcome some of the issues in the investigation of the genetic basis of depression (Goldstein & Klein, 2014). Primary emotions promote a neuroscientific and evolution-based understanding of the associations between personality and MDD and may therefore be better suited for depression research than broader personality dimensions (Karterud et al., 2016; Montag & Panksepp, 2017). Further, 45 % of genetic liability to depression is not shared between sexes (Kendler et al., 2001, 2006a; Weissman et al., 1996), which is why sex differences should necessarily be investigated. Even prenatal exposure of the embryo to testosterone relative to estrogen might be associated with depression development (Smedley et al., 2014). Additionally, environmental influences especially SLEs along with their interactions with an individual’s genotype have to be taken into account (Kendler et al., 2010; Montag et al., 2020). Epigenetic regulation, e.g., in form of DNA-methylation, bridges the gap between genes and the environment providing an explanation for an individual’s adaptability to its environmental conditions (Goldberg et al., 2007).

Building upon the theory of depression development postulated by Watt and Panksepp (2009) and the three-hit concept of vulnerability and resilience (Daskalakis et al., 2013) I propose a model for depression development that combines environmental, psychological and biological factors (Figure 1). In this model, the individual genome in combination with early environmental influences and epigenetic programming of gene transcription result in interindividually different primary emotional systems. Moreover, some environmental influences the developing embryo is exposed to like the prenatal exposure to testosterone relative to estrogen might not only influence primary emotional systems via epigenetic pathways but could also be associated with stable and measurable physical changes, e.g., of the 2D:4D ratio. As such, the 2D:4D ratio could be a first indicator of the risk for depression development. In the model, the developing individual with his or her individual phenotype and profile of primary emotions is subsequently exposed to a potentially stressful environment in early stages of development. Experiences of separation in early developmental stages lead to an upregulation of the SADNESS and subsequently the SEEKING system with their biological correlate in a downregulated oxytocin system and an increased activity of the HPA-axis both affecting the serotonin system (and other systems not investigated in this work) leading to a downregulation of 5-HTergic neurotransmission. If the individual fails at eliminating the environmental stressor
(e.g., not finding the mother), adaptive changes emerge in the individual. Consequently, SEEKING is reduced while SADNESS remains high. On a biological level, epigenetic alterations result in adaptive changes to survive adverse environmental conditions, resulting in lasting alterations in systems relevant for the stress response. If later environmental conditions mismatch the phenotype thus created, the described upregulation of SADNESS and downregulation of SEEKING encoded epigenetically occurs again. This response pattern, however, might not be adaptive anymore. It may lead to social withdrawal, anhedonia and depressed mood. This might be a stressful experience on its own shaped by the previously established primary emotional response to the SLE (an upregulation of SADNESS and downregulation of SEEKING). Once again, epigenetic processes unfold leading to alterations in the stress system. This vicious circle might ultimately culminate in depression development.

Figure 1. A comprehensive model of depression development. There are three developmental stages. Further, there are three levels comprising biological, psychological and environmental factors at which the predispositions for the risk of developing depression come to a head as the developmental stages progress. Each phase affects the subsequent phase and determines the psychology and the biology of an individual. An individual’s psychological and biological constitution interacts with environmental challenges. This leads to a match or mismatch of individual and environmental characteristics. Mismatches cause stress and activate the stress system. Separation distress (high SADNESS) might be especially relevant for depression development. After an initial protest phase (high SEEKING), epigenetic adaption processes take place programming the stress system (downregulation of SEEKING) and prepare the individual for future experiences of separation distress. The emerging phenotype is confronted with subsequent SLEs. The formerly adaptive upregulation of SADNESS and downregulation of SEEKING encoded in the individual’s epigenetic profile is recruited. This response pattern, however, might not be adaptive anymore. It may lead to social withdrawal, anhedonia and depressed mood, which may ultimately culminate in depression development. Bold type indicates the factors investigated in this work.
1.6. Aims and outline of the present work
The current work in accordance with the above model aims to provide evidence regarding the correlates of depression on an environmental, psychological and biological level. In detail, this dissertation aims at filling important gaps in knowledge and collect evidence for the comprehensive model of depression development: (i) The comprehensive model of depression development suggests prenatal exposure to androgens to affect depression development later in life. Since the 2D:4D ratio is a potential marker for prenatal androgen exposure and has not been examined in a sample of patients suffering from depression, the present work aims at investigating whether the 2D:4D ratio or its interactions with sex are associated with depression diagnosis or severity. An association of the 2D:4D ratio and depression would provide evidence for a presumed physical marker of prenatal testosterone to estrogen exposure representing an initial indicator of the risk for depression development. (ii) In line with the above model, the oxytocin system should be downregulated in depressed individuals, which is presumably indicated by an increased methylation of the OXT promoter. Previous studies focused on OXTR methylation in the investigation of the epigenetic regulation of the oxytocin system. This dissertation aims to provide first evidence that epigenetic alterations of OXT are associated with SLEs, depression severity and MDD diagnosis. (iii) With regard to the comprehensive model of depression development, SLEs should be associated with high SADNESS and low SEEKING in individuals suffering from depression. In addition, SLEs, SADNESS and SEEKING should be significant predictors of depression onset. In line with these assumptions, previous work found a positive association between SADNESS and depression and a negative association between SEEKING and depression (Montag et al., 2017). The present work aims at extending the findings of Montag and colleagues (2017) by examining the interrelations of primary emotions, SLEs and depression onset. (iv) In accordance with the presented model of depression development, SLEs as well as SADNESS should be positively associated with SLC6A4 methylation. SEEKING on the other hand should be negatively associated with SLC6A4 methylation. There are, to the best of my knowledge, no studies investigating primary emotions in combination with epigenetic profiles in individuals suffering from depression. The current work aims at elucidating the interrelations of SLEs, primary emotions, depression and methylation profiles of SLC6A4. (v) Based on the above model, SLEs should be associated with decreased SEEKING and increased SADNESS. SLEs, decreased SEEKING and increased SADNESS should be vulnerability factors for increased fear of the pandemic and thus increased depressive symptoms. The prospective influence of SLEs and depression-associated alterations in primary emotional systems on the perception of stressful life events like the respiratory disease called COVID-19, which is a consequence of the infection with the novel coronavirus SARS-CoV-2, has not yet been investigated. The present dissertation longitudinally examines associations between SLEs, primary emotions and the current pandemic. Thereby, I aim to elucidate how previous SLEs together with the associated
alterations in primary emotions predict fear and depressive symptomatology in the face of subsequent stressors.

This dissertation consists of five studies. Each study tests the following aspects of my model:

(i) Study I uses a matched case-control design to investigate the 2D:4D ratio in inpatients suffering from depression and matched healthy controls. This study extends previous research considering the 2D:4D ratio by examining a sample inpatients diagnosed with MDD for the first time. In addition, not only linear associations between the 2D:4D ratio and depression severity but also group differences between inpatients and controls with respect to the 2D:4D ratio were investigated. This is important since depression severity fluctuates while the 2D:4D ratio does not, which necessarily limits the strength of an association between these variables.

(ii) In study II, associations between SLEs, methylation of OXT and depression severity are explored in a sample of inpatients suffering from depression. This is the first study examining OXT methylation in depression research. In addition, sex differences in methylation patterns and their associations with SLEs and depression severity are reported.

(iii) After testing linear relationships between OXT methylation, SLEs and depression severity, study III aims to shed light on group differences and potential group-by-sex interactions with respect to SLEs and OXT methylation using a matched case-control design.

(iv) In study IV associations between SLEs, SEEKING as well as SADNESS, depression onset and methylation status of SLC6A4 are explored in a sample of inpatients suffering from depression. Additionally, genetic influences of the 5-HTTLPR potentially affecting the variables of interest are taken into account. A hierarchical linear regression analysis aims at elucidating which combination of the investigated factors (sex, the 5-HTTLPR, SLEs, SEEKING/SADNESS or methylation status of SLC6A4) predict age at depression onset. This is the first study including a joint investigation of stress, epigenetic profiles and primary emotions.

(v) Finally, study V explores the prospective associations between SLEs, primary emotions and fear of COVID-19 in inpatients suffering from depression and in healthy controls matched for age and sex. This study thereby examines how SLEs, depression severity and (group differences in) primary emotional systems might affect the perception of the danger to society and the individual that the current pandemic represents.
2. Original research articles

2.1. Study I - Depression is associated with the absence of sex differences in the 2D:4D ratio of the right hand


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Depression Is Associated With the Absence of Sex Differences in the 2D:4D Ratio of the Right Hand

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The 2D:4D digit ratio reflects prenatal testosterone relative to estradiol exposure of a developing embryo. Higher levels of prenatal testosterone have been related to lower 2D:4D ratios. In addition, higher 2D:4D ratios have been associated with female gender, neuroticism, and depression severity. Therefore, the present study investigated whether 2D:4D ratios differ between inpatients with major depression and matched healthy controls and whether 2D:4D ratios correlate with depression severity. We examined 139 inpatients diagnosed with major depression according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria and 137 healthy controls regarding 2D:4D ratios of both hands and BDI-II scores. While we observed significant sex differences in the 2D:4D ratio of the right hand in the healthy control group (women on average showed a significantly higher 2D:4D ratio), no such differences were found in the group of depressed patients. The 2D:4D digit ratios did not correlate with depression severity even when examined for group and sex separately. We conclude that major depression is associated with an absence of sex differences in the 2D:4D ratio.

Keywords: prenatal testosterone, 2D:4D ratio, case-control, depression severity, major depression, sex, gender role

INTRODUCTION

Major depression is a burden for affected individuals, their families, and the society due to costs for treatment, hospitalization, and loss of productivity (1). Depressive disorders are characterized by prolonged or permanent feelings of sadness, lack of interest, hopelessness, and exhaustion (2). This mirrors also in lower SEEKING and higher FEAR/SADNESS, hence primary emotional systems according to affective neuroscience theory (3) [ANT; for a recent introduction into ANT, please see Ref. (4)]. Women have a two-fold higher risk to develop episodes of depression than do men and in addition experience more severe episodes than men (5). It has been suggested that the observed sex-related differences in the prevalence of psychiatric disorder might be associated with the exposure of the embryo to testosterone, the male sex hormone, during development (6). The ratio of the index finger (2D) relative to the ring finger (4D) has been found to be associated with androgen-to-estrogen signaling during digit development in an animal model. Digit 4 was affected more strongly than digit 2 by both androgen receptor and estrogen receptor a activity. Inactivation of the androgen receptor decreased growth of digit 4 resulting in a higher 2D:4D ratio. In contrast, inactivation of estrogen receptor a increased growth of digit 4, which led to a lower 2D:4D ratio (7).
To date, there are six studies investigating the associations between prenatal/perinatal androgen/estrogen concentrations and 2D:4D ratios in humans. Three studies found an association (8–10), while three studies did not find a significant association (11–13). Lower 2D:4D ratios have previously been shown to be associated with male sex (14). Sex differences in 2D:4D ratios have been found to be more pronounced in the right hand (14, 15). Exposure to higher levels of prenatal testosterone is associated with the development of stereotypically male personality traits (16) coinciding with findings showing an association of lower 2D:4D ratios and aggressive tendencies (17) as well as addictive tendencies towards gaming disorder (18, 19). A study by Montag and colleagues (20) highlighted that female persons who stutter suffer more from stuttering when they had more male hands. On the other hand, a higher, more feminine, 2D:4D ratio has been associated with schizotypal personality traits (21) and neuroticism in women (22, 23).

However, only a small number of studies with heterogeneous results examined whether a non-clinical population with 2D:4D digit length is associated with depressive symptoms (the latter also being linked to trait neuroticism) (24, 25). In a sample of 298 psychology students, Bailey and Hurd found that males with higher, more feminine 2D:4D ratios have higher depression scores. This association was only observed for the digit ratio of the right hand (26). Martin and coworkers (27) investigated the finger length ratio and depression severity in a sample of 52 men and 50 women. The authors observed a non-significant negative correlation between 4D digit length and Beck Depression Inventory (BDI) scores in men and suggested that high prenatal testosterone might predispose men to depression.

In a study with 128 undergraduate students, Smedley and coworkers (6) investigated whether the 2D:4D digit ratio is predictive for depression severity and found that a more female (higher) 2D:4D digit ratio is correlated with more depressive symptoms in females only. Austin et al. (28) did not find a sex difference in the 2D:4D digit ratios of 79 male and 86 female students. Furthermore, males and females did not differ in depression ratings, while 2D:4D ratios were not associated with state depression, neither in males nor in females.

The heterogeneity of previous results is not surprising in light of the fact that sample sizes as well as effect sizes are mostly small in 2D:4D literature. Furthermore, the relation between 2D:4D ratio and depression has only been assessed in non-clinical samples. Therefore, further investigation of the associations between 2D:4D ratio and depression is clearly needed. We investigated the associations between 2D:4D ratios and major depression using a case–control design. Thus, we assessed the relation of 2D:4D ratios of both hands to depression by comparing inpatients diagnosed with major depression according to DSM-IV criteria with 137 matched (for gender, handedness, and age as far as possible) healthy controls.

In a first step, we compared 2D:4D ratios between patients with depression and control participants as a function of sex. In a second step, we related 2D:4D ratios to severity of depressive symptoms in both groups.

Basing on previous results, 1) we hypothesized that women report higher depressive symptom severity than do men (5). 2) We assumed that the 2D:4D ratio is positively correlated with depression severity in both depressed inpatients and controls with stronger associations for women than men based on the results of Smedley and colleagues (6). 3) We also hypothesized that patients suffering from depression have a higher 2D:4D ratio than have healthy controls based on the results of Smedley et al. (6). 4) Taking into account that the vast majority of previous studies examining 2D:4D ratios reported sex differences, we also expected sex differences when examining 2D:4D ratios with women showing higher 2D:4D ratios than men. 5) Last, we expected the correlations and group differences to be more pronounced in the right hand in line with the previous literature outlined above. Furthermore, we explored whether there is an interaction between group (depressed inpatients vs healthy controls) and sex in the prediction of 2D:4D ratios.

METHODS

Participants

A total of 139 Caucasian patients with major depression, 90 women (64.7%) and 49 men, were recruited at the Clinic for Psychiatry and Psychotherapy III, University Hospital Ulm. All patients were diagnosed by resident psychiatrists according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (29) criteria based on a structured interview. Two patients were excluded from analyses with 2D:4D ratio because handscans were missing. All patients received stable antidepressant medication. Depression course (age of onset, number of depressive episodes, age of first inpatient treatment, number of inpatient treatments, and average duration of inpatient treatments) and dose equivalents of antidepressants (30) are presented in Table 1. Patients completed the BDI-II (31), a self-report questionnaire measuring depression severity by paper and pencil as well as an online in-house questionnaire containing a detailed assessment of medical history such as neurological and psychiatric disorders in addition to general medical status (number of participants in each of the BDI-II groups are presented in Table 2). Subthreshold depressive symptom severity in six patients may be explained by antidepressant treatment in the depression group.

| TABLE 1 | Descriptive statistics of depression history and dose equivalents for antidepressants for the depressive inpatients. |
|----------|----------------------|------------------|------------------|------------------|------------------|
|          | n        | Min | Max | Mean | SD    |
| Age of onset | 137     | 2   | 65  | 26.74 | 13.96 |
| Number of depressive episodes | 115     | 1   | 156 | 7.48  | 17.27 |
| Age of first inpatient treatment | 136     | 11  | 61  | 34.58 | 14.13 |
| Number of inpatient treatments | 136     | 1   | 80  | 2.96  | 6.97  |
| Average duration of inpatient treatments | 130     | 7.85 | 147.00 | 34.54 | 19.34 |
| Dose equivalents AD | 107     | 5.56 | 172.43 | 41.59 | 27.11 |

Age of onset in years; age of first inpatient treatment in years; average duration of inpatient treatment in days; dose equivalents AD: dose equivalent to 40 mg fluoxetine. Age of onset as well as number of depressive episodes was assessed according to patients’ self-report, with one patient reporting age 2 as age of onset and one patient reporting 156 depressive episodes. Since Hayasaka and colleagues (33) did not report dose equivalents for all available antidepressants, medication of n = 32 patients was excluded.
The six patients having subthreshold depressive symptom severity may be explained by antidepressant medication in the depression group.

For the control group, data sets of healthy Caucasian participants were drawn from the database of the Ulm Gene Brain Behavior Project (UGBBP). All healthy participants in the large group completed the BDI-II via online questionnaire without missing items as well as an online in-house questionnaire containing a detailed assessment of medical history such as neurological and psychiatric disorders in addition to general medical status. The control group comprised 137 healthy controls, 88 women (64.2%) and 49 men, without a history of psychiatric illness according to self-report and with a BDI-II score ≤13, which is below the cutoff for mild depression (31).

The Table 2 shows the different BDI-II groups. TABLE 3 displays the descriptive statistics of the examined variables for both groups separately. All participants signed an informed written consent. Procedures of the study have been approved by the ethical review board of Ulm University.

The two groups differed significantly in age, with the depressed participants being significantly older than the healthy control group \([F(1,272) = 53.17, p < .001]\). The reason for the difficulty of matching age between both groups was that mean age in the large group comprising the UGBBP was much lower than mean age in the group of depressed inpatients. Age correlated significantly negatively with depression severity in the whole sample \((r = -.24, p < .001)\) and in the group of depressive inpatients \((r = -.22, p = .01)\) but not in the group of matched controls \((r = -.08, p = .37)\). The 2D:4D ratio, however, is considered an age-independent trait (33, 34), which was substantiated by a control analysis resulting in non-significant correlation coefficients (Pearson, two-tailed) between age and 2D:4D ratio in the depression (left hand, \(r = .07, p = .44\); right hand, \(r = .07, p = .48\)) as well as in the control group (left hand, \(r = .09, p = .30\); right hand, \(r = .05, p = .54\)). Nonetheless, we used age as a covariate in all analyses performed.

### Questionnaires

The BDI-II (31) is a self-report questionnaire comprising 21 questions for the assessment of depression severity. The items are sadness, pessimism, feelings of failure, loss of pleasure, feelings of guilt, punishment feelings, self-dislike, self-criticalness, suicidal thoughts, crying, agitation, loss of interest, indecisiveness, worthlessness, loss of energy, change of sleeping habits, irritability, change of appetite, concentration difficulty, tiredness or fatigue, and loss of interest in sex. Cutoff scores differentiate between no depression (0–12), mild depression (13–19), moderate depression (20–29), and severe depression (30–63) (Table 2). Participants were instructed that the answers should reflect the previous 2 weeks.

### 2D:4D Ratio

A Canon Scanner (Canoscan LiDE110, Canon, Tokyo, Japan) with a resolution of 400 DPI was used for the scans of the right and left hands for the measurement of the length of ring fingers (4D) and index fingers (2D) of depressed patients and healthy participants. Two independent raters digitally measured the length from the middle of the basal crease to the tip of these fingers using GIMP2.8 Software (The GIMP Team, available online at www.gimp.org). The on-screen resolution when measuring the finger lengths of 2D and 4D was 100%. The absolute finger length was measured in pixels. 2D:4D ratios were calculated for the right and left hands, resulting in two ratios for each hand due to two raters. Intraclass correlations between the two raters in the depressed sample were \(\alpha = .95, 95\%\ CI [.92; .96]\) for the right hand and \(\alpha = .96, 95\%\ CI [.95; .97]\) for the left hand. In the healthy control group, intrarater correlations were \(\alpha = .95, 95\%\ CI [.92; .96]\) for the right hand and \(\alpha = .96, 95\%\ CI [.94; .97]\) for the left hand (all \(p\)-values <.001). As interrater reliability was high, mean ratios of the two raters were calculated for each hand, resulting in one 2D:4D ratio for each of the two hands of each patient and each healthy participant. Additionally, we analyzed

### Table 2

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<th>Group</th>
<th>Depression</th>
<th>Control</th>
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<tr>
<td>n</td>
<td>%</td>
<td>n</td>
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<tr>
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<tr>
<td>Moderate depression</td>
<td>34</td>
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<td>Severe depression</td>
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### Table 3

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<th>Max</th>
<th>Mean</th>
<th>SD</th>
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<td>-0.0461</td>
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<td>0.0041</td>
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Statistical Analyses

We conducted statistical analysis using R (35) and IBM SPSS Statistics (version 25, IBM, USA). Statistical significance was determined at $p < .05$ (two-sided test level). We first assessed whether there were significant sex differences in depression severity in depressed inpatients and in the healthy control group using a one-way ANCOVA, with gender as independent variable and age as covariate. Note that even though BDI-II scores were non-normally distributed according to the Shapiro–Wilk test in the group of matched controls ($Shapiro–Wilk = 0.94, df = 137, p < .001$), visual inspection of the boxplot and Q–Q plot did reveal neither the presence of outliers nor a severe deviation from normal distribution. Moreover, analyses of variance are not considered to be sensitive to moderate deviations from normality. Simulation studies showed that using a variety of non-normal distributions did not affect the rate of false-positive results (36–38). Furthermore, variances were homogenous across groups [studentized Breusch–Pagan test: inpatients, $BP(4) = 4.59, p = .33$; controls, $BP(4) = 1.38, p = .85$]. We therefore decided to use parametric tests. In order to assess whether depression severity was associated with the 2D:4D ratios, we calculated partial Pearson correlation coefficients between BDI-II score and 2D:4D ratios of both hands, with age as covariate. Note that since partial Spearman’s rank correlations provided similar results, we decided to report the results of the analyses with partial Pearson correlation coefficients. We analyzed the whole sample as well as the two experimental groups and sexes separately to see whether the association between BDI-II score and 2D:4D ratio differed between group and sex.

Moreover, we performed three $2 \times 2$ two-way ANCOVAs to assess the effects of group and sex on 2D:4D ratio. We conducted one ANCOVA each for the difference in 2D:4D ratio (L–R) and 2D:4D ratios of the right and left hands as dependent variables. Group and sex as well as their interaction term were entered as predictors. Age was used as covariate. The model therefore was as follows: 2D:4D ratio = constant + age + sex + group + sex*group. According to visual inspection of the boxplots, there were no outliers. There was homogeneity of error variances [Levene’s test: Difference (L – R), $F(3,224) = 0.46, p = .71$; left hand, $F(3,233) = 2.47, p = .06$; right hand, $F(3,237) = 2.54, p = .06$]. Significant interaction terms were additionally analyzed with Bonferroni post hoc tests in order to reveal mean differences between conditions.

We additionally performed a $2 \times 2 \times 2$ mixed ANCOVA to assess the effects of group, sex, and hand on 2D:4D ratio. 2D:4D ratio was the dependent variable. Group and sex as well as their interaction term were entered as between-subjects variables. Hand (left vs right) was entered as within-subjects variable. We also included age as covariate. According to visual inspection of the boxplots, there were no outliers. There was no homogeneity of error variances [Levene’s test: $F(3,224) = 2.84, p = .04$]. There also was no homogeneity of covariances, as assessed by Box’s test ($p = .03$). Thus, results of this analysis have to be interpreted with caution because the prerequisites of an ANCOVA were not met. Nevertheless, this analysis can be found in the Supplementary Material. Bonferroni post hoc tests can still be interpreted and match the results of the aforementioned two-way ANCOVAs.

RESULTS

Sex Differences in Depression Severity

The one-way ANCOVA for the healthy control group revealed no significant sex difference in BDI-II scores [male, $M = 4.47, SD = 3.52$; female, $M = 5.22, SD = 3.46$; $F(1,134) = 1.05, p = .31$].

In contrast, we found a significant sex difference regarding depression severity in the group of depressed inpatients [male, $M = 27.34, SD = 11.13$; female, $M = 33.25, SD = 11.03$; $F(1,130) = 7.76, p = .006$; $\eta^2_p = .061$]. Women reported more depressive symptoms than men.

Partial Correlations Between 2D:4D and Depression Severity

Contrary to our expectations, there was no significant association between BDI-II score and 2D:4D ratios, neither for the difference in 2D:4D ratio (L–R) nor for the absolute ratios of the left or right hands (Table 4). There were also no significant associations between BDI-II score and 2D:4D ratios when looking at groups, sexes, or the right-handed participants (the number of left-handed individuals only was $n = 22$) separately. Partial correlation coefficients ranged from $r = -.15$ to $r = .18$ (all $p$-values <.10, two-sided testing). Subgroup analyses of BDI-II groups (no depression, moderate depression, and severe depression; the group suffering from mild depressive symptom severity was omitted due to the small number of individuals comprising this group) did also not yield any significant associations (correlation coefficients ranged from $r = -.14$ to $r = .18$; all $p$-values >.10) (for scatterplots, see the Supplement). Inclusion of dose equivalents for antidepressants as
covariate in the group of depressed inpatients did not yield any significant correlations between BDI-II scores and 2D:4D ratio (results not shown).

**Group and Sex Differences in 2D:4D Ratio**

The two-way ANCOVA with the difference in 2D:4D ratio (L − R) as dependent variable revealed no significant main effects [group, \(F(1,223) = 2.24, p = .14, \text{n.s.} \); sex, \(F(1,223) = 0.03, p = .87, \text{n.s.} \)]. The interaction term of group and sex had also no significant effect on the difference in 2D:4D ratio, \(F(1,223) = 1.94, p = .17 \text{(n.s.)} \).

The two-way ANCOVA with the 2D:4D ratio of the left hand as dependent variable revealed no significant main effects [group, \(F(1,232) = 0.46, p = .50, \text{n.s.} \); sex, \(F(1,232) = 2.86, p = .09, \text{n.s.} \)]. The interaction term of group and sex had also no significant effect on 2D:4D ratio of the left hand, \(F(1,232) = 1.69, p = .20, \text{n.s.} \) (Figure 1).

The two-way ANCOVA with the 2D:4D ratio of the right hand as dependent variable revealed a significant main effect of sex \([F(1,236) = 4.28, p = .04, \eta^2 = .018 \)]. In line with our assumptions, women had significantly higher 2D:4D ratios for the right hand. The main effect of group was not significant \([F(1,236) = 0.10, p = .76] \). Moreover, there was a significant interaction between group and sex \([F(1,236) = 7.77, p = .01, \eta^2 = .032] \). This interaction is presented in Figure 2. Bonferroni post hoc tests showed that 2D:4D ratio for women and men differed significantly only in the control group \((M_{\text{male}} = 0.965, SE_{\text{male}} = 0.004; M_{\text{female}} = 0.985, SE_{\text{female}} = 0.003; \text{Difference} = -0.020, p < .001, \eta^2 = .052) \), whereas there was no significant sex difference in the depression group \((M_{\text{male}} = 0.978, SE_{\text{male}} = 0.005; M_{\text{female}} = 0.975, SD_{\text{female}} = 0.004; \text{Difference} = 0.003, p = .64, \text{n.s.} \) Within both sexes, 2D:4D ratio did not significantly differ between patients and controls, but there was a non-significant trend with males suffering from depression having a higher 2D:4D ratio than healthy men and females suffering from depression showing a lower 2D:4D ratio than healthy women \((\text{Difference}_{\text{male}} = 0.013; p = .06, \text{n.s.}; \text{Difference}_{\text{female}} = 0.010; p = .06, \text{n.s.}) \).

**DISCUSSION**

The aim of our study was to assess whether 2D:4D finger ratios, reflecting prenatal testosterone exposure (39), differ between inpatients diagnosed with depression and healthy participants. 1) We hypothesized that women report higher depressive symptom severity than men (5). 2) We assumed that the 2D:4D ratio is positively correlated with depression severity in both depressed inpatients and controls, with stronger associations for women than men based on the results of Smedley and colleagues (6). 3) We also hypothesized that patients suffering from depression have a higher 2D:4D ratio than healthy controls based on the results of Smedley et al. (6). 4) Taking into account that the vast majority of previous studies examining 2D:4D ratios reported sex differences, we also expected sex differences when examining 2D:4D ratios with women showing higher 2D:4D ratios than do men. 5) Last, we expected the correlations and group differences to be more pronounced in the right hand in line with the previous literature outlined above. Furthermore, we explored whether there is an interaction between group (depressed inpatients vs healthy controls) and sex in the prediction of 2D:4D ratios.

We did not find a significant sex difference in depression severity in the healthy control group, which is not surprising due to the limited variance in BDI-II scores. However, female depressed inpatients showed significantly higher BDI-II scores when compared with male depressed inpatients. The non-significant sex difference in depression severity in healthy controls...
is in line with the results of Bailey and Hurd (26) reporting no significant sex difference in trait depression in a sample of undergraduate students. Moreover, Thayer and colleagues (40) found no significant sex difference in depressive symptom severity when examining individuals with low BDI-II scores (BDI-II score ≤ 6). However, when examining the group of individuals with relatively high depression severity (BDI-II score > 6), females showed significantly higher depression scores than males. According to Thayer and colleagues (40), healthy women exhibit greater emotional information processing due to high attention to emotions (41) paired with good emotional clarity and sufficient emotional repair strategies. In contrast, for depressed women, the high emotional attention combined with impaired anti-rumination strategies might lead to an emotional downward spiral and therefore higher depression severity (40).

Contrary to our expectation and some earlier work (6), in the present study, the 2D:4D ratio was not generally higher in patients with depression compared with healthy controls. Hence, there were no signs of a generally lower prenatal testosterone exposure in patients with depression. Instead, we found a significant interaction of group and sex for the 2D:4D ratio of the right hand. Significant sex differences in 2D:4D ratios were observed in healthy controls as expected, whereas in the group of depressed inpatients, sex differences in 2D:4D ratios were absent. This, in part, contrasts with the results of Bailey and Hurd (26) reporting that depression in men is associated with a higher more feminine 2D:4D digit ratio for the right hand. In our study, we found no significant difference in 2D:4D ratio between men in the depression and control groups. There was, however, a non-significant trend: depressive men tended to have a higher, more feminine 2D:4D digit ratio than have healthy men. Furthermore, we observed a non-significant difference, being lower in patients compared with controls, in the 2D:4D ratio within the group of women. These observations in combination with the significant interaction between depression diagnosis and sex indicate that depression is associated with the absence of sex differences in 2D:4D ratio of the right hand, i.e., 2D:4D ratio of the right hand is not an indicator of sex in patients suffering from depression. Our findings might be the result of prenatal levels of testosterone to estradiol influencing personality: high prenatal levels of testosterone relative to prenatal levels of estradiol are associated with lower 2D:4D ratios (39). Lower 2D:4D ratios, in turn, are associated with personality traits considered typically male such as aggressiveness (17) and a more masculine gender role identity. Conversely, higher 2D:4D ratios are related to a higher self-reported femininity (42). Thus, the absence of sex differences in 2D:4D ratios as found in our group of patients with depression might be associated with gender-atypical personality traits not matching societal gender stereotypes leading to a higher gender role conflict. Gender role conflict in turn is considered a predictor of depression and anxiety (43, 44). On the other hand, there are doubts concerning the robustness of within-sex correlations between 2D:4D ratios and personality traits considered typically male/female (45). The study by Manning and colleagues (42), however, has a large sample size and shows a consistent negative association between the 2D:4D ratio and self-reported masculinity.

The interaction of sex and group regarding 2D:4D ratio was significant only for the right hand (females had a higher 2D:4D ratio than men in the healthy control group but not in the group of depressed inpatients). This is in line with previous research reporting that the sex difference in 2D:4D is more prominent in the right hand than in the left hand (14, 15). Moreover, the difference in 2D:4D ratio between the right and left hands has been shown to be higher in right-handed than in left-handed participants (46). Like
the vast majority of previous studies, our sample comprised mostly right-handed participants tending to have greater sex differences in 2D:4D ratios especially in the right hand. Therefore, the observed differences between the right and left hands in the analysis of the 2D:4D ratios as a function of sex and group might be caused by the right-handed participants. Since the number of left-handed participants was low in both the patient and control groups, a separate analysis of the left-handed subgroup was not possible.

Contrary to our expectations, correlation analyses between 2D:4D ratios of both hands and depression severity did not reveal any significant associations, neither in the entire sample nor in subsamples of patients and controls or women and men. In light of the lacking linear relationship between 2D:4D ratio and depression severity and our previous assumptions, 2D:4D might not be a linear predictor of depression severity. Instead, there might be a certain threshold of gender role conflict, which has to be exceeded in order to predispose an individual to the development of depressive symptomatology. We presumed that the absence of sex differences in 2D:4D ratios of the right hand is associated with sex atypical levels of prenatal testosterone (39) and results in sex atypical personality traits (17) and thereby in a higher gender role conflict (42–44). Gender role conflict has been shown to be associated with depression (43, 44).

It should also be noted that previous reports of an association between depression severity and 2D:4D digit ratio were heterogeneous (6, 26, 27). These heterogeneous findings might be a result of sample composition. In the present study, 139 inpatients who were diagnosed with major depression by psychiatrists according to DSM-IV criteria as well as 137 healthy controls were analyzed regarding associations of BDI-II scores and 2D:4D digit ratio. The participants in the earlier studies did not involve inpatients with a clinical diagnosis of depression. Thus, the samples of the presented study and those of earlier studies are not comparable. Whereas we investigated the two extremes regarding depression severity (very low depression severity in the control group and highly depressive inpatients in the depression group), previous studies most likely had a sample comprising mainly participants with no or mild depression as indicated, e.g., by the BDI-II means and standard deviations reported in the study of Smedley and colleagues (6): $M_{male} = 6.12, SD_{male} = 4.99; M_{female} = 9.31, SD_{female} = 7.12$. Furthermore, as depression severity is certainly a state variable changing over time whereas 2D:4D digit ratio is a stable trait variable, the association between both measures is necessarily limited.

Compared to previously published studies (6, 26, 27), the present investigation is based on a case-control design including depressed patients diagnosed according to DSM-IV criteria and healthy controls who had to pass an extensive screening procedure to exclude a possibly undiagnosed depression. The depressed patient group and the control group were matched for sex and handedness. Although the depressed patients and the healthy controls differed significantly in age, a correction regarding the age difference is negligible since the 2D:4D ratio is a stable age-independent trait (33, 34). But to avoid any confounding influences, we controlled for age in all our analyses.

Even though we examined inpatients diagnosed with depression and a sample of healthy controls, there are some limitations that need to be considered when interpreting the results of the current study. First, a study with monozygotic and dizygotic adult twins showed that genetic and environmental effects both affect the 2D:4D ratio (47). Due to the study design, the separation between genetics and environment was not possible in the current investigation. In addition, in the present study, all patients were treated with antidepressant medication and psychotherapy, thereby reducing depressive symptom severity. Antidepressant treatment or psychotherapy could have reduced the correlation between 2D:4D ratios and depressive symptoms. This limitation of our study, however, cannot be avoided, since depressive and potentially suicidal patients have to be treated adequately. Furthermore, there were procedural differences between depressive inpatients and healthy controls. While controls completed an online questionnaire, control cases completed a paper–pencil version. We used a paper–pencil version for inpatients to enhance commitment in this group burdened by difficulties in concentration and decision making. However, usage of different methods of measurement could have a confounding effect, even though there are studies suggesting invariance across different measurement methods at least for some instruments (48, 49). Moreover, handedness was assessed via self-report without using a validated instrument. There are, however, doubts that the usage of handedness scales yields reliable results (50). Last, we did not consider personality or gender role, variables potentially mediating the association between 2D:4D ratios and depression.

In conclusion, the present study is, to the best of our knowledge, the first to investigate 2D:4D ratios in inpatients with major depression using a case-control design. In patients with major depression, sex differences in the 2D:4D ratio of the right hand were absent, whereas in healthy controls, 2D:4D ratio was smaller in men than in women, as expected. 2D:4D ratios have previously been associated with masculinity/femininity traits (17, 42). We therefore speculate that the absence of sex differences in 2D:4D ratios of the right hand might result in an increased gender role conflict, because of the incongruence of one’s personality and the still prevailing but outdated societal definitions of masculinity and femininity. Gender role conflict might constitute a risk factor predisposing for depressive symptomatology. Future research should investigate 2D:4D ratios in a longitudinal design elucidating the predictive role of 2D:4D ratios for the development of personality traits and depression risk.

**ETHICS STATEMENT**

This study was carried out in accordance with the recommendations of the Declaration of Helsinki, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Ulm University.

**AUTHOR CONTRIBUTIONS**

MK and CM conceived the study. KW-M and SS collected the data of the patients. JW and CS collected the data of the control participants and measured the finger length of all participants.
SS and KW-M analyzed the data and wrote the first draft of the manuscript. All authors revised the manuscript and approved the final version.

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**REFERENCES**


**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00483/full#supplementary-material


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.2. Study II - Relation of promoter methylation of the oxytocin gene to stressful life events and depression severity


Relation of Promoter Methylation of the Oxytocin Gene to Stressful Life Events and Depression Severity

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Abstract
Oxytocin (OT) is a neuropeptide associated with trauma, sociality, and depression. Despite the widely accepted assumption of OT playing a role in the etiology of mood and anxiety disorders, associations between stressful life events, depression, and epigenetic regulation of the gene coding for OT (OXT) have not yet been investigated. We therefore aimed to examine the interrelations of stressful life events, depression severity, and methylation of the promoter region of OXT in a sample of \( N = 146 \) inpatients suffering from major depression. We found significant negative associations of stressful life events with mean methylation status as well as with methylation status of single CpG sites in the promoter region of OXT. There was no association between depression severity and OXT methylation. However, there were significant sex differences in methylation status of OXT with women showing higher methylation rates than men, putatively suggesting that in depression OXT is less activated in females compared to males. These results speak against an association of OXT methylation and depression severity, but support the assumption of a dysregulation of the OT system due to life stress. Our findings further emphasize the importance of including sex as an important factor in the investigation of the interrelations between OXT, stress, and depression.

Keywords Major depression · Oxytocin · Epigenetics · DNA methylation · Sex

Introduction
The neuropeptide oxytocin (OT) was originally known for its role in lactation (for a review, see Richard et al. 1991) and parturition (Russell et al. 2003). However, in humans, OT also acts as a neuromodulator in neural networks associated with trust (Kosfeld et al. 2005; for replication problems, see Nave et al. 2015), empathy (Decety and Batson 2009), and sociality (Jack et al. 2012). Therefore, it is a key element fostering the social bond between caretaker and infant as well as cohesion of families and social groups (Montag and Davis 2018; Waller et al. 2015). OT is synthesized as inactive precursor in the hypothalamus along with its carrier protein neurophysin I. The oxytocin-neurophysin I gene (OXT) is localized on human chromosome 20p13 (Rao et al. 1992).

Even though humans rely most strongly for their survival on social interactions and communication, they also have a history of within-species aggression, abuse, and warfare (Hrdy 2009). These are extreme forms of situations causing discomfort by shuttering an individual’s sense of security or invulnerability to harm. Such stressful life events (SLEs) are assumed to trigger long-lasting to persistent hyper(re-)activity of the hypothalamic–pituitary–adrenal (HPA) axis via epigenetic mechanisms (McGowan et al. 2009; Murgatroyd et al. 2009; Roth et al. 2009). Persistent HPA hyper(re-)activity, which in turn increases stress vulnerability, has been identified as an important mechanism for the development of depression (Heim and Binder 2012; Heim et al. 2000). Depending on the nature of the stressor (e.g., psychosocial stress or physical experience), OT is released in stress-sensitive brain areas, thereby modulating the stress response (de Jong et al. 2015; Pierrehumbert et al. 2010; Winter and Jurek 2019). Also, the affective neuroscience theory by Panksepp (2004) suggests that in situations of sadness (caused by separation-distress),
the administration of OT might be able to down regulate the activity of the SADNESS\(^1\) circuitry. Even though the exact functional relevance of OT in HPA axis activity is not yet fully understood (Jurek et al. 2015), SLEs are associated with dysregulation of the endogenous OT system (Donadon et al. 2018) and are well-established major risk factors for the development of major depression (Culverhouse et al. 2018; Heim and Nemeroff 2001). SLEs may exert their pathogenic effect via epigenetic pathways (Szöenyi and Bick 2013).

Epigenetics is considered bridging the gap between genotype and phenotype. This term is used to describe changes in gene expression without changes in the underlying DNA sequence. An example of epigenetics is cell differentiation: Nearly all cells of a multicellular organism share one identical genotype. Nonetheless, a diversity of cell types with disparate, yet stable, profiles of gene expression and, therefore, distinct cell functions emerges during the development of an organism (Goldberg et al. 2007). However, a gene’s activity may also be influenced by environmental signals, thus depending upon interindividual context (Weaver et al. 2004). Epigenetic regulation comprises mechanisms like DNA methylation, histone modifications, and noncoding RNAs. Probably, the most extensively investigated and characterized epigenetic biochemical modification of chromatin is DNA methylation (Feil and Fraga 2012). DNA methylation has been largely examined at the 5′-position of cytosine residues of CpG dinucleotides. Genomic regions with a high density of CpG sites are referred to as CpG islands and can be found in the promoter regions of many genes (Bird 1986). DNA methylation can modify histone–DNA interactions, thus altering a gene’s accessibility for transcription factors and thereby gene expression (Meany and Szöenyi 2005). Methylation of CpG sites in the promoter region of a gene can for example prevent (but may also enhance) transcription factor binding and lead to a decline (or an increase) in transcription rate (Lim and Maher 2010; Watt and Molloy 1988). The role of DNA methylation in gene transcription inspired many scientists searching for potentially reversible biomarkers for disease risk and maintenance in humans (McGowan et al. 2009; Murgatroyd et al. 2009; Roth et al. 2009). An early study focused on the examination of the glucocorticoid receptor gene (NR3C1) (Weaver et al. 2004) being closely associated with the HPA axis (Liu et al. 1997). In that study investigating the interaction of early life stress, DNA methylation, and HPA reactivity later in life, early life stress was operationalized as poor maternal care in a rat model (Weaver et al. 2004). Parenting in mammals is affected by hormones with OT playing a vital role (Feldman and Bakermans-Kranenburg 2017). OT is also associated with trauma (Donadon et al. 2018), HPA axis activity (Winter and Jurek 2019), and depressive-like behavior (Bosch and Young 2017; Jurek and Neumann 2018).

The focus of previous research regarding stress, DNA methylation, and depression has been on the oxytocin receptor gene (OXTR). Results of previous studies with regard to the association between exposure to prenatal stress and OXTR methylation were heterogeneous: One study found significant hypermethylation of the OXTR to be associated with maternal perinatal depressive symptoms (King et al. 2017). Another study reports that the total number of maternal adversities was negatively associated with OXTR methylation in cord blood (Unternaehrer et al. 2016). Last, there are studies reporting no significant association between prenatal exposure to maternal stress and OXTR methylation in cord blood (Rijlaarsdam et al. 2017) and between depressive symptoms in pregnancy and placental OXTR methylation (Galbally et al. 2018). On the other hand, early life adversity as well as persistent stressors has been shown to be associated with OXTR methylation (Gouin et al. 2017; Simons et al. 2017). Associations between methylation patterns of OXTR and depression diagnosis also differ across studies: Whereas two studies report hypermethylation of CpG sites in the OXTR (Bell et al. 2015; Chagnon et al. 2015), other studies found a negative association of OXTR methylation and depressive symptoms (Kimmel et al. 2016) as well as depression diagnosis (Reiner et al. 2015). However, the only two studies we are aware of that investigated OXT methylation in humans examined OXT methylation and sociability (Haas et al. 2016) as well as dynamic DNA methylation changes in mothers and postpartum maternal intrusiveness (Toepfer et al. 2019). In the first study, OXT methylation was negatively associated with a secure attachment style, the ability to recognize emotional facial expressions, and greater superior temporal sulcus activity during two social–cognitive functional MRI tasks (Haas et al. 2016). The second study found OXT methylation to decrease from early to mid-pregnancy and no further change until late pregnancy. Additionally, intrusive compared to noninvasive mothers had 6% higher methylation of one CpG site in the OXT promoter in late pregnancy (Toepfer et al. 2019). The interrelations between SLEs, DNA methylation of OXT, and depression have not yet been examined.

Therefore, we examined the associations between DNA methylation of the promoter region of OXT and SLEs as well as current depressive symptoms in a sample of \(N = 146\) inpatients suffering from major depression. Since there are considerable sex differences in stress response (Bale 2011; Bale and Epperson 2015), we also explored whether there are sex differences in OXT promoter methylation and whether sex interacts with stressful life events in the prediction of OXT promoter methylation. We further explored correlational patterns between SLEs, DNA methylation, and depression severity, controlling for other variables potentially confounding these associations, such as age, body mass index (BMI), current

\(^1\) Written in capital letters in order to refer to the Pankseppian nomenclature (Davis and Montag 2019).
medication, and substance use (Abraham and Fava 1999; de Wit et al. 2009; Dick et al. 2014; Ernst and Angst 1995; Feinberg et al. 2010; Lee and Pausova 2013; Philibert et al. 2012). Based on previous findings (Heim and Binder 2012; Heim and Nemeroff 2001), we assumed a positive association between SLEs and depression severity (see also the new Culverhouse et al. 2018 meta-analysis). Our overarching hypothesis for the association between OXT promoter methylation and SLEs or depression severity was that a higher number of SLEs is associated with high OXT methylation, presumably an indicator of lower transcription rate and therefore lower OT. This would coincide with the previously observed negative association between trauma and endogenous OT levels (Donadon et al. 2018). Since SLEs are positively associated with depression severity and presumably with OXT promoter methylation, one would assume OXT methylation and depression severity to be positively correlated as well. However, previous research showed an increased number of OXT-expressing neurons in the paraventricular nucleus in postmortem tissue of depressed patients (Purba et al. 1996) and an increase of OXT mRNA in melancholic type depressed patients (Meynen et al. 2007). Based thereon, low OXT methylation could also be associated with high depression severity. A previous study reported a correlation between SLEs and depression severity of $r = 0.24$ (Plieger et al. 2015). This indicates that there is a considerable proportion of unshared variance between the two variables. Consequentially, it is possible that there are diametrically opposed correlational patterns comparing the association between SLEs and OXT methylation to the association between OXT methylation and depression severity.

**Patients and Methods**

**Participants**

Data of $N = 146$ depressed inpatients (98 females) were collected. All participants were diagnosed for major depression (and no other mental illness) by a senior resident supervised by a psychiatrist at admission to the hospital using the Structured Clinical Interview for DSM-IV (American Psychiatric Association 2003). All participants were inpatients at the Department of Psychiatry at Ulm University and individually recruited. We calculated dose equivalents for antidepressants (weighted mean dose/fluoxetine 40 mg) (Hayasaka et al. 2015), received at the day of assessment. After completing the questionnaires described below, whole blood samples were taken and a standardized interview was conducted comprising a standardized semistructured inhouse questionnaire on sociodemographic variables and the Montgomery Asberg Depression Rating Scale (MADRS) to assess depression severity (Montgomery and Asberg 1979).

We also assessed the BMI measuring the height and weight of the participants. As a measure for substance use, we assessed frequency and dose of consumption as well as the kind of alcoholic drinks and caffeine containing products consumed in the reported frequency and dose. We then calculated grams per day for alcohol, cigarettes per day for nicotine, and milligrams per day for caffeine use. All participants provided written consent prior to participation (Table 1). The ethics committee of Ulm University, Ulm, Germany, approved the study.

**Questionnaires**

**CLEQ**

The Critical Life Events Questionnaire (CLEQ) assesses 30 potentially traumatic life events, such as experience of violence, natural disaster, man-made disaster, or death of a close person (Plieger et al. 2015). A weighted score was calculated adding up the product of the occurrence of each event and the experienced severity. If there were nine or more incompletely answered events, participants were excluded from further analysis with the CLEQ.

**BDI-II**

We also administered the Beck depression inventory (German version, BDI-II) to assess individual differences in the severity of depressive symptoms (Beck et al. 2006). The BDI-II consists of 21 items each assessing the current state of a symptom of depression with four given options (0–3). A total score is calculated adding up the scores of the 21 items. Higher scores indicate higher depression severity. Internal consistency was excellent with $\alpha = 0.91$.

**Analyses of OXT Methylation**

We selected the target region for methylation analysis based upon a previous study (Haas et al. 2016). Methylation status of the CpG-rich regions in the promoter of OXT (Fig. 1) was quantified by varionostic GmbH (Ulm, Germany) using the Sequenom Epityper MassArray System (San Diego, CA, USA). At first, genomic DNA from peripheral blood samples was automatically purified by means of the MagNA Pure® 96 system using a commercial extraction kit (MagNA Pure 96 DNA kit; Roche Diagnostics, Mannheim, Germany). Afterwards, genomic DNA was bisulfite treated. The bisulfite treatment and all steps of the EpiTYPER assay were performed under routine conditions as outlined in the manufacturer’s suggested protocol. For the region of interest, amplicons were designed using Agena’s Epi DESIGNER software (San Diego, CA, USA). These amplicons were PCR amplified using the following primers: forward
In the next step, in vitro RNA transcription with subsequent base-specific cleavage using RNase A was performed resulting in fragmented RNA molecules. These RNA molecules may contain more than one CpG site. Cleavage products derived from methylated and unmethylated DNA are of identical length and differ only in their nucleotide composition due to bisulfite treatment. After sample conditioning, products were processed on a MALDI-TOF platform (Agena; MassARRAY 4). The different cleavage products created from methylated or unmethylated regions generated characteristic signal patterns that provided the basis for analysis by MALDI-TOF mass spectrometry. In analyzing the mass spectrum, the relative amount of methylation was calculated by comparing the difference in signal intensity between mass signals of the cleavage products and mass signals derived from completely methylated and unmethylated template DNA. Multiple CpG sites on one RNA molecule were analyzed as CpG unit.

Methylation status of the analyzed regions of the promoter OXT was examined with respect to single CpG sites or units as well as weighted (CpG units of two CpG sites were doubly weighted) mean methylation status across all 14 sites/units ($\alpha = 0.97$; mean DNA methylation: $M = 0.38; SD = 0.07; \min = 0.20; \max = 0.59$). Figure 2 presents boxplots of the methylation status for all examined CpG sites showing that there were no outliers or severe deviations from normality.

## Statistical Analysis

Statistical analysis was conducted using R (R Development Core Team 2008). We computed partial Spearman’s rank correlation coefficients ($r_p$) with sex, age, BMI, substance use (alcohol, nicotine, caffeine), and dose equivalents of antidepressants (weighted mean dose/fluoxetine 40 mg) (Hayasaka et al. 2015) as covariates to explore the association between the CLEQ score and depression severity (MADRS, BDI-II). We used the same approach to examine the associations of the methylation status of single CpG units in the promoter region of OXT and SLEs. Missing data was deleted pairwise. We used Spearman’s rank correlation because some of the examined variables had outliers.

We additionally performed a hierarchical multiple regression analysis with mean methylation of OXT as dependent variable; age, BMI, substance use, and dose equivalents of antidepressants as covariates; and sex as well as CLEQ score as independent variables. In the next step, we added the interaction between sex and CLEQ score. Statistical significance was determined at $p < 0.05$.

## Results

### SLEs and Depression Severity

After controlling for sex, age, BMI, substance use, and dose equivalents of antidepressants, we found no significant correlation between the CLEQ score and depression severity according to the MADRS ($r_p = 0.17, p = 0.050$, one-tailed, n.s.). However, the CLEQ score was significantly positively correlated with depression severity assessed with the BDI-II ($r_p = 0.27, p = 0.003$, one-tailed). The result pattern did not change with separate analysis of the correlations in women and men ($r_{women} = 0.23; p = 0.036; r_{men} = 0.39, p = 0.024$).

### Mean Methylation of the Promoter Region of OXT

There was no significant association between mean methylation status of OXT and depression severity, neither for

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### Table 1 Descriptive statistics of the examined variables

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>146</td>
<td>18.00</td>
<td>65.00</td>
<td>39.08</td>
<td>14.35</td>
</tr>
<tr>
<td>Nicotine</td>
<td>146</td>
<td>0.00</td>
<td>24.00</td>
<td>4.97</td>
<td>7.24</td>
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<tr>
<td>Caffeine</td>
<td>146</td>
<td>0.00</td>
<td>650.00</td>
<td>99.51</td>
<td>139.51</td>
</tr>
<tr>
<td>Alcohol</td>
<td>136*</td>
<td>0.00</td>
<td>57.14</td>
<td>3.09</td>
<td>8.33</td>
</tr>
<tr>
<td>BMI</td>
<td>139*</td>
<td>0.00</td>
<td>42.45</td>
<td>25.75</td>
<td>5.79</td>
</tr>
<tr>
<td>Dose equivalents of antidepressants</td>
<td>116*</td>
<td>0.00</td>
<td>172.43</td>
<td>39.93</td>
<td>27.62</td>
</tr>
<tr>
<td>CLEQ</td>
<td>139*</td>
<td>0.00</td>
<td>112.00</td>
<td>22.88</td>
<td>19.94</td>
</tr>
<tr>
<td>MADRS</td>
<td>146</td>
<td>7.00</td>
<td>48.00</td>
<td>25.26</td>
<td>9.39</td>
</tr>
<tr>
<td>BDI</td>
<td>140*</td>
<td>10.00</td>
<td>59.00</td>
<td>32.28</td>
<td>11.13</td>
</tr>
</tbody>
</table>

Nicotine: cigarettes per day, caffeine: milligrams per day, alcohol: grams per day, BMI (body mass index): kg/m², antidepressants: dose equivalent to 40 mg fluoxetine

*Differing n reflect patients failing to complete all items of the inhouse questionnaire, CLEQ, or BDI-II or patients receiving medication not available in Hayasaka et al. (2015)
MADRS ($r_p = 0.02, p = 0.416$, one-tailed) nor for BDI-II scores ($r_p = 0.04, p = 0.330$, one-tailed).

The regression model with age, BMI, substance use, and dose equivalents of antidepressants as covariates and sex as well as the CLEQ score as independent variables explained a significant amount of variance in mean OXT methylation ($\text{adjusted } R^2 = 0.15, \text{F}(8, 90) = 3.25, p = 0.003$). Sex and the CLEQ score were significant predictors of mean OXT methylation (Table 2). Females had significantly higher methylation status than males. Further, higher CLEQ scores indicating more stressful life events were associated with low mean OXT promoter methylation.

Adding the interaction of sex and CLEQ score to our model did not result in a significant increase in explained variance ($\text{adjusted } R^2 = 0.17, \text{F}(2, 86) = 0.13, p = 0.71$). Hence, there was no significant interaction of sex and CLEQ score in the prediction of mean OXT methylation ($b = -0.001, \text{SE} = 0.001, t(89) = -1.45, p = 0.15$).

**Single CpG Sites**

To further explore the association of OXT methylation and depression severity, we performed partial correlation analyses examining the association between the methylation of single CpG units in the OXT promoter and depression severity (as measured with the MADRS and the BDI-II) controlling for sex, age, BMI, substance use, and antidepressive medication. None of the single CpG sites or units was significantly associated with depression severity with age, BMI, substance use, and antidepressive medication.

Fig. 1 CpG island and target region in OXT (chr20:3,071,425–3,071,795, hg38; CpG 1.2 corresponds to rs3,071,452–3,071,455). Light gray marks the region 5′ of the first exon of OXT. Black letters mark the first exon. Italic letters mark the untranslated region (UTR) within the first exon. Nonitalic letters mark the coding sequence (CDS) within the first exon. Methylation status of CpG sites marked in red was not analyzable. Haas et al. (2016) examined an averaged methylation score of CpG sites corresponding to CpG 1.2, 3, 4, 6, 11, 12, 15, 16, and 20. Toepfer et al. (2019) examined changes in the methylation status of one CpG site corresponding to CpG 5. CpG 1.2, 4, 5, 7.8, 9.10, and 14.15 were associated with SLEs in the whole sample and in men (for the latter only before controlling FDR). Further, CpG 29 was also associated with SLEs in the whole sample and in women (for the latter only before controlling FDR). CpG 19 and 24.25 were associated with depression in the whole sample but only before controlling FDR.

Fig. 2 Methylation status of the examined CpG units in the promoter region of OXT. Boxes cover methylation data between 25th and 75th quantile (median ± 1 interquartile range). Whiskers represent values falling within 1.5-fold the interquartile range. The dashed line represents the 5% detection limit for the Sequenom EpiTYPER platform. Methylation seemed to be lower within the CDS of exon 1.
We additionally performed partial correlation analyses for the association of the methylation of single CpG sites with CLEQ score. Before controlling FDR, eight of the 14 CpG units correlated significantly negative with the CLEQ score (Table 3). Even after controlling FDR, seven of these eight CpG units were significantly negatively correlated with critical life events. Correlation coefficients ranged from $r_p = -0.29$ to $r_p = 0.04$ and all but two correlation coefficients were negative.

When looking at correlations of single CpG sites in the OXT promoter and CLEQ score for men and women separately (with age, BMI, substance use, and dose equivalents of antidepressants as covariates), all but two correlation coefficients were negative (Table 4). In men, correlation coefficients were higher, but sample size was smaller than in women. After controlling FDR, none of the CpG units was significantly associated with the CLEQ score. This could be due to the smaller sample size.

**Table 2** Regression coefficients for the prediction of mean OXT promoter methylation

<table>
<thead>
<tr>
<th>Variable</th>
<th>$b$</th>
<th>SE</th>
<th>$t$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.3970</td>
<td>0.0043</td>
<td>9.201</td>
<td>0.000</td>
</tr>
<tr>
<td>Age</td>
<td>−0.0008</td>
<td>0.0005</td>
<td>−1.671</td>
<td>0.098</td>
</tr>
<tr>
<td>Nicotine</td>
<td>−0.0003</td>
<td>0.0009</td>
<td>−0.315</td>
<td>0.754</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.597</td>
<td>0.552</td>
</tr>
<tr>
<td>Alcohol</td>
<td>−0.0005</td>
<td>0.0008</td>
<td>−0.694</td>
<td>0.489</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.0019</td>
<td>0.0014</td>
<td>−1.373</td>
<td>0.173</td>
</tr>
<tr>
<td>DE of antidepressants</td>
<td>0.0002</td>
<td>0.0002</td>
<td>0.755</td>
<td>0.452</td>
</tr>
<tr>
<td>Sex</td>
<td>0.0410</td>
<td>0.0140</td>
<td>2.937</td>
<td>0.004</td>
</tr>
<tr>
<td>CLEQ score</td>
<td>−0.0008</td>
<td>0.0003</td>
<td>−2.353</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Numbers in italics correspond to significant predictors

$DE$, dose equivalents; $CLEQ$, Critical Life Events Questionnaire; $SE$, standard error

**Discussion**

We examined the associations between critical life events, depression severity, and methylation of the promoter region of OXT in a sample of $N = 146$ inpatients suffering from major depression. SLEs were significantly positively associated with depression severity as measured by means of the BDI-II. Contradictory to our hypothesis, we did not find an association of OXT promoter methylation and severity of depressive symptoms after controlling FDR. Nevertheless, OXT promoter methylation at two CpG units was significantly positively associated with depression severity measured with the BDI-II before controlling FDR. Furthermore, we observed a significant negative association between SLEs and mean OXT methylation. The negative association was also obtained for seven out of 14 single CpG units. The correlation coefficients between single CpG units and SLEs were negative for all but two CpG units. Sex and SLEs were significant predictors of mean OXT promoter methylation even after controlling for age, BMI, substance use, and antidepressive medication. Females showed a significantly higher methylation status of the OXT promoter.

**OXT Methylation and Depression Severity**

We did not observe a significant association between OXT promoter methylation and depression severity after controlling FDR. In accordance, a recent meta-analysis concluded that there is no association between the endogenous OT concentration and depression diagnosis (Engel et al. 2019b). However, since nonsignificance of this association after FDR correction could also be a result of our sample size, we briefly discuss the findings without FDR correction: Two CpG sites were significantly positively associated with depression severity as measured with the BDI-II. The associations’ direction was contradictory to previous results (Meynen et al. 2007; Purba et al. 1996). However, provided that high OXT promoter methylation is associated with low transcription rate and finally with low brain oxytocin, our finding would fit theories postulating depression to be a shutdown mechanism to terminate protracted separation distress (Panksepp and Watt 2011; Watt and Panksepp 2009). This is also in line with a previous study reporting a negative association between plasma levels of oxytocin and depressive symptom severity (Scantamburlo et al. 2007). Further, there is considerable heterogeneity in the results regarding the association between the methylation status of the OXTR and depression. While some studies report a hypermethylation of the OXTR to be related to depression diagnosis (Bell et al. 2015; Chagnon et al. 2015), others found a negative association between OXTR methylation and depressive symptoms (Kimmel et al. 2016) as well as depression diagnosis (Reiner et al. 2015). This heterogeneity could be an artifact of sample composition and operationalization. While three studies focused on middle aged women (Bell et al. 2015; Kimmel et al. 2016; Reiner et al. 2015), one study investigated women aged 65 or older (Chagnon et al. 2015). Additionally, one study investigated depression and/or dysthymia (Reiner et al. 2015), one study focused on major depressive episodes as well as minor depression (Chagnon et al. 2015), and the other two studies examined postpartum depression (PPD) (Bell et al. 2015; Kimmel et al. 2016). However, it could also reflect the heterogeneity of depression itself: Patients suffering from depression often show different symptoms (even diametrically opposed in case of appetite and sleep). Perhaps, different subtypes of depression might be associated with a distinct etiology and with specific methylation patterns.

**Sex and OXT Methylation**

Sex and SLEs were significant predictors of mean OXT promoter methylation even after controlling for age, BMI,
substance use, and antidepressive medication. Females showed a significantly higher methylation status of the OXT promoter. This observation is in line with the assumption of biological sex differences in brain functioning contributing to sex differences in the prevalence of major depression (Albert 2015). Supporting evidence for OXT playing a role in sex differences considering the prevalence of mood disorders stems from research postulating OT to be involved in the development of postpartum depression (Jobst et al. 2016; Lara-Cinisomo et al. 2017). In these studies, postpartum depressive symptoms were associated with lower levels of plasma OT (Jobst et al. 2016; Lara-Cinisomo et al. 2017). This is in line with our results of higher OXT promoter methylation status in women, since promoter methylation is often suggested being a mechanism of gene silencing (Jones and Takai 2001). Alternatively, sex differences in the OT system could be independent of the diagnosis of major depression.

Moreover, endogenous OT concentrations fluctuate together with concentrations of female sex hormones. A recent meta-analysis showed a significant increase of OT concentrations from the early follicular phase to ovulation and a significant decrease from ovulation to the mid-luteal phase (Engel et al. 2019a). Additionally, a recent study showed that OXT promoter methylation changed dynamically throughout pregnancy (Toepfer et al. 2019) rendering a menstrual cycle-dependent change in OXT methylation possible. However, it is, to the best of our knowledge, unknown how OXT promoter methylation is affected by female sex hormones and associated with immediate changes in endogenous OT concentrations. Further, it is unknown how epigenetic modifications of other genes playing a role for the endogenous OT system (e.g., the OTR) interact in this regard. Nevertheless, there is a possibility that the observed sex difference in OXT promoter methylation is due to the fact that we did not control for phases of the menstrual cycle in women. Thus, the result of a sex difference in mean OXT methylation should be considered preliminary and needs further examination.

### SLEs and OXT Methylation

Contradictory to our hypotheses, we found a significant negative association between SLEs and mean OXT methylation. The CLEQ score assesses stressors across the lifespan. Thus, the CLEQ score being associated with reduced OXT promoter methylation with respect to mean methylation as well as single CpG sites is in line with previous results showing significant associations between early life adversities as well as persistent adverse environments and OXTR methylation (Gouin et al. 2017; Simons et al. 2017). It is, however, worth mentioning that future investigations of stressors occurring early versus late in life could shed light on different OXT and OXTR methylation patterns depending on the timing of the stressor.

Our results are also in line with a recent review suggesting trauma to be associated with dysregulation of the endogenous OT system (Donadon et al. 2018). Even though trauma showed a moderate negative association with endogenous OT concentrations in this review, the suggested relationship

### Table 3 Partial Spearman’s correlation coefficients for the association between CLEQ scores and methylation status of single CpG sites in the OXT promoter

<table>
<thead>
<tr>
<th>OXT CpG</th>
<th>r_p</th>
<th>p</th>
<th>p(BH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG 1.2</td>
<td>-0.25</td>
<td>0.007</td>
<td>0.043</td>
</tr>
<tr>
<td>CpG 3</td>
<td>-0.17</td>
<td>0.046</td>
<td>0.076</td>
</tr>
<tr>
<td>CpG 4</td>
<td>-0.22</td>
<td>0.014</td>
<td>0.043</td>
</tr>
<tr>
<td>CpG 5</td>
<td>-0.21</td>
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<td>0.045</td>
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<tr>
<td>CpG 7.8</td>
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<td>0.050</td>
</tr>
<tr>
<td>CpG 9.10</td>
<td>-0.14</td>
<td>0.089</td>
<td>0.113</td>
</tr>
<tr>
<td>CpG 14.15</td>
<td>0.21</td>
<td>0.016</td>
<td>0.045</td>
</tr>
<tr>
<td>CpG 16</td>
<td>-0.16</td>
<td>0.054</td>
<td>0.076</td>
</tr>
<tr>
<td>CpG 18</td>
<td>-0.16</td>
<td>0.052</td>
<td>0.076</td>
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<td>CpG 19</td>
<td>0.04</td>
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<td>0.378</td>
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<tr>
<td>CpG 20</td>
<td>-0.29</td>
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<td>0.027</td>
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<tr>
<td>CpG 24.25</td>
<td>0.01</td>
<td>0.462</td>
<td>0.462</td>
</tr>
<tr>
<td>CpG 26</td>
<td>-0.11</td>
<td>0.145</td>
<td>0.169</td>
</tr>
</tbody>
</table>

Covariates: sex, age, BMI, substance use (alcohol, nicotine, caffeine), and dose equivalents for antidepressants. One-tailed p values. Correlation coefficients in italics (with their respective p values) stayed significant after FDR correction

### Table 4 Partial Spearman’s correlation coefficients for the association between CLEQ scores and methylation status of single CpG sites in the OXT promoter separated by sex

| OXT CpG | Men | | | | Women | | |
|---------|-----|------|-------| |       |------|-------|
| CpG 1.2 | -0.38 | 0.024 | 0.080 | | -0.19 | 0.065 | 0.282 |
| CpG 3   | -0.31 | 0.061 | 0.107 | | -0.14 | 0.142 | 0.282 |
| CpG 4   | -0.40 | 0.015 | 0.080 | | -0.13 | 0.150 | 0.282 |
| CpG 5   | -0.37 | 0.029 | 0.080 | | -0.14 | 0.141 | 0.282 |
| CpG 7.8 | -0.40 | 0.018 | 0.080 | | -0.11 | 0.200 | 0.282 |
| CpG 9.10| -0.34 | 0.043 | 0.099 | | -0.15 | 0.125 | 0.282 |
| CpG 13  | -0.28 | 0.077 | 0.108 | | -0.06 | 0.309 | 0.309 |
| CpG 14.15| -0.31 | 0.056 | 0.107 | | -0.15 | 0.113 | 0.282 |
| CpG 16  | -0.38 | 0.026 | 0.080 | | -0.09 | 0.247 | 0.289 |
| CpG 18  | -0.24 | 0.116 | 0.147 | | -0.11 | 0.192 | 0.282 |
| CpG 19  | 0.16  | 0.219 | 0.219 | | -0.08 | 0.276 | 0.297 |
| CpG 20  | -0.20 | 0.074 | 0.108 | | -0.30 | 0.008 | 0.106 |
| CpG 24.25| 0.19 | 0.167 | 0.195 | | -0.11 | 0.201 | 0.282 |
| CpG 26  | -0.16 | 0.214 | 0.219 | | -0.09 | 0.242 | 0.289 |

Covariates: age, BMI, substance use (alcohol, nicotine, caffeine), and dose equivalents for antidepressants. One-tailed p values. Correlation coefficients in italics (with their respective p values) stayed significant before FDR correction.
between trauma and OT is considered multifaceted and complex (Donadon et al. 2018). In line with this, OT can also promote “antisocial” behavior with its effects depending on various contextual and individual factors like gender or psychopathology (Shamay-Tsoory and Abu-Akel 2016). In accordance, increased OT has been associated with symptoms of social detachment (Munro et al. 2013). Trauma is assumed to promote social avoidance (Casslen et al. 2001) and negative assumptions regarding interpersonal relationships (DePrince et al. 2009). OT is associated with agonistic tendencies in individuals chronically predisposed to perceive the social milieu as unsafe (Olf et al. 2013). Thus, it is possible that an interaction of SLEs and stress-induced low OXT promoter methylation is associated with a higher vulnerability for developing depressive disorders. In more detail, SLEs were associated with lower OXT methylation (and presumably higher OT) in our sample of depressed inpatients. Since trauma is also associated with perceiving social situations in negative terms (Casslen et al. 2001; DePrince et al. 2009), the “antisocial” effects of OT may be triggered in individuals having low stress-induced OXT promoter methylation which may predispose these individuals to depression development. After all, depression often is associated with social withdrawal, feelings of inferiority, and social anxiety (Gilbert 2000). On the other hand, the negative association between OXT promoter methylation and SLEs could be an adaptive reaction counteracting trauma-induced hyper(re-)activity of the HPA axis (Heim et al. 2000). This hypothesis is congruent with the results of one study, which found lower OXT methylation to be associated with more secure attachment styles and an improved ability to recognize emotional facial expressions, both arguably features of human sociability (Haas et al. 2016). Insecure attachment styles are a potential mediator for the relationship between SLEs and depressive symptoms later in life (Hankin 2005). OT, on the other hand, can enhance the experience of attachment security (Buchheim et al. 2009). Thus, it would be an adaptive process to have higher OT-induced sociability and concomitantly recruit social resources after trauma exposure. After all, poor social support following trauma is among the greatest risk factors for the development of posttraumatic stress disorder (Ozer et al. 2003). However, these hypotheses should be tested using a longitudinal case-control design and comparison of populations suffering from different psychiatric disorders with respect to SLE-associated methylation patterns in the OXT promoter.

Limitations

There are some limitations that need to be considered when interpreting the results of the present study. First, we assessed methylation data by analyzing peripheral leukocytes possibly not providing a direct index of methylation status in the central nervous system. However, there are findings indicating high convergences between CpG island methylation levels across different tissues (Byun et al. 2009; Smith et al. 2015; Tylee et al. 2013). Second, although we investigated a relatively large number of CpG sites in our target region, our investigation is not exhaustive in terms of the CpG-rich region in OXT. However, we decided on investigating the only region that has been examined in previous studies in terms of DNA methylation (Haas et al. 2016; Toepfer et al. 2019). Third, we did not assess OXT mRNA or protein levels. Therefore, our study has no implications for OXT transcription. Lastly, data of a control group is missing so that any conclusions about the specificity of the findings with regard to depression are not warranted.

Conclusion

In conclusion, methylation status of two single CpG units was positively associated with depression severity before controlling FDR. Moreover, mean methylation as well as methylation status of single CpG sites in the OXT promoter was negatively associated with SLEs in a sample of depressed inpatients. These findings support the importance of the OT system in the development of or the resilience to psychopathology. In addition, there were sex differences in the epigenetic regulation of OXT promoting the assumed sex-specific effects of OT. This is important since sex-dependent regulation of the OT system could be relevant for the etiology and treatment of major depression. Therefore, future studies should examine the interrelations between SLEs, epigenetic regulation of OXT, and depression with respect to sex differences using a case-control design.

Author Contributions S.S., C.M., and M.K. designed the present study. S.S. analyzed the data and wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of Interest There authors declare that they have no competing interests.

Ethical Approval All procedures performed in this study were in accordance with the ethical standards of the ethics committee of Ulm University, Ulm, Germany (reference number: 25/18), and with the 1964 Helsinki Declaration and its later amendments.

References


Philibert RA, Plume JM, Gibbons FX, Brody GH, Beach SRH (2012) The impact of recent alcohol use on genome wide DNA methylation


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2.3. Studies III - Relation of promoter methylation of the structural oxytocin gene to critical life events in major depression: A case control study


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Research paper

Relation of promoter methylation of the structural oxytocin gene to critical life events in major depression: A case control study

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ABSTRACT

Background: Stressful life events (SLEs) are associated with hyper(re-)activity of the HPA-axis. HPA-axis hyper (re-)activity is thought to be a major risk factor for depression development. SLEs may induce changes in an organism's stress system via epigenetic mechanisms. The neuropeptide oxytocin (OT) is able to attenuate the stress response, and OT pathways are dysregulated in individuals suffering from Major Depressive Disorder (MDD). Therefore, the gene coding for oxytocin (OXT) is a possible target for the investigation of depression development.

Methods: We collected data on SLEs, OXT promoter methylation (Sequenom Epityper MassArray) and depression severity from 90 MDD inpatients and 90 matched healthy controls.

Results: We found MDD inpatients to have a significantly lower OXT methylation than healthy controls. Methylation status was significantly negatively associated with SLEs but only in the group of MDD inpatients. There were no associations between methylation status and depression severity.

Limitations: Methylation in blood samples is only a proxy for epigenetic profiles in brain tissue. We did not assess mRNA or protein levels and cannot draw conclusions regarding the functionality or specificity of differences in OXT methylation between groups.

Conclusion: SLEs leave their traces in the epigenetic profiles of the OT system of MDD inpatients. Alterations in epigenetic profiles of the OXT system could constitute a vulnerability factor predisposing individuals for depression development. Better understanding of DNA methylation profiles of depression-associated genes could serve as basis for a personalized medicine, in which pharmacological or psychotherapeutic treatment of depression is tailored to the patient's individual characteristics.

1. Introduction

In recent years, the prevalence of depression increased (Mojtabai et al., 2016) and depressive disorders have become the world's second leading cause of years lived with disability (Ferrari et al., 2013). In 2018, an epidemiology analysis postulated a lifetime prevalence of DSM-5 major depressive disorder (MDD) of 20.6% and a 12-month prevalence of 10.4%. The heritability of MDD estimated from twin studies is about 37% (Sullivan et al., 2000). For complex traits such as MDD, the risk and development do not follow classic genetic heritability (Berdasco and Esteller, 2019). Therefore, even though some progress has been reported, the genetic basis of depression is still poorly understood (Mullins and Lewis, 2017). A frequently observed finding in the investigation of MDD development is the co-occurrence of MDD and traumatic or stressful life events (SLEs). Especially childhood maltreatment but also other kinds of traumatic stressors across the lifespan (e. g. divorce, sexual abuse, life-threatening or chronic illness or injuries) are associated with the diagnosis of MDD and depressive symptom severity (Dunn et al., 2017; Norman et al., 2012; Sanwald et al., 2019). However, individuals react differently to SLEs, which is why a gene-environment (G × E) interaction has been suggested (Assary et al., 2018). The idea is that the individual genome forms a vulnerability which, in combination with SLEs, can lead to persistent hyper(re-)activity of the hypothalamic-pituitary-adrenal (HPA) axis. HPA hyper(re-)activity in turn might increase stress vulnerability and thereby depression...
susceptibility (Heim and Binder, 2012; Shapero et al., 2019). HPA axis hyper(re-)activity may result from stress induced changes in the expression of HPA axis associated genes via epigenetic mechanisms (Ramón-Fernández et al., 2019; Suderman et al., 2012). This notion comes from observations of stressors determining patterns of gene expression in response to but also beyond the duration of a stressor (De Nadal et al., 2011; Weaver et al., 2004). Since an organism adapts to its environment on the level of gene expression, there have to be mechanisms that enable the plasticity of transcriptional activity. Epigenetics provides an explanation for environmentally induced changes in transcriptional activity, thus bridging the gap between genome and environment (Goldberg et al., 2007).

Epigenetic regulation of gene expression can be defined as the structural adaptation of chromosomal regions that leads an identical combination of genes to produce a different outcome (Bird, 2007). The most extensively investigated mechanism of epigenetic regulation is DNA methylation (O'Donnell and Meaney, 2020). DNA methylation in mammals was mainly examined in cytosine-guanine dinucleotides (CpGs) where a methyl group is added to the 5'-position of the cytosine residue (O'Donnell and Meaney, 2020). DNA regions with a high density of CpG sites are referred to as CpG islands. CpG islands can be found in the promoter region of many genes (Bird, 1986). Due to methylation associated changes in histone DNA interactions and therefore changes in a gene's accessibility for transcription factors, altered methylation profiles in a gene's promoter are associated with altered gene transcription (up- or downregulation) leading to an up- or downregulated synthesis of the corresponding protein (Lim and Maher, 2010; O'Donnell and Meaney, 2020).

Associations between the methylation status of gene promoters and HPA axis hyper(re-)activity have already been found for a variety of genes, e.g. the glucocorticoid receptor gene NR3C1 (Farrell et al., 2018; Perroud et al., 2011) and the oxytocin receptor gene (OXTR) (Fujisawa et al., 2019). While the glucocorticoid receptor gene NR3C1 in the development of MDD has been intensively studied in the past (Chen et al., 2017), the involvement of oxytocin (OT) in affective disorders has received growing interest in the recent years. However, results are heterogeneous and further research is needed to elucidate the role of OT in MDD (Ciobica et al., 2016; Engel et al., 2019). Central OT has been shown to attenuate the stress response (Amico et al., 2004), presumably by modulation of cortisol levels. Even though this relationship is strongly supported by previous evidence, its dynamic and context specific nature is not yet fully understood (Brown et al., 2016).

In the brain, OT is synthesized in the hypothalamus (Landgraf and Neumann, 2004). The synthesis and release of OT within the brain can be triggered by stressful stimuli (Kagerbauer et al., 2019; Slattery and Neumann, 2010). Further, OT pathways are dysregulated in patients suffering from MDD (Parker et al., 2011; Thomas and Larkin, 2020), and OT is assumed being potentially effective in the treatment of MDD due to its prosocial effects (Neumann and Landgraf, 2012). While there is a considerable body of evidence linking the OXTR gene to stress and depression (Reiner et al., 2015; Simons et al., 2017; Unternaehrer et al., 2012), other genes along the OT pathway have received little attention (e.g. CD38) (Sauer et al., 2012). This represents a knowledge gap, since not only OT receptor expression but also OT delivery may be involved in the development of psychosocial disorders (Grinevich et al., 2016).

Thus, we conducted our study to investigate the role of the OT neurophysin I gene (OXT). It is located on chromosome 20p13 and codes for an inactive precursor protein producing both OT and its transporter protein neurophysin I (Rao et al., 1992). Increased promoter methylation of OXT has already been associated with lower sociability (Haas et al., 2016) and higher maternal intrusiveness (Toepfer et al., 2019). In a sample of inpatients suffering from MDD, OXT DNA methylation was negatively related to SLEs, but not to depression severity (Sanwald et al., 2019). However, as methylation data for a matched control sample was not available, differences between patients suffering from MDD and healthy controls regarding OXT methylation could not be examined.

In the present study, we therefore analyzed data on SLEs, OXT promoter methylation and depression severity from 90 MDD inpatients diagnosed according to DSM-IV criteria and 90 healthy controls matched for sex, age, substance use (alcohol, nicotine) and BMI. We assumed (1) MDD inpatients to have experienced more SLEs and (2) SLEs to be positively associated with depression severity in both samples with a higher correlation in the MDD group. We also hypothesized that (3) the OXT promoter is hypomethylated in the MDD group as compared to the control group based on the finding of Sanwald et al. (2019) that OXT methylation was negatively associated with SLEs. In addition, we explored sex differences in SLEs, OXT methylation profiles and depression severity.

2. Patients and methods

2.1. Participants

Data of n = 157 inpatients suffering from MDD and n = 109 controls was taken from the database of the Ulm Gene Brain Behavior Project (UGBBP). Data of the patient group therefore partially overlapped with those of the earlier study, whereas the data of the control group was not available previously (Sanwald et al., 2019). All participants of the sample of MDD inpatients were recruited at the Department of Psychiatry at Ulm University, Ulm, Germany. Inpatients were diagnosed for MDD by a psychiatrist at admission to the hospital using the Structured Clinical Interview for DSM-IV (SCID-I) (American Psychiatric Association, 2003). For inpatients we calculated dose equivalents for current antidepressants (weighted mean dose/fluoxetine40 mg) (Hayasaka et al., 2015) and neuroleptics (weighted mean dose/Chlorpromazine100 mg) (Benkert and Hippius, 2012; Schneider and Niebling, 2008; Woods, 2003). Further information on medication in the group of MDD inpatients can be found in Table 1.

The sample of healthy controls was recruited by postings in public areas and online advertisement. The control group underwent a diagnostic interview comprising the Mini-DIPS (Mraguf, 1994) and SCID-II (American Psychiatric Association, 2003) to exclude participants potentially suffering from any kind of mental illness. An additional exclusion criterion was a lifetime diagnosis of any kind of mental illness or any kind of past psychiatric inpatient treatment or psychotherapy. Data of 90 participants of the control sample met inclusion criteria and was further analyzed. All procedures performed in this study were in accordance with the ethical standards of the ethics committee of Ulm University, Ulm, Germany and with the 1964 Helsinki declaration and its later amendments. Informed consent of the participants was obtained after the nature of the procedures had been fully explained.

Table 1

<table>
<thead>
<tr>
<th>Medication in the group of MDD inpatients.</th>
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<tr>
<td>Antidepressants</td>
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<tr>
<td>SSRIs</td>
</tr>
<tr>
<td>SNRIs</td>
</tr>
<tr>
<td>TCAs</td>
</tr>
<tr>
<td>TCA's</td>
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<td>Atypical</td>
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<table>
<thead>
<tr>
<th>Neuroleptics</th>
<th>n</th>
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<tbody>
<tr>
<td>MARIAs</td>
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<td>27.78</td>
</tr>
<tr>
<td>Selective D2/D3 antagonists</td>
<td>1</td>
<td>1.11</td>
</tr>
<tr>
<td>Partial dopamine receptor agonists</td>
<td>1</td>
<td>1.11</td>
</tr>
<tr>
<td>Typical</td>
<td>1</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Note: SSRIs: selective serotonin reuptake inhibitors; SNRI: serotonin-norepinephrine reuptake inhibitors; TCA: tricyclic antidepressants; TCA: tetracyclic antidepressants; MARIAs: multi acting receptor targeted antipsychotics. Weighted mean dose/fluoxetin40 mg: Mean = 35.69, Standard Deviation = 24.55; weighted mean dose/CPZ100 mg: Mean = 21.31, Standard Deviation = 43.31. * some inpatients received more than one antidepressant.
Both groups were administered the questionnaires described below. Depression severity was rated by a trained psychologist using the Montgomery Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979). Sociodemographic variables were obtained with a standardized semi-structured interview based on an in-house questionnaire. Furthermore, we assessed age, Body Mass Index (BMI in kg/m²), consumed alcohol (grams/day) and nicotine (cigarettes/day). Each of the n = 90 healthy control individuals was matched with one of the n = 157 inpatients using exact matching in case of sex and nearest neighbor matching (propensity score) in case of age, BMI, alcohol and nicotine (see Statistical Analyses). The resulting paired samples of n = 90 depressed inpatients and n = 90 healthy controls (50 females each) did not differ significantly in any of the variables used for matching (Table S1).

2.2. Questionnaires

2.2.1. CLEQ

The Critical Life Events Questionnaire (CLEQ) assesses 30 potentially traumatic life events (such as experience of violence, natural disaster, man-made disaster or death of a close person). The participants answered the questions of whether they had ever experienced the concerning event and, if so, how traumatic they felt about it on a scale from 1 (not traumatic) to 6 (very traumatic) (Plieger et al., 2015). We calculated a weighted score adding up the product of the occurrence of each event and the experienced severity. If there were 9 or more incompletely answered events, participants were excluded from further analyses with the CLEQ.

2.2.2. BDI-II

Individual severity levels of depressive symptoms were explored by using the Beck Depression Inventory (German version, BDI-II) (Beck et al., 2006). The BDI-II is an internationally recognized clinic and research and standardized psychopathological-psychometric instrument that records the severity of a depressive syndrome. It is a self-assessment scale containing 21 items, in which for each item ratings between 0 (not at all) and 3 (very intensive) are given depending on the symptom complaint. A total of 63 points can be reached; the following cut-off values are used: 0–8: no depression; 9–19 mild depressive syndrome; 20–28: moderate depressive syndrome; 29–63: severe depressive syndrome. Internal consistency was excellent in the group of individuals suffering from MDD with α = .91 and good in the group of healthy controls, α = .84.

2.3. Quantification of OXT promoter methylation

Methylation status of the OXT promoter (Fig. 1) was quantified by Varionostic GmbH (Ulm, Germany; 124 samples, 90 inpatients and 34 controls) and Seq-IT GmbH & Co.KG (Kaiserslautern, Germany; 56 samples, 56 controls). Control analysis took into account different plates and laboratories for the analysis of DNA methylation did not show any significant effects of these variables and revealed excellent reliability (see the supplementary material, Table S2). Both laboratories used the Sequenom Epityper MassArray System (San Diego, CA, USA). All steps of the Epityper assay were performed under routine conditions as described by Suchiman et al. (2015). In short, genomic DNA from peripheral blood was bisulfite treated. For the CpG-rich region in the OXT promoter (chr20:3071,425-3071,795, hg38), an amplicon was designed using Agena’s Epidesigner software (San Diego, CA, USA). Thereafter, the amplicon was PCR amplified using the following primers: forward (aggaagaagATTAGGGTTGGGATTTTTTTTG; lower case letters indicate T7 primers) and reverse (cagtaatacgactcaatacaggagagtcACCTCTATACCCAAACCATTACC; lower case letters indicate T7 primers).

Next, in-vitro RNA transcription with subsequent base-specific cleavage using RNase A was performed. The resulting fragmented RNA molecules were of identical length and differed only in their nucleotide composition due to bisulfite treatment. After sample conditioning, products were processed on a MALDI-TOF platform (Agena; Massarray 4). Methylation status was quantified by analysis of the mass spectra. Resulting data from the mass spectrometer was preprocessed using the Epityper Analyser.

We assessed methylation status with respect to single CpG sites and units (some CpG sites lay on the same RNA fragment and methylation status reflects the mean of all sites on this fragment). Additionally, in order to have a single measure for methylation status, we calculated a weighted mean across all CpG sites and units (e. g. units of two CpG sites were doubly weighted). To justify that this was an adequate approach, we first performed a reliability analysis for all CpG sites and units (see the supplementary material, Table S3). Methylation status of CpG 19 and CpG 24.25 was least associated with methylation status of all other CpG units. For simplicity, however, we still included CpG 19 and CpG 24.25 when calculating mean OXT methylation. Reliability across CpG sites was excellent for both the group of individuals suffering from MDD (α = .91) and for healthy controls (α = .91). Descriptive statistics of all variables relevant for further analyses are in Table S2.

We used BECon (Edgar et al., 2017) to get an approximate idea of the correlations between methylation profiles in peripheral blood and brain tissue. BECon provides metrics on the concordance of DNA methylation between tissues based on paired samples of 16 individuals from three brain regions and whole blood. We searched for all available CpG sites in OXT. Data was available for six of the CpG sites that were analyzable in our study (CpG 3, 5, 9.10, 13 and 19). The highest correlation coefficients (between methylation status in peripheral blood and brain tissue of Brodmann area 10, 20 or 7) for each CpG unit ranged from r = 0.20–0.47.

2.4. Statistical analysis

Statistical analysis was conducted using R (R Development Core Team, 2008) with the packages psych (Revelle, 2018), matchIt (Ho et al., 2011), lme4 (Bates et al., 2015) and ggplot2 (Wickham, 2016) as well as JASP (JASP Team, 2019). Matching was performed calculating propensity scores for inpatients and controls. Thereafter, each control participant was matched one inpatient of the same sex using the nearest neighbor method (Ho et al., 2011). In order to ensure that differences between cases and controls were not only artifacts of participants being analyzed on different plates during the analysis of DNA methylation, we calculated two linear mixed effects models. In the first model, we only added plate as random intercept to predict mean OXT methylation. In the second model, we added age, sex, BMI, alcohol use, nicotine use and group as fixed effects. These analyses, which did not yield any effects for plate, can be found in the supplementary material (Table S4). Furthermore, we wanted to ensure that differences between MDD inpatients and healthy controls regarding methylation status of OXT were not associated with antidepressive medication in the MDD group. We therefore calculated Spearman’s correlation coefficients between methylation status of the OXT promoter and antidepressive medication in the group of MDD inpatients. Thereafter, we examined differences in critical life events and methylation status between the groups performing independent-sample Mann-Whitney U-tests (we also performed ANCOVAs to test whether differences in methylation status are also present when comparing the group of n = 146 inpatients from Sanwald et al. (2019) to the n = 90 healthy controls, Table S5). Further, we examined within-group correlations between SLEs, depression severity and OXT methylation for inpatients and controls. Therefore, we calculated partial Spearman’s correlation coefficients to control for potentially confounding effects of the covariates within-group associations. We added sex, age, BMI and substance use (alcohol and nicotine) as covariates in both groups regarding SLEs, methylation status and depression severity. Therefore, we calculated independent-
sample Mann-Whitney U-tests. To test for an interaction between group and sex regarding mean methylation of OXT, we ran a two-way ANOVA with group and sex as between subject factors. Mean methylation of OXT was the dependent variable.

We controlled false discovery rate (FDR) using Benjamini-Hochberg correction (Benjamini and Hochberg, 2000). Statistical significance was determined at \( p < .05 \); all tests two-tailed.

3. Results

3.1. Medication

After controlling FDR, there was only one significant association between methylation status of CpG 16 and dose equivalents for antidepressants (\( r = -0.27, p_{BH} = 0.021 \)). Nevertheless, we excluded four CpG units (CpG 1.2, CpG 7.8, CpG 16 and CpG 18) from calculation of mean methylation and any further analysis, since the methylation status of these units was significantly associated with dose equivalents before controlling FDR (Table 2). Mean methylation of OXT was neither significantly associated with dose equivalents for antidepressants (\( r = -0.21, p = .054, \) not FDR corrected) nor with dose equivalents for neuroleptics (\( r = 0.09, p = .429, \) not FDR corrected).

3.2. Group differences

MDD inpatients as compared to healthy controls had experienced significantly more critical life events and showed significantly lower methylation status with regard to mean methylation of the OXT promoter (Table 3; Fig. 2). Further, inpatients suffering from MDD showed a significantly lower methylation status than controls in all but three CpG units (CpG 9.10, CpG 13 and CpG 14.15). These results consistently show hypomethylation of the examined sites in the OXT promoter of inpatients suffering from MDD (Fig. 2). All effects were small to medium. This result pattern was also observed when performing ANCOVAs comparing the group of \( n = 146 \) inpatients from

![Fig. 1. A schematic representation of OXT. The target region (chr20:3,071,425-3,071,795, target length: 371 bp, hg38) starts 5’ of exon 1 (black font). Blue rectangles mark primer annealing sites. Non-italic letters mark the coding sequence (CDS) within exon 1. CpG 1-2 corresponds to chr20:3,071,452-3,071,455. Methylation status of red marked CpG sites was not analyzable. CpG site 20 had to be excluded due to too many missing values. Adapted from Sanwald and colleagues (2019). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.]

<table>
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<th>Target region</th>
<th>CpG island</th>
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<tr>
<td>ACCTCTG</td>
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<td>CTGACCCTG</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Spearman correlation coefficients between OXT methylation and medication in the group of MDD inpatients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>weighted mean dose/flu40 mg</td>
<td>weighted mean dose/CPZ100 mg</td>
</tr>
<tr>
<td>( r )</td>
<td>( p )</td>
</tr>
<tr>
<td>CpG 1.2</td>
<td>-0.22*</td>
</tr>
<tr>
<td>CpG 3</td>
<td>-0.21</td>
</tr>
<tr>
<td>CpG 4</td>
<td>-0.14</td>
</tr>
<tr>
<td>CpG 5</td>
<td>-0.17</td>
</tr>
<tr>
<td>CpG 7.8</td>
<td>-0.22*</td>
</tr>
<tr>
<td>CpG 9.10</td>
<td>-0.18</td>
</tr>
<tr>
<td>CpG 13</td>
<td>-0.16</td>
</tr>
<tr>
<td>CpG 14.15</td>
<td>-0.20</td>
</tr>
<tr>
<td>CpG 16</td>
<td>-0.27*</td>
</tr>
<tr>
<td>CpG 18</td>
<td>-0.22*</td>
</tr>
<tr>
<td>CpG 19</td>
<td>-0.16</td>
</tr>
<tr>
<td>CpG 24.25</td>
<td>-0.16</td>
</tr>
<tr>
<td>CpG 26</td>
<td>-0.09</td>
</tr>
</tbody>
</table>

Note. \( p \) values not FDR corrected. * \( p < .05 \).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Group differences in critical life events and methylation of the OXT promoter between MDD inpatients and healthy controls.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( W )</td>
<td>( p_{BH} )</td>
</tr>
<tr>
<td>CLEQ</td>
<td>2256.00</td>
</tr>
<tr>
<td>CpG 3</td>
<td>5013.50</td>
</tr>
<tr>
<td>CpG 4</td>
<td>4764.00</td>
</tr>
<tr>
<td>CpG 5</td>
<td>5033.00</td>
</tr>
<tr>
<td>CpG 9.10</td>
<td>4558.00</td>
</tr>
<tr>
<td>CpG 13</td>
<td>4607.00</td>
</tr>
<tr>
<td>CpG 14.15</td>
<td>4504.50</td>
</tr>
<tr>
<td>CpG 19</td>
<td>4894.50</td>
</tr>
<tr>
<td>CpG 24.25</td>
<td>4952.00</td>
</tr>
<tr>
<td>CpG 26</td>
<td>4927.00</td>
</tr>
</tbody>
</table>

Note. \( W \) Mann–Whitney U test, \( p_{BH} \) \( p \)-values controlled for FDR, effect size is given by the rank-biserial correlation.
Sanwald et al. (2019) to the group of \( n = 90 \) healthy controls (see Supplementary Material Table S5).

### 3.3. Correlation analyses

In MDD inpatients Spearman’s correlation analyses (Table 4) showed that SLEs were not significantly associated with mean methylation status of the examined region in the OXT promoter (\( r = -0.23, p_{BH} = 0.059 \) n.s.). Exclusion of CpG 19 and CpG 24.25 – these CpG units were least associated with all the other CpG units – yielded a significant negative association of \( r = -0.26, p_{BH} = 0.032 \). Further, in MDD inpatients SLEs were not significantly positively associated with depression severity (\( r = 0.24, p_{BH} = 0.053 \) n.s.). Note that this association only being marginally significant could be due to the smaller sample of inpatients as compared to the study by Sanwald et al. (2019), which showed a significantly positive association between SLEs and depression severity. Mean methylation was not significantly associated with depression severity (\( r = 0.00, p_{BH} = 0.997 \) n.s.). We consistently found small to medium size negative correlation coefficients between SLEs and OXT methylation in MDD inpatients. Only for the associations between CpG 19/CpG 24.25 and SLEs, whose methylation status deviated from the remaining investigated CpG sites (Table S3), there were very small positive correlation coefficients.

In the group of healthy controls, SLEs were not significantly associated with mean methylation status of the examined region in the OXT promoter (\( r = -0.07, p_{BH} = 0.620 \) n.s.). Exclusion of CpG 19 and CpG 24.25 did not yield a significant association (\( r = -0.09, p_{BH} = 0.499 \) n.s.). In healthy controls, SLEs were not significantly associated with depression severity (\( r = 0.21, p_{BH} = 0.089 \) n.s.). Mean methylation was not significantly associated with depression severity (\( r = 0.01, p_{BH} = 0.924 \) n.s.).

### 3.4. Sex differences

The explorative two-way ANOVA with group and sex as between subjects factor revealed significant main effects of group (\( F(1176) = 22.39, p < .00001, \eta^2 = 0.107 \)) and sex (\( F(1176) = 9.55, p = 0.002, \eta^2 = 0.046 \)) on mean OXT methylation but no significant interaction of group and sex. Women showed higher OXT methylation than men, and controls showed higher OXT methylation than MDD inpatients.

In the group of MDD inpatients independent sample Mann-Whitney U tests revealed no significant sex differences in SLEs or depression severity (Table 5). However, there were significant sex differences in mean OXT methylation and in seven of the nine CpG units with women showing higher OXT methylation.

In the group of healthy controls, there was only one significant sex difference. Women showed significantly higher methylation status of CpG 26 than men (Table 6).
Sex differences in critical life events, methylation of the OXT promoter and depression severity in the group of MDD inpatients.

Table 5

<table>
<thead>
<tr>
<th></th>
<th>Male M(SD)</th>
<th>Female M(SD)</th>
<th>N(male/ female)</th>
<th>W</th>
<th>P_BH</th>
<th>Rank-biserial correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>28.97(12.27)</td>
<td>33.68(9.92)</td>
<td>88(40/48)</td>
<td>728.500</td>
<td>0.071</td>
<td>−0.241</td>
</tr>
<tr>
<td>CLEQ</td>
<td>17.95(18.93)</td>
<td>21.46(15.21)</td>
<td>86(38/48)</td>
<td>699.000</td>
<td>0.077</td>
<td>−0.234</td>
</tr>
<tr>
<td>CpG 3</td>
<td>0.524(0.069)</td>
<td>0.585(0.095)</td>
<td>90(40/50)</td>
<td>599.000</td>
<td>0.006</td>
<td>−0.401</td>
</tr>
<tr>
<td>CpG 4</td>
<td>0.497(0.085)</td>
<td>0.549(0.102)</td>
<td>90(40/50)</td>
<td>670.500</td>
<td>0.014</td>
<td>−0.339</td>
</tr>
<tr>
<td>CpG 5</td>
<td>0.648(0.086)</td>
<td>0.702(0.095)</td>
<td>90(40/50)</td>
<td>679.500</td>
<td>0.014</td>
<td>−0.321</td>
</tr>
<tr>
<td>CpG 9.10</td>
<td>0.373(0.077)</td>
<td>0.418(0.081)</td>
<td>90(40/50)</td>
<td>675.000</td>
<td>0.014</td>
<td>−0.325</td>
</tr>
<tr>
<td>CpG 13</td>
<td>0.301(0.066)</td>
<td>0.363(0.069)</td>
<td>90(40/50)</td>
<td>511.500</td>
<td>0.001</td>
<td>−0.488</td>
</tr>
<tr>
<td>CpG 14.15</td>
<td>0.365(0.065)</td>
<td>0.412(0.077)</td>
<td>90(40/50)</td>
<td>640.000</td>
<td>0.012</td>
<td>−0.360</td>
</tr>
<tr>
<td>CpG 19</td>
<td>0.145(0.061)</td>
<td>0.140(0.047)</td>
<td>90(40/50)</td>
<td>978.000</td>
<td>0.093</td>
<td>−0.322</td>
</tr>
<tr>
<td>CpG 24.25</td>
<td>0.145(0.047)</td>
<td>0.141(0.035)</td>
<td>90(40/50)</td>
<td>997.000</td>
<td>0.964</td>
<td>−0.003</td>
</tr>
<tr>
<td>CpG 26</td>
<td>0.183(0.032)</td>
<td>0.208(0.045)</td>
<td>90(40/50)</td>
<td>670.000</td>
<td>0.014</td>
<td>−0.330</td>
</tr>
<tr>
<td>mean methylation</td>
<td>0.339(0.047)</td>
<td>0.374(0.059)</td>
<td>90(40/50)</td>
<td>646.500</td>
<td>0.012</td>
<td>−0.353</td>
</tr>
</tbody>
</table>

Note. Mann–Whitney U test. $p_{BH}$ refers to $p$-values controlled for FDR, effect size is given by the rank-biserial correlation.

Sex differences in critical life events, methylation of the OXT promoter and depression severity in the group of healthy controls.

Table 6

<table>
<thead>
<tr>
<th></th>
<th>Male M(SD)</th>
<th>Female M(SD)</th>
<th>N(male/ female)</th>
<th>W</th>
<th>P_BH</th>
<th>Rank-biserial correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>4.80(4.85)</td>
<td>5.16(4.73)</td>
<td>90(40/50)</td>
<td>939.000</td>
<td>0.621</td>
<td>−0.061</td>
</tr>
<tr>
<td>CLEQ</td>
<td>7.42(2.78)</td>
<td>11.70(9.63)</td>
<td>88(38/50)</td>
<td>660.000</td>
<td>0.090</td>
<td>−0.305</td>
</tr>
<tr>
<td>CpG 3</td>
<td>0.589(0.089)</td>
<td>0.603(0.089)</td>
<td>90(40/50)</td>
<td>876.000</td>
<td>0.473</td>
<td>−0.124</td>
</tr>
<tr>
<td>CpG 4</td>
<td>0.546(0.089)</td>
<td>0.581(0.100)</td>
<td>88(38/49)</td>
<td>752.500</td>
<td>0.245</td>
<td>−0.212</td>
</tr>
<tr>
<td>CpG 5</td>
<td>0.726(0.087)</td>
<td>0.739(0.085)</td>
<td>85(38/47)</td>
<td>810.000</td>
<td>0.507</td>
<td>−0.093</td>
</tr>
<tr>
<td>CpG 9.10</td>
<td>0.403(0.070)</td>
<td>0.430(0.085)</td>
<td>90(40/50)</td>
<td>791.500</td>
<td>0.245</td>
<td>−0.206</td>
</tr>
<tr>
<td>CpG 13</td>
<td>0.342(0.072)</td>
<td>0.366(0.078)</td>
<td>90(40/50)</td>
<td>816.000</td>
<td>0.254</td>
<td>−0.184</td>
</tr>
<tr>
<td>CpG 14.15</td>
<td>0.388(0.082)</td>
<td>0.420(0.083)</td>
<td>90(40/50)</td>
<td>798.500</td>
<td>0.245</td>
<td>−0.202</td>
</tr>
<tr>
<td>CpG 19</td>
<td>0.219(0.124)</td>
<td>0.203(0.157)</td>
<td>80(36/44)</td>
<td>787.500</td>
<td>0.480</td>
<td>0.110</td>
</tr>
<tr>
<td>CpG 24.25</td>
<td>0.206(0.068)</td>
<td>0.188(0.078)</td>
<td>74(34/40)</td>
<td>765.000</td>
<td>0.479</td>
<td>0.125</td>
</tr>
<tr>
<td>CpG 26</td>
<td>0.198(0.047)</td>
<td>0.234(0.056)</td>
<td>90(40/50)</td>
<td>568.000</td>
<td>0.005</td>
<td>−0.432</td>
</tr>
<tr>
<td>mean methylation</td>
<td>0.389(0.058)</td>
<td>0.411(0.077)</td>
<td>90(40/50)</td>
<td>821.500</td>
<td>0.254</td>
<td>−0.178</td>
</tr>
</tbody>
</table>

Note. Mann–Whitney U test. $p_{BH}$ refers to $p$-values controlled for FDR, effect size is given by the rank-biserial correlation.

The comparison of MDD men to healthy men revealed significant group differences in SLEs with more SLEs in MDD men than in healthy men. In addition, MDD men had significantly lower mean OXT methylation and significantly lower methylation levels in all but three CpG units (Table 7).

The comparison of MDD women to healthy women revealed significant group differences in SLEs with more SLEs in MDD women than in healthy women. In addition, MDD women had significantly lower mean OXT methylation and significantly lower methylation levels in three CpG units (Table 7).

4. Discussion

In the present study, we examined whether MDD is associated with SLEs and methylation status of the OXT promoter. As expected, depressed inpatients reported having experienced more SLEs. Even though SLEs are a rather unspecific risk factor being associated with the development of many mental disorders such as schizophrenia (Gallagher et al., 2016) and posttraumatic stress disorder (Cromer and Sachs-Ericsson, 2006), past SLEs are consistently found in individuals suffering from MDD (Dunn et al., 2017; Norman et al., 2012). According to the diathesis-stress model of depression, an individual’s vulnerability and the experienced environmental or social stressors interact thereby forming an individual’s risk for depression development (Colodro-Conde et al., 2018). Adverse family history and SLEs early in life are not only predictors for depression development but may also result in worse stress coping strategies, resulting in the perception of current stressors as more challenging and finally in the perpetuation of depressive symptomatology (Flammer et al., 1992).

As expected, depressed inpatients consistently showed significantly lower methylation levels across the examined region in the OXT promoter compared with healthy controls. This effect was found for mean OXT methylation of both men and women. At first glance, the results of earlier studies that analyzed an overlapping region in the OXT promoter (Haas et al., 2016; Toepfer et al., 2019) seem to be in contrast to our findings. In these studies, higher methylation of the OXT promoter was associated with a more anxious attachment style, less accuracy in emotion recognition (Haas et al., 2016) and intrusiveness in mothers (Toepfer et al., 2019). These studies, however, did not examine MDD and their results are therefore hardly generalizable to our patient population. Previously, we examined the association of OXT methylation and depression severity in a sample of inpatients suffering from MDD (Sanwald et al., 2019). In the earlier study, including a larger patient sample than the present, there was also no significant association between OXT methylation and depressive symptom severity, but a significant negative association between SLEs and OXT methylation: MDD inpatients experienced more SLEs, and SLEs were associated with less OXT methylation. In confirming and extending this earlier work, the present study demonstrates a lower OXT methylation status in patients with MDD compared to a matched control sample, whose data was not available previously.

An explanation for hypomethylation of OXT in MDD could be related to depression development: In Affective Neuroscience Theory by Panksepp (2004, for principles of Pankseppian Neuroscience see (Davis and Montag, 2019)) depression is assumed to be a shutdown mechanism following pathologically prolonged separation distress. An initial loss initiates a protest phase in which the individual actively searches to soothe separation distress (in humans, this loss or separation may also be symbolic, i.e. without any overt or observable loss). If this goal cannot be achieved for a long time, the organism enters a despair phase. In this phase, the individual shuts down SEEKING behavior to preserve resources (Zellner et al., 2011). The OT system is one factor able to soothe separation distress (Davis and Montag, 2019; Panksepp, 1992). Thus, an upregulation of the OT system – low OXT
promoter methylation may be an indicator of higher OT expression – could be an attempt to counteract feelings of prolonged separation distress. This assumption, however, needs to be tested in future studies. Studies investigating the association of OXTR promoter methylation and depression show heterogeneous results. While three studies report an increased OXTR promoter methylation to be associated with depressive symptoms in women (Bell et al., 2015; Chagnon et al., 2015; Reiner et al., 2015), a fourth study reports less methylation being associated with symptoms of depression in a cohort of African Americans (Smerman et al., 2016).

As expected, in MDD inpatients OXT promoter methylation of three CpG units was significantly associated with SLEs. No such associations were found in the group of healthy controls. The higher the number of SLEs experienced by the group of MDD inpatients, the lower their methylation levels in the OXT promoter. All correlation coefficients were small to medium, which shows that stress is not the only factor associated with OXT methylation. However, in line with other research, our results provide evidence for an association between SLEs and DNA methylation (Essex et al., 2013; Unternaehrer et al., 2012). More specifically, our findings demonstrate that SLEs are related to changes in the endogenous OT system. However, unlike earlier studies suggesting SLEs to be associated with lower levels of OT (Donadon et al., 2018), we found lower OXT promoter methylation, i.e. presumably higher OT activity, to be related to SLEs. It is worth noting, that there is also considerable heterogeneity of findings with respect to OT levels in depression. While some studies suggest an inverse relationship of OT levels and depression (Scantamburlo et al., 2007; Yuen et al., 2014), other studies suggest the opposite (Parker et al., 2010). In addition, not only findings with regard to OT levels, but also findings with regard to OXTR methylation are heterogeneous: On the one hand, one study reported lower OXTR methylation to be decreased as a function of distinct prenatal adversities (Unternaehrer et al., 2016). On the other hand, another recent study reported female PTSD patients to have higher OXTR methylation than male PTSD patients or healthy controls (Nawijn et al., 2019). For the negative association of OXTR methylation and prenatal adversities it is argued that it could reflect an epigenetic adaption to an adverse environment (Unternaehrer et al., 2016). Possibly, OT initially dampens the acute stress response (Windle et al., 1997) and promotes prosocial behavior after a SLE preventing social avoidance (Lukas et al., 2011), i.e. it would be an adaptive process to release OT directly after experiencing a stressor. Chronically high availability of OT, however, has been shown to induce an anxiogenic phenotype and reduced OT receptor binding (Peters et al., 2014). Therefore, SLEs could trigger OXT demethylation to prepare the organism for dampening the stress response. Resulting chronically higher availability of OT, however, could be maladaptive increasing an individual's risk for depression development by inducing an anxiogenic phenotype and reduced OT receptor binding. This explanation is in line with results showing acute anxiolytic effects of OT (Blume et al., 2008; Martinetz et al., 2019; Radtke et al., 2011). By contrast, other studies showed an OT upregulation after chronic stress (Ondrejkova et al., 2010) as well as dose dependent effects of OT indicating that chronic infusions of high OT concentrations but not of low OT concentrations induce an anxiogenic phenotype (Peters et al., 2014). One way to better understand the heterogeneous effects of OT for depression would be the comparison of MDD patients with healthy controls in longitudinal studies investigating how changes in depressive symptoms are related to changes in OXT (and/or OXTR) methylation, corresponding mRNA levels and OT concentrations.

We found sex differences in mean OXT methylation only in the group of MDD inpatients. Women suffering from depression showed significantly higher OXT methylation than MDD men did. This could point towards sex differences in depression being associated with OXT methylation. Our finding, however, conflicts with studies reporting higher OT plasma levels in women than in men (Maraziti et al., 2019). However, there is a large number of studies reporting no sex difference with respect to OT mRNA expression or plasma OT (e.g. Dumais et al., 2013; Gordon et al., 2008). Furthermore, women were shown to have higher OXTR methylation than men (Nawijn et al., 2019; Rubin et al., 2017). Apart from that, our results are in line with the associations of postpartum depressive symptoms with lower levels of plasma OT (Jobst et al., 2016; Lara-Cinismo et al., 2017), if OXT promoter methylation is associated with transcriptional inactivity as it is often suggested (Jones and Takai, 2001).

Even though we used a matched case control design, some limitations need to be considered when interpreting the results of our study. First, even though there seem to be medium size associations between OXT methylation in blood and in different brain areas as indicated by BECon (Edgar et al., 2017), methylation in whole blood samples can only be considered a proxy for epigenetic profiles in brain tissue. Second, we did not assess mRNA or protein levels and therefore cannot draw conclusions regarding the functionality of the observed differences in OXT methylation between inpatients suffering from MDD and healthy controls. Third, we cannot draw conclusions about hypo-methylation being OXT specific, i.e. other genes or even the whole genome could be hypomethylated in MDD patients. For example global DNA hypermethylation has been observed for Alzheimer’s disease (Di Francesco et al., 2015). However, there are first indications that depressive symptoms are associated with DNA methylation levels of genes implicated in response to stress, depressive-like behaviors, and recurrent depression in patients, but not with global DNA methylation changes across the genome (Starnawska et al., 2019). Last, we used an unspecified assessment of life stress regarding the timing of the stressor, which did not allow for differentiation between early and late life.
stressors. This could be of interest in light of the fact that timing specific effects on methylation status have been suggested for other genes, e. g. NR3C1 (Palma-Gudiel et al., 2015).

Nonetheless, our study provides first indications that MDD is associated with alterations in OXT methylation and supports the notion of SLEs being a major risk factor for developing psychopathology. More specifically, traumatic events leave their traces in the epigenetic profiles of individuals suffering from MDD. Thus, investigating the epigenetic landscape could contribute to a better understanding of the influence of stress on a molecular level and shed light on the biological underpinnings of MDD and resilience to psychopathology.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the ethics committee of Ulm University, Ulm, Germany (reference number: 25/18) and with the 1964 Helsinki declaration and its later amendments.

CRediT authorship contribution statement

Simon Sanwald: Conceptualization, Writing - original draft, Formal analysis, Writing - review & editing. Katharina Widhenhorn-Müller: Writing - review & editing. Christian Montag: Conceptualization, Writing - review & editing. Markus Kiefer: Conceptualization, Writing - review & editing.

Declaration of Competing Interest

There are no competing financial interests to be disclosed.

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Supplementary materials


References


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Katharina Widenhorn-Müller:

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Jobst, A., Krause, D., Maiwald, C., Härtl, K., Myint, A.-.M., Kästner, R., Obermeier, M., JASP Team, 2019. JASP (Version 0.11.1).


2.4. Study IV - Factors related to age at depression onset: The role of SLC6A4 methylation, sex, exposure to stressful life events and personality in a sample of inpatients suffering from major depression


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Factors related to age at depression onset: the role of SLC6A4 methylation, sex, exposure to stressful life events and personality in a sample of inpatients suffering from major depression

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Abstract

Background: An early onset of depression is associated with higher chronicity and disability, more stressful life events (SLEs), higher negative emotionality as described by the primary emotion SADNESS and more severe depressive symptomatology compared to depression onset later in life. Additionally, methylation of the serotonin transporter gene (SLC6A4) is associated with SLEs and depressive symptoms.

Methods: We investigated the relation of SLEs, SLC6A4 methylation in peripheral blood, the primary emotions SADNESS and SEEKING (measured by the Affective Neuroscience Personality Scales) as well as depressive symptom severity to age at depression onset in a sample of \( N = 146 \) inpatients suffering from major depression.

Results: Depressed women showed higher SADNESS (\( t (91.05) = -3.17, p = 0.028, d = -0.57 \)) and higher SLC6A4 methylation (\( t (88.79) = -2.95, p = 0.02, d = -0.55 \)) compared to men. There were associations between SLEs, primary emotions and depression severity, which partly differed between women and men. The Akaike information criterion (AIC) indicated the selection of a model including sex, SLEs, SEEKING and SADNESS for the prediction of age at depression onset. SLC6A4 methylation was not related to depression severity, age at depression onset or SLEs in the entire group, but positively related to depression severity in women.

Conclusions: Taken together, we provide further evidence that age at depression onset is associated with SLEs, personality and depression severity. However, we found no associations between age at onset and SLC6A4 methylation. The joint investigation of variables originating in biology, psychology and psychiatry could make an important contribution to understanding the development of depressive disorders by elucidating potential subtypes of depression.

Keywords: SLC6A4, DNA methylation, 5-HTTLPR, Stress, Major depression, Primary emotions, Age at onset

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† Christian Montag and Markus Kiefer contributed equally to this work.
Background
Despite the high prevalence of Major Depressive Disorder (MDD) [1], there has only been limited success in identifying reliable biomarkers [2]. Reasons for difficulties in the identification and replication of depression associated genetic risk loci are now gradually becoming apparent. One complicating factor is that depression is a polygenic disorder with single genes only explaining small amounts of variance [3, 4]. Second, depression has a high lifetime prevalence of about 15% [5]. Since depression is a common disorder showing one of the highest prevalences regarding psychiatric disorders, there is only a small mean difference in phenotypic liability between case/patient and control groups and thus reduced power to detect differences in allele frequencies between them [3]. Last, there is an ongoing debate about depression being one homogeneous clinical syndrome or a subsumption of distinct phenomenological entities under one diagnostic label [2]. Twin studies indicate that 45% of genetic liability to depression is not shared between sexes [6–8]. In addition, it has been shown that subgrouping depression according to recurrence or early age at depression onset yielded higher heritability estimations in case of recurrent depressive episodes and early onset depression [9, 10].

Early-onset depression may also differ from late-onset depression with respect to the course and symptoms of the disorder: Patients who had their first depressive episode early in life showed a shorter time to relapse and more residual symptoms after recovery [11]. Moreover, they reported higher chronicity and disability [12], had experienced more stressful life events (SLEs) and differed in depressive symptomatology from patients with adult-onset depression [13]. High scores on the personality dimension neuroticism is considered a vulnerability factor associated with early age at depression onset [13–15]. The association of depression onset with SLEs and neuroticism is in line with a theory of depression development taking into account mammalian-brain emotional systems, bridging the gap between a dysregulation of the bodily stress system and affective changes of depression [16]. In short, this theory suggests a stressor to cause initially elevated efforts to terminate the stress response. When termination fails, a behavioral shutdown protects the individual against the fatal consequences of wasting too much resources but leaving it with lassitude and despair [17]. This theory builds upon the framework of affective neuroscience, a term coined by Panksepp [18]. Affective neuroscience theory (see also [19]) promotes the view that human personality is anchored in primary emotional systems comprising the fundamental emotional tendencies common among all mammals [20–22]. According to the above theory of depression development, depression is mainly associated with two primary emotions: SEEKING and SADNESS [16]. The SEEKING system is utilized by other primary emotions and is defined as the effort made to mitigate a negative emotional state or the search for vital resources. SADNESS on the other hand is triggered by separation distress such as the loss of a child or being apart from a loved one (in humans the separation or loss may also be a symbolic one) and eventually precipitates depression [20]. Both, low SEEKING and high SADNESS are associated with depression severity [23] but whether they are associated with age at depression onset has, to the best of our knowledge, not yet been investigated.

At the same time the serotonin (5-HT) system has a long history in the research on the pathophysiology of affective disorders [24]. Early theories of depression development postulate depression to originate from a dysfunction of monoaminergic neurotransmission. More recent theories suggest a modulating and not a causal role of 5-HT in the etiology of depression [24]. Considering its important role for early theories of depression development, it is not surprising that the 5-HT system has often been investigated in search for genetic markers of depression risk. The serotonin transporter (5-HTT) is one of the most investigated parameters of the 5-HT system in depression research [25, 26]. 5-HTT is one of several structurally similar transporter proteins having a high affinity for monoamines. These presynaptic membrane proteins transport their substrate from the extracellular space to the cytoplasm terminating serotonergic neurotransmission and recycling presynaptic supplies of serotonin [27]. The interest in the 5-HTT originates from antidepressants such as selective serotonin reuptake inhibitors (SSRIs) directly binding to 5-HTT inhibiting 5-HT reuptake [28]. As a consequence of SSRI treatment, 5-HTT is highly regulated and undergoes adaptive changes [29]. The SLC6A4 gene encoding the 5-HTT, is located on chromosome 17 q.11.1-q12 [30], has a length of 31 kilo bases and contains 14 exons [31]. The serotonin transporter linked polymorphic region (5-HTTLPR) – a repeat length polymorphism – is one of the most investigated polymorphisms in depression research [32]. The short (S) allele is associated with a lower transcription rate of the 5-HTT compared with the long (L) allele [33]. Former reports of carriers of the S-allele being more likely to develop MDD as a function of SLEs (and sometimes sex) could not be confirmed in a recent meta-analysis [32].

However, in the last years another field of research emerged impressively demonstrating that the transcriptional apparatus and thus the phenotype is not hardwired by the genome. In fact, the genome is the individual starting point for adaptation processes becoming necessary during cellular differentiation and due to individually different environmental challenges. The name epigenetics became established as term for
processes bridging the gap between genotype and phenotype [34]. The most investigated epigenetic mechanism is DNA-methylation [35]. DNA-methylation has mostly been examined in cytosine-phosphate-guanine (CpG) dinucleotides. In CpG dinucleotides a methyl group is added to the 5′-position of the cytosine residue [35]. CpG-methylation affects histone DNA interactions thereby modulating a gene’s accessibility for transcription factors and thus transcriptional activity [36, 37]. This is not surprising in light of the fact that CpG rich regions are frequently located in the promoter regions of genes [38].

Increased methylation of the whole CpG island or specific CpG sites in the promoter region of the SLC6A4 gene has already been associated with decreased transcription rate [39], lower mRNA concentrations [40], SLEs and recent depressive symptoms [41, 42], family history of depression [43] as well as post-stroke depression [44]. There are, however, ambiguous results regarding the association between SLC6A4 promoter methylation and depression severity [42, 43, 45–47]. In addition, there are reports of an interaction of 5-HTTLPR genotype and DNA-methylation in the investigation of the effects of stress on stress-related phenotypes [40, 48]. Thus, variations in SLC6A4 expression need to be integrated with the contribution arising from genetic as well as epigenetic mechanisms [49]. However, DNA methylation may also be sex specific. Especially in depression with its sexually dimorphic risk for depression development [50]. Accordingly, CpG sites in the SLC6A4 gene have been reported to be differentially methylated as a function of sex with females exhibiting higher SLC6A4 methylation than men [51]. Therefore, sex by genotype interactions should be explored when investigating SLC6A4 methylation in depression.

Taken together, depression can be understood as reduced SEEKING/higher SADNESS as a consequence of chronically prolonged separation distress [16]. The 5-HT system plays an important role in affective disorders [52] with the 5-HTT being the target for a large group of antidepressants [28]. Furthermore, SLEs are associated with SLC6A4 methylation and early-onset depression [13, 41]. Early-onset depression has been shown to have a higher heritability compared to the investigation of depression independent of age at onset [10]. There are findings of interactions between sex and genetic as well as epigenetic layers of SLC6A4 regulation [40, 48, 51]. Thus, we wanted to examine the relation of SLEs, primary emotions, DNA-methylation of SLC6A4 and their interactions to age at depression onset in a sample of inpatients suffering from MDD. We assumed depression onset to be positively associated with SLEs, SADNESS and depression severity and negatively associated with SEEKING. We also assumed an association between age at onset and SLC6A4 methylation. Considering the heterogeneity of findings in previous studies, we did not infer a directional hypothesis for associations between age at onset and SLC6A4 methylation. In addition, we wanted to explore possible sex differences between SLEs, primary emotions, age at depression onset, SLC6A4 methylation and depression severity. Last, we wanted to examine which factors (sex, SLEs, primary emotions, 5-HTTLPR genotype and SLC6A4 methylation) or interactions of factors predict age at depression onset.

**Methods**

**Participants**

Data of $N = 146$ inpatients ($n = 95$ females, age: $M = 38.74, SD = 14.25$) diagnosed for major depression at the time of admission to the hospital was taken from the database of the Ulm Gene Brain Behavior Project (UGBBP). Data from this sample was used for earlier studies focusing on other parameters [23, 53, 54]. All inpatients were recruited at the Department of Psychiatry and Psychotherapy III at Ulm University, Ulm, Germany. They were diagnosed for Major Depression by a psychiatrist at admission to the hospital using the Structured Clinical Interview for DSM-IV (SCID-I) [55]. Participants were administered the self-assessment questionnaires described below. Depression severity was rated by a trained interviewer using the Montgomery Asberg Depression Rating Scale (MADRS) [56]. Sociodemographic data was collected with a standardized semi-structured interview based on an in-house questionnaire. Further, we assessed age, Body Mass Index (BMI in kg/m$^2$), consumed alcohol (grams/day) and nicotine (cigarettes/day). Patients were asked when they had had their first episode of at least 2 weeks suffering from depressive mood, loss of interest and other depressive symptoms. We calculated dose equivalents for current antidepressants (weighted mean dose/fluoxetine 40 mg) [57] and neuroleptics (weighted mean dose/chlorpromazine 100 mg) [58–60]. Two patients fulfilled the DSM-IV criteria [55] for alcohol abuse but not alcohol dependence. We decided to include them in our analyses since we controlled for alcohol consumption. One patient had the diagnosis of a sexual dysfunction not otherwise specified. 56.8% of inpatients ($n = 83$) reported to know about the presence of psychiatric disorders in their family (depression: $n = 56$ with $n = 38$ in parents or siblings and $n = 18$ in more distant relatives or not otherwise specified; schizophrenia: $n = 5$ with $n = 2$ in parents or siblings; anxiety: $n = 4$ with $n = 2$ in parents or siblings; personality disorders: $n = 3$ with $n = 1$ in siblings; bipolar disorder: $n = 2$ in parents or siblings; eating disorders: $n = 3$ in more distant relatives; substance abuse: $n = 2$ with $n = 1$ in both parents and siblings; obsessive compulsive disorder: $n = 1$ in parents; attention deficit syndrome:}
n = 1 in siblings; not otherwise specified: n = 19). Median age at depression onset was 21 years. Please note that there is an overlap between the present manuscript and older publications [23], where the ANPS has been investigated in the context of BDI scores in a case-control design (but without SLEs and epigenetic variables).

**Questionnaires**

**CLEQ**
The Critical Life Events Questionnaire (CLEQ) assesses 30 traumatic life events such as sexual abuse, experience of violence or death of a close person. The participants answered a question of whether they had ever experienced the concerning event [61]. We calculated a score adding up the experienced events. If there were 9 or more unanswered events, participants were excluded from further analysis with the CLEQ.

**Affective neuroscience personality scales (ANPS)**
The ANPS German version [62] comprises 110 items assessing individual tendencies in six primary emotional systems: SEEKING, CARE, PLAY (positive emotionality) and FEAR, ANGER, SADNESS (negative emotionality). The primary emotion of LUST may potentially have negative carry over effects on the remaining items, if items on one’s own sexual behavior would be filled in. All items are answered on a four point Likert scale ranging from strongly disagree [1] to strongly agree [4]. Internal consistency of the SEEKING scale was acceptable (α = .77, n = 110, n = 36 were excluded listwise), internal consistency of the SADNESS scale was also acceptable (α = .73, n = 116, n = 30 were excluded listwise).

**BDI-II**
Severity levels of depressive symptoms were explored by using the Beck Depression Inventory (German version, BDI-II) [63]. The BDI-II is an internationally recognized clinical and research psychopathological-psychometric instrument recording the severity of a depressive syndrome. The BDI-II is a self-assessment scale and comprises 21 items. For each item ratings between 0 (not at all) and 3 (very intensive) are given depending on the symptom severity. A maximum of 63 points in total can be reached. Internal consistency was good with α = .84.

**Genotyping of 5-HTTLPR**
DNA extraction from whole blood samples was performed on Magna Pure 96 using a commercial extraction kit (Roche, Mannheim, Germany). Genotyping of the 5-HTTLPR including rs25531 was carried out as described in Lachmann and colleagues [64]. The combination of information from the 5-HTTLPR and rs25531 results in the distinction between the variants L_A and L_G. L_G is functionally similar to the S allele [65]. Frequencies of 5-HTTLPR/rs25531 genotype fulfilled Hardy-Weinberg-Equilibrium expectations with respect to 5-HTTLPR (49 L/L, 72 L/S, 23 S/S; χ²(1) = 0.16, p = 0.69). To maximize statistical power, groups were dichotomized into L_ALA homozygotes and S/L_G carriers (42 L_ALA, 102 S/L_G).

**Quantification of SLC6A4 promoter methylation**
Methylation status of the SLC6A4 gene (Fig. 1) was quantified by Varionostic GmbH (Ulm, Germany) using the Sequenom Epityper MassArray System (San Diego, CA, USA). Genomic DNA from peripheral blood was bisulfite treated. Amplicons of the CpG-rich region in the SLC6A4 promoter (chr17:30235345–30,236,068; amplicon 1: chr17:30235345–30,235,765; amplicon 2: chr17:30235734–30,236,068; hg38) were designed using Agena’s Epidesigner software (San Diego, CA, USA). These amplicons were PCR amplified using the following primers: amplicon 1: forward (aggaagagag GTTATTTAGAGAGGATATGTTGAG GTG) and reverse (cagtaatcactacagggagaagctCAACAATAAAAACCCCCTCA); amplicon 2: forward (aggaagagag GGTATTTTATATGGTTTGATTTT TAG) and reverse (cagtaatcactacagggagaagctCACCTACTCCTTTTACACCTCC).

In a next step, in-vitro RNA transcription with subsequent base-specific cleavage using RNase A was performed. This procedure resulted in fragmented RNA molecules of identical length. RNA molecules differed in their nucleotide composition due to bisulfite treatment. After sample preparation, a MALDI-TOF platform (Agena; MassArray 4) was used to process the probes. Resulting data from the mass spectrometer was preprocessed using the EpiTyPER Analyser. Methylation status was quantified by analyzing the mass spectra.

We assessed methylation status regarding single CpG units (some CpG sites lay on the same RNA fragment and methylation status reflects the mean of all sites on this fragment). Boxplots for CpG units with analyzable methylation status are depicted in Fig. 2. Since we wanted to have a joint measure of SLC6A4 DNA methylation, we first analyzed the associations between all CpG sites and units (see the supplementary material, Table S1). Reliability across CpG sites was good (α = .82). However, there were very low correlation coefficients between some of the CpG units. Therefore, we performed a Principal Component Analysis (PCA) to extract the most important independent factors for SLC6A4 methylation. The Kaiser–Meyer–Olkin measure of sampling adequacy was .698, representing a relatively good factor analysis, and Bartlett’s test of sphericity was
significant \( p < .001 \). Only factors explaining more than 10% of variance in the methylation data were considered [67], resulting in a two factor solution. The two factors accounted for 31.44% of the total variance in SLC6A4 methylation. Among the factor solutions, the varimax-rotated two-factor solution yielded the most interpretable solution (factor 1: centric to terminal CpG units of the investigated region; factor 2: anterior and centric CpG units), and most CpG units loaded highly on only one of the two factors (for further information and the varimax-rotated two factor solution see supplementary material Tables S2). These two factors of SLC6A4 methylation were entered in the statistical analyses.

**Statistical analysis**

Statistical analyses were conducted using R [68] with the packages **psych** [69], and **ggplot2** [70] as well as IBM SPSS Statistics for Windows, version 25. To control for confounding variables associated with SLC6A4 methylation or age at depression onset, we tested associations between potential covariates (age, BMI, substance use, dose equivalents of antidepressants and neuroleptics) and SLC6A4 methylation as well as age at depression onset using Spearman’s correlation coefficients. Next, we investigated sex differences and differences between inpatients without and with a family history of psychiatric disorders performing Welch’s t-tests (note that U-tests provided similar results). Thereafter, we calculated

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**Fig. 1** Schematic representation of the SLC6A4 gene. The position of the examined CpG island is marked by a light grey box. CpG sites are marked in yellow color and numbered. Bold and italic letters mark the putative TATA-box. Exon 1A is underlined.

**Fig. 2** Boxplots for the methylation status of the examined CpG Sites in the SLC6A4 CpG island.
partial Spearman’s correlation coefficients between the variables of interest controlling for potentially confounding variables. In detail, we examined associations between depression severity (BDI-II and MADRS), CLEQ score, SEEKING, SADNESS, age at depression onset as well as both factors of SLC6A4 methylation. In addition, we wanted to explore, which of the examined variables is a predictor of depression onset and whether interactions between sex, 5-HTTLPR genotype and SLC6A4 methylation affect early depression onset. Thus, we performed a stepwise hierarchical linear regression analysis with sex, CLEQ, SEEKING, SADNESS, 5-HTTLPR genotype (L/L vs. S+), SLC6A4 factor 1, SLC6A4 factor 2 as well as interactions between DNA methylation and sex as well as 5-HTTLPR genotype as predictors in the full model. Age at depression onset was the dependent variable. We used an automatic stepwise model selection by the Akaike information criterion (AIC) allowing for iteratively adding and removing predictors. Benjamini-Hochberg correction was used controlling false discovery rate (FDR) [71]. Statistical significance was determined at p < .05; all tests were two-tailed.

Results
Control analyses
Age and BMI were significantly associated with SLC6A4 methylation and/or age at depression onset (Table 1). Therefore, age and BMI were added as covariates in all analyses reported below. 5-HTTLPR genotype groups did not differ in SLC6A4 methylation neither for methylation factor 1 (L ALA: n = 35, M = 0.06, SD = 1.04; S/L G: n = 91, M = −0.08, SD = 0.93; t (56.06) = 0.68, p = .50) nor for methylation factor 2 (L ALA: n = 35, M = −0.22, SD = 0.09; S/L G: n = 91, M = 0.09, SD = 1.08; t (73.96) = −1.62, p = .11) nor for age at depression onset (L ALA: M = 26.02, SD = 12.72; S/L G: M = 25.78, SD = 13.44; t (80.49) = 0.11, p = .92). There were no significant genotype differences in any of the investigated variables even before controlling FDR (see supplementary material Table S4).

Sex differences and differences between inpatients without and with a family history of psychiatric disorders
The investigation of sex differences revealed women to score higher than men with respect to the primary emotion SADNESS. Furthermore, women showed higher methylation of centric to terminal CpG sites as indexed by factor 1 of SLC6A4 methylation. There were no sex differences in any of the other variables. Exact descriptive and inferential statistics can be found in (Table 2). There were no significant differences between inpatients without and inpatients with a family history of psychiatric disorders after FDR correction (Table 3).

Correlation analyses
Partial Spearman’s correlation analyses of the whole sample (Table 4) showed that after FDR correction there was a significantly positive association between CLEQ and depression severity (with both BDI-II and MADRS) as well as SADNESS. In addition, the CLEQ was significantly negatively associated with age at depression onset. SEEKING was significantly negatively associated with depression severity (with both BDI-II and MADRS) and SADNESS. SADNESS on the other hand was significantly positively associated with depression severity (with both BDI-II and MADRS) and significantly negatively associated with age at depression onset. Besides the significantly negative associations between age at onset and CLEQ as well as SADNESS, age at onset was also

<table>
<thead>
<tr>
<th>Variable</th>
<th>SLC6A4 factor 1</th>
<th>SLC6A4 factor 2</th>
<th>age at onset</th>
</tr>
</thead>
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<tr>
<td>Age</td>
<td>0.41</td>
<td>0.21</td>
<td>0.60</td>
</tr>
<tr>
<td>BMI</td>
<td>0.000</td>
<td>0.019</td>
<td>0.000</td>
</tr>
<tr>
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</tr>
<tr>
<td>cigarettes/day</td>
<td>−0.05</td>
<td>−0.17</td>
<td>−0.04</td>
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<td>DE antidepressants</td>
<td>−0.14</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>DE neuroleptics</td>
<td>0.165</td>
<td>0.324</td>
<td>0.149</td>
</tr>
<tr>
<td>DE dose equivalents</td>
<td>0.05</td>
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<td>0.05</td>
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<tr>
<td>P-values not FDR corrected</td>
<td>0.623</td>
<td>0.110</td>
<td>0.585</td>
</tr>
</tbody>
</table>

DE dose equivalents. Spearman’s correlation coefficients. P-values not FDR corrected.
significantly negatively associated with depression severity (BDI-II only).

Analysis of both sexes separately revealed similar result patterns. However, the significantly positive association between CLEQ and depression severity as well as SADNESS was present only in men. In addition, men as compared to women showed medium size negative associations between age at depression onset and depression severity. This association was significant for the MADRS. In women, we found a significantly positive association of medium size between methylation factor 2 of SLC6A4 and depression severity (MADRS only). This association was not present in men.

For associations between single CpG sites/units and depression severity, CLEQ, SEEKING, SADNESS and age at onset, see the Supplementary Material (Table S3).

**Hierarchical linear regression analysis**

In order to determine which predictors explain a significant amount of variance considering age at depression onset, we performed a stepwise regression analysis (both directions). We included age and BMI as covariates and sex, CLEQ, SEEKING, SADNESS, 5-HTTLPR genotype, SLC6A4 factor 1, SLC6A4 factor 2 as well as interactions between DNA methylation, sex and 5-HTTLPR genotype as predictors in the full model. All metric variables

### Table 2 Sex differences in the covariates and variables of interest

<table>
<thead>
<tr>
<th>Covariate</th>
<th>n (m/f)</th>
<th>M_men</th>
<th>SD_men</th>
<th>M_women</th>
<th>SD_women</th>
<th>t</th>
<th>df</th>
<th>p_BH</th>
<th>d</th>
</tr>
</thead>
<tbody>
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<td>Age</td>
<td>51/95</td>
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<td>13.98</td>
<td>38.44</td>
<td>14.46</td>
<td>0.35</td>
<td>105.44</td>
<td>0.785</td>
<td>0.06</td>
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<tr>
<td>BMI</td>
<td>51/95</td>
<td>26.48</td>
<td>4.68</td>
<td>25.66</td>
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<td>0.85</td>
<td>137.30</td>
<td>0.557</td>
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<tr>
<td>alcohol (grams/day)</td>
<td>49/89</td>
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<td>20.76</td>
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<td>53.55</td>
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</tr>
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<td>cigarettes/day</td>
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<td>10.53</td>
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<td>0.028</td>
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<tr>
<td>age at onset</td>
<td>51/95</td>
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<td>88.47</td>
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<td>0.13</td>
<td>0.91</td>
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<td>88.79</td>
<td>0.028</td>
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<tr>
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<td>1.58</td>
<td>79.43</td>
<td>0.280</td>
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</tbody>
</table>

Welch’s t-test

### Table 3 Differences between inpatients without (no) and with (yes) family history of psychiatric disorders

<table>
<thead>
<tr>
<th>Covariate</th>
<th>n (no/yes)</th>
<th>M_no</th>
<th>SD_no</th>
<th>M_yes</th>
<th>SD_yes</th>
<th>t</th>
<th>df</th>
<th>p_BH</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44/83</td>
<td>41.68</td>
<td>12.93</td>
<td>37.51</td>
<td>14.31</td>
<td>1.67</td>
<td>95.74</td>
<td>0.453</td>
<td>0.31</td>
</tr>
<tr>
<td>BMI</td>
<td>44/83</td>
<td>26.96</td>
<td>6.56</td>
<td>25.60</td>
<td>6.18</td>
<td>1.13</td>
<td>83.27</td>
<td>0.453</td>
<td>0.21</td>
</tr>
<tr>
<td>alcohol (grams/day)</td>
<td>42/78</td>
<td>7.25</td>
<td>22.07</td>
<td>2.85</td>
<td>7.78</td>
<td>1.25</td>
<td>46.56</td>
<td>0.453</td>
<td>0.27</td>
</tr>
<tr>
<td>cigarettes/day</td>
<td>44/81</td>
<td>4.80</td>
<td>6.70</td>
<td>6.03</td>
<td>9.61</td>
<td>−0.84</td>
<td>115.37</td>
<td>0.566</td>
<td>−0.15</td>
</tr>
<tr>
<td>DE antidepressants</td>
<td>35/73</td>
<td>35.05</td>
<td>21.48</td>
<td>37.43</td>
<td>31.67</td>
<td>−0.46</td>
<td>93.71</td>
<td>0.697</td>
<td>−0.09</td>
</tr>
<tr>
<td>DE neuroleptics</td>
<td>43/81</td>
<td>24.42</td>
<td>51.90</td>
<td>29.32</td>
<td>47.73</td>
<td>−0.52</td>
<td>79.75</td>
<td>0.697</td>
<td>−0.10</td>
</tr>
<tr>
<td>BDI-II</td>
<td>43/79</td>
<td>30.21</td>
<td>10.78</td>
<td>33.09</td>
<td>12.08</td>
<td>−1.35</td>
<td>95.12</td>
<td>0.453</td>
<td>−0.25</td>
</tr>
<tr>
<td>MADRS</td>
<td>44/83</td>
<td>23.27</td>
<td>9.49</td>
<td>25.59</td>
<td>9.77</td>
<td>−1.30</td>
<td>90.05</td>
<td>0.453</td>
<td>−0.24</td>
</tr>
<tr>
<td>CLEQ</td>
<td>43/80</td>
<td>7.28</td>
<td>4.33</td>
<td>8.21</td>
<td>5.19</td>
<td>−1.06</td>
<td>100.09</td>
<td>0.453</td>
<td>−0.20</td>
</tr>
<tr>
<td>SEEKING</td>
<td>42/80</td>
<td>2.41</td>
<td>0.31</td>
<td>2.43</td>
<td>0.42</td>
<td>−0.33</td>
<td>107.29</td>
<td>0.745</td>
<td>−0.06</td>
</tr>
<tr>
<td>SADNESS</td>
<td>43/80</td>
<td>2.88</td>
<td>0.40</td>
<td>2.96</td>
<td>0.38</td>
<td>−1.11</td>
<td>82.57</td>
<td>0.453</td>
<td>−0.21</td>
</tr>
<tr>
<td>age at onset</td>
<td>44/83</td>
<td>29.43</td>
<td>13.12</td>
<td>23.75</td>
<td>12.74</td>
<td>2.35</td>
<td>85.53</td>
<td>0.294</td>
<td>0.44</td>
</tr>
<tr>
<td>SLC6A4 factor 1</td>
<td>37/72</td>
<td>0.04</td>
<td>1.10</td>
<td>−0.06</td>
<td>0.83</td>
<td>0.48</td>
<td>58.08</td>
<td>0.697</td>
<td>0.10</td>
</tr>
<tr>
<td>SLC6A4 factor 2</td>
<td>37/72</td>
<td>0.22</td>
<td>0.99</td>
<td>−0.17</td>
<td>1.06</td>
<td>1.85</td>
<td>77.27</td>
<td>0.453</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Welch’s t-test
were standardized. The Akaike information criterion (AIC) indicated the selection of a model including sex, SLEs, SEEKING and SADNESS for the prediction of age at depression onset. This model is shown in Table 5 and explained a significant amount of variance in age at depression onset ($R^2 = 0.60$, $F(6,110) = 29.44$, $p < .000001$). Higher age at the time of measurement was associated with lower age at depression onset. Female sex and experience of SLEs were associated with lower age at depression onset. Higher SEEKING was associated with higher age at depression onset.

**Discussion**

In the present study, we examined the role of SLEs, primary emotions, DNA-methylation of SLC6A4 and depression severity for depression onset. We assumed

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**Table 4** Partial Spearman’s correlation coefficients (below the diagonal) and $p$-values (above the diagonal for the whole sample and both sexes separately)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>whole sample (N = 146)</th>
<th>BDI-II</th>
<th>MADRS</th>
<th>CLEQ</th>
<th>SEEKING</th>
<th>SADNESS</th>
<th>age at onset</th>
<th>SLC6A4 factor 1</th>
<th>SLC6A4 factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.025</td>
<td>0.700</td>
<td>0.700</td>
<td>0.700</td>
</tr>
<tr>
<td>MADRS</td>
<td>0.62***</td>
<td>0.035</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.160</td>
<td>0.700</td>
<td>0.123</td>
<td></td>
</tr>
<tr>
<td>CLEQ</td>
<td>0.33***</td>
<td>0.21*</td>
<td>0.700</td>
<td>0.011</td>
<td>0.001</td>
<td>0.869</td>
<td>0.598</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEEKING</td>
<td>−0.32***</td>
<td>−0.32***</td>
<td>0.05</td>
<td>0.008</td>
<td>0.102</td>
<td>0.998</td>
<td>0.416</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SADNESS</td>
<td>0.49***</td>
<td>0.42***</td>
<td>0.25*</td>
<td>−0.26**</td>
<td>&lt; 0.001</td>
<td>0.998</td>
<td>0.755</td>
<td></td>
<td></td>
</tr>
<tr>
<td>age at onset</td>
<td>−0.22*</td>
<td>−0.14</td>
<td>−0.31**</td>
<td>0.17</td>
<td>−0.35***</td>
<td>0.220</td>
<td>0.700</td>
<td>0.372</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 1</td>
<td>0.06</td>
<td>0.05</td>
<td>0.02</td>
<td>−0.00</td>
<td>−0.00</td>
<td>−0.14</td>
<td>0.05</td>
<td>−0.11</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 2</td>
<td>−0.05</td>
<td>0.17</td>
<td>−0.08</td>
<td>0.10</td>
<td>0.04</td>
<td>0.05</td>
<td>−0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**women (n = 95)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>BDI-II</th>
<th>MADRS</th>
<th>CLEQ</th>
<th>SEEKING</th>
<th>SADNESS</th>
<th>age at onset</th>
<th>SLC6A4 factor 1</th>
<th>SLC6A4 factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>&lt; 0.001</td>
<td>0.065</td>
<td>0.006</td>
<td>&lt; 0.001</td>
<td>0.437</td>
<td>0.479</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td>MADRS</td>
<td>0.66***</td>
<td>0.562</td>
<td>0.012</td>
<td>&lt; 0.001</td>
<td>0.851</td>
<td>0.851</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>CLEQ</td>
<td>0.25</td>
<td>0.826</td>
<td>0.769</td>
<td>0.042</td>
<td>0.361</td>
<td>0.479</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEEKING</td>
<td>−0.36**</td>
<td>−0.33*</td>
<td>0.05</td>
<td>0.026</td>
<td>0.361</td>
<td>0.945</td>
<td>0.479</td>
<td></td>
</tr>
<tr>
<td>SADNESS</td>
<td>0.47***</td>
<td>0.47***</td>
<td>0.07</td>
<td>−0.30**</td>
<td>0.494</td>
<td>0.479</td>
<td>0.785</td>
<td></td>
</tr>
<tr>
<td>age at onset</td>
<td>−0.14</td>
<td>−0.04</td>
<td>−0.27*</td>
<td>0.16</td>
<td>−0.26*</td>
<td>0.769</td>
<td>0.907</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 1</td>
<td>−0.13</td>
<td>−0.04</td>
<td>−0.17</td>
<td>0.01</td>
<td>−0.13</td>
<td>0.07</td>
<td>0.945</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 2</td>
<td>−0.00</td>
<td>0.31*</td>
<td>−0.12</td>
<td>0.13</td>
<td>0.06</td>
<td>0.03</td>
<td>−0.02</td>
<td></td>
</tr>
</tbody>
</table>

**men (n = 51)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>BDI-II</th>
<th>MADRS</th>
<th>CLEQ</th>
<th>SEEKING</th>
<th>SADNESS</th>
<th>age at onset</th>
<th>SLC6A4 factor 1</th>
<th>SLC6A4 factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>&lt; 0.001</td>
<td>0.014</td>
<td>0.180</td>
<td>0.006</td>
<td>0.181</td>
<td>0.829</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>MADRS</td>
<td>0.61***</td>
<td>0.011</td>
<td>0.124</td>
<td>0.047</td>
<td>0.047</td>
<td>0.956</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>CLEQ</td>
<td>0.45*</td>
<td>0.46*</td>
<td>0.975</td>
<td>0.001</td>
<td>0.026</td>
<td>0.401</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>SEEKING</td>
<td>−0.27</td>
<td>−0.30</td>
<td>0.00</td>
<td>0.578</td>
<td>0.101</td>
<td>0.962</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>SADNESS</td>
<td>0.50**</td>
<td>0.36*</td>
<td>0.56**</td>
<td>−0.15</td>
<td>0.014</td>
<td>0.956</td>
<td>0.703</td>
<td></td>
</tr>
<tr>
<td>age at onset</td>
<td>−0.26</td>
<td>−0.36*</td>
<td>−0.40*</td>
<td>0.32</td>
<td>−0.43*</td>
<td>0.233</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 1</td>
<td>0.09</td>
<td>0.05</td>
<td>0.20</td>
<td>−0.02</td>
<td>−0.05</td>
<td>−0.25</td>
<td>0.616</td>
<td></td>
</tr>
<tr>
<td>SLC6A4 factor 2</td>
<td>−0.02</td>
<td>−0.02</td>
<td>0.06</td>
<td>0.04</td>
<td>0.12</td>
<td>−0.03</td>
<td>−0.14</td>
<td></td>
</tr>
</tbody>
</table>

Covariates: age and BMI. FDR corrected $p$-values (for each sample separately). *** $p < .001$, ** $p < .01$, * $p < .05$

**Table 5** Final model of the stepwise regression analysis with age at onset as dependent variable

<table>
<thead>
<tr>
<th>Predictor</th>
<th>$b$</th>
<th>SE</th>
<th>$t$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>0.49</td>
<td>0.22</td>
<td>2.21</td>
<td>0.029</td>
</tr>
<tr>
<td>Age</td>
<td>0.69</td>
<td>0.06</td>
<td>10.75</td>
<td>0.000</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.06</td>
<td>0.06</td>
<td>−0.93</td>
<td>0.356</td>
</tr>
<tr>
<td>Sex</td>
<td>−0.30</td>
<td>0.13</td>
<td>−2.30</td>
<td>0.024</td>
</tr>
<tr>
<td>CLEQ</td>
<td>−0.16</td>
<td>0.06</td>
<td>−2.61</td>
<td>0.010</td>
</tr>
<tr>
<td>SEEKING</td>
<td>0.13</td>
<td>0.06</td>
<td>2.09</td>
<td>0.039</td>
</tr>
<tr>
<td>SADNESS</td>
<td>−0.11</td>
<td>0.07</td>
<td>−1.57</td>
<td>0.119</td>
</tr>
</tbody>
</table>

The covariates age and BMI were included by default.
early-onset depression to be associated with more SLEs, less SEEKING, higher SADNESS and higher depression severity. We also assumed an association between age at onset and SLEs in patients with early as compared to patients with adult-onset depression [13] and with the association of stress with first depression onset [72, 73]. The association between stress and depression is a well-established finding that has been documented for various stressors [74, 75], recent and early SLEs [76, 77] and in a variety of samples with different age groups [53, 78, 79]. In the adolescent period, the individual could be especially vulnerable to stressors since this period is considered important for the organization of behavioral and endocrine responses to stress. In addition, the maturation of brain systems involved in the control of the HPA-axis takes place during this transition period [80]. It is possible that inpatients with an early depression onset had an adverse environment in early developmental stages [81]. However, as data about early life adversities was not available in the present sample, this hypothesis needs to be tested in future studies.

SLEs in these early developmental stages could be a predictor of current depressive symptom severity because of their long lasting effects on the bodily stress system. Daskalakis and colleagues [82] suggest a three-hit concept of vulnerability and resilience in the face of early life adversity: They postulate an interaction of genetic factors (hit-1) with early environmental factors (hit-2) to be reflected in epigenetic modifications and altered endocrine regulations. This interaction programs gene expression patterns, which are relevant for an evolving phenotype during brain development. The emerging phenotype with altered stress axis regulation and sensitivity is exposed to the later-life environment (hit-3). Depending on the type of later-life challenge, the individual is either vulnerable or resilient to the development of psychiatric symptoms [82]. The three-hit concept provides a framework for the interpretation of early as well as later-life SLEs’ association with depressive symptoms [83] and takes into consideration why some individuals develop depression after the experience of early adversity and some do not. This in turn is an explanation for the finding of a medium size positive association between SLEs and depressive symptoms, which is in line with previous studies [13].

High SADNESS scores were associated with younger age at depression onset. This is in line with previous studies reporting high scores on the personality dimension neuroticism to be a vulnerability factor for early depression onset [13, 14]. However, after taking into account other predictors, SADNESS did not explain a significant amount of variance in age at depression onset. An explanation could be that there are associations between SLEs and SADNESS, between SEEKING and SADNESS and between sex and SADNESS. Thus, SADNESS shares variance with three of the other predictors included in the final model. However, SADNESS was still included in the model with the lowest AIC.

While there was a small to medium size positive correlation coefficient between SEEKING and age at depression onset, this association was not significant. In the regression model predicting age at onset, however, SEEKING explained a significant amount of variance in the dependent variable beyond the variance explained by other predictors. Thus, looking at the model best suited for the prediction of age at depression onset, our findings are in line with the theory of depression development by Watt and Panksepp [16]. Therefore, we want to give a possible explanation for our findings with reference to their theory: If an individual has high SADNESS scores arising from social loss or defeat [17], low reward-SEEKING protects against new social losses or defeats. However, social isolation also prevents positive social experiences, which in turn can be considered a social loss if it is a permanent condition. This vicious circle might culminate in a major depressive episode in line with Lewinsohn’s [84] social reinforcement theory. Therefore, SLEs, SADNESS and SEEKING might serve for early identification of individuals at risk of developing depression. Since the ANPS provides the opportunity for detecting individuals scoring high on two main symptoms of depression, depressive mood and loss of interest/energy, it could be better suited for the detection of individuals at risk for depression development than examining Neuroticism only. Of note, Neuroticism represents a super-factor, being not only associated with SADNESS, but also FEAR and ANGER [20, 22]. In terms of Affective Neuroscience theory, SADNESS should be the core dimension to understand depression, although strong overlaps with FEAR have been observed as well [23]. At this point we emphasize that it is not clear whether associations between age at onset or depression severity and SEEKING as well as SADNESS are an expression of primary emotions predisposing for depression or for depression having an impact on primary emotions. This needs to be evaluated using a longitudinal design.
Our findings are not only in line with interventions used for the treatment of depression, they could additionally provide an evolution based and easy to understand explanation of why these interventions are effective. For instance, behavioral activation is an effective initial intervention for the treatment of depression [85]. This may be explained by an activation of the downregulated SEEKING system thereby enabling the patient to experience positive reinforcements [85]. In addition, psychodynamic therapies or the cognitive behavioral analysis system of psychotherapy (CBASP) focusing on the experience of past and current relationships could teach patients to become connected with the depressogenic consequences of their interpersonal behavior [86, 87] potentially counteracting an upregulated SADNESS system by enabling rewarding social interactions. In addition, we found female gender to be a risk factor for early age at depression onset, which is in line with previous studies on sex differences in depression development [88].

It is also worth mentioning that we discovered slightly different methylation patterns between the variables of interest when investigating men and women separately. The expected positive association between SLEs and SADNESS was found only in men. Further, the negative association between depression severity and SEEKING was only present in women. This could mean one of four things: for one, depressive symptoms in women could differ from depressive symptoms in men in line with previous findings [89]. Second, women could have different risk factors predisposing them for depression, e.g. a more strongly expressed shutdown following separation distress as proposed by Watt and Panksepp [16]. Third, depressive symptoms could have a different impact on primary emotions as a function of sex. Fourth, effects of SLEs could differ from depressive symptoms in men in line with previous studies on sex differences in depression development. Overall, our findings do not support the notion of a strong linear association between SLC6A4 methylation and depression severity [42, 43, 45–47]. Depending on the specific CpG site or cluster of CpG sites examined, there may be positive or negative associations between SLC6A4 methylation, personality and depression. In line with a previous study [94] we found women to have higher SLC6A4 methylation of centric to terminal CpG sites as indexed by methylation factor 1. Since methylation of factor 1 is not associated with any of the investigated variables, this sex difference in SLC6A4 methylation does not seem to be related to SLEs or depressive symptoms. Furthermore, SLC6A4 methylation was not included in the model predicting age at depression onset. Therefore, SLC6A4 methylation does not seem strongly related to depression, which questions the usefulness of SLC6A4 methylation as biomarker for depression onset. But again, our insights are also limited by the rather small sample of patients investigated. Beyond that, it is possible that SLC6A4 methylation was associated with depression severity at the time of onset of the first depressive episode. Furthermore, it is possible that SLC6A4 methylation differs between individuals developing a depressive episode and individuals that do not develop a depressive episode. Another interesting question is whether and when a stressor affects SLC6A4 methylation. It is possible that depression itself is stressful [95] and leaves traces in epigenetic signatures of the serotonin system of affected individuals which would make it more difficult to distinguish epigenetic mechanisms involved in depression development from signatures of depression itself. Therefore, prospective studies investigating epigenetic signatures before and after depression onset as well as during the course of the disorder are needed.

We did not find any significant association between the examined variables and 5-HTTLPR genotype (see supplementary material Table S4). Even though our sample size is too small for making conclusions on the presence or absence of a genotype effect, this is in line with the meta-analysis by Culverhouse and colleagues [32], who did not find strong evidence of a main effect or interaction of 5-HTTLPR in the development of depression. Overall, our findings do not support the notion of a strong linear association between SLC6A4 methylation and depression severity in women. This association is in line with previous studies reporting higher SLC6A4 methylation being associated with depression severity [42, 44]. There are, however, also studies reporting negative associations between SLC6A4 methylation and depression severity [42, 43, 45–47]. Depending on the specific CpG site or cluster of CpG sites examined, there may be positive or negative associations between SLC6A4 methylation, personality and depression. In line with a previous study [94] we found women to have higher SLC6A4 methylation of centric to terminal CpG sites as indexed by methylation factor 1. Since methylation of factor 1 is not associated with any of the investigated variables, this sex difference in SLC6A4 methylation does not seem to be related to SLEs or depressive symptoms. Furthermore, SLC6A4 methylation was not included in the model predicting age at depression onset. Therefore, SLC6A4 methylation does not seem strongly related to depression, which questions the usefulness of SLC6A4 methylation as biomarker for depression onset. But again, our insights are also limited by the rather small sample of patients investigated. Beyond that, it is possible that SLC6A4 methylation was associated with depression severity at the time of onset of the first depressive episode. Furthermore, it is possible that SLC6A4 methylation differs between individuals developing a depressive episode and individuals that do not develop a depressive episode. Another interesting question is whether and when a stressor affects SLC6A4 methylation. It is possible that depression itself is stressful [95] and leaves traces in epigenetic signatures of the serotonin system of affected individuals which would make it more difficult to distinguish epigenetic mechanisms involved in depression development from signatures of depression itself. Therefore, prospective studies investigating epigenetic signatures before and after depression onset as well as during the course of the disorder are needed.

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regulation and depression. However, there could still be differences in SLC6A4 methylation when comparing in-patients suffering from depression to healthy controls. Nevertheless, SLC6A4 appears to be associated with depression, but in a more complex fashion. There might be moderating factors other than sex and 5-HTTLPR that make it difficult to understand the exact function of SLC6A4 in the development and maintenance of depression. However, if the lack of a direct association between SLC6A4 or the 5-HTT and depression replicates in future studies, the mechanism of action of SSRIs and the role of the serotonin system in the development and maintenance of depression as a whole would need to be re-discussed. After all, it has been shown that neurogenesis mediates some beneficial effects of antidepressant treatment [96]. Additionally, first steps towards a joint explanation of the effectiveness of different kinds of antidepressants have already been taken and point towards a role of sphingolipid-controlled autophagy as an important target for antidepressive treatment [97]. A better understanding of the mechanism of action of antidepressants and the role of the 5-HTT in depression and antidepressive treatment could clarify the question of whether current antidepressants are effective in the treatment of depression and how they exert their antidepressive effect [98–101].

Some limitations need to be considered when interpreting the results of our study. First, methylation in whole blood samples is only a proxy for epigenetic profiles in brain tissue. Second, we cannot draw conclusions regarding the functionality of the observed alterations in SLC6A4 methylation since we did neither assess mRNA nor 5-HTT levels. Third, statistical power could be too low to detect associations between SLC6A4 methylation and age at depression onset or depression severity. A post-hoc power analysis, however, revealed that given our total sample size of $N = 146$ and $\alpha = .05$ (two-tailed) power to detect a medium-sized effect [102] was determined to be 0.97. However, we cannot rule out the possibility that there are small associations between SLC6A4 methylation and age at depression onset. Fourth, we did not assess the timing of the stressor, which is why we cannot differentiate between early and late life stressors. Last, we assessed depression onset retrospectively. In future, studies with prospective longitudinal designs are needed to confirm and extend our results.

Taken together, we provide evidence that young age at depression onset is associated with depressive symptom severity. In addition, we found that a considerable amount of variance in depression onset can be explained by sex, the experience of SLEs and personality traits comprising high SADNESS and low SEEKING. Thus, our work can serve as starting point for future studies using a longitudinal design for the investigation of the causal role of sex, primary emotions, SLEs and epigenetic factors for depression development in young age. As the number of people suffering from depression rises, early identification of at-risk individuals is becoming increasingly important for establishing prevention interventions alleviating the burden that depression imposes on individuals, their social environment and society.

Abbreviations
S-HT: Serotonin; 5-HT: Serotonin transporter; 5-HTTLPR: Serotonin transporter linked polymorphic region; AIC: Akaike information criterion; ANPS: Affective Neuroscience Personality Scales; BD-II: Beck Depression Inventory; BMI: Body Mass Index; CLEQ: Critical Life Events Questionnaire; CpG: Cytosine-phosphate-guanine dinucleotides; DE: Dose equivalents; FDR: False discovery rate; MADRS: Montgomery Asberg Depression Rating Scale; MDD: Major Depressive Disorder; SLE: Stressful life event; SSRI: Selective serotonin reuptake inhibitor

Supplementary Information
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Additional file 1.

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None.

Authors’ contributions
S.S., C.M., M.K. and the GenEmo Research group designed the present study. S.S. analyzed the data and wrote the first draft of the manuscript. C.M., M.K., K-W.M. and C.S-L. commented on and improved previous versions of the manuscript. All authors contributed to and approved the manuscript.

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Availability of data and materials
The datasets analyzed during the current study are not publicly available since the authors do not have permission to publish the data. However, data are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All procedures performed in this study were in accordance with the standards of the ethics committee of Ulm University, Ulm, Germany and with the 1964 Helsinki declaration and its later amendments. The experimental protocols were approved by the ethics committee of Ulm University, Ulm, Germany. Written informed consent of the participants was obtained after the procedures had been fully explained.

Consent for publication
Not applicable.

Competing interests
There are no competing financial interests to be disclosed.

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4. Montag C, Ebstein R, Jav里面的文本已经翻译成英文，可以继续阅读。
2.5. Study V - When a playful personality counteracts fear: Predictors for fear of COVID-19 in former inpatients with major depressive disorder and healthy control participants

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When a playful personality counteracts fear: Predictors for fear of COVID 19 in former inpatients with major depressive disorder and healthy control participants

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Running head: Coronavirus and Depression

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Abstract
Depressive symptoms and fear might have increased during the COVID-19 pandemic. Well-established risk factors for developing depression or anxiety disorders comprise stressful life events (SLEs), high scores on the primary emotion SADNESS and low scores with respect to SEEKING as well as dysfunctional emotion regulation strategies, i.e. high suppression and low reappraisal. Therefore, we investigated the predictive value of previously experienced SLEs, primary emotions and emotion regulation strategies for fear of COVID-19.

We examined data of $n = 44$ former inpatients suffering from depression and $n = 49$ healthy controls in a longitudinal design with two measurement points. Before the pandemic, we assessed SLEs, primary emotions, emotion regulation and depression severity. During the pandemic, we assessed COVID-19 associated stressors and life events, emotion regulation, depression severity and fear of COVID-19.

Fear of COVID-19 and depression severity during the pandemic were significantly higher in former inpatients than in healthy controls. Depression diagnosis, SLEs and depression severity before the pandemic were significant (positive) predictors of fear of COVID-19. The primary emotion PLAY was a significantly negative predictor of fear of COVID-19. Depressive symptom severity did not change significantly in healthy controls.

Our results show that risk factors for depression might be valuable predictors of who is at risk of high fear of COVID-19. In addition, a playful personality could help preventing mental stress in pandemic situations. Thus, positivity based interventions could counteract elevated fear scores during a pandemic.

Keywords: COVID-19, corona, depression, fear, stress, emotion regulation, primary emotions
Introduction

In December 2019, a novel respiratory disease emerged in Wuhan China. In January, Chinese authorities reported the respiratory disease to be the consequence of the infection with the novel coronavirus SARS-CoV-2. The same day, the first person was reported dead. In February, the respiratory disease was called COVID-19. Initial confidence in being adequately prepared for the outbreak of an infectious disease quickly waned due to the rapid spread of the virus. On March 11, the World Health Organization (WHO) declared a pandemic. Since then, public health systems of some countries were on the verge of collapse, numerous people died world-wide (WHO) and it is becoming apparent that the pandemic is far from over since the number of new cases rises rapidly. In order to avoid overburdening public health systems, governments took unprecedented measures, restricting travelling and freedom of assembly, culminating in shutting down whole areas. As undeniably important as measures to prevent the uncontrolled spread of Coronavirus are, it is also important to consider the effects of such a threatening situation in combination with major restrictions for the individual on mental health.

Accordingly, there are reports of an increase of fear and depressive symptoms during the current pandemic (Ettman et al., 2020; Lippold et al., 2020) and a lot of cross-sectional studies reporting elevated prevalence ratios of anxiety and depressive symptoms (Bäuerle et al., 2020; Fitzpatrick et al., 2020; Fullana et al., 2020; Hyland et al., 2020; Salari et al., 2020). Whether individuals with preexisting depression diagnosis are especially vulnerable to fear of the current pandemic, to the best of our knowledge, has not yet been investigated. However the high comorbidity between anxiety and depressive disorders (Lamers et al., 2011) point towards a vulnerability to fear of the current pandemic for individuals suffering from depression. Therefore, we examined whether there are differences with respect to fear of COVID-19 comparing former inpatients suffering from depression to healthy controls.

Major Depressive Disorder (MDD) being the world’s second leading cause of years with disability is a major burden to affected individuals, their social environment and society (Ferrari et al., 2013). Depression has been consistently found to be associated with traumatic or stressful life events (SLEs) (Sanwald et al., 2020, 2019) and hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Heim and Binder, 2012; Shapero et al., 2019). Moreover, reports of an increased
prevalence of depressive symptoms is one of the most common findings after a disaster (Goldmann and Galea, 2014). According to the diathesis-stress model of depression, a stressor alone does not cause depression. Only the interplay of stress and an individual’s vulnerability leads to depression development (Hyde et al., 2008). Personality factors describing a tendency to experience negative effect but also dysfunctional strategies in regulating negative emotions are well established candidates making an individual vulnerable for depression development (Goldstein and Klein, 2014; Joormann and Michael Vanderlind, 2014).

Affective Neuroscience (AN) postulates individual differences in emotionality to represent the oldest part of human personality (Panksepp, 1992). There are seven primary emotional systems (positive emotions: SEEKING, CARE, PLAY and LUST; negative emotions: FEAR, SADNESS and ANGER) having their well-documented neural substrate in subcortical brain areas (Davis and Montag, 2019). For research linking human personality to psychopathology, primary emotions provide a more direct biopsychological view than classical language-derived approaches to model personality (Davis et al., 2003; Montag and Davis, 2018; Montag and Panksepp, 2017). Thus, the investigation of primary emotions might shed light on fundamental elements of mammalian personality associated with the development and maintenance of mental disorders (Davis and Montag, 2019). Of note, Montag and Elhai (2020) recently also proposed that Panksepp’s Affective Neuroscience Theory is very helpful to better understand the impact of COVID-19 on children/adolescents’ mental health including their caretakers.

According to Watt and Panksepp’s (2009) theory of depression development, there are two primary emotional systems of major importance for depression development: On the one hand, SEEKING is defined as the effort made to alleviate negative emotions or the drive to search for vital resources. On the other hand, SADNESS is best described as the emotional state evoked after separation from a loved one. The aforementioned theory postulates that an initial stressor provoking separation distress (in humans the separation may also be a symbolic one) results in a protest phase characterized by an individual making efforts to relieve emotional stress. When the individual has no success in soothing separation distress, an emotional shutdown takes place. Thus, the individual saves vital resources. If separation distress in
combination with the emotional shutdown is chronically prolonged, however, it will eventually culminate in depression. In accordance with this theory, previous studies found associations between both, low SEEKING as well as high SADNESS and depression severity (Fuchshuber et al., 2019; Montag et al., 2017). Accordingly, SEEKING and SADNESS but also the primary emotion FEAR are obvious candidates for the prediction of fear scores during the current pandemic (again see Montag & Elhai, 2020). On the other hand, positivity has been found to be a predictor of happiness during the current pandemic (Yıldırım and Güler, 2021) and humor has been shown to be negatively associated with fear of COVID-19 (Saricali et al., 2020). The primary emotion PLAY is defined as social joy adding fun in adulthood (Davis and Montag, 2019) and could therefore be a resilience factor regarding fear of COVID-19. Accordingly, the PLAY system is postulated to have potential for helping patients in adult psychotherapy to reintegrate troublesome emotional experiences towards more adaptive affective trajectories in a playful way (Davis and Panksepp, 2011).

In line with chronically prolonged separation distress, it has been hypothesized that ineffective emotion regulation strategies are risk factors for depression as well as anxiety disorders (Hofmann et al., 2012; Joormann and Michael Vanderlind, 2014) and thus relevant for the investigation of predictors for fear of COVID-19. Previous studies suggest two emotion regulation strategies, high suppression and low reappraisal, are associated with current (suppression and reappraisal) as well as remitted (only suppression) depression (Visted et al., 2018). Suppression describes the inhibition of emotion expressing behavior or emotional reactions (Gross and John, 2003; Hayes et al., 2004; Visted et al., 2018). Reappraisal involves changing the interpretation of a situation eventually eliciting an emotion thereby changing the situation’s emotional impact (Gross and John, 2003). This strategy is especially important for cognitive behavioral therapy, where patients learn how to interpret situations in ways provoking less negative emotions.

In summary, fear together with depressive symptoms seem to have increased during the current pandemic (Ettman et al., 2020; Lippold et al., 2020; Montag and Elhai, 2020). Accordingly, risk factors for depression development like SLEs (Sanwald et al., 2020, 2019), high SADNESS and low SEEKING (Montag et al., 2017), high suppression and low reappraisal
(Visted et al., 2018) but also resilience factors like positivity (Yıldırım and Güler, 2021) or
PLAY are potentially relevant for the prediction of fear of COVID-19. Therefore, we assessed
SLEs, primary emotions, emotion regulation and depression severity before the pandemic in
former inpatients (at previous admission to the hospital) as well as in healthy controls. At a
second point of measurement during the pandemic we again collected data in these participant
groups on emotion regulation and depression severity and additionally assessed COVID-19
associated life events and stressors as well as fear of COVID-19. We wanted to investigate the
predictive value of SLEs, primary emotions and emotion regulation strategies for fear of
COVID-19 in depressive inpatients as well as in healthy controls. In addition, we wanted to
examine the association of these variables with the change in depressive symptom severity in
mentally healthy individuals. Changes in depressive symptoms in former inpatients cannot be
attributed to the current pandemic since this group received treatment after the first assessment
of depression severity. We assumed former inpatients to have higher suppression, lower
reappraisal and more severe depressive symptoms than healthy controls during the pandemic.
Furthermore, we hypothesized depressive symptom severity to have increased in healthy
individuals as compared to depressive symptom severity before the current pandemic. We
hypothesized that healthy controls show an increase in suppression and a decrease in reappraisal
during the pandemic. We predicted suppression (during the pandemic) to be positively
associated with fear of COVID-19 and depressive symptom severity. We predicted reappraisal
(during the pandemic) to be negatively associated with fear of COVID-19 and depressive
symptom severity. In addition, we wanted to investigate which combination of variables is best
suited for predicting fear of COVID-19 in MDD patients and in healthy controls.
Methods

Participants

We recruited \( n = 44 \) former inpatients suffering from MDD (age: \( M = 42.32 \) years, \( SD = 13.34 \) years; 24 females) and \( n = 49 \) controls (age: \( M = 38.46 \) years, \( SD = 13.95 \) years; 31 females) from the database of the Ulm Gene Brain Behavior Project (UGBBP). Groups did not differ significantly with respect to age (\( t(87) = -1.33, p = 0.188 \)) or with regard to the frequencies of sexes (\( \chi^2(1) = 0.73, p = 0.393 \)). With respect to the first point of measurement before the pandemic, data partially overlapped with those of earlier studies, whereas data of the second point of measurement (during the pandemic) was not available previously (Montag et al., 2017; Sanwald et al., 2020, 2019). For further information on the two measurement points see Study Design. All procedures performed in this study were in accordance with the ethical standards of the ethics committee of Ulm University, Ulm, Germany and with the 1964 Helsinki declaration and its later amendments. Informed consent of the participants was obtained after the procedures had been fully explained.

Questionnaires

CLEQ

The Critical Life Events Questionnaire (CLEQ) comprises 60 items concerning 30 potentially traumatic life events (such as natural disaster, man-made disaster or death of a close one). There are two questions each for all 30 events assessing whether participants ever experienced the concerning event and, if so, how traumatic they felt about it on a scale from 1 (not traumatic) to 6 (very traumatic) (Plieger et al., 2015). We added up the product of the occurrence of each event and the experienced severity, thereby calculating a weighted mean. In case of nine or more incompletely answered events, participants were excluded from further analysis with the CLEQ.
The Affective Neuroscience Personality Scales (ANPS) German version (Reuter et al., 2017) assesses individual tendencies in six primary emotional systems with 110 items. The assessed primary emotions are SEEKING, CARE, PLAY (positive emotionality) and SADNESS, FEAR, ANGER, (negative emotionality). The seventh primary emotion of LUST is not assessed by the ANPS since it may potentially have negative carry over effects on the remaining items. The items are answered on a four point Likert scale (strongly disagree (1) to strongly agree (4)). Internal consistencies for inpatients were acceptable or good (SEEKING: $\alpha = .82$; CARE: $\alpha = .87$; PLAY: $\alpha = .84$; SADNESS: $\alpha = .68$; FEAR: $\alpha = .87$; ANGER: $\alpha = .77$) as they were for healthy controls (SEEKING: $\alpha = .71$; CARE: $\alpha = .78$; PLAY: $\alpha = .76$; SADNESS: $\alpha = .58$; FEAR: $\alpha = .86$; ANGER: $\alpha = .85$).

The Emotion Regulation Questionnaire (ERQ) German version (Abler and Kessler, 2009) assesses two common emotion regulation strategies: suppression and reappraisal. It comprises ten items that are answered on a seven point Likert scale from (1) strongly disagree to (7) strongly agree. Suppression is measured with four and reappraisal with six items. A mean is calculated as long as all items covering the respective emotion regulation strategies are answered. For inpatients suffering from depression internal consistency was good or excellent (measurement point 1: suppression: $\alpha = .78$, reappraisal: $\alpha = .84$; measurement point 2: suppression: $\alpha = .76$, reappraisal: $\alpha = .92$). For healthy controls, internal consistencies were good (measurement point 1: suppression: $\alpha = .77$, reappraisal: $\alpha = .84$; measurement point 2: suppression: $\alpha = .76$, reappraisal: $\alpha = .81$).
Severity levels of depressive symptoms were assessed using the Beck Depression Inventory (BDI-II) German version (Beck et al., 2006). The BDI-II is a self-assessment scale comprising 21 items. For each item ratings between 0 (not at all) and 3 (very intensive) are given depending on the symptom complaint. A sum is calculated by adding up ratings of all items. Thus, a maximum of 63 points can be reached. Internal consistency was excellent in the group of individuals suffering from depression with $\alpha = .91$ for the first point of measurement and $\alpha = .95$ for the second point of measurement. Reliability was good for both points of measurement in healthy controls (measurement point 1: $\alpha = .77$; measurement point 2: $\alpha = .84$).

The Fear of COVID-19 Scale (FCV-19S) measures the severity of the fear of COVID-19 (Ahorsu et al., 2020b) comprising 7 items that are answered on a five point Likert scale from (1) strongly disagree to (5) strongly agree. A total is calculated by adding up the scores for each item. Thus, the total score ranges from seven to 35. Higher scores indicate greater fear of COVID-19. Internal consistency was excellent for former inpatients ($\alpha = .93$) and good for healthy controls ($\alpha = .78$). The German version was forth- and back-translated independently by two persons speaking both English and German. The items can be found in the Supplementary Material (Table S1).

To control for COVID-19 related life events, we asked participants whether they experienced the following events, which they answered with yes or no: infection with SARS-CoV-2, infection of a close person with SARS-CoV-2, death of a close person due to infection with SARS-CoV-2, short time work due to the pandemic and job loss due to the pandemic.
addition, we assessed how much participants suffered from certain stressors associated with the pandemic using six items: I am very afraid of infecting others with the new corona virus; I have great financial worries due to the current corona pandemic; I am very worried about losing my job because of the current corona pandemic; social distancing in the context of the current corona pandemic is a great burden for me; the restrictions in the organization of my free time due to the current corona pandemic are a great burden for me; I am currently stressed for reasons that have nothing to do with the corona pandemic. These items were rated on a five point scale from 1 (strongly disagree) to 5 (strongly agree). We did not calculate a score since we wanted to examine which specific aspects of the current pandemic are associated with fear of COVID-19.

**Study Design**

The first period of measurement took place from September 2015 to February 2020, i.e. before the WHO called out a pandemic. The sample of depressed inpatients was recruited at the Department of Psychiatry and Psychotherapy at Ulm University, Ulm, Germany. Inpatients were diagnosed for MDD by a psychiatrist at admission to the hospital using the Structured Clinical Interview for DSM-IV (SCID-I) (American Psychiatric Association, 2003).

The sample of healthy controls was recruited by postings in public areas and online advertisement. The control group underwent a diagnostic interview comprising the Mini-DIPS (Margraf, 1994) and SCID-II (American Psychiatric Association, 2003) to exclude participants potentially suffering from any kind of mental illness. An additional exclusion criterion was a lifetime diagnosis of any kind of mental or neurological illness or any kind of past psychiatric inpatient treatment or psychotherapy. Both, inpatients and controls, were administered the CLEQ, the BDI-II, the ERQ and the ANPS; all described above. Sociodemographic variables were assessed with a standardized semi-structured interview based on an in-house
questionnaire. All participants of both groups gave their written consent to be contacted again for follow-up investigations.

The second period of measurement was from July 14 to September 23 of 2020. Participants (both groups) were contacted via E-Mail and answered four questionnaires. The four questionnaires comprised the BDI-II, the ERQ, the FCV19-S and additional five items covering specific COVID-19 associated life events as well as six items covering specific potential stressors associated with COVID-19.

The difference in time (in days) between the two points of measurement was initially included in all longitudinal analyses as a covariate.

**Statistical analysis**

Statistical analysis was conducted using R (R Development Core Team, 2008) with the packages *psych* (Revelle, 2018), *MASS* (Venables and Ripley, 2002) and *ggplot2* (Wickham, 2016) as well as *JASP* (JASP Team, 2019). We performed a stepwise hierarchical linear regression analysis with Fear of COVID-19 as the dependent variable. SLEs, primary emotions, emotion regulation and depression severity as well as their interactions with group were predictors. These predictors were measured before the COVID-19 pandemic. In addition, we used current age, sex and time difference between measurements as well as their interactions with group as covariates. Then we automatically and iteratively added and removed predictors and covariates based on AIC comparisons.

We performed $\chi^2$-tests to test whether there are group differences in stressful events associated with the COVID-19 pandemic. Using independent sample Welch’s t-tests, we examined differences between former inpatients and healthy controls with respect to SLEs, emotion regulation, primary emotions, depression severity, fear of COVID-19 and COVID-19 associated stressors (note that U-tests provided similar results). We calculated repeated measures ANCOVAs to examine changes in emotion regulation strategies and depressive
symptom severity controlling for sex, age and time between the two points of measurement (in days). We did not use group as between subjects factor since former inpatients had been treated and dismissed from the hospital, therefore group differences with respect to changes in emotion regulation or depressive symptom severity are confounded by treatment effects and do not genuinely represent group differences in reactions to the current pandemic situation. To investigate associations with fear of COVID-19 and depression severity during the pandemic, we calculated partial Spearman’s correlation coefficients between fear of COVID-19, depression severity, emotion regulation as well as COVID-19 associated stressors controlling for sex and age (even though groups did not differ with respect to these variables, sex and age could still affect within-group associations) for each group separately. We controlled false discovery rate (FDR) using Benjamini-Hochberg correction (Benjamini and Hochberg, 2000). Statistical significance was determined at $p < .05$.

**Results**

**Experience of COVID-19 related stressful life events**

Only one participant of the healthy control group and two participants of the group of former inpatients were infected with SARS-CoV-2, hence there was no significant difference in infection frequencies ($\chi^2(1) = 0.47, p = 0.495$). Four healthy controls and five former inpatients indicated that a close person was infected with SARS-CoV-2 ($\chi^2(1) = 0.27, p = 0.602$). No participant stated to have lost a close person due to COVID-19. Nine controls and five former inpatients were affected by short-time work due to the pandemic ($\chi^2(1) = 0.89, p = 0.346$). Three controls and zero former inpatients lost their job due to the pandemic ($\chi^2(1) = 2.78, p = 0.095$).

**Group differences**

Before the Corona pandemic, inpatients reported that they experienced significantly more SLEs than healthy controls. Additionally, inpatients showed significantly higher suppression and less
reappraisal than controls did. As expected, inpatients suffering from MDD scored significantly lower on the primary emotion SEEKING but also on the primary emotion PLAY than controls did. Inpatients as compared to healthy controls had significantly higher scores with respect to FEAR and SADNESS (Table 1). Group differences in depression severity before the pandemic are not reported since we consider it trivial that inpatients suffering from depression have a higher BDI-II score than healthy controls.

Table 1
Group differences between healthy controls and inpatients before the pandemic (age and sex needed not to be controlled for in these analyses, because these variables differed not significantly between control and MDD group).

<table>
<thead>
<tr>
<th></th>
<th>Control M(SD)</th>
<th>MDD M(SD)</th>
<th>df</th>
<th>t</th>
<th>p_BH</th>
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<tr>
<td>CLEQ</td>
<td>49 8.25(7.13)</td>
<td>44 25.00(23.89)</td>
<td>49.88</td>
<td>-4.48</td>
<td>0.000</td>
<td>-0.95</td>
</tr>
<tr>
<td>Suppression</td>
<td>49 3.47(1.30)</td>
<td>42 4.63(1.51)</td>
<td>81.41</td>
<td>-3.90</td>
<td>0.000</td>
<td>-0.83</td>
</tr>
<tr>
<td>Reappraisal</td>
<td>49 4.72(1.01)</td>
<td>42 3.61(1.29)</td>
<td>77.22</td>
<td>4.50</td>
<td>0.000</td>
<td>0.96</td>
</tr>
<tr>
<td>SEEKING</td>
<td>48 2.85(0.32)</td>
<td>43 2.46(0.46)</td>
<td>73.56</td>
<td>4.71</td>
<td>0.000</td>
<td>1.00</td>
</tr>
<tr>
<td>CARE</td>
<td>48 3.02(0.38)</td>
<td>43 2.95(0.55)</td>
<td>74.17</td>
<td>0.73</td>
<td>0.551</td>
<td>0.51</td>
</tr>
<tr>
<td>PLAY</td>
<td>48 2.97(0.36)</td>
<td>43 2.27(0.47)</td>
<td>78.34</td>
<td>7.94</td>
<td>0.000</td>
<td>-1.98</td>
</tr>
<tr>
<td>FEAR</td>
<td>48 2.30(0.41)</td>
<td>43 3.21(0.51)</td>
<td>80.12</td>
<td>-9.37</td>
<td>0.000</td>
<td>-1.98</td>
</tr>
<tr>
<td>ANGER</td>
<td>48 2.41(0.43)</td>
<td>44 2.49(0.46)</td>
<td>87.88</td>
<td>-0.88</td>
<td>0.508</td>
<td>-0.81</td>
</tr>
<tr>
<td>SADNESS</td>
<td>48 2.24(0.30)</td>
<td>44 2.89(0.42)</td>
<td>77.52</td>
<td>-8.51</td>
<td>0.000</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Note. p_BH refers to p-values (two-tailed) controlled for FDR.

During the pandemic, former inpatients still showed significantly more severe depressive symptoms than controls. In addition, former inpatients reported to experience significantly more fear of COVID-19 than did healthy controls and showed significantly higher suppression scores. There were no group differences with respect to corona-associated fears such as fear of financial hardship or of unemployment. However, former inpatients reported to have elevated psychological strain due to circumstances not associated with the current pandemic (Table 2). Note, that U-tests comparing former inpatients with depression severity below a BDI-II score of 20 (n = 17) to healthy controls revealed only one significant group difference: Former inpatients reported more psychological strain not associated with the current pandemic (p = .017).
Table 2.

Group differences between healthy controls and former inpatients during the pandemic (age and sex needed not to be controlled for in these analyses, because these variables differed not significantly between control and MDD group).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MDD</th>
<th>df</th>
<th>t</th>
<th>(p_{BH})</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI-II</td>
<td>49 4.96(4.86)</td>
<td>44 23.68(14.80)</td>
<td>51.31</td>
<td>-8.01</td>
<td>0.000</td>
<td>-1.70</td>
</tr>
<tr>
<td>FCV-19S</td>
<td>49 9.78(3.18)</td>
<td>44 13.27(6.65)</td>
<td>60.19</td>
<td>-3.18</td>
<td>0.004</td>
<td>-0.67</td>
</tr>
<tr>
<td>Suppression (t2)</td>
<td>49 3.37(1.35)</td>
<td>44 4.25(1.44)</td>
<td>88.32</td>
<td>-3.03</td>
<td>0.005</td>
<td>-0.63</td>
</tr>
<tr>
<td>Reappraisal (t2)</td>
<td>49 4.55(1.10)</td>
<td>44 4.01(1.57)</td>
<td>76.23</td>
<td>1.89</td>
<td>0.102</td>
<td>0.40</td>
</tr>
<tr>
<td>Fear of infecting others</td>
<td>49 2.37(1.27)</td>
<td>44 2.61(1.40)</td>
<td>87.28</td>
<td>-0.89</td>
<td>0.508</td>
<td>-0.18</td>
</tr>
<tr>
<td>Financial hardships</td>
<td>49 1.78(0.99)</td>
<td>44 1.82(1.15)</td>
<td>85.29</td>
<td>-0.19</td>
<td>0.894</td>
<td>-0.04</td>
</tr>
<tr>
<td>Fear of unemployment</td>
<td>49 1.74(1.06)</td>
<td>44 1.66(1.03)</td>
<td>90.32</td>
<td>0.35</td>
<td>0.809</td>
<td>0.07</td>
</tr>
<tr>
<td>Stress due to social distancing</td>
<td>49 2.76(1.20)</td>
<td>44 2.98(1.52)</td>
<td>81.75</td>
<td>-0.78</td>
<td>0.549</td>
<td>-0.16</td>
</tr>
<tr>
<td>Stress due to restrictions with respect to leisure time activities</td>
<td>49 2.78(1.18)</td>
<td>44 2.77(1.61)</td>
<td>78.02</td>
<td>0.01</td>
<td>0.993</td>
<td>0.00</td>
</tr>
<tr>
<td>Psychological strain due to other circumstances</td>
<td>49 1.90(1.25)</td>
<td>44 3.59(1.34)</td>
<td>88.22</td>
<td>-6.30</td>
<td>0.000</td>
<td>-1.31</td>
</tr>
</tbody>
</table>

Note. \(p_{BH}\) refers to \(p\)-values (two-tailed) controlled for FDR.

Change in symptoms of depression, suppression and reappraisal

After controlling for sex, age and time between the two points of measurement, there were no significant changes in depression severity, neither for healthy controls (BDI-II before the pandemic: \(M = 4.53, SD = 4.02, F(1,44) = 1.33, p = .254\)) nor for former inpatients (BDI-II before the pandemic: \(M = 32.01, SD = 11.39, F(1,34) = 0.58, p = .451\)). There were no changes in suppression or reappraisal, neither for healthy controls (suppression: \(F(1,44) = 0.24, p = .626\); reappraisal: \(F(1,44) = 0.16, p = .688\)) nor for former inpatients (suppression: \(F(1,34) = 0.14, p = .714\); reappraisal: \(F(1,34) = 0.13, p = .722\)).

Correlation analyses

During the pandemic, in former inpatients fear of COVID-19 (FCV-19S) was significantly positively associated with the fear of infecting others, financial hardship due to the current...
pandemic and depression severity during the pandemic. Depression severity during the pandemic on the other hand was significantly positively associated with psychological strain due to other circumstances. Depression severity during the pandemic was significantly negatively associated with the emotion regulation strategy of reappraisal. Correlation coefficients for the hypothesized associations were of small to medium size and had the predicted polarities (Table 3).

**Table 3**

Spearman’s correlation coefficients between FCV-19S, BDI-II and the other variables in former inpatients controlling for age and sex.

<table>
<thead>
<tr>
<th></th>
<th>FCV-19S</th>
<th>BDI-II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r((p_{BH}))</td>
<td>r((p_{BH}))</td>
</tr>
<tr>
<td>BDI-II</td>
<td>0.54(.006)**</td>
<td>0.35(.119)</td>
</tr>
<tr>
<td>Fear of infecting others with coronavirus</td>
<td>0.60(.001)**</td>
<td>0.12(.628)</td>
</tr>
<tr>
<td>Financial hardships due to pandemic</td>
<td>0.51(.011)*</td>
<td>0.20(.360)</td>
</tr>
<tr>
<td>Fear of unemployment due to pandemic</td>
<td>0.38(.097)</td>
<td>0.29(.194)</td>
</tr>
<tr>
<td>Stress due to social distancing</td>
<td>0.40(.068)</td>
<td>0.29(.194)</td>
</tr>
<tr>
<td>Stress due to restrictions with respect to leisure time activities</td>
<td>0.35(.119)</td>
<td>0.07(.779)</td>
</tr>
<tr>
<td>Psychological strain due to other circumstances</td>
<td>0.21(.350)</td>
<td>0.69(.000)**</td>
</tr>
<tr>
<td>Suppression</td>
<td>0.31(.184)</td>
<td>0.21(.350)</td>
</tr>
<tr>
<td>Reappraisal</td>
<td>-0.26(.263)</td>
<td>-0.47(.018)*</td>
</tr>
</tbody>
</table>

*Note. \(p_{BH}\) refers to \(p\)-values (two-tailed) controlled for FDR. BDI-II, suppression and reappraisal during the pandemic. * \(p_{BH}\) < .05, ** \(p_{BH}\) < .01, *** \(p_{BH}\) < .001*

In the group of healthy controls, fear of COVID-19 was significantly positively associated with the fear of infecting others. Depression severity during the pandemic was significantly positively associated with psychological strain due to restrictions in leisure time activities and other circumstances not associated with COVID-19. There were no other significant associations (Table 4).

For associations between fear of COVID-19 and the ANPS see Supplementary Material (Table S2).
Table 4
Spearman’s correlation coefficients between FCV-19S, BDI-II and the other variables in healthy controls controlling for age and gender.

<table>
<thead>
<tr>
<th></th>
<th>FCV-19S</th>
<th>BDI-II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r(p_{BH})$</td>
<td>$r(p_{BH})$</td>
</tr>
<tr>
<td>BDI-II</td>
<td>0.17(.746)</td>
<td>0.18(.746)</td>
</tr>
<tr>
<td>Fear of infecting others</td>
<td>0.55(.001)**</td>
<td>0.18(.746)</td>
</tr>
<tr>
<td>Financial hardships</td>
<td>-0.06(.960)</td>
<td>-0.04(.976)</td>
</tr>
<tr>
<td>Fear of unemployment</td>
<td>0.12(.850)</td>
<td>0.26(.469)</td>
</tr>
<tr>
<td>Stress due to social distancing</td>
<td>0.16(.794)</td>
<td>0.36(.115)</td>
</tr>
<tr>
<td>Stress due to restrictions with respect to leisure time activities</td>
<td>0.18(.746)</td>
<td>0.42(.043)*</td>
</tr>
<tr>
<td>Psychological strain due to other circumstances</td>
<td>-0.06(.960)</td>
<td>0.64(.000)***</td>
</tr>
<tr>
<td>Suppression</td>
<td>0.01(.999)</td>
<td>0.27(.442)</td>
</tr>
<tr>
<td>Reappraisal</td>
<td>-0.01(.999)</td>
<td>0.01(.999)</td>
</tr>
</tbody>
</table>

Note. $p_{BH}$ refers to $p$-values (two-tailed) controlled for FDR. BDI-II, suppression and reappraisal during the pandemic. * $p_{BH} < .05$, ** $p_{BH} < .01$, *** $p_{BH} < .001$
After stepwise model reduction, the model with the lowest AIC explained a significant amount of variance in fear of COVID-19 ($R^2 = 36.94$, $F(10,72) = 4.22$, $p = 0.0001$; Table 5). The experience of SLEs (CLEQ), a diagnosis of depression and high depression severity before the current pandemic were significantly positively associated with fear of COVID-19. The primary emotion PLAY was significantly negatively associated with fear of COVID-19. Last, there was a significant interaction: While there was a (non-significant) positive association between SADNESS and fear of COVID-19 in healthy controls, SADNESS was negatively associated with fear of COVID-19 in former inpatients (Figure 1).

**Table 5.**
Final model of the stepwise regression analysis with age at onset as dependent variable.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>$b$</th>
<th>$b$ (std.)</th>
<th>SE</th>
<th>$t$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>7.92</td>
<td>0.23</td>
<td>5.66</td>
<td>1.40</td>
<td>0.166</td>
</tr>
<tr>
<td>Group</td>
<td>15.49</td>
<td>-0.37</td>
<td>7.44</td>
<td>2.08</td>
<td>0.041*</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.45</td>
<td>0.08</td>
<td>1.19</td>
<td>-0.38</td>
<td>0.705</td>
</tr>
<tr>
<td>CLEQ</td>
<td>0.09</td>
<td>0.39</td>
<td>0.03</td>
<td>3.22</td>
<td>0.002**</td>
</tr>
<tr>
<td>PLAY</td>
<td>-2.32</td>
<td>0.28</td>
<td>1.16</td>
<td>-2.01</td>
<td>0.048*</td>
</tr>
<tr>
<td>SADNESS</td>
<td>1.192</td>
<td>-0.13</td>
<td>1.86</td>
<td>1.03</td>
<td>0.306</td>
</tr>
<tr>
<td>Reappraisal</td>
<td>0.83</td>
<td>0.09</td>
<td>0.55</td>
<td>1.50</td>
<td>0.138</td>
</tr>
<tr>
<td>BDI-II</td>
<td>0.15</td>
<td>0.54</td>
<td>0.06</td>
<td>2.48</td>
<td>0.015*</td>
</tr>
<tr>
<td>Group*sex</td>
<td>2.70</td>
<td>0.15</td>
<td>1.80</td>
<td>1.50</td>
<td>0.137</td>
</tr>
<tr>
<td>Group*SADNESS</td>
<td>-7.06</td>
<td>-0.41</td>
<td>2.64</td>
<td>-2.67</td>
<td>0.009**</td>
</tr>
<tr>
<td>Group*reappraisal</td>
<td>-1.19</td>
<td>-0.17</td>
<td>0.74</td>
<td>-1.60</td>
<td>0.113</td>
</tr>
</tbody>
</table>

*Note.* B (std.) represents standardized coefficients. **$p < .01$, *$p < .05$. 
Discussion

In the current study, we wanted to investigate the predictive value of SLEs, primary emotions and emotion regulation strategies for fear of COVID-19 in former inpatients with major depressive disorder as well as in healthy controls. There were no group differences with respect to the experience of COVID-19 related SLEs. This is worth noting, since group differences in fear of COVID-19 or stress experience related to the current pandemic are unlikely to originate from one group living in a high risk environment for COVID-19 related SLEs. Overall, there were very few controls or inpatients who experienced any of the events in question (please see also that fear of COVID-19 scores were not overly high in both groups). This can be explained by the relatively low rate of infection in Germany in the period from July to September.

The investigation of group differences in variables examined before the current pandemic revealed the expected result pattern: Depressive inpatients reported having experienced more SLEs, show more suppression, less reappraisal, lower SEEKING, lower PLAY, higher FEAR and higher SADNESS than healthy controls did. These findings are in line with previous research (Montag et al., 2017; Sanwald et al., 2020; Visted et al., 2018). Note that at the first point of measurement, the sample of inpatients suffering from depression partially overlapped with samples analyzed in previous studies (Montag et al., 2017; Sanwald et al., 2020). SLEs like sexual abuse are associated with an increased risk of a lifetime diagnosis of depression (Chen et al., 2010). Depression in turn is associated with a higher reactivity to stressors (Burke et al., 2005; Shapero et al., 2014).

Higher stress reactivity could be additionally explained by the use of ineffective emotion regulation strategies, i.e. less reappraisal and more suppression as compared to non-depressed individuals (Moore et al., 2008; Troy et al., 2010). This is also in line with Watt and Panksepp's (2009) theory of depression development. They postulate depression to originate from a shutdown mechanism terminating chronically prolonged separation distress (Watt and Panksepp, 2009). The emotional shutdown is characterized by a tendency to experience less SEEKING and more SADNESS. In sum, SLEs may induce the tendency to experience more emotions that are negative due to subsequent stressors. This in combination with ineffective emotion regulation strategies could lead to an increase in stress experience leaving an individual...
vulnerable to depression development. We want to highlight that we only have cross-sectional data and therefore cannot make causal conclusions. However, there is longitudinal data supporting the causal link between stress, negative affect and coping strategies with the development of depressive symptoms (Evans et al., 2014; Kendler et al., 2006, 1999).

During the pandemic, former inpatients still showed higher depression severity and a higher use of suppression than did healthy controls. In addition, they experienced more fear of COVID-19 compared to healthy controls. Contrary to our expectations, former inpatients did not experience more concerns due to specific aspects coinciding with the pandemic.

Depression severity was significantly positively associated with psychological strain due to circumstances not associated with the current pandemic in both groups. In addition, reappraisal was significantly negatively associated with depression severity in the group of former inpatients. These findings highlight that depression can be explained by an interaction of vulnerability – resulting from intra-individual (biological and psychological) as well as social interaction factors – and stressful life events.

It seems that former inpatients with still elevated scores of depression severity are more burdened than controls by a fearful view of the pandemic situation, but not by restrictions to limit the incidence of infection. The group difference considering fear of COVID-19 was absent when comparing patients without or with mild depressive symptom complaint to healthy controls. These findings are in line with the results of a recent study reporting more COVID-19 related fear of individuals with mental disorders as compared to healthy participants (Skoda et al., 2020). Our results extend these findings by showing group differences between healthy individuals and individuals previously diagnosed for depression.

In healthy controls, we did not find significantly more severe depressive symptoms or significant changes in emotion regulation strategies during than before the pandemic. This finding at first glance seems to contradict the many reports of an increase in depressive symptoms (Ettman et al., 2020; Skoda et al., 2020) or relatively high rates of depression during the pandemic (for a systematic review, see Xiong et al., 2020). It is, however, worth noting that the sample of healthy individuals in our control group underwent an extensive screening procedure to exclude individuals with potential signs of mental disorders. Therefore, it is
possible that we examined a sample of individuals with a high degree of resilience to stressors associated with the current pandemic. Our sample of former inpatients, on the other hand, was assessed before the pandemic when they were in need of an inpatient treatment. After the first point of measurement, they received treatment and at the second point of measurement during the pandemic, they were already dismissed from the hospital. Treatment effects therefore might mask a potential worsening of depressive symptoms in the former inpatient group.

Fear of COVID-19 was significantly positively associated with the fear of infecting others in both groups and with financial hardships as well as with depression severity in former inpatients. This is in line with a recent study reporting perceived risk for loved ones to be related to fear of COVID-19 (Mertens et al., 2020). The significantly positive association between fear of COVID-19 and depression severity is also in line with recent findings of a positive association between fear of COVID-19 and depression in pregnant wives and their husbands (Ahorsu et al., 2020a). The fact that the association between depression severity and fear of COVID-19 was present only in former inpatients could reflect the long known comorbidity of depression and anxiety disorders (Lamers et al., 2011). The positive association between fear of COVID-19 and financial hardships in the group of former inpatients can be explained by depression affecting an individual’s job performance even after symptom improvement (Adler et al., 2006) in combination with a pessimistic bias in the prediction of future events (Strunk et al., 2006). Contrary to our expectations, there were no associations of fear of COVID-19 with emotion regulation in the control group. In healthy controls, low scores and small variances considering fear of COVID-19 could be responsible for small correlation coefficients in correlational analyses using this variable. It is, however, worth noting that we found small to medium size associations in the hypothesized direction between emotion regulation and fear of COVID-19 in the group of inpatients. Therefore, the associations between emotion regulation and fear of COVID-19 should be tested in a larger sample of individuals with a diagnosis of depression.

Fear of COVID-19 was predicted by the presence of a diagnosis of depression and higher depression severity. In addition, it is in line with the stable finding of associations between
depression and fear as well as anxiety which has been argued to result from common etiologic factors like negative affectivity and neural substrate (Garber and Weersing, 2010).

We found PLAY, a primary emotion characterized by a humorous and light-hearted way of dealing with circumstances, to be significantly negatively associated with fear of COVID-19 independent of group. This finding supports findings from a recent study reporting positivity to have a significant effect on death distress and happiness during the current pandemic (Yıldırım and Güler, 2021). Benign, i.e. self-enhancing humor, has been shown to be effective in down regulating negative and upregulating positive emotions (Samson and Gross, 2012). A playful personality represents a trait of having fun in life and can thus be considered a self-enhancing form of humor. Self-enhancing humor helps in dealing with difficult situations in everyday life (Booth-Butterfield et al., 2007) and is an important coping strategy in case of SLEs (Boerner et al., 2017). PLAY is known to be a bottom-up driver of Extraversion (Montag & Panksepp, 2017) and Extraversion itself has been associated with higher life satisfaction in many studies (e.g. Lachmann et al., 2017). Taking all this together, even though the effect size is small we consider the PLAY finding of major importance since it might help not only healthy but especially mentally burdened individuals dealing with the current pandemic (see Supplementary Material Table S2). Humor or positivity based interventions could help to prevent the stress level in such difficult situations from rising above a critical point. In addition, humor-based online interventions could be especially important in times of a pandemic, being available everywhere at a low threshold while enabling the maintenance of social distance. Such humor-based online interventions have already been shown to effectively increase happiness and reduce depressive symptoms (Wellenzohn et al., 2018). Therefore, the development of online interventions tailored to pandemic situations could be worthwhile in order to maintain not only the physical but also the mental health of the population.

The association of group and SADNESS is an interesting finding we did not expect. A possible explanation is that SADNESS or separation distress causes a time-limited response to loss in healthy individuals and initially an increase of fear of COVID-19. If, however, SADNESS is transformed into something recurrent because of avoidance behavior, a depressive episode develops (Leventhal, 2008). Avoidance behavior is known to reduce fear in short-term but to
be responsible for the maintenance of fear in the long-term (Mowrer, 1956). Therefore, increased SADNESS associated avoidance behavior in former inpatients may explain the contradictory associations in the comparison of both groups. Nevertheless, and this limits the interpretation, the SADNESS measure of the ANPS is known to be stable and to measure a trait (Orri et al., 2018).

SLEs were also a factor explaining a significant amount of variance in fear of COVID-19. Research postulating the experience of SLEs to increase an individual’s vulnerability to subsequent stressors and risk of developing a MDD or anxiety disorder (McLaughlin et al., 2010) provides an explanation for increased fear during a pandemic as a function of SLEs.

Some limitations need to be considered when interpreting the results of our study. First, our sample size was rather small and results should be replicated in a larger sample. However, our sample underwent an extensive screening procedure ensuring that there was no suspicion of mental disorder in healthy controls. Furthermore, a diagnosis of MDD in the sample of former inpatients was carefully confirmed during the hospital stay before the pandemic. Second, the carefully screened sample of healthy participants reduces generalizability of our results to the common population. Third, assessment during the pandemic was during the summertime when the infection rate was rather low in Germany. Thus, restrictions were not as strict as they were in spring or in winter. Therefore, the questions examined in our study should be reconsidered in times with high infection rates and major restrictions.

In summary, our study provides novel findings highlighting that humor could be a valuable ally in the development of preventive strategies to combat mental stress in pandemic situations. The development and use of online interventions aiming at increasing humor and happiness tailored to pandemic situations could have beneficial effects on mental health when social distancing is needed to counteract the spread of an infectious disease. SLEs, depressive symptoms, primary emotions and emotion regulation might be valuable predictors of who is at risk of high fear levels during a pandemic.
Author Contributions

S.S., C.M. and M.K. designed the present study. S.S. analyzed the data and wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the manuscript.

Acknowledgements

None.

Conflict of Interest

There are no competing financial interests to be disclosed.

Ethical Approval

All procedures performed in this study were in accordance with the ethical standards of the ethics committee of Ulm University, Ulm, Germany (reference number: 25/18) and with the 1964 Helsinki declaration and its later amendments.

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JASP Team, 2019. JASP (Version 0.11.1).


https://doi.org/10.1016/j.comppsych.2016.11.007


Figure 1. Interaction between group and SADNESS in the prediction of fear of COVID-19.
3. General discussion and future directions
The present dissertation aimed at providing evidence regarding the correlates of depression at the biological, psychological and environmental level as described in the comprehensive model of depression development. This dissertation complements and extends previous research by conducting a joint investigation of SLEs, epigenetic alterations, primary emotions and depression. According to the comprehensive model of depression development, I hypothesized that the 2D:4D ratio, a potential marker of prenatal androgen exposure, is associated with depression later in life. Furthermore, in line with the model, I assumed a positive association between SLEs and OXT as well as SLC6A4 methylation. In accordance with the model, SLEs and depression should be positively associated with SADNESS and negatively associated with SEEKING. Stress associated alterations in epigenetic profiles and primary emotions should predict depression onset and the perception of and reaction to future stressors like the current pandemic.

As predicted by the comprehensive model of depression development, the current dissertation demonstrates that the 2D:4D ratio is related to depression diagnosis. However, there was no direct association between the 2D:4D ratio and depression in study I. Instead, study I yielded no sex differences in the 2D:4D ratio of patients suffering from depression, while there were sex differences in the group of healthy controls (Sanwald et al., 2019). This is in line with predictions derived from the model since prenatal androgen exposure in combination and interaction with other prenatal environmental, psychological and biological factors represents an individual’s initial vulnerability for depression development. Many intermediate factors influence whether a vulnerability leads to the manifestation of a disease. According to the comprehensive model of depression development, methylation status of OXT should be positively associated with SLEs and depression severity. However, studies II and III demonstrated that OXT methylation was not only negatively associated with SLEs and depression severity but was also significantly lower in inpatients suffering from MDD as compared to healthy controls. The correlational pattern of negative associations between SLEs and OXT methylation was consistent across both studies and was found for the majority of individual CpG units (Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). Therefore, while the assumption of an association between SLEs and OXT methylation was correct, the direction of the association was contrary to what I predicted. Study IV tested the comprehensive model of depression development’s assumption that a combination of environmental, psychological and biological factors predicts a significant amount of variance in depression onset. Study IV showed that sex, SLEs, SEEKING and SADNESS but not SLC6A4 methylation were significant predictors of age at depression onset. SLC6A4 methylation, however, was positively associated with depression severity in women (Sanwald et al., 2021). A positive association of SLC6A4 methylation and depression is in line with the predictions derived from my model. These results demonstrate the importance of the three
levels, i.e., environmental, psychological and biological factors, and their joint influence on depression onset. Basing on the model, past SLEs should be associated with alterations in primary emotions. These stress-associated alterations in primary emotions should be predictive for the reaction to future stressors. Study V demonstrated that SADNESS and PLAY among other variables assessed before the pandemic explained a significant amount of variance in fear of COVID-19 (Sanwald et al., under review). Therefore, the five studies comprising this work show that depression can be understood as a disorder that emerges from a vulnerability formed by the interaction of an individual’s environment, the individual’s personality affecting the interpretation and reaction to the environment and the biological makeup. The notion of the comprehensive model of depression development is that initially biology and environment shape an individual’s primary emotional basis of personality. Primary emotions in turn shape the individual’s perception of and reaction to its environment and thereby coin epigenetic alterations associated with environmental influences. If the experience of a SLE elicits a pattern of high SADNESS and low SEEKING previously established by early separation distress, an individual may ultimately develop an episode of Major Depression. The implications and limitations of the five studies are further integrated with the comprehensive model of depression development in the following sections. To this end, after describing the associations of single factors with depression, a section on the interrelations and interactions of the investigated variables transitions to suggestions for future research and an overview of practical implications of the findings of the five studies comprising this dissertation.

3.1. The 2D:4D ratio and MDD
A significant interaction between sex and group indicated that sex differences in the 2D:4D ratio of the right hand are less pronounced in patients suffering from depression as compared to healthy controls (Sanwald et al., 2019). However, the effect size was small and the interaction should therefore be interpreted with caution. We found neither conclusive evidence of a direct association between the 2D:4D ratio and depression severity nor with a diagnosis of MDD. This result pattern is in accordance with a recent study reporting no significant associations between the 2D:4D ratio and depression severity as well as depression history (Lautenbacher & Neyse, 2020). It is, however, worth mentioning that this study used self-report to assess depression history retrospectively. Furthermore, a systematic review on case-control studies found no significant difference between patients with depression or bipolar disorder and healthy controls (Fusar-Poli et al., 2021). This review included only two studies on mood disorders, one of them being our study. Therefore, the authors highlight that data is sparse and conclusions on the associations between the 2D:4D ratio and mood disorders are preliminary. More data are needed for a well-powered meta-analytic evaluation. Taking into consideration the comprehensive model of depression development, the 2D:4D ratio should only be indirectly associated with depression since there are many intermediate steps between prenatal
exposure to testosterone relative to estradiol and the full-blown disorder. Therefore, preliminary analyses considering associations between the 2D:4D ratio and primary emotions are described below.

It is worth mentioning that there is an ongoing discussion regarding the assumption of prenatal exposure to androgens affecting digit ratio. On the one hand the genes implicated in the differentiation of genitals have also been implicated in the growth of the digits (Kondo et al., 1997). In addition, there is a stable sex difference in the 2D:4D ratio (Hönekopp & Watson, 2010) and there are studies indicating a link between sex-hormones in amniotic fluid and the 2D:4D ratio albeit with less clear result pattern than expected (Lutchmaya et al., 2004; Ventura et al., 2013). Furthermore, experimental studies in rodents support the link between prenatal androgen exposure and the 2D:4D ratio (Zheng & Cohn, 2011). On the other hand, in humans there are also studies that report null-findings with regard to the association of prenatal androgen exposure and digit ratio (Nave et al., 2021; Van Hemmen et al., 2017; Voracek et al., 2019).

However, despite the ongoing debate of prenatal androgen exposure affecting the human 2D:4D ratio, our results indicate that men and women with a sex-neutral 2D:4D ratio could be at risk for developing depression later in life. Therefore, future studies should investigate a combination of amniotic fluid androgen concentration, 2D:4D ratio and depression to further elaborate the results presented in this dissertation.

3.2. SLEs and MDD

The association between SLEs and depression development is a well-established finding in depression research. The experience of early adversity is postulated to sensitize for the influence of SLEs on the biological and mental states in adulthood (Harkness et al., 2006). For instance, recent stressors might be associated with a higher risk for depression development if an individual was exposed to childhood adversity (McLaughlin et al., 2010). In the comprehensive model of depression development, SLEs across the life span are associated with depression severity and development. In our studies, we used a relatively unspecific measure of SLEs and our results showed that SLEs irrespective of their timing are significantly positively associated with depression severity (Sanwald et al., under review, 2021; Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). Therefore, an unspecific measure of SLEs is in line with the model’s assumption of SLEs irrespective of their timing being associated with depression development. However, this approach did not allow for investigating the differential influence of SLEs in early developmental stages and SLEs in later stages of development. According to the model, early experiences of separation distress should result in a protest phase with a subsequent shutdown (Watt & Panksepp, 2009). The shutdown mechanism might be reactivated in response to subsequent SLEs. To cut the chase: early adversity results in a vulnerability while subsequent stressors may lead to depression.
development because of the previously established emotional shutdown. These assumptions are in line with a recent longitudinal study postulating early SLEs to contribute to a vulnerability for depression while recent SLEs resulted in greater depressive symptom risk in a dose dependent manner (Arpawong et al., 2021). Since the comprehensive model of depression development assumes all SLEs across the lifespan to impact an individual’s personality and biology and as a result the individual’s interpretation of and reaction to subsequent SLEs, this makes it difficult to retrospectively separate the influence of early-life and later-life SLEs. Longitudinal studies in birth cohorts are better suited to shed light on the three phases postulated by the model. In our studies, inpatients reported having experienced significantly more SLEs as compared to healthy controls. This was true for two case-control studies (study III and study V). The combination of a linear association between SLEs and depressive symptoms as well as an association between SLEs and depression diagnosis indicates that the experience of SLEs is not only a factor associated with the risk of developing depression but may also predict the severity of an episode of MDD (Sanwald et al., under review; Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). After all, the previous experience of stressors is known to be associated with an elevated risk for experiencing subsequent events as more stressful (Stroud et al., 2011). Not only childhood adversity but also SLEs later in life might sensitize for subsequent stressors at least for a period of time after the initial SLE (Carroll et al., 2005; Smid et al., 2012). An episode of depression itself can be considered a stressor (Sachar et al., 1973) and individuals suffering from MDD show higher cortisol levels during stress recovery as compared to non-depressed controls (Burke et al., 2005). This could explain why individuals who experienced more SLEs are more vulnerable for depression development and report higher depressive symptom severity, which is in line with the comprehensive model of depression development. However, according to this explanation, individuals who experienced more SLEs should also feel more threatened in the face of current stressors like the COVID-19 pandemic. Indeed, the results of study V are in line with this explanation demonstrating that the experience of more SLEs significantly predicts higher fear of COVID-19 even after controlling for group and depression severity (Sanwald et al., under review).

The comprehensive model of depression development also implicates that SLEs (in combination with psychological and biological factors) predict depression onset. Study IV demonstrates that SLEs predict a significant amount of variance in depression onset (Sanwald et al., 2021). Even though we used a retrospective measure of age at depression onset, our results are in line with previous studies postulating a (causal) relationship between SLEs and depression onset (Kendler et al., 1999, 2010).

In summary, as predicted by the comprehensive model of depression development, SLEs are a major risk factor for depression development. This finding was homogeneous across all four
studies investigating SLEs within this dissertation. Furthermore, SLEs were significantly associated with epigenetic alterations and primary emotions.

3.3. Primary emotions associated with MDD
In terms of psychological factors, this work focused on primary emotions, since in line with the theory of depression development by Watt and Panksepp (2009) I consider primary emotions of utmost importance for understanding emotional changes in the context of depression. The importance of primary emotions was substantiated by the results of studies IV and V. As in a previous study by Montag and colleagues (2017), SADNESS was significantly positively and SEEKING significantly negatively associated with depression severity or the diagnosis of depression. Furthermore, in study IV we showed that SADNESS and SEEKING were not only associated with a self-report measure of depression severity (the Beck Depression Inventory; Beck, Steer, & Brown, 2006) but also with depression severity externally assessed by a trained interviewer using the Montgomery Asberg Depression Rating Scale (Montgomery & Asberg, 1979). Additionally, SEEKING and SADNESS were both included in the model predicting depression onset as indicated by the Akaike information criterion (Sanwald et al., under review, 2021).

The finding of lower PLAY in the group of inpatients as compared to healthy controls in study V is also in line with Watt and Panksepp (2009) postulating depression to be associated with diminished PLAY. In addition, our results extend the theory by Watt and Panksepp (2009) demonstrating a prospective association between the primary emotion PLAY and fear of COVID-19. We argued that this is in line with other studies reporting positivity to have a significant effect on distress and happiness during the corona pandemic (Sanwald et al., under review; Yıldırım & Güler, 2021). Furthermore, self-enhancing humor might be an effective strategy to upregulate positive and downregulate negative emotion and might therefore help dealing with difficult situations like a pandemic (Boerner et al., 2017; Booth-Butterfield et al., 2007; Samson & Gross, 2012).

However, there was no prospective association between SEEKING, SADNESS and depression severity after discharge from the hospital. Performing an additional stepwise regression analysis with the sample of inpatients of study V revealed that in the final model (indicated by the Akaike information criterion; the starting model included all variables assessed in study V as predictors, i.e., age, sex, time difference between the two points of measurement, SEEKING, PLAY, CARE, FEAR, ANGER, SADNESS, reappraisal, suppression, BDI-II before the pandemic, CLEQ and FCV19S) in terms of primary emotions only FEAR predicted a significant amount of variance in depression severity measured later in life (Table 1). Higher scores on FEAR predicted higher depression severity later in life.
Table 1.
Regression model predicting depression severity in the sample of inpatients of study V.

<table>
<thead>
<tr>
<th></th>
<th>b</th>
<th>SE</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>2.815</td>
<td>5.540</td>
<td>0.508</td>
<td>.615</td>
</tr>
<tr>
<td>sex</td>
<td>-6.440</td>
<td>3.029</td>
<td>-2.126</td>
<td>.042*</td>
</tr>
<tr>
<td>SEEKING</td>
<td>2.894</td>
<td>1.693</td>
<td>1.709</td>
<td>.098</td>
</tr>
<tr>
<td>PLAY</td>
<td>-3.373</td>
<td>2.456</td>
<td>-1.373</td>
<td>.180</td>
</tr>
<tr>
<td>FEAR</td>
<td>5.825</td>
<td>2.074</td>
<td>2.808</td>
<td>.009**</td>
</tr>
<tr>
<td>BDI-II</td>
<td>6.830</td>
<td>2.640</td>
<td>2.587</td>
<td>.015*</td>
</tr>
<tr>
<td>FCV19S</td>
<td>4.779</td>
<td>1.735</td>
<td>2.755</td>
<td>.010*</td>
</tr>
</tbody>
</table>

Note. Standard error of the mean (SE), Beck Depression Inventory (BDI-II). Fear of COVID-19 Scale (FCV19S). $R^2 = 0.71$, $F(6,29) = 11.80$, $p < .00001$. *$p < .05$, **$p < .01$. *BDI-II assessed before the pandemic.

These results, however, are only preliminary since the sample size was small and the time between the two points of measurement varied. Even though the inclusion of time between measurement points did not change the results, these results should be validated in a larger sample with a fixed time interval between the two points of measurement. Nevertheless, these first exploratory findings are interesting and could point towards different roles of primary emotional systems for depression etiology or first onset of depression, for depression severity during a depressive episode and for the prediction of future depressive episodes. In fact, similar findings have been shown by other studies: One study concludes that stress associated alterations in HPA activity may play different roles for first onset and for the onset of recurrent depressive episodes (Mazurka et al., 2016). Another study found negative emotionality to be associated with first onset of depression while trait anxiety was associated with recurrent MDD (Wilson, Vaidyanathan, Miller, McGue, & Iacono, 2014). These results are in line with the results of studies IV and V and with the exploratory analysis shown above.

In summary, increased SADNESS and decreased SEEKING were associated with depression severity during an episode of depression, depression diagnosis and predicted depression onset. SEEKING and SADNESS were not associated with depression severity after discharge from the hospital. FEAR predicted depression severity after hospital discharge. Therefore, the results of studies IV and V support the comprehensive model of depression development suggesting primary emotions to be predictive for depression development and extend the model by FEAR being a predictor of depression severity later in life.

3.4. Biological factors associated with MDD

In terms of biological factors, we investigated the 5-HTTLPR and methylation status of SLC6A4 as well as OXT. In line with the early interest in serotonin regarding depression research, we start with our findings considering SLC6A4, followed by an integration of our findings considering OXT, which received more interest only in recent years.
3.4.1. SLC6A4 and MDD

Despite its long tradition in theories of depression development, a direct association of the methylation of SLC6A4 or alleles of the 5-HTTLPR and MDD is not supported by the current literature. For instance, a recent genome-wide meta-analysis of depression-associated genes did not identify any genes linked with the serotonergic system. The authors suggested that genetic pathways to depression might be functionally separated from pathways of antidepressant treatment (Howard et al., 2019). In addition, a recent study on induced pluripotent stem cell-derived serotonergic neurons found no significant differences in 5-HT release and reuptake comparing MDD patients treatment-resistant considering SSRIs, MDD patients with remittent depressive symptoms after treatment with SSRIs and healthy controls (Vadodaria et al., 2019). Furthermore, SSRIs are moderately effective with delayed treatment effects (Gaynes et al., 2009) and might produce unfavorable sometimes even paradoxical long-term effects (Carvalho et al., 2016). On the other hand, however, there are many studies, especially animal studies, demonstrating the involvement of almost all serotonin receptors in antidepressant effects (Żmudzka et al., 2018). Despite the fact that the role of 5-HT receptors for depression is not yet fully understood, the serotonin system seems to be involved in MDD but in a more complex fashion than initially postulated by the monoamine hypothesis (Krystal et al., 2019; Pitsillou et al., 2020). Serotonin receptors might even interact with oxytocin receptors potentially contributing to psychiatric disorders (Borroto-Escuela et al., 2021). An involvement of SLC6A4 methylation in MDD could also be found in our study. We did not find an association between SLEs and SLC6A4 methylation but showed that SLC6A4 methylation was significantly positively associated with externally assessed depression severity in female inpatients (Sanwald et al., 2021). We found a correlation coefficient of moderate size according to Cohen’s criteria (Cohen, 1988). This result is in line with other recent studies reporting an association of increased SLC6A4 methylation and depression (Bakusic et al., 2020; Park et al., 2019). However, the directionality of the association is heterogeneous across studies as well as the exact CpG sites or clusters of CpG sites that are differentially methylated (Mendonça et al., 2019; Park et al., 2019). Therefore, as the number of studies investigating SLC6A4 methylation in depression increases, meta-analyses are needed to elucidate which CpG sites of SLC6A4 are altered in MDD patients. We did not find a significant effect of the 5-HTTLPR in our study (Sanwald et al., 2021). Even though our sample size was much too small to make definitive conclusions, our results are in line with the current literature, which suggests no or only a small effect considering the association of the 5-HTTLPR and MDD (Border et al., 2019; Culverhouse et al., 2018; Howard et al., 2019).

Taken together, study IV is in line with the comprehensive model of depression development which postulates the serotonin system to be indirectly related to stress via the bodily stress or the oxytocin system since we did not find an association between SLEs and SLC6A4 methylation. However, SLC6A4 methylation was associated with depression severity in
women. It is worth mentioning that our results are in line with the suggestion of a functional separation of depression etiology from pathways of antidepressant treatment (Howard et al., 2019). Additionally, our results do not support the notion of a strong association between depression and SLC6A4 methylation or the 5-HTTLPR. Therefore, the theory-driven investigation of other neurotransmitter systems potentially involved in the pathogenesis of MDD is important. The following section describes our findings with regard to oxytocin, which sparked researchers' interest after initial animal studies demonstrated its stress attenuating properties (Pitsillou et al., 2020).

3.4.2. OXT and MDD
Our results of studies II and III extend previous reports of dysregulated oxytocin pathways in depression (Cyranowski et al., 2008; Thomas & Larkin, 2020). We investigated for the first time OXT (the gene coding for oxytocin) methylation in patients suffering from MDD. As predicted by Watt and Panksepp (2009) and the comprehensive model of depression development, we found SLEs to be significantly associated with OXT methylation (Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). We also found OXT to be hypomethylated in MDD patients as compared to healthy controls (Sanwald, Widenhorn-Müller, et al., 2020). The pattern of a negative association between SLEs and OXT methylation was consistently found for mean methylation and for the majority of single CpG units. This was contrary to the initially expected downregulation of OXT following SLEs as described in the comprehensive model of depression development. However, it points towards an important role of alterations of the epigenetic regulation of the oxytocin system following SLEs for depression development. There are no other studies having investigated OXT methylation, however, there are numerous studies showing an association of early adversity and peripheral oxytocin concentrations. Most of these studies suggest early adversity to be inversely related to peripheral oxytocin levels, even though there are also contradictory findings and it is currently unknown how different types of SLEs influence peripheral oxytocin (for a review, see Londono Tobon et al., 2018). Whilst inverse associations of early adversity and peripheral oxytocin seem to contrast our findings – if lower methylation levels are associated with a higher translation rate, more mRNA and thus more oxytocin – an initial upregulation of the OXT system following separation distress could also promote an urge for social support, i.e., an upregulation of OXT could be an adaptive mechanism following SLEs. This notion is supported by studies reporting SLEs to promote short term rather than long term relationships (Hill et al., 1994) and is also in line with reports of the ability of individuals with relatively high exposure to SLEs early in life to build secure attachments (Thomson & Jaque, 2017). However, chronic oxytocin hyperactivity could also be associated with anxiogenic or depressogenic effects (Peters et al., 2014; Sanwald, Widenhorn-Müller, et al., 2020).
Furthermore, we found significant sex differences in OXT methylation with women exhibiting higher OXT methylation than did men in the majority of CpG units investigated (Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). This could point towards a role of OXT methylation or function of the oxytocin system for sex differences in depression (Sanwald, Widenhorn-Müller, et al., 2020). The investigation and identification of sex differences in epigenetic markers associated with MDD is important since only if we understand these differences we will be able to develop treatments for everyone (Hodes et al., 2017).

Taken together, SLEs were significantly negatively associated with OXT methylation contrary to our initial hypotheses. Furthermore, inpatients suffering from depression exhibited decreased OXT methylation as compared to healthy controls. The following section elaborates how the 2D:4D ratio, SLEs, primary emotions and OXT methylation are interrelated and interact comparing the group of inpatients to healthy controls.

3.5. Interrelations and interactions of environmental, psychological and biological factors

In study I, we found depression to be associated with the absence of sex differences in the 2D:4D ratio (Sanwald et al., 2019). The comprehensive model of depression development suggests many intermediate factors like primary emotions between the potential marker of prenatal androgen exposure and the development of a depressive episode. This is in line with previous studies. For instance, there are findings of associations between the 2D:4D ratio and personality traits (Manning et al., 2017; Turanovic et al., 2017). In addition, another study suggested fetal testosterone to program the reward system in males since increasing levels of fetal testosterone predicted increased behavioral approach tendencies (Lombardo et al., 2012). Therefore, higher levels of fetal testosterone presumably associated with a lower, more masculine 2D:4D ratio could be a prenatally determined resilience factor against depression development in males. The integration of these findings and the results of study I with the comprehensive model of depression development suggests associations between the 2D:4D ratio and depression associated primary emotions. Since the SEEKING system coordinates approach behavior, there should be negative associations between SEEKING and the 2D:4D ratio if high levels of fetal testosterone were associated with a lower 2D:4D ratio. A preliminary analysis of the data of study I revealed that there was indeed a significantly negative association between the 2D:4D ratio of the left hand and SEEKING in healthy men (Table 2). The same was not true for men suffering from depression. Instead, men suffering from depression showed a positive and marginally significant association between the 2D:4D ratio of the right hand and SADNESS (n = 45, p = .078, n.s.). In women suffering from depression, albeit non-significant, there was a positive association between the 2D:4D ratio of the left hand and SEEKING (n = 71, p = .074, n.s.). In line with previous findings (Lombardo et al., 2012), the presented results indicate higher SEEKING to be associated with higher levels of prenatal
testosterone as indicated by a lower 2D:4D ratio in healthy men. In summary, the 2D:4D ratio was directly associated with primary emotions and indirectly associated with depression diagnosis. This is in line with the comprehensive model of depression development.

**Table 2.** Pearson correlation coefficients between the 2D:4D ratio and primary emotions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEEKING</td>
<td>SADNESS</td>
</tr>
<tr>
<td>men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left 2D:4D</td>
<td>-0.37*</td>
<td>0.06</td>
</tr>
<tr>
<td>Right 2D:4D</td>
<td>-0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>women</td>
<td>SEEKING</td>
<td>SADNESS</td>
</tr>
<tr>
<td>Left 2D:4D</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Right 2D:4D</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Note.* *p* (two-sided) < .05.

The comprehensive model of depression development postulated SLEs to be associated with higher SADNESS and after an initial protest phase with lower SEEKING. The investigation of the associations between SLEs and SEEKING as well as SADNESS in study IV revealed that only SADNESS was negatively associated with SLEs (Sanwald et al., 2021). A recent review on stress induced anhedonia concludes that different stressful events may produce heterogeneous outcomes with regard to motivation and reward processing (Stanton et al., 2019). The fact that we used an unspecific measure of SLEs could explain decreased associations between stress and SEEKING. Furthermore, according to Watt and Panksepp (2009) a stressor causes separation distress, i.e., an upregulation of the SADNESS system. The SADNESS system on the other hand recruits SEEKING circuits thus triggering initially elevated efforts to terminate separation distress. The shutdown is only subsequent to the protest phase. Therefore, decreased SEEKING as opposed to SADNESS should only be indirectly related to SLEs.

It is worth mentioning that in study V we expected SEEKING and SADNESS to be associated with fear of COVID-19 since we expected SLEs and depression severity to be associated with fear of COVID-19. However, there were significant associations between neither SEEKING nor SADNESS and fear of COVID-19 (Sanwald et al., under review). This could be explained by SADNESS being triggered mainly by loss-associated stressors. In our sample, no one had lost anyone due to the pandemic and only three individuals of the control group had lost their job due to the pandemic. The lack of significant associations could therefore indicate chronically prolonged separation distress and the emotional shutdown to be depression specific and triggered only in case of separation or loss (Watt & Panksepp, 2009). This explanation is in line with attachment theories of depression development (Dagan et al., 2018; Spruit et al., 2020) and with a recent mouse model of depression which impressively demonstrated not only depressive symptoms but also a sex difference in depression risk following a specific early stressor described as fragmented maternal care (Goodwill et al., 2019). Reduced parental care has also been observed to be especially prevalent among
General discussion and future directions

individuals suffering from MDD (Lemoult et al., 2020). Therefore, alterations in the SEEKING and SADNESS system subsequent to early experiences of loss or reduced parental care might only be predictive for future stressors characterized by at least a symbolic loss for the individual. Thus, the comprehensive model of depression development should highlight that loss-associated stressors might be of particular importance.

The comprehensive model of depression development also postulates OXT methylation to be associated with primary emotions. Studies II and III, however, only showed that OXT methylation was significantly negatively associated with SLEs and depression diagnosis (Sanwald, Gahr, et al., 2020; Sanwald, Widenhorn-Müller, et al., 2020). An exploratory analysis on OXT methylation and primary emotions in patients suffering from depression (Table 3) revealed significantly negative associations between OXT methylation and the CARE system. Accordingly, stress associated lower OXT methylation could promote a more active CARE system. CARE urges might be essential for satisfying relationships (Montag & Panksepp, 2017). Good quality relationships are a resilience factor for psychopathology (Collishaw et al., 2007) and an upregulation of the CARE system can downregulate SADNESS (Montag & Panksepp, 2017). This should reflect in inverse associations between OXT methylation and SADNESS. Even though we consistently found small negative correlation coefficients for the associations between OXT methylation and SADNESS, these associations were not significant. However, our sample could be too small to detect a small effect of OXT methylation on SADNESS. Therefore, the positive association between OXT methylation and SADNESS postulated by the comprehensive model of depression development is not supported by the above results. However, taking into consideration the negative associations between SLEs and OXT methylation reported in studies II and III, OXT methylation should be negatively associated with SADNESS. This association should be analyzed in a larger sample providing the power to detect even small effects. After all, OXT as a single gene can explain only a small amount of variance in human personality (Montag et al., 2020).

There was also a significantly positive association between OXT methylation and ANGER (Table 2). This finding is in line with the current view that downregulation of the oxytocin system is associated with increased aggression (de Jong & Neumann, 2018). Last, it is worth mentioning that even though we provide promising results indicating an important role for OXT methylation in depression, the time courses of stress-associated alterations in OXT methylation and primary emotions remain speculative on the basis of our investigations and should be studied prospectively in the future.
Table 3.
Partial Spearman’s correlation coefficients between OXT methylation and primary emotions in the sample of inpatients from study II.

<table>
<thead>
<tr>
<th>CpGs</th>
<th>SEEKING</th>
<th>CARE</th>
<th>PLAY</th>
<th>FEAR</th>
<th>ANGER</th>
<th>SADNESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG_3</td>
<td>-0.02</td>
<td>-0.20*</td>
<td>0.06</td>
<td>-0.13</td>
<td>0.25**</td>
<td>-0.18</td>
</tr>
<tr>
<td>CpG_4</td>
<td>-0.09</td>
<td>-0.20*</td>
<td>-0.04</td>
<td>-0.05</td>
<td>0.15</td>
<td>-0.11</td>
</tr>
<tr>
<td>CpG_5</td>
<td>-0.02</td>
<td>-0.20*</td>
<td>-0.01</td>
<td>-0.10</td>
<td>0.13</td>
<td>-0.11</td>
</tr>
<tr>
<td>CpG_7.8</td>
<td>0.01</td>
<td>-0.14</td>
<td>0.03</td>
<td>-0.15</td>
<td>0.12</td>
<td>-0.18</td>
</tr>
<tr>
<td>CpG_9.10</td>
<td>-0.05</td>
<td>-0.20*</td>
<td>0.04</td>
<td>-0.05</td>
<td>0.18</td>
<td>-0.10</td>
</tr>
<tr>
<td>CpG_13</td>
<td>-0.06</td>
<td>-0.17</td>
<td>-0.02</td>
<td>-0.03</td>
<td>0.16</td>
<td>-0.16</td>
</tr>
<tr>
<td>CpG_14.15</td>
<td>-0.02</td>
<td>-0.16</td>
<td>0.02</td>
<td>-0.11</td>
<td>0.19*</td>
<td>-0.11</td>
</tr>
<tr>
<td>CpG_26</td>
<td>-0.17</td>
<td>-0.27**</td>
<td>-0.15</td>
<td>0.06</td>
<td>0.11</td>
<td>0.01</td>
</tr>
</tbody>
</table>

mean OXT methylation | -0.06  | -0.20* | -0.00 | -0.09 | 0.19* | -0.12  |

Note. Covariates: sex, age, body mass index (kg/m²), cigarettes per day, alcohol in grams per day. N = 121. *p < .05, **p < .01.

OXT methylation was negatively associated with CARE and positively associated with ANGER. This is in line with the negative associations between SLEs and OXT methylation and our above interpretation of an initial upregulation of the OXT system after a SLE promoting an urge for social support. Furthermore, this result pattern could point towards a stress-induced upregulation of the OXT system in depression being an important part of the epigenetic basis of an activation of CARE urges promoting the search for social support and relationships. Thus, lower OXT methylation could be an adaptive mechanism to counteract experiences of separation distress at least immediately after exposure to a SLE.

Taken together, the results are consistent with the notion of the comprehensive model of depression development that environmental, psychological and biological factors associated with depression are interrelated. This dissertation showed that the 2D:4D ratio was negatively associated with SEEKING in men. In addition, SLEs were associated with alterations in the oxytocin system and alterations in SADNESS. OXT methylation on the other hand was significantly negatively associated with CARE and significantly positively associated with ANGER. Thus, prenatal and epigenetic factors as well as SLEs are associated with primary emotions and all of these factors have predictive value for the course of MDD.

3.6. Implications of the current findings for research and clinical practice
This dissertation provides novel results that are incompatible with the view of a solely intraindividual and biological genesis of MDD. Instead, the results of our studies underscore the importance of SLEs in the etiology of MDD and show that there are stress associated epigenetic alterations in the oxytocin system comparing patients with a diagnosis of MDD to healthy controls. Thus, epigenetic mechanisms could indeed be one link between genes and the environment, which is needed to explain gene by environment interactions as foundation of vulnerability-stress-models of depression development. There is, however, a second link
between environment and an individual’s biology aside from epigenetics that has been known much longer but seems to be forgotten in the current trend to explain psychiatry with biology: personality. This is why the comprehensive model of depression development emphasizes the importance of primary emotions in the investigation of gene by environment interactions, especially since it becomes more and more apparent that depression is characterized by a plethora of alterations in a huge variety of biological variables. If an individual’s primary emotional characteristics are omitted, there is a risk of difficult-to-interpret and contradictory findings, because the step between environment and epigenetic adaptation is ignored, namely the perception and processing of environmental stimuli by an individual with its very own interpretation and emotional response. The investigation of primary emotional systems is only a starting point for future research. Attachment to caregivers and present partners, instruments assessing emotion regulation strategies and abilities, cognitive resources as well as course of the disorder have already been associated with MDD and could provide valuable information to better characterize samples, interpret biological correlates of MDD and to generate and falsify novel hypotheses (Bifulco et al., 2002; Gabrys et al., 2018; Pettit et al., 2009; Spruit et al., 2020; Visted et al., 2018). This dissertation shows that primary emotional systems provide a possibility to explain affective consequences of SLEs, may bring us closer to the interpretation of alterations in methylation profiles and might be useful for the prediction of depression onset as well as depression severity later in life. Therefore, future research on depression development should not only focus on larger samples and modern technology to decipher more and more biological alterations associated with depression but should also aim at understanding the individual perceptions, interpretations and emotional consequences associated with these alterations in well-characterized samples.

Furthermore, the sex differences in OXT methylation and with regard to the differential associations between SLC6A4 methylation and MDD in women as compared to men underscores the importance of the inclusion of sex as a moderating factor in future epigenetic studies. The investigation of sex differences in the epigenetic landscape of male and female patients suffering from MDD may even shed light on differences in the effectiveness of current antidepressant treatments (Khan et al., 2005; Young et al., 2009).

A comprehensive model of depression development combining environmental, psychological and biological factors could not only be beneficial for future research but also promote a more direct translation of research into clinical practice. For instance, the assessment of a combination of early and recent SLEs and primary emotions can help nurses, psychiatrists and psychologists to understand a patient’s affective reactions to upcoming events interpreted by the patient in a similar way to past events. Furthermore, the assessment of SLEs and primary emotions might be helpful for setting goals regarding a stepwise change of situation-specific affective reactions so that the patient not only experiences improvement on a symptom level.
but can begin a personal development that equips him or her for upcoming challenging situations. Furthermore, the consideration of personality in etiological models of MDD gives the patient the possibility to actively contribute to a positive change of his situation and prevents the mindset of being a victim of one’s biology and environment. After all, it is long known that a sense of uncontrollability of a situation can deprive individuals of their motivation and can thus be a pathway to depression (Maier & Seligman, 1976).

The investigation of epigenetic alterations associated with MDD will be of major importance for developing new therapeutic strategies since they are potentially reversible as it has already been shown in animal studies (Weaver et al., 2004). With respect to oxytocin, there are heterogeneous effects for the treatment of depression with the neuropeptide (Peled-Avron et al., 2020). The results of studies II and III do not support the notion of oxytocin being an effective long-term treatment for depression since OXT methylation was lower in inpatients which is presumably associated with higher OXT transcription. However, there are many intermediate steps between DNA methylation and the resulting protein. In addition, other factors like the availability of oxytocin receptors influence the effects of the neuropeptide. Therefore, it would be interesting to investigate medication with oxytocin alongside epigenetic profiles of OXT and OXTR, mRNA levels and oxytocin concentrations. This approach could help to understand which patients profit from oxytocin treatment and how the treatment interacts with the epigenetic landscape of the oxytocin system. The additional assessment of primary emotions in future research and clinical practice could help to further understand these interactions since OXT methylation was negatively associated with the CARE system. It is possible that primary emotions in combination with epigenetic profiles of the oxytocin system provide a useful tool for predicting a patient’s treatment response. In this regard, such studies would be a first step towards a treatment tailored to the patient’s individual characteristics.

3.7. Limitations and future directions

The current dissertation provides evidence for a comprehensive model of depression development comprising environmental, psychological and biological factors that are interrelated and in combination have considerable predictive value for depression course. However, four of the five studies presented have a cross-sectional design thus providing correlational information on the investigated variables. Future investigations should therefore use prospective designs comparing inpatients receiving treatment to well-matched healthy controls over several points in time.

The fifth study, while having a longitudinal design, did not investigate epigenetic factors and is an observational study. Even though we controlled a set of covariates matching cases to controls and performing additional control analyses, there are still many potentially confounding variables making causal conclusions problematic. In addition, the correlative studies and also the fifth study with its prospective design do not allow for testing the three
phases postulated in the comprehensive model of depression development. A solution to these issues could lie in the case co-twin study paradigm (Turner et al., 2020). This paradigm suggests the investigation of disease-discordant twins for the identification of disease associated epigenetic processes. By examining monozygotic twin pairs over the course of their lives it becomes possible to identify environmental events that induced epigenetic changes that are associated with for example depression development (Castillo-Fernandez et al., 2014). Performing adequate statistics can further support interpretations of possible causal relationships, e.g., the use of a general model that includes separate regression coefficients for within-twin-pair and between-pair effects (Carlin et al., 2005). It is also important to examine psychological properties of the twin-pairs since it has been shown that similar events are interpreted differently and thus may have a different impact on epigenetic alterations even in monozygotic twins (Turner et al., 2020).

Moreover, since SLEs and depression onset were assessed retrospectively there could be inaccuracies in participant's retrieval of the concerning events. Accordingly, future studies should prospectively investigate cohorts and document SLEs as soon as they are experienced. This would also allow for the prospective investigation of factors influencing differences in stress experience, which could be important since an individual's coping strategies have been suggested to be a mediator between SLEs and depression (Evans et al., 2014). Another limitation of our studies was that we used a measure of SLEs that was unspecific regarding the timing of events. Future studies should investigate both early life adversity and SLEs later in life to shed light on the differential effects of SLEs throughout the lifespan.

Furthermore, conclusions about the functional relevance of DNA methylation cannot be drawn since neither mRNA nor protein levels were assessed in the presented studies. Therefore, future studies should investigate epigenetic alterations alongside mRNA and protein levels to get an idea of the functional relevance of certain epigenetic mechanisms for the final product of a gene.

Last, the measurement of methylation profiles in peripheral blood makes conclusions about central methylation speculative. There are, however, first efforts to develop tools giving an approximate idea of associations between peripheral and central methylation of single CpG sites (Edgar et al., 2017). Additionally, overlapping results for differentially methylated regions in blood and brain when comparing individuals suffering from MDD to healthy controls have been reported (Aberg et al., 2020).

3.8. Conclusions
The studies comprising this work support the basic assumption of the comprehensive model of depression development that depression is the endpoint of vulnerability factors and stress associated alterations in biological and primary emotional systems that influence one another. The biological sex, the 2D:4D ratio, SLEs, primary emotions and epigenetic alterations of
SLC6A4 and OXT were not only associated with depression severity, diagnosis of depression or depression onset but were also interrelated, interacted and incrementally explained variance in MDD. Therefore, our results are in line with the three levels postulated by the comprehensive model of depression development. The three phases of the model, however, need further examination in future studies.

In conclusion, an individual’s sex and primary emotional systems predict the emotional and biological response to SLEs as seen in associations between SLEs, primary emotions, epigenetic alterations and MDD. This response might be epigenetically and psychologically encoded and result in individual patterns of perception, interpretation and reaction. In the presence of later life SLEs, these patterns might be reactivated in a complex interplay between environmental influences, primary emotions and their biological underpinnings. A reactivation of these patterns might allow for experience based interpretations of and reactions to the environment, which in turn influence further adaption processes on the psychological and epigenetic level. Therefore, the biology of MDD is highly complex and it is unlikely that we will be able to make accurate predictions about how a particular event will affect an individual’s biology in the near future if we ignore an individual’s personality and the events that contributed to shaping this personality. Personality constructs such as primary emotions might help us access the underlying biology of a complex disorder like MDD. Since primary emotions are less complex than depression, differences in biological systems associated with primary emotions are more easily interpreted and can help generate novel hypotheses regarding the emotional consequences of SLE associated epigenetic alterations. Therefore, SLEs and primary emotions have a central role in the comprehensive model of depression development and the consideration of psychological mechanisms in etiological research regarding MDD will be of major importance in future studies. After all, depression is the result of past events, their perception and according biological alterations. Thus, personality is the link between the environment and epigenetic adaption to the environment and only the joint investigation of environment, personality and biology might bring us closer to understanding the development of MDD.
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