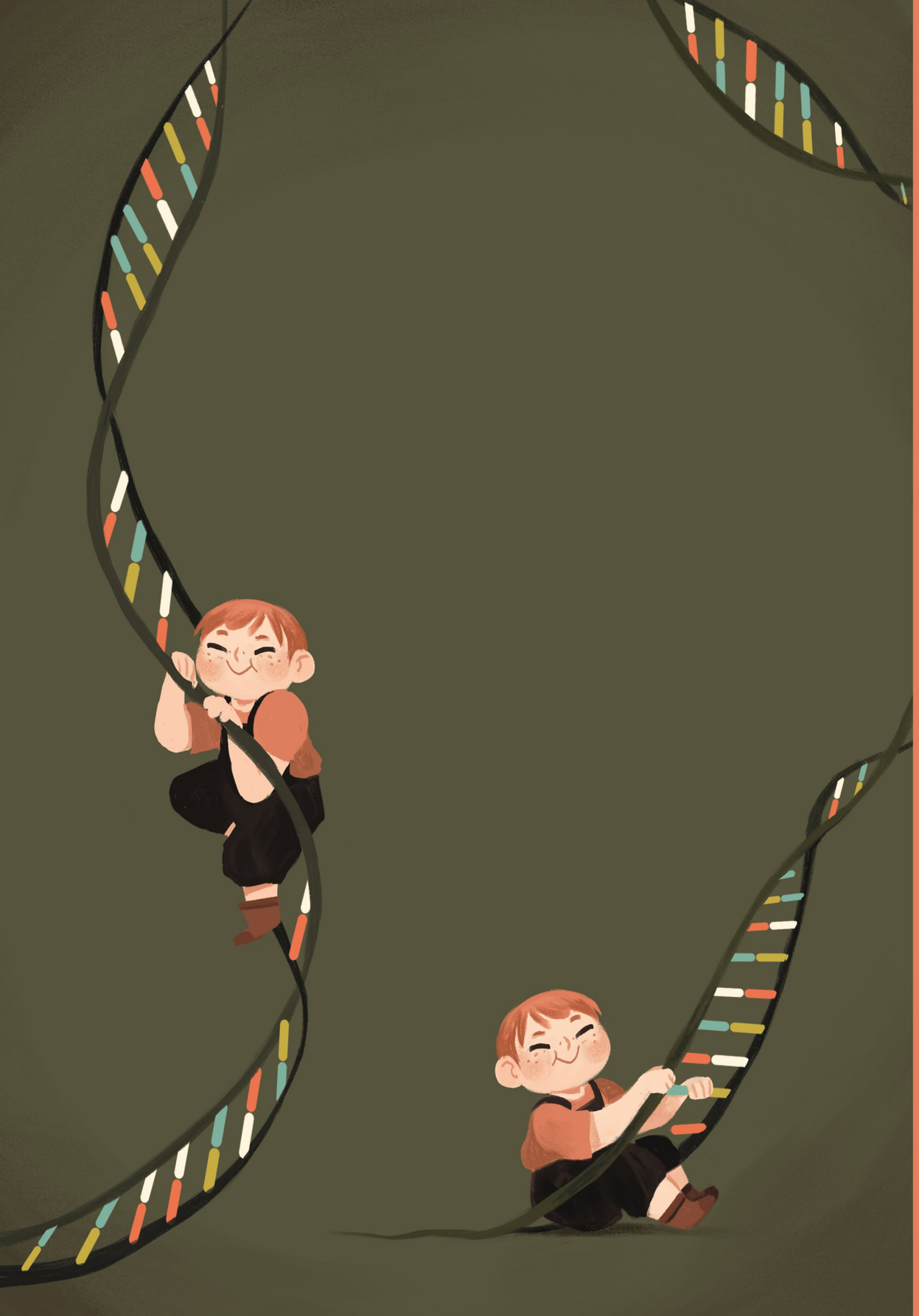
UNIVERSITAT DE BARCELONA

From early life stress to adult psychopathology: An epigenetic view from monozygotic twin based approaches

Helena Palma Gudiel



DOCTORAL THESIS ◆ HELENA PALMA GUDIEL ◆ 2019

FROM EARLY LIFE STRESS TO ADULT PSYCOPHATOLOGY

AN EPIGENETIC VIEW FROM MONOZYGOTIC TWIN BASED APPROACHES

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UNIVERSITAT DE BARCELONA

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From early life stress to adult psychopathology: An epigenetic view from monozygotic twin based approaches

Doctoral Thesis presented by
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In partial fulfillment of the requirements for the degree of PhD
by the University of Barcelona

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INTRODUCTION

1. The nature of mental health

There is a myriad of approaches to address mental health issues; thereby, one can speak of psychiatric disorders from a clinical point of view, but also delve into etiological research, epidemiological data, social disadvantage or even evolutionary hypothesis for their origin. These aspects are briefly addressed in the introduction of this thesis, starting with several remarks about the affliction experienced by those individuals enduring mental health issues.

Those who suffer from mental illness bear psychological distress, feelings of guilt and dreadful stigma. Unlike other complex diseases highly prevalent in our society, such as cardiovascular disease or cancer, psychiatric disorders are still feared and misunderstood by the public opinion and the media. Mental disorders are commonly regarded either as dangerous—in the case of psychotic and manic phenotypes—or simply nonexistent—like depression or anxiety. Those affected by psychopathology in limited resource settings often suffer demeaning treatment such as incarceration or torture (Patel et al., 2018).

People diagnosed with psychiatric disorders are exposed to discrimination in the main spheres of their life: relationships, family, education and work. A recent report published by the Catalan mental health observatory *Obertament* (2016) revealed statistics for such unfair and biased treatments merely based on the existence of a mental diagnostic. According to the report, 61.9% of people with a psychiatric disorder were unemployed, 20.1% had been ridiculed or scorned by members of their nuclear family, 19.2% did not have a romantic partner since the diagnosis and a 20.3% had received pressure to not have children by either a member of their nuclear family, their romantic partner or a mental health specialist.

The lack of validated biomarkers for psychiatric disorders may be the origin of widespread ignorance regarding psychopathology–which inevitably leads to discrimination, stigma and false attributions. Although patients are swiftly *tagged* based on the subjective evaluation of reported symptoms, there is no measurable biological alteration leading to a robust diagnosis or treatment of choice. Nevertheless, the joint effort of clinicians and researchers during the last decades has shed some light on the patho-

physiological mechanisms underlying psychiatric disorders (Borenstein, 2018). These advances come from research fields as diverse as molecular biology, pharmacology, neuroimaging, genetics, transcriptomics, proteomics, metabolomics and, more recently, epigenetics.

1.1. Evolutionary view

Humans (*Homo sapiens*) are a predominantly social species. So much so that the human brain has been shaped to be able to interact, understand and emotionally bond to their peers; accordingly, loneliness and social isolation predict premature mortality (Holt-Lunstad et al., 2015). One of the key differences between humans and our evolutionary closest relatives, chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*), is encephalization. Notably, the human brain has the highest ratio between neocortex volume and the volume of the rest of brain. **Figure 1** displays the linear relationship between the neocortex ratio and the typical size of social groups within primate species (Dunbar, 2009).

From an evolutionary point of view, it is difficult to ascertain when did psychiatric disorders emerge in the history of hominids. Genomic and epigenomic analysis of Neandertal and Denisovan[1] DNA have unraveled some intriguing cues. Genomic DNA retrieved from fossil records has allowed (i) the study of the epigenomic landscape of our far-relatives, (ii) the identification of Neandertal- and Denisovan- introgressed[2] sequences in modern humans, and (iii) the study of Neanderthal selective sweep (NSS).

Take schizophrenia for example, one of the most detrimental psychiatric disorders. Whereas heritability studies have revealed its genetic component to be as high as 70%, its worldwide prevalence around 1% suggests it originated early in human evolution (McGrath et al., 2008). Notably, subjects affected by schizophrenia exhibit reduced reproductive success when compared to control subjects (Fañanás and Bertranpetit, 1995; MacCabe et al., 2009). This incongruity led to the postulation of the so-called schizophrenia evolutionary paradox (van Dongen and Boomsma, 2013). In this regard, Timothy J. Crow suggested the roots of schizophrenia to be intertwined with the development of language (Crow, 1997); in this

[1] Neandertal and Denisovan are two extinct species of archaic humans.
[2] Introgression refers to acquisition of DNA from other species by interbreeding.

framework, schizophrenia would have emerged as a disadvantageous-by-product of language and social brain development. Actually, the study of NSS has recently unfolded an enrichment for genetic variants of risk for schizophrenia in recent human evolution suggesting a positive selection process (Srinivasan et al., 2016).

Unlike the psychosis spectrum, major depressive disorder exhibits much more moderated heritability estimates, around 37% (Sullivan et al., 2000), along with a substantially higher prevalence (Kessler et al., 2012) and great variation across countries highlighting the involvement of sociocultural factors in its etiology (Kessler and Bromet, 2013). However, depression is a commonly occurring disorder in all countries where its prevalence has been documented; thus, genetic liability for depression must have low pene-

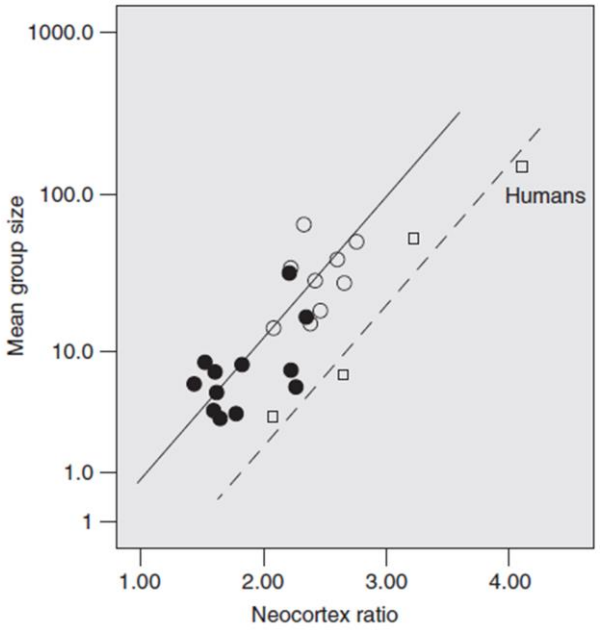


Figure 1. Mean social group size for different primate species plotted against relative neocortex volume. Solid symbols are New World monkeys; open circles are Old World monkeys; squares are apes. Note that apes lie on a separate grade to monkeys: they seem to require more computing power to handle groups of a particular size than monkeys do, suggesting that their social life is cognitively more complex. Reprinted with permission from Dunbar R. Social Brain: Evolution (2009).

trance[3] but widespread distribution, probably as a result of positive selection. Contrary to schizophrenia, depressive phenotypes could have been positively selected across evolution due to the adaptive advantage conferred by core depression symptoms in the context of an infection (Raison and Miller, 2017). This theory builds upon the observed behavioral response to illness, referred to as *sickness behavior*, involving characteristic symptoms of depression such as social withdrawal, lethargy and depressed mood (Dantzer and Kelley, 2007). Intriguingly, meta-analytic evidence suggests heightened inflammatory states in subjects suffering depression (Dowlati et al., 2010). Additionally, the study of introgressed sequences revealed Neanderthal SNPs to increase risk for depression (Simonti et al., 2016); this legacy seems to operate through Neanderthal alleles in genes involved in circadian rhythms since depression is highly influenced by sunlight exposure.

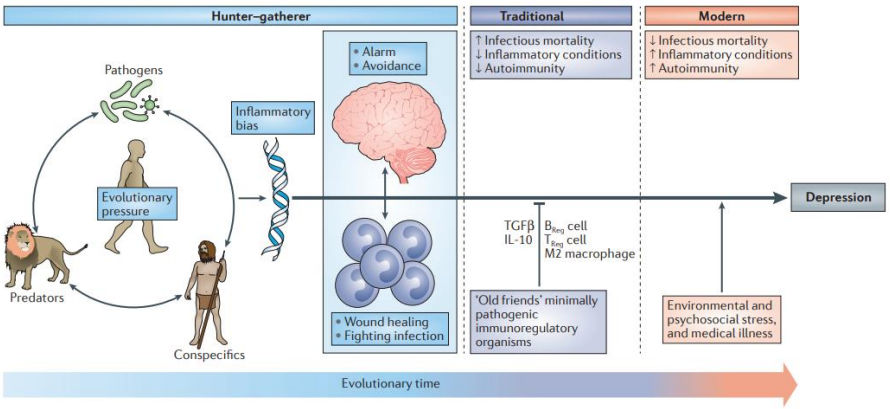


Figure 2. Evolutionary legacy of an inflammatory bias. Early evolutionary pressures derived from interactions with pathogens, predators and human conspecifics resulted in an inflammatory bias intended for fighting infection and healing wounds, while maintaining vigilance against attack. In modern times, sanitized urban environments characterized by continuous psychological challenges instigate ancestral immunological and behavioral repertoires contributing to inflammation-related disorders liability (including depression). Reproduced with permission from Miller A & Raison C. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nature Reviews Immunology 16, 22-34 (2016).

[3] In genetics, penetrance refers to the impact on the phenotype of a certain genetic variant.

1.2. Epidemiology

From an epidemiological point of view, psychiatric disorders are one of the leading causes of morbidity and disease burden worldwide, as estimated from disability-adjusted life years (DALYs). Moreover, several independent reports have documented a dramatic rise in psychiatric disorders prevalence (Compton et al., 2006; Goldney et al., 2010; Mojtabai, 2011). As a matter of fact, environmental factors conferring risk for psychopathology such as urbanicity and exposure to early stress are on the rise, coupled with a dearth of knowledge regarding etiology of psychiatric disorders. Thus, while the number of diagnosed cases gradually increases, the availability of successful treatment strategies has been halted for decades. In this regard, a report on drug development success rates revealed psychiatric drugs to be the second lowest category regarding those who made it from phase I to approval, with a success rate of 6.2% (Hay et al., 2014).

This public health scenario is further overshadowed by the chronicity and resistance to treatment of a substantial proportion of subjects affected by psychiatric disorders. Current pharmacological treatments only target the alleviation of symptoms. Clinical remission is unpredictable and does not often match with self-reported remission; i.e. scoring lower in clinical scales such as the Hamilton rating scale for depression does not correlate with similar improvements in quality of life assessments, revealing the long and chronic impact of the disorder for a majority of affected subjects (Zimmerman et al., 2012).

1.3. The p factor

Defining and categorizing mental diseases has been challenging since their acknowledgment by the medical community in the beginning of the nineteenth century—when the term *psychiatry* was coined. Nowadays, the Diagnostic and Statistical Manual of Mental Disorders (DSM) is one of the key resources to diagnose individuals presenting symptoms of the psychopathological domain. Nevertheless, all editions of the DSM so far have been criticized as a non-reliable tool to accurately categorize psychiatric disorders. This criticism is partially based on the lack of scientific evidence supporting its historically accepted nosology.

However, since there are no known biomarkers for any psychiatric disorder, DSM continues to be the gold standard for psychiatrists. No research field to date has been able to shed light on the roots of psychopathology, neither neuroimaging, genetics, epigenetics nor proteomics. Hence, the only resource to tell apart two separate conditions relies on symptomatology alone. However, the frontiers between similar disorders blur easily and some researchers and clinicians suggest mental health issues to be part of a spectrum (Doherty and Owen, 2014). Thus, psychiatric symptoms have been hypothesized to belong to three main categories or, more appropriately, dimensions, as depicted in **Figure 3** (Patel et al., 2018).

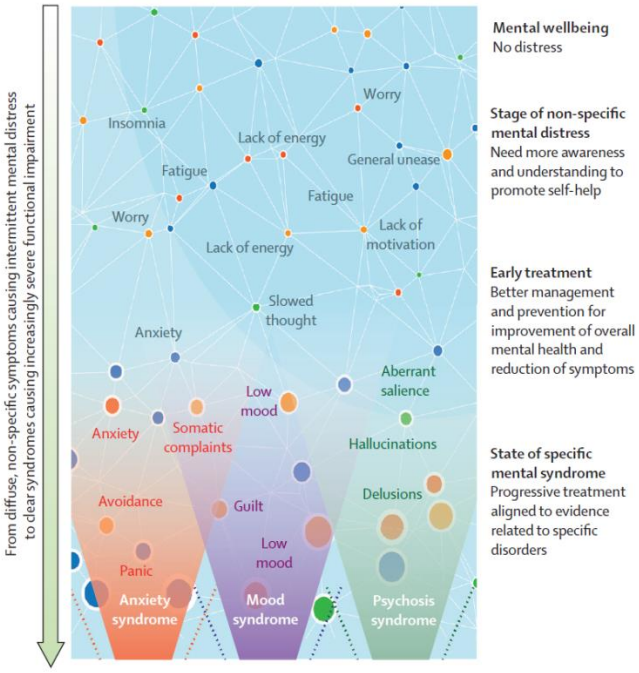


Figure 3. Spectrum and overlap of psychiatric manifestations. Psychopathological symptoms are present in the general population manifested as mental distress. Increasing number and severity of presented symptoms leads to subsyndromal profiles. Finally, psychiatric conditions are diagnosed when the accumulation and chronicity of symptoms impacts social and occupational spheres. Modified with permission from Patel et al. The Lancet Commission on global mental health and sustainable development. *The Lancet* (2018).

As a matter of fact, psychopathology as a whole has been suggested to be comprised of one single dimension: the *p* factor (Caspi and Moffitt, 2018). This revolutionary hypothesis builds on: (i) the overlap of symptoms between different diagnoses, (ii) the high rates of comorbidity[4], and (iii) the curious patterns of heredity observed (offspring of parents affected by psychiatric disorders are more prone to develop a psychiatric disorder but not necessarily the same as their progenitors).

Interestingly, acknowledged risk factors for psychopathology such as exposure to childhood trauma or certain genetic variants confer risk unspecifically to several psychiatric disorders at the same time, further supporting the notion of one single psychopathology dimension—p—rather than several categorical labels (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Teicher and Samson, 2013). Likewise, antipsychotic and antidepressant medications are prescribed to a wide range of patients with different categorical psychiatric diagnoses.

Despite its shortcomings, DSM-based categorical diagnoses continue to be the standard in both clinical and research forums. Therefore, the main body of the present dissertation relies on such categorization. In this regard, specific categorical psychiatric entities discussed throughout the thesis are briefly described in the following sections.

1.4. Depression and anxiety

Depressive and anxiety disorders are the most prevalent of all psychiatric disorders. They are particularly challenging to study due to their low heritabilities and the preponderant role of stress in their etiology.

1.4.1 Depression

Major Depressive Disorder (MDD) is the non-communicable disease with the highest impact worldwide (Friedrich, 2017). Unipolar depressive disorders have been predicted to be the second leading cause of disease burden worldwide by 2030 and the first in high-income countries (Mathers and Loncar, 2006). According to the World Health Organization, depression affected an estimated 322 million people in 2015 (representing 4.4% of the

[4] Comorbidity refers to the experience of more than one categorical disorder simultaneously, e.g. depression and anxiety.

global population), which supposed an absolute 18.4% increase with regard to 2005 estimates (World Health Organization, 2017).

Major depressive disorder is characterized by the presence of pathologic sadness and inability to experience pleasure, along with a plethora of emotional, neurovegetative and cognitive symptoms. DSM-IV-TR criteria for major depressive disorder are included in **Table 1**. Remarkably, DSM-5 was released in 2013, with slight changes with regard to depression diagnostic criteria. Nevertheless, DSM-IV-TR criteria were used to diagnose subjects analyzed in the present dissertation as they were recruited prior to DSM-5 release.

Epidemiological studies undoubtedly show depression to be more common among women than among men. Specifically, women are at a doubled risk of depression when compared to men; these differences arise after puberty (Kuehner, 2003). This difference in prevalence has been suggested to rely on both sociocultural and biological factors (Kuehner, 2017). On the one hand, women worldwide are at a higher risk of interpersonal violence including childhood sexual abuse, sexual partner violence and forced marriage, among others. Furthermore, balancing full-time employment and motherhood leads to role overload in women. On the other hand, sex differences regarding physiological stress response (see section 2.2.3) together with hormonal influences, personality traits and coping styles, could also contribute to this sex-biased prevalence of MDD.

As introduced in section 1.1, several hypotheses have been suggested for explaining the origin of depressive disorders. The first of them being the monoaminergic hypothesis, which postulates depressive symptomatology to be caused by a deficiency in monoamines—serotonin, norepinephrine and dopamine. Favoring this hypothesis, all antidepressant medications target monoamine receptors or transporters, modulating monoaminergic neurotransmission to revert the depressive phenotype. Accordingly, selective serotonin reuptake inhibitors (SSRIs), the most prescribed antidepressants worldwide, specifically target the serotonin transporter. Despite the perennial controversy regarding the actual usefulness of antidepressant medication (Andrews et al., 2015), a recent meta-analysis revealed all antidepressant drugs to be more efficacious than placebo (Cipriani et al., 2018).

Table 1. DSM-IV-TR diagnostic criteria for Major Depressive Disorder

A – Presence of a minimum of five of the following symptoms have been present during a period of at least 2 weeks and represent a change from previous functioning. One of the symptoms has to be (1) or (2). Symptoms due to a general medical condition or mood-incongruent delusions or hallucinations are not included. 1) depressed mood for most of the day, nearly every day, as indicated by either subjective report or observation made by others (in children and adolescents, the mood can be irritable) 2) markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others) 3) significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day 4) insomnia or hypersomnia nearly every day 5) psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down) 6) fatigue or energy loss nearly every day 7) feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick) 8) diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others) 9) recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide
B – Symptoms do not meet criteria for a mixed episode.
C – Symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
D – Symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).
E – Symptoms are not better accounted by bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than 2 months or are characterized by a marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation.

However, due to the high proportion of treatment resistant subjects, the monoaminergic theory was soon followed by alternative complementary explanations such as the neuroendocrine, circadian, neuroimmune and microbiota hypotheses for depression.

Due to the preeminent role of the environment in the causality of MDD—as derived from low heritability estimates—, psychosocial stress has been suggested as the main triggering factor for depressive episodes. Thus, abnormalities in the major stress response system, the hypothalamic-pituitary-adrenal (HPA) axis, have been suggested as putative mediators of depressive vulnerability (Holsboer, 2000) (more detailed description of HPA axis involvement in psychiatric disorders will be discussed in section 2.2.3). Empirical studies testing HPA axis regulation in depressed subjects by means of the dexamethasone suppression test[5] sum up evidence towards neuroendocrine involvement in depressive liability (Mokhtari et al., 2013).

Likewise, disruption of the circadian rhythms has been suggested to underlie some depressive symptom domains like sleep disturbances (Courtet and Olié, 2012). Evidence supporting an immune role in the pathophysiology of depression comes from studies of non-psychiatric patients who after receiving interferon alpha (IFN-α) treatment develop depressive-like phenotypes meeting DSM criteria for major depressive disorder (**Table 1**) (Capuron and Miller, 2004). Complementarily, immunological dysfunction in depressed patients would dialogue with disbalanced gut microbiota, another etiological factor recently involved in the pathophysiology of MDD (Koopman et al., 2017; Mayer, 2011).

Finally, two recent genome-wide association studies (GWAS) have revealed the involvement of excitatory neurotransmission—specifically glutamatergic pathways—, mechanosensory behavior and, generally, genes involved in neuron differentiation and morphogenesis in depression (Howard et al., 2018; Wray et al., 2018). These genomic findings are especially relevant in light of recent clinical trials testing the efficacy of ketamine as a pharmacological approach in treatment-resistant depressive subjects (Fava et al., 2018).

[5] The dexamethasone suppression test consists in the administration of a synthetic steroid, dexamethasone, to assess HPA axis negative feedback (analyzing whether cortisol production is suppressed or not).

1.4.2 Anxiety

As opposed to *major* psychiatric disorders—MDD, schizophrenia or bipolar disorder—, anxiety disorders as a whole are characterized by a lower burden of disease. Nevertheless, they constitute the most prevalent group of psychiatric disorders affecting one in 14 people worldwide (Baxter et al., 2013; Kessler et al., 2010). Thus, anxiety disorders ranked in the 9th position as one of the top leading causes of disease burden worldwide, as measured by years of life lived with disability (Vos et al., 2015). Just like depression, anxiety disorders are characterized by low heritability estimates (Kessler et al., 2005a), revealing the role of stress not just as a contributor but as an essential factor in their pathophysiology (Teicher and Samson, 2013).

As categorized in both the recently released DSM-5 and ICD-11, anxiety disorders encompass a number of distinct categorical disorders including: separation anxiety disorder, selective mutism, specific phobias, social anxiety disorder, panic disorder, agoraphobia and generalized anxiety disorder ("Anxiety Disorders," 2013). The core of most anxiety disorders is the presence of marked and irrational fear. Remarkably, the aforementioned DSM-5 listing of anxiety disorders moderately differs from anxiety disorders as categorized by the DSM-IV-TR. **Table 2** highlights specific anxiety disorders as included in the DSM-IV-TR as these criteria were used to diagnose the adult twin sample assessed in the context of this dissertation.

Table 2. DSM-IV-TR classification of anxiety disorders

Generalized anxiety disorder	
Panic disorder	With agoraphobia
	Without agoraphobia
Agoraphobia without history of panic disorder	
Specific phobia	
Social phobia	
Obsessive-compulsive disorder	
Post-traumatic stress disorder	
Acute stress disorder	

Due to the anxiolytic efficacy of benzodiazepines, the involvement of GABAergic transmission in the pathophysiology of anxiety has been traditionally accepted. However, due to the side effects of benzodiazepines long-term use, other psychoactive drugs such as SSRIs have proven the same efficacy in alleviating anxious symptoms (Craske and Stein, 2016).

Given the heterogeneity of this many anxiety disorders, they are particularly challenging to study. Most studies focusing on anxiety disorders analyze only one of the aforementioned categories in terms of either genetic underpinnings, neuroimaging traits, response to treatment or environmental risk factors. However, anxiety disorders are characterized by outstanding comorbidity rates; thus, excluding from analysis comorbid subjects results in recruitment bias, probably neglecting the most severe forms of anxiety disorders. Fewer are the studies that consider the category as a mixture of diverse disorders. Dimensional approaches by means of quantitative and continuous scores constitute a suitable design to address this issue.

1.4.3. Comorbidity and symptom overlap between depression and anxiety

Following the rationale presented in section 1.3 regarding the clinical, genetic and molecular overlap between different psychiatric categories, depression and anxiety have been repeatedly described to co-occur in a considerable proportion of psychiatric patients (**Figure 4**).

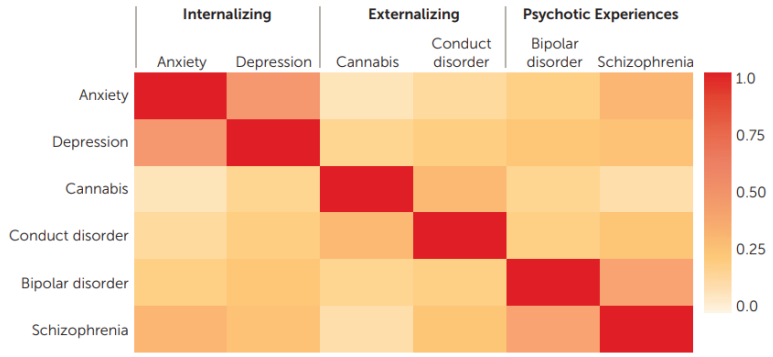


Figure 4. Psychiatric comorbidity is ubiquitous. Heatmap of correlations displaying comorbidity between several psychiatric disorders. Data derive from the last wave of the Dunedin Longitudinal Study. Reproduced with permission from: Caspi A & Moffitt T. All for One and One for All: Mental Disorders in One Dimension. *The American Journal of Psychiatry* 175(9): 831-844 (2018).

As a matter of fact, several researchers have previously considered depression and anxiety as a single clinical group (Ressler and Mayberg, 2007). This decision arises from several clinical and biological observations briefly discussed below. On the one hand, anxiety and depression have been repeatedly described to co-occur, either in the same individuals at the same time, consecutively or in different subjects from the same family (Fava et al., 2000; Kessler et al., 2005b; Lamers et al., 2011). On the other hand, the combination of subsyndromal depressive and anxiety symptoms has been recognized as a nosological entity of its own, known as mixed anxiety-depression (Hettema et al., 2015).

One of the samples analyzed in the context of the present thesis dissertation was drawn from the general population; thus, the number of subjects affected by psychiatric disorders was fairly low with most of the affected subjects presenting either depressive or anxious symptomatology, and a significant subset of those presenting comorbid anxious-depressive patterns. Thus, patients affected by either depression or anxiety disorders were considered as a single group of anxious-depressive subjects.

1.5. Schizophrenia

Schizophrenia is regarded as the most severe and recognizable psychiatric disorder due to the presence of hallucinations and delusions, perhaps the most noticeable psychiatric symptoms. Nevertheless, schizophrenia spectrum disorders (SSD) can be characterized by three main domains or dimensions: positive, negative and cognitive. While positive or psychotic symptoms include all behavioral abnormalities most striking to neurotypical subjects such as hallucinations, delusions, disordered thought and movement disorders; negative symptoms refer to the lack of energy and motivation, flattened affect and anhedonia—which is a core feature of severe depression. Finally, cognitive symptoms comprise deficits in working memory and executive functioning together with attention deficits.

In this regard, findings from a polynosologic study of multiplex families affected by psychotic disorders revealed the lack of familiality[6] for either

[6] *Familiality* refers to the heritable component of a certain trait as measured by its transmission in family studies. Familiality is used instead of heritability to refer to the combined effect of shared genetics and environment between the members of the same family.

delusions or hallucinations (Peralta et al., 2016). Conversely, deficit and negative syndromes exhibited striking familiality estimates suggesting the genetic basis for SSD to rely on genetic liability for the negative dimension. Although negative and cognitive symptoms have been shown to be the most pervasive and deleterious for patients with SSD, almost all antipsychotic treatment are only effective in diminishing the impact of positive symptoms.

While depression and anxiety are not only highly comorbid but also share genetic and neurobiological bases, SSD would be in the other extreme of the psychopathological spectrum, in a position between (i) autism spectrum disorders (ASD) and other neurodevelopmental disorders, and (ii) bipolar disorder (**Figure 3**). SSD, and particularly schizophrenia, are characterized by the highest heritability estimate of all psychiatric disorders pointing to the preeminent role of genetics in their pathophysiology (Sullivan et al., 2012)(**Figure 5**).

Accordingly, genome-wide association studies (GWAS) have revealed one of the most robust genetic architectures in psychiatric genetics so far, with 113 loci significantly associated with this disorder (Li et al., 2017; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Nevertheless, all risk alleles identified so far confer low-to-moderate risk (all ORs lower than 1.2), and the combined effects of all loci included in the polygenic risk score (PRS)[7] account for an approximate 18.4% of the disorder variance (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Therefore, several environmental risk factors have been highlighted as putative triggering factors for schizophrenia in genetically vulnerable individuals (Alemany et al., 2011). In this context, the neurodevelopmental hypothesis of schizophrenia was born relying on three main ideas: (i) evidence for a prodromal cognitive delay prior to the onset of schizophrenia (Reichenberg et al., 2010), (ii) the association between obstetric complications (OCs) and schizophrenia incidence (Cannon et al., 2002), and (iii) the obvious gap left by genetic studies pointing to the existence of environmental risk factors.

[7] The polygenic risk score used in this analysis included all genetic variants associated with schizophrenia under a 0.05 level of significance as opposed to the validated 113 loci, which surpassed the $5x10^{-8}$ threshold of significance.

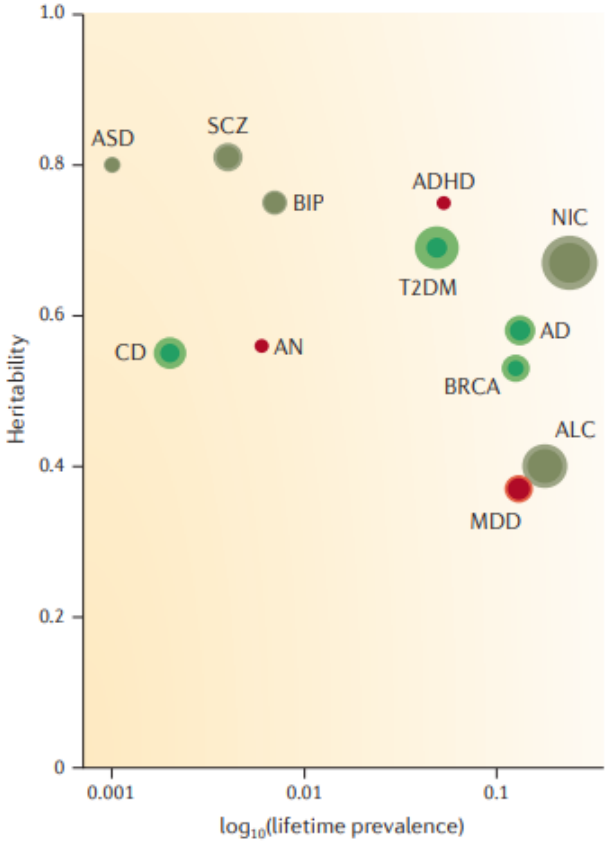


Figure 5. Genetic architecture of psychiatric disorders. Plot of heritability by $\log_{10}$ (lifetime prevalence) for nine psychiatric disorders and three complex diseases characterized by a successful genetic dissection. Color indicates qualitative success in identifying etiological genetic variation (bright green meaning notably successful and red highlighting minimal or no clear success). The bubble sizes are proportional to the number of cases studied in genome-wide association studies. Abbreviations: AD, Alzheimer's disease; ADHD, attention-deficit hyperactivity disorders; ALC, alcohol dependence; AN, anorexia nervosa; ASD, autism spectrum disorder; BIP, bipolar disorder; BRCA, breast cancer; CD, Crohn's disease; MDD, major depressive disorder; NIC, nicotine usage; SCZ, schizophrenia; T2DM, type 2 diabetes mellitus. Reproduced with permission from Sullivan P, Daly M & O'Donovan M. Genetic architectures of psychiatric disorders: the emerging picture and its implications. *Nature Reviews Genetics* 13, 537-551 (2012).

This conceptualization of psychosis had been already presented by the Scottish psychiatrist Thomas Clouston back in 1891 who referred to what we now know as schizophrenia as «the last cortical developmental disease» (Clouston, 1891). One century later, this hypothesis was refined by several researchers leaving an unresolved issue: the latency between the perinatal insult and the onset of frank symptomatology—around late adolescence and early adulthood (Murray, 2010; Weinberger, 1996). This perspective leads to the consideration of the first stages of life and neurodevelopment as crucial time windows for the exploration of such hypothesis. Actually, a recent study has highlighted the role of the prenatal environment as an essential factor interacting with the genetic architecture of schizophrenia by means of placental regulation (Ursini et al., 2018).

2. The quest for risk factors underlying psychopathological manifestations

As discussed in section 1.2, the lack of validated biomarkers for any psychiatric disorder forces psychiatrists to exclusively rely on diagnostic manuals—such as the DSM-5. Nevertheless, several decades worth of scientific research have revealed robust evidence regarding environmental and genetic factors that modestly increase the risk for suffering a mental disorder.

2.1. The human genome

The central dogma of molecular biology as postulated by Francis Crick posits that genetic information encoded in the DNA is transcribed into RNA to later be translated into protein, which will then exert its function (Crick, 1970). Although this dogma has been revisited several times in the light of later findings regarding gene regulation and non-coding DNA regions, it provided the starting point for genetic studies on any known phenotype, including human disorders and traits.

Whereas there is a considerable number of monogenic diseases in which a single mutation on a single gene is sufficient to induce a pathologic phenotype, the most prevalent disorders are complex in nature, i.e. they are caused by numerous mutations in several genes, thus exhibiting a polygenic architecture. In this regard, the penetrance of a certain genetic variant is inversely associated with its prevalence in the general population (**Figure 6**). Such a negative correlation follows the laws of natural selection considering that deleterious mutations are suppressed from the genetic pool of a species whenever they reduce an individual's fitness[8]. Importantly, this polygenic architecture is also present in continuous human traits—such as intelligence, height or skin color—for which the endless combinations of alleles determine the specific phenotype.

[8] The concept of *fitness* is crucial in evolutionary biology and has been a recurring topic of discussion since psychiatric disorders often debut in juvenile stages (teen to early 20s), co-occurring with the peak of reproductive activity.

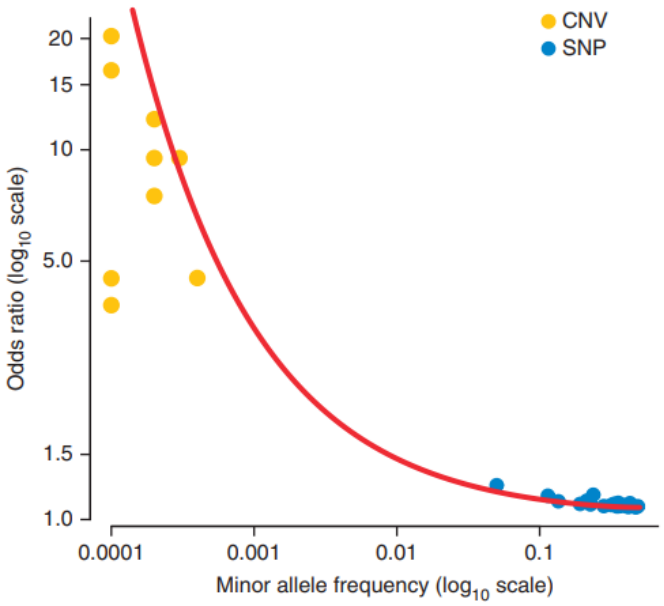


Figure 6. Inverse relationship between population frequency of a certain genetic variance and its contribution to disease (displayed data derives from schizophrenia studies). At one extreme, rare variants of large effect are carried by few individuals. At the other extreme, common variants of small effect are carried by lots of people; the increased risk of disease to an individual from such variants may be trivial but the effect on population variance can be the same as for the rare variant of large effect. Abbreviations: CNV, copy number variant; SNP, single nucleotide polymorphism. Reproduced with permission from Gratten J, Wray N, Keller M & Visscher P. Large-scale genomics unveils the genetic architecture of psychiatric disorders.

An initial study design to ascertain which genetic variants could be underlying a certain complex trait consists of candidate approaches in which the genes under investigation have been selected following functional criteria. At the beginning of psychiatric genetic exploration, it was logical to start analyzing genes involved in the different neurotransmission systems targeted by available psychoactive drugs. Thus, studies focusing on genes encoding dopamine receptors 2, 3 and 4—*DRD2*, *DRD3* and *DRD4*, respectively—, the serotonin transporter—*SLC6A4*—, or the brain-derived neurotrophic factor—*BDNF*—, among others, proliferated during the 90s and 00s (Farrell et al., 2015). Nevertheless, such approaches have yielded mostly negative or

non-replicable findings; hence, candidate gene study designs are slowly falling into disuse with the advent of the genomic era.

More recently, the successful[9] sequencing of the human genome back in 2001 (The International Human Genome Mapping Consortium et al., 2001) allowed the development of genome-wide association studies (GWAS) to find out which genes are involved in a certain complex trait or disorder. Briefly, GWAS consist of hypothesis-free approaches in which thousands of SNPs[10] are tested comparing groups of subjects informative for the phenotype of interest being researched. Typically, the subject of study is a complex disorder; thus, the dependent variable in the analysis is qualitative and dichotomic—according to presence/absence of categorical diagnosis—and the GWAS is conducted in two differentiated groups of healthy controls and affected subjects. Due to the multiple comparisons conducted in such massive analyses, sample size must reach at least several thousand subjects in order for the GWAS to have sufficient statistical power.

Paradigmatically, psychiatric disorders are highly polygenic and multiple GWAS have been conducted in the last years in an attempt to identify genetic variants of risk for several categorical diagnoses such as schizophrenia, bipolar disorder, major depressive disorder, and autism, among others (Anney et al., 2017; Deckert et al., 2017; Hyde et al., 2016; Li et al., 2017). To summarize, there are three main findings that deserve attention: (i) the number of significant hits depends on the heritability of the disorder being analyzed, (ii) the proportion of the variance explained by the sum of all risk alleles is low-to-moderate, and (iii) there is a shared genetic basis for the major psychiatric disorders.

Firstly, the notably higher number of significant loci described in schizophrenia when compared to major depressive disorder came as no

[9] Although the publication of the first map for the human genome was a research landmark and it served as the cornerstone for the developing of genomics as a new biology field, the reported sequencing did not cover the entire human genome but an approximate 90% including ~150,000 gaps (International Human Genome Sequencing Consortium, 2004)

[10] Single nucleotide position or SNP refers to genetic mutations affecting one single nucleotide of the DNA sequence giving rise to two alternative genetic variants known as alleles. Specifically, a genetic variant of one single nucleotide is considered as a SNP when the minor allele frequency—the frequency of the less common variant—can be found in % of the population.

surprise since such results paralleled the different heritability estimates for both disorders (section 1.4). However, even when summarizing the odds ratio (OR) conferred by the 108 identified loci associated with schizophrenia, the accumulated risk only accounted for a 18.4% of the total variance of the disorder (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Such paucity of results was generalizable to other complex non-psychiatric disorders and was recognized as the missing heritability problem (Manolio et al., 2009). On the other hand, the shared loci for several psychiatric disorders is in line with the p factor hypothesis presented in section 1.3.

2.2. Environmental stress

The inability to find genetic variants unambiguously associated with psychiatric disorders lead to a growing interest in the study of the environment. Actually, the preeminent role of external factors is nothing but restricted to psychopathology; virtually all polygenic traits are influenced by environmental factors such as diet, sunlight exposure, lifestyle and socioeconomic status. Environmental factors considered as major risk factors for a given psychiatric disorder include exposure to traumatic stress (especially during early development), substance abuse, low socioeconomic status and urbanicity (Patel et al., 2018).

Threatening environmental insults are most deleterious when they occur during ontogenic windows of development such as the prenatal period or childhood.

2.2.1. Prenatal stress

As briefly introduced in section 1.5, prenatal stress has been hypothesized to play a key role in the etiology of neurodevelopmental disorders, including ASD and schizophrenia, and other psychiatric disorders such as depression. The neurodevelopmental hypothesis of schizophrenia was first hinted in the late 19th century by the Scottish psychiatrist Thomas Clouston who referred to what we now know as psychosis as «the last cortical developmental disease» (Clouston, 1891). It was not until the 80s when this hypothesis was regained based on exploratory analyses testing the association between several obstetric complications and the development of schizophrenia in later stages of life (Lewis and Murray, 1987). Such theories were

often questioned due to the substantial latency between the risk factor—during the perinatal period—and the onset of the disorder—around the early twenties (Paus et al., 2008). Nevertheless, there is no longer doubt about the extant association between obstetric complications and schizophrenia, with an associated OR of 2 (Belbasis et al., 2018).

Likewise, psychosocial stress as experienced by the expectant mother has been described to influence postnatal behavior, the infant's stress response and his/her long-term psychopathological liability. This kind of prenatal stress ranges from low-to-moderate daily stressors to the most severe forms of intimate partner violence and war trauma. Additionally, maternal psychopathology during pregnancy entails considerable emotional suffering which has also been incorporated in the notion of prenatal stress (O'Donnell et al., 2012). Evidence from animal and human studies reveals the potential role of prenatal stress in the regulation of placental gene expression, particularly of genes involved in the stress response system which may shape fetal long-term stress responsivity (Welberg et al., 2005).

Furthermore, maternal immune activation (MIA) during pregnancy due to either viral, bacterial or parasitic infections has been epidemiologically linked to an increase in neurodevelopmental disorders, particularly ASD and schizophrenia (Estes and McAllister, 2016). A most recent outbreak exemplifying the extreme risks of MIA for fetal neural development is the association between Zika virus infection and congenital abnormalities including microcephaly (Melo et al., 2016; Wen et al., 2017). Accordingly, animal models for neurodevelopmental disorders rely on experimentally induced MIA. One of the most commonly used models in psychiatric research consists of poly(I:C) inoculation in pregnant dams, which triggers an acute immune response (Meyer and Feldon, 2012).

Prenatal insults have been also associated with a number of non-psychiatric phenotypes such as cardiovascular and metabolic disorders. The developmental origins of health and disease (DOHaD) hypothesis sumps up this evidence suggesting fetuses have the ability to program themselves to adapt to the prenatal environment on the assumption that it will be a proxy for postnatal environment (Barker, 1998). Such calibration was first suggested to explain metabolic programming of a *thrifty* phenotype leading to type 2 diabetes and obesity whenever the fetus is first exposed to prenatal

famine but is later reared in a nutrient rich environment (Hales and Barker, 1992). More recently, the DOHaD hypothesis has been explored in the context of psychiatric disorders (Robinson et al., 2018).

2.2.2. Childhood adversity

Childhood is another highly vulnerable period for the development of psychiatric disorders. Although several milestones of human brain development such as neurulation, neuronal proliferation and neural migration take place during prenatal stages, there are as much brain maturation events in postnatal life including apoptosis, synaptogenesis, myelination and synaptic pruning (**Figure 7**). Thus, human brain continues to mature until the early twenties.

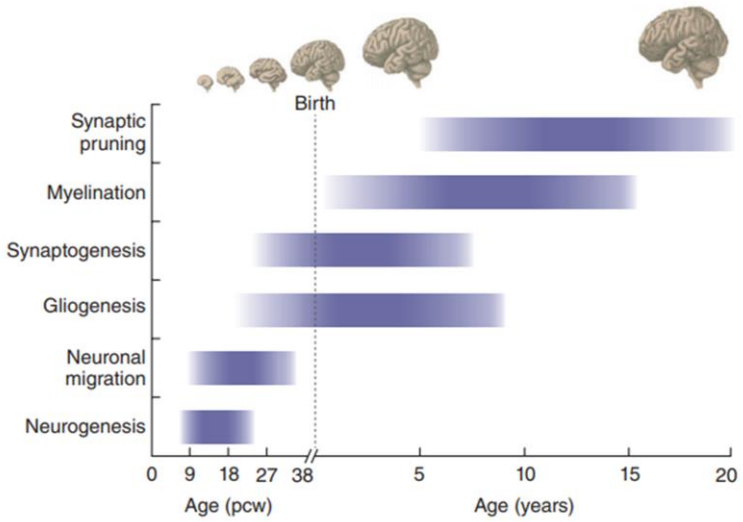


Figure 7. Key processes in human neurodevelopment. Timeline of human development during prenatal (in postconception weeks, pcw) and postnatal (in years) periods, in which the horizontal bars represent the approximate timing of key neurobiological processes and developmental milestones. Reproduced with permission from Marín O. Developmental timing and critical windows for the treatment of psychiatric disorders. *Nature Medicine* 22, 1229-1238 (2016).

Childhood adversity encompasses any circumstance that a child could experience as a threat to the self and thus includes childhood mal-

treatment, poverty, loss of a parent, witnessing domestic violence, and exposure to natural disasters, among others.

One of the strongest environmental predictors of psychiatric illness is early exposure to trauma, adversity or maltreatment. Analogous to genetic predisposition, childhood maltreatment exerts a transdiagnostic effect increasing the risk to suffer any psychiatric disorder, which further supports the hypothesis of the p factor (section 1.3) (Teicher and Samson, 2013). Furthermore, presence of childhood maltreatment has been associated with worse course of illness and treatment response (Nanni et al., 2012).

Of note, stress during childhood has more subtle effects in neural functioning when compared to prenatal exposure to stress according to the neural processes taking place during this period (**Figure 7**). The individual impact of childhood adversity depends on both genetic liability to stress-related disorders, prior exposure to stress in the prenatal period and the availability of a support network provided by family, caretakers and peers.

Specifically, schizophrenia was the first psychiatric disorder suggested to be caused by the cumulative effects of several insults occurring at different developmental stages (Bayer et al., 1999). This principle is currently known as the double hit hypothesis, which dialogues with the neurodevelopmental hypothesis for schizophrenia (section 1.5) positing prenatal stress to act as the first hit. Consequently, postnatal stressful situations would operate upon a sensitized state, finally triggering the onset of a disorder (Giovanoli et al., 2013).

The cumulative risk conferred by multiple adversities during childhood was also described to directly correlate with the number of comorbid mental health disturbances in a dose-response fashion (Anda et al., 2006). This finding was independent of either genetic liability or prenatal environment, highlighting the developmental relevance of childhood in shaping adult mental health and wellbeing. Of note, clinical outcomes of interest in this study included depressed mood, somatic complaints, substance abuse, impaired memory, promiscuity and perceived stress.

Nevertheless, the biological mechanisms mediating the association between childhood trauma and later vulnerability for psychiatric disorders remain unknown.

2.2.3. Brain areas involved in the stress response

Exposure to stress has been associated with several neuroimaging abnormalities including volumetric and connectivity changes. Specifically, different types of childhood maltreatment have been associated with differential gray matter loss, reduced cortical surface and thickness, and decreased network connectivity (Teicher et al., 2016). Globally, maltreatment is clearly associated with alterations in brain regions and pathways involved in both the perception and memory of threatening/emotional situations.

The amygdala and the hippocampus, as the main components of the limbic system, are critically involved in socioemotional functioning and stress response (**Figure 8**). While the hippocampus is essential for learning, memory and neuroendocrine regulation of the stress response; emotional and social information is primarily processed by the amygdala (Hanson et al., 2015).

The hippocampus is particularly sensitive to heightened circulating levels of cortisol. Thus, exposure to toxic stress has been described to decrease hippocampal volume; meta-analytic evidence revealed significant

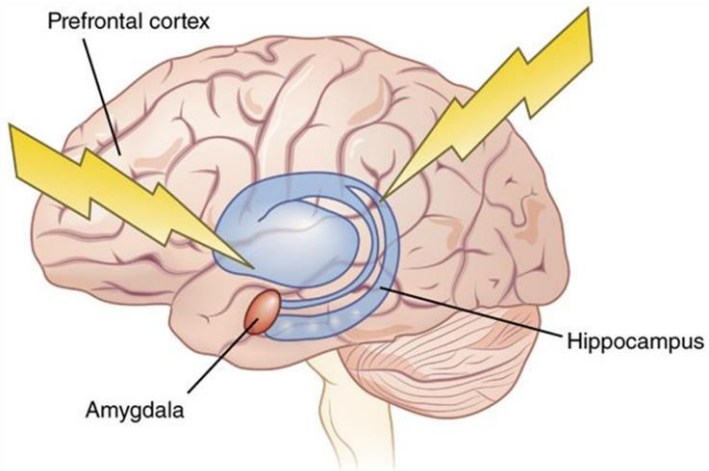


Figure 8. Mechanisms underlying stress effects on the brain. In the hippocampus, stress and glucocorticoids cause dendritic shrinkage and loss of spines while stress on the amygdala is associated with increased spine density and dendrites' expansion. Reproduced with permission from: McEwen B et al. Mechanisms of stress in the brain. Nature Neuroscience 18, 1353-1363 (2015).

hippocampal reductions in association with childhood maltreatment, particularly when it was experienced during middle childhood (Riem et al., 2015). Remarkably, hippocampal gray matter loss is a neuroimaging finding specific to depression, as opposed to a majority of neuroimaging findings overlapping across different categorical diagnoses (Goodkind et al., 2015).

Beyond volumetric alterations, brain wiring and activation patterns have also been widely researched in the context of psychiatry. Functional magnetic resonance imaging allows us to study both brain connectivity and activity by means of blood-oxygen-level-dependent (BOLD) contrast. This methodology has been instrumental in the description of several neural networks affected in psychiatric disorders. In this regard, heightened amygdala reactivity in healthy subjects was described as a reliable biomarker of stress-triggered anxiety and depressive disorder up to four years later (Swartz et al., 2015). Higher amygdala reactivity and activity have been also described in traumatized individuals in response to negative valence stimuli presented in experimental settings.

Interestingly, integration between genetic and neuroimaging approaches has led to a new research field, imaging genetics and, more recently, to imaging epigenetics. Such new approaches allow the exploration of stress vulnerability, resilience and plasticity mechanisms.

2.2.4. Stress response: the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is one of the main stress response systems; its activation allows resource reallocation to appropriately react before a threat, including regulation of digestion, energy expenditure and immune response. Exposure to threatening situations mediates the secretion of corticotropin-releasing hormone (CRH) by the paraventricular nucleus of the hypothalamus. Once in the anterior pituitary, CRH promotes the release of adrenocorticotropic hormone (ACTH) to the adrenal glands, which finally release cortisol—the end product of human HPA axis activation (**Figure 9**). Once in the bloodstream, this steroid hormone is distributed systemically, including the hypothalamus and the pituitary where it suppresses CRH and ACTH production, terminating the stress response. As a lipophilic molecule, cortisol crosses the cellular membrane by passive diffusion and can bind to both mineralocorticoid and glucocorticoid receptors

(MR and GR, respectively) in the cytoplasm. While the MR has a higher affinity for glucocorticoids, the GR is characterized by a higher threshold for activation and thus responds to heightened cortisol states indicative of stress exposure.

When cortisol binds to the GR, the later changes its conformation, gets rid of co-chaperones preventing the receptor from translocating to the nucleus and directly accesses the DNA sequence, functioning as a transcription factor. The GR-cortisol complex then binds to glucocorticoid responsive elements (GRE) in the genome activating and repressing gene expression. Of

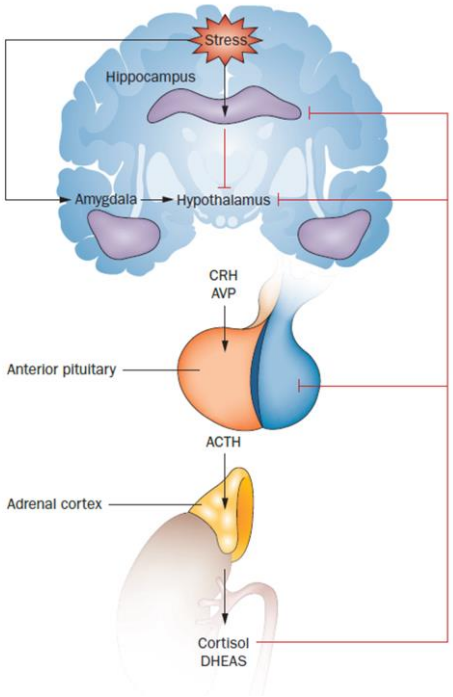


Figure 9. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the parvocellular neurons in the parvocellular neurons in the paraventricular nucleus of the hypothalamus leads to synthesis of CRH and AVP, which stimulate release of ACTH at the pituitary, which in turn acts at the adrenals to stimulate synthesis and release of corticosteroids. While the hippocampus negatively regulates HPA axis functioning, amygdala activity triggers its activation. Reproduced with permission from: Papadopoulos A & Cleare A. Hypothalamic–pituitary–adrenal axis dysfunction in chronic fatigue syndrome. Nature Reviews Endocrinology 8, 22-32 (2012).

note, the GR is ubiquitously distributed throughout the organism and cortisol release from the adrenal glands results in homeostasis regulation at both peripheral and central levels.

Interestingly, one of the key actions of cortisol consists in the termination of the stress response by means of a negative feedback mediated by GRs located at hypothalamic and pituitary regions. Deterioration of this feedback leads to glucocorticoid resistance, giving rise to chronically heightened levels of circulating cortisol, followed by immune dysregulation and altered stress response (Cohen et al., 2012; Pariante, 2017). Glucocorticoid resistance can be monitored by administration of a synthetic glucocorticoid, dexamethasone, and later measuring of cortisol circulating levels (Leistner and Menke, 2018).

Effects of excess cortisol can be easily monitored in Cushing's syndrome patients. Cushing's syndrome is a medical condition characterized by chronically high levels of circulating cortisol due to either corticosteroid medication or the presence of tumors in any of the organs involved in the HPA axis (Lacroix et al., 2015). Hypercortisolism observed in Cushing syndrome patients causes a wide range of symptoms such as weight gain, headache, muscle weakness or hypertension. Of note, Cushing syndrome also presents with psychological symptoms including depression, anxiety and cognitive difficulties, further confirming the deleterious role of hypercortisolism in mood and cognition.

HPA axis functioning and GR itself have been thoroughly analyzed in both psychiatric patients and subjects exposed to severe trauma. Human response to acute stress can be measured in a lab setting by means of experimentally controlled challenges such as the Trier Social Stress Test (TSST), a validated procedure shown to reliably activate stress response systems in both children and adult subjects (Allen et al., 2014). Such approaches have revealed abnormal cortisol outputs after exposure to acute stress in both depressed and maltreated subjects. However, whether HPA axis alterations are causal or consequential in such associations remains a controversial matter (Zorn et al., 2017).

From puberty onward, men and women display significant differences in their HPA axis reactivity in front of threatening stimuli. In this re-

gard, men have been described to show greater increases in peripheral cortisol after exposure to acute stress than women (Eisenberger et al., 2007). From an evolutionary point of view, males are expected to prioritize competition and aggression in threatening situations while females favor investing in offspring protection (Glover and Hill, 2012). Remarkably, prenatal stress induces sex-specific adaptations, contributing to the fetal programming theory (section 2.2.1). Interestingly, these differences have been hypothesized to be caused by sexual dimorphism of the human placenta; while male fetuses prioritize growth at the expense of survival, females adapt to prenatal environment increasing their overall survival rate but limiting their potential to grow (Clifton, 2010).

2.2.4.1. The glucocorticoid receptor gene

GR is encoded by the nuclear receptor subfamily 3 group C member 1—*NR3C1*—gene, located in chromosome 5q31. *NR3C1* gene transcription gives rise to 16 splice variants and several isoforms (the most commonly expressed being GR-α and GR-β). While GR-α is the predominant isoform, carrying out GR physiological activity, GR-β is shorter and resides mainly in the cell nucleus. Since GR-β cannot bind to glucocorticoids, it inhibits GR-α by forming heterodimers and preventing GR-α-mediated transcription regulation (Oakley et al., 1999). Interestingly, decreased monocytic expression of GR-α in depressed patients was reported together with an upregulation of pro-inflammatory pathways (Carvalho et al., 2014).

NR3C1 gene is comprised of 8 coding exons and 9 alternate non-coding first exons (1_A, 1_B, 1_C, 1_D, 1_E, 1_F, 1_H, 1_I and 1_J). Non-coding exons are located in the gene promoter region and are transcribed into different mRNA variants, but they have not been described to be translated into any of the protein isoforms (**Figure 10**). Molecular studies have revealed each of the non-coding exons transcripts to display a characteristic tissue distribution (Turner and Muller, 2005).

Given the essential role of GR in the regulation of HPA axis functioning, *NR3C1* genetic variants have been widely analyzed in the context of psychiatric genetics research, with a focus on depression (see section 1.4).

Accordingly, *NR3C1* SNPs and haplotypes[11] have been associated with: MDD liability and antidepressant response (van Rossum et al., 2006), predominance of depressive symptoms in bipolar disorder (Szczepankiewicz et al., 2011), disease severity as reflected by hospital admissions (Lahti et al., 2011), hippocampal volume reduction (Zobel et al., 2008), and HPA axis reactivity as measured by cortisol output after acute exposure to stress (Plieger et al., 2018).

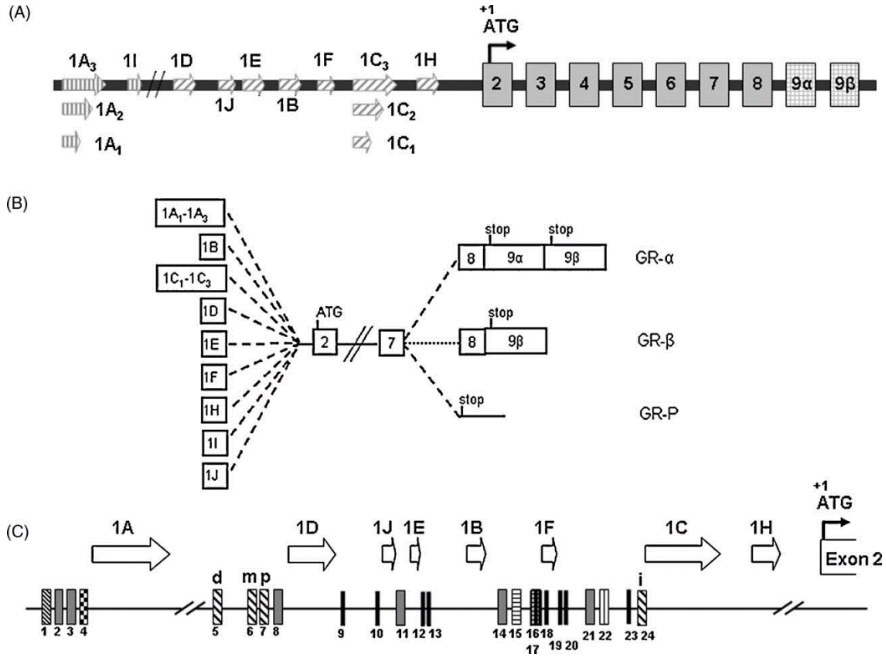


Figure 10. Structure of the *NR3C1* gene, its potential mRNA transcripts and the binding sites within the CpG island. (A) Genomic structure of NR3C1 gene including 5' untranslated exons (arrows), common exons and 3' alternatively spliced exons (9α and 9β). **(B)** Potential mRNA transcripts encoding the three of the GR isoforms (GRα, GRβ and GR-P). **(C)** Location of known transcription factor binding sites. Reproduced with permission from: Turner et al. Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. *Biochemical Pharmacology* 80 (12): 1860-1868.

[11] Haplotype refers to a group of alleles inherited together due to their proximity in the chromosome (high linkage disequilibrium)

Nevertheless, such studies have also revealed inconsistent results regarding the specific effects of particular SNPs. Anyway, *NR3C1* genetic variation does not seem to fully account for observed variability in HPA axis functioning and regulation, suggesting the involvement of additional mechanisms yet to be identified.

2.2.4.2. Serotonergic involvement in HPA axis regulation

Serotonin (5-HT) regulates a plethora of physiological processes including mood, aggression, memory consolidation, food intake and stress response. As introduced in section 1.4, 5-HT is one of the key neurotransmitters altered in depression, with a serotonergic deficit suggested as one of the main etiological factors for MDD.

Additionally, evidence points to synergistic regulation of stress response by HPA axis and serotonergic pathways. For instance, experimental activation of the HPA axis in animal models has been described to increase 5-HT release in several brain areas (Lanfumey et al., 2008). Similarly, the study of genetically modified mice with defective serotonergic transmission revealed increased stress sensitization, decreased GR expression and blunted response to dexamethasone administration (Jiang et al., 2009).

***SLC6A4* gene and 5-HTTLPR**

The serotonin transporter—also known as 5-HTT or SERT—is encoded by the solute carrier family 6 member 4—*SLC6A4*—gene, located in chromosome 17q11.2. SERT reuptakes serotonin from the synaptic cleft into the pre-synaptic neuron, thus terminating serotonergic transmission. Selective serotonin reuptake inhibitors (SSRIs), the most widely prescribed antidepressants worldwide, act by pharmacologically blocking this transporter.

A long polymorphic region in *SLC6A4* promoter—5-HTTLPR—consisting of a short (*s*) and a long (*l*) alleles has been characterized to influence SERT expression and thus has been thoroughly researched in the context of psychiatric genetics (**Figure 11**). Accordingly, s carriers exhibit poorer regulation of the HPA axis and increased vulnerability to early life stress when compared to *ll* homozygotes (Gunnewiek et al., 2018). Furthermore, 5-HTTLPR has also been associated with course of illness and antidepressant response (Arias et al., 2003). Nevertheless, association between both varia-

bles remains controversial due to inconsistent results across studies (Taylor et al., 2010).

Additionally, 5-HTTLPR has also been described to alter brain functioning, particularly in the amygdala, a brain area involved in emotion processing and response to aversive stimuli. Specifically, *s* carriers exhibit heightened amygdala reactivity to environmental threat when compared to l homozygotes (Munafò et al., 2008).

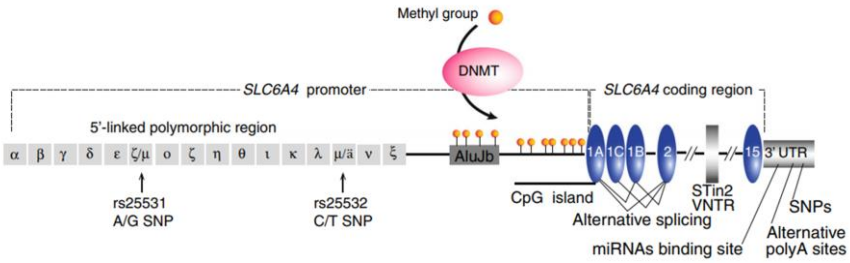


Figure 11. Structure of the *SLC6A4* gene, including its promoter-located CpG island, the 5-HTTLPR, alternative splicing and several SNPs. The depicted 5-HTTLPR corresponds to the long allele (16 repeats designated by Greek letters according to Nakamura et al.'s nomenclature). DNA methylation at an AluJb element and a CpG island may regulate *SLC6A4* expression. Reproduce with permission from: Iurescia S, Seripa D & Rinaldi M. Looking Beyond the 5-HTTLPR Polymorphism: Genetic and Epigenetic Layers of Regulation Affecting the Serotonin Transporter Gene Expression. *Molecular Neurobiology* 54 (10): 8386-8403.

2.3. Nature versus Nurture: a paradigm for the understanding of psychiatric phenotypes

Throughout section 2.1. the genetic bases of mental disorders have been discussed revealing the moderate risk conferred by the genetic variants identified so far. Conversely, section 2.2 considers the role of the environment in psychiatric disorders unfolding a similar lack of definitive knowledge to be used as either a biomarker or a target for treatment. Finally, section 2.3 presents the inconsistent genetic associations between variants at candidate genes involved in HPA axis regulation—*NR3C1*, *FKBP5*, *SKA2*, *SLC6A4*—and a number of psychiatric, cognitive and stress outcomes.

This framework lead to the so-called nature versus nurture debate, applied to several human phenotypes, particularly those of a psychological nature, such as intelligence, personality or psychopathology. Currently, it is widely accepted that both innate and acquired factors are required to the development of such complex traits.

2.3.1. Gene per environment interactions

The simultaneous and synergistic involvement of genes and environment (GxE interaction) in psychiatric phenotypes was firstly recognized by Caspi and colleagues in their seminal works regarding the interaction between (i) monoamine oxidase A (MAOA) risk alleles and exposure to severe childhood maltreatment to give rise to antisocial behavior (Caspi et al., 2002), and (ii) the 5-HTTLPR and exposure to stressful life events to trigger depression (**Figure 12**) (Caspi et al., 2003). Such interaction models broke new ground and lead to a new conceptualization of psychiatric disorders and related phenotypes.

Thenceforth, several GxE interactions have been suggested in the psychiatric field. Nevertheless, candidate approaches are limited by its own simplistic conceptualization consisting of one single genetic variant of risk and one unique environmental factor. Unlike solely genetic designs, massive exploration of the environment is restrained by methodological and statistical limitations.

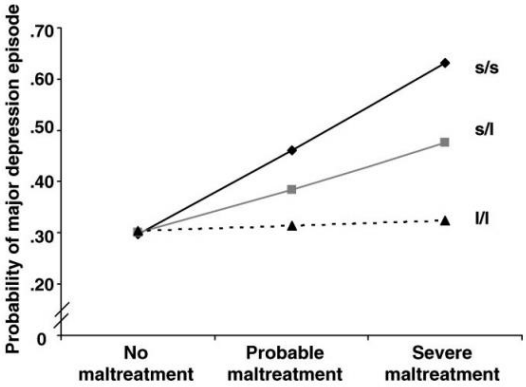


Figure 12. GxE interaction between childhood maltreatment and adult depression as a function of 5-HTTLPR. Reproduced with permission from: Caspi A. Influence of Life Stress on Depression: Moderation by a polymorphism in the 5-HTT gene. *Science* 301, 386 (2003).

3. Epigenetics

In this maelstrom of inconsistent findings and elusive etiologies, epigenetics emerged as a *jack of all trades* which could potentially explain at the same time (i) the dialogue between genes and environment, (ii) the latency from distant exposure to proximal symptomatology, (iii) the sex-biased prevalence of most psychiatric disorders, and (iv) the observed phenotypic discordance between monozygotic, or identical, twins.

The term epigenetics was coined by Waddington back in 1942, to refer to the developmental processes operating from the genotype to the phenotype (Waddington, 2012). Since then, epigenetics definition has been reshaped several times to better reflect the nature of the mechanisms and modifications considered to be *epigenetic*. From the many definitions of

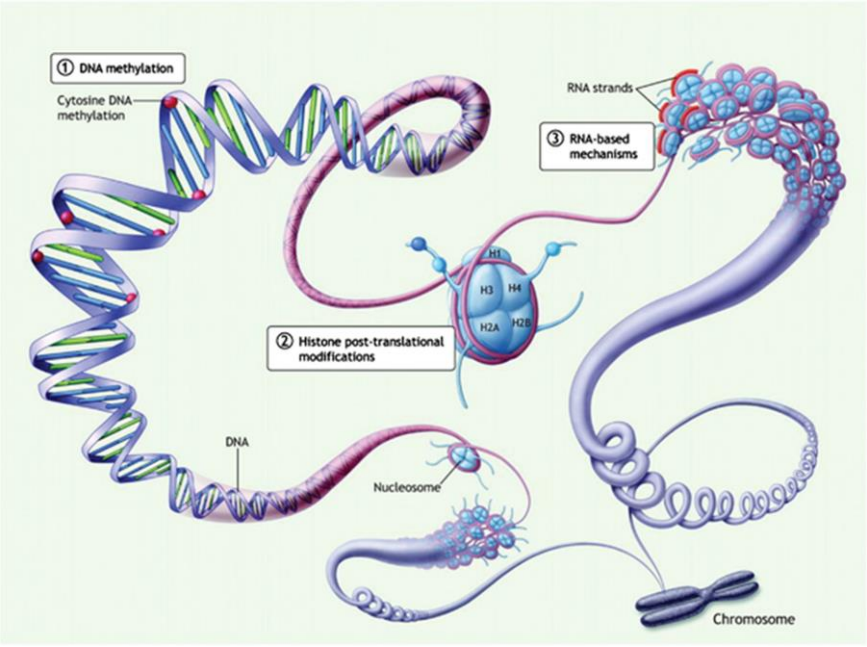


Figure 13. Main epigenetic mechanisms. DNA methylation, histone post-translational modifications and RNA-based mechanisms. Reproduced with permission from Lee J, Murphy G & Lian C. Melanoma epigenetics: novel mechanisms, markers, and medicines. *Laboratory Investigation* 94(8): 822-838 (2014).

what epigenetics means, and for the purpose of this thesis dissertation, epigenetics refers to «*any heritable change in gene expression that does not affect the DNA sequence itself*» (Jaenisch and Bird, 2003).

This definition contains three main features shared by all epigenetic modifications, the first of them being their impact on chromatin conformation and subsequent gene expression and regulation. Additionally, neither of them affects the DNA sequence although epigenetic marks are passed from one cell to another, making such mechanisms heritable. Of note, the heritable component of epigenetic mechanisms refers to the maintenance of epigenetic signatures along subsequent cell divisions. Heritability of epigenetic patterns across different generations will be discussed in more detail in section 3.4. This epigenetic maintenance is confronted with its plasticity and responsivity to the environment; thus, acquired epigenetic changes have been acknowledged as contributors to health and disease (Romanoski et al., 2015).

Histone tail modifications (acetylation, phosphorylation and ubiquitination, among others), DNA methylation and microRNAs constitute the major epigenetic modifications (**Figure 13**). Such mechanisms are involved in key physiological pathways such as cell lineage determination or X chromosome inactivation in females (Riggs, 1975). By modulating the compaction of the chromatin, DNA methylation and histone acetylation, among other mechanisms, dictate which genes are transcriptionally silenced. Similarly, miRNA directly bind to complementary mRNA molecules thus silencing gene expression (without modifying chromatin structure).

3.1. DNA methylation

DNA methylation usually occurs in cytosines adjacent to guanines, also known as CpG dinucleotides[12], although it has also been described to occur in non-CpG dinucleotides, particularly in brain tissue (Guo et al., 2013). Of note, although the GC content spans an approximate 40% of the human genome, CpG dinucleotides are underrepresented (Josse et al., 1961). This phenomenon is thought to operate through spontaneous mutations of methylated cytosines into thymines. Interestingly, CpG dinucleotides tend to cluster together in small regions of the DNA, known as CpG

[12] The “p” in the CpG refers to the phosphate group between both nucleotides

islands; specifically, CpG islands and first exons occupy less than 0.5% of the genome but contain 4.5 of the total CpG content of the genome. CpG dinucleotides within CpG islands are characterized by their low levels of DNA methylation. In this regard, CpG islands are typically located near gene promoter regions—containing 75% of all human gene promoters—and their methylation has been reliably associated with gene expression (Edwards et al., 2017; Jones, 2012). Thus, there is an interest in analyzing DNA methylation of CpG islands close to candidate gene promoter regions since it could be mediating the pernicious role of the environment upon disease vulnerability.

DNA methyltransferases (DNMTs) mediate both *de novo* and maintenance methylation across the genome. Somatic inheritance of methylation patterns is mediated by DNMT1, an enzyme that preferentially binds to hemimethylated DNA (i.e. when only one of the two strands of the DNA is methylated) (Edwards et al., 2017). Demethylation processes are less understood and have been described to occur either passively or actively mediated by ten-eleven translocation—TET—methylcytosine dioxygenase enzymes (Wu and Zhang, 2017).

Briefly, DNA methylation can be experimentally measured by means of bisulfite treatment. This substance triggers conversion of any unmethylated cytosine to uracil while methylated cytosines remain the same. Hence, sequencing of bisulfite treated DNA allows the detection of methylated cytosines. Based on this procedure, DNA methylation can be assayed either in candidate gene—where bisulfite treated DNA is pyrosequenced—or massive approaches—including an array of CpG sites distributed throughout the whole genome. Other methods to measure methylation include methylation-specific PCR, luminometric methylation assay and mass spectrometry based approaches, among many others (Kurdyukov and Bullock, 2016).

3.2. Epigenetic clock

Time is one of the modulators of DNA methylation patterns. So much so that an epigenetic clock was developed exclusively based on DNA methylation patterns. The notion of biological age as opposed to chronological age emerged as a more accurate estimation of age where environmental exposures and disease states could be considered (**Figure 14**). In this

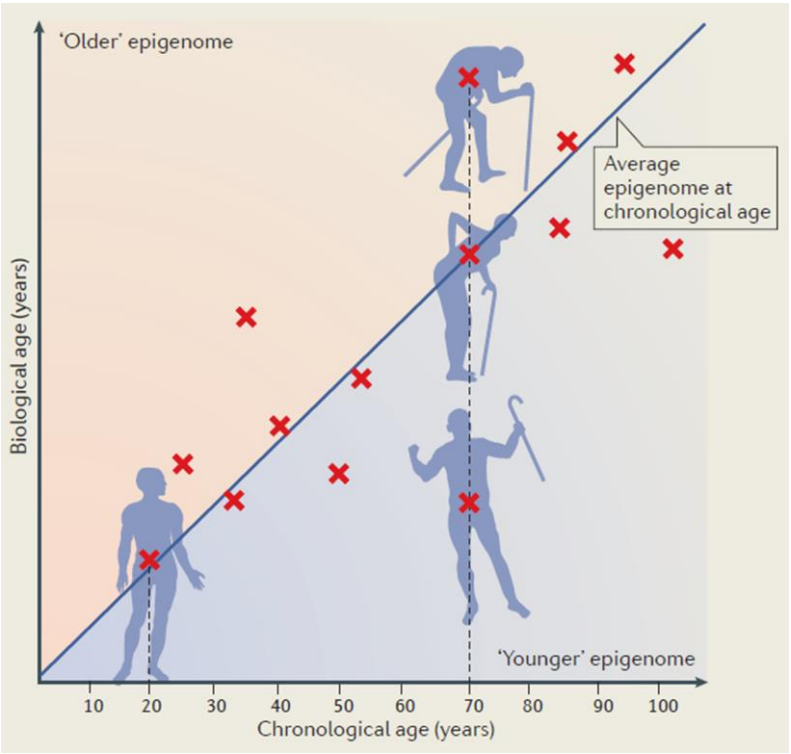


Figure 14. Epigenetic modifications as aging biomarkers. DNA methylation-based age estimations accurately predict chronological or *real* age. Deviations between chronological and biological age might reflect underlying pathologic effects and/or accelerated senescence. Reproduced with permission from: Benayoun B, Pollina E & Brunet A. Epigenetic regulation of ageing: linking environmental inputs to genomic stability. Nature Reviews Molecular Cell Biology 16, 593-610 (2015).

regard, several attempts have been made to estimate biological age based on telomere length, proteomics or metabolomics. Nevertheless, DNA methylation based epigenetic estimation has proven most accurate (Jylhävä et al., 2017).

Currently, there are three different estimators of epigenetic age based on DNA methylation. Horvath's clock was the first to be developed, it was trained in samples of multiple tissues ranging from 0 to 100 years old and it can predict chronological age with an accuracy of 3.6 years (Horvath, 2013).

Concurrently, a second clock was built in a dataset of adult blood samples; despite its low reliability in children and non-blood samples, it has been described to better predict time to death (B. Chen et al., 2016; Hannum et al., 2013). Finally, a third clock was developed in cord blood and neonatal blood spots in order to predict gestational age at birth (Knight et al., 2016). Due to the limited range of human gestational ages at birth, the latter clock is characterized by a more precise accuracy of only 1.24 weeks of median error.

Epigenetic age estimation allows the calculation of age acceleration whenever epigenetic age is higher than chronological age. In this regard, age acceleration has been associated with time to death, body mass index, infections, cancer and menopause; of note, age acceleration has also been associated with exposure to trauma (Wolf et al., 2018). Thus, it could be underlying the so far discussed long-term consequences of adverse events such as heightened immune activation and, particularly, increased vulnerability to psychiatric disorders.

Interestingly, CpG sites included in Horvath's clock have been described to be enriched in glucocorticoid responsive element (GRE) sequences, 85 out of 353 clock CpG sites colocalized with GREs (Zannas et al., 2015). Additionally, dexamethasone administration induced significant changes in gene expression of 81.7% of probes colocalizing with clock CpG sites further suggesting glucocorticoid responsivity of the epigenetic clock.

3.3. Sex-specific methylation

Another obvious modulator of DNA methylation patterns is sex. Sex-biased methylation is highly suggestive in the context of psychopathology due to the profoundly biased prevalence ratios of most psychiatric disorders. Specifically, depression and anxiety disorders (section 1.4) are far more prevalent in women than men while the opposite is true for neurodevelopmental disorders.

Remarkably, sex-based methylomic signatures have been described in prefrontal cortex (Xu et al., 2014); up to 5.1% of CpG sites investigated were found to be differentially methylated between men and women (n = 22,124 CpG sites). Shortly after, another study also explored the role of sex in DNA methylation differences in a peripheral blood based genome-wide

approach with a significant overlap with CpG sites identified in prefrontal cortex (Inoshita et al., 2015). Interestingly, both studies found a majority of sex-differentially methylated CpG sites to be hypermethylated in females when compared to males. Such differences have been hypothesized to rely on hormonal mechanisms.

3.4. Stochasticity

Epigenetic modifications are usually understood from a functional and adaptive point of view. Whenever an epigenetic mark is associated with an environmental exposure it is hypothesized to arise from such exposure as a sort of adaptive feature. Nevertheless, stochastic epigenetic modifications had been largely neglected from epigenetic research until recently.

In a general sense, stochasticity can refer to biological processes that arise from randomness or noise. The notion of stochastic epigenetics builds upon the observation that genetically identical organisms exposed to the same environment experimentally exhibit epigenetic differences that cannot be attributed to either genes or (macroscopic) environment. Interestingly, there are genomic regions more susceptible to stochastic epigenetic variation, which are referred to as variably methylated regions or VMRs (Feinberg and Irizarry, 2010). Epigenetic plasticity has been suggested to be favored in the context of natural selection since it allows better adaptation in changing environments.

Just like the epigenetic field itself, stochastic epigenetic modifications with potential pathogenic effects were first described in cancer (Hansen et al., 2011). In this regard, epimutations giving rise to tumors would emerge as a side-effect of increased epigenetic plasticity in the evolutionary timescale. Specifically, repeatedly changing environments throughout human lifespan would induce cumulative stochastic epigenetic effects finally giving rise to tumorigenic activity (Feinberg and Irizarry, 2010). Accordingly, epimutations have been described to accumulate with age in humans (Gentilini et al., 2015).

Exploration of epigenetic stochasticity has been scarcely explored in other complex traits such as obesity of depression revealing suggestive findings. Preliminary studies point to the presence of higher methylation varia-

bility and methylation outliers (suggesting stochastic effects) in subjects affected by depression (Oh et al., 2015).

3.5. Transgenerational epigenetics

Although the heritable component of epigenetic mechanisms present in the aforementioned definition refers to the maintenance of epigenetic patterns through subsequent cell divisions, there has been extensive—and controversial—research regarding transgenerational transmission of epigenetic signatures. Such studies suggest acquired epigenetic modifications can be transmitted to subsequent generations in organisms with sexual reproduction, such as *Caenorhabditis elegans*, mice and humans.

While some researchers argue that epigenetic reprogramming in the germline precludes epigenetic transmission from progenitors to offspring, others have developed thorough experimental designs to empirically demonstrate the existence of such transgenerational effects. Evidence comes from modifications of the same paradigm: one generation is experimentally exposed to stress (e.g. thermal stress in *C. elegans* and odor fear conditioning in mice) and the subsequent generations are investigated for behavioral adaptations and the allegedly underlying epigenetic marks (Klosin et al., 2017; Szyf, 2013).

In this regard, trans- and inter- generational effects must be told apart. While transgenerational refers to the transmission through multiple generations, intergenerational effects are not necessarily transmitted by inherited epigenetic embedding but by direct exposure of the F_1 and F_2 at the same time than the F_0. That is possible when a pregnant female (F_0) is exposed to an environmental threat since both the fetus (F_1) and the gametes[13] to give rise to the F_2 are simultaneously exposed (Bowers and Yehuda, 2016). Intergenerational effects can also be transmitted through offspring rearing since progenitors exposed to traumatic stress will be more prone to develop psychiatric symptoms during the early postnatal period giving rise to insecure attachment (Choi et al., 2017).

[13] The primary oocytes, which would give rise to mature gametocytes, are already formed during the fetal period.

Whether exposure to trauma can lead to transgenerational inheritance via epigenetic mechanisms in humans is currently under debate. However, intergenerational effects have been demonstrated after exposure to (i) the Holocaust (Yehuda et al., 2016), (ii) intimate partner violence (Serpeloni et al., 2017), and other types of traumatic stress during pregnancy (Bowers and Yehuda, 2015). Currently, epigenetic inheritance is thought to be passed on to the next generation by RNAs contained in the sperm rather than by DNA methylation (Q. Chen et al., 2016).

3.6. Stress-dependent methylation: a model for GxE interactions

As introduced in section 2.3.1, GxE interaction models were developed to resolve the longstanding debate of whether genes or environment were the causative agents underlying psychiatric physiopathology. However, after describing several of such interactions, their mediator biological mechanisms remained largely unknown. In this regard, epigenetic mechanisms, and DNA methylation in particular, have been suggested to bridge the environment and the genome to give rise to psychiatric disorders. Favoring this hypothesis, epigenetic embedding of early life exposures could explain the latency observed between epidemiologically described associations between obstetric complications and later risk for neurodevelopmental disorders of late onset such as schizophrenia (section 1.5).

Recently, a paradigmatic GxE interaction was described to be mediated by differential DNA methylation (Klengel et al., 2013). FKBP5 polymorphisms had been already described to interact with childhood abuse in the onset of depression, PTSD symptoms and increasing the risk for attempted suicide (Binder et al., 2008; Roy et al., 2010; Zimmermann et al., 2011). Exploration of DNA methylation patterns at *FKBP5* gene revealed the presence of a CpG-specific allele-specific demethylation in response to childhood trauma (Klengel et al., 2013). This demethylation was associated with HPA axis dysregulation

3.7. An epigenetic view of NR3C1 gene

Biological and clinical relevance of HPA axis disturbances and, more specifically, of *NR3C1* gene variation have been discussed in section 2.2.3. In the context of epigenetic research, *NR3C1* gene emerges as the most promising candidate gene due to its crucial role in stress response and regulation

and its well-described sensitivity to chronic stress. In this regard, glucocorticoid resistance observed in psychiatric patients has been suggested to be mediated by increased DNA methylation in its promoter region.

To test this hypothesis, a research group lead by Meaney focused on an animal model naturally displaying two distinct maternal rearing behaviors. While some rats repeatedly lick and groom (LG) their pups and acquire an arched back position to nurse them (ABN), others do not. Thus, rats were dichotomically classified as either high or low LG-ABN dams based on their displayed rearing behavior. Several studies revealed offspring from low LG-ABN mothers to (i) be more fearful, (ii) exhibit increased HPA axis activity, and (iii) show lower hippocampal expression of GR, when compared to those reared by high LG-ABN (Caldji et al., 1998; Liu et al., 1997). Furthermore, the same research group demonstrated the nongenomic inheritance of such behaviors by means of cross-fostering experiments (Francis et al., 1999). Finally, DNA hypermethylation at a promoter region of the *Nr3c1* gene was found only in pups from low LG-ABN dams setting precedent for myriad studies exploring DNA methylation at *NR3C1* gene in humans.

NR3C1 gene has a long CpG island—spanning 3kb and 303 CpG sites—in its promoter region encompassing seven out of its nine first non-coding exons (1_D, 1_J, 1_E, 1_B, 1_F, 1_C, 1_H). The complexity of the *NR3C1* promoter region and, more specifically, enrichment in CpG sites of the seven exons, suggests DNA methylation to play a role in the tissue distribution of the different transcripts.

3.8. An epigenetic view of SLC6A4 gene

Another candidate gene of interest in the context of psychiatric epigenetics is the *SLC6A4* gene. Due to the targeting of the serotonin transporter by SSRIs, *SLC6A4* methylation has been hypothesized to mediate treatment response. Furthermore, like *NR3C1*, *SLC6A4* methylation has also been suggested to be responsive to environmental threats and to mediate psychopathological liability.

SLC6A4 contains a CpG island in its promoter region encompassing the first coding exon. Interestingly, DNA methylation at this CpG island has been reliably described to decrease SERT expression, following the widely

accepted notion that CpG island methylation inversely correlates with gene expression.

3.9. Limitations

However, the nature of DNA methylation comes along with a number of limitations specific to the field. As opposed to the genomic sequence, DNA methylation varies from cell to cell, with each cell tissue being characterized by a particular epigenomic signature. This posits two major challenges: (i) confounding by cellular heterogeneity and (ii) dubious replication of methylation signatures across different tissues.

Scientific evidence of epigenetic modulation in association with psychopathology mostly comes from studies focusing on the study of peripheral blood samples due to invasiveness of sampling brain tissue in living subjects. On the one hand, peripheral blood consists of a mixture of cell types such as lymphocytes, monocytes and NK cells with differing epigenetic patterns each (Houseman et al., 2012). On the other hand, brain and blood tissue exhibit low-to-moderate methylation correlation making interpretation of results challenging (Hannon et al., 2015).

When a genetic variant is associated with a certain disorder, it is assumed that either the variant itself or one in linkage disequilibrium plays a causal role in the phenotype of interest. Since DNA methylation can be modified due to environmental exposures, whenever an association is described in a cross-sectional study, causality cannot be stablished. Thus, epigenetic approaches allow the identification of both causes and consequences of the disorder. In this regard, an epigenomic wide association study in schizophrenia revealed the top findings to be derived from higher smoking in cases than controls (Hannon et al., 2016).

Finally, DNA methylation is partially determined by the genetic sequence. The identification of methylation quantitative trait loci (mQTL), i.e. genetic variants that directly influence DNA methylation, highlights the importance of incorporating genetic influences into epigenetic research (Gaunt et al., 2016; Hannon et al., 2018a). Discerning the influence of environmental and genetic factors in epigenetic studies is essential for understanding how methylation signatures associated with a certain trait are established.

4. Twin studies

As introduced in section 3.4, genetically identical subjects experimentally exposed to the same environment exhibit differential epigenetic patterns. Furthermore, across sections 3.1 and 3.2, both age and sex have been described to influence epigenetic patterns. Additionally, epigenetic variability is known to depend on the underlying genetic sequence. In this framework, human twins emerge as an excellent resource to disentangle the role of genes and environment on a phenotype for which they are discordant.

4.1. Human twinning and twin-based studies

There are two types of human twins: monozygotic (MZ) and dizygotic (DZ). MZ twins origin from a single zygote that splits into two individuals in an early phase of embryonic development. Depending on the timing of

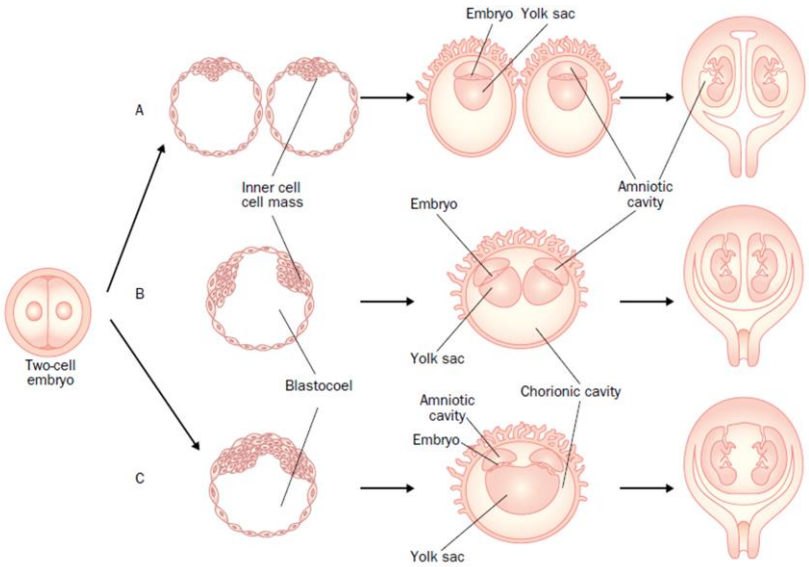


Figure 15. Formation of three patterns of MZ twins. (A) Early twinning, occurring in the first cell divisions, leads to diamniotic dichorionic twins. **(B)** When twinning takes place during the early blastocyst stages, it gives rise to diamniotic monochorionic twins. **(C)** Finally, twinning occurring in the post-implantation blastocyst results in monochorionic monoamniotic twins.

this division, MZ twins can share or not the amnion and the chorion; very late or partial splitting gives rise to conjoined twins. **Figure 15** illustrates the three most common twinning processes giving rise to the three main types of human MZ twins. Molecular mechanisms mediating twinning processes in humans remain unknown (Blickstein and Keith, 2007; Steinman, 2000).

In turn, DZ twins develop from different zygotes due to the maturation and release of more than one oocyte in the same menstrual cycle. Remarkably, although DZ twins arise from different zygotes, there have been several case reports of monochorionicity in DZ twin pregnancies highlighting the need to test zygosity instead of assuming monochorionic twins to be always MZ (Ekelund et al., 2008; Hackmon et al., 2009). An increase in monochorionic dizygotic twins has been suggested to be mediated by a rise in the use of assisted reproduction technologies (Miura and Niikawa, 2004). Interest in the prevalence of monochorionic dizygotic pregnancies derives from the higher frequency of OCs observed in monochorionic versus dichorionic twin pregnancies (Coutinho Nunes et al., 2016; D'Antonio et al., 2012).

Traditionally, both MZ and DZ twins have been employed to estimate the heritability of complex traits and disorders. In order to do so, the phenotypic variance (P) of a certain trait is considered to arise from three additive terms: (i) additive genetic influences (A), (ii) shared environmental influences (C), and (iii) non-shared or unique environmental influences (E). More complex models including, for example, the effects of non-additive genetic influences (D), due to interactions between alleles, have also been developed but the more simplistic view of P = A + C + E is sufficient to understand preliminary calculations of heritability (**Figure 16**). According to Falconer's formula, heritability (h^2) can be estimated as follows:

$$h^2 = 2(r_{MZ} - r_{DZ})$$

where rMZ and rDZ refer to the mean phenotypic concordance for the trait of interest between monozygotic and dizygotic twins, respectively (Falconer and Mackay, 1996).

A recent meta-analysis of all heritability studies based on twin samples revealed an outstanding interest in psychiatric traits with 5 million subjects being analyzed with regard to this category (Polderman et al., 2015).

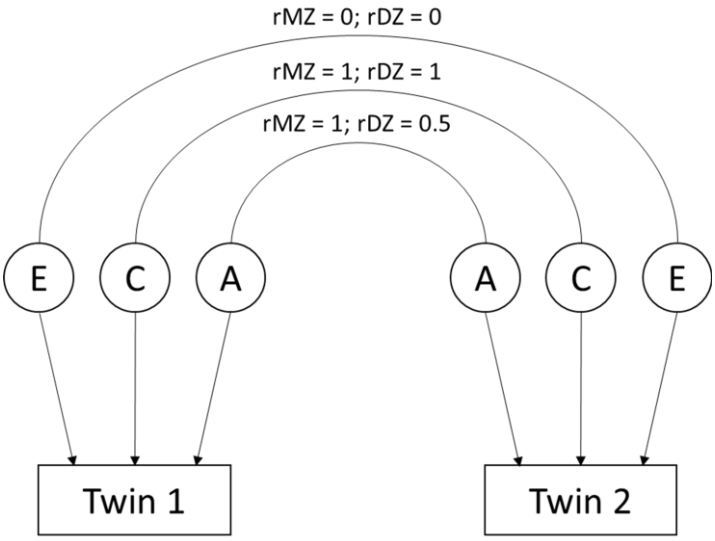


Figure 16. Path diagram for the basic univariate twin model. The phenotype of each twin is decomposed into A, C and E variance components corresponding to additive genetics, common environment and unique environment, respectively. Adapted from Rijsdijk and Sham (2002).

4.2. Intrapair designs

Nevertheless, alternative study designs exclusively based on MZ twin pairs have also been developed to disentangle the role of genes and environment in complex traits. The high degree of intrapair discordance for a number of traits of interest allows comparison between healthy, discordant and concordant twin pairs. In such studies, healthy twin pairs carry neither genetic nor environmental liability while discordant and concordant pairs carry the unique environment and genetic liability, respectively. Although the effects of additive genetics cannot be dissected from shared environment, both drivers of phenotypic variance are merged into a *familial* component.

Interestingly, discordant monozygotic twins can be analyzed in terms of intrapair differences. Specifically, testing the association between two variables of interest by including in the statistical model the intrapair difference values for each twin pair instead of the actual values for each subject, removes sex and age confounding from the analysis together with

any other variable shared by both twins of a pair. The downside of this study design is that the sample size is halved, thus decreasing the power of the analysis.

4.3. Obstetric complications in twin pregnancies

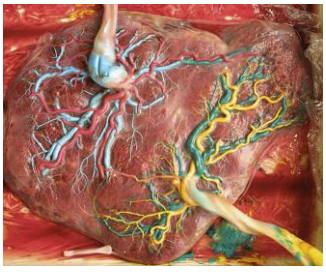
Remarkably, MZ twin pregnancies give rise to differential co-twin specific intrauterine microenvironments. Thus, the presence of non-shared or unique environment (E) is already apparent during prenatal stages of life. Interestingly, methylomic exploration of MZ twins at birth revealed monochorionic—shared placenta—twins to be more discordant in terms of DNA methylation than dichorionic—non-shared placenta—twins (Gordon et al., 2012); additionally, an independent study in adolescent subjects revealed these differences to be maintained in buccal epithelial cells (Kaminsky et al., 2009). These effects have been hypothesized to arise from the struggle for resources in co-twins that share placenta. Consequently, epigenetic exploration of birth samples from monochorionic monozygotic twins could help unravel how prenatal stress gets biologically embedded to later give rise to psychopathological outcomes as postulated by the DOHaD hypothesis (section 2.2.1).

As a matter of fact, around 30% of all MZ pregnancies result in specific pregnancy complications; two of the most common are: (i) selective intrauterine growth restriction (sIUGR), and (ii) twin-to-twin transfusion syndrome (TTTS). Both conditions are associated with increased mortality and morbidity of exposed individuals including lower birthweight, premature birth, and cardiac failure, among others.

TTTS consists in the existence of intrauterine vascular anastomoses between MZ twins leading to an unequal blood supply (**Figure 17**). These communications decompensate blood flow between twins giving rise to a donor and a recipient twin. Briefly, the donor twin becomes hypovolemic while the recipient twin suffers hypervolemia (Bebbington, 2010). TTTS is particularly severe and both the donor and the recipient conditions are lethal in the absence of prenatal surgery (Chalouhi et al., 2010; Rossi and D'Addario, 2008). In this regard, laser photocoagulation of vascular anastomoses between co-twins is the current procedure of choice to treat TTTS (Khalek et al., 2013).

Meanwhile, sIUGR arises from asymmetric placental distribution in monochorionic (MC) twin pregnancies (**Figure 18**). Specifically, it is diagnosed when one of the co-twins has an estimated fetal weight under the 10th percentile (Valsky et al., 2010). As opposed to TTTS cases, sIUGR is maintained alongside the whole pregnancy due to the lack of intrauterine treatment options. Actually, the growth discordance is present at birth with both twins of a pair exhibiting significant weight differences.

Birthweight (BW) has been reliably associated with long-term complex traits such as all-cause mortality, overweight and obesity, and coronary heart disease (Belbasis et al., 2016). Nevertheless, the association between BW and psychiatric conditions remains controversial; a recent meta-analysis of the relationship between birthweight and depression concluded there is only weak evidence to support this association (Wojcik et al., 2013). Twin studies might aid to unravel the actual risk conferred by non-genetically determined low birthweight; accordingly, a MZ twin approach further supported lack of association between BW and anxious-depressive disorders



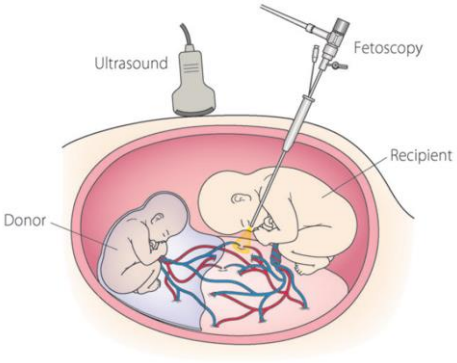


Figure 17. Twint-to-twin transfusion syndrome. **(Left)** A monochorionic twin placenta following vascular injection studies, demonstrating multiple vascular anastomoses, including both clear arterioarterial and arteriovenous communications. Reproduced with permission from: Weber M & Sebire N. Genetics and developmental pathology of twinning. *Seminars in Fetal & Neonatal Medicine* 15: 313-318 (2010). **(Right)** Schematic representation of fetoscopic laser photocoagulation in a TTTS pregnancy. Reproduced with permission from Khalek N, Johnson M & Bebbington M. Fetoscopic laser therapy for twin-to-twin transfusion syndrome. *Seminars in pediatric surgery* 22 (1): 18-23 (2013).

(Córdova-Palomera et al., 2014). Nevertheless, intrauterine unique environment leading to BW differences has been associated with brain morphological alterations and cortical surface area (Raznahan et al., 2012).

Consequently, subjects previously exposed to sIUGR exhibit short and long-term alterations at both neurodevelopmental and cognitive levels. These effects have been hypothesized to rely in abnormal brain circuitry. In this regard, alterations in functional connectivity, neurobehavior and neurodevelopment have been described in both neonates and 1-year old toddlers who experienced IUGR (Batalle et al., 2016; Eixarch et al., 2016).

The key parameters used in routine clinical visits to assess the presence of either TTTS or sIUGR are measured by means of Doppler ultrasound. The pulsatility index (PI)—a hemodynamic parameter reflecting blood flow—of the umbilical artery (UA) can be used as a surrogate marker of placental vascular resistance, i.e. decreased blood supply to the fetus. Complementarily, a decreased middle cerebral artery (MCA) PI indicating increased blood flow to the fetal brain can be used as a proxy of fetal hypoxia; the organism prioritizes the most hypoxia-compromised organ: the brain. The ratio between both UA and MCA PIs, cerebroplacental ratio (CPR), is widely used in clinical settings as a good estimator of fetal well-being and perinatal morbidity (DeVore, 2015).

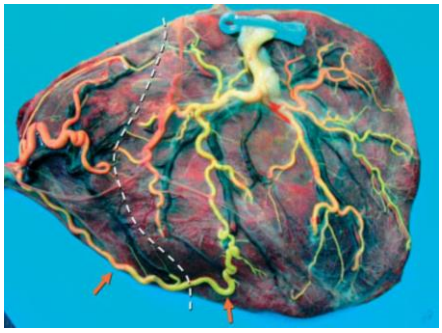


Figure 18. A monochorionic placenta of a type III sIUGR. The dotted white line indicates the vascular equator. Note the small placental area of the IUGR fetus (left). Reproduced with permission from: Valsky D, Eixarch E, Martinez J & Gratacós E. Prenatal Diagnosis 30: 719-726 (2010).

4.4. Twin epigenetics

In the context of epigenetic research, MZ twins are instrumental not only for the identification of biomarkers and risk factors but also for a better understanding of epigenetic dynamics in humans. For instance, MZ twins have been described to grow epigenetically apart; as they get older they accumulate epigenetic intrapair differences (Fraga et al., 2005). Such findings revealed the plasticity of epigenetic mechanisms and their responsiveness to environmental agents. As shown in **Figure 19**, a methylomic approach developed in MZ and DZ twins revealed the impact of A, C and E variance components and analyzed their association with methylation variability (Hannon et al., 2018b).

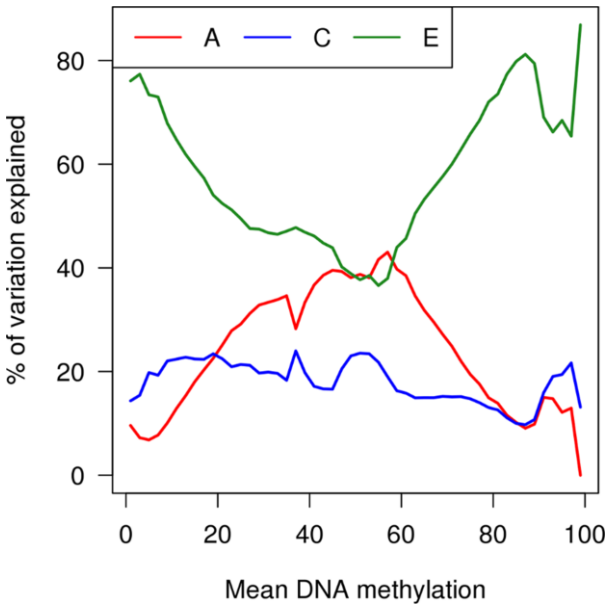


Figure 19. The contribution of genetic and environmental influences on DNA methylation differs as a function of average DNA methylation level at that location. The most heritable sites are characterized by intermediate levels of DNA methylation. Reproduced from Hannon E et al. Characterizing genetic and environmental influences on variable DNA methylation using monozygotic and dizygotic twins. PLOS Genetics 14(8): e1007544 (2018) under the terms of the Creative Commons Attribution 4.0 International (https://creativecommons.org/licenses/by/4.0/).

Remarkably, a recent study sought to determine whether epigenetic similarity observed in MZ twins was due to either genetic or environmental influences. Surprisingly, heritability values greater than 1 were found for several CpG probes revealing the so-called epigenetic supersimilarity of MZ twins (Van Baak et al., 2018). These unprecedented findings were complemented by a wide array of analyses finally demonstrating (i) temporal metastability of such epialleles and (ii) the potential of the periconceptional environment to induce epigenetic changes prior to the embryo cleavage into MZ twins. This phenomenon is a most likely contributor of missing heritability and a contributor of the inflated heritability estimates obtained in twin studies when compared to family studies.

In this framework, MZ twin based designs constitute quasi-experimental studies where the role of genetic and environmental factors can be dissected. Specifically, MZ twins are ideal for the exploration of the epigenetic landscape and its involvement in the etiology of complex traits and disorders. A better definition and development of tools to assess and measure the environment remain a current research challenge.

HYPOTHESIS AND OBJECTIVES

General hypothesis:

Epigenetic mechanisms might underlie anxious-depressive liability and neurodevelopmental consequences arising after exposure to stress.

Particularly, three specific hypotheses were proposed:

(i) DNA methylation at candidate genes of interest for stress responsivity—the glucocorticoid receptor gene and the serotonin transporter gene—could explain differential vulnerability to anxious-depressive disorders and related traits.

(ii) Epigenetic stochasticity, as measured by DNA methylation outliers, could also be involved in the pathophysiology of anxious-depressive disorders.

(iii) The fetal epigenome might be modified in response to prenatal insults, such as placental insufficiency or hypoxia, and alter neurodevelopmental trajectories of relevance for mental disorders.

Specific objectives

To address the first specific hypothesis (i), five different objectives were developed in five independent manuscripts:

(1) To systematically review all scientific papers analyzing the association between human *NR3C1* methylation and both exposure to stress and risk for stress-related disorders.

(2) To meta-analyze the impact of maternal psychosocial stress as experienced during pregnancy upon newborn *NR3C1* methylation profile.

(3) To explore DNA methylation at a novel promoter region of the *NR3C1* gene, and its association with (a) anxious-depressive disorders and (b) hippocampal connectivity, in a sample of adult monozygotic twins from the general population affected and non-affected by anxious-depressive disorders, with availability for fMRIs.

(4) To systematically review all scientific papers analyzing the impact of *SLC6A4* methylation in response to early life stress and in conferring risk for psychiatric conditions, and the role of 5-HTTLPR in such associations.

(5) To explore *SLC6A4* methylation with regard to internalizing symptoms dimensions in a sample of adult monozygotic twins from the

general population affected by anxious-depressive categorical diagnoses.

In order to test the second specific hypothesis (ii), a sixth objective was developed in another manuscript:

(6) To analyze the contribution of stochastic epigenetic signatures in the risk for anxious-depressive liability in a sample of adult monozygotic twins discordant for such psychiatric conditions.

Finally, the third specific hypothesis (iii) was tested through the last objective in a seventh manuscript:

(7) To identify DNA methylation signatures arising from exposure to prenatal stress measured in a sample of newborn monochorionic twins enriched for severe obstetric complications.

SUPERVISOR'S REPORT ON IMPACT FACTOR

Supervisor's Report on Impact Factor

The doctoral thesis "From prenatal stress to adult psychopathology: an epigenetic view from a monozygotic twin approach" is based on the original results obtained by Helena Palma Gudiel. These results are based on the epigenetic analysis in i) a sample encompassing adult Spanish monozygotic twins from the general population exhaustively researched in terms of psychopathology and risk factors; and ii) a sample comprised of Spanish monozygotic twin newborns thoroughly examined with regard to obstetric complications.

Complementarily, this doctoral thesis also comprises several systematic reviews and meta-analysis performed by Helena Palma Gudiel which aided the construction of a conceptual framework to develop the subsequent empirical research.

These results and reviews have been published or have been submitted to international peer reviewer journals. The impact factors of these journals demonstrate the quality of the research conducted, and are as following:

1. **Glucocorticoid receptor gene (*NR3C1*) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review**, published in *Neuroscience and Biobehavioral Reviews*. This journal publishes review articles which are original and significant and deal with all aspects of neuroscience, where the relationship to the study of psychological processes and behavior is clearly established. This journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 8.037 and classified in the first decile of the area of Neurosciences (ranking: 17/261). The scientific value and interest of this systematic review is further corroborated by the high number of citations it has received; 78 as of 13th December 2018.

2. **Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: A meta-analysis**, published in *Epigenetics*. This journal published peer-reviewed original research and review articles that provide an unprecedented forum where epigenetic mechanisms and

their role in diverse biological processes can be revealed, shared and discussed. This journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 5.167 and classified in the first quartile of the area of Genetics & Heredity (ranking: 30/171). The scientific value and interest of this meta-analysis is further corroborated by the high number of citations it has received; 56 as of 13th December 2018.

3. **Increased methylation at an unexplored glucocorticoid responsive element within exon 1_D of *NR3C1* gene is related to anxious-depressive disorders and decreased hippocampal connectivity**, published in *European Neuropsychopharmacology*. This journal focuses on clinical and basic science contributions that advance our understanding of brain function and human behavior and enable translation into improved treatments and enhanced public health impact in psychiatry. This journal is indexed in the Journal Citation Reports (Science Edition) with a current impact factor of 4.129 and classified in the first quartile of the area of Psychiatry (ranking: 29/142).

4. **An integrative of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR**, published in *Neuroscience and Biobehavioral Reviews*. This journal publishes review articles which are original and significant and deal with all aspects of neuroscience, where the relationship to the study of psychological processes and behavior is clearly established. This journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 8.037 and classified in the first decile of the area of Neurosciences (ranking: 17/261).

5. **Epigenetics-by-sex interaction for somatization conferred by methylation at the promoter region of *SLC6A4* gene**, published in *Progress in Neuropsychopharmacology and Biological Psychiatry*. This international and multidisciplinary journal aims to ensure the rapid publication of authoritative reviews and research papers dealing with experimental and clinical aspects of neuro-

psychopharmacology and biological psychiatry. This journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 4.185 and is classified in the first quartile of the area of Psychiatry (ranking: 28/142).

6. **Epigenetic outlier profiles in depression: A genome-wide DNA methylation analysis of monozygotic twins**, accepted for publication (in press) in *PLOS ONE*. This was the world's first multidisciplinary Open Access journal, it accepts scientifically rigorous research, regardless of novelty. Its broad scope provides a platform to publish primary research, including interdisciplinary and replication studies as well as negative results. This journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 2.766 and is classified in the first quartile of the area of Multidisciplinary Sciences (ranking: 15/64).

7. **Prenatal suffering is associated with epigenetic age deceleration and differential methylation in hypoxia-responsive *EP300* gene**, currently submitted to *Clinical Epigenetics*. This open access journal is the official journal of the Clinical Epigenetics Society, it encompasses all aspects of epigenetic principles and mechanisms in relation to human disease, diagnosis and therapy. The journal is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 6.091 and is classified in the first quartile of the area of Oncology (ranking: 37/223).

Accordingly, I confirm the quality of the published and submitted articles.

Signed by Prof. Lourdes Fañanás

Barcelona, 13th December 2018

PUBLICATIONS

Glucocorticoid receptor gene (*NR3C1*) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review.

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Review

Glucocorticoid receptor gene (*NR3C1*) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review

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ABSTRACT

Early life stress (ELS) is a known risk factor for suffering psychopathology in adulthood. The hypothalamic–pituitary–adrenal (HPA) axis has been described to be deregulated in both individuals who experienced early psychosocial stress and in patients with a wide range of psychiatric disorders. The *NR3C1* gene codes for the glucocorticoid receptor, a key element involved in several steps of HPA axis modulation. In this review, we gather existing evidence linking *NR3C1* methylation pattern with either ELS or psychopathology. We summarize that several types of ELS have been frequently associated with *NR3C1* hypermethylation whereas hypomethylation has been continuously found to be associated with post-traumatic stress disorder. In light of the reported findings, the main concerns of ongoing research in this field are the lack of methodological consensus and selection of CpG sites. Further studies should target individual CpG site methylation assessment focusing in biologically relevant areas such as transcription factor binding regions whereas widening the examined sequence in order to include all non-coding first exons of the *NR3C1* gene in the analysis.

Contents

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1. Introduction

Mental disorders affect around one third of Europe's population and cause the greatest effect in terms of disability-adjusted life-years (DALYs) (Kaplan and Laing, 2004). Anxious-depressive disorders have particularly high prevalence ratios as well as the highest associated risk of suicide (for a review, see Miret et al., 2013). The lifetime prevalence of major depression disorders (MDD) ranges from 1% to 29.9% depending on biological, geographical and sociocultural variables (Kessler and Bromet, 2013; Kessler et al., 2012). The lifetime prevalence for anxiety disorders is around 30% and its lifetime morbid risk is estimated to be as much as 41.7% in the US (Kessler et al., 2012).

In contrast, although post-traumatic stress disorder (PTSD) has a moderately low prevalence in developed countries (around 1%), its incidence increases dramatically in subjects exposed to a number of severe traumatic experiences (for a review, see Keane et al., 2006). Indeed, PTSD patients must have experienced recent severe trauma in order to meet the recent DSM5 criteria (American Psychiatric Association, 2013). Finally, borderline personality disorder (BPD) affects 0.5–5% of the general population; many authors have reported its association with childhood maltreatment, especially sexual abuse (Leichsenring et al., 2011).

Epidemiological studies have reported that psychosocially stressful events are a necessary triggering factor for the adult onset of an extremely high percentage of the aforementioned mental disorders, which can be referred as "stress-related disorders" (for a review, see Slavich et al., 2010). Of particular interest is the extensive scientific literature on the major causal role of early adversity in the sensitization to adult psychopathology, with special emphasis on the impact of childhood maltreatment on vulnerability to stress-related disorders in adulthood (for reviews of this topic, see Carr et al., 2013; Strüber et al., 2014; Teicher et al., 2003).

Childhood maltreatment prevalence ratios are estimated to be as high as anything from 4% right up to 16% in developed countries, depending on the type of abuse (Gilbert et al., 2009); it thus constitutes a major health concern in developed societies.

The long latency between early exposure to environmental risk factors and the late onset of a pathological status is widely observed in several complex disorders such as cancer, metabolic diseases and psychiatric conditions. Early life events, such as prenatal stress or childhood adversity, have the potential to modify later vulnerability to complex disorders due to *developmental plasticity*, i.e., the ability to develop in various ways depending on the early environment allows organisms to adapt. This evolutionary competence is probably mediated at least in part by epigenetic mechanisms such as histone modifications and DNA methylation (Gluckman et al., 2008; Petronis, 2010; Ptak and Petronis, 2010).

The hippocampus is a brain area that is crucial in the modulation of the hypothalamic–pituitary–adrenal (HPA) axis: our primary stress response system. Alterations in both hippocampal volume and functionality have been associated with psychiatric disorders, especially with major depression (Anacker et al., 2011). Indeed, prenatal and childhood trauma can modulate the HPA axis (Ehlert, 2013). Briefly, upon exposure to stress, the paraventricular nucleus of the hypothalamus activates and secretes corticotropin-releasing hormone (CRH) which promotes the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary to the adrenal glands, which finally release glucocorticoids. In humans, cortisol distributes systemically and executes a wide range of functions involving the immune, digestive and endocrine systems, including HPA axis self-regulation. As a lipophilic molecule, cortisol crosses the cellular membrane by passive diffusion and binds to the cytoplasmic mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR has a higher affinity for glucocorticoids than GR; thus, GR activation is key for an individual to appropriately cope with stress.

Once bound to cortisol, GR can translocate to the nucleus, where it acts both as a transcription factor and as a repressor. This coupling enables the dissociation of a chaperone complex, which is preventing nuclear translocation of GR in the absence of glucocorticoids. Interestingly, both epigenetic modifications and genetic variability of a certain co-chaperone of this complex, FKBP5, have been associated with stress-related disorders, specifically PTSD (Binder, 2009; Klengel et al., 2013).

The human glucocorticoid receptor is encoded by the *NR3C1* gene, spanning almost one half mega base pairs of chromosome 5. It contains 17 exons, nine of them are non-coding exons located at the gene promoter (Fig. 1). Seven of these non-coding exons are clustered along the same CpG island (see Fig. 2 for the complete sequence).

A decade ago, Weaver et al. (2004) successfully found in rodents an epigenetic modification of the *NR3C1* gene that was indicative of differential maternal rearing behavior. Specifically, rat pups raised by mothers that exhibited more nursing behavior exhibited remarkable hypomethylation at a specific CpG site located at an NGFI-A binding site; which suggested it was involved in transcription modulation.

Based on prior evidence from animal studies, over the last decade, a significant number of authors have focused their research on the epigenetic modulation of the *NR3C1* gene in humans in association with early stress, as well as with additional variables such as stress reactivity and different psychopathological conditions. DNA methylation has been explored in a myriad of tissues and clinical profiles, including both peripheral tissues (blood and saliva) and central nervous system samples from patients as well as controls.

Nevertheless, many concerns regarding particular epigenetic modifications of the *NR3C1* gene, the relevance and nature of early stressors and the specific clinical profile associated with these variables, remain to be elucidated.

Within this framework, the main goals of this review are: (i) to ascertain which methodology should be used to assess DNA methylation in the *NR3C1* gene; (ii) to determine which CpG sites within the *NR3C1* gene are definitely relevant for the etiology of stress-related disorders, according to published data; and (iii) to discuss the rationale driving current research on this topic in order to address future analysis of subjects suffering from stress-related disorders.

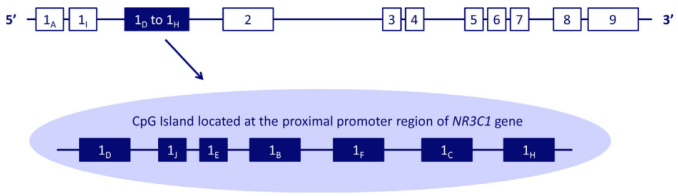

Fig. 1. *NR3C1* gene structure. The *NR3C1* gene consists of eight coding exons numbered 2–9 and nine non-coding first exons referred to as A–J (excluding "G") which are thought to act as alternate promoters. Exons 1_D, 1_J, 1_E, 1_B, 1_F, 1_C and 1_H are located within a CpG island spanning 3 kb along the proximal promoter region of *NR3C1* gene. The disarrayed numbering is due to the staggered discovery of the exons, which occurred at three different times. CpG dinucleotides with differential methylation may be located all along the *NR3C1* gene sequence but epigenetic research focuses on the only CpG island described in this gene, which is located at a promoter region.

2. Methodology

2.1. Selection criteria

Research was conducted via a systematic literature search in the PubMed, PsycINFO and Web of Science databases. The search terms were the following: "*NR3C1* or *glucocorticoid receptor*", "*methylation*" and "*stress* or *childhood adversity* or *childhood abuse* or *maternal disorder*". The English language filter was activated, which excluded 3 of the 103 papers that the defined search retrieved. We then excluded from the resulting pool: all papers reporting research on animals ($n = 37$); and papers which did not report original research ($n = 30$). To be considered suitable for the present review, the papers needed to include either an early or a late stress measure in association with a thorough methylation assessment of a CpG island located at the *NR3C1* gene promoter (Figs. 1 and 2). The final set consisted of 23 papers to be reviewed (Table 1).

2.2. Brief introduction to the assessment instruments and scales for measuring psychopathology and experienced adversity

Psychiatric diagnoses need to rely on instruments and scales that measure symptoms, since there is no known biomarker for any mental disorder. The Diagnostic and Statistical Manual of Mental Disorders (DSM) contains the reference criteria for diagnosing psychiatric conditions and it is widely used in both health care and

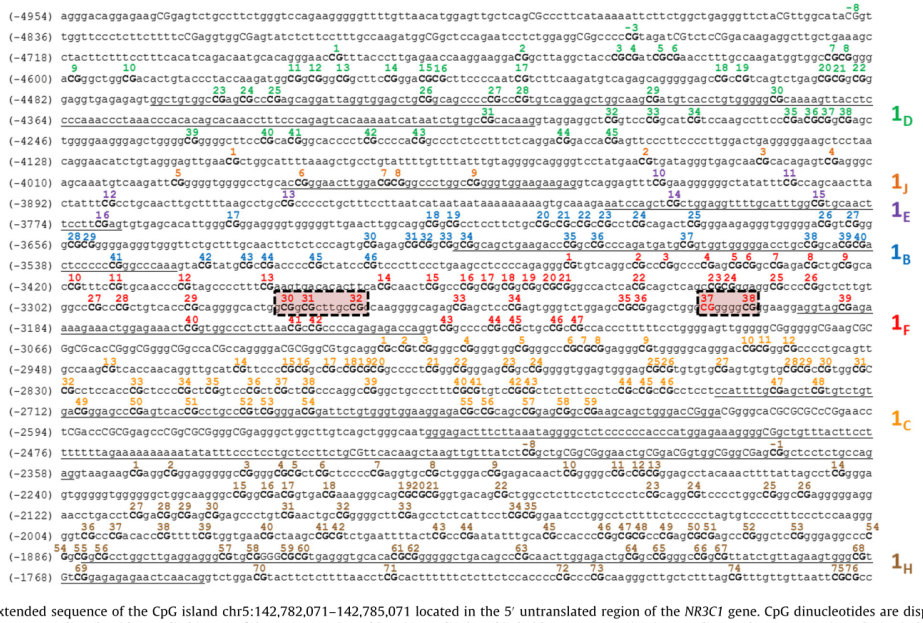

Fig. 2. Extended sequence of the CpG island chr5:142,782,071–142,785,071 located in the 5′ untranslated region of the *NR3C1* gene. CpG dinucleotides are displayed in capital letters. CpG dinucleotides studied in any of the papers reviewed herein are displayed in bold. CpG numeration is according to the papers reviewed. Five independent numbering systems are included in order to adjust to previous research: (a) 1–45 referring to the region 1D (colored in green); (b) 1–46 covering the regions 1J (colored in dark orange), 1E (colored in purple) and 1B (colored in blue), due to their proximity; (c) 1–47 as placed in region 1F (colored in red); (d) 1–59 located in region 1C (colored in light orange); and (e) 1–76 including the region1H (colored in brown). Negative numeration in three individual CpG sites corresponds with dinucleotides assayed in the Illumina BeadChip which were not included in the analysis performed by the other authors. Underlined sequences correspond to exons as annotated by Turner and Muller (2005) and Presul et al. (2007). Parenthetical numbering is of gene locations with respect to the translation start site within exon 2. Dashed boxes delineate NGFI-A binding regions. Red has been used to mark 1F CpG #37, as it is the most widely reported in the literature, in both animal and human studies.

Table 1
Summary of studies analyzing *NR3C1* methylation as associated to both psychosocial stress and psychopathological condition.

Study	Sample size/characterization	Tissue	Analyzed CpG sites	Methylation assessment	Expression	Stress measure	Epigenetic findings	Extent of the reported methylation differences	Correlated biomarkers (i.a., cortisol measurements)
Oberlander et al. (2008)	*n* = 82 mother-child dyads 33 depressed and treated mothers 13 depressed and not treated mothers 36 controls	Cord blood	Exon 1_F (and partial promoter 1_F) 13 CpG sites analyzed	Pyrosequencing	No	Prenatal stress (mood disorder during pregnancy) and infant stress challenge	Maternal depression severity was associated with neonatal 1_F promoter methylation.	<5%	CpG specific methylation predicted HPA reactivity
McGowan et al. (2009)	*n* = 36 deceased donors 12 suicide victims with history of childhood abuse 12 suicide victims without history of childhood abuse 12 controls without history of childhood abuse	Brain (hippocampus)	Promoter 1_F 38 CpG sites analyzed	Sodium bisulfite mapping, cloning and Sanger sequencing	Yes	Childhood adversity (CECA adapted for psychological autopsies)	History of childhood abuse was associated with higher methylation at the promoter 1_F. Total GR mRNA expression was decreased in abused suicide victims.	<20% >30% (in CpG 32)	–
Radtke et al. (2011)	*n* = 25 mother-child dyads 8 women suffered IPV during pregnancy	Peripheral blood (whole blood)	Promoter 1_F 10 CpG sites analyzed	Sodium bisulfite mapping, cloning and Sanger sequencing	No	Prenatal stress (mother's exposure to intimate partner violence)	The presence of methylated residues at promoter 1_F was associated with maternal exposure to IPV during pregnancy.	<5%	–
Perroud et al. (2011)	*n* = 215 101 BPD subjects 99 MDD subjects without past/current PTSD 15 MDD subjects with past/current PTSD	Peripheral blood (leukocytes)	Exon 1_F 8 CpG sites analyzed	Pyrosequencing	No	Childhood adversity (CTQ)	Exon 1_F methylation correlated with abuse severity and number of maltreatment types.	<5%	–
De Rooij et al. (2012)	*n* = 675 healthy subjects	Peripheral blood (whole blood)	Exon 1_C amplicon included 30 CpG sites	Methylation sensitive PCR assay	No	Stress reactivity (psychological stress protocol)	An adverse lifestyle was associated with lower methylation of GR 1_C promoter.	NA Mean methylation range = 0.064	Lower methylation was associated with decreased stress reactivity

Table 1 (*Continued*)

Study	Sample size/characterization	Tissue	Analyzed CpG sites	Methylation assessment	Expression	Stress measure	Epigenetic findings	Extent of the reported methylation differences	Correlated biomarkers (i.a., cortisol measurements)
Tyrka et al. (2012)	*n* = 99 healthy subjects	Peripheral blood (whole blood)	Exon 1_F (and partial promoter 1_F) 13 CpG sites analyzed	Pyrosequencing	No	Childhood adversity (CTQ, Childhood Parental Loss and Parental Bonding Index)	Childhood adversity was associated with promoter 1_F methylation.	<5%	CpG specific methylation was negatively associated with cortisol response after Dex/CRH test
Labonté et al. (2012)	*n* = 56 deceased donors 21 suicide victims with history of childhood abuse 21 suicide victims without history of childhood abuse 14 controls	Brain (hippocampus)	Promoters 1_B, 1_C and 1_H 43 CpG sites analyzed	Pyrosequencing	Yes	Childhood adversity (CECA adapted for psychological autopsies)	Relative expression levels of total GR, $GR1_B$, $GR1_C$ and $GR1_H$ were decreased in abused suicides. Methylation at specific CpG sites of 1_B and 1_C promoters negatively correlated with expression. 1_B and 1_C specific CpG site methylation was associated with suicide completion and history of childhood abuse. 1_H promoter showed hypomethylation in abused suicides.	<5%	–
Mulligan et al. (2012)	*n* = 25 mother-newborn dyads Sample native to Democratic Republic of Congo (common exposure to war and violence against women)	Cord blood	Promoter 1_F 39 CpG sites analyzed	Sodium bisulfite mapping, cloning and sequencing	No	Prenatal stress (maternal exposure to war during pregnancy)	Mother's exposure to stress during pregnancy correlated with newborn methylation at promoter 1_F, which inversely correlated to birth weight.	NA	–
Edelman et al. (2012)	*n* = 92 healthy subjects 50% female	Saliva	Promoter 1_F 39 CpG sites analyzed	Pyrosequencing	No	Stress reactivity (TSST)	Women showed greater methylation at promoter 1_F than men.	<5%	1_F methylation was correlated to stress reactivity in women
Melas et al. (2013)	*n* = 176 women 93 depressed 83 controls	Saliva	Promoter and exon 1_F 47 CpG sites analyzed	Sequenom EpiTYPER platform (MALDI-TOF mass spectrometry)	No	Childhood adversity (childhood parental loss)	EPD was associated with 1_F CpG-specific hypermethylation.	<10%	–
Hompes et al. (2013)	*n* = 83 mother-child dyads	Cord blood	Promoters 1_D, 1_B and 1_F 94 CpG sites analyzed	Sequenom EpiTYPER platform (MALDI-TOF mass spectrometry)	No	Prenatal stress (mood disorder during pregnancy)	PRAQ scores were correlated to 1_D and 1_F methylation.	NA	–

Hogg et al. (2013)	*n* = 161 mother-child dyads 19 early onset pre-eclampsia 18 late onset pre-eclampsia 13 normotensive intrauterine growth restriction 111 controls	Placenta	37 CpG sites distributed all across alternate non-coding *NR3C1* exons	Illumina 450K Methylation BeadChip plus additional pyrosequencing for further verifying array findings	Yes	Prenatal stress (diagnosis of pre-eclampsia)	1_D CpG-specific hypermethylation was found in pre-eclampsia placentae. The expression of *NR3C1* mRNA was not altered in pre-eclampsia cases. Glucocorticoid signaling and steroidogenic pathway genes are differentially methylated in pre-eclampsia.	<10%	–
Conradt et al. (2013)	*n* = 482 mother-child dyads 27 depressed mothers 18 anxious mothers 39 mothers with comorbid depression and anxiety 482 controls	Placenta	Exon 1_F (and partial promoter 1_F) 13 CpG sites analyzed	Pyrosequencing	No	Prenatal stress (mood disorder during pregnancy)	Maternal depression during pregnancy was associated with newborn's 1_F CpG-specific hypermethylation, which correlated to several neurobehavioral variables. Anxiety during pregnancy was associated with newborn's *11β-HSD-2* specific CpG site methylation, which correlated with newborn hypotonia.	<5%	–
Steiger et al. (2013)	*n* = 96 32 BN women with childhood abuse 32 BN women without childhood abuse 32 controls Within BN sample: 14 subjects with comorbid BPD and 14 subjects with history of suicidality	Peripheral blood (whole blood)	Promoters 1_B, 1_F, 1_C and 1_H 195 CpG sites analyzed	Sequenom EpiTYPER platform (MALDI-TOF mass spectrometry)	No	Childhood adversity (Childhood Trauma Interview)	BN subjects with comorbid BPD showed higher methylation at specific CpG sites of 1_C promoter and lower methylation at 1_H promoter. BN subjects with previous history of suicidality showed elevated methylation at specific CpG sites of 1_C promoter.	<5%	–
Labonté et al. (2014)	*n* = 43 27 PTSD subjects 16 never exposed to trauma controls	Peripheral blood (T lymphocytes)	Promoters 1_B and 1_C 83 CpG sites analyzed	Sequenom EpiTYPER platform (MALDI-TOF mass spectrometry)	Yes	Childhood and adulthood adversity (an index of traumatic events)	PTSD subjects showed higher 1_B and 1_C expression together with lower methylation.	<5% (overall percentage across the region)	PTSD subjects showed lower levels of basal cortisol correlating with 1_B methylation

Table 1 (*Continued*)

Study	Sample size/characterization	Tissue	Analyzed CpG sites	Methylation assessment	Expression	Stress measure	Epigenetic findings	Extent of the reported methylation differences	Correlated biomarkers (i.a., cortisol measurements)
Weder et al. (2014)	*n* = 190 94 maltreated children 96 controls	Saliva	41 CpG sites distributed all across alternate non-coding *NR3C1* exons	Illumina 450K Methylation BeadChip	No	Childhood adversity (child protective services reports; CTQ; mother reports of domestic violence)	Maltreated children showed differential CpG-specific methylation at the *NR3C1* locus. *FKBP5* methylation was also associated to maltreatment.	NA	Specific CpG site methylation predicted morning cortisol values
Yehuda et al. (2014)	*n* = 122 61 PTSD subjects 61 controls	Peripheral blood (PBMC)	Promoter 1_F 39 CpG sites analyzed	Sodium bisulfite mapping, cloning and sequencing	No	Childhood adversity (Early Trauma Inventory)	PTSD subjects showed lower methylation. Promoter 1_F methylation negatively correlated to clinical severity.	<5%	PTSD subjects showed greater GR sensitivity; methylation negatively correlated with cortisol decline
Perroud et al. (2014)	*n* = 50 mother-child dyads 25 widows pregnant during the Rwanda genocide 25 controls	Peripheral blood (leukocytes)	Exon 1_F 10 CpG sites analyzed	Pyrosequencing	Yes	Prenatal stress (maternal exposure to genocide during pregnancy)	Exposed mothers and their children showed higher 1_F methylation and lower GR levels. Methylation negatively correlated to expression.	<15%	Exposed mothers and their children exhibited lower basal cortisol levels
Van der Knaap et al. (2014)	*n* = 468 adolescents informative for perinatal stress and stressful life events experienced either in childhood or adolescence	Peripheral blood (whole blood)	Promoters 1_D, 1_F and 1_H 108 CpG sites analyzed	Sequenom EpiTYPER platform (MALDI-TOF mass spectrometry)	No	Prenatal stress (preterm delivery, low birth weight, maternal alcohol use or smoking during pregnancy, etc.); Childhood adversity (a list of stressful life events experienced between the ages 0 and 15 years; the number of traumatic youth experiences before the age of 16)	Exposure to stress either in childhood or adolescence was associated with higher methylation in 1_F and 1_H regions.	NA	–

Stroud et al. (2014)	$n = 100$ mother-child dyads 53 smokers during pregnancy	Placenta	Exon 1_F (and partial promoter 1_F) 13 CpG sites analyzed	Pyrosequencing	No	Prenatal stress (maternal smoking during pregnancy) and newborn stress reactivity (NNNS)	Maternal smoking during pregnancy negatively correlated with newborn 1_F specific CpG site methylation.	<5%	Maternal smoking during pregnancy was associated with attenuated baseline cortisol and cortisol reactivity over the first postnatal month
Martín-Blanco et al. (2014)	$n = 281$ BPD subjects	Peripheral blood (leukocytes)	Exon 1_F 8 CpG sites analyzed	Pyrosequencing	No	Childhood adversity (CTQ)	1_F exon methylation correlated with both childhood maltreatment and clinical severity.	NA	–
Romens et al. (2014)	$n = 56$ children 18 physically maltreated children 38 controls	Peripheral blood (whole blood)	Exon 1_F (and partial promoter 1_F) 13 CpG sites analyzed	Pyrosequencing	No	Childhood adversity (records reporting childhood physical maltreatment)	History of maltreatment correlated with methylation at specific CpG sites of exon 1_F.	<10%	–
Vukojevic et al. (2014)	$n = 152$ survivors from the 1994 Rwandan genocide 93 PTSD subjects 59 controls	Saliva	Exon 1_F 8 CpG sites analyzed	Pyrosequencing	Yes	Childhood and adolescent adversity (checklist of 36 traumatic event types)	1_F CpG-specific methylation negatively correlated to both symptom severity and GR expression (in saliva).	NA	Specific CpG site methylation correlated with brain activity and memory performance, only in men

Abbreviations: BN, bulimia nervosa; BPD, borderline personality disorder; CTQ, childhood trauma questionnaire; EPD, early parental death; IPV, intimate partner violence; MDD, major depressive disorder; NA, non-available; NNNS, NICU network neurobehavioral scale; PBMC, peripheral blood mononuclear cell; PRAQ, pregnancy related anxiety questionnaire; PTSD, post-traumatic stress disorder; TSST, Trier social stress yest.
Extended results and further discussion can be found in Section 3.

research. Additionally, there is a number of scales designed for characterize specific disorders and/or target populations, which have been utilized in the studies analyzed in this review such as: (i) Edinburgh Depression Scale (EDS); (ii) Hamilton rating scale for anxiety (HAM-A); (iii) Hamilton rating scale for depression (HAM-D); and (iv) Pregnancy Related Anxiety Questionnaire (PRAQ).

Early life stress (ELS) can be also measured by a number of scales. The Childhood Trauma Questionnaire (CTQ) is a validated instrument commonly used for research purposes which measures five types of childhood adversity: physical, sexual and emotional abuse and physical and emotional neglect. Papers reviewed include several measures of early adversity such as the Childhood Trauma Interview (CTI), the Early Trauma Inventory – Self Report (ETI-SR) and the Childhood Experience of Care and Abuse (CECA), among others.

Prenatal stress measures can be derived from Obstetrical Complications (OCs). None of the articles reviewed herein included a comprehensive examination of OCs but of specific maternal conditions that may be deleterious for fetal growth and adequate development such as mood disorders experienced during pregnancy and pre-eclampsia.

Finally, stress reactivity can be quantified by means of several challenges such as: (i) the Trier Social Stress Test (TSST), where cortisol levels are monitored in subjects exposed to psychosocially stressful situations; (ii) the dexamethasone suppression test (DST), where subjects are administered with a synthetic glucocorticoid, dexamethasone, in order to measure HPA axis functioning; and (iii) the combined dexamethasone/CRH (dex/CRH) challenge, where following dexamethasone administration, subjects also receive a CRH dose for a more sensitive measure of HPA axis activity.

2.3. NR3C1 gene and CpG island structure

The *NR3C1* gene codes for the glucocorticoid receptor and is located in the reverse strand of chromosome 5q31. It has 16 splice variants, 3 isoforms which are derived from 3′ splicing (GR-α, GR-β and GR-P) and 8 coding exons plus 9 alternate non-coding first exons which can be transcribed to the mRNA level but are not translated to any known protein. The first exons have different expression ratios along with distinct tissue distributions and their function remains widely unknown. In the 5′ untranslated region of the *NR3C1* gene, there is a CpG island spanning 3 kb located at chr5:142,782,071–142,785,071 (GRCh17/hg19 assembly) which includes seven of the aforementioned nine first exons: 1_D, 1_J, 1_E, 1_B, 1_F, 1_C (which has three splice variants) and 1_H. A schematic view of *NR3C1* gene structure can be seen in Fig. 1. In addition, a detailed view of the whole sequence of this CpG island is depicted in Fig. 2, where all of its individual CpG sites have been highlighted. NGFI-A binding sites within exon 1_F promoter are emphasized with red boxes as they constitute the main reported findings across the literature in both humans and rats.

3. Results and discussion

3.1. Variables assessed by papers reviewed

Table 1 summarizes the sample description, tissues analyzed, methodology used to assess DNA methylation, targeted regions of the *NR3C1* gene and stress variables considered in the 23 papers eligible for this review, along with their main findings. 21 papers analyzed some sort of ELS: 8 of them focused on prenatal stress, 12 examined childhood and/or adolescent trauma, while one additional paper took into account both prenatal stress and childhood adversity. Thirteen of the studies were performed on healthy subjects, while the remaining ten examined several psychiatric disorders including major depressive disorder (MDD), post-traumatic stress disorder (PTSD), borderline personality disorder (BPD), bulimia nervosa (BN) and suicide completion (SC). The samples analyzed included post-mortem brain tissue ($n = 2$), saliva ($n = 4$), cord blood and placenta ($n = 7$) and peripheral blood ($n = 10$), while one of the studies specifically examined T cells.

3.2. NR3C1 methylation, ELS and psychopathological condition

Seventeen of the 21 papers accounting for ELS found significant correlations between ELS exposure and DNA methylation at a number of CpG sites located at several of the alternate first exons of the *NR3C1* gene. The findings are reported below, sorted by region. The paradigmatic region encompassing exon and promoter 1_F will be the first to be reviewed.

3.2.1. NR3C1 gene – 1_F region

The region 1_F (including both promoter and exon) contains 47 CpG sites; 25 of them have been reported at least once as being differentially methylated. Although nearly all the research groups (20 out of 23) considered methylation of the *NR3C1* region 1_F, not all of them analyzed the same section (Table 2). As shown in Fig. 3, most researchers included the region immediately anterior to exon 1_F in their analyses; this genomic locus is particularly intriguing since it contains the NGFI-A binding region which was reported to be epigenetically modified by Weaver et al. (2004). Accordingly, methylation of this promoter region (comprising CpG sites from 35 to 37) has regularly been associated with both ELS and psychopathological condition.

3.2.1.1. 1_F region – CpG 35. CpG site 35 (also referred to as 1_F CpG 1 or *F13* depending on the study) has been reported to be differentially methylated by five research groups in association with several phenotypes: (i) prenatal stress as measured by maternal anxious-depressed condition during pregnancy (Hompes et al., 2013; Oberlander et al., 2008); (ii) early parental loss and low levels of parental care (Melas et al., 2013; Tyrka et al., 2012); and (iii) childhood maltreatment (Weder et al., 2014). Hypermethylation was always associated with early stress exposure, with the exception of the results of Weder et al., who did not report the direction of the effect.

3.2.1.2. 1_F region – CpG 36. 1_F CpG 36 (also referred to as 1_F CpG 2 or *F12*) newborn hypermethylation has been associated with maternal psychopathology during pregnancy in three separate studies, as assessed by clinical records of depression diagnosis, EDS, HAM-A, HAM-D and PRAQ fear of delivery and fear of integrity scores (Conradt et al., 2013; Hompes et al., 2013; Oberlander et al., 2008).

3.2.1.3. 1_F region – CpG 37. 1_F CpG 37 (also referred to as 1_F CpG 3 or *F11*) methylation has been associated with both ELS and psychopathology in humans by six distinct research groups. It corresponds to the paradigmatic CpG dinucleotide reported by Weaver et al. (2004) to be strikingly hypomethylated in rats which received higher maternal care as pups. Increased 1_F CpG 37 newborn methylation has been associated with maternal EDS and HAM-D scores during pregnancy (Oberlander et al., 2008). Similarly, early parental loss and childhood maltreatment were associated with hypermethylation at this CpG site in healthy adults (Romens et al., 2014; Tyrka et al., 2012). Finally, 1_F CpG 37 methylation was found to be decreased in PTSD subjects who exhibited higher severity of intrusive memories (Vukojevic et al., 2014), and in newborns either formerly exposed to maternal smoking during pregnancy (Stroud et al., 2014) or to a maternal anxious-depressed condition during pregnancy, as measured by EDS and PRAQ fear of integrity scores

Table 2
Main epigenetic findings in the region 1_F.

Region 1_F	Main findings
1–29	Isolated associations between methylation at particular CpG sites and early psychosocial stress (see Table S3 for further detail).
30–32	Putative NGFI-A binding region. Increased methylation at this region was associated with both prenatal stress and childhood abuse in three independent studies. One additional study reported decreased methylation in association with prenatal stress (see Table S1 for further detail).
33–34	Methylation at CpG sites 33 and 34 was not reported as associated with any stress or clinical variable in any of the papers reviewed.
35–38	NGFI-A binding region. CpG site 37 is homologous to rat's CpG site 16 as identified by Weaver et al. (2004) to be differentially methylated according to maternal behavior. Methylation at CpG sites 35–37 positively correlates to early stress exposure, as reported by six independent research groups. One additional study reported this association to be negative when analyzing a CpG unit which included CpG sites 37 and 38. Methylation negatively correlated to PTSD severity.
39–47	Methylation at this region positively correlates to early stress exposure as experienced either prenatally or during childhood. Altered methylation at CpG sites 40–47 has been reported in BPD samples (see Table S2 for further detail).

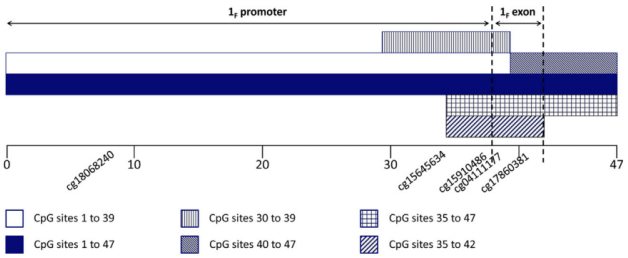

Fig. 3. CpG site distribution among exon and promoter 1_F. Template coded boxes correspond to regions analyzed, depending on research group. Some authors focused on the region immediately anterior to the 1_F exon (Radtke et al., 2011; Vukojevic et al., 2014), others centered on the exon itself and even posterior sections (Martín-Blanco et al., 2014; Perroud et al., 2014, 2011), a major group of authors took into account both regions (Conradt et al., 2013; Oberlander et al., 2008; Romens et al., 2014; Stroud et al., 2014; Tyrka et al., 2012), and still another group took into consideration a major region spanning the 1_F exon promoter (Edelman et al., 2012; McGowan et al., 2009; Mulligan et al., 2012; Van der Knaap et al., 2014; Yehuda et al., 2014) or the whole subdivision of the CpG island including all of the regions cited above (Hompes et al., 2013; Melas et al., 2013; Steiger et al., 2013). Both papers assessing methylation by means of the Illumina 450K BeadChip included data on 5 isolated CpG sites within the 1_F promoter and exon: cg18068240, cg15645634, cg15910486, cg04111177 and cg17860381, which correspond to CpG sites 9, 35, 38, 39 and 41 (as numbered in Fig. 2) respectively (Hogg et al., 2013; Weder et al., 2014).

(Hompes et al., 2013). CpG 37 methylation will be discussed below in association with secondary variables.

Remarkably, the results reported by Hompes et al. (2013) refer to the CpG units F12.13 and F10.11, which include CpG sites 35 and 36, and CpG sites 37 and 38, respectively.

3.2.1.4. 1_F region – CpG sites from 30 to 32. A second cluster of differentially methylated CpG sites consists of the CpG dinucleotides from 30 to 32 which are located at a putative non-canonical NGFI-A binding site (McGowan et al., 2009). Findings in this region are not always reported as CpG-specific but map to a small cluster of CpG dinucleotides (Table S1). Three out of four papers found higher levels of methylation in this region associated with either childhood abuse or prenatal stress.

3.2.1.5. 1_F region – CpG sites from 39 to 47. Thirdly, a region spanning CpG sites 39–47, which are located in the exonic 1_F sequence and beyond (toward the 1_C promoter), has also been widely reported as associated with several early stressor and psychopathological outcomes by a number of authors (Table S2). Two separate groups found associations between average methylation and either childhood maltreatment (Perroud et al., 2011) or clinical severity (Martín-Blanco et al., 2014) in BPD samples in a region encompassing 1_F CpG sites 40–47. One study reported significantly higher methylation percentages in a region spanning 1_F CpG sites 30–39 (Radtke et al., 2011). Tyrka et al. (2012) found a high intercorrelation between methylation at CpG sites from 41 to 47; that is to say, methylation at these CpG sites fluctuate in a coordinated fashion.

3.2.1.6. Prior 1_F regions – CpG sites 1–29. CpG-specific methylation findings in the region spanning CpG sites 1–29 are scarce (Table S3). Globally, increased methylation has been associated with several measures of early stress (Hompes et al., 2013; Van der Knaap et al., 2014).

Intriguingly, Edelman et al. found CpG site 12 to be differentially methylated in males and females; while its methylation was a significant predictor of cortisol output after the Trier Social Stress Test (TSST) only in females (Table 3), as discussed below in Section 3.4. These results may point to gender-specific regulation of the HPA axis driven by subtle epigenetic differences in the *NR3C1* gene. Accordingly, males and females respond very differently to a stress challenge (Edelman et al., 2012). Differential modulation of the stress response in males and females may be involved in the large dependence of the prevalence of psychiatric illnesses on gender.

PTSD continues to be associated with hypomethylation (Yehuda et al., 2014). CpG sites 12 and 13 are of special interest since they constitute a non-canonical NGFI-A binding site (McGowan et al., 2009).

3.2.1.7. Mean methylation of other 1_F subregions. Specific CpG data were not available in four papers that assessed 1_F methylation (Edelman et al., 2012; Mulligan et al., 2012; Radtke et al., 2011; Van der Knaap et al., 2014): the experience of ELS—such as maternal exposure to intimate partner violence (Radtke et al., 2011), war stress (Mulligan et al., 2012) during pregnancy or childhood experiences of sexual abuse (Van der Knaap et al., 2014)—was associated with average hypermethylation of 1_F regions (see Fig. 3 for a detailed description of assayed 1_F regions by paper). Furthermore, Perroud et al. (2011) performed most of their statistical analysis in relation to the mean methylation value and found associations between 1_F hypermethylation and both the severity and frequency of different types of childhood maltreatment (specially

Table 3
Correlations between peripheral cortisol measurements in either basal or stress-related conditions and *NR3C1* methylation.

Study	Methodology	Findings
Oberlander et al. (2008)	Saliva samples were collected at baseline and at post stress recovery following a stress challenge	Greater methylation at 1_F CpG 37 associates with greater HPA reactivity (increasing cortisol after the stress challenge)
De Rooij et al. (2012)[a]	Saliva samples were collected at 5 and 20 min during baseline, and in five occasions following several steps of a psychological stress protocol	1_C exon methylation was positively associated with stress reactivity cortisol levels (peak) and self-perceived stress variables: perceived performance and control. It was negatively associated with perceived stress.
Tyrka et al. (2012)	Dex/CRH test: following administration of dexamethasone and CRH, blood samples were collected at several time points for cortisol determination	Cortisol concentration negatively correlated to methylation at 1_F CpG sites 36, 39 and the mean of CpG sites 41–47
Edelman et al. (2012)	Trier Social Stress Test (TSST): salivary cortisol was sampled 8 times (twice before the test and six times after the stress task)	Only for women, average methylation of 1_F promoter (CpG sites 1–39) negatively correlated to salivary cortisol after the TSST
Labonté et al. (2014)	Basal cortisol levels were measured across daytime	Cortisol levels were lower in the PTSD group, and 1_B CpG-specific methylation positively correlated to cortisol levels
Weder et al., 2014	Basal cortisol levels were measured in the morning	CpG sites 1_F 39 and 1_C 11 predicted morning cortisol value
Yehuda et al. (2014)	DEX Suppression Test (DST) and 24-h urine collection	PTSD subjects exhibited a hypersuppression after DST together with lower urinary cortisol excretion; 1_F methylation negatively correlated to cortisol decline
Perroud et al. (2014)	Basal cortisol levels were measured in the morning	Exposed mothers and their children exhibited lower cortisol levels; 1_F methylation negatively correlated to cortisol levels
Stroud et al. (2014)	Cortisol stress response was elicited using the NICU Network Neurobehavioral Scale (NNNS)	MSDP-exposed children exhibited attenuated baseline cortisol and cortisol reactivity together with 1_F CpG 37 decreased methylation

[a] Significance fades when adjusting for sex, educational level, smoking and physical activity suggesting that these variables may be driving methylation differences or stress reactivity outcomes.

sexual abuse and physical neglect). Additionally, Yehuda et al. (2014) reported mean hypomethylation of 1_F promoter in PTSD subjects. In contrast to the findings reviewed above, Steiger et al. (2013) found no associations between 1_F methylation and either BPD, history of suicidality or childhood abuse.

3.2.2. *NR3C1 gene – 1_B region*

Two of the four studies that examined region 1_B methylation found no association with prenatal stress (Hompes et al., 2013) or either childhood abuse or clinical severity (Steiger et al., 2013). Intriguingly, both the papers that did report associations between psychopathology (either suicide completion or PTSD) and DNA methylation were authored by Labonté et al. (2014, 2012) and cover a gene region placed immediately before exon 1_E, including its putative promoter and the whole exon 1_J. Labonté et al. (2014) showed a clear association between PTSD and overall hypomethylation of the 1_B promoter region. In contrast, when studying suicide completers with and without a history of childhood abuse, results are not so straightforward. Suicide completers exhibited CpG-specific hypermethylation at two CpG sites (one of them was found to be associated with decreased GR expression) and hypomethylation at a third CpG site when compared to controls, independently of history of childhood abuse (Labonté et al., 2012). The CpG sites reported by the two studies do not match; even though exon 1_B is one of the most studied exons, there is no replication among studies.

3.2.3. *NR3C1 gene – 1_C region*

Three of the four studies specifically examining promoter and region 1_C methylation were performed by the same research group (Labonté et al., 2014, 2012; Steiger et al., 2013) and cover similar regions encompassing 59 CpG sites; 11 of them have been reported to be individually associated with either stress or psychiatric variables. Additionally, a second research group (De Rooij et al., 2012) analyzed a region specifically spanning exon 1_C (excluding its promoter region from the analysis). Steiger et al. (2013) found no epigenetic correlates of experiencing childhood abuse in BN subjects with or without BPD. Nevertheless, they did observe a set of differential CpG-specific hypermethylation patterns in both BN-BPD subjects when compared to controls without eating disorders, and in BN individuals with a history of suicidality when compared to controls. Labonté et al. (2012) identified four differentially methylated CpG sites in a suicide sample: one hypermethylated CpG site and one hypomethylated CpG site in suicide completers with a history of childhood abuse when compared to controls and non-abused suicide completers; and two hypermethylated CpG sites in suicide completers when compared to controls independently of history of childhood abuse. When examining PTSD subjects, significant global hypomethylation was revealed in a region encompassing 54 CpG sites in the exon 1_C promoter. Remarkably, one specific CpG site was found hypermethylated in PTSD patients (Labonté et al., 2014). Among healthy individuals, De Rooij et al. (2012) described a positive association between 1_C mean methylation and levels of both education and physical activity; whereas methylation in this region negatively correlated to smoking. Additionally, a fifth study reported differential methylation at a specific CpG site (cg11152298) to be associated with childhood maltreatment. Nevertheless, this study did not report the direction of the effect and it is unknown whether early trauma is associated with hypermethylation or hypomethylation (Weder et al., 2014). Once again, there is no replication among studies of region 1_C differential methylation.

3.2.4. *NR3C1 gene – 1_H region*

Two of the studies that examined exon 1_H methylation analyzed a total of 69 CpG sites with a little overlap: just 6 CpG sites were examined by both. They reported mean hypomethylation values in either suicide completers with a history of childhood abuse (Labonté et al., 2012) or BN-BPD individuals (Steiger et al., 2013) compared to controls. Nevertheless, a third study extended the region analyzed beyond exon 1_H and reported higher methylation values in association with several measures of early trauma, such as repeated exposure to sexual and physical abuse or stressful life events experienced during adolescence (Van der Knaap et al., 2014). The different methods used in prior studies preclude the replication of results.

3.2.5. *NR3C1 gene – 1_D region*

Hompes et al. (2013) found significant correlations between several measures of prenatal stress and levels of methylation at 10 CpG sites in exon 1_D (pertaining to 4 methylation units identified by MALDI-TOF) as measured in cord blood from newborns. Additionally, Van der Knaap et al. (2014) observed a significant negative correlation between repeated exposure to "other trauma" (non-sexual and non-physical) and overall methylation of a region spanning exon 1_D and beyond. Furthermore, Hogg et al. (2013) detected significant associations between methylation at CpG sites cg21702128, cg14558428, cg24026230 and cg13648501—which are located in the 1_D promoter—and early-onset pre-eclampsia (EOPE), together with higher mean methylation values at exon 1_D in EOPE subjects compared to controls. Lastly, Weder et al. (2014) also found differential methylation at CpG sites cg21702128 and cg13648501 in association with childhood maltreatment. Nevertheless, these associations lack the direction of the effect and it is unknown whether early trauma is associated with hypermethylation or hypomethylation. In this case, the 1_D CpG dinucleotides included in the Illumina BeadChip do not match those assessed by Hompes et al. or van der Knaap et al., which contributes to the lack of replicated results (the equivalence of the Illumina nomenclature and the CpG site numbering adopted in the current review is displayed in Table S4).

3.3. NR3C1 *promoter methylation inversely correlates with GR expression*

Six of the 23 papers reviewed explored GR expression coupled with epigenetic analysis (Hogg et al., 2013; Labonté et al., 2014, 2012; McGowan et al., 2009; Perroud et al., 2014; Vukojevic et al., 2014). Five of them described negative correlations between *NR3C1* methylation at different first exons (including CpG sites in the 1_B, 1_C and 1_F exons) and GR expression (either total GR mRNA or specific GR transcripts) in both brain and peripheral tissues (T lymphocytes and saliva). Intriguingly, Labonté et al. (2012) reported a positive rather than negative association between 1_H methylation and expression which draws our attention back to Section 3.2.4, since 1_H methylation values were the only ones to be negatively associated with early stress.

Conversely, Hogg et al. (2013) did not find any relationship between *NR3C1* methylation and expression. However, they did not assay 1_D containing transcripts but generic *NR3C1* expression, which may have diluted moderate methylation changes of a scarcely expressed transcript. Nevertheless, there is no data on the expression of GR transcripts containing the 1_F exon in peripheral blood in relation to 1_F methylation.

3.4. NR3C1 *methylation and stress reactivity*

Nine of the 23 papers reviewed measured cortisol levels either at baseline or after a stress challenge—such as the Trier Social Stress Test (TSST) or the Dex/CRH challenge—in order to measure basal and stress-related HPA axis functioning. These studies produced inconsistent data (Table 3). While five papers point to a positive association between methylation and cortisol levels, either at baseline or after a stress challenge (De Rooij et al., 2012; Labonté et al., 2014; Oberlander et al., 2008; Stroud et al., 2014; Yehuda et al., 2014), three studies claim these associations to be negative (Edelman et al., 2012; Perroud et al., 2014; Tyrka et al., 2012). Furthermore, methylation at two CpG sites as analyzed by Weder et al. (2014) were found to predict morning cortisol values but the direction of the effect is not reported. Controversial findings regarding cortisol measurements are in line with previous studies (for a review, see Strüber et al., 2014).

3.5. *Both hypermethylation and hypomethylation of the* NR3C1 *gene may be maladaptive*

While hypermethylation at specific CpG sites has been linked to ELS (see Section 3.2), a global pattern of hypomethylation has been found in PTSD subjects. Intriguingly, depressed patients are widely reported to show non-suppression after the dex/CRH test (Heim et al., 2008); while individuals suffering from PTSD exhibit a hypersuppression after the DST (McFarlane et al., 2011). This divergence suggests that the timing of the stress exposure could determine differential modulations of HPA axis functioning: (i) early adversity, such as prenatal stress or childhood maltreatment, increases methylation in promoter regions of the *NR3C1* gene which dampens GR expression, promotes glucocorticoid resistance and may trigger several psychiatric disorders, such as a depressive episode or BPD in adulthood; (ii) late and acute exposure to trauma may induce demethylation in the same regions, thus increasing GR expression and leading to a sensitization of the HPA axis.

Alternatively, subjects with hypomethylation of the *NR3C1* gene prior to trauma may suffer stress coping difficulties together with an increased recall of negative memories, thus becoming more susceptible to developing PTSD after being exposed to severe trauma during adulthood. In fact, Perroud et al. (2014) recently reported the percentage of methylation of the *NR3C1* exon 1_F across a sample of genocide-exposed mothers and their children, thus providing methylation measures that can be associated with late stress. Intriguingly, the exposed mothers showed increased CpG-specific methylation compared to non-exposed mothers, which suggests stress-associated *NR3C1* hypermethylation at any stage of life and further supports a role of basal hypomethylation prior to trauma in triggering PTSD.

3.6. *Biological relevance of methylation at a NGFI-A binding site*

Intriguingly, four papers reported separate biomarker-like features associated with exon 1_F CpG 37 methylation. As discussed in Section 3.4, Oberlander et al. (2008) reported an association between methylation at this particular CpG site and HPA axis responsiveness: infants with increased cortisol responses after a stress challenge had higher methylation. Meanwhile, Stroud et al. (2014) found CpG 37 methylation to be positively correlated with cortisol reactivity. Accordingly, Yehuda et al. (2014) found the same association in PTSD subjects whose CpG 37 methylation was lower and predicted cortisol levels after the DST. Subsequently, Vukojevic et al. (2014) investigated the putative interaction between *NR3C1* methylation and memory performance during a fMRI picture-recognition task in healthy subjects; they found a correlation between CpG 37 methylation and brain activity related to the successful recognition of previously seen pictures in men but not in women. Strikingly, CpG 37 methylation was negatively correlated with the correct recognition of previously seen pictures in men.

3.7. *Tissue distribution of alternative non-coding first exons*

The tissue distribution of GR first exons has been extensively examined. In this regard, mRNA containing exons 1_B or 1_C in any of its splice variants (1_{C1}–1_{C3}) constitute the most widely expressed *NR3C1* transcripts as they are detected in the hippocampus, T cells, B cells, monocytes, heart muscle, lungs, liver and skin, among other tissues examined (Presul et al., 2007; Turner and Muller, 2005). Moreover, mRNAs containing either 1_B or 1_C are the most abundant transcripts in several brain areas including the hippocampus (Alt et al., 2010). In contrast, exons 1_E and 1_J are the least abundant in brain, with marginal expression ratios accounting for 0.1% of total GR expression (Alt et al., 2010). Strikingly, intronic upstream regions preceding both the 1_E and 1_J exons do not exhibit

promoter activity as opposed to the corresponding regions of the five remaining first exons located in the same CpG island (Cao-Lei et al., 2011). Exons 1_D, 1_F and 1_H exhibit intermediate expression ratios. Exon 1_D has been reported to be expressed in brain and placenta (Hogg et al., 2013); exon 1_F is expressed in immune cells and brain (Turner and Muller, 2005); and exon 1_H has been found to be expressed in several tissues including liver, lung, heart muscle and a number of blood cell subpopulations (Turner and Muller, 2005). Interestingly, the human hippocampus was the only tissue analyzed where all the non-coding exons are present at the mRNA level (Turner and Muller, 2005).

4. Conclusions

This study is the first to systematically review all the scientific literature regarding *NR3C1* non-coding first exon methylation in association with both psychosocial stress at several stages of life (including fetal development, early childhood, adolescence and adulthood) and a broad range of stress-related disorders. Even when accounting for moderate differences, early life adversity has been repeatedly shown to be associated with hypermethylation at several CpG sites located in the non-coding first exons of the *NR3C1* gene. Such epigenetic modifications may impair HPA axis functioning and further predispose early stress-exposed subjects to a wide range of psychiatric conditions, such as major depression or borderline personality disorder, in adulthood. Taking into account all the findings discussed in this review, some methodological issues should be noted for further research (as summarized in Fig. 4).

4.1. How do we assess DNA methylation?

Our first concern regard the differences observed in the assessment of methylation across the various studies, which preclude meta-analysis. On the one hand, the methods used to measure methylation differed greatly between studies including: bisulfite pyrosequencing; cloning and further Sanger sequencing of regions of interest; the use of genome-wide methylation arrays; and mass spectrometry (MS) analyses. Because of the limited capacity of MS to discriminate between specific CpG dinucleotides, studies using MALDI-TOF tend to report average methylation values in minor sets spanning two or more individual CpG sites, rather than CpG-specific results (Hompes et al., 2013; Melas et al., 2013; Van der Knaap et al., 2014). This methodological discrepancy could be the primary cause of the apparently controversial findings of two separate research groups focusing on the same 1_F region while analyzing almost identical prenatal stress variables (Hompes et al., 2013; Oberlander et al., 2008); as discussed in Section 3.2.1. Their opposing findings also correspond to different stages of pregnancy, which may have contributed to the heterogeneity of the results. However, the results reported by Hompes et al. correspond to a CpG unit including CpG sites 37 and 38; thus, CpG 37 methylation could be masked by a marked hypomethylation of CpG 38.

On the other hand, regardless of the method selected, while some authors reported average methylation of assayed regions, others displayed their data in a CpG by CpG fashion, while a third group reported both measures of methylation. The authors reporting both types of data reveal the seeming biological relevance of assaying methylation in a CpG-specific manner. For instance, McGowan et al. (2009) detected a higher global methylation percentage in abused suicide victims than in either controls or non-abused suicide victims. Strikingly, when examining individual CpG sites, it seems that this overall difference is mainly driven by two out of 38 assayed CpG sites (CpG sites 30 and 32). Thus, mean methylation differences could be driven by a very few truly informative CpG sites placed in biologically relevant positions, such as transcription factor binding sites, such as, the widely reported nerve growth factor-inducible protein A (NGFI-A) binding site in both rats (Weaver et al., 2004) and humans (McGowan et al., 2009).

However, once the specific methylation value is available, the statistical approach is still challenging. There a number of confounders which may directly influence methylation, mainly (i) genetic variability in the DNA methylation machinery genes, and (ii) genetic variability affecting the epigenetically regulated region of interest. Genetic variability in epigenetic machinery genes has been widely studied regarding psychiatric disorders (Murgatroyd and Spengler, 2012) and genetic variation of a DNA methyltransferase (*DNMT3B*) has been associated with environmental sensitivity affecting cognitive plasticity (Córdova-Palomera et al., 2015). In this regard, genetic variation directly affecting CpG sites will modify methylation percentage as these regions will be unable to be chemically modified to incorporate a methyl group. Nevertheless, there are almost no described SNPs overlapping with CpG sites of interest at the *NR3C1* gene and, when present, they display very low minor allele frequencies (such as rs562481368 or rs573565118, both located at region 1F, overlapping with CpG sites 39 and 46 respectively).

4.2. Where do we look?

Secondly, there is no agreement regarding the delimitation of *NR3C1* first exon promoter regions. This could be the reason why authors who allegedly focus on the same exons have actually analyzed distinct CpG sites. This may be due to late annotations of exons 1_D–1_J compared to the initial report of exons 1_A–1_C, which lead to several misconceptions regarding the topic. In fact, another controversial issue is whether to explore promoter or exonic regions; or both. Most of the papers reviewed rely on epigenetic findings reported in rats (Weaver et al., 2004) and consequently concentrate on human exon 1_F and its associated promoter. Hence, region 1_F should be further analyzed as this exon may be key for the modulation of stress responses. Furthermore, some authors examined wider regions according to expression studies in brain tissue (Alt et al., 2010). Exons 1_B and 1_C should be examined in detail since they are the most expressed *NR3C1* first exons throughout the body, including the brain. Intriguingly, findings reported for these exons—although limited—seem to be associated with final psychopathological phenotype (either suicide completion or PTSD) rather than with any early life stressor; which further suggests a possible role as biomarkers.

Moreover, the region 1_E emerges as an elusive target. Although it has not been directly addressed by any research group, Labonté et al. examined its putative promoter methylation, while theoretically focusing on the 1_B promoter. Recently, Cao-Lei et al. (2011) examined all the intronic regions immediately upstream of exons 1_B–1_F plus 1_H and 1_J and reported promoter activity for all of them except those preceding exons 1_E and 1_J. Further studies need to be performed on the $1_{J/E}$ region in order to determine whether epigenetic modifications have the potential to modulate the expression of the least abundant first exons of the *NR3C1* gene; and whether such variations have any functional repercussion on GR activity and further HPA dysregulation. As they are the least expressed exons, they could promote the transcription of the least abundant isoform of the glucocorticoid receptor: GR-β, whose function and relevance are still largely unknown, but which seems to antagonize GR-α thereby promoting glucocorticoid resistance (Turner et al., 2010; Webster et al., 2001). Interestingly, transcripts containing exon 1_E have been reported to be expressed in immune cells, particularly in monocytes and CD8+ T cells (Turner and Muller, 2005). Indeed, a decreased GRα/GRβ expression ratio has been identified in monocytes from MDD patients, which suggests a further role of *NR3C1* modulation in ongoing psychopathological status

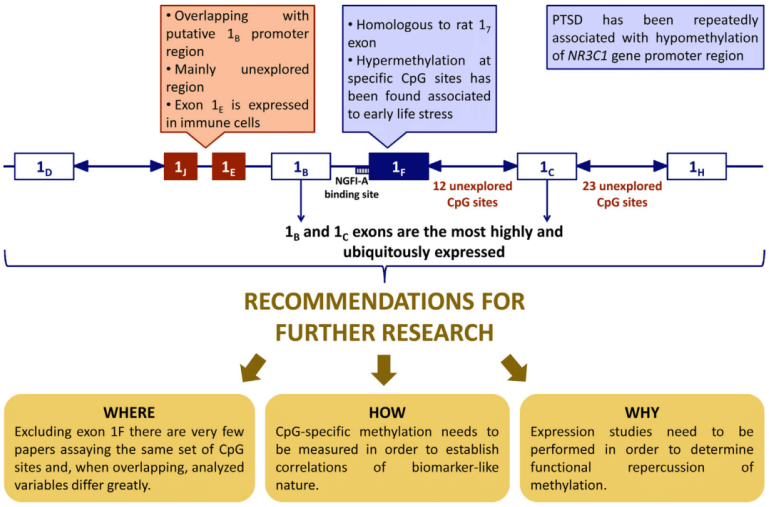

Fig. 4. Box diagram representing the main findings reported and suggested future directions to improve our knowledge of the role of early life stress upon *NR3C1* methylation and psychopathological outcomes. The *NR3C1* gene contains a CpG island which encloses non-coding alternate first exons 1_D–1_J spanning 3 kb. This CpG island consists of around 300 CpG sites, i.e., 300 methylation-sensitive loci.

(Carvalho et al., 2014). There is much literature regarding the complex interplay between the HPA axis and immune system, e.g., the janus-faced actions of glucocorticoids on the modulation of inflammatory response (Sorrells et al., 2009), as well as the putative engagement of inflammation in the etiology of psychiatric disorders (Haroon et al., 2012). Cortisol can act as an anti-inflammatory molecule by means of peripheral GR located in immune cells, which should encourage future research to focus on the study of the epigenetic signature of exon 1_E in peripheral samples, as it may be involved in the neuroendocrine modulation of inflammation.

In addition, exons 1_D and 1_H need to be studied further since the evidence regarding their association with early stress is very limited. These exons reach transcription rates comparable to those of exon 1_F. Finally, a certain pool of CpG sites remains unexplored. Although they are placed within the CpG island containing exons 1_B–1_F, 1_H and 1_J, they have not been targeted by any study to date (Fig. 4).

Furthermore, researchers in this field do not only need to choose carefully which exons—and which CpG sites along with them—should be analyzed, but also in which tissues. As exemplified in several reviews but particularly in the current one, epigenetic research has been conducted on a number of tissues including postmortem brain samples together with peripheral tissues such as saliva and blood. Epigenetic signatures are known to differ from one tissue to another; nevertheless, recent studies encourage the common assumption of epigenetic hallmarks in blood being replicable in brain tissues (Ewald et al., 2014; Masliah et al., 2013).

Finally, it is important to keep in mind that DNA methylation does not operate on its own but in conjunction with a number of additional epigenetic mechanisms such as histone modification and microRNA-mediated gene silencing. While there is no research yet regarding the role of histone modifications in *NR3C1* regulation, microRNAs are gaining popularity and will be studied extensively in the next decade. As proposed by Turner et al. (2010) in a previous review, microRNAs may play an essential role in regulating translation of GR. Recently, several authors have suggested the role of microRNAs in either the regulation of a GR-dependent signaling network (Vallès et al., 2014) or the development of glucocorticoid resistance after exposure to chronic stress (Jung et al., 2015). Interestingly, it has been also described that expression of a specific microRNA (miR-137, which is a hit of a GWAS for schizophrenia) can be regulated by methylation in adult neural stem cells from mice (Szulwach et al., 2010) thus illustrating the crosstalk between distinct epigenetic marks in neural tissue.

4.3. *Why do we analyze* NR3C1 *methylation?*

Identifying epigenetic signatures associated with early stress or psychopathology does not imply causality. A limited number of papers examine mRNA expression in relation to epigenetic modifications (Hogg et al., 2013; Labonté et al., 2014, 2012; McGowan et al., 2009; Perroud et al., 2014; Vukojevic et al., 2014). Such research is essential in order to establish functional correlations between methylation patterns and phenotypic variation. The expression studies reviewed here were performed in either brain tissue, saliva or blood samples. Notably, five of the six studies that assessed expression together with methylation reported an inverse association between the aforementioned variables. Further studies examining both DNA methylation and gene expression are needed to elucidate the functional repercussions of epigenetic modifications of the exon 1_F in relation to ELS.

There is still a major concern regarding the extent of the reported methylation differences (Table 1). DNA methylation is an absolute modification: a CpG site is either methylated or unmethylated. Accordingly, methylation percentages ranging from 0% to 100% indicate uneven methylation across different cells. It is worth noting that most of the reported significant differences included in this review are values either under 5% or merely not specified (nor suitable to be extrapolated from figures). This may raise three concerns: (i) between-group differences of 5% are certainly close to the detection sensitivity limits of the methylation assessment techniques employed (Klengel et al., 2014); (ii) these small changes are likely to be caused by marginal cellular subsets (accounting for a tiny fraction of the total cell population), which may have

been differentially methylated after a stressor; and (iii) since the link between DNA methylation and gene expression is relatively continuous (Siegfried and Simon, 2010), these minor methylation differences may induce only relatively small transcriptional changes.

Nevertheless, several reports have consistently found this CpG island to be widely unmethylated with average methylation levels below 5% suggesting that small methylation increases in crucial CpG sites may have the potential to influence transcription greatly. In this regard, it is worth mentioning that some novel statistical approaches to analyze DNA methylation changes between groups, with a special emphasis on large methylation differences, have been able to retrieve biologically-feasible results (Dempster et al., 2014) and may thus be useful in this context.

These moderate methylation differences could be due to differential methylation patterns across distinct cell types. Both blood and brain samples comprise a number of separate cell types such as monocytes, T-lymphocytes and B-lymphocytes, and neurons, astrocytes and microglia, respectively. Focusing on epigenetic signatures of specific cell types rather than measuring methylation in mixed tissues may elucidate not only which genes are deregulated in pathological conditions but also which cell types are more vulnerable to stress and thus involved in the etiology of these disorders.

4.4. Future directions

All of the foregoing should be taken into account in future studies that examine the methylation of the *NR3C1* gene promoter region. Likewise, the exons to be studied should be selected in accordance with transcriptional studies, providing mRNA expression and tissue distribution data in order to focus on regions that are biologically relevant for HPA axis functioning. In this regard, exon 1_F showed a significantly lower expression in the hippocampi of MDD subjects than in controls; which suggests its involvement in the etiology of mood disorders (Alt et al., 2010). Future selection of the CpG sites to be analyzed should prioritize those located in transcription factor (TF) binding regions, since methylation can prevent TF binding and thus impair gene expression. Additionally, specific cell types are more likely to exhibit homogenous epigenetic signatures rather than mixtures such as peripheral blood or brain homogenate.

With regard to study design, it would be very informative to analyze several variables simultaneously. Some such suggestions are: (i) to study both prenatal and childhood stress in order to assess whether they have additive or synergistic effects on *NR3C1* methylation; (ii) to collect lifestyle information such as drug exposure, smoking habits and other factors known to modify epigenetic signatures as they can confound the results; (iii) to assess immune function by means of cytokine determination in plasma, to determine the extent to which psychosocial stress-induced epigenetic modifications of the *NR3C1* gene can modulate the inflammatory response; and (iv) to study monozygotic twins discordant for either ELS or psychopathology in order to rule out heterogeneity due to genetic variability and non-shared environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neubiorev.2015.05.016

References

Alt, S.R., Turner, J.D., Klok, M.D., Meijer, O.C., Lakke, E.A., Derijk, R.H., Muller, C.P., 2010. Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. Psychoneuroendocrinology 35, 544–556.

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders, 5th ed.

Anacker, C., Zunszain, P.A., Carvalho, L.A., Pariante, C.M., 2011. The glucocorticoid receptor: pivot of depression and of antidepressant treatment? Psychoneuroendocrinology 36, 415–425.

Binder, E.B., 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology 34 (Suppl. 1), S186–S195.

Cao-Lei, L., Leija, S.C., Kumsta, R., Wüst, S., Meyer, J., Turner, J.D., Muller, C.P., 2011. Transcriptional control of the human glucocorticoid receptor: identification and analysis of alternative promoter regions. Hum. Genet. 129, 533–543.

Carr, C.P., Martins, C.M.S., Stingel, A.M., Lemgruber, V.B., Juruena, M.F., 2013. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. J. Nerv. Ment. Dis. 201, 1007–1020.

Carvalho, L.A., Bergink, V., Sumaski, L., Wijkhuijs, J., Hoogendijk, W.J., Birkenhager, T.K., Drexhage, H.A., 2014. Inflammatory activation is associated with a reduced glucocorticoid receptor alpha/beta expression ratio in monocytes of inpatients with melancholic major depressive disorder. Transl. Psychiatry 4, e344.

Conradt, E., Lester, B.M., Appleton, A.A., Armstrong, D.A., Marsit, C.J., 2013. The roles of DNA methylation of NR3C1 and 11β-HSD2 and exposure to maternal mood disorder in utero on newborn neurobehavior. Epigenetics 8, 1321–1329.

Córdova-Palomera, A., Fatjó-Vilas, M., Kebir, O., Gastó, C., Krebs, M.O., Fañanás, L., 2015. Polymorphic variation in the epigenetic gene DNMT3B modulates the environmental impact on cognitive ability: a twin study. Eur. Psychiatry 30, 303–308.

De Rooij, S.R., Costello, P.M., Veenendaal, M.V.E., Lillycrop, K.A., Gluckman, P.D., Hanson, M.A., Painter, R.C., Roseboom, T.J., 2012. Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. Psychoneuroendocrinology 37, 782–788.

Dempster, E.L., Wong, C.C.Y., Lester, K.J., Burrage, J., Gregory, A.M., Mill, J., Eley, T.C., 2014. Genome-wide methylomic analysis of monozygotic twins discordant for adolescent depression. Biol. Psychiatry 76, 977–983, http://dx.doi.org/10.1016/j.biopsych.2014.04.013

Edelman, S., Shalev, I., Uzefovsky, F., Israel, S., Knafo, A., Kremer, I., Mankuta, D., Kaitz, M., Ebstein, R.P., 2012. Epigenetic and genetic factors predict women's salivary cortisol following a threat to the social self. PLoS ONE 7, e48597.

Ehlert, U., 2013. Enduring psychobiological effects of childhood adversity. Psychoneuroendocrinology 38, 1850–1857.

Ewald, E.R., Wand, G.S., Seifuddin, F., Yang, X., Tamashiro, K.L., Potash, J.B., Zandi, P., Lee, R.S., 2014. Alterations in DNA methylation of Fkbp5 as a determinant of blood–brain correlation of glucocorticoid exposure. Psychoneuroendocrinology 44, 112–122.

Gilbert, R., Widom, C.S., Browne, K., Fergusson, D., Webb, E., Janson, S., 2009. Burden and consequences of child maltreatment in high-income countries. Lancet 373, 68–81.

Gluckman, P.D., Hanson, M.A., Cooper, C., Thornburg, K.L., 2008. Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med., 61–73.

Haroon, E., Raison, C.L., Miller, A.H., 2012. Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. Neuropsychopharmacology 37, 137–162.

Heim, C., Mletzko, T., Purselle, D., Musselman, D.L., Nemeroff, C.B., 2008. The dexamethasone/corticotropin-releasing factor test in men with major depression: role of childhood trauma. Biol. Psychiatry 63, 398–405.

Hogg, K., Blair, J.D., McFadden, D.E., von Dadelszen, P., Robinson, W.P., 2013. Early onset pre-eclampsia is associated with altered DNA methylation of cortisol-signalling and steroidogenic genes in the placenta. PLOS ONE 8, e62969.

Hompes, T., Izzi, B., Gellens, E., Morreels, M., Fieuws, S., Pexsters, A., Schops, G., Dom, M., Van Bree, R., Freson, K., Verhaeghe, J., Spitz, B., Demyttenaere, K., Glover, V., Van den Bergh, B., Allegaert, K., Claes, S., 2013. Investigating the influence of maternal cortisol and emotional state during pregnancy on the DNA methylation status of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood. J. Psychiatr. Res. 47, 880–891.

Jung, S.H., Wang, Y., Kim, T., Tarr, A., Reader, B., Powell, N., Sheridan, J.F., 2015. Molecular mechanisms of repeated social defeat-induced glucocorticoid resistance: role of microRNA. Brain. Behav. Immun. 44, 195–206.

Kaplan, W., Laing, R., 2004. Priority Medicines for Europe and the World. Geneva.

Keane, T.M., Marshall, A.D., Taft, C.T., 2006. Posttraumatic stress disorder: etiology, epidemiology, and treatment outcome. Annu. Rev. Clin. Psychol. 2, 161–197.
Kessler, R.C., Bromet, E.J., 2013. The epidemiology of depression across cultures. Annu. Rev. Public Health 34, 119–138.
Kessler, R.C., Petukhova, M., Sampson, N.A., Zaslavsky, A.M., Wittchen, H.-U., 2012. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. Int. J. Methods Psychiatr. Res. 21, 169–184.
Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16, 33–41.
Klengel, T., Pape, J., Binder, E.B., Mehta, D., 2014. The role of DNA methylation in stress-related psychiatric disorders. Neuropharmacology 80, 115–132.
Labonté, B., Azoulay, N., Yerko, V., Turecki, G., Brunet, A., 2014. Epigenetic modulation of glucocorticoid receptors in posttraumatic stress disorder. Transl. Psychiatry 4, e368.
Labonté, B., Yerko, V., Gross, J., Mechawar, N., Meaney, M.J., Szyf, M., Turecki, G., 2012. Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. Biol. Psychiatry 72, 41–48.
Leichsenring, F., Leibing, E., Kruse, J., New, A.S., Leweke, F., 2011. Borderline personality disorder. Lancet 377, 74–84.
Martín-Blanco, A., Ferrer, M., Soler, J., Salazar, J., Vega, D., Andión, O., Sanchez-Mora, C., Arranz, M.J., Ribases, M., Feliu-Soler, A., Pérez, V., Pascual, J.C., 2014. Association between methylation of the glucocorticoid receptor gene, childhood maltreatment, and clinical severity in borderline personality disorder. J. Psychiatr. Res. 57, 34–40.
Masliah, E., Dumaop, W., Galasko, D., Desplats, P., 2013. Distinctive patterns of DNA methylation associated with Parkinson disease. Epigenetics 8, 1030–1038.
McFarlane, A.C., Barton, C.A., Yehuda, R., Wittert, G., 2011. Cortisol response to acute trauma and risk of posttraumatic stress disorder. Psychoneuroendocrinology 36, 720–727.
McGowan, P.O., Sasaki, A., D'Alessio, A.C., Dymov, S., Labonté, B., Szyf, M., Turecki, G., Meaney, M.J., 2009. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat. Neurosci. 12, 342–348.
Melas, P.A., Wei, Y., Wong, C.C.Y., Sjöholm, L.K., Åberg, E., Mill, J., Schalling, M., Forsell, Y., Lavebratt, C., 2013. Genetic and epigenetic associations of MAOA and NR3C1 with depression and childhood adversities. Int. J. Neuropsychopharmacol. 16, 1513–1528.
Miret, M., Ayuso-Mateos, J.L., Sanchez-Moreno, J., Vieta, E., 2013. Depressive disorders and suicide: epidemiology, risk factors, and burden. Neurosci. Biobehav. Rev. 37, 2372–2374.
Mulligan, C.J., D'Errico, N.C., Stees, J., Hughes, D.A., 2012. Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. Epigenetics, 853–857.
Murgatroyd, C., Spengler, D., 2012. Genetic variation in the epigenetic machinery and mental health. Curr. Psychiatry Rep. 14, 138–149, http://dx.doi.org/10.1007/s11920-012-0255-261
Oberlander, T.F., Weinberg, J., Papsdorf, M., Grunau, R., Misri, S., Devlin, A.M., 2008. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics, 97–106.
Perroud, N., Paoloni-Giacobino, A., Prada, P., Olié, E., Salzmann, A., Nicastro, R., Guillaume, S., Mouthon, D., Stouder, C., Dieben, K., Huguelet, P., Courtet, P., Malafosse, A., 2011. Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. Transl. Psychiatry 1, e59.
Perroud, N., Rutembesa, E., Paoloni-Giacobino, A., Mutabaruka, J., Mutesa, L., Stenz, L., Malafosse, A., Karege, F., 2014. The Tutsi genocide and transgenerational transmission of maternal stress: epigenetics and biology of the HPA axis. World J. Biol. Psychiatry 15, 334–345.
Petronis, A., 2010. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 465, 721–727.
Presul, E., Schmidt, S., Kofler, R., Helmberg, A., 2007. Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. J. Mol. Endocrinol. 38, 79–90.
Ptak, C., Petronis, A., 2010. Epigenetic approaches to psychiatric disorders. Dialogues Clin. Neurosci. 12, 25–35.
Radtke, K.M., Ruf, M., Gunter, H.M., Dohrmann, K., Schauer, M., Meyer, A., Elbert, T., 2011. Transgenerational impact of intimate partner violence on methylation in the promoter of the glucocorticoid receptor. Transl. Psychiatry 1, e21.
Romens, S.E., McDonald, J., Svaren, J., Pollak, S.D., 2014. Associations between early life stress and gene methylation in children. Child Dev., 1–7.
Siegfried, Z., Simon, I., 2010. DNA methylation and gene expression. Wiley Interdiscip. Rev. Syst. Biol. Med. 2, 362–371, http://dx.doi.org/10.1002/wsbm.64
Slavich, G.M., O'Donovan, A., Epel, E.S., Kemeny, M.E., 2010. Black sheep get the blues: a psychobiological model of social rejection and depression. Neurosci. Biobehav. Rev. 35, 39–45, http://dx.doi.org/10.1016/j.neubiorev.2010.01.003
Sorrells, S.F., Caso, J.R., Munhoz, C.D., Sapolsky, R.M., 2009. The stressed CNS: when glucocorticoids aggravate inflammation. Neuron 64, 33–39.
Steiger, H., Labonté, B., Groleau, P., Turecki, G., Israel, M., 2013. Methylation of the glucocorticoid receptor gene promoter in bulimic women: associations with borderline personality disorder, suicidality, and exposure to childhood abuse. Int. J. Eat. Disord. 46, 246–255.
Stroud, L.R., Papandonatos, G.D., Rodriguez, D., Mccallum, M., Salisbury, A.L., Phipps, M.G., Lester, B., Huestis, M.A., Niaura, R., Padbury, J.F., Marsit, C.J., 2014. Maternal smoking during pregnancy and infant stress response: test of a prenatal programming hypothesis. Psychoneuroendocrinology 48, 29–40.
Strüber, N., Strüber, D., Roth, G., 2014. Impact of early adversity on glucocorticoid regulation and later mental disorders. Neurosci. Biobehav. Rev. 38, 17–37.
Szulwach, K.E., Li, X., Smrt, R.D., Li, Y., Luo, Y., Lin, L., Santistevan, N.J., Li, W., Zhao, X., Jin, P., 2010. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J. Cell Biol. 189, 127–141.
Teicher, M.H., Andersen, S.L., Polcari, A., Anderson, C.M., Navalta, C.P., Kim, D.M., 2003. The neurobiological consequences of early stress and childhood maltreatment. Neurosci. Biobehav. Rev. 27, 33–44.
Turner, J.D., Alt, S.R., Cao, L., Vernocchi, S., Trifonova, S., Battello, N., Muller, C.P., 2010. Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem. Pharmacol. 80, 1860–1868.
Turner, J.D., Muller, C.P., 2005. Structure of the glucocorticoid receptor (NR3C1) gene 5′ untranslated region: identification, and tissue distribution of multiple new human exon 1. J. Mol. Endocrinol. 35, 283–292.
Tyrka, A.R., Price, L.H., Marsit, C., Walters, O.C., Carpenter, L.L., 2012. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. PLoS ONE 7, e30148.
Vallès, A., Martens, G.J.M., De Weerd, P., Poelmans, G., Aschrafi, A., 2014. MicroRNA-137 regulates a glucocorticoid receptor-dependent signalling network: implications for the etiology of schizophrenia. J. Psychiatry Neurosci. 39, 312–320.
Van der Knaap, L.J., Riese, H., Hudziak, J.J., Verbiest, M.M.P.J., Verhulst, F.C., Oldehinkel, A.J., van Oort, F.V.A., 2014. Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence The TRAILS study. Transl. Psychiatry 4, e381.
Vukojevic, V., Kolassa, I.-T., Fastenrath, M., Gschwind, L., Spalek, K., Milnik, A., Heck, A., Vogler, C., Wilker, S., Demougin, P., Peter, F., Atucha, E., Stetak, A., Roozendaal, B., Elbert, T., Papassotiropoulos, A., de Quervain, D.J.-F., 2014. Epigenetic modification of the glucocorticoid receptor gene is linked to traumatic memory and post-traumatic stress disorder risk in genocide survivors. J. Neurosci. 34, 10274–10284.
Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854.
Webster, J.C., Oakley, R.H., Jewell, C.M., Cidlowski, J.A., 2001. Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative β isoform: a mechanism for the generation of glucocorticoid resistance. Proc. Natl. Acad. Sci. U. S. A. 98, 6865–6870.
Weder, N., Zhang, H., Jensen, K., Yang, B.Z., Simen, A., Jackowski, A., Lipschitz, D., Douglas-palumberi, H., Ge, M., Perepletchikova, F., O'Loughlin, K., Hudziak, J.J., Gelernter, J., Kaufman, J., 2014. Child abuse, depression, and methylation in genes involved with stress, neural plasticity, and brain circuitry. J. Am. Acad. Child Adolesc. Psychiatry 53, 417–424.e5.
Yehuda, R., Flory, J.D., Bierer, L.M., Henn-Haase, C., Lehrner, A., Desarnaud, F., Makotkine, I., Daskalakis, N.P., Marmar, C.R., Meaney, M.J., 2014. Lower methylation of glucocorticoid receptor gene promoter 1F in peripheral blood of veterans with posttraumatic stress disorder. Biol. Psychiatry, 1–8.

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Glucocorticoid receptor gene (*NR3C1*) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review" included the following tasks:

- Participation in the conception and design of the study
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: a meta-analysis.

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REVIEW

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Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: a meta-analysis

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Keywords: epigenetics, DNA methylation, glucocorticoid receptor, HPA axis, maternal stress, NR3C1 gene

Prenatal stress has been widely associated with a number of short- and long-term pathological outcomes. Epigenetic mechanisms are thought to partially mediate these environmental insults into the fetal physiology. One of the main targets of developmental programming is the hypothalamic-pituitary-adrenal (HPA) axis as it is the main regulator of the stress response. Accordingly, an increasing number of researchers have recently focused on the putative association between DNA methylation at the glucocorticoid receptor gene (*NR3C1*) and prenatal stress, among other types of psychosocial stress. The current study aims to systematically review and meta-analyze the existing evidence linking several forms of prenatal stress with DNA methylation at the region 1_F of the *NR3C1* gene. The inclusion of relevant articles allowed combining empirical evidence from 977 individuals by meta-analytic techniques, whose methylation assessments showed overlap across 5 consecutive CpG sites (GRCh37/hg19 chr5:142,783,607-142,783,639). From this information, methylation levels at CpG site 36 displayed a significant correlation to prenatal stress ($r = 0.14$, 95% CI: 0.05–0.23, $P = 0.002$). This result supports the proposed association between a specific CpG site located at the *NR3C1* promoter and prenatal stress. Several confounders, such as gender, methylation at other glucocorticoid-related genes, and adjustment for pharmacological treatments during pregnancy, should be taken into account in further studies.

Introduction

The prenatal period constitutes a crucial ontogenic developmental window where environmental influences have a preponderant effect on shaping fetal physiology by means of epigenetic mechanisms.[1] One of the most complex organs to rapidly develop is the brain, which presents its fastest rate of expression change during the fetal period.[2,3] Furthermore, the intrauterine environment is thought to be essential not only for fetal development itself but also for long-term health and disease, as prenatal environment risk factors, such as fetal nutrition, have been associated with a number of adult conditions, such as cardiovascular disease, which has encouraged the notion of a certain *programming*.[4,5] Although early plasticity allows a proper adaptation to the environment that enables survival until reproductive age, it might be deleterious for both mental and physical long-term health.

Maternal stressors experienced during pregnancy that may influence offspring health include: i) physical stressors, such as malnutrition and toxins[6] like alcohol, nicotine, or polychlorinated biphenyls; ii) psychosocial chronic stressors,[7] such as suffering from a psychiatric disorder, taking care of terminally ill relatives,[8] or being exposed to continuous violence (e.g., domestic violence or living in a war zone) or poverty (e.g., famine[9] or low socioeconomic status[10]); and iii) being exposed to severe incontrollable acute trauma (e.g., natural disaster,[11] terrorism,[12] or genocide[13]), which may result in post-traumatic stress disorder (PTSD) development. It is important to note that these prenatal stressors do not present isolated but rather tend to converge. Intriguingly, the experience of chronic stress together with the development of anxious-depressive spectrum disorders have been associated with the onset of glucocorticoid resistance and higher circulating levels of cortisol,[14,15] whereas PTSD has been consistently described to involve a higher glucocorticoid sensitivity and decreased cortisol levels.[16] Due to this dual nature of cortisol effects after exposure to psychosocial stressors, we will focus on chronic conditions that have been described to increase circulating cortisol levels of pregnant women.

The human fetal hypothalamic-pituitary-adrenal (HPA) axis is developed and functioning at week 22 of pregnancy, although its plasticity is maintained during the first 2 years of life.[17,18] Thus, maternal experiences of stress during pregnancy

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have the potential to permanently alter the physiology of their offspring, especially to program the HPA axis functioning. As the HPA axis regulates a myriad of biological processes, such as metabolism, blood pressure, and the immune response, these alterations can predispose prenatally stressed individuals to suffer metabolic, cardiovascular, and mental disorders in adulthood.[19]

Typically, after exposure to an acute stressor, the hypothalamus releases corticotropin-releasing hormone (CRH), which promotes the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. Once in the adrenal glands, ACTH triggers the release of cortisol. This system is self-regulated by a negative feedback in which cortisol inhibits CRH and ACTH release from the central nervous system. Nevertheless, and as discussed above, during the exposure to chronic stress, this feedback may be impaired by the development of glucocorticoid resistance mediated by glucocorticoid receptor (GR) desensitization.[20] This situation leads to an overactivation of the HPA axis that has been suggested to underpin the development of depressive symptoms at least in a subset of patients showing alterations of the HPA axis functioning.[21]

Hence, the GR is essential in the modulation and functioning of the HPA axis. The *NR3C1* gene, which codes for the GR, has extensively been studied regarding its role in several psychiatric disorders characterized by HPA axis dysfunction and in subjects exposed to several forms of early stress by means of both genetic and epigenetic approaches.[22,23]

In this regard, several authors have analyzed DNA methylation at a CpG island located at the promoter region of this gene in different paradigms of early stress exposure. First, the discovery of a CpG-specific hypermethylation as found in exon 1_7 of postnatally neglected rat pups when compared to pups reared by mothers exhibiting high licking, grooming, and arched-back nursing (LG-ABN) behavior, prompted a new research field that has extended to human populations in the last decade.[22,24] Special attention has been paid to exon 1_F due to its homology with rat exon 1_7. Nevertheless, there is still no consensus about the effects of early stress on *NR3C1* methylation due to the heterogeneity of assessed stress variables, regions analyzed, and subjects included in human studies.[22] Although animal studies have largely focused in the study of postnatal stress as a modulator of epigenetic patterns, prenatal stages arise as a key ontogenic window of development where stress sensitization can also occur and become embedded in the epigenome; in fact, there is one study developed in mice that reported an increase in methylation in males exposed to early prenatal stress, which was restricted to an NGF1A binding region within GR promoter exon 1_7, thus paralleling prior results obtained in postnatal paradigms.[25]

By reviewing and meta-analyzing all previously published empirical reports in human populations, the current work aims to determine whether there is enough evidence to support a link between maternal chronic psychosocial stress (as experienced during pregnancy) and site-specific *NR3C1* promoter methylation (as assessed in their offspring).

Results

Eligibility of studies and specific CpG sites to be meta-analyzed

Twenty-five scientific papers concurrently covering for the 3 topics of interest as described in the methodology section were retrieved by our defined search strategy for further assessment of eligibility. As can be seen in **Figure 1**, 15 articles were excluded due to the lack of new human data reported (reviews, animal studies, and human reports where there was no prenatal stress assessment); a total of 10 papers analyzed diverse prenatal stressors in relation to *NR3C1* promoter methylation. In order to homogenize the final sample, 3 additional papers were excluded from the meta-analysis: one of them since smoking during pregnancy was not considered as a psychosocial stressor,[26] and the remaining 2 since, as discussed in the introduction, PTSD status during pregnancy has been described to upregulate cortisol inactivation and decrease cortisol circulating levels, and not the other way around.[12,13] The resulting pool consisted of 7 selected papers for the meta-analysis. Five of them analyzed maternal anxious-depressive status during pregnancy.[27-31] The remaining 2 papers focused on the effect of maternal exposure to violence (either intimate partner violence or war-related events) during pregnancy.[32,33]

As depicted in **Figure 2**, there is an overlap of 5 analyzed CpG sites (35 to 39) at the promoter of the exon 1_F among 6 of the 7 papers meta-analyzed herein (see also **Figure 3** for specific location in the sequence). Thus, 5 meta-analyses were carried out individually for each CpG site (**Fig. 4**) since previous literature supports the association between distinct early stressors and specific CpG sites methylation rather than the mean methylation of a region containing several CpG sites.[24,34]

Since all of the reviewed articles included several CpG sites in their analyses, which led to a mixture of positive and negative findings, we expected publication bias to be avoided. Indeed, this hypothesis has been statistically assessed by funnel plot asymmetry tests for each individual CpG site included in the meta-

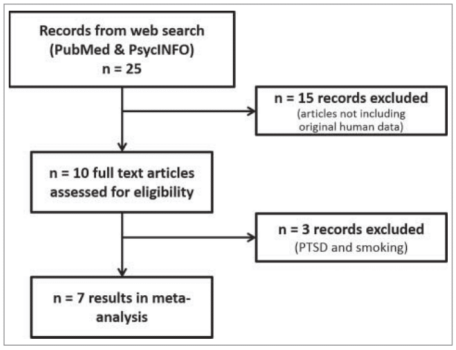

Figure 1. Flowchart of study selection and inclusion of results. Data from 7 papers were included in the meta-analysis.

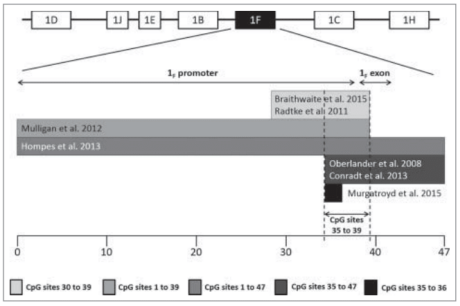

Figure 2. CpG site distribution and overlap among included papers in the meta-analysis. Research groups focused on distinct regions of promoter and exon 1_F. Six out of the 7 papers included in the meta-analysis assessed the DNA methylation of a small cluster of CpG sites located immediately preceding exon 1_F, from 35 to 39; additionally, Murgatroyd et al. limited their analyses to CpG sites 35 and 36 as they had been already pointed out as informative in the previous papers. The whole sequence of this region is displayed in **Figure 3**.

analysis, further confirming the lack of publication bias (data not shown). In addition, presence of heterogeneity was statistically tested by means of 4 different estimators as reported below and described in the methodology.

NR3C1 exon 1_F CpG site 35 methylation

DNA methylation at CpG site 35 of the *NR3C1* gene was examined in 977 subjects across the 7 studies. We detected no significant correlation between offspring methylation and prenatal stress ($r = 0.10$, 95% CI: −0.01–0.21, $p_r = 0.08$; τ^2: 0.01, I^2: 54.7%, $H^2 = 1.49$, $Q = 13.23$, $p_Q = 0.04$).

NR3C1 exon 1_F CpG site 36 methylation

DNA methylation at CpG site 36 of the *NR3C1* gene was examined in 977 subjects across the 7 studies. We detected a significant correlation between offspring's methylation and prenatal stress ($r = 0.14$, 95% CI: 0.05–0.23, $p_r = 0.002$; τ^2: 0, I^2: 34.7%, $H^2 = 1.24$, $Q = 9.18$, $p_Q = 0.16$) pointing to an

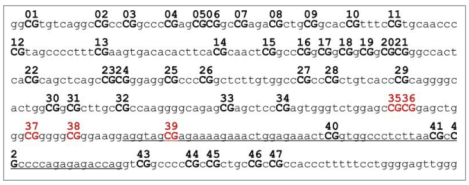

Figure 3. Exon 1_F (*NR3C1* gene) structure. Exon is underlined. All CpG sites (CG) are numbered, remarked in bold and capital letters. Reviewed CpG sites herein (35 to 39) are highlighted in dark red.

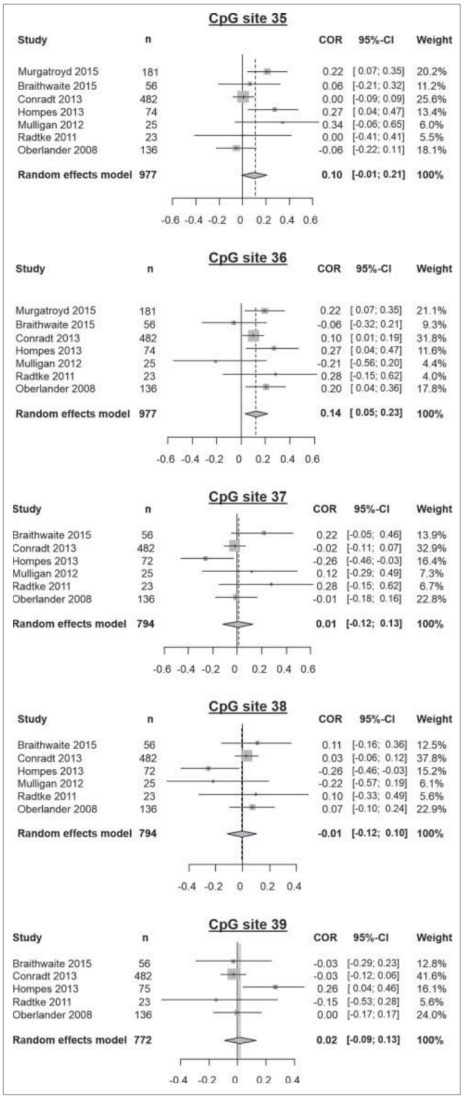

Figure 4. Results of meta-analyses of prenatal stress exposure influence on methylation of the CpG sites 35 to 39. From left to right, displayed variables consist of: study, sample size (n), a graphical representation of the computed correlations, correlation values between methylation and the assessed prenatal stress variable, their corresponding confidence intervals and the weight of each study in the meta-analysis as determined by a random effects model.

increased methylation of CpG site 36 after prenatal stress exposure. Final correlation values obtained by means of both random effects model and fixed effects model are the same (data not shown).

***NR3C1* exon 1_F CpG site 37 methylation**

DNA methylation at CpG site 37 of the *NR3C1* gene was examined in 794 subjects across the 6 studies; Murgatroyd et al. did not provide methylation percentages for this CpG site. We detected no significant correlation between offspring methylation and prenatal stress ($r = 0.01$, 95% CI: −0.12–0.13, $p_r = 0.91$; τ^2: 0.01, I^2: 47.6%, $H^2 = 1.38$, $Q = 9.54$, $p_Q = 0.09$).

***NR3C1* exon 1_F CpG site 38 methylation**

DNA methylation at CpG site 38 of the *NR3C1* gene was examined in 794 subjects across 6 studies; Murgatroyd et al. did not provide methylation percentages for this CpG site. We detected no significant correlation between offspring methylation and prenatal stress ($r = -0.01$, 95% CI: −0.12–0.10, $p_r = 0.91$; τ^2: 0, I^2: 35.7%, $H^2 = 1.25$, $Q = 7.78$, $p_Q = 0.17$).

***NR3C1* exon 1_F CpG site 39 methylation**

DNA methylation at CpG site 39 of the *NR3C1* gene was examined in 769 subjects across 5 studies; Murgatroyd et al. and Mulligan et al. did not provide methylation percentages for this CpG site. We detected no significant correlation between offspring methylation and prenatal stress ($r = 0.02$, 95% CI: −0.09–0.13, $p_r = 0.54$; τ^2: 0, I^2: 35.9%, $H^2 = 1.25$, $Q = 6.24$, $p_Q = 0.18$).

Discussion

By meta-analyzing data available in the literature we found a significant correlation between psychosocial maternal stress and offspring methylation at a specific CpG site located in the exon 1_F of the human glucocorticoid receptor gene *NR3C1*. Interestingly, we report a significant correlation between DNA methylation and presence of prenatal stress only in CpG site 36 but not in its adjacent positions 35, 37, 38, or 39. These results provide further evidence of the previously suggested biological relevance of CpG site-specific methylation when compared with average methylation of a region spanning several CpG sites.[22]

Interest in the methylation signature of *NR3C1* exon 1_F followed the publication of the seminal work developed in rats by Weaver and collaborators.[24] They described a maternally mediated change in DNA methylation in the rat homolog of human CpG site 37, which is located at a transcription factor (NGFI-A)-binding region, thus suggesting the functional relevance of this modification. Although most of the human studies have focused on this specific CpG site due to its biological relevance, it is interesting to note that, in their animal model, Weaver et al. also found significant methylation differences in the adjacent CpG sites, including those who are homologous to human CpG sites 35 and 36. These unprecedented results in rats support human findings reported herein as other CpG sites located within the promoter of exon 1_F could alter its transcription efficacy.

Of note, there was only one human study that paralleled the experimental conditions tested in rats. Murgatroyd and colleagues[31] took into account both early postnatal depression and maternal tactile stimulation when analyzing newborn methylation. This design allowed the researchers to describe a mediating effect of both variables in altering DNA methylation at CpG sites 35 and 36 where postnatal depression in newborns non-exposed to prenatal depression increased methylation while maternal stroking decreased it. These results are in line with previous research from this group where maternal stroking has been reported to lessen the influence of prenatal depression upon infant behavioral and physiological outcomes.[35]

The evidence of a replicated correlation between prenatal stress and *NR3C1* methylation is particularly relevant in the context of 3 infant neurobehavioral and neuroendocrine studies developed by Marsit and Lester's research groups, whose findings point to a significant effect of placental *NR3C1* methylation in both newborn neurobehavior and cortisol reactivity after a newborn-adjusted stress procedure (the still-face paradigm).[36-38] It is worth noting that the statistically significant correlations between *NR3C1* methylation and neurobehavioral parameters were restricted to either (i) CpG sites 39 to 47, which are located within the exon 1_F and beyond (instead of localizing to a promoter region as CpG sites 35 to 38), or (ii) average methylation across 13 CpG sites (35 – 47) instead of providing CpG specific associations. Anyhow, longitudinal follow-up of these infants will be crucial in order to assess the long-term impact of these methylation patterns and whether they last into adulthood. Interestingly, an independent study measuring HPA axis functioning in adults as associated with *NR3C1* methylation also reported a correlation between methylation at 1_F CpG sites 41 to 47 and cortisol levels after the Dex/CRH challenge, further supporting the role of epigenetic modifications at this region in neuroendocrine systems.[39] Additionally, it would be interesting to assess DNA methylation in prenatally stressed newborns in CpG sites 40 to 47, as it seems to be associated with several neurobehavioral and neuroendocrine outcomes.[36-38] Nevertheless, meta-analyzed papers which already assessed methylation in these extended regions did not report any correlation between pregnant anxious-depressive symptomatology and methylation at CpG sites 40–47, thus implying that prenatal stress may not modulate neurobehavior and neuroendocrine response as mediated by *NR3C1* methylation.[27-29]

Prenatal stress-induced methylation changes are thought to partially arise due to increased fetal circulating cortisol levels. Recent evidence points to the essential role of the placental enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in regulating the cortisol flux from the mother to the fetus. Maternal cortisol levels increase progressively during pregnancy. Since high concentrations of cortisol are deleterious to fetal development, 11β-HSD2 is required to protect the fetus by oxidizing cortisol into its less active metabolite cortisone.[40] Accordingly, evidence supports a critical role of this enzyme in preventing cortisol damaging effects; lower 11β-HSD2 activity has been

associated with lower birth weight in both humans and rodents.[41,42] Generally, the experience of psychosocial stress entails the overproduction of cortisol. As previously stated, 11β-HSD2 protects the fetus from the increased cortisol levels present in maternal blood; nevertheless, it has been described in rats that, in the presence of chronic maternal stress exposure, 11β-HSD2 activity gets downregulated;[43] furthermore, a posterior study determined that the placental effects of this enzyme come from the fetus and not the mother.[44] Interestingly, a recent study reported similar effects due to exposure to maternal prenatal anxiety in humans, prenatal trait anxiety scores negatively correlated to placental 11β-HSD2 mRNA expression.[45] This decrease on 11β-HSD2 expression is thought to be mediated, at least partially, by epigenetic mechanisms such as DNA methylation.[46] This evidence points to a fetal overexposure to cortisol in stressed pregnant mothers, which may induce fetal epigenetic changes in glucocorticoid-related genes as the one reported herein. Thus, further studies analyzing the interaction between *HSD11B2* and *NR3C1* methylation are needed to disentangle the role of each gene in mediating prenatal experiences into adulthood.

Besides, as pointed out by Braithwaite and colleagues,[30] newborn gender may be a key factor in order to assimilate prenatal stress into the epigenome. As they discuss, only male subjects exhibited higher *NR3C1* methylation after prenatal exposure to depressive maternal status during pregnancy whereas females tended to be hypomethylated when exposed to prenatal depression. In this sense, the sex-specific programming effects of prenatal stress have been widely discussed in a number of reviews, as they have been repeatedly reported during the last decade in both rodents and humans.[25,47-50] Interestingly, the placenta behaves differently depending on the fetal sex,[51] which could be one of the mediating mechanisms of the increased male mortality after exposure to stressful experiences during pregnancy.[52] This sexual dimorphism is currently under study, as its underlying causal mechanisms remain unknown. This topic is of major relevance in the psychiatric field, as most of the more prevalent mental disorders are sex-biased and this different sensitivity to maternal prenatal stress could be one of the involved mechanisms. From an evolutionary point of view, females have adapted in order to protect their offspring under a threat, which can be promoted by anxious behavior (tend and befriend hypothesis), while males have adapted to abandon their offspring and look for new mates, which may involve aggression (flight or fight hypothesis).[53] Nevertheless, these hypotheses cannot be tested in this meta-analysis as Braithwaite and colleagues[30] were the only group to study methylation in separate analyses depending on newborn gender. As a consequence, when averaging male and female methylation values, the significance of their findings is lost (see **Fig. 4**). Thus, gender-specific effects should be further examined in future studies assessing *NR3C1* methylation.

Notoriously, methylation measures reported by Oberlander and colleagues[27] differ considerably from the ones collected in this meta-analysis. This is due to a new calculation of unadjusted correlation coefficients, provided by Oberlander for proper meta-analysis calculation, instead of analyzing the previously reported adjusted regressions. As a consequence, exposure to serotonin reuptake inhibitors during pregnancy was neglected from the meta-analysis in order to compare data from the different papers meta-analyzed herein. Combining adjusted and unadjusted findings in mixed research synthesis can lead to artificial associations; given the nature of meta-analyzed studies, inclusion of exclusively unadjusted results appeared more appropriate.[54] Nevertheless, this divergence is intriguing as it could suggest a protective role for serotonin reuptake inhibitor treatment during pregnancy, which may be minimizing the anxious-depressed-mediated methylation observed in offspring. Therefore, antidepressant treatment should be thoroughly investigated as a potential protective factor in further studies.

In the frame of epigenome-wide association studies (EWAS) examining prenatal stress, it is surprising to find no reference to either exon 1_F of the *NR3C1* gene or other related genes such as *HSD11B2*. On the contrary, these studies detected non-replicated significant hits in a number of genes such as *MORC1, LTA, SGC5, TNFRSF21, CHRNA2,* or *COL7A1*, among others.[55-58] Interestingly, antidepressant treatment during pregnancy seems to be a major influencing factor, which would be in line with the discussion above. In this regard, Non and colleagues reported several changes in methylation only in the non-medicated depressed group when compared to healthy controls.[57] On the other hand, Schroeder and colleagues found a significant methylation change at 2 CpG sites only in newborns exposed to antidepressants, while no global effect of maternal depression could be found. One of the limitations of this study is that the comparison group was recruited at a psychiatric center and, therefore, all controls had a lifetime history of at least one psychiatric disorder.[58] Of note, 2 EWAS detected associations between *NR3C1* CpG-specific methylation and prenatal environment: Non et al.[57] found an association between methylation at an exon 1_C located CpG site and non-medicated anxious/depressed group; Teh et al.[59] found a gene-environment effect explaining a variably methylated CpG site located at an intronic region of *NR3C1* gene; one the environmental variables assessed in this study was maternal depression during pregnancy as measured by EDPS.

As briefly stated in the introduction, we excluded 2 papers analyzing *NR3C1* methylation in the offspring of mothers suffering from PTSD; their results were not eligible to be meta-analyzed herein due to a number of issues. On the one hand, Perroud et al.[13] studied CpG site-specific methylation in the offspring of mothers exposed and non-exposed to the Rwandan genocide. Nevertheless, they analyzed a posterior region of exon 1_F that exceeds its coding region, starting in CpG site 38. Although they reported a clear hypermethylation in this region associated with maternal PTSD status, there was no effect in CpG sites 38 and 39. Furthermore, this hypermethylation appeared coupled with lower cortisol levels, which cannot be explained by lower GR expression; the authors discussed a preponderant role of the mineralocorticoid receptor rather than GR in the pathology of PTSD. On the other hand, Yehuda et al.[12] assessed the effects of transgenerational PTSD; however, they did not study prenatal stress as their included subjects were born after the World War II and the transversal nature of the study does not

allow knowing whether parents were suffering from PTSD specifically before, during, or after pregnancy. In any case, maternal PTSD was associated with lower *NR3C1* methylation, thus supporting opposite directions of the effect of maternal depression- and PTSD-derived epigenetic modifications affecting HPA axis functioning. Intriguingly, paternal rather than maternal PTSD status was associated with their offspring's higher methylation thus suggesting that the sex-specific effects discussed above are not limited to the assessed subjects but also affect their progenitors. Finally, both studies analyzed peripheral blood of either children or adult subjects, thus including a potential confounder due to immeasurable effects of life events experienced during later stages of life.

Interestingly, the own nature of the meta-analyzed data points to a major homogenous effect of anxious-depressive spectrum disorders in increasing CpG-specific methylation at the *NR3C1* exon 1_F promoter. Both war stress and intimate partner violence may be too different from diagnoses of either anxiety or depression and include a number of additional confusing factors into the analysis such as ethnicity (since Mulligan et al. study was performed in the Democratic Republic of Congo and Radtke et al. assessed a very reduced sample with a wide ethnic heterogeneity, further detailed in **Table 1**) and assessment timing (since Radtke et al. analyzed methylation in children instead of newborns).

There are some limitations to be noted regarding the reviewed papers herein. First, each paper assessed DNA methylation by means of distinct technologies including pyrosequencing, mass spectrometry, and Sanger sequencing of cloned vectors. This variability might be biasing the meta-analysis results as each technique has its own limitations. As a matter of fact, both Murgatroyd et al.[31] and Hompes et al.[29] reported methylation values as for pairs of CpG sites due to the reduced resolution of mass spectrometry analyses thus compromising the comparison. Second, none of the studies explored paired GR expression in association with methylation; therefore, the phenotypic effect of CpG site 36 methylation remains unknown. Third, the timing of the psychiatric assessment is fundamental since the developmental effects of a single stressor fluctuate substantially according to different windows of susceptibility.[19] Accordingly, the epigenetic modulation caused by anxious-depressive diagnosis during pregnancy seems to impact harder the fetuses when it takes place during the early stages of pregnancy as shown both in animal and human studies.[25,27,29] Thus, the restricted correlations reported by Conradt et al.[28] could be due to inclusion of women who experienced either depression or anxiety at any time during pregnancy without specifying the trimester. Fourth, given the length of the *NR3C1* promoter area and the preliminary results correlating DNA methylation at additional CpG sites to distinct early stressors, other regions besides exon 1_F could have been investigated. Fifth, all the methylation analyses were performed in peripheral tissues such as cord blood or saliva (see **Table 1** for further details), which calls into question the comparability with brain measures; nevertheless, similar *NR3C1* methylation findings have been found in both peripheral and brain tissues in relation with early adversity thus suggesting the tissue unspecificity of the signatures elicited by psychosocial stress in this gene.[22] Furthermore, *NR3C1* peripheral methylation and its correlated GR differential expression may be informative on their own as a substantial component of cortisol's actions occurs at a peripheral level. Finally, the overall methylation differences reported by all the reviewed papers, when specified, are modest (below 5 percent) and significance should be interpreted with caution.

There are also several limitations to be noted in this meta-analysis. To begin with, prenatal anxious-depressive disorder and exposure to violence may exert differential effects upon fetal methylation; we have tried to avoid this confounder by excluding certain prenatal stressors such as smoking or PTSD status as their long-term effects on offspring's health are unclear. Furthermore, the limited number of papers on this topic restricts the statistical power of this meta-analysis; nevertheless, the relatively large final sample size (977 subjects), together with the finding of replicated and significant results, strengthens the notion of epigenetically-driven changes modulated by very early environment. Finally, these results should be interpreted with caution since the correlation value is low (0.14). Further studies assessing newborn methylation with a CpG-specific level resolution controlling for prenatal maternal psychiatric diagnosis at specific time points of pregnancy are needed to replicate this correlation, whereas longitudinal studies are required to disentangle the long-term effects of this methylation in adult health.

Materials and Methods

Search strategy and exclusion criteria

Research was conducted via a systematic literature search in the PubMed and PsycINFO databases. The search terms were the following: «"*NR3C1* or glucocorticoid receptor" and "methylation" and "prenatal stress or maternal psychiatric disorder"».

When necessary, corresponding authors of the selected papers were contacted in order to obtain data required for the meta-analysis. Selection of specific CpG sites to be studied was in accordance with the reviewed papers coverage.

NR3C1 gene and CpG island structure

NR3C1 gene contains 9 exons. The first of them includes 9 alternate untranslated variants (1A to 1J, excluding 1G) that are thought to influence GR tissue distribution, mRNA stability and translational efficiency. [60] Seven of these 9 alternate first exons are located within a CpG island spanning 3 kb that has been extensively studied regarding its methylation.

Statistical analysis

All statistical analyses were performed in R.[61] Since both psychosocial stress and methylation levels are typically measured in continuous scales and, thus, authors typically report correlation coefficients, meta-analyses of correlations were performed. However, as not all manuscripts provide the same effect size measure (for instance, authors may report mean differences or t-statistics), correlation coefficients were estimated when necessary using R's compute.es package.[62] This package allows statistics from one

Table 1. Summary of data considered for meta-analysis and potential confounders.

Authors	Main stress outcome	Timing of the stressor	n	Tissue	Methylation assessment	Ethnicity	Observations
Oberlander et al. 2008[27]	Maternal depression or anxiety during pregnancy, as assessed by HAM-D, HAM-A and EPDS	Psychiatric assessments at 2nd and 3rd trimesters	136	Cord blood	Pyrosequencing	Canadian recruited sample	This is the first study to explore *NR3C1* methylation in humans in relation to early stress. Loss of significance due to unadjusted nature of the meta-analyzed results.
Radtke et al. 2011[33]	Prenatal exposure to intimate partner violence, as assessed by CAS	During pregnancy (all trimesters)	23	Whole blood (at 14.1 ± 0.5 years)	Sodium bisulfite mapping, cloning and Sanger sequencing	Mixed origin of the sample including Turkey, Iraq and Kosovo, among others	This is the only study that studied pre-adolescent and adolescent subjects instead of newborns (or infants).
Mulligan et al. 2012[32]	War stress, as assessed by trauma surveys	During pregnancy (all trimesters)	25	Cord blood	Sodium bisulfite mapping, cloning and Sanger sequencing	100% of the sample was recruited at the Democratic Republic of Congo	Although Mulligan et al. reported an overall significant effect of war stress upon NR3C1 methylation, they found no significant effect of individual CpG sites.
Hompes et al. 2013[29]	Maternal depression or anxiety during pregnancy, as assessed by EPDS and PRAQ	Psychiatric assessments at 1st, 2nd and 3rd trimesters	74	Cord blood	MALDI-TOF mass spectrometry	93% of the sample was Belgian	Conflicting results may be due to the study of methylation in CpG units rather than in individual CpG sites.
Conradt et al. 2013[28]	Maternal depression or anxiety during pregnancy, as assessed retrospectively from clinical records and interviews	Retrospective psychiatric assessment (all trimesters)	482	Placenta(fetal origin)	Pyrosequencing	73% of the sample was Caucasian	Since the included sample in Conradt et al. study is several folds higher than the others, their reported associations drive the meta-analysis results, even in a random effects model as can be seen by the accounted weight.
Braithwaite et al. 2015[30]	Maternal depression during pregnancy, as assessed by EPDS	2nd or 3rd trimester of pregnancy	56	Buccal swabs (collected at 53.6 ± 9.99 days)	Pyrosequencing	89% of the sample was Caucasian	They successfully replicated Weaver and colleagues' findings in rats in male subjects but not in the females.As DNA methylation was examined in buccal swabs instead of cord or whole blood, replication of results suggests inter-reliability between tissues.
Murgatroyd et al. 2015[31]	Maternal depression during pregnancy, as assessed by EPDS	2nd trimester of pregnancy (20 weeks)	181	Saliva (collected at 14 months)	MALDI-TOF mass spectrometry	96% of the sample identified themselves as white British	This is the only study to date to examine both prenatal and postnatal depression and their interaction on offspring methylation. They also described a moderating role of maternal stroking in final methylation values.As DNA methylation was examined in saliva instead of cord or whole blood, replication of results suggests inter-reliability between tissues.

Abbreviations: CAS, composite abuse scale; EPDS, Edinburgh Postnatal Depression Scale; HAM-A, Hamilton Rating Scale for Anxiety; HAM-D, Hamilton Rating Scale for Depression; PRAQ, pregnancy related anxiety questionnaire.

study to be converted to many other effect size estimates, using standard procedures on meta-analysis methodology.[63]

The meta-analyses were conducted following conventional protocols as implemented in R's meta package.[64] The inverse variance weighting method was used for pooling. For comparison, sensitivity analyses were conducted using both fixed and random effects. Since there were no large differences between them, and as random effects models are especially suitable for sets of studies with different methods and samples, the Results section only shows the outcome of random effects models.[65]

Considering that sample sizes of the studies in the meta-analyses are relatively small, the Fisher's z transformation of correlations was conducted, and residual heterogeneity was accounted for through the DerSimonian-Laird estimator, in accordance with ordinary guidelines on meta-analysis methods.[63,64]

To assess between-study differences, the next measures of heterogeneity and variability were computed: τ^2 (estimated amount of total heterogeneity), I^2 (total heterogeneity / total variability), H (square root of the χ^2 heterogeneity statistic / degrees of freedom), and Cochran's Q (evaluation of whether the outcome variability is greater than expected based on sampling variability).[66]

Publication bias was assessed by funnel plot asymmetry tests. In the present context, these tests should be regarded as exploratory due to the small number of studies in each meta-analysis.[64,67]

Due to the presence of several stress/psychopathology assessment scales in most of the studies, selection of one informative scale per study was performed if necessary. For Oberlander et al. data, Edinburgh Postnatal Depression Scale (EPDS) measurements at 2nd trimester of pregnancy were selected (so Ham-A and EPDS scores at 3rd trimester were dismissed) in order to reduce heterogeneity between studies given (i) the major force of Conradt et al. results driving the meta-analyses' final results, which were obtained in depressed pregnant mothers, (ii) the timing of Hompes et al. and Murgatroyd et al. results, which were restricted to the 2 first trimesters of pregnancy, and (iii) the timing of the effects reported in animal models, which also are restricted to early stages of pregnancy.[25] For Hompes et al. study, available data was extracted from their published paper thus selecting average PRAQ scores for CpG sites 35–36 analyses and EPDS scores for CpG sites 37–39, following the aforementioned criteria. For Mulligan et al. paper, war stress was selected as the variable of interest as it had the largest effect on birth weight.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Kofink D, Boks MPM, Timmers HTM, Kas MJ. Epigenetic dynamics in psychiatric disorders: environmental programming of neurodevelopmental processes. Neurosci Biobehav Rev 2013; 37:831-45; PMID:23567520; http://dx.doi.org/10.1016/j.neubiorev.2013.03.020
2. Naumova OY, Lee M, Rychkov SY, Vlasova NV, Grigorenko EL. Gene expression in the human brain: the current state of the study of specificity and spatiotemporal dynamics. Child Dev 2013; 84:76-88; PMID:23145569; http://dx.doi.org/10.1111/cdev.12014
3. Colantuoni C, Lipska BK, Ye T, Hyde TM, Tao R, Leek JT, Colantuoni EA, Elkahloun AG, Herman MM, Weinberger DR, et al. Temporal dynamics and genetic control of transcription in the human prefrontal cortex. Nature 2011; 478:519-23; PMID:22031444; http://dx.doi.org/10.1038/nature10524
4. Hanson M a, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? Physiol Rev 2014; 94:1027-76; PMID:25287859; http://dx.doi.org/10.1152/physrev.00029.2013
5. Delisle H. Programming of chronic disease by impaired fetal nutrition. Evidence and implications for policy and intervention strategies. World Health Organization 2001; Retrieved from: http://www.who.int/nutrition/publications/obesity/WHO_NHD_02.3/en/
6. Williams JHG, Ross L. Consequences of prenatal toxin exposure for mental health in children and adolescents. Eur Child Adolesc Psychiatry 2007; 16:243-53; PMID:17200791; http://dx.doi.org/10.1007/s00787-006-0596-6
7. Glover V. Annual research review: prenatal stress and the origins of psychopathology: an evolutionary perspective. J Child Psychol Psychiatry 2011; 52:356-67; PMID:21250994; http://dx.doi.org/10.1111/j.1469-7610.2011.02371.x
8. Miller GE, Murphy MLM, Cashman R, Ma R, Ma J, Arevalo JMG, Kobor MS, Cole SW. Greater inflammatory activity and blunted glucocorticoid signaling in monocytes of chronically stressed caregivers. Brain Behav Immun 2014; 41:191-9; PMID:25242587; http://dx.doi.org/10.1016/j.bbi.2014.05.016
9. Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol 2005; 20:345-52; PMID:15893910; http://dx.doi.org/10.1016/j.reprotox.2005.04.005
10. Miller GE, Chen E, Fok AK, Walker H, Lim A, Nicholls EF, Cole S, Kobor MS. Low early-life social class leaves a biological residue manifested by decreased glucocorticoid and increased proinflammatory signaling. 2009; 106:14716-21; PMID:19617551; http://dx.doi.org/10.1073/pnas.0902971106
11. King S, Dancause K, Turcotte-Tremblay A-M, Veru F, Laplante DP. Using natural disasters to study the effects of prenatal maternal stress on child health and development. Birth Defects Res C Embryo Today 2012; 96:273-88; PMID:24203917; http://dx.doi.org/10.1002/bdrc.21026
12. Yehuda R, Daskalakis NP, Lehrner A, Desarnaud F, Bader HN, Makotkine I, Flory JD, Bierer LM, Meaney MJ. Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in holocaust survivor offspring. Am J Psychiat 2014; 171:872-80; PMID:24832930; http://dx.doi.org/10.1176/appi.ajp.2014.13121571
13. Perroud N, Rutembesa E, Paoloni-Giacobino A, Mutabaruka J, Mutesa L, Stenz L, Malafosse A, Karege F. The Tutsi genocide and transgenerational transmission of maternal stress: epigenetics and biology of the HPA axis. World J Biol Psychiat 2014; 15:334-45; PMID:24690014; http://dx.doi.org/10.3109/15622975.2013.866693
14. Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS, Turner RB. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proc

Natl Acad Sci U S A 2012; 109:5995-9; PMID:22474371; http://dx.doi.org/10.1073/pnas.1118355109
15. Lopez-Duran NL, Kovacs M, George CJ. Hypothalamic-pituitary-adrenal axis dysregulation in depressed children and adolescents: a meta-analysis. Psychoneuroendocrinology 2009; 34:1272-83; PMID:19406581; http://dx.doi.org/10.1016/j.psyneuen.2009.03.016
16. Lehrner A, Bierer LM, Passarelli V, Pratchett LC, Flory JD, Bader HN, Harris IR, Bedi A, Daskalakis NP, Makotkine I, et al. Maternal PTSD associates with greater glucocorticoid sensitivity in offspring of Holocaust survivors. Psychoneuroendocrinology 2014; 40:213-20; PMID:24485493; http://dx.doi.org/10.1016/j.psyneuen.2013.11.019
17. Mesiano S, Jaffe RB. Developmental and functional biology of the primate fetal adrenal cortex. Endocr Rev 1997; 18:378-403; PMID:9183569
18. Tarullo AR, Gunnar MR. Child maltreatment and the developing HPA axis. Horm Behav 2006; 50:632-9; PMID:16876168; http://dx.doi.org/10.1016/j.yhbeh.2006.06.010
19. Reynolds RM. Glucocorticoid excess and the developmental origins of disease: two decades of testing the hypothesis–2012 Curt Richter Award Winner. Psychoneuroendocrinology 2013; 38:1-11; PMID:22998948; http://dx.doi.org/10.1016/j.psyneuen.2012.08.012
20. Yang N, Ray DW, Matthews LC. Current concepts in glucocorticoid resistance. Steroids 2012; 77:1041-9; PMID:22728894; http://dx.doi.org/10.1016/j.steroids.2012.05.007
21. Holsboer F. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 2000; 23:477-501; PMID:11027914; http://dx.doi.org/10.1016/S0893-133X(00)00159-7
22. Palma-Gudiel H, Córdova-Palomera A, Leza JC, Fañanás L. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. Neurosci Biobehav Rev 2015; 55:520-35; PMID:26073068; http://dx.doi.org/10.1016/j.neubiorev.2015.05.016
23. Van Rossum EFC, Binder EB, Majer M, Koper JW, Ising M, Modell S, Salyakina D, Lamberts SWJ, Holsboer F. Polymorphisms of the glucocorticoid receptor gene and major depression. Biol Psychiatry 2006; 59:681-8; PMID:16580345; http://dx.doi.org/10.1016/j.biopsych.2006.02.007
24. Weaver ICG, Cervoni N, Champagne F a, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. Epigenetic programming by maternal behavior. Nat Neurosci 2004; 7:847-54; PMID:15220929; http://dx.doi.org/10.1038/nn1276
25. Mueller BR, Bale TL. Sex-specific programming of offspring emotionality after stress early in pregnancy. J Neurosci 2008; 28:9055-65; PMID:18768700; http://dx.doi.org/10.1523/JNEUROSCI.1424-08.2008
26. Stroud LR, Papandonatos GD, Rodriguez D, Mccallum M, Salisbury AL, Phipps MG, Lester B, Huestis MA, Niaura R, Padbury JF, et al. Maternal smoking during pregnancy and infant stress response: test of a prenatal programming hypothesis. Psychoneuroendocrinology 2014; 48:29-40; PMID:24999830; http://dx.doi.org/10.1016/j.psyneuen.2014.05.017
27. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 2008; 3:97-106; PMID:18536531; http://dx.doi.org/10.4161/epi.3.2.6034
28. Conradt E, Lester BM, Appleton AA, Armstrong DA, Marsit CJ. The roles of DNA methylation of NR3C1 and 11β-HSD2 and exposure to maternal mood disorder in utero on newborn neurobehavior. Epigenetics 2013; 8:1321-9; PMID:24135662; http://dx.doi.org/10.4161/epi.26634
29. Hompes T, Izzi B, Gellens E, Morreels M, Fieuws S, Pexsters A, Schops G, Dom M, Van Bree R, Freson K, et al. Investigating the influence of maternal cortisol and emotional state during pregnancy on the DNA methylation status of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood. J Psychiatr Res 2013; 47:880-91; PMID:23566423; http://dx.doi.org/10.1016/j.jpsychires.2013.03.009
30. Braithwaite EC, Kundakovic M, Ramchandani PG, Murphy SE, Champagne F a. Maternal prenatal depressive symptoms predict infant NR3C1 1F and BDNF IV DNA methylation. Epigenetics 2015; 10:408-17; PMID:25875334; http://dx.doi.org/10.1080/15592294.2015.1039221
31. Murgatroyd C, Quinn JP, Sharp HM, Pickles A, Hill J. Effects of prenatal and postnatal depression, and maternal stroking, at the glucocorticoid receptor gene. Transl Psychiatry 2015; 5:e560; PMID:25942041; http://dx.doi.org/10.1038/tp.2014.140
32. Mulligan CJ, D'Errico NC, Stees J, Hughes DA. Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. Epigenetics 2012; 7:853-7; PMID:22810058; http://dx.doi.org/10.4161/epi.21180
33. Radtke KM, Ruf M, Gunter HM, Dohrmann K, Schauer M, Meyer A, Elbert T. Transgenerational impact of intimate partner violence on methylation in the promoter of the glucocorticoid receptor. Transl Psychiat 2011; 1:e21; PMID:22832523; http://dx.doi.org/10.1038/tp.2011.21
34. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, Turecki G, Meaney MJ. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 2009; 12:342-8; PMID:19234457; http://dx.doi.org/10.1038/nn.2270
35. Sharp H, Pickles A, Meaney M, Marshall K, Tibu F, Hill J. Frequency of infant stroking reported by mothers moderates the effect of prenatal depression on infant behavioural and physiological outcomes. PLoS One 2012; 7:e45446; PMID:23091594; http://dx.doi.org/10.1371/journal.pone.0045446
36. Bromer C, Marsit CJ, Armstrong D a, Padbury JF, Lester B. Genetic and epigenetic variation of the glucocorticoid receptor (NR3C1) in placenta and infant neurobehavior. Dev Psychobiol 2013; 55:673-83; PMID:22714792
37. Appleton AA, Lester BM, Armstrong DA, Lesseur C, Marsit CJ. Examining the joint contribution of placental NR3C1 and HSD11B2 methylation for infant neurobehavior. Psychoneuroendocrinology 2015; 52:32-42; PMID:25459891; http://dx.doi.org/10.1016/j.psyneuen.2014.11.004
38. Conradt E, Fei M, LaGasse L, Tronick E, Guerin D, Gorman D, Marsit CJ, Lester BM. Prenatal predictors of infant self-regulation: the contributions of placental DNA methylation of NR3C1 and neuroendocrine activity. Front Behav Neurosci 2015; 9:130; PMID:26074794; http://dx.doi.org/10.3389/fnbeh.2015.00130
39. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. PLoS One 2012; 7:e30148; PMID:22295073; http://dx.doi.org/10.1371/journal.pone.0030148
40. Murphy B, Clark S, Donald I, Pinsky M, Velady D. Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. Am J Obstet Gynecol 1974; 118:538-41; PMID:4812574
41. Benediktsson R, Lindsay R, Noble J, Seckl JR, Edwards C. Glucocorticoid exposure in utero: New model for adult hypertension. Lancet 1993; 341:339-41; PMID:8094115; http://dx.doi.org/10.1016/0140-6736(93)90138-7
42. Murphy VE, Zakar T, Smith R, Giles WB, Gibson PG, Clifton VL. Reduced 11β-hydroxysteroid dehydrogenase type 2 activity is associated with decreased birth weight centile in pregnancies complicated by asthma. J Clin Endocrinol Metab 2002; 87:1660-8; PMID:11932298
43. Welberg L a M, Thrivikraman KV, Plotsky PM. Chronic maternal stress inhibits the capacity to up-regulate placental 11beta-hydroxysteroid dehydrogenase type 2 activity. J Endocrinol 2005; 186:R7-R12; PMID:16135661; http://dx.doi.org/10.1677/joe.1.06374
44. Holmes MC, Abrahamsen CT, French KL, Paterson JM, Mullins JJ, Seckl JR. The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids. J Neurosci 2006; 26:3840-4; PMID:16597738; http://dx.doi.org/10.1523/JNEUROSCI.4464-05.2006
45. O'Donnell KJ, Bugge A, Freeman L, Khalife N, Connor TGO, Glover V. Maternal prenatal anxiety and downregulation of placental 11 b -HSD2. Psychoneuroendocrinology 2012; 37:818-26; PMID:22001010; http://dx.doi.org/10.1016/j.psyneuen.2011.09.014
46. Jensen Peña C, Monk C, Champagne F a. Epigenetic effects of prenatal stress on 11β-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. PLoS One 2012; 7:e39791; PMID:22761903; http://dx.doi.org/10.1371/journal.pone.0039791
47. Bale TL. Sex differences in prenatal epigenetic programming of stress pathways. Stress 2011; 14:348-56; PMID:21663536
48. Goel N, Bale TL. Examining the intersection of sex and stress in modelling neuropsychiatric disorders. J Neuroendocrinol 2009; 21:415-20; PMID:19187468; http://dx.doi.org/10.1111/j.1365-2826.2009.01843.x
49. Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne F a. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proc Natl Acad Sci U S A 2013; 110:9956-61; PMID:23716699; http://dx.doi.org/10.1073/pnas.1214056110
50. Van Os J, Selten JP. Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 Invasion of The Netherlands. Br J Psychiat 1998; 172:324-6
51. Clifton VL. Review: sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta 2010; 31 Suppl:S33-9; PMID:20004469; http://dx.doi.org/10.1016/j.placenta.2009.11.010
52. Catalano R, Yorifuji T, Kawachi I. Natural selection in utero: evidence from the Great East Japan Earthquake. Am J Hum Biol 2013; 25:555-9; PMID:23754635; http://dx.doi.org/10.1002/ajhb.22414
53. Glover V, Hill J. Sex differences in the programming effects of prenatal stress on psychopathology and stress responses: an evolutionary perspective. Physiol Behav 2012; 106:736-40; PMID:22353310; http://dx.doi.org/10.1016/j.physbeh.2012.02.011
54. Voils CI, Crandell JL, Chang Y, Leeman J, Sandelowski M. Combining adjusted and unadjusted findings in mixed research synthesis. J Eval Clin Pract 2011; 17:429-34; PMID:21040243; http://dx.doi.org/10.1111/j.1365-2753.2010.01444.x
55. Cao-Lei L, Massart R, Suderman MJ, Machnes Z, Elgbeili G, Laplante DP, Szyf M, King S. DNA methylation signatures triggered by prenatal maternal stress exposure to a natural disaster: project ice storm. PLoS One 2014; 9:e107653; PMID:25238154; http://dx.doi.org/10.1371/journal.pone.0107653
56. Nieratschker V, Massart R, Gilles M, Luoni A, Suderman MJ, Krumm B, Meier S, Witt SH, Nöthen MM, Suomi SJ, et al. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Transl Psychiat 2014; 4:e429; PMID:25158004; http://dx.doi.org/10.1038/tp.2014.75
57. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates exposed

to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics 2014; 9:964-72; PMID:24751725; http://dx.doi.org/10.4161/epi.28853
58. Schroeder JW, Smith AK, Brennan P a, Conneely KN, Kilaru V, Knight BT, Newport DJ, Cubells JF, Stowe ZN. DNA methylation in neonates born to women receiving psychiatric care. Epigenetics 2012; 7:409-14; PMID:22419064; http://dx.doi.org/10.4161/epi.19551
59. Teh AL, Pan H, Chen L, Ong M, Dogra S, Wong J, Macisaac JL, Mah SM, Mcewen LM, Saw S, et al. The effect of genotype and in utero environment on interindividual variation in neonate DNA methylomes. Genome Res 2014; 24:1064-74; PMID:24709820; http://dx.doi.org/10.1101/gr.171439.113
60. Turner JD, Vernocchi S, Schmitz S, Muller CP. Role of the 5′-untranslated regions in post-transcriptional regulation of the human glucocorticoid receptor. Biochim Biophys Acta 2014; 1839:1051-61; PMID:25150144; http://dx.doi.org/10.1016/j.bbagrm.2014.08.010
61. R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014. Retrived from: http://www.r-project.org
62. Del Re AC. Compute.es: compute effect sizes. 2014; Retrieved from: http://cran.r-project.org/web/packages/compute.es
63. Cooper H, Hedges LV, Valentine JC. The Handbook of Research Synthesis and Meta-Analysis. Second. New York: Russell Sage Foundation; 2009.
64. Schwarzer G. Meta: general package for meta-analysis. 2015; Retrieved from: https://cran.r-project.org/web/packages/meta/
65. Viechtbauer W. Conducting meta-analyses in R with the metafor package. J Stat Softw 2010; 36:1-48.
66. Cochran W. The combination of estimates from different experiments. Biometrics 1954; 10:101-29; http://dx.doi.org/10.2307/3001666
67. Sterne JAC, Sutton AJ, Ioannidis JPA, Terrin N, Jones DR, Lau J, Carpenter J, Rücker G, Harbord RM, Schmid CH, et al. Recommendations for examining and interpreting funnel plot asymmetry in meta-analyses of randomised controlled trials. BMJ 2011; 343:d4002; PMID:21784880; http://dx.doi.org/10.1136/bmj.d4002

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: a meta-analysis" included the following tasks:

- Participation in the conception and design of the study
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

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Increased methylation at an unexplored glucocorticoid responsive element within exon 1_D of *NR3C1* gene is related to anxious-depressive disorders and decreased hippocampal connectivity

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Abstract
Among the major psychiatric disorders, anxious-depressive disorders stand out as one of the more prevalent and more frequently associated with hypothalamic-pituitary-adrenal (HPA) axis abnormalities. Methylation at the exon 1_F of the glucocorticoid receptor gene *NR3C1* has been associated with both early stress exposure and risk for developing a psychiatric disorder; however, other *NR3C1* promoter regions have been underexplored. Exon 1_D emerges as a suggestive new target in stress-related disorders epigenetically sensitive to early adversity.

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After assessment of 48 monozygotic twin pairs (n=96 subjects) informative for lifetime history of anxious-depressive disorders, they were classified as concordant, discordant or healthy in function of whether both, one or neither twin in each pair had a lifetime diagnosis of anxious-depressive disorders. DNA for epigenetic analysis was extracted from peripheral blood. Exon 1_F and exon 1_D CpG-specific methylation was analysed by means of pyrosequencing technology. Functional magnetic resonance imaging was available for 54 subjects (n=27 twin pairs). Exon 1_D CpG-specific methylation within a glucocorticoid responsive element (GRE) was correlated with familial burden of anxious-depressive disorders (r=0.35, z=2.26, p=0.02). Right hippocampal connectivity was significantly associated with CpG-specific GRE methylation ($\beta = -2.33$, $t = -2.85$, p=0.01). Exon 1_F was uniformly hypomethylated across all subgroups of the present sample. GRE hypermethylation at exon 1_D of the *NR3C1* gene in monozygotic twins concordant for anxious-depressive disorders suggests this region plays a role in increasing vulnerability to psychosocial stress, partly mediated by altered hippocampal connectivity.

1. Introduction

Hypothalamic-pituitary-adrenal (HPA) axis abnormalities have been reported in a number of psychiatric disorders, with a preponderant involvement in stress-related disorders such as depression, anxiety and post-traumatic stress disorder. In this regard, the clinical heterogeneity exhibited by patients meeting diagnostic criteria for anxious-depressive psychopathology might depend on a number of biological correlates such as HPA axis functioning and inflammation (Lamers et al., 2012). Specifically, the HPA axis is known to be overactivated in a subset of anxious-depressed subjects, such that these patients exhibit increased cortisol levels and impaired stress reactivity mediated by glucocorticoid resistance (Pariante, 2017). Furthermore, excess cortisol has been described to compromise hippocampal integrity, measured in terms of both volume and connectivity, in depression (Campbell et al., 2004; Córdova-Palomera et al., 2016a).

Glucocorticoid resistance refers to the inability of body homeostasis to downregulate HPA axis activity after a stressor has ended; this downregulation occurs after binding of cortisol to the glucocorticoid receptor (GR) by means of a negative feedback mechanism. Glucocorticoid resistance has been suggested to be mediated by epigenetic desensitization of the GR via methylation in the promoter region of the *NR3C1* gene, which codes for the glucocorticoid receptor. In this regard, Weaver and colleagues found robust CpG-specific *Nr3c1* hypermethylation in rat pups reared by mothers exhibiting little maternal behavior (Weaver et al., 2004). The same research group had previously reported that rats reared by low-caring mothers exhibit decreased hippocampal GR expression, decreased glucocorticoid feedback sensitivity, and increased fearfulness under conditions of novelty; resembling an anxious-depressive-like phenotype (Francis et al., 1999). Human studies in postmortem brain tissue similarly described increases in *NR3C1* exon 1_F methylation coupled with decreased GR expression in the hippocampus of abused subjects who had committed suicide (McGowan et al., 2009).

In the light of these results, methylation of the human *NR3C1* gene has been extensively analysed in association with several psychiatric disorders and stressful stimuli (Palma-Gudiel et al., 2015b). Despite the complexity of the *NR3C1* promoter region, that research effort has focused on exon 1_F (the human homologue of exon 1_7 in rats). However, the promoter region of the *NR3C1* gene includes a total of nine alternative non-coding first exons (Turner and Muller, 2005); their tissue specificity and differential function is still under study and limited attention has been paid to methylation at the first exons other than 1_F (Palma-Gudiel et al., 2015b). Notably, exon 1_D is located in the 5′ end of a large CpG island spanning some 3 kb within the promoter region of the *NR3C1* gene (Fig. 1). CpG island shores have previously been highlighted as intermediate regions where methylation is more variable than within CpG islands themselves or in the so-called "open sea" (Ziller et al., 2013). Thus, exon 1_D emerges as a widely unexplored potential target whose methylation may contribute to modulating GR expression and to the physiopathology of stress-related disorders.

As methylation is known to modulate gene expression, differential methylation of the *NR3C1* gene may mediate brain changes, particularly in brain areas enriched in the GR, such as the hippocampus. Alterations in hippocampal functional connectivity have already been associated with major depressive disorder (Cao et al., 2012), anxiety disorder (Cui et al., 2016) and HPA axis reactivity in a healthy sample (Kiem et al., 2013). Nevertheless, few studies to date have analyzed the impact of *NR3C1* methylation on brain circuitry with a substantial scarcity of studies simultaneously assessing methylation, psychopathology and neuroimaging. Specifically, Na and colleagues were the only research group to focus on hippocampus as a target brain area putatively associated with both *NR3C1* methylation and depression in humans, reporting a positive association between *NR3C1* methylation and hippocampal subfields (Na et al., 2014). Hence, we aimed to analyze for the first time hippocampal functional connectivity, rather than

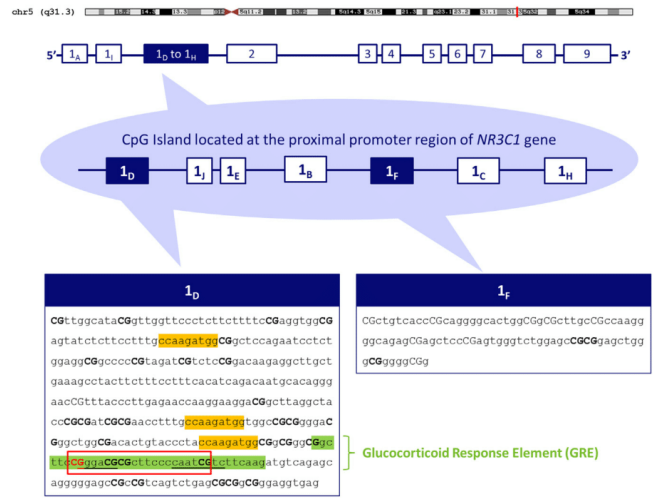

Fig. 1 Detailed sequence of CpG sites analyzed. *Top*: The *NR3C1* gene is located on chromosome 5, spanning around 150 kb (chr5:143,277,931-143,435,512; hg38 assembly). The *NR3C1* gene includes 9 alternative non-coding first exons and 8 coding exons. *Middle*: The *NR3C1* gene contains a CpG island (highlighted in light blue) spanning 3 kb, which includes 7 of the 9 non-coding first exons (1_D, 1_J, 1_E, 1_B, 1_F, 1_C, 1_H) of the *NR3C1* gene. The exons analyzed in the present study, 1_D and 1_F, are highlighted with dark blue boxes. *Bottom*: The genetic sequences of the regions assessed are shown; the CpG sites analyzed are highlighted with bold capital letters. The differentially methylated CpG site 21 is highlighted in red. The glucocorticoid responsive element (GRE) binding region is highlighted in green. Several transcription factor Yin Yang 1 (YY1) binding regions are highlighted in orange.

morphological features, as an explanatory variable underlying previously reported associations between peripheral *NR3C1* methylation and anxious-depressive vulnerability.

In order to analyse the putative role of exon 1_D methylation in the aetiology of anxious-depressive disorders, we examined a monozygotic (MZ) twin sample, comprised of discordant and concordant twin pairs for lifetime anxious-depressive disorders; and a control group of healthy twin pairs. We hypothesized that exon 1_D methylation would show higher variability than exon 1_F methylation, and that methylation at the former exon would be associated with both increased risk for suffering anxious-depressive disorders and reduced hippocampal connectivity in a sample of MZ twins.

2. Experimental procedures

2.1. Participants

Analysis was performed on a subset of 96 MZ twins (n=48 twin pairs) belonging to the UB twin register. This register consists of 230 adult Spanish twins (84.8% MZ; 66.1% female) from the general population who gave their authorization to be contacted for research purposes between May 2007 and April 2009; further details on this sample can be obtained elsewhere (Córdova-Palomera et al., 2016b). Briefly, lifetime psychiatric history was recorded, face-to-face, by means of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) administered by a trained psychologist (First et al., 1996). The subset (n=96) of the sample assessed for epigenetics of the *NR3C1* gene consisted of 13 concordant (n=26), 14 discordant (n=28) and 21 healthy (n=42) MZ twin pairs, based on whether both, one or neither twin met the DSM-IV criteria for a lifetime diagnosis of depression, anxiety or comorbid anxious-depressive psychopathology (see Table 1 for further demographic and diagnostic characteristics). Additionally, the Brief Symptom Inventory (BSI) was administered to all the subjects in order to assess psychiatric symptoms experienced during the last month, thus working as a state measure (Derogatis and Spencer, 1982). Peripheral blood samples were obtained from all the twins on the same day as the psychometric evaluation.

2.2. Methylation analysis

DNA methylation was quantified by means of bisulfite pyrosequencing. Two candidate regions within the 5′ UTR of the *NR3C1* gene were analyzed: *(i)* the classical exon 1_F, including three CpG sites found to be hypermethylated after prenatal stress in humans (Palma-Gudiel et al., 2015a) and homologous to those associated with perinatal stress in rats (Weaver et al., 2004); and *(ii)* the suggestive exon 1_D, including 30 CpG sites encompassing the whole exon. Details of the analysed sequence and CpG sites assayed can be found in Fig. 1.

Table 1 Demographic and clinical characteristics of assessed twin subjects.

Demographics	**Healthy twins**	**Discordant twins**	**Concordant twins**	
n (pairs)	42 (21)	28 (14)	26 (13)	
Gender (M/F)	18/24	10/18	2/24	
Mean age at assessment (SD)	31.1 (8.3)	32.4 (12)	36.7 (14.3)	
Age Range	19-46	18-50	20-54	
Lifetime DSM-IV diagnoses[a]	**Healthy twins**	**Discordant healthy**	**Discordant affected**	**Concordant twins**
MDD (single episode)	-	-	6	9
MDD (recurrent)	-	-	0	4
MDD (not otherwise specified)	-	-	3	4
Specific phobia	-	-	1	9
Social phobia	-	-	1	0
Anxiety disorder with agoraphobia	-	-	2	4
Anxiety disorder without agoraphobia	-	-	4	6
Anxiety disorder not otherwise specified	-	-	2	1
Number of subjects with comorbidities	-	-	4	10
Clinical variables	**Healthy twins**	**Discordant healthy**	**Discordant affected**	**Concordant twins**
Mean BSI score at assessment	10.4	17	21	40.5

Abbreviations: *SD* standard deviation; *BSI* brief symptom inventory; *MDD* major depressive disorder.
[a]The total sum of different diagnostic categories is higher than the number of subjects within the group due to the high prevalence of psychiatric comorbidities in our sample.

Genomic DNA (50 ng/μL) was extracted from the peripheral blood samples with a Realpure DNA SSS kit (Durviz). The EZ-96 DNA Methylation™ Kit (Zymo Research) was used for bisulfite conversion. Primers for the amplification of bisulfite-converted DNA were designed using Pyromark Assay Design 2.0 software (Qiagen). Further details of primer design for the PCR and sequencing reactions for all the regions of interest is included in Supplementary Table S1. All the pyrosequencing reactions were carried out in a PyroMark Q96 system (Qiagen). The pyrosequencing method allows quantification of the degree of methylation over selected CpG sites. CpG-specific methylation data were assessed using the Pyro Q-CpG software (Qiagen), expressed as percentages from 0 to 100%. This quantitative variable reflects the percentage of cells in which the CpG site of interest is methylated, since methylation is a dichotomous chemical modification (a CpG site is either methylated or non-methylated, with the exception of imprinted regions).

A total of 33 CpG sites were assessed, including (i) 3 CpG sites in exon 1_F which were previously identified as being associated with either early-life stress or psychopathology, and (ii) 30 CpG sites spanning the newly explored promoter region of exon 1_D (see Fig. 1 for details). Cases of potential errors due to incomplete bisulfite conversion or low signal peaks, detected by the Pyro Q-CpG software, were discarded. Six CpG sites located at exon 1_D were further excluded from analysis due to high number of missing values, defined as more than 30% of the total sample; thus, the final number of CpG sites included in statistical analysis consisted of (i) 3 CpG sites in exon 1_F and (ii) 24 CpG sites in exon 1_D.

2.3. Brain imaging acquisition and measures of hippocampal connectivity

A subset of 54 twins from the former cohort agreed to participate in the brain imaging section of the current study. These included 6 concordant twin pairs (n=12), 10 discordant twin pairs (n=20), and 11 healthy twin pairs (n=22). The participants included in the neuroimaging study did not differ from the total sample in terms of age or BSI scores (data not shown); since neuroimaging features are known to differ as a function of gender, the participants of this subsample were selected for a more balanced gender ratio than the total sample (58% of female participants versus 69% in the total sample). The scans were acquired at *Centro de Diagnóstico por la Imagen Clínic* (CDIC, Hospital Clínic de Barcelona), using an 8-channel head coil TIM TRIo 3 T scanner (Siemens, Erlangen, Germany). Each resting-state functional magnetic resonance (fMRI) dataset comprised 210 echo-palanar (EPI) blood-oxygen-level dependent (BOLD) sensitive volumes (TR=2790 ms, TE=30 ms, 3 mm slice thickness, no gap, 45 axial slices parallel to anterior-posterior commissure plane acquired in interleaved order, FOV=2075 × 1344 mm^2, voxel size=2.67 × 2.67 × 3 mm^3). In addition, high-resolution 3D anatomical reference datasets were acquired, using a T1-weighted magnetization prepared rapid gradient echo (MPRAGE sequence, TR=2300 ms, TE=3.03 ms, TI=900 ms, 192 sagital slices, 1 mm^3 isometric voxel, matrix size=256 × 256, flip angle=9°).

Briefly, brain functional connectivity was estimated from resting-state time series extracted via the Statistical Parametric Mapping software, version 8 (SPM8; Friston et al., 1995) in MATLAB (The Mathworks, Natick, MA). The fMRI slices were co-registered to the anatomical reference (3D, T1) and to the mean functional image after correction of slice-timing differences and head motion. The images were then normalized to the standard stereotaxic space MNI (Evans et al., 1993). Blood pulsation and instrumental artifacts were removed from the BOLD time series in MNI space, using independent component analysis

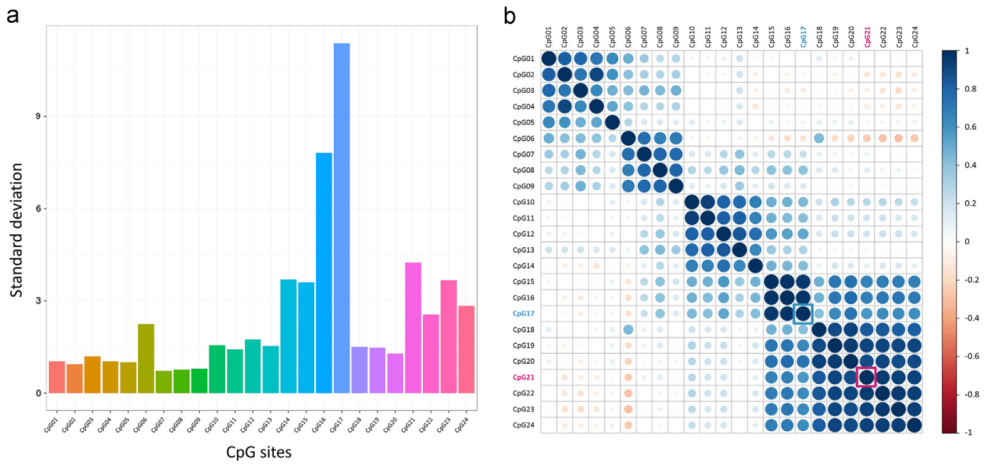

Fig. 2 Methylation variability across exon 1_D CpG sites. (a) Standard deviations for CpG-specific methylation at all selected *NR3C1* exon 1_D CpG sites (n=24). Higher standard deviation values correspond to CpG sites 16, 17 and 21; CpG site 16 was excluded from further analysis due to extremely high correlation with CpG site 17 values (r=0.97; see Supplemental Table S3). (b) CpG-specific methylation correlations among selected *NR3C1* exon 1_D CpG sites. Methylation values of all the CpG sites analyzed (n=24) were assessed for correlation across the region of interest in order to identify clusters of neighboring CpG sites with highly correlated methylation percentages. The methylation variable positions (MVPs) selected for analysis, CpG sites 17 and 21, are highlighted in blue and magenta respectively.

as implemented in GIFT (Calhoun et al., 2009; Sui et al., 2009). Mean time-series were then extracted from the regions of interest included in the Automatic Anatomical Labeling (AAL) atlas (Tzourio-Mazoyer et al., 2002), masked with binarized individual tissue probability maps. Further details on the imaging acquisition and pre-processing, together with extraction of functional connectivity networks for each individual have been reported previously (Córdova-Palomera et al., 2016b).

Hippocampal connectivity was analysed within the so-called emotional network, operationalized following neurosynth (Yarkoni et al., 2011) meta-analysis data on the emotional term (see Supplementary Table S2 for a detailed list of nodes included in the analysed network). To estimate connectivity within the emotional network, the fMRI time series were band-pass filtered within the narrowband from 0.04 to 0.07 Hz, and a partial correlation matrix was computed for each individual (Glerean et al., 2012). The functional connectivity matrices were normalized with Fischer's z transform; negative edges were removed with soft thresholding, since their network topology can affect the overall topology of fMRI networks (Schwarz and McGonigle, 2011). Finally, hippocampal connectivity was independently computed in right and left hippocampal nodes as the nodal strength within the aforementioned network. Nodal strength was selected as a proxy for connectivity since it directly reflects the overall connectivity of the node of interest and hence provides a simple interpretation of results from a biological point of view.

2.4. Statistical analysis

A number of linear models were devised to assess whether exon 1_D methylation of *NR3C1* could predict psychopathological vulnerability and/or hippocampal connectivity within the emotional network.

Most of the statistical analysis was performed using an MZ twin-based approach, thus working with the intrapair average value of each variable of interest, within each MZ twin pair. Briefly, MZ twins have been widely used in heritability studies in order to disentangle the extent to which a complex disorder is bound to additive genetic influences, given that MZ twins share 100% of the genome but partially differ in their environmental exposure. In fact, epigenetic differences between MZ co-twins increase with age (Fraga et al., 2005). In this regard, it has been suggested that concordant twins for a complex disorder of interest, i.e. anxious-depressive disorders, bear a greater genetic (or familial) burden for the disorder. Thus, comparison between concordant MZ twin pairs and discordant or healthy MZ twin pairs may help to identify new genetic (and epigenetic) candidates for involvement in complex disorders (Wolfensberger et al., 2008).

Complementarily, additional analysis was performed where the subjects were considered in a case-control approach (affected and non-affected subjects were compared regardless of their twin nature). Gender and age were included as predictor variables in all models. All statistical analysis was performed with R programming (R Development Core Team, 2011).

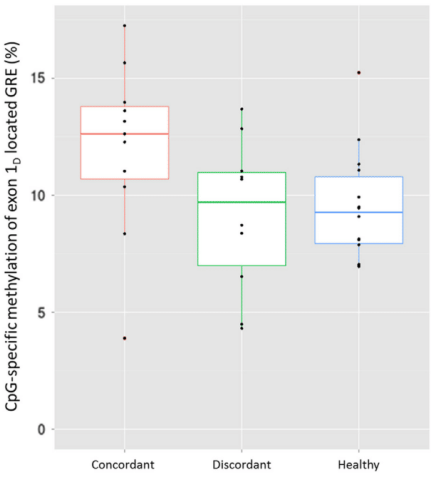

Fig. 3 Exon 1_D CpG-specific GRE methylation is higher in concordant twins. CpG site 21 methylation is higher in concordant twin pairs (mean methylation=12.0%) than in either healthy (9.5%) or discordant (9.5%) twin pairs. Each point of the graphic corresponds to the mean methylation value of a twin pair. Boxplot graphs display data distribution within each subgroup; thick horizontal lines correspond to median methylation values for each subgroup; boxes include all values within the interquartile range for each subgroup.

3. Results

3.1. Methylation profile of selected CpG sites in exon 1_F

The DNA methylation percentage was assessed in three consecutive CpG sites of exon 1_F previously identified to be associated with a number of early stressors. Exon 1_F did not reveal enough methylation variability, exhibiting uniformly low levels of methylation throughout the whole sample (0 to 3.4% methylation, SD=0.4). These CpG sites located in exon 1_F were thus excluded from further analysis, since the very limited methylation variability could not yield relevant results in the present sample (n=77).

3.2. Methylation profile of the newly explored exon 1_D

Regarding exon 1_D probes, statistical analysis was based on methylation variable positions (MVPs) reflecting CpG sites whose methylation pattern is particularly dynamic and which may reflect functional changes in gene activity and disease state (Gu et al., 2016). After a preliminary analysis of the methylation data, CpG sites 17 and 21 were selected for further analysis, since: (i) they exhibited the highest methylation variability across our sample (SD_{16}=11.38; SD_{21}=4.25; Fig. 2a); (ii) their methylation percentages were highly correlated to those of neighboring CpG sites (Fig. 2b); and (iii) the amount of missing values across the sample was modest (NA_{17}=11; NA_{21}=15). Detailed information of methylation data for each CpG site analyzed is further displayed in Supplementary Table S3.

3.3. Case-control analysis of exon 1_D methylation

When examining MVP methylation in relation to lifetime DSM-IV diagnosis of anxious-depressive disorders in a case-control approach (regardless of the twin nature of the sample), no association was found with either CpG site 17 (r=0.03; z=1.04; p=0.3) or CpG site 21 (r=0.09; z=1.41; p=0.16).

3.4. MZ twin based analysis of exon 1_D methylation

Subsequently, MVP methylation and psychopathological data were analyzed in a twin-based approach as described above. The MVP methylation percentage for each twin of a pair was averaged to give a single mean value per twin pair. Participants pertaining to concordant twin pairs were considered as subjects with high familial vulnerability to anxious-depressive disorders; while discordant and healthy twin pairs were considered as a comparison group with low familial vulnerability to anxious-depressive disorders. In this twin pair-based approach, concordant twins exhibited significantly higher CpG site 21 methylation than discordant and healthy twin pairs (r=0.35; z=2.26; p=0.02; Fig. 3). No significant methylation differences were found at CpG site 16 (r=0.06; z=1.24; p=0.21).

CpG site 21 methylation was further characterized as a function of both age and gender as they are known confounders in epigenetic research. CpG site 21 methylation was not significantly associated with either gender ($\beta=-0.02$; p=0.95) or age (r=0.12; p=0.3; see Supplementary Fig. S1). CpG site 21 hypermethylation of concordant twin pairs with regard to discordant and healthy twin pairs was also observed when testing only-females subjects; the male sub-sample was unpowered for such approach (data not shown).

3.5. Description of increased methylation at a glucocorticoid responsive element within exon 1_D promoter in twin pairs with high familial vulnerability to anxious-depressive disorders

Further bibliographic enquiring into this region revealed that the MVP CpG site 21 is located within a glucocorticoid response element (GRE); that is, a transcription factor binding region directly involved in HPA axis regulation. This GRE is composed of four partially overlapping transcription factor binding sites spanning 35 bp and includes four CpG sites (Fig. 1). Thus, a new analysis was performed with the average methylation value of these 4 neighbouring CpG sites (CpG sites 18, 19, 20 and 21) which will be referred to as GRE methylation hereinafter. GRE methylation ranged across the subjects from 2.53% to 11.7% methylation (x=6.07, SD=2.01). GRE methylation predicted higher vulnerability to anxious-depressive disorders in our MZ twin pair based approach (β=0.16, t=2.48, p=0.03).

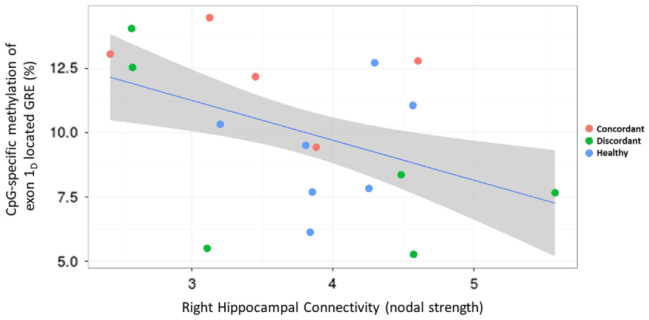

Fig. 4 Exon 1_D CpG-specific GRE methylation is negatively correlated to right hippocampal connectivity. Mean intrapair right hippocampal connectivity within the emotional network predicted mean intrapair CpG-specific GRE methylation of *NR3C1* exon 1_D. Each dot represents one of the twin pairs analysed for who both variables were available for both twins in the pair. Each dot has been colour-coded to show the concordant, discordant or healthy nature of the twin pairs. It should be noted that all the concordant twin pairs cluster towards the upper left sector of the graphic, thus highlighting the increased methylation and decreased connectivity in the subgroup exhibiting high familial vulnerability to anxious-depressive disorders.

3.6. Hippocampus connectivity and CpG-specific GRE methylation

Given the association between GRE methylation and familial burden for anxious-depressive disorders, hippocampal connectivity was incorporated into the statistical model. Due to the limited sample size (n=18 twin pairs in which both neuroimaging and methylation data were available for both co-twins), an interaction model could not be devised. Accordingly, due to the higher amount of missing values for CpG site 20 with regard to the other GRE CpG sites, mean GRE methylation could not be included in the model (see Supplementary Table S3). Nonetheless, a linear model was fitted to predict CpG-specific GRE methylation (CpG 21) as a function of both hippocampal connectivity and familial vulnerability to anxious-depressive disorders as independent predictor variables together with age and gender. Right and left hippocampal connectivity were analyzed separately. Right hippocampal connectivity within the emotional network and low familial vulnerability to anxious-depressive disorders were significantly associated with CpG-specific GRE methylation ($\beta=-2.33$, $t=-2.85$, $p=0.01$ and $\beta=-3.48$, $t=-2.73$, $p=0.02$, respectively; Fig. 4). Left hippocampal connectivity was not associated with CpG-specific GRE methylation ($\beta=-0.08$, $t=-0.13$, $p=0.9$).

4. Discussion

This is the first study to date of the putative implication of methylation at a functional GRE located within the hitherto unexplored exon 1_D of the *NR3C1* gene in the etiology of stress-related disorders. GRE methylation was found to be positively associated with both an increased familial burden of anxious-depressive disorders and poorer right hippocampal connectivity in an MZ twin sample.

Methylation of the *NR3C1* gene has been a prominent focus of epigenetics research into mental health for over a decade. Nevertheless, this research effort has mainly concentrated on exon 1_F, which only represents a limited fraction of the promoter region of the gene. Notably, *NR3C1* transcripts containing the herein described as differentially methylated exon 1_D were found to encode the membrane isoform of GR (mGR) more frequently than any other alternate first exon of the *NR3C1* gene (Turner et al., 2014). This widely unknown mGR has the capacity to bind to cortisol from the cell membrane, which allows the very rapid *non-genomic* effects of glucocorticoids to occur (Vernocchi et al., 2013). Additionally, tissue-specificity analysis revealed *NR3C1* transcripts containing exon 1_D to be enriched in peripheral tissues (Presul et al., 2007). The differentially methylated GRE described herein was found to be essential for glucocorticoid-mediated apoptosis in lymphoblasts (Geng et al., 2008) suggesting the functional implication of this GRE in mediating downstream effects of glucocorticoid signaling, i.e. unavailability of this GRE would involve immune dysfunction, which has been described as a clinical feature of a subset of depressed patients (Dowlati et al., 2010). Given that accessibility to this GRE is required in order for transcription factors to bind to it and trigger autoregulation of the GR after cortisol release, methylation at this particular region of exon 1_D may result in a profound change in HPA axis functioning.

To the best of our knowledge, no previous research design has combined DNA methylation measurements of the *NR3C1* gene with resting-state functional connectivity in anxious-depressive psychopathology. Nevertheless, the observed association between increased GRE methylation and decreased hippocampal connectivity is in line with prior research. In a sample of healthy males, lower hippocampal connectivity correlated to higher cortisol and ACTH concentration after dexamethasone challenge (Kiem et al.,

2013); these results point to the presence of decreased hippocampal connectivity in the context of impaired HPA axis regulation. Furthermore, baseline activation of the HPA axis has been reported to influence amygdala connectivity within emotion processing networks (Veer et al., 2012). In the context of our current findings, we hypothesize differential *NR3C1* DNA methylation to impair HPA axis regulation; afterwards, overexposure to cortisol would decrease hippocampal connectivity and, ultimately, negatively impact mood and behavior. Thus, disrupted hippocampal connectivity patterns might underlie the association between increased DNA methylation at exon 1_D of the *NR3C1* gene and anxious-depressive psychopathology in adults, probably through decreased peripheral GR expression and a subsequent overexposure to cortisol in response to stressful events.

To our knowledge, only two previous studies have reported exon 1_D methylation in humans. First, Hogg and colleagues found increased placental exon 1_D methylation in women suffering early onset pre-eclampsia during pregnancy when compared to healthy pregnant women (Hogg et al., 2013). More recently, Monk and colleagues reported placental methylation patterns in glucocorticoid pathway genes, including *NR3C1*, to be associated with maternal distress during pregnancy (Monk et al., 2016). Although the authors did not report any significant association between maternal cortisol during pregnancy and methylation at any CpG block assayed, they did observe two significant negative correlations between CpG-specific methylation and maternal cortisol. Strikingly, these associations lay in two nearby CpG sites located within exon 1_D. Therefore, methylation at exon 1_D might only be plastic (i.e. susceptible to environmental insults) during the earliest stages of life.

Alternatively, higher methylation at exon 1_D could have been inherited directly from exposed progenitors. Although the search for biological mechanisms and clinically relevant outcomes of transgenerational inheritance of epigenetic patterns is quite controversial (Mill and Heijmans, 2013), it is increasingly acknowledged in a number of fields and has been suggested to mediate exposure to stressful events across several generations in rodents (Franklin et al., 2010) and humans (Yehuda et al., 2016). The fact that CpG-specific GRE methylation was not associated with age further points to its long-term stability, at least throughout adulthood (age range of the current sample included 18 to 54 years old). Consequently, our results point to the existence of differential pathways leading to anxious-depressive disorders depending on the familial burden of the disorder. While concordant twins would share an epigenetic pattern of risk, discordant twins would exhibit distinct life trajectories – including life events, trauma, drug use, among others – which have the capacity to modify the epigenetic pattern at later stages (Kendler and Halberstadt, 2013). Thus, the differences in methylation identified in this work highlight the putative prenatal origin of epigenetic patterns relevant for later anxious-depressive disorders.

There are several limitations of this study to be noted. First, due to the moderate sample size (n=96), our results are to be interpreted with caution; in this regard, the non-significant association between methylation and connectivity in the concordant subgroup is probably due to insufficient effect size. Nevertheless, the sample studied was comprised of MZ twins, which removes genetic confounding from the analysis. Second, DNA methylation was measured at the peripheral level; however, methylation of the *NR3C1* gene in peripheral blood is meaningful on its own, since both the mGR and cGR are well known to be functional in peripheral blood cells. Third, DNA methylation was not measured in a cell-type specific manner; thus, differences in DNA methylation could be due to different blood cell composition across diagnostic subgroups; in this regard, methylation assessment at different regions of *NR3C1* gene has been frequently analyzed in heterogeneous tissues such as brain, placenta or peripheral whole blood as in the present study (Palma-Gudiel et al., 2015b). Furthermore, absolute values of methylation differences are small and their biological relevance could thus be brought into question; nevertheless, although mGR is expressed at very low levels, it is still described as clinically relevant, which opens the way to speculation concerning small methylation-mediated perturbations in its expression also being clinically relevant. Finally, we do not have twin-derived data on either GR expression or maternal stress experienced during early life or during pregnancy to test our hypotheses regarding the functional consequences of exon 1_D GRE methylation and its putative origin, respectively; further expression studies testing the functional correlates of methylation at *NR3C1* exon 1_D GRE are needed together with longitudinal prospective studies to explore the role of environmental risk factors in triggering methylation at this region.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

Lourdes Fañanás designed the study and revised and edited the manuscript. Helena Palma-Gudiel and Aldo Córdova Palomera performed the epigenetic analyses, analyzed the data and drafted the manuscript. Cristian Tornador and Carles Falcón performed the neuroimaging acquisition procedures. Núria Bargalló and Gustavo Deco revised and edited the manuscript. All authors approved the final manuscript.

Role of the funding source

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2018.03.015.

References

Calhoun, V.D., Liu, J., Adali, T., 2009. A review of group ICA for fMRI data and ICA for joint inference of imaging, genetic, and ERP data. Neuroimage 45, S163–S172. http://dx.doi.org/10.1016/J.NEUROIMAGE.2008.10.057.

Campbell, S., Marriott, M., Nahmias, C., MacQueen, G.M., 2004. Lower hippocampal volume in patients suffering from depression: a meta-analysis. Am. J. Psychiatry 161, 598–607. http://dx.doi.org/10.1176/appi.ajp.161.4.598.

Cao, X., Liu, Z., Xu, C., Li, J., Gao, Q., Sun, N., Xu, Y., Ren, Y., Yang, C., Zhang, K., 2012. Disrupted resting-state functional connectivity of the hippocampus in medication-naïve patients with major depressive disorder. J. Affect. Disord. 141, 194–203. http://dx.doi.org/10.1016/j.jad.2012.03.002.

Córdova-Palomera, A., de Reus, M.A., Fatjó-Vilas, M., Falcón, C., Bargalló, N., van Den Heuvel, M.P., Fañanás, L., 2016a. FKBP5 modulates the hippocampal connectivity deficits in depression: a study in twins. Brain Imaging Behav.. http://dx.doi.org/10.1007/s11682-015-9503-4.

Córdova-Palomera, A., Tornador, C., Falcón, C., Bargalló, N., Brambilla, P., Crespo-Facorro, B., Deco, G., Fañanás, L., 2016b. Environmental factors linked to depression vulnerability are associated with altered cerebellar resting-state synchronization. Sci. Rep. 6, 37384. http://dx.doi.org/10.1038/srep37384.

Cui, H., Zhang, J., Liu, Y., Li, Q., Li, H., Zhang, L., Hu, Q., Cheng, W., Luo, Q., Li, J., Li, W., Wang, J., Feng, J., Li, C., Northoff, G., 2016. Differential alterations of resting-state functional connectivity in generalized anxiety disorder and panic disorder. Hum. Brain Mapp. 37, 1459–1473. http://dx.doi.org/10.1002/hbm.23113.

Derogatis, L., Spencer, M., 1982. The Brief Symptom Inventory (BSI): Administration, Scoring, and Procedures Manual-I. Johns Hopkins University School of Medicine, Clinical Psychometrics Research Unit, Baltimore.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., Lanctôt, K.L., 2010. A meta-analysis of cytokines in major depression. Biol. Psychiatry 67, 446–457. http://dx.doi.org/10.1016/j.biopsych.2009.09.033.

Evans, A.C., Collins, D.L., Mills, S.R., Brown, E.D., Kelly, R.L., 1993. 3D statistical neuroanatomical models from 305 MRI volumes. Nuclear Science Symposium and Medical Imaging Conference, IEEE Conference Record. pp. 1813–1817.

First, M.B., Spitzer, R.L., Gibbon, M., Williams, J.B.W., 1996. Structured Clinical Interview for DSM-IV Axis I Disorders, Clinician Version (SCID-CV). American Psychiatric Press, Washington, D.C..

Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suñer, D., Cigudosa, J.C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T.D., Wu, Y.-Z., Plass, C., Esteller, M., 2005. Epigenetic differences arise during the lifetime of monozygotic twins. Proc. Natl. Acad. Sci. USA 102, 10604–10609. http://dx.doi.org/10.1073/pnas.0500398102.

Francis, D., Diorio, J., Liu, D., Meaney, M.J., 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science (80-) 286, 1155 LP–1158.

Franklin, T.B., Russig, H., Weiss, I.C., Grff, J., Linder, N., Michalon, A., Vizi, S., Mansuy, I.M., 2010. Epigenetic transmission of the impact of early stress across generations. Biol. Psychiatry 68, 408–415. http://dx.doi.org/10.1016/j.biopsych.2010.05.036.

Friston, K.J., Holmes, A.P., Poline, J.-B., Grasby, P.J., Williams, S.C.R., Frackowiak, R.S.J., Turner, R., 1995. Analysis of fMRI time-series revisited. Neuroimage 2, 45–53. http://dx.doi.org/10.1006/NIMG.1995.1007.

Geng, C., Schwartz, J.R., Vedeckis, W.V., 2008. A conserved molecular mechanism is responsible for the auto-up-regulation of glucocorticoid receptor gene promoters. Mol. Endocrinol. 22, 2624–2642. http://dx.doi.org/10.1210/me.2008-0157.

Glerean, E., Salmi, J., Lahnakoski, J.M., Jääskeläinen, I.P., Sams, M., 2012. Functional magnetic resonance imaging phase synchronization as a measure of dynamic functional connectivity. Brain Connect. 2, 91–101. http://dx.doi.org/10.1089/brain.2011.0068.

Gu, J., Stevens, M., Xing, X., Li, D., Zhang, B., Payton, J.E., Oltz, E.M., Jarvis, J.N., Jiang, K., Cicero, T., Costello, J.F., Wang, T., 2016. Mapping of variable DNA methylation across multiple cell types defines a dynamic regulatory landscape of the human genome. G3 Genes|Genomes|Genet. 6, 973–986. http://dx.doi.org/10.1534/g3.115.025437.

Hogg, K., Blair, J.D., McFadden, D.E., von Dadelszen, P., Robinson, W.P., 2013. Early onset pre-eclampsia is associated with altered DNA methylation of cortisol-signalling and steroidogenic genes in the placenta. PLoS One 8, e62969.

Kendler, K.S., Halberstadt, L.J., 2013. The road not taken: life experiences in monozygotic twin pairs discordant for major depression. Mol. Psychiatry 18, 975–984. http://dx.doi.org/10.1038/mp.2012.55.

Kiem, S.A., Andrade, K.C., Spoormaker, V.I., Holsboer, F., Czisch, M., Sämann, P.G., 2013. Resting state functional MRI connectivity predicts hypothalamus-pituitary-axis status in healthy males. Psychoneuroendocrinology 38, 1338–1348. http://dx.doi.org/10.1016/j.psyneuen.2012.11.021.

Lamers, F., Vogelzangs, N., Merikangas, K., De Jonge, P., Beekman, A., Penninx, B., 2012. Evidence for a differential role of HPA-axis function, inflammation and metabolic syndrome in

melancholic versus atypical depression. Mol. Psychiatry 18, 692–699. http://dx.doi.org/10.1038/mp.2012.144.

McGowan, P.O., Sasaki, A., D'Alessio, A.C., Dymov, S., Labonté, B., Szyf, M., Turecki, G., Meaney, M.J., 2009. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat. Neurosci. 12, 342–348.

Mill, J., Heijmans, B.T., 2013. From promises to practical strategies in epigenetic epidemiology. Nat. Rev. Genet. 14, 585–594. http://dx.doi.org/10.1038/nrg3405.

Monk, C., Feng, T., Lee, S., Krupska, I., Champagne, F.A., Tycko, B., 2016. Distress during pregnancy: epigenetic regulation of placenta glucocorticoid-related genes and fetal neurobehavior. Am. J. Psychiatry 173, 705–713. http://dx.doi.org/10.1176/appi.ajp.2015.15091171.

Na, K.S., Chang, H.S., Won, E., Han, K.M., Choi, S., Tae, W.S., Yoon, H.K., Kim, Y.K., Joe, S.H., Jung, I.K., Lee, M.S., Ham, B.J., 2014. Association between glucocorticoid receptor methylation and hippocampal subfields in major depressive disorder. PLoS One 9, 1–9. http://dx.doi.org/10.1371/journal.pone.0085425.

Palma-Gudiel, H., Córdova-Palomera, A., Eixarch, E., Deuschle, M., Fañanás, L., 2015a. Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: a meta-analysis. Epigenetics 10, 893–902. http://dx.doi.org/10.1080/15592294.2015.1088630.

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J.C., Fañanás, L., 2015b. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. Neurosci. Biobehav. Rev. 55, 520–535. http://dx.doi.org/10.1016/j.neubiorev.2015.05.016.

Pariante, C.M., 2017. Why are depressed patients inflamed? A reflection on 20 years of research on depression, glucocorticoid resistance and inflammation. Eur. Neuropsychopharmacol., 554–559. http://dx.doi.org/10.1016/j.euroneuro.2017.04.001.

Presul, E., Schmidt, S., Kofler, R., Helmberg, A., 2007. Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. J. Mol. Endocrinol. 38, 79–90.

R Development Core Team, 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. doi:10.1007/978-3-540-74686-7.

Schwarz, A.J., McGonigle, J., 2011. Negative edges and soft thresholding in complex network analysis of resting state functional connectivity data. Neuroimage 55, 1132–1146. http://dx.doi.org/10.1016/j.neuroimage.2010.12.047.

Sui, J., Adali, T., Pearlson, G.D., Calhoun, V.D., 2009. An ICA-based method for the identification of optimal FMRI features and components using combined group-discriminative techniques. Neuroimage 46, 73–86. http://dx.doi.org/10.1016/j.neuroimage.2009.01.026.

Turner, J.D., Muller, C.P., 2005. Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. J. Mol. Endocrinol. 35, 283–292.

Turner, J.D., Vernocchi, S., Schmitz, S., Muller, C.P., 2014. Role of the 5'-untranslated regions in post-transcriptional regulation of the human glucocorticoid receptor. Biochim. Biophys. Acta 1839, 1051–1061.

Tzourio-Mazoyer, N., Landeau, B., Papathanassiou, D., Crivello, F., Etard, O., Delcroix, N., Mazoyer, B., Joliot, M., 2002. Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. Neuroimage 15, 273–289. http://dx.doi.org/10.1006/NIMG.2001.0978.

Veer, I.M., Oei, N.Y.L., Spinhoven, P., Van Buchem, M.A., Elzinga, B.M., Rombouts, S.A.R.B., 2012. Endogenous cortisol is associated with functional connectivity between the amygdala and medial prefrontal cortex. Psychoneuroendocrinology 37, 1039–1047. http://dx.doi.org/10.1016/j.psyneuen.2011.12.001.

Vernocchi, S., Battello, N., Schmitz, S., Revets, D., Billing, A.M., Turner, J.D., Muller, C.P., 2013. Membrane glucocorticoid receptor activation induces proteomic changes aligning with classical glucocorticoid effects. Mol. Cell. Proteom., 1764–1779. http://dx.doi.org/10.1074/mcp.M112.022947.

Weaver, I.C.G., Cervoni, N., Champagne, F. a, D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854.

Wolfensberger, S.P.A., Veltman, D.J., Hoogendijk, W.J.G., Boomsma, D.I., de Geus, E.J.C., 2008. Amygdala responses to emotional faces in twins discordant or concordant for the risk for anxiety and depression. Neuroimage 41, 544–552. http://dx.doi.org/10.1016/j.neuroimage.2008.01.053.

Yarkoni, T., Poldrack, R.A., Nichols, T.E., Van Essen, D.C., Wager, T.D., 2011. Large-scale automated synthesis of human functional neuroimaging data. Nat. Methods 8, 665–670. http://dx.doi.org/10.1038/nmeth.1635.

Yehuda, R., Daskalakis, N.P., Bierer, L.M., Bader, H.N., Klengel, T., Holsboer, F., Binder, E.B., 2016. Holocaust exposure induced intergenerational effects on FKBP5 methylation. Biol. Psychiatry 80, 372–380. http://dx.doi.org/10.1016/j.biopsych.2015.08.005.

Ziller, M.J., Gu, H., Müller, F., Donaghey, J., Tsai, L.T.-Y., Kohlbacher, O., De Jager, P.L., Rosen, E.D., Bennett, D.A., Bernstein, B.E., Gnirke, A., Meissner, A., 2013. Charting a dynamic DNA methylation landscape of the human genome. Nature 500, 477–481, http://dx.doi.org/10.1038/nature12433.

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Increased methylation at an unexplored glucocorticoid responsive element within exon 1_D of *NR3C1* gene is related to anxious-depressive disorders and decreased hippocampal connectivity" included the following tasks:

- Participation in the conception and design of the study
- Statistical analyses
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR

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An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR

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ABSTRACT

Gene—environment (G × E) interactions have largely been regarded as the root of many complex disorders, including several psychiatric disorders. In this regard, it has been hypothesized that epigenetic mechanisms may be the main mediators of such interactions. Of particular interest is the previously described interaction between psychosocial stress and genetic variability of the serotonin transporter gene (*SLC6A4*) in its polymorphic region 5-HTTLPR. Here we review the literature concerning *SLC6A4* methylation in association with environmental, clinical or genetic variables. While *SLC6A4* hypermethylation has typically been described to be independently associated with both early life stress and depressive disorders, only a few papers address whether methylation could mediate the interaction between stress and 5-HTTLPR in predicting psychopathological risk. Nevertheless, research preliminarily indicates a methylation-driven increased vulnerability of carriers of the short allele of 5-HTTLPR to psychiatric disorders when exposed to early stress or soon after exposure to stress.

Contents

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1. A serotoninergic story

5-Hydroxytryptamine (5-HT), also known as serotonin, is a key neurotransmitter involved in several brain processes such as mood regulation, memory consolidation, aggression and stress response. Nevertheless, its range of action is not limited to the central nervous system (CNS) but expands to cardiovascular and gastrointestinal systems, among others (Berger et al., 2009). In fact, 90% of total serotonin in the organism is stored at enterochromaffin cells, located in the gastrointestinal tract (Gershon, 2004).

In the mid-twentieth century, a serotonergic deficit was postulated as the causative agent of depression thus giving rise to the so-called monoaminergic hypothesis of depression. Soon thereafter, selective serotonin reuptake inhibitors (SSRI) were developed in order to treat depression and, nowadays, they continue to be the first-choice pharmacological treatment for this disorder (Millan et al., 2015). Serotonin was also hypothesized to play a role in the etiology of other psychiatric conditions such as anxiety disorders and schizophrenia (Geyer and Vollenweider, 2008; Graeff, 2002).

As their name indicates, SSRIs act by inhibiting the serotonin transporter, 5-HTT or SERT, which reuptakes secreted 5-HT from the synaptic cleft and returns it to the presynaptic neuron. Thus, SERT became a target not only of drug development, but also of genetic and pharmacogenetic research (Arias et al., 2003). In this regard, the *SLC6A4* gene, which codes for 5-HTT, has been extensively studied, with special interest paid to its promoter-located linked polymorphic region also known as 5-HTTLPR. Functional polymorphism in this region consisting of an insertion/deletion has two major variants: the short (*s*) and the long (*l*) alleles; with the *s* allele being the less active variant that leads to decreased mRNA expression and reduced serotonin clearance at the synaptic cleft (Heils et al., 1996; Lesch et al., 1996). The *s* allele was thus hypothesized to confer risk for a number of psychiatric conditions, including mood disorders, obsessive-compulsive disorder and suicidal tendencies, among others, with the notable exception of schizophrenia (Serretti et al., 2006, 2002). Intriguingly, the *s* allele is often seen as a putative risk allele for a range of psychiatric disorders, although its associated decreased SERT expression would seem to mimic the effect of SSRI-based antidepressant therapy (which blocks this transporter). However, pharmacogenetic studies suggest that carrying the *s* allele is a predictor of worse antidepressant response since the pharmacologic target of SSRIs is already under-expressed. Hence, 5-HTTLPR was also studied in connection with antidepressant response, leading to controversial results (Porcelli et al., 2012; Taylor et al., 2010). From an epidemiological point of view, this polymorphism is relevant due to the high prevalence of both alleles, with an estimated occurrence of the *s* allele around 42% in Caucasian populations (Murphy and Moya, 2011; Serretti et al., 2007).

Caspi et al. shed some light on this conundrum by revealing an insightful gene—environment (G × E) interaction by means of a large longitudinal study in 847 subjects where they observed that the increased depression risk conferred by the *s* allele was only observed in subjects exposed to psychosocial stress if they had distally experienced childhood maltreatment or they reported proximal stressful life events (Caspi et al., 2003). Several meta-analyses have since been published once again leading to conflicting interpretations of the literature: while some papers support the unprecedented results described by Caspi et al. (Sharpley et al., 2014), others find no evidence of a G × E interaction between 5-HTTLPR and exposure to stress in mediating depression liability, arguing that the observed associations are likely to be statistical artifacts (Munafò et al., 2009; Risch et al., 2009). Moreover, the aforementioned phenotypic effects of 5-HTTLPR have been suggested to operate by means of abnormal brain functioning. Briefly, *s* carriers exhibit heightened amygdala reactivity to environmental threat compared to *l* homozygotes, which could be a risk endophenotype for developing stress-related disorders after exposure to stressful experiences (Munafò et al., 2008). Hence, the next step was to explore the mechanisms that could be mediating this interaction; thereby providing a more complex framework that would explain the conflicting results.

Epigenetic mechanisms, defined as any process that alters gene expression without changing the DNA sequence, have been hypothesized to be responsible for embedding contextual cues and thus mediating the biological impact of the environment on an exposed individual (Petronis, 2010). DNA methylation is one of the epigenetic modifications that has received most attention from the scientific community due to the methodological ease with which it can be studied and the balance between stability and reversibility of methylation markers. Generally, DNA methylation occurs in cytosines that are adjacent to guanines forming the so-called CpG sites; although, it has recently been reported that DNA methylation can also occur in non-CpG cytosines. Interestingly, although CpG sites can be found isolated within or between genes, they tend to cluster in the promoter regions of genes; these clusters of CpG sites are known as CpG islands. CpG islands are naturally unmethylated, which has been associated with high expression levels by research in several fields. Briefly, methylation at GC-rich DNA fragments increases DNA attraction and thus alters chromatin accessibility (Yoo et al., 2016). Conversely, methylation at CpG sites located in CpG island shores or intragenic regions seems to be associated with higher rather than lower expression, although the underlying mechanisms are still under discussion (Ball et al., 2009; Edgar et al., 2014).

In the particular case of SERT, variability at both *SLC6A4* methylation and 5-HTTLPR have independently been associated with changes in *SLC6A4* expression: specifically, higher methylation and

the *s* allele are independently associated with lower expression of the transporter (Heils et al., 1996; Philibert et al., 2007). Hence, the joint contribution of both epigenetic and genetic variability might be relevant to predict final expression of SERT and so one variable should not be analyzed without the other.

To better illustrate how DNA methylation can mediate the effect of G × E interactions with regard to stress reactivity and stress-related disorders, recent evidence by Klengel et al. is briefly discussed. Focusing in *FKBP5* gene, which codes for a co-chaperone that prevents the GR from entering the nucleus and thus acts as a glucocorticoid inhibitor, they described the exact DNA methylation modification that could explain the previously reported G × E interaction between early adversity and an FKBP5 genetic polymorphism (rs1360780) resulting in increased risk for psychopathology. Briefly, carriers of the risk allele exhibit a distinct three-dimensional conformation of FKBP5 which, only in the presence of childhood trauma (which promotes exacerbated release of cortisol), allows the demethylation of an intronic region of the gene. This demethylation is stable in the long-term and so further deregulates stress response and increases vulnerability to psychopathology in adulthood after further exposure to stress, such as PTSD resulting from war (Klengel et al., 2013).

Hence, epigenetic modulation could mediate the interaction between environmental cues and genetic polymorphisms; specifically, DNA methylation could mediate the interplay between childhood trauma and 5-HTTLPR. Accordingly, several authors have assessed *SLC6A4* methylation in association with both: (i) experience of psychosocial stress and (ii) the development of psychopathology. Within this framework, the primary objective of this review is to understand: (i) how the methylation pattern of the *SLC6A4* gene fluctuates after exposure to environmental factors, with special emphasis on psychosocial stress; (ii) whether this methylation pattern has the capacity to influence psychopathology risk; and (iii) the role 5-HTTLPR plays in the aforementioned associations. To this end, all the papers that assess *SLC6A4* methylation in human populations have been thoroughly reviewed.

2. Methodology

2.1. Bibliographic search

The serotonin transporter is referred to in the scientific literature under a number of names: 5-HTT, SLC6A4 and SERT are the most widespread. Thus, in order to define our search parameters, we performed four parallel searches as follows: "serotonin transporter methylation" (93 hits), "5-HTT methylation" (18 hits), "SERT methylation" (20 hits) and "SLC6A4 methylation" (66 hits). After removing duplicates from this initial pool of 197 papers, it decreased to 104 papers. Exclusion criteria consisted of research performed in animal models, papers where there was no *SLC6A4* methylation assessment and reviews. The final selection consisted of 49 papers to be reviewed, published between 2008 and 2016 (see Fig. 1 and Table 1 for further details). Notably, some of the included papers targeted the same populations as part of ongoing projects publishing methylation data from the same cohorts in separate papers as the authors focused on different variables in their analysis.

2.2. The SLC6A4 gene

The *SLC6A4* gene is located in the reverse strand of chromosome 17q11 spanning about 40 kb. It has about 30 genetic variants including SNPs, VNTRs and deletion/insertion variations. As mentioned above, an insertion/deletion located at the promoter region of the gene, called 5-HTTLPR, has received most attention from the scientific community since it was reported to have an effect on final mRNA expression. Regarding epigenetic modulation of *SLC6A4*, one CpG island can be found in the 5′ end of the gene, considered as a promoter region for this gene, encompassing its non-coding exon 1A and containing 81 CpG sites, approximately 1 kb from 5-HTTLPR (see Fig. 2).

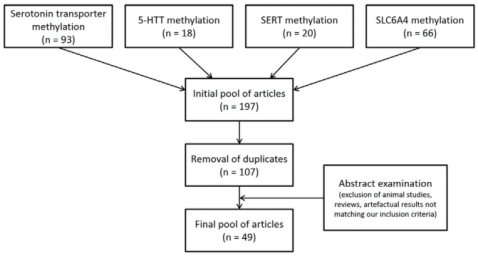

Fig. 1. Flow diagram of literature search. Independent searches were performed in order to ensure all papers assessing methylation at the *SLC6A4* gene were included in the initial pool. This was mandatory as the *SLC6A4* gene has a number of aliases and can be referred to by any of them in the scientific literature.

2.3. Instruments, questionnaires and scales

Most of the papers included in the present review studied either psychopathological or environmental variables; or both. Briefly, psychopathology was accounted for by DSM criteria (from the III, III-R, IV or IV-R editions) by means of semi-structured interviews and different scales based on DSM symptoms. Likewise, experience of stressful or traumatic events such as childhood maltreatment, financial hardship, parental loss or being a victim of bullying were assessed by personalized interviews with study-tailored questions. Meanwhile, environmental factors were assessed by a range of validated scales such as the childhood trauma questionnaire (CTQ), which is commonly used for research purposes and includes 5 different types of maltreatment: sexual, physical and emotional abuse and physical and emotional neglect (Bernstein et al., 1994).

3. Non-integrative approaches

Due to the nature of the data reviewed and although the main focus of the present review is the integration of environmental, genetic and epigenetic data in a single model to explain psychopathology, it is first necessary to discuss the individual associations with *SLC6A4* methylation one by one. Therefore, this section is devoted to analysis of the interplay between epigenetics and several environmental, psychopathological and clinical variables regardless of the putative genetic moderation (which will be discussed in the next section). Our results will be displayed in accordance with a conceptual framework in which environment first shapes the epigenetic code (Section 3.1) to later modify a number of clinical outcomes, with special emphasis on psychopathology (Section 3.2). Finally, other variables of interest such as neuroimaging correlates and *SLC6A4* expression that may be viewed as explanatory variables mediating the effect of methylation on psychopathology is also discussed (Section 3.3). These associations must be considered with caution as they consider isolated factors that belong to far more complex interactive networks. For a schematic view of the papers included and their main results listed according to publication date, see Table 1.

Table 1
Outline of papers reviewed including the nature of the samples, main variables assessed and main epigenetic findings.

Study	Variable of interest	Sample	Tissue	Methylation assessment (# of analyzed CpG sites)	Findings	Extent of the reported differences	5-HTTLPR
Philibert et al., 2008	MDD and AD liability	192 adults (mostly of Caucasian origin)	Lymphoblast cell lines	Mass spectrometry (71 CpG sites)	There was a non-significant trend for subjects with a lifetime history of MD to have higher methylation than those without	Average methylation levels < 2%	*l* allele homozygotes produce more mRNA than *ss*
Beach et al., 2010	Child abuse	192 adults (mostly of Caucasian origin)	Lymphoblast cell lines	Mass spectrometry (71 CpG sites)	Significant effect of child abuse on overall methylation levels	NA	Not analyzed
Devlin et al., 2010	Maternal mood	82 mother-newborn dyads	Peripheral blood (leucocytes)	Pyrosequencing (10 CpG sites)	*SLC6A4* methylation is lower in mothers with increased depressive symptoms and their newborns	<5%	Not analyzed
Olsson et al., 2010	Depression	150 young adults	Buccal swabs	Mass Spectrometry (86 CpG sites)	Joint effect of methylation and *s* allele carriage on risk for depression.	<10%	*s* carriers with high methylation had 5-fold more depressive symptoms
van IJzendoorn et al., 2010	Unresolved loss	143 adults (mostly of Caucasian origin)	Lymphoblast cell lines	Mass spectrometry (71 CpG sites)	*s* homozygotes exhibit higher unresolved loss scores except those with higher methylation. *l* homozygotes exhibit higher unresolved loss scores only when densely methylated.	NA	*s* homozygotes show decreased methylation when there is more trauma and viceversa
Beach et al., 2011	Child sex abuse	155 adults (mostly of Caucasian origin)	Lymphoblast cell lines	Mass spectrometry (71 CpG sites)	Child sex abuse increases methylation of *SLC6A4* which in turn increases prevalence of ASPD in women	NA	The correlation between ASPD and methylation was greater in *s* homozygotes
Koenen et al., 2011	Risk for PTSD	100 adults from Detroit	Peripheral blood	Illumina Bead Chip 27K (2 CpG sites)	Interaction between number of traumatic events and cg22584138 methylation predicted PTSD diagnosis, symptom severity and number of symptoms	NA	Not analyzed
Park et al., 2011	Alcohol dependence	42 subjects (27 male patients and 15 controls)	Peripheral blood	Pyrosequencing (7 CpG sites)	No differences were found when comparing alcohol-dependent patients with controls	<5%	Not analyzed
Sugawara et al., 2011	Bipolar disorder	4 (2 pairs of MZ twins) BD-discordant	Lymphoblast cell lines	Tiling array + pyrosequencing to confirm	Methylation at two CpG sites out of the CpG island, inside the *SLC6A4* gene, is higher in the affected co-twin	>20%	Among the *s* homozygotes, there was lower mRNA expression and higher methylation in BD patients
	Bipolar disorder	40 subjects	Lymphoblast cell lines	Pyrosequencing (5 CpG sites)	Methylation at the same CpG sites is higher in patients than in controls	<20%	DNA methylation correlated with mRNA only in *s* homozygotes.
	Bipolar disorder	63 donors	Post-mortem brain tissue (PFC)	Pyrosequencing (5 CpG sites)	Methylation at the same CpG sites is higher in patients than in controls	<10%	
Alasaari et al., 2012	Environmental stress	49 female health-care professionals	Peripheral blood	Cycle sequencing + 450K BeadChip to confirm (5 CpG sites)	Exposure to a high-stress work environment (in adulthood) was associated with lower methylation while higher methylation correlated to higher burnout scores	<10%	Non-significant increase of methylation in *s* homozygotes
Vijayendran et al., 2012	Child sex abuse	152 subjects	Lymphoblast cell lines	Illumina Human-Methylation450 BeadChip (16 CpG sites)	Methylation at individual CpG sites downstream of exon 1A (*SLC6A4*) is associated with either 5-HTTLPR or child sex abuse	NA	Significant G × E interaction at cg18584905 methylation (direction was not specified)

Table 1 (*Continued*)

Study	Variable of interest	Sample	Tissue	Methylation assessment (# of analyzed CpG sites)	Findings	Extent of the reported differences	5-HTTLPR
Wang et al., 2012	Childhood physical aggression	25 healthy adult males	Peripheral blood (T cells and monocytes)	Pyrosequencing (24 CpG sites)	Higher CpG-specific methylation was associated with childhood aggression and negatively correlated to 5-HT synthesis at the OBFC	<10%	No effect
Kang et al., 2013	Childhood adversity	108 MDD patients	Peripheral blood	Pyrosequencing (7 CpG sites)	Childhood adversity was associated with higher *SLC6A4* promoter methylation	<5%	Not analyzed
Kim et al., 2013	Post stroke depression (PSD)	286 South Koreans	Peripheral blood	Pyrosequencing (7 CpG sites)	Higher *SLC6A4* methylation was associated with PSD (this association was stronger in *ss* individuals)	<20%	*s* homozygotes showed increased methylation Association between methylation and PSD was stronger in *ss*
Ouellet-Morin et al., 2013	Bullying	56 10-year-old MZ twins	Saliva	Mass spectrometry	Bullied twins had higher SERT methylation than their non-bullied MZ co-twins	<10%	Not analyzed
Pjetri et al., 2013	Anorexia nervosa	90 subjects (45 lifetime AN and 45 controls)	Peripheral blood	Mass spectrometry	There was no detectable association between *SLC6A4* methylation and either AN diagnosis or BMI.	NA	Not analyzed
Xu et al., 2013	Depression in SLE	192 subjects (96 SLE patients and 96 controls)	Peripheral blood	Cloning + Sanger sequencing (15 CpG sites)	No significant differences between depressed and non-depressed SLE patients nor between SLE and controls were observed	NA	*s* homozygotes were more prevalent in the depressed SLE group
Zhao et al., 2013a	Depressive symptoms	168 male veterans (84 MZ twin pairs)	Peripheral blood	Pyrosequencing (20 CpG sites)	Intrapair differences in DNA methylation variation were correlated with intrapair differences in BDI scores at 10/20 CpG sites assayed	An 10% increase in mean methylation was associated with a 4.4 increase in the BDI	Non-significant higher intrapair BDI difference in *s* homozygotes twin pairs
Zhao et al., 2013b	Obesity measures	168 male veterans (84 MZ twin pairs)	Peripheral blood	Pyrosequencing (20 CpG sites)	Promoter methylation of SLC6A4 is associated with several obesity measures.	A 1% increase in mean methylation was associated with 1.16 kg increase in body weight	Not analyzed
Abdolmaleky et al., 2014	Schizophrenia and bipolar disorder	205 subjects	Frontal dissects (n = 105); saliva (n = 100)	Illumina arrays + quantitative methylation specific PCR	Drug naïve schizophrenia patients exhibited *SLC6A4* hypermethylation	<30%	Not analyzed
Alexander et al., 2014	Stress reactivity	186 healthy Caucasians	Peripheral blood	Pyrosequencing (83 CpG sites)	Interaction of 5-HTTLPR and *SLC6A4* methylation on cortisol stress reactivity	NA	Only when methylation is low, *s* homozygotes have higher cortisol levels
Beach et al., 2014	Cumulative SES in childhood	388 young adults	Peripheral blood	Illumina Human-Methylation450 BeadChip	Cumulative SES risk altered CpG-specific *SLC6A4* methylation in a gender-dependent fashion	NA	5-HTTLPR genotype altered CpG-specific *SLC6A4* methylation in a G × E fashion
Chau et al., 2014	Neonatal pain-related stress	111 children: 61 born very preterm and 50 born full-term	Saliva	Pyrosequencing (10 CpG sites)	Very preterm children had higher *SLC6A4* methylation than full-term children both at birth and at age 7 years. CBCL Total Problems score correlated with *SLC6A4* methylation.	<5%	No effect

Dannlowski et al., 2014	Hippocampal gray matter volume	189 healthy participants	Peripheral blood	Cycle sequencing (8 CpG sites)	Mean methylation in an AluJb element in the promoter region of *SLC6A4* gene correlated with hippocampal gray matter volume.	There were no comparison groups. Differential methylation around 20%	The association between methylation and gray matter was stronger in *s* carriers
Domschke et al., 2014	Antidepressant response	94 German MDD patients	Peripheral blood	Cycle sequencing (9 CpG sites)	Higher *SLC6A4* methylation was associated with better antidepressant treatment response	NA (CpG-specific methylation range = 0%–18.5%; mean methylation = 3.6%)	Carriers of the less active 5-HTTLPR/rs25531 haplotypes with lower methylation were at increased risk of poor treatment response
Nikolova et al., 2014	Brain function	Discovery (n = 80), replication (n = 96), and postmortem cohorts (n = 35)	Saliva, whole blood & postmortem brain (depending on the cohort)	Pyrosequencing (20 CpG sites)	CpG-specific methylation at the *SLC6A4* promoter predicts threat-related left amygdala reactivity and negatively correlates with *SLC6A4* mRNA levels	<5%	No effect
Okada et al., 2014	Therapeutic response	100 Japanese participants (50 controls and 50 MDD patients)	Peripheral blood	Mass spectrometry (81 CpG sites)	Methylation at two individual CpG sites was correlated to early adversity, severity of MD symptoms and/or therapeutic response	<5%	No effect
Roberts et al., 2014	CBT response	116 children with anxiety disorders	Saliva	Mass spectrometry (14 CpG sites)	Change of CpG-specific methylation at the *SLC6A4* promoter after CBT differed between responders and non-responders to therapy.	<10%	Not analyzed
Tahara et al., 2014	Functional dyspepsia	157 Japanese participants (79 cases and 78 controls)	Gastric biopsy	Pyrosequencing (11 CpG sites)	Average methylation at *SLC6A4* promoter was lower in FD patients than controls while *SLC6A4* exon 9 methylation was higher in FD patients	<5%	Not analyzed
van Mil et al., 2014	ADHD symptoms	426 Dutch children	Cord blood	Mass spectrometry (60 CpG sites)	Negative correlation between *SLC6A4* methylation and ADHD symptom score	NA	*s* carriers had lower levels of methylation and an increase of ADHD symptoms
Wankerl et al., 2014	Early trauma	133 healthy Caucasians	Peripheral blood	Pyrosequencing (83 CpG sites)	Both prenatal and early-stress were associated with lower mRNA levels. CTQ scores were inversely correlated with SERT mRNA levels. Methylation levels at 10 out of the 83 CpG sites correlated with lower SERT mRNA. Prenatal stress was found to be associated with higher methylation levels at 4 specific CpG sites.	NA	Lower levels of mRNA in *s* carriers. G × E interact: the *s* allele and ELS associate with decreased mRNA in an additive fashion.
Booij et al., 2015	Hippocampal volume	69 adults	Peripheral blood	Pyrosequencing (11 CpG sites)	Hippocampal volume inversely correlated with SLC6A4 promoter methylation. Physical abuse was strongly associated with methylation when compared to other maltreatment subtypes.	<5%	*ll* genotype and a history of abuse had greater methylation than *s* carriers
Chagnon et al., 2015	Late-life anxiety/depression	43 older women (19 cases and 24 controls)	Saliva	Pyrosequencing (6 CpG sites)	No difference was observed	NA	Not explored
Dukal et al., 2015	Prenatal stress	45 high ELS and 45 low ELS infants	Cord blood	Pyrosequencing (4 CpG sites)	Neither prenatal stress nor 5-HTTLPR altered *SLC6A4* methylation but newborn gender did	<10%	No effect

Table 1 (*Continued*)

Study	Variable of interest	Sample	Tissue	Methylation assessment (# of analyzed CpG sites)	Findings	Extent of the reported differences	5-HTTLPR
Duman and Canli, 2015	Life stress (both early and recent) and stress reactivity	105 healthy Caucasian males	Peripheral blood	Mass spectrometry (79 CpG sites)	Both early life stress and chronic stress correlated with SLC6A4 methylation only in *s* carriers (not in *ll* participants)	NA	Only *s* carriers are epigenetically susceptible to ELS
Frodl et al., 2015	MDD, emotion processing	60 adult participants	Peripheral blood	Pyrosequencing (11 CpG sites)	Higher SLC6A4 methylation was associated with greater childhood maltreatment. Methylation was associated with complex patterns of specific brain region activation in response to emotion processing tasks.	<5%	Not analyzed
Lee et al., 2015	Peri-conceptional alcohol consumption	164 triads (father, mother and newborn)	Peripheral blood and cord blood	Methylation-specific digestion plus PCR (2 CpG sites)	Babies of mothers with a light drinking pattern either before or during pregnancy exhibited lower *SLC6A4* methylation	NA	Not analyzed
Lei et al., 2015	Depressive symptoms and neighborhood crime	99 African American women	Peripheral blood	Illumina Human-Methylation450 BeadChip (7 CpG sites)	Correlation between *SLC6A4* methylation and depressive symptoms. *s* carriers living in high-crime neighborhoods exhibited higher *SLC6A4* methylation together with increased depressive symptoms when compared to *ll* subjects	NA	*s* carriers were more susceptible to environment
Muehlhan et al., 2015	Brain functional connectivity	74 healthy Caucasian participants	Peripheral blood	Pyrosequencing(83 CpG sites)	*SLC6A4* methylation correlated with amygdala rsFC with several nodes of the salience network	<5%	No effect
Park et al., 2015	ADHD	102 ADHD children	Peripheral blood	Pyrosequencing (8 CpG sites)	CpG-specific and mean methylation were associated with higher hyperactive-impulsive scores, ARS scores and commission error scores; inverse correlation between cortical thickness and *SLC6A4* methylation	NA	No effect
Provenzi et al., 2015	Neonatal pain-related stress	88 newborns (VPT infants followed until NICU discharge)	Cord blood and peripheral blood	Next-generation sequencing of a DNA library (20 CpG sites)	CpG-specific *SLC6A4* methylation increased after frequent exposure to skin-breaking procedures	<5%	Not analyzed
Schneider et al., 2015	Suicide	6 suicide completers & 6 controls	Post-mortem brain tissue (BA10)	Illumina Human-Methylation450 BeadChip	*SLC6A4* gene belonged to the top 1000 promoter DMRs between cases and controls	<5%	Not analyzed
van der Knaap et al., 2015a	Adverse life events	939 adolescents	Peripheral blood	Mass spectrometry (41 CpG sites of which 21 could be analyzed in 11 CpG units)	Exposure to stressful life events during childhood and especially adolescence predicted higher methylation.	<5%	*l* homozygotes showed increased methylation after exposure to stress

van der Knaap et al., 2015b	Internalizing problems	945 adolescents	Peripheral blood	Mass spectrometry (41 CpG sites of which 21 could be analyzed in 11 CpG units)	SLC6A4 methylation was not associated with lifetime internalizing disorders nor anxiety symptom scores but with higher risk of anxiety and depressive symptom scores at follow-up.	<5%	No effect
Gartstein et al., 2016	Prenatal SSRI exposure	115 newborns (46 SSRI exposed and 69 non-exposed)	Cord blood	Pyrosequencing (10 CpG sites)	Higher SSRI consumption was associated with both neonatal SLC6A4 methylation and higher soothability after 3 and 6 months' follow-up	NA	Not analyzed
Montirosso et al., 2016	Neonatal intensive care unit stay	78 newborns (48 preterm and 30 full-term infants)	Cord blood (and peripheral blood only in preterm infants)	Next generation sequencing of a DNA library (20 CpG sites)	Higher *SLC6A4* CpG-specific methylation at NICU discharge when compared to methylation at birth, in preterm infants	<5%	Not analyzed
Non et al., 2016	Early life institutionalization	117 children (84 EIG and 35 NIG)	Buccal samples	Pyrosequencing (6 CpG sites)	Lower SLC6A4 CpG-specific methylation was associated with longer institutionalization periods	<5%	Not analyzed
Iga et al., 2016	MDD and antidepressant response	57 subjects (28 MDD patients and 29 controls)	Peripheral blood	Pyrosequencing (9 CpG sites)	Both *SLC6A4* mean methylation and mRNA expression were increased in MDD patients compared with controls. CpG-specific lower methylation predicted better antidepressant response.	<5%	There was an *I* dosage effect on SLC6A4 methylation (*I* homozygotes had greater methylation)
Swartz et al., 2016	Adolescent SES and prospective amygdala reactivity and depressive symptomatology	132 adolescents at Wave 2	Peripheral blood	Pyrosequencing (20 CpG sites)	Lower SES predicted an *SLC6A4* methylation increase which correlated to a simultaneous increase of amygdala reactivity in the same period which further predicted an increase in depressive symptoms one year later	<5%	Not explored due to poor model fit

Abbreviations: 5-HT, serotonin; AD, alcohol dependence; ADHD, attention deficit-hyperactivity disorder; AN, anorexia nervosa; ARS, ADHD rating scale-IV; ASD, autism spectrum disorder; ASPD, antisocial personality disorder; BA10, Brodmann area 10; BD, bipolar disorder; BDI, Beck depressive inventory; CBCL, children behavior checklist; CBT, cognitive behavior therapy; DMR, differentially methylated region; EIG, ever institutionalized group of children; ELS, early life stress; FD, functional dyspepsia; MDD, major depressive disorder; MZ, monozygotic twins; NA, not available; NIG, never institutionalized group of children; OBFC, orbitofrontal cortex; PFC, prefrontal cortex; PTB, preterm birth; rsFC, resting state functional coupling; SES, socio-economic status; SLE, systemic lupus erythematosus; SSRI, selective serotonin reuptake inhibitor; VPT, very preterm.

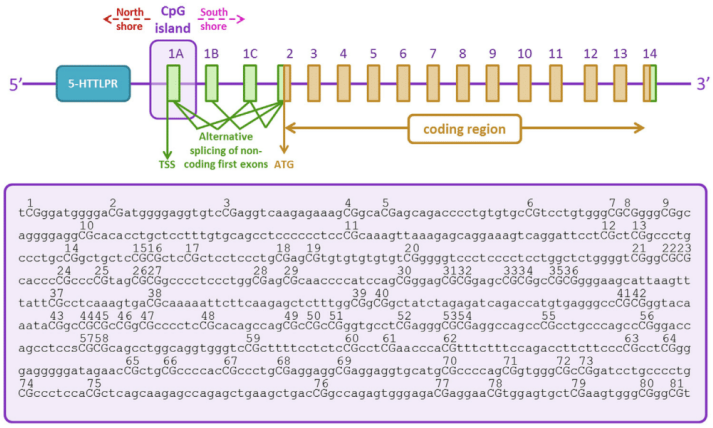

Fig. 2. *SLC6A4* gene structure. Top: schematic representation of the *SLC6A4* gene; 5-HTTLPR and CpG island locations within the gene are highlighted. Bottom: DNA sequence of the CpG island; CpG sites within the CpG island are marked with capital letters and numbered. Abbreviations: TSS, transcriptional start site; ATG, refers to the translation initiation codon.

3.1. Association between psychosocial stress and SLC6A4 methylation

Psychosocial stress in relation to *SLC6A4* methylation was studied in 26 out of the 49 articles reviewed. The main findings are disclosed below, divided in three main sections depending on whether the assessed stressor was experienced during the perinatal period, childhood or adulthood. This compartmentalization of results is based on the widespread notion that there are different developmental stages during which an individual is more or less prone to environmental influences.

3.1.1. Perinatal

According to the developmental origins of health and disease (DOHaD) hypothesis, also known as the fetal programming hypothesis, the intrauterine environment is of outstanding relevance not only to the short-term health of the fetus, but also to long-term outcomes. Thus, prenatal insults can compromise adult wellbeing and have been reported to increase vulnerability to cardiovascular, metabolic and psychiatric disorders, among others (Cottrell and Seckl, 2009).

In this regard, Devlin et al. reported lower methylation levels in newborns whose mothers experienced depressive symptoms during pregnancy (Devlin et al., 2010); intriguingly, they did not find SSRI consumption during pregnancy to have any effect on newborn *SLC6A4* methylation. Meanwhile, Gartstein et al. found higher SSRI consumption during pregnancy to be associated with higher newborn *SLC6A4* methylation levels; interestingly, this higher methylation was associated with higher soothability – i.e. better self-regulation – only in SSRI-exposed infants while non-exposed offspring born to mothers with internalizing symptoms exhibited lower soothability (Gartstein et al., 2016).

Additionally, Wankerl and colleagues described an association between CpG-specific methylation and prenatal stress, defined as maternal experience of stressful life events during pregnancy (Wankerl et al., 2014); whereas Dukal and colleagues found no effect of prenatal stress on newborn *SLC6A4* methylation in the north shore of the CpG island of interest (Dukal et al., 2015). In contrast, Lee et al. examined the effect of parental alcohol intake during the periconceptional period on methylation at the south shore of the *SLC6A4* CpG island in newborns (Lee et al., 2015). They reported a decrease in *SLC6A4* methylation in babies whose mothers had drunk either before pregnancy or during early pregnancy, compared with babies of non-drinking mothers. The heterogeneity of these results might be explained by the inherent heterogeneity in sampling and assessment in these studies, as the experience of psychiatric symptoms during pregnancy, as opposed to the exposure to psychosocial stress factors of a sociocultural nature, might have different epigenetic consequences for the fetus. Moreover, different cell types were assessed in epigenetic analysis (these shortcomings will be further discussed in Section 5).

The perinatal period, including immediate post-natal stages, has also been studied as a key developmental window. In fact, both extremely premature infants and newborns exposed to pain-related stress in the neonatal intensive care unit exhibited higher *SLC6A4* methylation than controls (Chau et al., 2014; Provenzi et al., 2015). Notably, Provenzi et al. assessed DNA methylation perinatally, while Chau et al. analyzed saliva from 7-year-old subjects. Moreover, Montirosso et al. found no methylation differences at birth between full-term and preterm infants; but, thanks to their longitudinal design, they reported an increase of *SLC6A4* methylation between birth and NICU release in preterm newborns, highlighting the major effect of postnatal (and not prenatal) stress in *SLC6A4* methylation (Montirosso et al., 2016). Overall, these results suggest that the methylation differences observed are due to postnatal stress experienced by premature infants and not by prematurity itself.

3.1.2. Childhood

Childhood constitutes a developmental window of outstanding interest in epigenetic research. Children are both directly exposed to a wide range of diverse socioeconomic cues (which can only be experienced indirectly during intrauterine life) and have the plasticity to assimilate them (a faculty which decreases as subjects approach and progress through adulthood). Thus, children harbor the potential to become vulnerable to certain complex disorders after exposure to a number of stressful events.

When focusing on childhood adversities (ranging from maltreatment, institutionalization and parental loss to low socioeconomic status and being a victim of bullying), ten of the 17 studies

found positive associations with *SLC6A4* methylation levels (Beach et al., 2011, 2010; Frodl et al., 2015; Kang et al., 2013; Ouellet-Morin et al., 2013; Swartz et al., 2016; van der Knaap et al., 2015a); two of them reported such an association only when accounting for 5-HTTLPR genotype (Duman and Canli, 2015; Lei et al., 2015); and another only when examining physical abuse, but none of the other subtypes of childhood maltreatment that were assessed in the same study (Booij et al., 2015).

In contrast, one study found no associations between childhood trauma and *SLC6A4* methylation (Wankerl et al., 2014); and another found no differences in the average CTQ scores when analyzed as a function of a dichotomous split of the *SLC6A4* methylation values (each half of the sample was assigned to either the low or the high methylation group, but they did not statistically test for the potential association between the two continuous variables) (Alexander et al., 2014). An additional pair of papers found associations but did not report whether they were positive or negative (Beach et al., 2014; Vijayendran et al., 2012); one article described a bimodal response depending on the participants' 5-HTTLPR genotype (van IJzendoorn et al., 2010); intriguingly, when assessing early life institutionalization, a negative association was found between longer time spent in foster care and *SLC6A4* methylation (Non et al., 2016); and finally, the remaining study reported both positive and negative associations depending on the specific CpG site examined (Okada et al., 2014).

3.1.3. Adulthood

Only four studies analyzed the effect of experiencing stress during adulthood on *SLC6A4* methylation. Unexpectedly, in a sample of 49 nurses, a high-stress work environment was associated with lower *SLC6A4* methylation levels, while intragroup work stress correlated positively to methylation (Alasaari et al., 2012). The lack of a more comprehensive measurement of stress, by means of either questionnaires concerning perceived stress or biological correlates such as cortisol secretion, hinders the interpretation of such results, which rely on a quite subjective measurement of stress based on the demand/control ratio in a particular ward (further discussion of these results is continued in Section 3.2.1.2).

In contrast, Duman et al. reported an association between chronic stress – as measured by the Trier Inventory of Chronic Stress (TICS) – and *SLC6A4* methylation (driven by *s* allele carriers) where higher TICS scores correlated with higher methylation levels (Duman and Canli, 2015). These results are in line with those reported by van der Knaap et al., thus further supporting the major effect of recent exposure to stress on *SLC6A4* methylation not only during adolescence but also encompassing adulthood as a window of susceptibility. However, Wankerl et al. found no effect of recent trauma on *SLC6A4* methylation (Wankerl et al., 2014). Finally, Koenen et al. reported an association between the number of traumatic events and the methylation percentage at a specific CpG site located in the *SLC6A4* promoter region; nevertheless, the authors did not specify the direction of the effect (Koenen et al., 2011).

3.2. Association between SLC6A4 methylation and clinical variables

3.2.1. Psychopathology

The psychiatric diagnoses assessed with regard to *SLC6A4* methylation included: major depressive disorder (MDD), post-traumatic stress disorder (PTSD), bipolar disorder (BD), schizophrenia (SZ), attention deficit-hyperactivity disorder (ADHD), alcohol dependence (AD), anorexia nervosa (AN), suicidal tendencies and internalizing disorders (including anxious-depressive spectrum disorders).

3.2.1.1. Depression. The term depression typically covers a wide spectrum of phenotypes from both clinical and research points of view. Nowadays, depression is one of the most widespread and severe complex disorders worldwide, affecting millions of people and causing a huge socioeconomic burden in terms of both medical expenses and disability-adjusted life years (DALYs) (Murray et al., 2012). Thus, the concept of *depression* has been addressed via a plethora of separate approaches in the literature including, but not limited to, diagnosis of MDD, subclinical depressive symptomatology as measured by distinct psychometric scales, and suicide (either attempted or completed) as its most extreme form.

Fifteen papers examined depression-related phenotypes in association with *SLC6A4* methylation. While some authors found clear associations between methylation of different regions of the *SLC6A4* promoter and depression (Iga et al., 2016; Lei et al., 2015; Okada et al., 2014; Philibert et al., 2008; Schneider et al., 2015; van der Knaap et al., 2015b), there were also models of indirect effects mediated by brain function (Swartz et al., 2016) and findings that only reached statistical significance when incorporating genotype (5-HTTLPR) into the statistical model (Kim et al., 2013; Olsson et al., 2010). Notably, using a monozygotic twin sample, Beck Depression Inventory (BDI) scores were found to correlate to *SLC6A4* methylation levels in an intrapair differences design, which allows genetic confounding to be avoided (Zhao et al., 2013a). Overall, higher depressive symptomatology scores correlated to higher methylation levels (in any region); in some cases, only in *s* allele carriers.

Interestingly, Kang et al. found a strong correlation between *SLC6A4* methylation and a family history of depression in a sample consisting entirely of MDD cases (Kang et al., 2013). This result is consistent with hypotheses regarding epigenetic inheritance, transgenerational effects of stress and an epigenetic contribution to the heritability of complex disorders (Bowers and Yehuda, 2016; Maher, 2008). Similarly, Swartz et al. only found an association between methylation-driven change in amygdala reactivity and change in depression symptoms in subjects with a family history of depression (as further discussed in Section 3.3.1.2).

Nevertheless, two of the studies reported no association between *SLC6A4* methylation and either anxiety or depression (Booij et al., 2015; Chagnon et al., 2015). Meanwhile, Alexander et al. did not find average depression scores to differ when comparing subjects with either "high" or "low" methylation values, but did not directly test for the association of the two quantitative variables (Alexander et al., 2014). Finally, another study reported a negative association between *SLC6A4* methylation and depressive symptomatology during pregnancy (Devlin et al., 2010).

3.2.1.2. Other psychiatric disorders. By means of a discordant monozygotic twin-based approach further replicated in a case-control approach in both peripheral blood and postmortem brain samples, Sugawara et al. found *SLC6A4* methylation to be increased in BD patients (Sugawara et al., 2011). *SLC6A4* hypermethylation was also observed in SZ patients, especially in those who were drug naïve (Abdolmaleky et al., 2014).

Higher *SLC6A4* methylation was also observed in women suffering from antisocial personality disorder, especially in those homozygous for the *s* allele and, to a lesser extent, in those heterozygous; intriguingly, the correlation between SLC6A4 methylation and symptoms of antisocial personality disorder was non-significant in *l* allele homozygous women (Beach et al., 2011).

Additionally, the child behavior checklist (CBCL) total problems score was significantly associated with higher *SLC6A4* methylation in preterm children, especially in those that were very preterm, when compared to full-term children (Chau et al., 2014).

Regarding ADHD, the literature shows conflicting results: on the one hand, van Mil et al. reported higher mean *SLC6A4* methylation

to be associated with lower ADHD symptom score (van Mil et al., 2014); on the other hand, Park et al. reported CpG-specific methylation to be positively associated with several ADHD variables such as ADHD rating scale (ARS) scores and ARS hyperactive-impulsive subscores (Park et al., 2015). This apparent contradiction could be due to sampling disparities since van Mil et al. analyzed DNA methylation in cord blood samples while Park et al. evaluated DNA extracted from peripheral blood of 6- to 15-years-old subjects.

As for burnout in a nurse cohort exposed to a high stress environment, moderate burnout symptoms correlated to *SLC6A4* methylation (Alasaari et al., 2012). Nevertheless, the subjects with the highest levels of methylation belonged to the low stress environment group and presented no symptoms of burnout. This puzzling finding could be an artifact due to either the small sample size (n = 25) or an inherent bias where the more burnout-susceptible subjects causally worked in the less stressful environments and/or the nurses working in high-stress environments were resilient. Alternatively, environmental stress could reduce *SLC6A4* methylation while the posterior development of burnout symptoms could increase these methylation levels.

Moreover, Koenen et al. investigated whether the risk of developing PTSD after exposure to traumatic events could be modulated by *SLC6A4* methylation. Whereas there was no direct association between *SLC6A4* methylation and PTSD, there was a significant interaction between methylation and the number of traumatic events in predicting PTSD diagnosis: only subjects with low levels of *SLC6A4* methylation were at increased risk of developing PTSD after exposure to a greater number of traumatic events, whereas subjects with high levels of *SLC6A4* methylation were resilient to PTSD symptomatology despite exposure to traumatic events (Koenen et al., 2011). The results are in line with the notion of hypomethylation occurring in individuals suffering from PTSD as previously observed for the *NR3C1* gene (Palma-Gudiel et al., 2015).

Finally, *SLC6A4* methylation was unrelated to either AN or alcohol dependence (Park et al., 2011; Pjetri et al., 2013).

3.2.2. *Other clinical outcomes associated with* SLC6A4 *methylation*

Monozygotic twin studies allow complex traits to be examined independently of genetic confounding. Briefly, the *intrapair* difference of the variable of interest can only be explained by non-shared environmental factors; often, this effect can be mediated by epigenetic mechanisms. That is why monozygotic twin studies are an excellent tool for disentangling the role of epigenetics in complex traits such as psychiatric disorders but also stress reactivity and obesity, as detailed below (Córdova-Palomera et al., 2015).

On the one hand, Zhao et al. described a correlation between: intrapair differences in several obesity measures, such as body mass index, body weight and waist circumference; and intrapair differences in *SLC6A4* methylation, such that the two types of measure were positively associated, i.e. higher methylation was associated with greater obesity (Zhao et al., 2013b).

On the other hand, Ouellet-Morin et al. explored stress reactivity by means of an adapted version of the Trier Social Stress Test (TSST), whereby subjects are exposed to a laboratory stressor in order to study their HPA axis response, and found a significant inverse correlation between *SLC6A4* CpG-specific methylation and cortisol levels during the stress procedure (Ouellet-Morin et al., 2013). Additionally, Alexander et al. explored the same variable by means of the TSST in a sample of the general population (non-twins) and obtained similar results: *SLC6A4* methylation at three specific CpG sites was associated with cortisol response (one direct and two inverse correlations were reported), although these results did not survive Bonferroni correction (Alexander et al., 2014).

As briefly discussed in the neuroimaging section, Wang et al. studied brain 5-HT synthesis and *SLC6A4* methylation in subjects who exhibited childhood-limited high physical aggression (C-LHPA); they reported higher methylation at specific CpG sites of C-LHPA subjects than in controls, in both T-lymphocytes and monocytes (Wang et al., 2012).

As previously mentioned, 5-HT is a key neurotransmitter whose range of action is not limited to the CNS but it is also involved in a number of peripheral processes such as gastrointestinal motility (Gershon and Biology, 2004). Thus, Tahara et al. analyzed the possible role of *SLC6A4* methylation at three different gene regions in gastric mucosa of functional dyspepsia (FD) patients. When compared to controls, FD patients exhibited lower *SLC6A4* methylation in the promoter region of the gene, while they also exhibited higher methylation in a region overlapping with exon 9 (Tahara et al., 2014).

In a sample of systemic lupus erythematosus (SLE) patients, there was no significant difference in SLC6A4 methylation either in SLE patients compared to controls, or in concomitantly depressed SLE patients compared to non-depressed SLE patients (Xu et al., 2013).

3.2.3. *The role of* SLC6A4 *methylation in pharmacological and psychotherapy responses*

Studies focusing on treatment response employed one of two approaches: assessing *SLC6A4* methylation either a priori, before treatment administration, or a posteriori, after treatment had been ceased.

3.2.3.1. Assessing the prognostic value of SLC6A4 *methylation.* Domschke et al. found higher *SLC6A4* methylation to be associated with better treatment response to SSRIs (Domschke et al., 2014). Similarly, Okada and colleagues found higher *SLC6A4* methylation at a specific CpG site to be positively correlated to the improvement ratio; interestingly, methylation at this particular CpG site was inversely correlated to early trauma in the study (Okada et al., 2014). Nevertheless, Kang et al. found less Hamilton Depression Rating Scale (HAM-D), Hamilton Anxiety Rating Scale (HAM-A) and Social and Occupational Functional Assessment Scale (SOFAS) improvement to be related to higher methylation (Kang et al., 2013); furthermore, Iga et al. reported higher CpG-specific methylation to be associated with less improvement in depressive symptoms as measured by the HAM-D scale (Iga et al., 2016).

3.2.3.2. Assessing the effect of treatment on SLC6A4 *methylation.* Booij et al. reported greater CpG-specific methylation in patients treated with SSRIs than in both patients taking dual antidepressants and subjects that did not take any medication, irrespective of treatment response (Booij et al., 2015). In this vein, Gartstein et al. reported higher methylation in newborns exposed to maternal consumption of SSRI during pregnancy (Gartstein et al., 2016); in contrast, Devlin et al. reported no methylation differences between SSRI-exposed and non-exposed pregnant women, or in their newborns (Devlin et al., 2010).

Notably, Roberts et al. reported that only responders to cognitive behavior therapy (CBT) showed increased *SLC6A4* methylation with respect to methylation values before starting CBT; in contrast, non-responders exhibited a methylation decrease at the same CpG site (Roberts et al., 2014).

3.3. *Explanatory variables*

3.3.1. *Neuroimaging correlates of* SLC6A4 *methylation*

Seven papers assessed several neuroimaging variables in association with *SLC6A4* methylation (Booij et al., 2015; Dannlowski et al., 2014; Frodl et al., 2015; Muehlhan et al., 2015; Nikolova et al., 2014; Swartz et al., 2016; Wang et al., 2012). The hippocampus and amyg-

dala received most attention as they are two of the main brain areas involved in HPA axis regulation (McEwen et al., 2015).

3.3.1.1. The hippocampus. Regarding hippocampal volume, whose reduction is one of the most replicated findings in depression, Dannlowski et al. found a positive correlation between *SLC6A4* methylation and bilateral hippocampal gray matter volume; while Booij et al. found a negative correlation between *SLC6A4* methylation and hippocampal volume (Booij et al., 2015; Dannlowski et al., 2014). Despite the seemingly controversial nature of this evidence, it is important to note that Dannlowski et al. focused on DNA methylation of an *AluJb* element (located outside the CpG island of interest) while Booij et al. analyzed DNA methylation of a sequence within this CpG island. Traditionally, CpG sites located within a CpG island have been described to be hypomethylated; while CpG sites located outside CpG islands, also described as the "open sea", usually appear hypermethylated. It is widely accepted that higher methylation within a promoter-located CpG island correlates with decreased gene expression; in the same manner, higher methylation in *Alu* elements could prevent their expression (Bakshi et al., 2016). Notably, the transcription of *Alu* elements competes with transcripts close by thus diminishing their expression (Kaer and Speek, 2013).

3.3.1.2. The amygdala and the salience network. The amygdala has also received widespread attention due to its involvement in emotion processing and response to aversive cues. Threat-related amygdala reactivity was associated with *SLC6A4* methylation in two independent cohorts, including one in a longitudinal study where the increase in methylation after a 2 to 3 years of follow-up correlated to an increase in threat-related amygdala reactivity in the same time period (Nikolova et al., 2014; Swartz et al., 2016). Moreover, Muehlhan et al. found the connectivity between the amygdala and key nodes of the salience network to be associated with *SLC6A4* methylation (Muehlhan et al., 2015). The nodes identified and functionally connected to the amygdala included anterior cingulate regions, the anterior insula, the operculum and the inferior frontal gyrus, which all belong to the salience network (Downar et al., 2016). Additionally, Dannlowski and colleagues not only found a positive correlation between *SLC6A4 AluJb* methylation and hippocampal volume, but also with the amygdala, insula and striatum. In agreement with this, by means of an emotion-processing task during fMRI, Frodl and colleagues found *SLC6A4* methylation to be associated with BOLD responses of both the anterior insula and the frontal operculum area only when elicited by negative emotional stimuli (Frodl et al., 2015). As previously stated, both independently identified areas belong to the salience network and were previously described as differentially connected to the amygdala depending on *SLC6A4* methylation.

3.3.1.3. 5-HT synthesis. Finally, Wang et al. studied brain 5-HT synthesis, by means of PET imaging, in relation to peripheral *SLC6A4* methylation at three specific CpG sites previously found to be associated with childhood aggression in the same study; specifically, higher methylation was associated with lower 5-HT synthesis (Wang et al., 2012).

3.3.2. Methylation and expression

By definition, epigenetic mechanisms have the capacity to modify gene expression (Jaenisch and Bird, 2003); thus, the scientific relevance of detecting differences in DNA methylation between cases and controls stems from the principle that the differences have an effect on gene expression. Twelve out of the 49 articles reviewed examined *SLC6A4* mRNA expression in relation to its methylation. In vitro studies using either luciferase reporter assays or lymphoblast cell line cultures, together with papers assessing mRNA expression in post-mortem brain tissue, all report an inverse correlation between methylation and expression of the *SLC6A4* gene (Abdolmaleky et al., 2014; Nikolova et al., 2014; Olsson et al., 2010; Philibert et al., 2008; Sugawara et al., 2011; Vijayendran et al., 2012; Wang et al., 2012). Additionally, Tahara et al. reported a direct correlation between mRNA expression and methylation at a non-CpG island region and a statistical tendency in the opposite direction when examining methylation at the CpG island (Tahara et al., 2014). No correlation was found when examining mean *SLC6A4* methylation and its expression directly in peripheral fresh blood (Booij et al., 2015; Duman and Canli, 2015; Wang et al., 2012; Wankerl et al., 2014). Notably, both Wankerl et al. and Iga et al. did find an association between CpG-specific methylation and mRNA expression in peripheral blood suggesting that correlations between methylation and expression cannot be obtained from average methylation percentages but can from CpG-specific analysis. Notably, Iga et al. found this association in patients but not in controls (Iga et al., 2016).

3.4. Conclusions

Psychosocial stress was studied in more than a half of the articles reviewed, highlighting the major interest in describing how the environment can become embedded under the skin by means of epigenetic mechanisms at any age. Based on the reported findings, early postnatal stages spanning to late childhood seem to be crucial windows of vulnerability to stressful environments, with a clear and replicated positive association where higher levels of trauma correlate to higher levels of *SLC6A4* methylation. Results from prenatal and adult studies are not so straightforward but also suggest a certain epigenetic susceptibility, with epigenetic modifications more easily detectable if the stressor occurred more recently.

Regarding the putative association between *SLC6A4* methylation and disease, there is enough evidence to support a link with depression such that higher methylation correlates with both higher severity and incidence of this disorder. Interestingly, this methylation could be inherited as supported by its association with familial aggregation of depression (Kang et al., 2013). Additionally, higher *SLC6A4* methylation has also been reported in isolated studies targeting other psychiatric diagnoses, such as SZ, BD and antisocial personality disorder; or other non-psychiatric conditions or symptoms, such as aggression, obesity and functional dyspepsia. Although these results require further replication, they suggest a non-categorical impact of *SLC6A4* methylation on the psychopathological continuum (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013) while it may also have an impact on other complex traits that have been reported to be highly comorbid with different psychiatric disorders (Shelton and Miller, 2010; Tillisch and Labus, 2011). However, due to the transversal nature of all the studies, it remains unclear whether heightened *SLC6A4* methylation confers increased vulnerability to psychopathology or the other way round, i.e. disorder onset itself induces the methylation of the *SLC6A4* gene.

Additionally, *SLC6A4* methylation seems to alter the functioning of the hypothalamic-pituitary-adrenal axis, as can be derived from the association between stress-reactive cortisol levels and *SLC6A4* methylation. These results were anticipated as serotonin is known to interact with the HPA axis and they further suggest that an abnormal stress response plays a role as one of the effectors of the G × E interactions reviewed (Miller et al., 2012). Unfortunately, regarding pharmacological/psychotherapy response, only one paper reported results from a longitudinal study where methylation was assessed both before and after the treatment took place. Research into the putative role of *SLC6A4* methylation in treatment response is scarce and heterogeneous (both in terms of regions assessed, timing of the sampling, the ethnicity of the participants and the outcome

variable of interest), showing conflicting results. In the light of the available data, no conclusions can be drawn, as it remains unclear whether methylation at the *SLC6A4* promoter can influence treatment response or mediate its effects.

The cross-sectional findings discussed above suggest a hypothetical framework where early exposure to severe psychosocial stress could trigger *SLC6A4* hypermethylation which would further increase vulnerability to developing a range of unspecific psychopathological manifestations in adulthood and other comorbidities known to be epidemiologically linked to exposure to stress. This hypermethylation would be associated with reduced expression of the serotonin transporter in the brain (suggesting that this effect might be present in peripheral tissues as well). The clinical and molecular consequences of the changes in function or expression of 5-HT receptors and transporters due to antidepressants have been discussed at length for over 50 years now. In this regard, there is considerable agreement concerning the existence of an optimal serotoninergic balance at both the pre- and post-synaptic levels in order for neurotransmission to be efficient (Blier and Montigny, 1999). Thus, the previously described stress-driven constitutive reduction of the serotonin transporter mediated by increased methylation is suggested to increase the synaptic concentration of 5-HT due to the reduced reuptake of this neurotransmitter. However, this abnormal serotoninergic tone most likely deregulates homeostatic pathways such as 5-HT_{1A} function and/or 5-HT synthesis, as suggested by the findings of Wang et al. in childhood-limited aggression. Neuroimaging evidence further complements this conceptual construct by revealing hippocampal deterioration together with heightened reactivity of the amygdala and connectivity to the salience network in association with increased *SLC6A4* methylation. Ultimately, these methylation-dependent molecular, neuroanatomical and functional alterations would be the drivers of cognitive, emotion-processing and salience deficits together with worse treatment response, thereby giving rise to and increasing the severity of psychiatric disorders.

4. Moderation of methylation by 5-HTTLPR

Assuming that there existed a G × E interaction between early trauma and 5-HTTLPR that increased psychopathology risk, as suggested by Caspi et al., could *SLC6A4* methylation be mediating it? Out of the 49 papers reviewed, 29 (61%) assessed the genotype for 5-HTTLPR.

4.1. Moderator effect of 5-HTTLPR in the reported association between psychosocial stress and SLC6A4 methylation

Fourteen studies simultaneously assessed *SLC6A4* methylation, genotyped 5-HTTLPR and determined psychosocial stress. Four of those papers found no 5-HTTLPR moderation independently of the timing of the stressor (Alasaari et al., 2012; Chau et al., 2014; Dukal et al., 2015; Swartz et al., 2016). An additional study did not find 5-HTTLPR to have an influence on methylation after controlling for diagnosis, so this polymorphism was excluded from further research into early trauma and the study did not explore the putative moderator effect of 5-HTTLPR on the reported association between methylation and trauma (Okada et al., 2014).

Lei et al. found *s* allele carriers to be more susceptible to the environment, such that only s carriers exhibited an increase in *SLC6A4* methylation when exposed to high neighborhood crime; while *s* carriers raised in a more positive environment exhibited lower methylation than *ll* homozygotes (Lei et al., 2015). Along these lines, Beach et al. reported a G × E interaction where *SLC6A4* methylation correlated with both child sex abuse and symptoms of antisocial personality disorder, with the second correlation only significant for *s* carriers in a dose-dependent manner (the strongest correlation was for *ss* homozygotes) (Beach et al., 2011). Additionally, Duman et al. reported early life stress to correlate with methylation only in *s* carriers (Duman and Canli, 2015).

In contrast, van der Knaap found *ll* homozygotes exposed to stressful life events to exhibit higher *SLC6A4* methylation; whereas there was no association between stressful life events and methylation in s carriers (van der Knaap et al., 2015a, 2015b). Moreover, van Ijzendoorn found densely methylated *ll* homozygotes to score higher for unresolved loss and trauma while ss homozygotes with higher methylation scored lower for this same variable (van IJzendoorn et al., 2010). Likewise, Booij et al. described *ll* homozygotes with a history of abuse to have greater *SLC6A4* methylation compared to both abused and non-abused *s* carriers; notably, only 7 out of 69 participating subjects were *ll* homozygotes with a history of abuse (Booij et al., 2015).

Wankerl et al. found 5-HTTLPR to interact with early trauma decreasing *SLC6A4* mRNA expression; however, they statistically ruled out *SLC6A4* methylation as a mediator of this interaction (Wankerl et al., 2014). Studies assessing methylation by means of genome-wide arrays reported significant interactions but did not specify the direction of the effect (Beach et al., 2014; Vijayendran et al., 2012).

4.2. Moderator effect of 5-HTTLPR in the reported association between SLC6A4 methylation and psychopathological risk and associated variables

Meanwhile, 8 of the 49 studies analyzed 5-HTTLPR genotype in relation to the methylation—psychopathology couple, regardless of environmental variables. As to the putative effect of 5-HTTLPR in depression susceptibility, Olsson et al. reported *s* carriers with the highest *SLC6A4* methylation levels to be at an increased risk of developing depression (Olsson et al., 2010); similarly, Kim et al. found the association between *SLC6A4* methylation and post stroke depression to be stronger in *s* homozygotes (Kim et al., 2013). In this vein, Sugawara et al. reported higher *SLC6A4* CpG-specific methylation together with lower expression among the *s* homozygotes with BD compared to controls; this association was not observed in *l* carriers (Sugawara et al., 2011).

Conversely, van Mil et al. reported *s* carriers to exhibit lower *SLC6A4* methylation and to be at increased risk of developing ADHD (van Mil et al., 2014); likewise, Iga and colleagues found *l* carriers to exhibit increased methylation but only in the depressed group (Iga et al., 2016).

Zhao and colleagues found no genetic moderation of 5-HTTLPR in either methylation or depressive symptoms (Zhao et al., 2013a, 2013b). Since no association between *SLC6A4* methylation and depression was observed in the study by Chagnon et al., no further genetic analysis was performed (Chagnon et al., 2015). In a similar fashion, there was no association between 5-HTTLPR and methylation in another study, so no moderation was explored (Park et al., 2015).

With respect to neuroimaging studies, there is quite some disparity. Muehlhan et al., Nikolova et al. and Swartz et al. all found an epigenetic preponderance over genetic sequence, as 5-HTTLPR did not seem to have an impact on overall findings (Muehlhan et al., 2015; Nikolova et al., 2014; Swartz et al., 2016). In contrast, the association between methylation and hippocampal volume reported by Dannlowski and colleagues was not significant in the L_aL_a group (Dannlowski et al., 2014).

Moreover, Alexander et al. described low methylation individuals to respond differently to stress as a function of their 5-HTTLPR genotype, in such a way that *s* homozygotes exhibited higher cortisol responses to the TSST than their *l* homozygote counterparts. Intriguingly, this difference vanished in subjects pertaining to the

high methylation group, irrespective of their genotype (Alexander et al., 2014). As mentioned in previous sections, subjects were assigned to either the low or the high methylation group, after the total sample was divided between the two.

Finally, Domschke et al. found the effect of low methylation on poorer antidepressant responses to be more pronounced in subjects with haplotypes associated with low *SLC6A4* mRNA expression (Domschke et al., 2014). Meanwhile Xu et al. reported *s* homozygotes to be more prevalent in the depressed group of systemic lupus erythematosus than among the non-depressed patients; however, 5-HTTLPR had no effect on *SLC6A4* methylation (Xu et al., 2013).

4.3. Moderator effect of 5-HTTLPR as part of a gene—environment interaction on psychopathological risk mediated by SLC6A4 methylation

Strikingly, only 9 papers included in their methodology an analysis of *SLC6A4* methylation, 5-HTTLPR genotyping, some sort of stress measure and psychopathological assessment simultaneously. Of these, only 3 considered all the variables at once in the same statistical approach, including some of them as covariates. Finally, only a couple of the studies directly examined a G × E model where 5-HTTLPR and methylation were introduced as moderator and mediator variables respectively. This paucity of studies explicitly exploring a G × E interaction was unexpected considering that most of the papers reviewed herein cite the work by Caspi et al. at some point. Interestingly, both these articles found s carriers to be more susceptible to early stress, showing greater *SLC6A4* methylation associated with higher depressive or ASPD symptoms respectively (Beach et al., 2011; Lei et al., 2015).

4.4. Conclusions

The evidence does not allow a straightforward conclusion regarding the role of 5-HTTLPR in moderating the epidemiological link between psychosocial stress and psychiatric disorders as mediated by *SLC6A4* methylation. Depending on the study, ss homozygotes, *s/l* heterozygotes, *ll* homozygotes or none of these are suggested to be more susceptible to the environment and more prone to developing psychopathological symptoms later. However, when examining only those studies that statistically modeled a G × E interaction mediated by *SLC6A4* methylation with psychopathology as the outcome, *s* carriers who had suffered stressful experiences during childhood were reported as the most vulnerable subgroup with the highest chance of developing psychopathology and highest *SLC6A4* methylation levels. These findings would be in line with traditional G × E studies performed without methylation data and with most of the papers reviewed in the present section; notwithstanding, these conclusions are derived from just two different studies and further replication is needed to disentangle this interaction. More importantly, the fact that numerous papers report no effect of 5-HTTLPR at all, explicitly stating in some cases the preponderance of epigenetics over genetics, means that the controversy over this polymorphism does not seem likely to end soon. Fig. 3 schematically summarizes the conceptual framework presented in this review and the strength of the associations, moderations and interactions discussed.

5. Methodological challenges

When it comes to DNA methylation studies, there are a number of pervasive issues that emerge in all discussions, calling into question the validity of the findings reported to date.

5.1. Methylation assessment and data processing

Since there is a wide range of possibilities to choose from when it comes to both assessing and statistically analyzing DNA methylation, it is essential to have a biological hypothesis *a priori* so the methylation data undergo logical processing. For example, if methylation at a specific transcription factor binding region is of interest for the working hypothesis, (i) technologies which do not allow CpG-specific methylation resolution, such as mass spectrometry, should be avoided; and (ii) statistical analysis must be computed with CpG-specific values rather than with mean percentages or principal component analysis (PCA). Additionally, when examining several CpG sites individually, multiple testing corrections must be employed to avoid an overload of false positives. For a detailed overview of specific CpG sites examined by each paper, see Fig. 4. Likewise, when analyzing DNA methylation in brain tissue, the high ratio of hydroxymethylation also has to be taken into account as bisulfite treatment does not allow discrimination between methylation and hydroxymethylation.

To better illustrate this problem, papers that assess the relationship between childhood trauma and *SLC6A4* methylation (Section 3.1) are further discussed below, with regard to the specific location of the CpG sites of interest. Of the ten papers reporting positive associations between childhood trauma and *SLC6A4* methylation, six only took into account average or composite methylation percentages as the dependent variable; while the other four also performed the analysis CpG site by CpG site. Intriguingly, significant associations are concentrated at both ends of the CpG island. On the one hand, four papers report clear associations between childhood adversity and methylation in the 5′ end of the CpG island (Booij et al., 2015; Frodl et al., 2015; Kang et al., 2013; Swartz et al., 2016); however, two of them studied the same cohort. The fact that only Kang et al. found CpG-specific effects while Booij et al. and Frodl et al. only found associations with mean methylation values could be due to both the larger sample and the wider definition of adversity used by the former. On the other hand, Beach et al. found clear CpG-specific effects only in females at the 3′ end of the CpG island (Beach et al., 2010). Later on, three additional papers found the same association between childhood adversity and mean methylation percentage in a wide region encompassing the 3′ end of the CpG island, analyzed by means of mass spectrometry which does not allow site-specific CpG resolution (Beach et al., 2011; Duman and Canli, 2015; van der Knaap et al., 2015a). Thus, this apparent replication of results could or could not be due to increased methylation specifically at the CpG sites primarily reported by Beach and colleagues: the method employed does not allow such discrimination. Additionally, Ouellet-Morin et al. assessed *SLC6A4* methylation in an intermediate region which does not encompass either of the CpG island ends, and still reported a site-specific CpG methylation at CpG #30, as numbered in Figs. 2 and 3 (Ouellet-Morin et al., 2013). As for Lei et al., since their analyses were performed with the composite index of seven probes of the Illumina array scattered all along the CpG island, their paper does not provide additional data regarding the specific CpG sites or CpG island regions of relevance regarding childhood adversity (Lei et al., 2015).

Thus, the nature of the available data does not allow a description of a particular biomarker of known genomic coordinates. In this sense, it is important to note the relevance of such markers as methylation events in neighboring dinucleotides could exert comparable effects on gene expression (this issue is further developed in Section 6).

5.2. Peripheral measures as a surrogate for brain tissue

With respect to extrapolation from peripheral to central methylation, it remains unclear whether certain epigenetic markers may

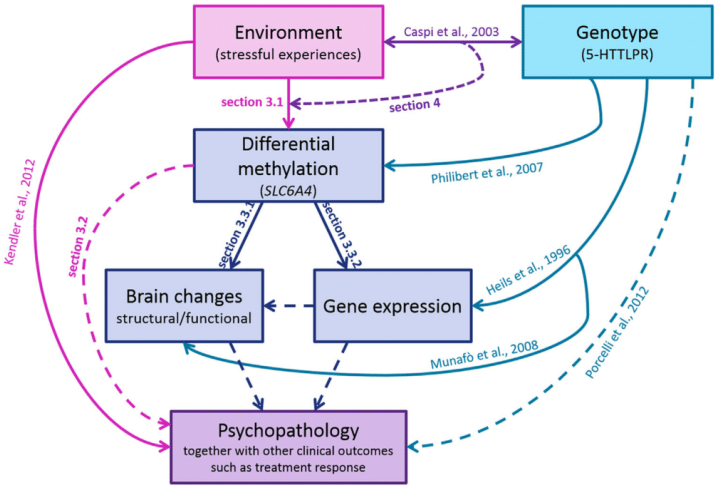

Fig. 3. Conceptual framework of the complex gene—environment interaction described in the present review. As previously reported, both psychosocial stress (Section 3.1) and 5-HTTLPR genotype (introduction) have been independently associated with *SLC6A4* methylation. Likewise, both environmental and genetic variables have been associated with the onset of psychopathology. Moreover, 5-HTTLPR genotype has been reported to modulate directly both *SLC6A4* expression and brain functioning (introduction). Finally, translational research shows evidence for a gene—environment interaction between exposure to stressful events and the *s* allele. This interaction might be mediated by *SLC6A4* methylation (Section 4). Specifically, *SLC6A4* methylation decreases gene expression (Section 3.3.2) and has been associated with abnormal brain structural and functional patterns (Section 3.3.1), which are most likely mediated by changes in *SLC6A4* expression. These changes in *SLC6A4* expression and brain wiring would be the ultimate effectors of increased psychopathological risk. To distinguish between evidence-based strong associations and those which are preliminary (reported but requiring further replication), the latter are displayed with dashed arrows.

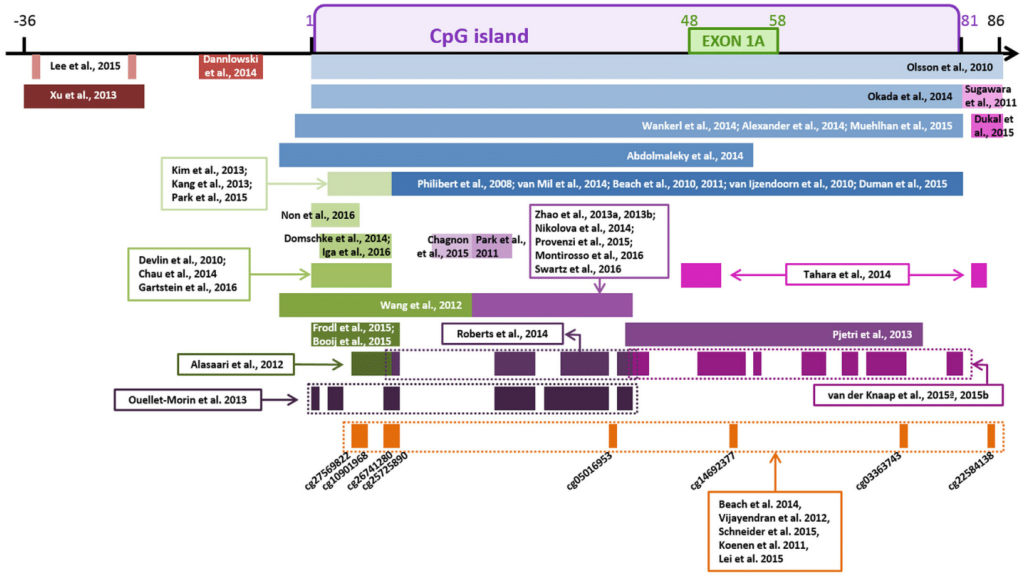

Fig. 4. Schematic representation of CpG sites assessed by each of the 49 papers included in the current review. Different colors indicate papers assessing: the whole CpG island located at *SLC6A4* promoter region (blue); its 5′end (green); its 3′ end (pink); internal regions not encompassing either end (purple); proximal regions not encompassed in the CpG island itself (red); and specific CpG sites included in epigenome-wide arrays (orange) with code numbers for the Infinium HumanMethylation450 BeadChip Kit (Illumina). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have an impact on several tissues simultaneously; but an increasing number of researchers suggest there is indeed a correlation. Nevertheless, epigenetic enquiry in rhesus macaques exposed to maternal separation showed differential methylation patterns in both brain tissue and peripheral cells, thus supporting a peripheral impact of early stress detectable in adult individuals (Provençal et al., 2012). Alternatively, the peripheral source of DNA may be informative on its own as candidate genes for neurobehavioral alterations have expression patterns surpassing the brain; for instance, *SLC6A4* is expressed and functioning in platelets. In contrast, if either the stressor is experienced prenatally or the methylation pattern is inherited, the observed pattern in peripheral tissue will definitely correspond to the central one. Evidently, post-mortem brain samples are also suitable for epigenetic analysis; but, no sample is optimal as brain tissue is comprised of a mixture of neurons, astrocytes, microglia and oligodendrocytes with cell-specific epigenetic patterns. Nevertheless, statistical tools have already been designed to avoid the confounding effect of cellular heterogeneity when working with epigenomic data in both brain tissue (Guintivano et al., 2013) and peripheral blood mixture (Houseman et al., 2012).

As a matter of fact, there was some active scientific correspondence a few years ago regarding the use of lymphoblast cell lines in DNA methylation research due to their viral transformation. Briefly, while Åberg and Van Den Oord argued that B lymphocytes transformed by the Epstein – Barr virus could undergo epigenetic reprogramming which would not reflect the original cellular signature, Philibert et al. defended the idea that low passage lymphoblast lines may be better proxies of cerebral DNA methylation than whole blood (Åberg and Van Den Oord, 2011; Philibert et al., 2011).

5.3. Potential confounding variables

As reviewed so far, most research has focused on the putative role of DNA methylation in mediating the effect of exposure to psychosocial stress on vulnerability to several complex traits and disorders, mostly of a psychiatric nature. However, attention must be paid to several confounding factors that could also be influencing methylation and hindering the correct interpretation of the results. This sort of analysis has been addressed by a number of authors, their main findings are detailed below.

Gender and age are the most evident confounders in methylation studies; that are two classic covariates usually included in all statistical approaches regardless of the topic (Fraga et al., 2005). Regarding gender, of the 49 papers reviewed, ten studies were only of male or only of female samples. Of the 39 mixed samples, seven papers reported that gender had a major effect on *SLC6A4* methylation (Beach et al., 2014, 2010; Booij et al., 2015; Dukal et al., 2015; Koenen et al., 2011; Philibert et al., 2008; van Mil et al., 2014). Two of those studies reported female subjects to exhibit higher *SLC6A4* methylation levels than male subjects; and this significant difference was observable from birth to adulthood (Dukal et al., 2015; Philibert et al., 2008). In contrast, Booij et al. reported higher methylation in male subjects (Booij et al., 2015). Notably, they studied non-overlapping regions of the *SLC6A4* gene. Likewise, Beach et al. reported several gender-specific associations between stress and methylation (Beach et al., 2014). Although the remaining 32 papers report no gender effect on methylation, several authors did not test for this association or included sex as a covariate in their statistical models without specifying the amount of the variance explained by this variable.

Additionally, Wankerl et al. found a significant association between the use of oral contraceptives and mean *SLC6A4* methylation (Wankerl et al., 2014); Lei et al. also found cigarette consumption and age to influence methylation in a comprehensive regression model including eight control variables besides the interaction of interest (Lei et al., 2015). Furthermore, the main findings already discussed by Zhao et al. suggest that BMI could be associated with *SLC6A4* methylation and thus should be considered as an additional confounder (Zhao et al., 2013b).

Although it was not directly addressed in any of the papers reviewed, an additional issue that may confound overall conclusions is the existence of sociocultural differences both across and within the countries of origin of the samples assessed. The subjective experience of stress may vary as a function of sociocultural background. Prior research has shown that socioeconomic status mediates the impact of childhood maltreatment on memory performance: briefly, childhood maltreatment only contributed to impaired memory performance in those subjects belonging to high-SES families (not to low-SES families), thus suggesting that environmental enrichment could predict worse outcomes after exposure to trauma, or that low-SES could trigger resilience to trauma; or a combination of the two (Goldberg et al., 2013). In this regard, it is fundamental to consider the different impact of different stressors since several authors design "trauma indexes" or "stress variables" simultaneously including in the same statistical construct data concerning explicit maltreatment, peer bullying victimization, parental loss and poverty, among others.

5.4. Small yet significant differences

Regarding the very limited extent of the reported differences in the psychiatric and neurobehavioral fields, there are two main concepts to be noted. First, aberrant methylation percentages exceeding a 30% methylation increase (or decrease) are only seen in pathological tissue such as cancer tissue. Here, basic cellular physiology and functioning has been compromised. In contrast, the changes expected when it comes to differentiating depressive patients from controls must me subtle, as the organism might be affected but continues to function normally. Subtle changes in methylation can indicate equally subtle changes in tissue composition. Most methylation research is based on the analysis of whole blood or brain tissue homogenates which both include a mixture of cells with different epigenetic patterns.

5.5. Study design: can we rely on the association between retrospective measures and dynamic markers?

Concerning study design, although transversal studies in informative samples yield suggestive findings, they do not allow us to establish causal relationships between trauma and methylation definitely nor between methylation and psychopathology. Notably, epigenetic markers are very dynamic and while it is uncertain whether perinatal stress can elicit an epigenetic signature that lasts until adulthood, the inheritance of epigenetic markers from parents has to be accounted for too. Thus, the optimal strategy to characterize DNA methylation dynamics fully is by means of longitudinal studies in large populations. Alternatively, assessing methylation proximally after the environmental variable of interest has taken place would be a suitable approach, as suggested by van der Knaap et al., who found higher correlations between stressful life events and methylation when they had been experienced recently rather than in earlier stages of life (van der Knaap et al., 2015a).

5.6. Can we assume that 5-HTTLPR reflects most SLC6A4 genetic variability?

Although the 5-HTTLPR insertion/deletion variant has received most attention in genetic studies focusing on the *SLC6A4* gene, more than twenty SNPs can be found alongside this sequence; some of them described to be functional (Murphy and Moya, 2011; Murphy et al., 2013). Given that in the epigenetic studies reported herein,

the whole CpG island initially described by Philibert and colleagues has been thoroughly assessed with regard to DNA methylation at all of its more than 80 CpG sites, it would be natural to study all the reported SNPs in the gene as well, or at least the functional ones. Accordingly, when examining samples of various ethnicities, it is important to consider the potential role of 5-HTTLPR in moderating the results derived from these studies due to the different frequencies of the *s*/*l* alleles among subjects of African, American, Asian or Caucasian genetic backgrounds (Murphy and Moya, 2011).

6. Further directions

Although methylation at the *SLC6A4* gene has been widely studied by a number of researchers in the last decade, further studies are still needed in order to disentangle its role as a mediator from stress and genetic vulnerability to psychopathology. However, there is overall agreement that higher stress tends to be paired with higher promoter methylation, especially if the stress is experienced in early stages of life or recently prior to sample extraction (as Caspi and colleagues had already guessed more than a decade ago). In this regard, *s* allele carriers seem to be more epigenetically susceptible to environmental cues following this trend; but the lack of papers directly addressing the interaction with proper statistical models together with the considerable number of authors reporting either no association or one in the opposite direction, continue to call the validity of this finding into question.

In order to better understand the dynamics of *SLC6A4* methylation and its interaction with 5-HTTLPR, there is a need for longitudinal studies where not only adverse but also positive lifestyles, such as social support and high socioeconomic status, are accounted for. 5-HTTLPR has repeatedly been suggested to be a plasticity gene rather than a vulnerability gene in such a way that *s* allele carriers are not only more susceptible to negative cues but also to positive ones in a for-better-or-for-worse manner (Belsky et al., 2009; Homberg and Hove, 2012).

Interestingly, gender is highlighted as a moderating factor of epigenetic associations from the very beginning of life as sex-specific methylation differences can already be detected at birth, with female newborns exhibiting higher *SLC6A4* methylation than their male counterparts (Dukal et al., 2015). These preliminary findings are in agreement with the epidemiological evidence of sex-specific differences in the prevalence of several psychiatric disorders, with women being two to three times more vulnerable to developing mood disorders. In this vein, the human stress response itself also exhibits critical differences between males and females: men typically exhibit higher cortisol release after exposure to acute stress. These differences may be related with gender-specific adaptations to threat, with men being more prone to a *fight-or-flight* response while women develop *tend-and-befriend* behavior (Del Giudice et al., 2011). As previously introduced in Section 3.4, the HPA axis interacts with serotoninergic pathways thus suggesting the different impact psychosocial stress may have on individuals depending on their gender partly due to *a priori* gender-specific epigenetic differences.

Furthermore, the epigenetic effect exerted by oral consumption of contraceptives is of great interest as it serves as evidence of the dynamics of epigenetic signatures, while it leaves the door open to pharmacological approaches to target epigenetic mechanisms as well. In this regard, different pharmacological treatments exert profound epigenetic modulation, which may be responsible for both therapeutic effects and side effects (Csoka and Szyf, 2009). Notably, the use of contraceptives has previously been studied with regard to depression scores, relapse and antidepressant response, yielding conflicting results (Damoiseaux et al., 2014; Gingnell et al., 2013; Wiebe, 2013).

At the core of this discussion is the notion of stress-driven methylation specificity. Research aims to determine methylation in candidate regions that have been selected due to their putative function in the etiology of some complex disorders. Nevertheless, it is hard to figure out how stress – or excessive cortisol release – could affect not just specific genes, but specific CpG sites within those genes, triggering epigenetic events only in those nucleotides where it would have a direct functional impact. It seems more likely that certain genomic regions may be more vulnerable to epigenetic events in certain developmental windows due to a particular, so to speak, *open* chromatin state that allows chemical modifications such as DNA methylation to occur. These modifications would act coordinately with other epigenetic mechanisms such as histone acetylation and deacetylation processes. Evolutionary processes would thus modulate stress-related genes to be more epigenetically susceptible to stress in order to provide better adaptation to changing environments that can be passed down to future generations. High throughput approaches exploring epigenome-wide arrays are needed to identify these stress-susceptible genes.

As well as genetic studies focusing on particular candidate genes being difficult to replicate, this could also be the case with epigenetic research. *In vitro* studies are needed to empirically prove the causative effect of CpG-specific methylation on gene expression and also to elucidate whether methylation in neighboring CpG sites could be a conceptual equivalent of linkage disequilibrium (and thus have no effect on its own but be of use as a biomarker) or if methylation itself at any CpG site has an additive effect. The latter would imply that average methylation percentages of particular regions of interest should be the target of further research rather than CpG-specific values. In order to achieve that goal, new tools for *in vivo* epigenetic editing are currently in development and will be crucial for elucidating the real consequences of DNA methylation at any region of interest (Konermann et al., 2013; Vojta et al., 2016).

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References

Åberg, K., Van Den Oord, E.J.C.G., 2011. Epstein-Barr virus transformed DNA as a source of false positive findings in methylation studies of psychiatric conditions. Biol. Psychiatry 70, e25–e26, http://dx.doi.org/10.1016/j.biopsych.2011.01.042.

Abdolmaleky, H.M., Nohesara, S., Ghadirivasfi, M., Lambert, A.W., Ahmadkhaniha, H., Ozturk, S., Wong, C.K., Shafa, R., Mostafavi, A., Thiagalingam, S., 2014. DNA hypermethylation of serotonin transporter gene promoter in drug naïve patients with schizophrenia. Schizophr. Res. 152, 373–380, http://dx.doi.org/10.1016/j.schres.2013.12.007.

Alasaari, J.S., Lagus, M., Ollila, H.M., Toivola, A., Kivimäki, M., Vahtera, J., Kronholm, E., Härmä, M., Puttonen, S., Paunio, T., 2012. Environmental stress affects DNA methylation of a CpG rich promoter region of serotonin transporter gene in a nurse cohort. PLoS One 7, e45813, http://dx.doi.org/10.1371/journal.pone.0045813.

Alexander, N., Wankerl, M., Hennig, J., Miller, R., Zänkert, S., Steudte-Schmiedgen, S., Stalder, T., Kirschbaum, C., 2014. DNA methylation profiles within the serotonin transporter gene moderate the association of 5-HTTLPR and cortisol

stress reactivity. Transl. Psychiatry 4, e443, http://dx.doi.org/10.1038/tp.2014.88.
Arias, B., Catalán, R., Gastó, C., Gutiérrez, B., Fañanás, L., 2003. 5-HTTLPR polymorphism of the serotonin transporter gene predicts non-remission in major depression patients treated with citalopram in a 12-weeks follow up study. J. Clin. Psychopharmacol. 23, 563–567.
Bakshi, A., Herke, S.W., Batzer, M.A., Kim, J., 2016. DNA methylation variation of human-specific Alu repeats. Epigenetics 11, 163–173, http://dx.doi.org/10.1080/15592294.2015.1130518.
Ball, M.P., Li, J.B., Gao, Y., Lee, J.-H., LeProust, E.M., Park, I.-H., Xie, B., Daley, G.Q., Church, G.M., 2009. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat. Biotechnol. 27, 361–368, http://dx.doi.org/10.1038/nbt.1533.
Beach, S.R.H., Brody, G.H., Todorov, A., Gunter, T.D., Philibert, R., 2010. Methylation at SLC6A4 is linked to family history of child abuse: an examination of the Iowa Adoptee sample. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 153B, 710–713, http://dx.doi.org/10.1002/ajmg.b.31028.
Beach, S.R.H., Brody, G.H., Todorov, A., Gunter, T.D., Philibert, R., 2011. Methylation at 5HTT mediates the impact of child sex abuse on women's antisocial behavior: an examination of the Iowa adoptee sample. Psychosom. Med. 73, 83–87, http://dx.doi.org/10.1097/PSY.0b013e3181fdd074.
Beach, S.R.H., Dogan, M.V., Brody, G.H., Philibert, R., 2014. Differential impact of cumulative SES risk on methylation of protein–protein interaction pathways as a function of SLC6A4 genetic variation in African American young adults. Biol. Psychol. 96, 28–34, http://dx.doi.org/10.1016/j.biopsycho.2013.10.006.
Belsky, J., Jonassaint, C., Pluess, M., Stanton, M., Brummett, B., Williams, R., 2009. Vulnerability genes or plasticity genes? Mol. Psychiatry 14, 746–754, http://dx.doi.org/10.1038/mp.2009.44.
Berger, M., Gray, J., Roth, B.L., 2009. The expanded biology of serotonin. Annu. Rev. Med. 60, 355–366, http://dx.doi.org/10.1146/annurev.med.60.042307.110802.
Bernstein, D.P., Fink, L., Handelsman, L., Foote, J., Lovejoy, M., Wenzel, K., Sapareto, E., Ruggiero, J., 1994. Initial reliability and validity of a new retrospective measure of child abuse and neglect. Am. J. Psychiatry 151, 1132–1136, http://dx.doi.org/10.1176/ajp.151.8.1132.
Blier, P., Montigny, C. De, 1999. Serotonin and drug-Induced therapeutic responses in major depression, obsessive–compulsive and panic disorders. Neuropsychopharmacology 21, 91S–98S.
Booij, L., Szyf, M., Carballedo, A., Frey, E.-M., Morris, D., Dymov, S., Vaisheva, F., Ly, V., Fahey, C., Meaney, J., Gill, M., Frodl, T., 2015. DNA methylation of the serotonin transporter gene in peripheral cells and stress-related changes in hippocampal volume: a study in depressed patients and healthy controls. PLoS One 10, e0119061, http://dx.doi.org/10.1371/journal.pone.0119061.
Bowers, M.E., Yehuda, R., 2016. Intergenerational transmission of stress in humans. Neuropsychopharmacology 41, 232–244, http://dx.doi.org/10.1038/npp.2015.247.
Córdova-Palomera, A., Fatjó-Vilas, M., Palma-Gudiel, H., Blasco-Fontecilla, H., Kebir, O., Fañanás, L., 2015. Further evidence of DEPDC7 DNA hypomethylation in depression: a study in adult twins. Eur. Psychiatry 30, 715–718, http://dx.doi.org/10.1016/j.eurpsy.2015.04.001.
Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., Mcclay, J., Mill, J., Martin, J., Braithwaite, A., Poulton, R., 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301, 386–389.
Chagnon, Y.C., Potvin, O., Hudon, C., Préville, M., 2015. DNA methylation and single nucleotide variants in the brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) genes are associated with anxiety/depression in older women. Front. Genet. 6, 230, http://dx.doi.org/10.3389/fgene.2015.00230.
Chau, C.M.Y., Ranger, M., Sulistyoningrum, D., Devlin, A.M., Oberlander, T.F., Grunau, R.E., 2014. Neonatal pain and COMT Val158Met genotype in relation to serotonin transporter (SLC6A4) promoter methylation in very preterm children at school age. Front. Behav. Neurosci. 8, 409, http://dx.doi.org/10.3389/fnbeh.2014.00409.
Cottrell, E.C., Seckl, J., 2009. Prenatal stress, glucocorticoids and the programming of adult disease. Front. Behav. Neurosci. 3, 19.
Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet 381, 1371–1379.
Csoka, A.B., Szyf, M., 2009. Epigenetic side-effects of common pharmaceuticals: a potential new field in medicine and pharmacology. Med. Hypotheses 73, 770–780, http://dx.doi.org/10.1016/j.mehy.2008.10.039.
Damoiseaux, V.A., Proost, J.H., Jiawan, V.C.R., Melgert, B.N., 2014. Sex differences in the pharmacokinetics of antidepressants: influence of female sex hormones and oral contraceptives. Clin. Pharmacokinet. 53, 509–519, http://dx.doi.org/10.1007/s40262-014-0145-2.
Dannlowski, U., Kugel, H., Redlich, R., Halik, A., Schneider, I., Opel, N., Grotegerd, D., Schwarte, K., Schettler, C., Ambrée, O., Rust, S., Domschke, K., Arolt, V., Heindel, W., Baune, B.T., Suslow, T., Zhang, W., Hohoff, C., 2014. Serotonin transporter gene methylation is associated with hippocampal gray matter volume. Hum. Brain Mapp. 35, 5356–5367, http://dx.doi.org/10.1002/hbm.22555.
Del Giudice, M., Ellis, B.J., Shirtcliff, E.A., 2011. The adaptive calibration model of stress responsivity. Neurosci. Biobehav. Rev. 35, 1562–1592, http://dx.doi.org/10.1016/j.neubiorev.2010.11.007.
Devlin, A.M., Brain, U., Austin, J., Oberlander, T.F., 2010. Prenatal exposure to maternal depressed mood and the MTHFR C677T variant affect SLC6A4 methylation in infants at birth. PLoS One 5, e12201, http://dx.doi.org/10.1371/journal.pone.0012201.
Domschke, K., Tidow, N., Schwarte, K., Deckert, J., Lesch, K.-P., Arolt, V., Zwanzger, P., Baune, B.T., 2014. Serotonin transporter gene hypomethylation predicts impaired antidepressant treatment response. Int. J. Neuropsychopharmacol. 17, 1167–1176, http://dx.doi.org/10.1017/S146114571400039X.
Downar, J., Blumberger, D.M., Daskalakis, Z.J., 2016. Trends in cognitive sciences the neural crossroads of psychiatric illness: an emerging brain stimulation target. Trends Cogn. Sci. 20, 107–120, http://dx.doi.org/10.1016/j.tics.2015.10.007.
Dukal, H., Frank, J., Lang, M., Treutlein, J., Gilles, M., Wolf, I.A., Krumm, B., Massart, R., Szyf, M., Laucht, M., Deuschle, M., Rietschel, M., Witt, S.H., 2015. New-born females show higher stress- and genotype-independent methylation of SLC6A4 than males. Borderline Personal. Disord. Emot. Dysregulation 2, 8, http://dx.doi.org/10.1186/s40479-015-0029-6.
Duman, E., Canli, T., 2015. Influence of life stress, 5-HTTLPR genotype, and SLC6A4 methylation on gene expression and stress response in healthy Caucasian males. Biol. Mood Anxiety Disord. 5, 2, http://dx.doi.org/10.1186/s13587-015-0017-x.
Edgar, R., Tan, P.P.C., Portales-Casamar, E., Pavlidis, P., 2014. Meta-analysis of human methylomes reveals stably methylated sequences surrounding CpG islands associated with high gene expression. Epigenetics Chromatin 7, 28, http://dx.doi.org/10.1186/1756-8935-7-28.
Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suñer, D., Cigudosa, J.C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T.D., Wu, Y.-Z., Plass, C., Esteller, M., 2005. Epigenetic differences arise during the lifetime of monozygotic twins. Proc. Natl. Acad. Sci. U. S. A. 102, 10604–10609, http://dx.doi.org/10.1073/pnas.0500398102.
Frodl, T., Szyf, M., Carballedo, A., Ly, V., Dymov, S., Vaisheva, F., Morris, D., Fahey, C., Meaney, J., Gill, M., Booij, L., 2015. DNA methylation of the serotonin transporter gene (SLC6A4) is associated with brain function involved in processing emotional stimuli. J. Psychiatry Neurosci. 40, 296–305, http://dx.doi.org/10.1503/jpn.140180.
Gartstein, M.A., Hookenson, K.V., Brain, U., Devlin, A.M., Grunau, R.E., Oberlander, T.F., 2016. Sculpting infant soothability: the role of prenatal SSRI antidepressant exposure and neonatal SLC6A4 methylation status. Dev. Psychobiol. 58, 745–758, http://dx.doi.org/10.1002/dev.21414.
Gershon, M.D., Biology, C., 2004. Review article: serotonin receptors and transporters—roles in normal and abnormal gastrointestinal motility. Aliment. Pharmacol. Ther. 20, 3–14.
Gershon, M.D., 2004. Review article: serotonin receptors and transporters—roles in normal and abnormal gastrointestinal motility. Aliment. Pharmacol. Ther. 20, 3–14.
Geyer, M., Vollenweider, F.X., 2008. Serotonin research: contributions to understanding psychoses. Trends Pharmacol. Sci. 29, 445–453, http://dx.doi.org/10.1016/j.tips.2008.06.006.
Gingnell, M., Engman, J., Frick, A., Moby, L., Wikström, J., Fredrikson, M., Sundström-Poromaa, I., 2013. Oral contraceptive use changes brain activity and mood in women with previous negative affect on the pill-A double-blinded, placebo-controlled randomized trial of a levonorgestrel-containing combined oral contraceptive. Psychoneuroendocrinology 38, 1133–1144, http://dx.doi.org/10.1016/j.psyneuen.2012.11.006.
Goldberg, X., Alemany, S., Fatjó-Vilas, M., González-Ortega, I., González-Pinto, A., Cuesta, M.J., Fañanás, L., 2013. Twin-based study of the complex interplay between childhood maltreatment, socioeconomic status and adult memory. Eur. Arch. Psychiatry Clin. Neurosci. 263, 435–440, http://dx.doi.org/10.1007/s00406-012-0382-z.
Graeff, F.G., 2002. On serotonin and experimental anxiety. Psychopharmacology (Berl.) 163, 467–476, http://dx.doi.org/10.1007/s00213-002-1112-4.
Guintivano, J., Aryee, M.J., Kaminsky, Z.A., 2013. A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. Epigenetics 8, 290–302.
Heils, A., Teufel, A., Petri, S., Stöber, G., Riederer, P., Bengel, D., Lesch, K.P., 1996. Allelic variation of human serotonin transporter gene expression. J. Neurochem. 66, 2621–2624.
Homberg, J.R., Hove, D.L.A. Van Den, 2012. Progress in Neurobiology The serotonin transporter gene and functional and pathological adaptation to environmental variation across the life span. Prog. Neurobiol. 99, 117–127, http://dx.doi.org/10.1016/j.pneurobio.2012.08.003.
Houseman, E.A., Accomando, W.P., Koestler, D.C., Christensen, B.C., Marsit, C.J., Nelson, H.H., Wiencke, J.K., Kelsey, K.T., 2012. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinf. 13.
Iga, J., Watanabe, S., Numata, S., Umehara, H., Nishi, A., Kinoshita, M., Inoshita, M., Shimodera, S., Fujita, H., Ohmori, T., 2016. Association study of polymorphism in the serotonin transporter gene promoter, methylation profiles, and expression in patients with major depressive disorder. Hum. Psychopharmacol. 31, 193–199, http://dx.doi.org/10.1002/hup.2527.
Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33 (Suppl), 245–254, http://dx.doi.org/10.1038/ng1089.
Kaer, K., Speek, M., 2013. Retroelements in human disease. Gene 518, 231–241, http://dx.doi.org/10.1016/j.gene.2013.01.008.
Kang, H.-J., Kim, J.-M., Stewart, R., Kim, S.-Y., Bae, K.-Y., Kim, S.-W., Shin, I.-S., Shin, M.-G., Yoon, J.-S., 2013. Association of SLC6A4 methylation with early adversity, characteristics and outcomes in depression. Prog.

Neuropsychopharmacol. Biol. Psychiatry 44, 23–28, http://dx.doi.org/10.1016/j.pnpbp.2013.01.006.
Kim, J.-M., Stewart, R., Kang, H.-J., Kim, S.-W., Shin, I.-S., Kim, H.-R., Shin, M.-G., Kim, J.-T., Park, M.-S., Cho, K.-H., Yoon, J.-S., 2013. A longitudinal study of SLC6A4 DNA promoter methylation and poststroke depression. J. Psychiatr. Res. 47, 1222–1227, http://dx.doi.org/10.1016/j.jpsychires.2013.04.010.
Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16, 33–41.
Koenen, K.C., Uddin, M., Chang, S.-C., Aiello, A.E., Wildman, D.E., Goldmann, E., Galea, S., 2011. SLC6A4 methylation modifies the effect of the number of traumatic events on risk for posttraumatic stress disorder. Depress. Anxiety 28, 639–647, http://dx.doi.org/10.1002/da.20825.
Konermann, S., Brigham, M.D., Trevino, A., Hsu, P.D., Heidenreich, M., Cong, L., Platt, R.J., Scott, D.A., Church, G.M., Zhang, F., 2013. Optical control of mammalian endogenous transcription and epigenetic states. Nature 500, 472–476.
Lee, B.-Y., Park, S.-Y., Ryu, H.-M., Shin, C.-Y., Ko, K.-N., Han, J.-Y., Koren, G., Cho, Y.-H., 2015. Changes in the methylation status of DAT, SERT, and MeCP2 gene promoters in the blood cell in families exposed to alcohol during the periconceptional period. Alcohol. Clin. Exp. Res. 39, 239–250, http://dx.doi.org/10.1111/acer.12635.
Lei, M.-K., Beach, S.R.H., Simons, R.L., Philibert, R., 2015. Neighborhood crime and depressive symptoms among African American women: genetic moderation and epigenetic mediation of effects. Soc. Sci. Med. 146, 120–128, http://dx.doi.org/10.1016/j.socscimed.2015.10.035.
Lesch, K.-P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274, 1527–1531.
Maher, B., 2008. The case of the missing heritability. Nature 456, 18–21, http://dx.doi.org/10.1038/456018a.
McEwen, B.S., Bowles, N.P., Gray, J.D., Hill, M.N., Hunter, R.G., Karatsoreos, I.N., Nasca, C., 2015. Mechanisms of stress in the brain. Nat. Neurosci. 18, 1353–1363, http://dx.doi.org/10.1038/nn.4086.
Millan, M.J., Goodwin, G.M., Meyer-Lindenberg, A., Ove Ogren, S., 2015. Learning from the past and looking to the future: emerging perspectives for improving the treatment of psychiatric disorders. Eur. Neuropsychopharmacol. 25, 599–656, http://dx.doi.org/10.1016/j.euroneuro.2015.01.016.
Miller, R., Wankerl, M., Stalder, T., Kirschbaum, C., Alexander, N., 2012. The serotonin transporter gene-linked polymorphic region (5-HTTLPR) and cortisol stress reactivity: a meta-analysis. Eur. J. Psychotraumatol. 3, 1–7, http://dx.doi.org/10.3402/ejpt.v3i0.19328.
Montirosso, R., Provenzi, L., Fumagalli, M., Sirgiovanni, I., Giorda, R., Pozzoli, U., Beri, S., Menozzi, G., Tronick, E., Morandi, F., Mosca, F., Borgatti, R., 2016. Serotonin transporter gene (*SLC6A4*) methylation associates with neonatal intensive care unit stay and 3-month-old temperament in preterm infants. Child Dev. 87, 38–48, http://dx.doi.org/10.1111/cdev.12492.
Muehlhan, M., Kirschbaum, C., Wittchen, H.-U., Alexander, N., 2015. Epigenetic variation in the serotonin transporter gene predicts resting state functional connectivity strength within the salience-network. Hum. Brain Mapp. 00, http://dx.doi.org/10.1002/hbm.22923.
Munafò, M.R., Brown, S.M., Hariri, A.R., 2008. Serotonin transporter (5-HTTLPR) genotype and amygdala activation: a meta-analysis. Biol. Psychiatry 63, 852–857, http://dx.doi.org/10.1016/j.biopsych.2007.08.016.
Munafò, M.R., Durrant, C., Lewis, G., Flint, J., 2009. Gene X environment interactions at the serotonin transporter locus. Biol. Psychiatry 65, 211–219, http://dx.doi.org/10.1016/j.biopsych.2008.06.009.
Murphy, D.L., Moya, P.R., 2011. Human serotonin transporter gene (SLC6A4) variants: their contributions to understanding pharmacogenomic and other functional G × G and G × E differences in health and disease. Curr. Opin. Pharmacol. 11, 3–10, http://dx.doi.org/10.1016/j.coph.2011.02.008.
Murphy, D.L., Maile, M.S., Vogt, N.M., 2013. 5HTTLPR: white knight or dark blight? ACS Chem. Neurosci. 4, 13–15.
Murray, C.J.L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., Aboyans, V., Abraham, J., Ackerman, I., Aggarwal, R., Ahn, S.Y., Ali, M.K., Alvarado, M., Anderson, H.R., Anderson, L.M., Andrews, K.G., Atkinson, C., Baddour, L.M., Bahalim, A.N., Barker-Collo, S., Barrero, L.H., Bartels, D.H., Basez, M.G., Baxter, A., Bell, M.L., Benjamin, E.J., Bennett, D., Bhalla, E., Bhandari, K., Bikbov, B., Abdulhak, B., Bin, A., Birbeck, G., Black, J.A., Blencowe, H., Blore, J.D., Blyth, F., Bolliger, I., Bonaventure, A., Boufous, S., Bourne, R., Boussinesq, M., Braithwaite, T., Brayne, C., Bridgett, L., Brooker, S., Brooks, P., Brugha, T.S., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Buckle, G., Budke, C.M., Burch, M., Burney, P., Burstein, R., Calabria, B., Campbell, B., Canter, C.E., Carabin, H., Carapetis, J., Carmona, L., Cella, C., Charlson, F., Chen, H., Cheng, A.T.A., Chou, D., Chugh, S.S., Coffeng, L.E., Colan, S.D., Colquhoun, S., Colson, K.E., Condon, J., Connor, M.D., Cooper, L.T., Corriere, M., Cortinovis, M., De Vaccaro, K.C., Couser, W., Cowie, B.C., Criqui, M.H., Cross, M., Dabhadkar, K.C., Dahiya, M., Dahodwala, N., Damsere-Derry, J., Danaei, G., Davis, A., De Leo, D., Degenhardt, L., Dellavalle, R., Delossantos, A., Denenberg, J., Derrett, S., Des Jarlais, D.C., Dharmaratne, S.D., et al., 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380, 2197–2223, http://dx.doi.org/10.1016/S0140-6736(12)61689-4.
Nikolova, Y.S., Koenen, K.C., Galea, S., Wang, C.-M., Seney, M.L., Sibille, E., Williamson, D.E., Hariri, A.R., 2014. Beyond genotype: serotonin transporter epigenetic modification predicts human brain function. Nat. Neurosci. 17, 1153–1155, http://dx.doi.org/10.1038/nn.3778.
Non, A.L., Hollister, B.M., Humphreys, K.L., Childebayeva, A., Esteves, K., Zeanah, C.H., Fox, N.A., Nelson, C.A., Drury, S.S., 2016. DNA methylation at stress-related genes is associated with exposure to early life institutionalization. Am. J. Phys. Anthropol. 00, http://dx.doi.org/10.1002/ajpa.23010.
Okada, S., Morinobu, S., Fuchikami, M., Segawa, M., Yokomaku, K., Kataoka, T., Okamoto, Y., Yamawaki, S., Inoue, T., Kusumi, I., Koyama, T., Tsuchiyama, K., Terao, T., Kokubo, Y., Mimura, M., 2014. The potential of SLC6A4 gene methylation analysis for the diagnosis and treatment of major depression. J. Psychiatr. Res. 53, 47–53, http://dx.doi.org/10.1016/j.jpsychires.2014.02.002.
Olsson, C., Foley, D.L., Parkinson-Bates, M., Byrnes, G., McKenzie, M., Patton, G.C., Morley, R., Anney, R.J.L., Craig, J.M., Saffery, R., 2010. Prospects for epigenetic research within cohort studies of psychological disorder: a pilot investigation of a peripheral cell marker of epigenetic risk for depression. Biol. Psychol. 83, 159–165, http://dx.doi.org/10.1016/j.biopsycho.2009.12.003.
Ouellet-Morin, I., Wong, C.C.Y., Danese, A., Pariante, C.M., Papadopoulos, S., Mill, J., Arseneault, L., 2013. Increased serotonin transporter gene (SERT) DNA methylation is associated with bullying victimization and blunted cortisol response to stress in childhood: a longitudinal study of discordant monozygotic twins. Psychol. Med. 43, 1813–1823, http://dx.doi.org/10.1017/S0033291712002784.
Palma-Gudiel, H., Córdova-Palomera, A., Leza, J.C., Fañanás, L., 2015. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. Neurosci. Biobehav. Rev. 55, 520–535, http://dx.doi.org/10.1016/j.neubiorev.2015.05.016.
Park, B.-Y., Lee, B.-C., Jung, K.H., Jung, M.H., Park, B.L., Chai, Y.G., Choi, I.-G., 2011. Epigenetic changes of serotonin transporter in the patients with alcohol dependence: methylation of an serotonin transporter promoter CpG island. Psychiatry Investig. 8, 130–133, http://dx.doi.org/10.4306/pi.2011.8.2.130.
Park, S., Lee, J.-M., Kim, J.-W., Cho, D.-Y., Yun, H.J., Han, D.H., Cheong, J.H., Kim, B.-N., 2015. Associations between serotonin transporter gene (SLC6A4) methylation and clinical characteristics and cortical thickness in children with ADHD. Psychol. Med., 1–9, http://dx.doi.org/10.1017/s003329171500094x.
Petronis, A., 2010. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 465, 721–727.
Philibert, R., Madan, A., Andersen, A., Cadoret, R., Packer, H., Sandhu, H., 2007. Serotonin transporter mRNA levels are associated with the methylation of an upstream CpG island. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 144B, 101–105, http://dx.doi.org/10.1002/ajmg.b.30414.
Philibert, R., Sandhu, H., Hollenbeck, N., Gunter, T., Adams, W., Madan, A., 2008. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 147B, 543–549, http://dx.doi.org/10.1002/ajmg.b.30657.
Philibert, R., Caspers, K., Beach, S.R.H., Bakermans-Kranenburg, M.J., van IJzendoorn, M.H., 2011. Reply to: Epstein-Barr virus transformed DNA as a source of false positive findings in methylation studies of psychiatric conditions. Biol. Psychiatry 70, e27–e28, http://dx.doi.org/10.1016/j.biopsych.2011.02.029.
Pjetri, E., Dempster, E., Collier, D., Treasure, J., Kas, M.J., Mill, J., Campbell, I.C., Schmidt, U., 2013. Quantitative promoter DNA methylation analysis of four candidate genes in anorexia nervosa: a pilot study. J. Psychiatr. Res. 47, 280–282, http://dx.doi.org/10.1016/j.jpsychires.2012.10.007.
Porcelli, S., Fabbri, C., Serretti, A., 2012. Meta-analysis of serotonin transporter gene promoter polymorphism (5-HTTLPR) association with antidepressant efficacy. Eur. Neuropsychopharmacol. 22, 239–258, http://dx.doi.org/10.1016/j.euroneuro.2011.10.003.
Provençal, N., Suderman, M.J., Guillemin, C., Massart, R., Ruggiero, A., Wang, D., Bennett, A.J., Pierre, P.J., Friedman, D.P., Côté, S.M., Hallett, M., Tremblay, R.E., Suomi, S.J., Szyf, M., 2012. The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. J. Neurosci. 32, 15626–15642, http://dx.doi.org/10.1523/JNEUROSCI.1470-12.2012.
Provenzi, L., Fumagalli, M., Sirgiovanni, I., Giorda, R., Pozzoli, U., Morandi, F., Beri, S., Menozzi, G., Mosca, F., Borgatti, R., Montirosso, R., 2015. Pain-related stress during the Neonatal Intensive Care Unit stay and SLC6A4 methylation in very preterm infants. Front. Behav. Neurosci. 9, 99, http://dx.doi.org/10.3389/fnbeh.2015.00099.
Risch, N., Herrell, R., Lehner, T., Liang, K.-Y., Eaves, L., Hoh, J., Griem, A., Kovacs, M., Ott, J., Merikangas, K.R., 2009. Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression. JAMA 301, 2462–2471.
Roberts, S., Lester, K.J., Hudson, J.L., Rapee, R.M., Creswell, C., Cooper, P.J., Thirlwall, K.J., Coleman, J.R.I., Breen, G., Wong, C.C.Y., Eley, T.C., 2014. Serotonin tranporter methylation and response to cognitive behaviour therapy in children with anxiety disorders. Transl. Psychiatry 4, e444, http://dx.doi.org/10.1038/tp.2014.83.
Schneider, E., El Hajj, N., Müller, F., Navarro, B., Haaf, T., 2015. Epigenetic dysregulation in the prefrontal cortex of suicide completers. Cytogenet. Genome Res. 146, 19–27, http://dx.doi.org/10.1159/000435778.
Serretti, A., Lilli, R., Lorenzi, C., Lattuada, E., Cusin, C., Smeraldi, E., 2002. Serotonin transporter gene (5-HTTLPR) and major psychoses. Mol. Psychiatry 7, 95–99, http://dx.doi.org/10.1038/sj/mp/4000936.

Serretti, A., Calati, R., Mandelli, L., De Ronchi, D., 2006. Serotonin transporter gene variants and behavior: a comprehensive review. Curr. Drug Targets 7, 1659–1669.
Serretti, A., Kato, M., De Ronchi, D., Kinoshita, T., 2007. Meta-analysis of serotonin transporter gene promoter polymorphism (5-HTTLPR) association with selective serotonin reuptake inhibitor efficacy in depressed patients. Mol. Psychiatry 12, 247–257, http://dx.doi.org/10.1038/sj.mp.4001926.
Sharpley, C.F., Palanisamy, S.K., Glyde, N.S., Dillingham, P.W., Agnew, L.L., 2014. An update on the interaction between the serotonin transporter promoter variant (5-HTTLPR), stress and depression, plus an exploration of non-confirming findings. Behav. Brain Res. 273, 89–105, http://dx.doi.org/10.1016/j.bbr.2014.07.030.
Shelton, R.C., Miller, A.H., 2010. Eating ourselves to death (and despair): the contribution of adiposity and inflammation to depression. Prog. Neurobiol. 91, 275–299, http://dx.doi.org/10.1016/j.pneurobio.2010.04.004.
Sugawara, H., Iwamoto, K., Bundo, M., Ueda, J., Miyauchi, T., Komori, A., Kazuno, A., Adati, N., Kusumi, I., Okazaki, Y., Ishigooka, J., Kojima, T., Kato, T., 2011. Hypermethylation of serotonin transporter gene in bipolar disorder detected by epigenome analysis of discordant monozygotic twins. Transl. Psychiatry 1, e24, http://dx.doi.org/10.1038/tp.2011.26.
Swartz, J.R., Hariri, A.R., Williamson, D.E., 2016. An epigenetic mechanism links socioeconomic status to changes in depression-related brain function in high-risk adolescents. Mol. Psychiatry, 1–6, http://dx.doi.org/10.1038/mp.2016.82.
Tahara, T., Shibata, T., Okubo, M., Sumi, K., Ishizuka, T., Nakamura, M., Nagasaka, M., Nakagawa, Y., Ohmiya, N., Arisawa, T., Hirata, I., 2014. Change in DNA methylation patterns of SLC6A4 gene in the gastric mucosa in functional dyspepsia. PLoS One 9, e105565, http://dx.doi.org/10.1371/journal.pone.0105565.
Taylor, M.J., Sen, S., Bhagwagar, Z., 2010. Antidepressant response and the serotonin transporter gene-linked polymorphic region. Biol. Psychiatry 68, 536–543, http://dx.doi.org/10.1016/j.biopsych.2010.04.034.
Tillisch, K., Labus, J.S., 2011. Advances in imaging the BrainGut axis: functional gastrointestinal disorders. Gastroenterology 140, 407–411, http://dx.doi.org/10.1053/j.gastro.2010.12.014.
Vijayendran, M., Beach, S.R.H., Plume, J.M., Brody, G.H., Philibert, R., 2012. Effects of genotype and child abuse on DNA methylation and gene expression at the serotonin transporter. Front. Psychiatry 3, 55, http://dx.doi.org/10.3389/fpsyt.2012.00055.
Vojta, A., Dobrinic, P., Tadic, V., Bockor, L., Korac, P., Julg, B., Klasic, M., Zoldos, V., 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucl. Acids Res. 44, 5615–5628, http://dx.doi.org/10.1093/nar/gkw159.
Wang, D., Szyf, M., Benkelfat, C., Provençal, N., Turecki, G., Caramaschi, D., Côté, S.M., Vitaro, F., Tremblay, R.E., Booij, L., 2012. Peripheral SLC6A4 DNA methylation is associated with in vivo measures of human brain serotonin synthesis and childhood physical aggression. PLoS One 7, e39501, http://dx.doi.org/10.1371/journal.pone.0039501.
Wankerl, M., Miller, R., Kirschbaum, C., Hennig, J., Stalder, T., Alexander, N., 2014. Effects of genetic and early environmental risk factors for depression on serotonin transporter expression and methylation profiles. Transl. Psychiatry 4, e402, http://dx.doi.org/10.1038/tp.2014.37.
Wiebe, E.R., 2013. Invited commentary: how can we reconcile the findings of Keyes et al.'s study with the experience of our patients in clinical practice? Am. J. Epidemiol. 178, 1389–1391, http://dx.doi.org/10.1093/aje/kwt186.
Xu, J., Cheng, Y.Q., Chen, B., Bai, R., Li, S., Xu, X.F., Xu, L., Wen, J.F., Lu, Z.P., Zeng, X.F., 2013. Depression in systemic lupus erythematosus patients is associated with link-polymorphism but not methylation status of the 5HTT promoter region. Lupus, 1001–1010.
Yoo, J., Kim, H., Aksimentiev, A., Ha, T., 2016. Direct evidence for sequence-dependent attraction between double-stranded DNA controlled by methylation. Nat. Commun. 7, 11045, http://dx.doi.org/10.1038/ncomms11045.
Zhao, J., Goldberg, J., Bremner, J.D., Vaccarino, V., 2013a. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. Psychosom. Med. 75, 523–529, http://dx.doi.org/10.1097/PSY.0b013e3182924cf4.
Zhao, J., Goldberg, J., Vaccarino, V., 2013b. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. Int. J. Obes. (Lond). 37, 140–145, http://dx.doi.org/10.1038/ijo.2012.8.
van IJzendoorn, M.H., Caspers, K., Bakermans-Kranenburg, M.J., Beach, S.R.H., Philibert, R., 2010. Methylation matters: interaction between methylation density and serotonin transporter genotype predicts unresolved loss or trauma. Biol. Psychiatry 68, 405–407, http://dx.doi.org/10.1016/j.biopsych.2010.05.008.
van Mil, N.H., Steegers-Theunissen, R.P.M., Bouwland-Both, M.I., Verbiest, M.M.P.J., Rijlaarsdam, J., Hofman, A., Steegers, E.A.P., Heijmans, B.T., Jaddoe, V.W.V., Verhulst, F.C., Stolk, L., Eilers, P.H.C., Uitterlinden, A.G., Tiemeier, H., 2014. DNA methylation profiles at birth and child ADHD symptoms. J. Psychiatr. Res. 49, 51–59, http://dx.doi.org/10.1016/j.jpsychires.2013.10.017.
van der Knaap, L.J., Riese, H., Hudziak, J.J., Verbiest, M.M.P.J., Verhulst, F.C., Oldehinkel, A.J., van Oort, F.V., 2015a. Adverse life events and allele-specific methylation of the serotonin transporter gene (SLC6A4) in adolescents. Psychosom. Med. 77, 246–255, http://dx.doi.org/10.1097/PSY.0000000000000159.
van der Knaap, L.J., van Oort, F.V., Verhulst, F.C., Oldehinkel, A.J., Riese, H., 2015b. Methylation of NR3C1 and SLC6A4 and internalizing problems. The TRAILS study. J. Affect. Disord. 180, 97–103, http://dx.doi.org/10.1016/j.jad.2015.03.056.

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR" included the following tasks:

- Participation in the conception and design of the study
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

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Epigenetics-by-sex interaction for somatization conferred by methylation at the promoter region of *SLC6A4* gene

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ABSTRACT

Background: Depression, anxiety and somatoform disorders are all more prevalent in women than in men. However, specific biological mechanisms contributing to such sex differences remain unknown. Serotonergic pathways are involved in mood and behavior regulation and thus have been suggested to be altered in several psychiatric disorders. The serotonin transporter (SERT), encoded by *SLC6A4* gene, has received major attention due to its crucial role in serotonergic transmission.
Methods: 148 monozygotic twin subjects were assessed for (i) lifetime categorical diagnosis of anxious-depressive disorders, following SCID-I-based DSM-IV criteria, and (ii) current psychiatric symptomatology, from a dimensional approach, by means of the Brief Symptom Inventory (BSI). *SLC6A4* gene methylation was analyzed by means of Infinium HumanMethylation450 in a subset of the sample. CpG-specific methylation at the promoter region of *SLC6A4* gene was further analyzed by means of pyrosequencing technology in the total sample.
Results: SLC6A4 methylation was found to be significantly higher in women when compared to men independent of DSM-IV diagnosis. *SLC6A4* methylation was further associated with the BSI-derived somatization dimension.
Conclusions: Female hypermethylation of a discrete region located within *SLC6A4* promoter region could underlie differential SERT expression in women when compared to men and could be one of the causative mechanisms by which women exhibit increased prevalence of somatic symptoms.

1. Introduction

There are well-established sex differences in the prevalence of certain mental disorders. Internalizing disorders are more common among women and externalizing disorders among men (Boyd et al., 2015). Within internalizing disorders, the burden of mood, anxiety and somatoform disorders is far greater among women than men (Kessler et al., 1994). What accounts for sex disparities in the prevalence of these disorders is currently unknown (Kuehner, 2017; van Loo et al., 2017). In this regard, epigenetic mechanisms and, particularly, DNA methylation pattern have been described to be greatly influenced by stochastic and environmental factors in a sex-specific manner (Van Dongen et al., 2016).

Serotonin (5-HT) is a neurotransmitter involved in the regulation of mood and behavior, among others, and is known to be altered in internalizing disorders, particularly in depression (Krishnan and Nestler, 2008). Moreover, the human serotonergic system displays striking sex differences in its homeostasis. Baseline 5-HT function has been hypothesized to be higher in women than in men based on findings of increased (i) whole blood 5-HT levels, and (ii) brain expression of 5-HT_{1A} receptors, in women when compared to men (Ortiz et al., 1988; Parsey et al., 2002).

The serotonin transporter (SERT or 5-HTT) is one of the key elements involved in the complex regulation of serotonergic pathways. In this regard, the most prescribed antidepressants worldwide are selective serotonin reuptake inhibitors (SSRIs) (Olfson and Marcus, 2009); additionally, there is some evidence for the efficacy of this type of pharmacological treatment in somatization disorders, such as chronic functional pain conditions and fibromyalgia (Patetsos and Horjales-Araujo, 2016). As their name indicate, SSRIs act by pharmacologically

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blocking SERT, which is accompanied by a marked increase of 5-HT in the synaptic cleft. Interestingly, SSRIs are also used to treat other psychiatric disorders such as anxiety and obsessive compulsive disorder.

SLC6A4 gene encodes the serotonin transporter and has been thus extensively analyzed at a genetic and epigenetic level with regard to several complex disorders, with special emphasis on depression (Arias et al., 2005; Palma-Gudiel and Fañanás, 2017). A long polymorphic region in its promoter region, 5-HTTLPR, has received particular attention due to its functional relevance: 5-HTTLPR short allele (*s*) has been associated with decreased SERT expression when compared to its long allele (*l*). Nevertheless, genetic variability at this locus does not seem to robustly explain either vulnerability or course of illness in depressive disorders across studies (de Vries et al., 2016). Hence, attention is being paid to epigenetic signatures at the *SLC6A4* promoter region as a putative causal mechanism in the development of psychopathology.

Epigenetic mechanisms refer to chemical modifications found directly in the DNA sequence itself or in its packaging proteins, histones. Specifically, DNA methylation consists in the addition of a methyl group onto a cytosine residue belonging to a CpG dinucleotide. Although individual CpG sites can be found throughout the whole genome, CpG sites tend to cluster in specific regions of the genome called CpG islands. CpG islands are usually found in the promoter regions of genes and are characterized by extensive hypomethylation (that is, lack of DNA methylation). Interestingly, CpG islands' hypermethylation has been associated with decreased gene expression. In this regard, the *SLC6A4* gene contains a CpG island in its promoter region.

SLC6A4's CpG island methylation has been found to be associated with early or recent exposure to psychosocial stress together with a number of psychiatric disorders following a transdiagnostic pattern (Palma-Gudiel and Fañanás, 2017); however causality of such associations remains elusive due to the lack of longitudinal approaches. Interestingly, *SLC6A4* methylation was described to be positively associated with the presence of family history of depression rather than with treatment response suggesting epigenetic patterns may be heritable (Kang et al., 2013).

The aim of the current study was to investigate the role of *SLC6A4* methylation in anxious-depressive disorders and six dimensions of psychopathology in a sample of monozygotic twin pairs from the general population. To analyze this relationship, *SLC6A4* methylation was assessed by means of CpG probes included in a genome-wide array and further replicated by means of pyrosequencing technology. We hypothesized that (i) anxious-depressed subjects would exhibit higher *SLC6A4* methylation levels when compared to otherwise healthy subjects, (ii) *SLC6A4* methylation would be differentially correlated to internalizing and externalizing dimensions of psychopathology, (iii) sex would be a moderator factor in all associations, and (iv) *SLC6A4* methylation patterns would be highly correlated across co-twins.

2. Methods

2.1. Sample selection

Participants in this study were part of a larger sample recruited at the University of Barcelona (UB Twin Register). This sample consists of 230 adult Spanish dizygotic and monozygotic twins pertaining to the general population. Written informed consent, as approved by the local Ethics Committee, was obtained from all participants before assessment. Peripheral blood was obtained from all participants. A detailed description of sampling and methods can be found elsewhere (Córdova-Palomera et al., 2015). Eligibility criteria for the current epigenetic approach included monozygotic twin pairs for which blood samples and psychometric data were available for both twins of each pair. One hundred forty-eight subjects were eligible for the current epigenetic approach (Table 1, Replication sample).

Table 1
Summary and description of samples included.

	Testing sample	Replication sample	Brain sample
n	34	148[a]	39[b]
Tissue	Peripheral blood	Peripheral blood	Postmortem brain
DNA methylation assessment	Illumina 450 K BeadChip	Bisulfite pyrosequencing	Illumina 450 K Beadchip
Mean age (range)	35.5 (19, 54)	35.5 (17, 68)	73 (15, 114)
Sex (F:M)	16:18	90:58	19:20

[a] One hundred forty-eight subjects included in the Replication sample comprise the thirty-four subjects of the testing sample.
[b] Actual number of samples assayed in the 450 K array in the Brain sample amounts to 260 since several samples from different brain sources were included for each subject.

2.2. Clinical evaluation

Lifetime psychiatric history was screened by trained psychologists following the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (First et al., 1996). Because of both the relatively low prevalence rates of individual psychiatric disorders in the general population and the higher prevalence of anxiety and depressive disorders relative to other disorders, these diagnoses were subsumed under a single diagnostic category of "anxious-depressive disorders". This classification has been used in previous studies conducted in the UB Twin Register sample revealing common biological pathways for such disorders (Córdova-Palomera et al., 2015). This category included the DSM-IV categorical diagnoses of: major depressive disorder (either single episode, recurrent or non-otherwise specified), anxiety disorder (with or without agoraphobia, and non-otherwise specified) and phobias (either specific or social). Specifically, in our sample 40.0% of women (n = 36) and 12.1.8% of men (n = 7) had a lifetime diagnostic of any anxious-depressive disorder; additionally, three subjects met DSM-IV criteria for anorexia nervosa, bulimia and psychotic disorder non-otherwise specified, respectively. Out of 46 subjects affected by any psychiatric disorder, 19 subjects exhibited DSM-IV Axis I comorbidity. Twenty-five subjects (54% of the affected ones) met DSM-IV Axis I criteria for current psychiatric disorders.

Additionally, on the same day of blood sampling, current psychiatric symptoms were evaluated using the Brief Symptom Inventory (BSI). The BSI is a self-administered scale consisting of 46 items to quantify the experience of psychopathological symptoms of a broad nature during the last 30 days including six subscales: depression, phobic anxiety, somatization, obsession-compulsion, hostility and paranoid ideation (Derogatis and Spencer, 1982). The BSI has been validated as a reliable tool for the assessment of symptom-based psychopathological dimensions in non-clinical samples (Ruipérez et al., 2001). Further details on specific BSI scores in the currently assessed sample can be found in Table 2.

Table 2
Age at assessment and BSI dimensions scores of assessed subjects (from the Replication sample) according to sex.

Mean age	Women (n = 90)	Men (n = 58)	p-Value
BSI Dimensions			
Mean age at assessment (SD)	34.4 (13.6)	37.3 (13.3)	0.2
BSI total score	22.8 (21.1)	14.3 (12.1)	0.002
BSI depression score	5.4 (5.4)	3.0 (2.9)	0.001
BSI anxiety score	1.8 (3.1)	0.8 (1.3)	0.006
BSI obsessive compulsive score	6.0 (5.5)	4.1 (4.1)	0.02
BSI somatization score	4.0 (4.2)	2.3 (2.6)	0.003
BSI hostility score	1.0 (2.0)	0.9 (1.2)	0.6
BSI paranoid score	4.8 (4.4)	3.4 (2.9)	0.02

2.3. 5-HTTLPR genotyping

Genomic DNA for genotypic analysis was extracted from peripheral blood with a Realpure DNA SSS kit (Durviz). The 5-HTTLPR polymorphism of the SLC6A4 gene was analyzed following the protocol previously described by Lesch et al. (1996).

2.4. DNA methylation analyses

Genomic DNA was extracted from peripheral blood with a Realpure DNA SSS kit (Durviz). Genome-wide methylation data was available from a subset of 34 subjects (17 twin pairs) using Illumina Infinium HumanMethylation450 (450 K) BeadChip technology (Table 1, Testing sample). This platform assays methylation at > 450,000 CpG sites across the genome at a single-base resolution, including 16 CpG sites located within *SLC6A4* gene (cg20592995, cg24984698, cg01330016, cg26126367, cg05951817, cg22584138, cg03363743, cg14692377, cg05016953, cg25725890, cg26741280, cg10901968, cg27569822, cg18584905, cg06841846, cg12074493; see Supplementary Table S1 for their chromosomal coordinates and gene location).

Pyrosequencing technology was employed in order to further explore DNA methylation in the vicinity of differentially-methylated cg22584138 probe. This approach allowed us to analyze CpG-specific methylation in a results-driven region of interest encompassing cg22584138 and four adjacent CpG sites which were not covered by the 450 K BeadChip (see Fig. 1 for details on the DNA sequence). The region of interest was PCR-amplified with forward primer AGTTGTTTGGGTATTTGTGTTAATT and reverse primer CTATTATTACAAAAACTTTACAACCTCT. Three independent pyrosequencing reactions were required to cover the region of interest. Sequencing primers used were (i) AGAATTTTTTTTTGTATAAAGTTA, (ii) AAGTAAAGATTAAATATAAATTATG and (iii) AGATTTTTTTTAAGGGGTTT. Pyrosequencing reactions were assayed without replicates. CpG-specific data were assessed using the Pyro Q-CpG software (Qiagen), expressed as percentages from 0 to 100%. Cases of potential errors due to incomplete bisulfe conversion or low signal peaks were discarded. Selected CpG sites correspond to CpG sites 82 to 86, as mentioned in our previous work (Palma-Gudiel and Fañanás, 2017) (see Fig. 1, for sequence details). Pyrosequencing data was available for 148 subjects (72 monozygotic twin pairs).

2.5. Brain replication

A 450 K BeadChip-derived methylomic dataset obtained in postmortem brain tissue (GEO access code GSE64509) was accessed in the NCBI Gene Expression Omnibus (Table 1, Brain sample). Briefly, the GSE64509 dataset consists of 260 postmortem brain samples obtained from 39 subjects, 19 females (21 subjects with Alzheimer's disease and 18 subjects without any neurodegenerative disease). Specifically, methylomic data in this sample was extracted from the following brain regions: caudate nucleus, cingulate gyrus, cerebellum, hippocampus, inferior parietal cortex, left frontal lobe, left occipital cortex, left temporal cortex, midbrain, middle frontal gyrus, motor cortex, right frontal lobe, right occipital cortex, right temporal cortex, sensory cortex, superior parietal cortex and visual cortex.

DNA methylation at the 16 CpG sites located within *SLC6A4* gene (see DNA methylation analysis for specific CpG probes' codes) was extracted from the dataset. Additionally, information regarding sex, age, postmortem interval and tissue source – brain region – of each sample were also extracted. All statistical models were fit including the aforementioned variables as predictors since they are all known to influence methylation levels.

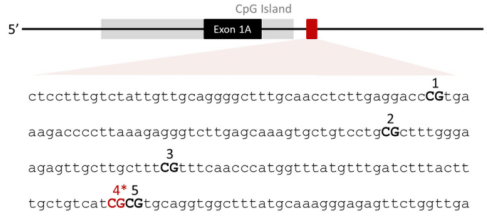

Fig. 1. Schematic view of SLC6A4 promoter region and CpG sites analyzed. The *SLC6A4* gene contains a CpG island in its promoter region (highlighted with a light grey box), spanning 799 base pairs and encompassing the exon 1A (black box). Based on array-derived findings at cg22584138, pyrosequencing technology was employed to replicate this result in the detailed sequence, which is located in the CpG island 3′ shore (dark red box). CpG-specific methylation values were available for CpG sites 1 to 5; CpG site 4* corresponding to the aforementioned cg22584138. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Statistical analysis

A linear regression model was fit in order to test whether methylation at the 16 CpG probes mapping to *SLC6A4* gene (as included in the 450 K BeadChip) could predict categorical diagnosis of anxious-depressive disorders in an initial subsample. Epigenomic data was available for a subset of 34 MZ twins. Additionally, *SLC6A4* methylation was analyzed as regards to sex, age and 5-HTTLPR genotype of the participants included in the subsample ($n = 34$).

Multiple linear regression model was built to analyze the correlation between BSI subscales scores and CpG-specific *SLC6A4* promoter methylation at the region of interest (consisting of cg22584138 and four adjacent CpG sites; details on the DNA sequence can be viewed in Fig. 1) as measured by pyrosequencing. This analysis was conducted in the total MZ twin sample ($n = 148$). Age of the participants at assessment, sex and DSM-IV based categorical diagnosis of anxious-depressive disorders were included as independent variables in all analysis as they are known to influence methylation status. All p values corresponding to correlations between different BSI subscales scores and *SLC6A4* methylation were adjusted by false discovery rate (FDR) procedures to correct for multiple testing.

A twin-based approach previously developed in our group (Palma-Gudiel et al., 2018) was also applied for this dataset in order to test whether the association between DNA methylation and clinical outcome relied on shared or non-shared environmental influences. Briefly, CpG-specific DNA methylation and BSI scores were averaged across all twin pairs in order to compare intrapair mean values of both variables.

All statistical analysis was performed with R programming (R Development Core Team, 2011).

3. Results

3.1. Array-based methylation in peripheral blood with regard to clinical and demographic variables

No associations were found between any of the *SLC6A4* CpG probes of the 450 K BeadChip and categorical diagnostic of anxious-depressive disorders according to DSM-IV criteria (the F statistic ranged from 0.02 to 3.25, all p values > .05 prior to correction for multiple testing) in the Testing sample (Table 1). Sex was significantly associated with methylation at three contiguous CpG probes: cg05951817, cg22584138 and cg03363743 ($\beta = -0.05$; $t = -3.03$, $p = .005$; $\beta = -0.09$; $t = -3.92$, $p = .0004$; $\beta = -0.03$, $t = -2.32$, $p = .03$; respectively) adjusting for age and categorical diagnosis. After correction for multiple testing, methylation at cg05951817 and cg22584138 remained significantly associated with sex ($q_{cg05951817} = 0.04$; $q_{cg22584138} = 0.007$). Absolute methylation difference between men and women is displayed in Fig. 2, highlighting increased female methylation at CpG probes cg05951817 and cg22584138. 5-HTTLPR

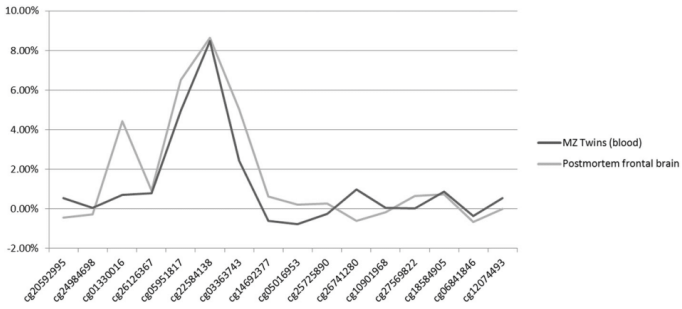

Fig. 2. Sex-differential methylation across the *SLC6A4* gene as measured in CpG probes included in the 450 K BeadChip in two independent samples. Subtraction of female minus male methylation at each CpG probe included in the 450 K BeadChip (n = 16 CpG sites) was analyzed in two independent samples: (i) peripheral blood from our current sample of monozygotic twins, and (ii) postmortem brain tissue freely accessible in the NCBI Gene Expression Omnibus (GEO access code GSE64509). Genomic location of each CpG probe can be found in Supplementary Table S1, CpG sites are shown ordered by genomic location, from 3-UTR to 5-UTR and CpG sites located close to Transcription Start Site (TSS). cg22584138 corresponds to CpG site 4 as measured via pyrosequencing. Methylation at cg22584138 was significantly associated with sex in both samples (p < .01). Methylation values derived from the current sample of monozygotic twins (n = 34) have been highlighted in dark grey (Δβ = 8.47%). Methylation values corresponding to postmortem frontal brain tissue (n = 41) have been highlighted in light grey (Δβ = 8.63%). This subsample was selected from the total brain sample since it was the more sex-balanced brain region (22 females/19 males).

genotype did not influence *SLC6A4* methylation at any of the CpG probes tested.

3.2. Sex-specific methylation at cg22584138 in brain tissue

Sex-specific methylation at *SLC6A4* gene was observed in an independent sample (Table 1, Brain sample) consisting of postmortem brain samples (n = 260) at three CpG probes: cg01330016, cg22584138 and cg03363743 (β = −0.02; t = −2.32, p = .021; β = −0.05, t = −5.49, p < .0001; β = −0.01, t = −2.0, p = .046; respectively) and at cg05951817 at a trend-level (β = 0.02; t = −1.96, p = .05). All analyses were controlled for age at death, postmortem interval, and brain region (as detailed in Methods). After correction for multiple testing, only cg22584138 methylation remained associated with sex at a statistically significant level (q < 0.0001). Absolute brain frontal methylation difference between men and women is displayed in Fig. 2, highlighting the similarity between sex-dependent differences across the two independent samples.

3.3. CpG-specific methylation at a region of interest including cg22584138 and its surrounding CpG sites is associated with sex

CpG-specific methylation at CpG sites surrounding cg22584138 was analyzed by means of pyrosequencing technology in 148 twin subjects (Table 1, Replication sample). Methylation at all CpG sites tested was significantly associated with sex when adjusting for age and categorical diagnostic (all p values < .0001; see Fig. 3). All associations remain significant after correction for multiple testing (all q values < 0.0001). Specifically, women exhibited higher methylation levels when compared to men. Since women exhibited significantly higher BSI scores than men in our sample (Table 2), all sub-scales of the BSI were also included as covariates in the model revealing the association between *SLC6A4* methylation and sex is not confounded by clinical status of the participants (all adjusted q values < 0.005).

3.4. CpG-specific methylation correlates to somatization symptoms

In the total sample of 148 MZ twins, *SLC6A4* methylation differed as a function of BSI total score. Specifically, BSI total score was significantly associated with methylation at CpG sites 3 and 4, adjusting for the effect of age and sex (β = 0.05, p < .01; β = 0.07, p = .01). When analyzing the specific associations between each of the BSI subscales, the somatization scale was significantly associated with methylation at all CpG sites tested (β = 0.13, t = 2.25, p_1 = 0.03; β = 0.36, t = 2.47, p_2 = 0.01; β = 0.30, t = 3.39, p_3 = 0.0009; β = 0.44, t = 3.35, p_4 = 0.001; β =0.24, t = 2.71, p_5 = 0.008). After FDR correction, only methylation at CpG sites 3 and 4 was significantly associated with somatization subscale (q_3 = 0.01; q_4 = 0.01). Positive correlation between somatization score and *SLC6A4* methylation seems to be driven by the female subjects of the study (Fig. 4). Thus, an interaction term between methylation and sex was included in the model to test whether the interaction between both variables could predict BSI somatization score. The interaction was significant at CpG sites 2 to 5 (β = −0.25, t = −2.03, p_2 = 0.04; β = −0.39, t = −2.47, p_3 = 0.01; β = −0.23, t = −2.12, p_4 = 0.04; β = −0.44, t = −2.69, p_5 = 0.008) but not CpG site 1 (β = −0.53, t = −1.86, p_1 = 0.06). After FDR correction, only the interaction between sex and methylation at CpG sites 3 and 5 remained significantly associated with the somatization BSI subscale (q_3 = 0.04; q_5 = 0.04).

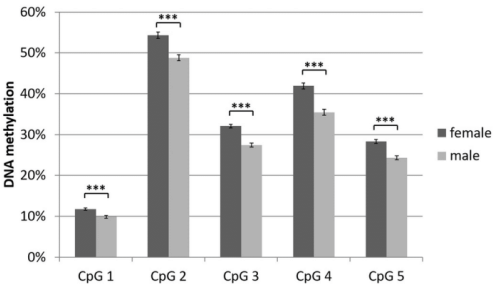

Fig. 3. Mean DNA methylation at each of the five SLC6A4 promoter CpG sites included in the region of interest surrounding cg22584138 as a function of sex. Female subjects exhibited significantly higher methylation at all CpG sites analyzed (all p values < .001). CpG site 4 corresponds to cg22584138. Note that DNA methylation at CpG sites 1 to 5 was measured by means of pyrosequencing. Gene location of CpG sites 1 to 5 have been displayed in Fig. 1.

3.5. SLC6A4 methylation and somatization symptoms score are highly correlated within twin pairs

SLC6A4 methylation at CpG sites 1 to 5 was highly correlated within twin pairs (r_1 = 0.81, p_1 < 0.001; r_2 = 0.78, p_2 < 0.001; r_3 = 0.67, p_3 < 0.001; r_4 = 0.49, p_4 < 0.001; r_5 = 0.49, p_5 < 0.001). Paired t-tests revealed no significant differences in DNA methylation values between co-twins (all p values > .54). Similarly, somatization symptoms BSI score was significantly correlated when comparing twin subjects with their co-twins (r = 0.45, p < .001). In order to account for co-twin similarity, the association between CpG-specific *SLC6A4*

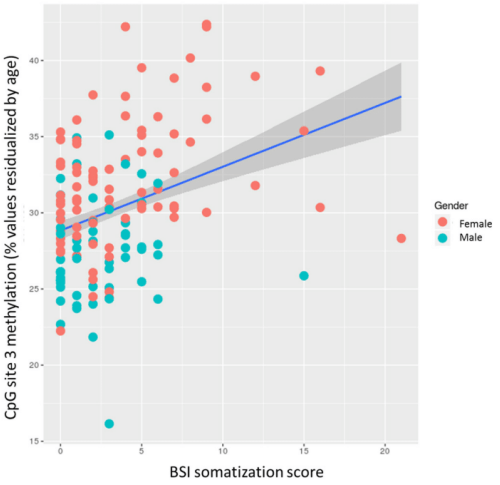

Fig. 4. BSI somatization score is positively associated with *SLC6A4* methylation.
Schematic representation of the positive association between DNA methylation at CpG site 3of our study, measured by pyrosequencing, and the BSI score of the subscale somatization (n = 148). Note that somatization scores correlated to methylation at all CpG sites tested (#1 to #5); CpG site 3 was selected for graphical representation as it was the CpG site exhibiting the higher sex-dependent methylation differences in our sample.

methylation and somatization score was tested considering twin pairs as a unit, that is, analyzing the association between mean intrapair values for both variables of interest (controlling for age and sex). This analysis yielded significant positive associations at all CpGs assayed ($\beta = 0.24$, $t = 2.71$, $p_1 = 0.008$; $\beta = 0.44$, $t = 3.35$, $p_2 = 0.001$; $\beta = 0.30$, $t = 3.39$, $p_3 = 0.001$; $\beta = 0.36$, $t = 2.47$, $p_4 = 0.015$; $\beta = 0.14$, $t = 2.25$, $p_5 = 0.026$). After FDR correction, all associations remain significant (all q values < 0.05).

4. Discussion

This is the first study to date to report an association between somatization score and methylation at *SLC6A4* gene. Interestingly, a sex-specific cross-tissue methylation signature was identified at the promoter region of *SLC6A4* gene. Methylation at five contiguous CpG sites was robustly increased in women when compared to men in (i) a sample of monozygotic twins from the general population, when analyzed in peripheral blood cells; and (ii) in an independent sample, when analyzed in different brain regions of postmortem tissue. Of note, methylation at the same region of interest analyzed herein (consisting of cg22584138 and its neighboring CpG sites) was described to exclusively differ with regard to sex in a sample of newborns born to women with differing levels of stress during pregnancy (Dukal et al., 2015). Thus, female hypermethylation at this region emerges as a distinctive sex-specific cross-tissue trait which may be stable throughout lifetime.

Therefore, *SLC6A4* female hypermethylation could be contributing to the observed differences between men and women in terms of psychopathological proneness. When considering (i) the robust female-specific increase in *SLC6A4* methylation already apparent in cord blood samples; (ii) its maintenance in postmortem brain samples from adult subjects; (iii) the consistently greater prevalence of somatic complaints in women when compared to men; and (iv) the herein described correlation between *SLC6A4* methylation and BSI somatization score; evidence suggests directionality of the effect such that increased *SLC6A4* methylation precedes somatization symptoms and could be playing a causal mechanism.

Additionally, in order to contextualize reported findings regarding sex-based differential methylation of *SLC6A4* gene, another candidate gene for stress-related disorders previously explored in a subset of the replication sample (Table 1) was also explored for putative effects of sex on DNA methylation. *NR3C1* gene, encoding the glucocorticoid receptor, CpG island shore methylation had been analyzed by means of pyrosequencing technology in a subset of the sample consisting of 96 subjects (Palma-Gudiel et al., 2018) thus being a suitable dataset for comparison with reported findings herein. In this regard, there was no association between sex and *NR3C1* methylation when adjusting for age and BSI scores (data not shown). Such null results further point to the role of sex on *SLC6A4* methylation and its relevance with regard to downstream associations with clinical outcomes.

Following the rationale for previously described gene—environment interactions underlying risk for psychopathology and, specifically, for stress-related disorders, we hypothesize sexual hormones could act as developmental mediators of observed differential *SLC6A4* methylation (Klengel et al., 2013; Palma-Gudiel et al., 2015). Accordingly, estrogen administration has been described to decrease brain expression of SERT in an animal model (Pecins-Thompson et al., 1998). As lipophilic molecules, estrogens cross the cell membrane and bind to the cytoplasm-located estrogen receptor (ER); once ERs are hormonally activated by its binding to estrogens, they form dimers and translocate to the cell nucleus where they recognize ERE motifs and bind to the DNA. Consequently, estrogen response elements (EREs) were searched in the proximity of CpG sites assayed. Nevertheless, no ERE motifs were found in the *SLC6A4* promoter region (data not shown).

Strikingly, no associations were found between anxious-depressive disorders and methylation at any of the *SLC6A4* CpG sites assayed. This negative finding could be due to the nature of the analyzed sample which was collected from the general population instead of a clinical setting. Furthermore, subjects with either prior or current history of anxious-depressive disorders were analyzed as affected in the study design. The fact that several DSM-IV-defined categorical diagnoses were merged into the "anxious-depressive" entity could be contributing to the heterogeneity of the sample in terms of both symptomatology and putative etiologic factors.

SLC6A4 CpG-specific methylation was associated with neither categorical diagnostic of anxious-depressive disorders nor stress during pregnancy but with somatization score. This result is of outstanding interest in the context of the NIMH Research Domain Criteria (RdoC) initiative, which posits biological psychiatric research should move away its focus from categorical to dimensional approaches (Wichers et al., 2010). Accordingly, somatization emerges as a shared dimension by several categorical entities, including depression, generalized anxiety disorder and somatoform disorders (Bekhuis et al., 2016), but also medical disorders of unknown etiology such as fibromyalgia, chronic fatigue syndrome or medically unexplained symptoms (Anderson et al., 2014) all of which are more common in woman than men. The involvement of serotonergic pathways not only in anxious-depressive spectrum disorders but also in somatoform disorders has been already acknowledged in the scientific literature (Koh et al., 2011). Thus, future studies should examine DNA methylation in these and other conditions with somatization symptoms in order to disentangle the extent to which the methylation-by-sex interaction for somatization has a transdiagnostic character.

In another vein, genetic studies focusing on the role of 5-HTTLPR in depression have revealed controversial results (de Vries et al., 2016). In our study, 5-HTTLPR was not associated with either psychiatric diagnostic, somatization or *SLC6A4* methylation in agreement with previous studies (Palma-Gudiel and Fañanás, 2017). Nevertheless, the high intrapair correlations observed for CpG-specific methylation at all CpG sites assessed by pyrosequencing suggests genetic variability could be a

major driver of *SLC6A4* methylation together with developmental shared exposures.

There are a number of limitations to the current study that should be acknowledged. First, actual downstream effects of observed methylation differences cannot be extrapolated from our results; however, increased methylation at the *SLC6A4* promoter region has been previously shown to be accompanied by decreased gene expression (Wang et al., 2012). Second, men and women from the investigated sample significantly differed in terms of psychiatric manifestations (Table 2); nevertheless, *SLC6A4* methylation and somatization score were positively correlated when analyzing only female subjects further strengthening the notion of both variables being associated. Additionally, BSI scores of all 6 dimensions were included as covariates of the primary analysis testing the association between sex and *SLC6A4* methylation. Third, 5-HTTLPR assessment comprised only its short and long variants, neglecting the additional allelic variants as previously described (Nakamura et al., 2000); however, the lack of association between 5HTTLPR and either methylation or psychopathology is in line with prior evidence suggesting epigenetic dominance over genetic variability (Palma-Gudiel and Fañanás, 2017). Finally, the limited sample size provides low to moderate statistical power to detect associations between categorical diagnosis and methylation; thus, lack of association reported herein could be due to type II errors.

5. Conclusion

Our findings sum up to the prior evidence that *SLC6A4* CpG island shore methylation is mainly associated with sex. Interestingly, our findings further revealed methylation at this region to be significantly associated with somatization symptoms which exhibit pronounced sex-based differences. While further studies are required to elucidate the downstream effects of such epigenetic pattern, our results point to the role of serotoninergic transmission in the development of somatization symptoms rather than mapping to a specific categorical disorder.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2018.09.002.

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Ethical statement

Written informed consent was obtained from all participants after a detailed description of the study aims and design, as approved by the Bioethics Committee of the University of Barcelona. All procedures contributing to this work were performed in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Data statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

References

Anderson, G., Berk, M., Maes, M., 2014. Biological phenotypes underpin the physio-somatic symptoms of somatization, depression, and chronic fatigue syndrome. Acta Psychiatr. Scand. 129, 83–97. https://doi.org/10.1111/acps.12182.

Arias, B., Catalán, R., Gastó, C., Gutiérrez, B., Fañanás, L., 2005. Evidence for a combined genetic effect of the 5-HT1A receptor and serotonin transporter genes in the clinical outcome of major depressive patients treated with citalopram. J. Psychopharmacol. 19, 166–172. https://doi.org/10.1177/0269881105049037.

Bekhuis, E., Schoevers, R.A., van Borkulo, C.D., Rosmalen, J.G.M., Boschloo, L., 2016. The network structure of major depressive disorder, generalized anxiety disorder and somatic symptomatology. Psychol. Med. 46, 2989–2998. https://doi.org/10.1017/S0033291716001550.

Boyd, A., Van de Velde, S., Vilagut, G., de Graaf, R., O'Neill, S., Florescu, S., Alonso, J., Kovess-Masfety, V., 2015. Gender differences in mental disorders and suicidality in Europe: results from a large cross-sectional population-based study. J. Affect. Disord. 173, 245–254. https://doi.org/10.1016/j.jad.2014.11.002.

Córdova-Palomera, A., Fatjó-Vilas, M., Gastó, C., Navarro, V., Krebs, M.-O., Fañanás, L., 2015. Genome-wide methylation study on depression: differential methylation and variable methylation in monozygotic twins. Transl. Psychiatry 5, e557. https://doi.org/10.1038/tp.2015.49.

Derogatis, L., Spencer, M., 1982. The Brief Symptom Inventory (BSI): Administration, Scoring, and Procedures Manual-I. Johns Hopkins University School of Medicine, Clinical Psychometrics Research Unit, Baltimore.

Dukal, H., Frank, J., Lang, M., Treutlein, J., Gilles, M., Wolf, I.A., Krumm, B., Massart, R., Szyf, M., Laucht, M., Deuschle, M., Rietschel, M., Witt, S.H., 2015. New-born females show higher stress- and genotype-independent methylation of SLC6A4 than males. Bord. Pers. Disord. Emot. Dysregul. 2, 8. https://doi.org/10.1186/s40479-015-0029-6.

First, M.B. et, Spitzer, R.L., Gibbon, M., Williams, J.B.W., 1996. Structured Clinical Interview for DSM-IV for Axis I Disorders (SCID-I) Clinician Version. for. DSMIV.

Kang, H.-J., Kim, J.-M., Stewart, R., Kim, S.-Y., Bae, K.-Y., Kim, S.-W., Shin, I.-S., Shin, M.-G., Yoon, J.-S., 2013. Association of SLC6A4 methylation with early adversity, characteristics and outcomes in depression. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 44, 23–28. https://doi.org/10.1016/j.pnpbp.2013.01.006.

Kessler, R.C., McGonagle, K., Zhao, S., Nelson, C.B., Hughes, M., Eshleman, S., Wittchen, H.-U., Kendler, K.S., 1994. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States: results from the national comorbidity survey. Arch. Gen. Psychiatry 51, 8–19.

Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16, 33–41.

Koh, K.B., Choi, E.H., Lee, Y. joon, Han, M., 2011. Serotonin-related gene pathways associated with undifferentiated somatoform disorder. Psychiatry Res. 189, 246–250. https://doi.org/10.1016/j.psychres.2011.04.002.

Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894–902. https://doi.org/10.1016/j.psc.2006.12.005.

Kuehner, C., 2017. Why is depression more common among women than among men? Lancet Psychiatry 4, 146–158. https://doi.org/10.1016/S2215-0366(16)30263-2.

Lesch, K.-P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 80 (274), 1527–1531.

van Loo, H.M., Aggen, S.H., Gardner, C.O., Kendler, K.S., 2017. Sex similarities and differences in risk factors for recurrence of major depression. Psychol. Med. 1–9. https://doi.org/10.1017/S0033291717003178.

Nakamura, M., Ueno, S., Sano, A., Tanabe, H., 2000. The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. Mol. Psychiatry 5, 32–38.

Olfson, M., Marcus, S.C., 2009. National Patterns in Antidepressant Medication Treatment. Arch. Gen. Psychiatry 66, 848. https://doi.org/10.1001/archgenpsychiatry.2009.81.

Ortiz, J., Artigas, F., Gelpi, E., 1988. Serotonergic status in human blood. Life Sci. 43, 983–990.

Palma-Gudiel, H., Fañanás, L., 2017. An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR. Neurosci. Biobehav. Rev. 72, 190–209. https://doi.org/10.1016/j.neubiorev.2016.11.011.

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J.C., Fañanás, L., 2015. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. Neurosci. Biobehav. Rev. 55, 520–535. https://doi.org/10.1016/j.neubiorev.2015.05.016.

Palma-Gudiel, H., Córdova-Palomera, A., Tornador, C., Falcón, C., Bargalló, N., Deco, G., Fañanás, L., 2018. Increased methylation at an unexplored glucocorticoid responsive element within exon 1 D of NR3C1 gene is related to anxious-depressive disorders and decreased hippocampal connectivity. Eur. Neuropsychopharmacol. 28, 579–588. https://doi.org/10.1016/j.euroneuro.2018.03.015.

Parsey, R.V., Oquendo, M.A., Simpson, N.R., Ogden, R.T., Van Heertum, R., Arango, V., Mann, J.J., 2002. Effects of sex, age, and aggressive traits in man on brain serotonin 5-HT1A receptor binding potential measured by PET using [C-11]WAY-100635. Brain Res. 954, 173–182. https://doi.org/10.1016/S0006-8993(02)03243-2.

Patetsos, E., Horjales-Araujo, E., 2016. Treating chronic pain with SSRIs: what do we know? Pain Res. Manag. 2016, 2020915. https://doi.org/10.1155/2016/2020915.

Pecins-Thompson, M., Brown, N.A., Bethea, C.L., 1998. Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. Mol. Brain Res. 53, 120–129.

R Development Core Team, 2011. R: a Language and Environment for Statistical Computing. R Found. Stat. Comput., R Foundation for Statistical Computinghttps://doi.org/10.1007/978-3-540-74686-7.

Ruipérez, M.Á., Ibáñez, M.I., Lorente, E., Moro, M., Ortet, G., 2001. Psychometric properties of the Spanish version of the BSI: contributions to the relationship between personality and psychopathology. Eur. J. Psychol. Assess. 17, 241–250. https://doi.org/10.1027//1015-5759.17.3.241.

Van Dongen, J., Nivard, M.G., Willemsen, G., Hottenga, J.J., Helmer, Q., Dolan, C.V., Ehli, E.A., Davies, G.E., Van Iterson, M., Breeze, C.E., Beck, S., Suchiman, H.E., Jansen, R., Van Meurs, J.B., Heijmans, B.T., Slagboom, P.E., Boomsma, D.I., 2016. Genetic and environmental influences interact with age and sex in shaping the human methylome. Nat. Commun. 7, 1–13. https://doi.org/10.1038/ncomms11115.

de Vries, Y.A., Roest, A.M., Franzen, M., Munafò, M.R., Bastiaansen, J.A., 2016. Citation bias and selective focus on positive findings in the literature on the serotonin transporter gene (5-HTTLPR), life stress and depression. Psychol. Med. 46, 2971–2979. https://doi.org/10.1017/S0033291716002178.

Wang, D., Szyf, M., Benkelfat, C., Provençal, N., Turecki, G., Caramaschi, D., Côté, S.M., Vitaro, F., Tremblay, R.E., Booij, L., 2012. Peripheral SLC6A4 DNA methylation is associated with in vivo measures of human brain serotonin synthesis and childhood physical aggression. PLoS One 7, e39501. https://doi.org/10.1371/journal.pone.0039501.

Wichers, M., Geschwind, N., Van Os, J., Peeters, F., 2010. Scars in depression: is a conceptual shift necessary to solve the puzzle? Psychol. Med. 40, 359–365. https://doi.org/10.1017/S0033291709990420.

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Epigenetics-by-sex interaction for somatization conferred by methylation at the promoter region of *SLC6A4* gene" included the following tasks:

- Participation in the conception and design of the study
- Statistical analysis
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

Epigenetic outlier profiles in depression: A genome-wide DNA methylation analysis of monozygotic twins

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RESEARCH ARTICLE

Epigenetic outlier profiles in depression: A genome-wide DNA methylation analysis of monozygotic twins

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Abstract

Recent discoveries highlight the importance of stochastic epigenetic changes, as indexed by epigenetic outlier DNA methylation signatures, as a valuable tool to understand aberrant cell function and subsequent human pathology. There is evidence of such changes in different complex disorders as diverse as cancer, obesity and, to a lesser extent, depression. The current study was aimed at identifying outlying DNA methylation signatures of depressive psychopathology. Here, genome-wide DNA methylation levels were measured (by means of Illumina Infinium HumanMethylation450 Beadchip) in peripheral blood of thirty-four monozygotic twins informative for depressive psychopathology (lifetime DSM-IV diagnoses). This dataset was explored to identify outlying epigenetic signatures of depression, operationalized as extreme hyper- or hypo-methylation in affected co-twins from discordant pairs that is not observed across the rest of the study sample. After adjusting for blood cell count, there were thirteen CpG sites across which depressed co-twins from the discordant pairs exhibited outlying DNA methylation signatures. None of them exhibited a methylation outlier profile in the concordant and healthy pairs, and some of these loci spanned genes previously associated with neuropsychiatric phenotypes, such as *GHSR* and *KCNQ1*. This exploratory study provides preliminary proof-of-concept validation that epigenetic outlier profiles derived from genome-wide DNA methylation data may be related to depression risk.

Introduction

Recent discoveries, mainly in the field of cancer research, highlight the importance of differentially variable methylation signatures as a valuable tool to understand cellular biology [1,2]. Accordingly, new studies are providing biologically-plausible frameworks to understand the origins and implications of such stochasticity-related epigenetic modifications [3–5]. Beyond environmental altering DNA methylation, recent data support the idea of epigenetic

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stochasticity as an important modifier of DNA methylation. Epigenetic stochasticity refers to the *mutation* of epigenetic marks in the absence of detectable environmental influences, such as in events where DNA methylation marks are not replicated [6,7].

Epigenetic stochasticity, as indexed by DNA methylation variability, has become a very popular candidate mechanism in studies of cancer cell biology [8–10]. Notably, the importance of DNA methylation variability to unravel disease aetiology and dynamics does not seem limited to the field of cancer. For instance, there is some evidence of increased DNA methylation variability in obesity [11] and in depression [12–16]. This clinical background confers particular importance to the study of stochastic epigenetic changes in diseased populations, as they may be linked to aberrant cell function and subsequent human pathology.

Due to the novelty of this subject, only a few tools to statistically assess differences in DNA methylation variability between health and disease have been developed to date [17–19]. Most of these tools have mainly been used in cancer, and incorporate statistical methods in which outlier observations in the healthy and affected DNA samples are purposefully removed before the analyses [17,18]. However, further research has indicated that epigenetic outliers may be frequent across a broad set of pathological states, and could probably function as disease markers [19].

The translatability of the above mentioned techniques to phenotypes apart from cancer comes from previous evidence of a sound epigenetic influence in a wide range of complex phenotypes [7,20]. In this sense, perhaps one of the clearest applications of these approaches outside the field of cancer is the work of Xu et al. [11], who showed an increase in the number of DNA methylation sites with outlier methylation within a group of obese individuals. Nevertheless, applying these methods to pathological states such as mental disorders is not straightforward, since their epigenetic dynamics and the statistical properties of the data extracted from them may have some particularities [16,21]. As described by Mill and Petronis [21], several environmental factors and gene-environment interactions associated with depression are hard to explain: e.g., the prevalence of depression in women almost doubles the prevalence in men after puberty, and depression has a sharp rise in prevalence in women after puberty. Additionally, epigenetic changes observed in psychiatric disorders are quite subtle, i.e. DNA methylation absolute changes reported in the literature are limited, typically under 5%; nevertheless, such small changes may be sufficient to impact mental health [22,23].

As reported by Oh et al. [16] in a sample of monozygotic twin pairs discordant for major depressive disorder, depressed individuals exhibited a statistically significant higher number of epigenetic outliers, in both gene coding and intergenic regions, when compared to healthy subjects. Although these previous findings indicate that there are epigenetic outliers spanning the whole (epi)genome of depressed individuals [16], further research may allow substantiate these findings with standard methods and to determine the precise genomic loci where DNA methylation outliers could be frequent in psychopathology.

The present work aims to confirm the biological feasibility of epigenetic outlier signatures in depressive disorders. To test for variable (outlying) methylation levels at the CpG level associated with depression, the authors analyzed a DNA methylation dataset from the Illumina Infinium HumanMethylation450 Beadchip, which covers >450,000 CpG sites across the human genome. Data for this pilot evaluation came mainly from a set of six monozygotic (MZ) adult twin pairs (12 individuals) discordant in their liability for depressive psychopathology, and groups of concordant and healthy individuals (4 and 7 MZ pairs, respectively) were used to further validate the findings. Using MZ twin samples to analyze methylation variability in disease status has the advantage of suppressing potential sources of methylation variance due to DNA sequence variation. Namely, the potential bias of single nucleotide polymorphism (SNP)-containing probes [24,25] is controlled.

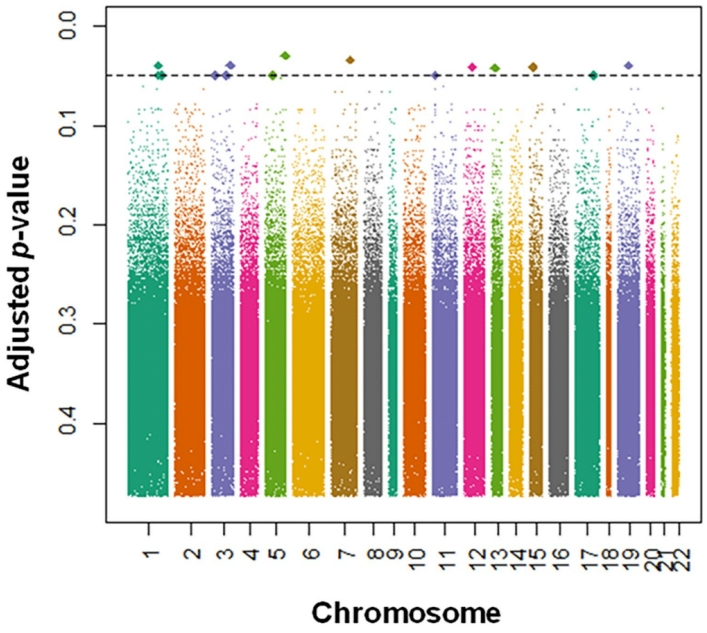

Fig 1. Sixteen DNA methylation probes across the genome exhibit larger methylation variance in the depression-affected co-twins than in their healthy counterparts. The statistical significance of these *p*-values is already adjusted for multiple comparisons using the FDR protocol proposed by Storey and Tibshirani (2003).

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Results

After multiple testing adjustments, affected co-twins from discordant MZ pairs showed increased DNA methylation variance at sixteen CpG probes spanning the whole genome (Fig 1).

As described in *Materials and methods*, an additional analysis step was conducted to discard CpG probes showing statistical significance due to potential technical artifacts, or perhaps lacking biological relevance. Specifically, former research indicates that the Illumina technology employed here is able to detect DNA methylation differences of 10% or more with a low probability of error, and there is also evidence showing that methylation differences above 10% are likely to have important functional consequences [13,26–28]. Accordingly, the ranges of DNA methylation values were estimated in both the healthy and affected co-twin subsets, for all 16 CpG probes with increased DNA methylation variance in affected individuals. Since 3 of these probes had only slight increases in DNA methylation ranges (< 10%) in the affected co-twins, they were discarded from the next discussion and analysis steps.

Fig 2 depicts the DNA methylation values observed in the 6 discordant twin pairs at the remaining 13 probes. Two main observations can be derived from that data. First, the DNA methylation variance increases in depression are driven by epigenetic outliers, rather than by a homogeneous distribution of the methylation values in the affected co-twins (i.e., typically only one of the six affected co-twins constitutes an outlier observation, increasing the overall

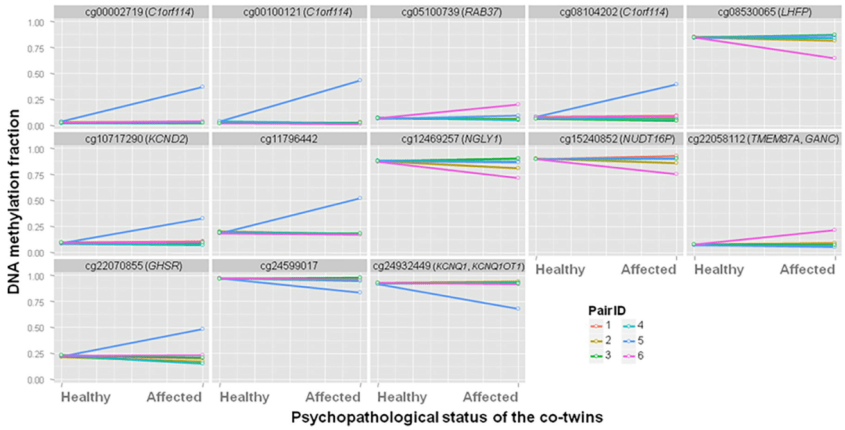

Fig 2. CpG probes with large and statistically significant DNA methylation variances in the diagnostic-discordant MZ twins. The thirteen probes displayed here are those with genome-wide statistically significant methylation variance increases in affected co-twins from the discordant pairs. PairID: randomly assigned pair number.

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variance of the group). This fact somehow confirms the feasibility of the adopted statistical protocol (*F*-tests of variance) to detect outlying observations. Secondly, across the 13 probes, it seems that only two out of the six affected co-twins show epigenetic outlier signatures (pairs 5 and 6: blue and pink lines).

Table 1 shows descriptive information on the 13 CpG probes across the genome showing an epigenetic outlier-like profile in depression. The names of the genes they span are also shown, as well as a brief overview of their potential involvement in depression and related brain and behavioral phenotypes.

As an additional validation procedure, the distributions of DNA methylation profiles for the same 13 probes were also analyzed in the subsets of healthy and depression-concordant MZ pairs. If the outlier methylation profiles observed in the affected co-twins from discordant pairs were solely due to technical artifacts or not related to the disease etiology/manifestation, healthy and concordant pairs may show high-variance distributions. The results of these analyses are depicted in Fig 3.

Discussion

The current study evaluated the feasibility of an epigenetic outlier structure in DNA methylation profiles of depressed individuals. The statistical approach adopted here was customized to account for the fact that, as previously indicated in the literature, both healthy and depressed co-twins may exhibit epigenetic outlier profiles at specific CpG sites across the genome [16]. Most of the CpG sites with outlier distributions in the affected co-twins from depression-discordant pairs were located at genes previously associated with neuropsychiatric and related phenotypes, likely indicating that they have functional consequences on relevant neuropsychiatric pathways. Hence, the results offer a preliminary proof-of-concept validation of a methylation outlier structure in depression, and propose data analysis guidelines to evaluate this epigenetic phenomenon in samples of depressed individuals, where the statistical distribution of DNA methylation levels could differ from other phenotypes.

Table 1. DNA methylation probes showing outlier distributions in the affected co-twins from the six adult MZ pairs discordant for depression, and potential neuropsychiatric relevance of their associated genes.

Probe name (TargetID)	Unadjusted *p*-value	Adjusted *p*-value (*q*-value)	*β* range (%): affected	*β* range (%): healthy	*β* range difference	Mean methylation (SD)	Coordinates (hg19)	Gene name (UCSC)	Gene region feature category (UCSC)	Brain-blood methylation correlation*	Potential relevance of the gene in neuropsychiatric disorders
cg00002719	9×10^{-7}	0.039	34.8	1.7	33.2	3.9	Chr1:169396706	*CCDC181*	TSS200	None	*CCDC181* methylation has been previously described to be associated with exposure to gestational diabetes mellitus highlighting the importance of prenatal environment in the programming of long-term health and disease [61].
cg00100121	3×10^{-6}	0.049	42.1	2.2	39.9	3.6	Chr1:169396635	*CCDC181*	1stExon; 5'UTR	Cerebellum correlation (p = 0.02)	
cg08104202	4×10^{-6}	0.049	35.1	2	33	8.4	Chr1:169396712	*CCDC181*	TSS200	None	
cg05100739	4×10^{-6}	0.049	15.3	1	14.4	5.7	Chr17:72733163	*RAB37*	TSS200; 1stExon; Body; TSS200	None	Gene expression correlated with brain resting-state oscillatory activity [62].
cg08530065	2×10^{-6}	0.042	22.5	1.3	21.3	86.6	Chr13:39980228	*LHFP*	Body	None	Epigenetic regulation of brain function after prenatal insults [63].
cg10717290	3×10^{-7}	0.034	25.6	1	24.6	9.2	Chr7:119913576	*KCND2*	TSS200	Prefrontal cortex (p = 0.03) and cerebellum (p = 0.02) correlations	Suggestive evidence of an etiological role in autism[36].
cg11796442	2×10^{-6}	0.049	35.2	1.8	33.4	17.8	Chr5:72593919	-	-	None	-
cg12469257	3×10^{-6}	0.049	19.2	1.2	18	88.5	Chr3:25761040	*NGLY1*	3'UTR; Body; Body; Body	None	Association with intellectual disability, neuromotor impairment and neuropathy[64].
cg15240852	3×10^{-6}	0.049	17.2	1	16.2	90.9	Chr3:131083585	*NUDT16P*	Body	None	-
cg22058112	1×10^{-6}	0.041	15.9	1	15.2	7.4	Chr15:42566300	*TMEM87A*; *GANC*	TSS1500; TSS200	None	-
cg22070855	8×10^{-7}	0.039	32.9	1.6	31.3	17.4	Chr3:172167527	*GHSR*	TSS1500	Prefrontal cortex ($p < 0.001$), entorhinal cortex (p = 0.02) and superior temporal gyrus ($p < 0.001$) correlations	Association with substance abuse [65]. Acts as ghrelin receptor, regulating important features in the central nervous system, such as sleep, mood, memory and reward[31].

(*Continued*)

Table 1. (Continued)

Probe name (TargetID)	Unadjusted *p*-value	Adjusted *p*-value (*q*-value)	*β* range (%): affected	*β* range (%): healthy	*β* range difference	Mean methylation (SD)	Coordinates (hg19)	Gene name (UCSC)	Gene region feature category (UCSC)	Brain-blood methylation correlation*	Potential relevance of the gene in neuropsychiatric disorders
cg24599017	1×10^{-7}	0.03	14.6	0.4	14.2	96.3	Chr5:178835885	-	-	None	-
cg24932449	4×10^{-6}	0.049	26.2	1.6	24.7	91.5	Chr11:2672613	*KCNQ1*; *KCNQ1OT1*	Body; Body	Prefrontal cortex correlation ($p = 0.03$)	Putative link with working memory, psychopathology and brain activity [33]. DNA methylation levels at birth may correlate with psychiatric symptoms later in life[35].

Note that no SNPs have been described in any of the CpG sites exhibiting outlier distributions. *Brain-blood correlations were retrieved from the *Blood Brain DNA Methylation Comparison Tool*, a publicly available database [56].

Abbreviations: TargetID, Illumina identifier; 1stExon, first exon; 5'UTR, 5' untranslated region; Body, within gene body; TSS200, within 200 bp of a TSS.

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DNA methylation levels at the thirteen identified CpG probes were retrieved for the groups of diagnostic-concordant and healthy pairs, to explore whether they also exhibited a similar outlier profile regardless of psychopathological status. As shown in Fig 3, most probes exhibit similar ranges of values (and, accordingly, similar variances) across pairs of co-twins, regardless of whether they are concordant or healthy. A number of specificities on the statistical distributions should be noted, as they may provide complementary information. For instance, both co-twins of one of the healthy pairs (pair ID: 13) exhibit an outlier-like profile at CpG sites cg00002719, cg00100121 and cg08104202. However, both co-twins in this pair have almost identical methylation levels at these sites suggesting that their particular genomic DNA sequences may contain low-frequency SNVs associated with hyper-methylation such as SNP-containing probes [24,25]. Alternatively, the shared methylation profile could have arised in response to an environmental exposure common to both co-twins of the pair [29]. Thus, the relatively high methylation levels at these probes are not actually indicating stochastic epigenetic effects in healthy pairs. Rather, the plots would somehow indicate that methylation levels at these three CpG sites can be genetically-regulated, but this observation does not invalidate the epigenetic-outlier pattern observed in the discordant subset. Namely, in the present sample, affected co-twins from discordant pairs showed an outlier profile regardless of their DNA sequence match with their healthy counterparts.

Besides, probes cg08530065, cg11796442, cg12469257 and cg22070855 also show relatively large variances (Fig 3). But as displayed, these CpG sites had similar methylation levels in both co-twins from each pair: the intrapair differences in DNA methylation are typically less than 5%, which may be due to technical measurement artifacts and/or have small functional effects. In this regard, 15 mQTLs have been described to influence cg08530065 methylation, as retrieved from the mQTL database [30]. In contrast, larger methylation differences were observed when comparing the affected co-twin with outlier profile with the healthy co-twin (Table 1 and Fig 2). Hence, analysis of DNA methylation profiles at the thirteen candidate probes (retrieved from the diagnostic-discordant pairs), suggest that outlying methylation profiles are related to diagnostic status. This analysis may also suggest that the outlier profiles are not due to technical artifacts.

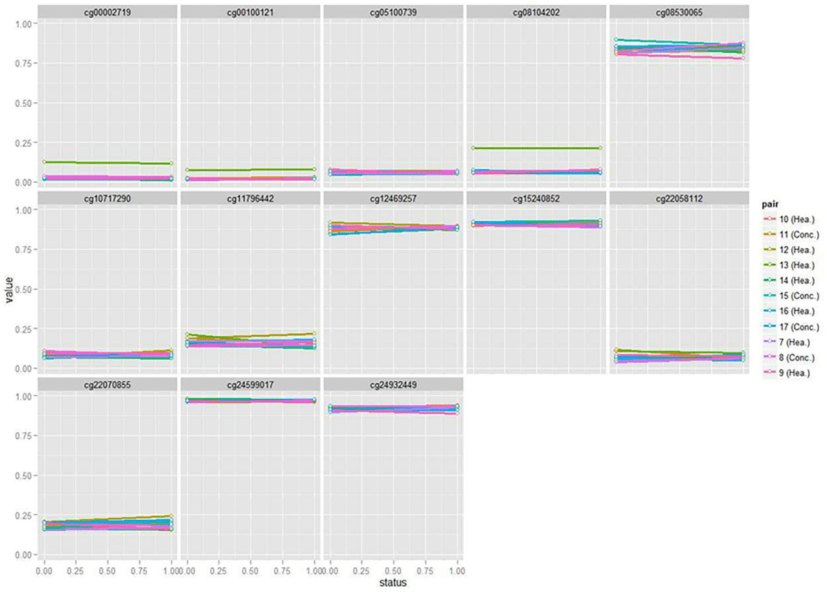

Fig 3. Assessment of DNA methylation levels in diagnostic-concordant and healthy pairs at the 13 CpG probes with epigenetic outlier profiles in affected co-twins from discordant pairs. The thirteen probes displayed here are those with genome-wide statistically significant methylation variance increases in affected co-twins from the discordant pairs (see Fig 2). PairID: randomly assigned pair number.

https://doi.org/10.1371/journal.pone.0207754.g003

Regarding similar research studies, previous population-based clinical reports had used analogous statistical approaches with data from genetically independent individuals with non-psychiatric phenotypes (i.e., singletons; for instance Xu et al. [11]). By definition, DNA sequence variants such as SNPs are equally represented across healthy and depressed co-twin samples. Accordingly, the current study takes advantage of the DNA sequence parity between MZ co-twins discordant for depression to show the presence of epigenetic outliers in affected co-twins, regardless of some SNPs that may be present across the general population.

Additionally, a recent report by Oh et al. [16] has found that epigenetic outliers can be found in both depressed and control populations–though they are more frequent in samples from depressed individuals–. The current report somehow expands on this topic by suggesting that DNA methylation variability due to epigenetic outliers may be related to the neurobiological mechanisms underlying depressive physiopathology. Although there is no clear mechanism about how these epigenetic changes can affect neurobiology downstream, it is important noticing that the identified probes were found within genes and may have relatively direct functional effects on those. Namely, as shown in Table 1, most of the CpG probes found with an epigenetic outlier profile in the affected co-twins from discordant pairs are located within the genomic coordinates of genes previously studied in the literature of psychiatric disorders. In agreement with the findings by Oh et al. [16], our results indicate that DNA methylation variance analyses in depressed individuals should be conducted using one-tailed tests, since some CpG probes with increased variance in normal *control* samples may be present and mislead

the biological meaning of the results. Perhaps the most suggestive genes from this set are the ghrelin receptor (*GHSR*), the potassium channel, voltage gated KQT-like subfamily Q, member 1 (*KCNQ1*) and the potassium voltage-gated channel subfamily D member 2 (*KCND2*). Ghrelin plays an important role in a broad spectrum of psychopathological outcomes, including stress, mood-and anxiety disorders [31], probably by modifying brain reward circuitry [32]. Similarly, there is evidence suggesting that *KCNQ1* may be related to psychopathological phenotypes [33], and peripheral tissue DNA methylation levels of *KCNQ1* have been shown to correlate with both adult personality traits [34] and psychiatric symptoms during the first years of life [35]. As opposed to *KCNQ1*, which is predominantly expressed in the adrenal glands and the thyroid, *KCND2* is most expressed in brain tissue; *KCND2* genetic variants have been associated with both epilepsy and autism [36]. Interestingly, CpG probes identified in this set of genes exhibit methylation correlation across blood and brain tissue (see Table 1).

There are several limitations of this study to be noted. First, due to the statistical approach focused in discordant twin pairs, the sample size to estimate DNA methylation outliers was limited to only 6 twin pairs. Each of the epigenetic changes reported here was observed on one discordant pair at a time, suggesting that stochastic factors could be underlying the results, rather than non-shared environment across discordant pairs. However, there is no conclusive evidence against the hypothesis of DNA methylation outliers caused by environmental factors in the current analysis. Additionally, due to the cross-sectional nature of the study and the inclusion of subjects with both prior and current history of anxious-depressive disorders, causality of observed DNA methylation outliers cannot be established. Finally, none of the reported hits have been previously described in association with depression.

In summary, the present results suggest that, alongside other methylation variability mechanisms recently shown in the literature of depression [12–15], epigenetic outliers may index biological disruptions underlying the etiopathology and clinical manifestation of depression.

Materials and methods

Subjects

The participants of this study were part of a larger twin sample (UB-Twin Registry) consisting of 242 Caucasian Spanish adult twins from the general population who gave permission to be contacted for research purposes. The exclusion criteria included age under 18 and over 65 years, a medical history of neurological disturbance, presence of sensory or motor alterations and current substance misuse or dependence. Written informed consent was obtained from all participants after a detailed description of the study aims and design, as approved by the Bioethics Committee of the University of Barcelona. All procedures contributing to this work were performed in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Trained psychologists applied face-to-face interviews to apply a battery of psychological and neurocognitive tests and to obtain medical records information. Additionally, peripheral blood or saliva samples were obtained from all 242 participants. The zygosity of the pairs was determined by genotyping 16 highly polymorphic microsatellite loci from DNA samples (SSRs; PowerPlex 16 System Promega Corporation). Identity on all the markers can be used to assign monozygosity with greater than 99% accuracy [37].

A group of 34 middle-aged participants (17 MZ twin pairs; age range 22–56, median age 38; 47% female) who were representative and informative for psychopathology, neurocognition and related factors was extracted from the above-described sample, to be investigated for brain function and genome-wide epigenetic signatures. Peripheral blood was available from all members of this group. Regarding depressive status of the participants, there were 6 discordant, 4 concordant and 7 healthy MZ pairs (12, 8 and 14 individuals). Further information

Table 2. Psychopathological, neurocognitive and demographic variables for DSM-IV diagnostic concordant, discordant and healthy MZ twin pairs.

	CONCORDANT (8 subjects, 8 female)		DISCORDANT (12 subjects, 4 female)		HEALTHY (14 subjects, 4 female)		Group comparison
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	X-squared[a]; *p*
Age	42.5 (13)	22–54	37 (10.9)	20–50	30.3 (7.3)	19–39	5.9; 0.052
IQ	105.1 (12.5)	87–127	108.1 (11.8)	87–131	110.5 (5.5)	103–118	1.9; 0.393
Current psycho-pathology (total BSI)	27.9 (16.5)	6–57	20.9 (13.3)	4–45	10.6 (9.3)	1–33	8.7; 0.013*
Current depressive symptoms (BSI subscale)	6.9 (6.5)	1–20	3.5 (2.7)	0–9	1.7 (1.8)	0–6	6.4; 0.04*

Subjects from discordant twin pairs exhibit intermediate BSI scores (as compared with subjects from healthy or concordant groups) since they constitute a 50% of affected and a 50% of non-affected subjects (their individual scores being averaged).

Notes: SD, standard deviation; IQ, intellectual quotient; BSI, Brief Symptom Inventory

[a], Kruskal-Wallis X-squared, as these variables were continuous

*, statistically significant *p*-value.

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about these participants can be found elsewhere [13,38]; the specific categorical DSM-IV based diagnoses of all subjects are detailed in S1 and S2 Tables. The main analyses described in this manuscript were conducted with the six discordant pairs, and complementary confirmatory analyses were carried out with the concordant and healthy pairs.

Clinical evaluation

A trained clinical psychologist applied the Structural Clinical Interview for DSM-IV Axis I Disorders (SCID-I) [39] in a face-to-face interview to screen for the presence of any lifetime depression or related anxiety spectrum disorder. Only two out of the twelve participants in this study had predominantly liability for anxious psychopathology. This apparently broad category of disorders was adopted in view of the evidence on comorbidity, shared etiopathology and diagnostic criteria overlap between depressive and anxious disorders [40–44], as well as taking into account evidences of some shared DNA methylation mechanisms in these diagnoses [40,45].

Complementarily, on the day of blood extraction, the current psychopathological status of all participants was evaluated with the Brief Symptom Inventory (BSI) [46,47]. This self-administered 46-item questionnaire is aimed at identifying the experience of psychopathological symptoms during the last 30 days. Its six subscales (depression, phobic anxiety, paranoid ideation, obsession-compulsion, somatization and hostility) were conceived for use in both clinical and non-clinical settings. All items are rated on a 5-point likert scale of distress, according to self-perception of symptoms. There were no between-group differences in intellectual quotient distributions, and the whole sample showed overall intelligence level profiles similar to those reported for demographically analogous samples [48]. Summarized information is shown in Table 2.

Methylation data

The Illumina Infinium HumanMethylation450 (450K) BeadChip [49,50] was employed with peripheral blood DNA samples for all participants. Specifically, by genotyping sodium bisulfite-treated DNA, DNA methylation is assayed by this platform at > 450 000 CpG sites across the genome at single-base resolution; next, bisulfite-converted DNA undergoes whole-genome amplification, before being fragmented and hybridized to microarray probes. The DNA methylation fraction of each CpG site is estimated as $\beta = M / (M + U + \alpha)$; M and U stand for methylated and unmethylated fluorescence intensities, and α is an arbitrary offset applied to stabilize β values with low intensities.

Infinium methylation data was processed with Methylation Module of GenomeStudio software using HumanMethylation450 manifest v1.1 following the instructions published by Bibikova et al. [49] CpG sites with poor detection quality ($p > 10^{-4}$) were removed from further analysis.

The obtained DNA methylation data was further processed to adjust for cell mixture distribution. Briefly, the proportions of different mononuclear cell populations and granulocytes were calculated following a previously published protocol [51,52]. By using 493 probes that matched the informative CpG sites reported by Houseman et al. [51], the proportions of six different cell types (B, $CD4^+$ T and $CD8^+$ T lymphocytes, plus monocytes, natural killer cells and granulocyte contamination) were estimated across the >450,000 measurements from the Illumina array. Afterwards, a penalized regression procedure allowed retrieving a β value representing the *average* cell. A software function to perform this cell mixture adjustment protocol is publicly available at https://gist.github.com/brentp/5058805#file-houseman-r. As expected, results using the adjusted β were more conservative than those using the unadjusted methylation values (i.e., there were less statistically significant CpG probes when using the adjusted β value).

Since the present MZ twin sample contains both male and female participants, probes in the X and Y chromosomes were removed from the analyses to avoid confounding. Likewise, in view of the relatively small sample size, all CpG probes for which at least one of the 12 diagnostic-discordant individuals had a missing value were removed, giving a final number of 473,864 probes.

The dataset supporting the results of this article have been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO SuperSeries accession number GSE120307.

Statistical analyses

In order to find CpG probes in which depressed co-twins from discordant MZ twin pairs exhibited outlier DNA methylation signatures, independent *F*-tests were conducted at each of the 473,864 probes across chromosomes 1 to 22 using var.test() in R. The F-test was implemented using standard procedures as follows. First, let $\bar{X} = \frac{1}{n}\sum_{i=1}^{n} X_i$ and $\bar{Y} = \frac{1}{m}\sum_{i=1}^{m} Y_i$ be the sample means and $S_X^2 = \frac{1}{n-1}\sum_{i=1}^{n} (X_i - \bar{X})^2$ and $S_Y^2 = \frac{1}{m-1}\sum_{i=1}^{m} (Y_i - \bar{Y})^2$ be the sample variances. The test statistic is computed as $F = \frac{S_X^2}{S_Y^2}$, and it has an F-distribution under the null hypothesis with n-1 and m-1 degrees of freedom. These tests allowed assessing the null hypothesis that the variances of both healthy and affected co-twin groups were equal. This test was chosen to detect epigenetic outlier measurements since it is highly sensitive to departures from normality in a statistical distribution (i.e., outliers) [53]. Considering the evidence of a large number of CpG probes with increased epigenetic outlier features in normal populations when compared to depressed individuals [16], it is necessary controlling for the fact that, in some cases, the control group may display greater variance than the affected group. Hence, one-tailed versions of the *F*-test were implemented.

Multiple testing adjustments were conducted using *q*-values, a measure based upon the false discovery rate (FDR) that has been shown useful in genome-wide statistical analyses and other large-scale multiple comparison settings [54,55]. Values of *q*–the multiple-comparison-adjusted version of *p*–below a 0.05 threshold were considered statistically significant.

An additional filter was applied to the CpG probes obtained from the former procedure. As previous reports indicate that methylation differences above 10% in Illumina assays may have important biological implications and show a low probability of being technical artifacts

[13,26–28], a DNA methylation measurement was considered an "outlier" if, apart from being statistically significant at $q \leq 0.05$, the between-group (healthy vs. depressed) difference in methylation ranges was above 10%.

Information regarding brain and blood correlation of DNA methylation values at the CpG probes meeting the aforementioned outlier criteria was retrieved from the *Blood Brain DNA Methylation Comparison Tool*, a publicly available database [56].

Finally, the names of the genes containing epigenetic outlier probes only within depressed individuals were retrieved to further evaluate the biological feasibility of the results. All analyses, as well as all data visualization procedures, were conducted using some packages for the R software [57–60].

Supporting information

S1 Table. DSM-IV based categorical diagnosis of affected subjects within discordant twin pairs. Pairs 5 and 6, exhibiting outlier methylation profiles have been highlighted in light grey. When a subject met criteria for several categorical entities, those were separated by "/". Abbreviations: NOS, not otherwise specified.
(DOCX)

S2 Table. DSM-IV based categorical diagnosis of subjects within concordant twin pairs. When a subject met criteria for several categorical entities, those were separated by "/". Abbreviations: NOS, not otherwise specified.
(DOCX)

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References

1. Massicotte R, Whitelaw E, Angers B. DNA methylation: A source of random variation in natural populations. Epigenetics. 2011; https://doi.org/10.4161/epi.6.4.14532
2. Teschendorff AE, Liu X, Caren H, Pollard SM, Beck S, Widschwendter M, et al. The Dynamics of DNA Methylation Covariation Patterns in Carcinogenesis. PLoS Comput Biol. 2014; https://doi.org/10.1371/journal.pcbi.1003709 PMID: 25010556
3. Feinberg AP. Epigenetic stochasticity, nuclear structure and cancer: The implications for medicine. J Intern Med. 2014; https://doi.org/10.1111/joim.12224 PMID: 24635672
4. Lévesque ML, Casey KF, Szyf M, Ismaylova E, Ly V, Verner MP, et al. Genome-wide DNA methylation variability in adolescent monozygotic twins followed since birth. Epigenetics. 2014; https://doi.org/10.4161/15592294.2014.970060 PMID: 25437055
5. Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. Nature Reviews Cancer. 2013. https://doi.org/10.1038/nrc3486 PMID: 23760024
6. Kebir O, Chaumette B, Krebs MO. Epigenetic variability in conversion to psychosis: Novel findings from an innovative longitudinal methylomic analysis. Transl Psychiatry. Springer US; 2018; 8. https://doi.org/10.1038/s41398-018-0138-2 PMID: 29695761
7. Petronis A. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature. 2010; 465: 721–7. https://doi.org/10.1038/nature09230 PMID: 20535201
8. Flanagan JM, Wilhelm-Benartzi CS, Metcalf M, Kaye SB, Brown R. Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients. Ann Oncol. 2013; https://doi.org/10.1093/annonc/mdt370 PMID: 24114859
9. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, et al. Increased methylation variation in epigenetic domains across cancer types. Nat Genet. 2011; https://doi.org/10.1038/ng.865 PMID: 21706001
10. Titus AJ, Way GP, Johnson KC, Christensen BC. Deconvolution of DNA methylation identifies differentially methylated gene regions on 1p36 across breast cancer subtypes. Sci Rep. Springer US; 2017; 7: 1–9. https://doi.org/10.1038/s41598-017-10199-z PMID: 28912426
11. Xu X, Su S, Barnes VA, De Miguel C, Pollock J, Ownby D, et al. A genome-wide methylation study on obesity: Differential variability and differential methylation. Epigenetics. 2013; https://doi.org/10.4161/epi.24506 PMID: 23644594
12. Byrne EM, Carrillo-Roa T, Henders AK, Bowdler L, McRae AF, Heath AC, et al. Monozygotic twins affected with major depressive disorder have greater variance in methylation than their unaffected co-twin. Transl Psychiatry. 2013; https://doi.org/10.1038/tp.2013.45 PMID: 23756378
13. Córdova-Palomera A, Fatjó-Vilas M, Gastó C, Navarro V, Krebs M-O, Fañanás L. Genome-wide methylation study on depression: differential methylation and variable methylation in monozygotic twins. Transl Psychiatry. 2015; 5: e557. https://doi.org/10.1038/tp.2015.49 PMID: 25918994
14. Davies MN, Krause L, Bell JT, Gao F, Ward KJ, Wu H, et al. Hypermethylation in the ZBTB20 gene is associated with major depressive disorder. Genome Biol. 2014; https://doi.org/10.1186/gb-2014-15-4-r56 PMID: 24694013
15. Dempster EL, Wong CCY, Lester KJ, Burrage J, Gregory AM, Mill J, et al. Genome-wide Methylomic Analysis of Monozygotic Twins Discordant for Adolescent Depression. Biol Psychiatry. Elsevier; 2014; 76: 977–983. https://doi.org/10.1016/j.biopsych.2014.04.013 PMID: 24929637
16. Oh G, Wang SC, Pal M, Chen ZF, Khare T, Tochigi M, et al. DNA modification study of major depressive disorder: Beyond locus-by-locus comparisons. Biol Psychiatry. 2015; https://doi.org/10.1016/j.biopsych.2014.06.016 PMID: 25108803
17. Jaffe AE, Feinberg AP, Irizarry RA, Leek JT. Significance analysis and statistical dissection of variably methylated regions. Biostatistics. 2012; https://doi.org/10.1093/biostatistics/kxr013 PMID: 21685414
18. Phipson B, Oshlack A. DiffVar: a new method for detecting differential variability with application to methylation in cancer and aging. Genome Biol. 2014; https://doi.org/10.1186/s13059-014-0465-4 PMID: 25245051
19. Teschendorff AE, Widschwendter M. Differential variability improves the identification of cancer risk markers in DNA methylation studies profiling precursor cancer lesions. Bioinformatics. 2012; https://doi.org/10.1093/bioinformatics/bts170 PMID: 22492641

20. Córdova-Palomera A, Fatjó-Vilas M, Palma-Gudiel H, Blasco-Fontecilla H, Kebir O, Fañanás L. Further evidence of DEPDC7 DNA hypomethylation in depression: A study in adult twins. Eur Psychiatry. 2015; 30: 715–718. https://doi.org/10.1016/j.eurpsy.2015.04.001 PMID: 25952135

21. Mill J, Petronis A. Molecular studies of major depressive disorder: The epigenetic perspective. Molecular Psychiatry. 2007. https://doi.org/10.1038/sj.mp.4001992 PMID: 17420765

22. Klengel T, Binder EB. Review Epigenetics of Stress-Related Psychiatric Disorders and Gene × Environment Interactions. Neuron. Elsevier Inc.; 2015; 86: 1343–1357. https://doi.org/10.1016/j.neuron.2015.05.036 PMID: 26087162

23. Palma-Gudiel H, Córdova-Palomera A, Leza JC, Fañanás L. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review. Neurosci Biobehav Rev. 2015; 55: 520–535. https://doi.org/10.1016/j.neubiorev.2015.05.016 PMID: 26073068

24. Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics. 2013; https://doi.org/10.4161/epi.23470 PMID: 23314698

25. Heyn H, Li N, Ferreira HJ, Moran S, Pisano DG, Gomez A, et al. Distinct DNA methylomes of newborns and centenarians. Proc Natl Acad Sci. 2012; https://doi.org/10.1073/pnas.1120658109 PMID: 22689993

26. Nakamura K, Aizawa K, Nakabayashi K, Kato N, Yamauchi J, Hata K, et al. DNA Methyltransferase Inhibitor Zebularine Inhibits Human Hepatic Carcinoma Cells Proliferation and Induces Apoptosis. PLoS One. 2013; https://doi.org/10.1371/journal.pone.0054036 PMID: 23320119

27. Yuen RKC, Pëaherrera MS, Von Dadelszen P, McFadden DE, Robinson WP. DNA methylation profiling of human placentas reveals promoter hypomethylation of multiple genes in early-onset preeclampsia. Eur J Hum Genet. 2010; https://doi.org/10.1038/ejhg.2010.63 PMID: 20442742

28. Zeller C, Dai W, Steele NL, Siddiq A, Walley AJ, Wilhelm-Benartzi CSM, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. Oncogene. 2012; https://doi.org/10.1038/onc.2011.611 PMID: 22249249

29. Palma-Gudiel H, Córdova-Palomera A, Tornador C, Falcón C, Bargalló N, Deco G, et al. Increased methylation at an unexplored glucocorticoid responsive element within exon 1 D of NR3C1 gene is related to anxious-depressive disorders and decreased hippocampal connectivity. Eur Neuropsychopharmacol. 2018; 28: 579–588. https://doi.org/10.1016/j.euroneuro.2018.03.015 PMID: 29650294

30. Gaunt TR, Shihab HA, Hemani G, Min JL, Woodward G, Lyttleton O, et al. Systematic identification of genetic influences on methylation across the human life course. Genome Biol. Genome Biology; 2016; 17: 61. https://doi.org/10.1186/s13059-016-0926-z PMID: 27036880

31. Wittekind DA, Kluge M. Ghrelin in psychiatric disorders–a review. Psychoneuroendocrinology. 2014; https://doi.org/10.1016/j.psyneuen.2014.11.013 PMID: 25459900

32. Russo SJ, Nestler EJ. The brain reward circuitry in mood disorders. Nat Rev Neurosci. 2013; https://doi.org/10.1038/nrn3381 PMID: 23942470

33. Heck A, Fastenrath M, Ackermann S, Auschra B, Bickel H, Coynel D, et al. Converging genetic and functional brain imaging evidence links neuronal excitability to working memory, psychiatric disease, and brain activity. Neuron. 2014; https://doi.org/10.1016/j.neuron.2014.01.010 PMID: 24529980

34. Teschler S, Bartkuhn M, Künzel N, Schmidt C, Kiehl S, Dammann G, et al. Aberrant methylation of gene associated CpG sites occurs in borderline personality disorder. PLoS One. 2013; https://doi.org/10.1371/journal.pone.0084180 PMID: 24367640

35. van Mil NH, Steegers-Theunissen RPM, Bouwland-Both MI, Verbiest MMPJ, Rijlaarsdam J, Hofman A, et al. DNA methylation profiles at birth and child ADHD symptoms. J Psychiatr Res. Elsevier Ltd; 2014; 49: 51–59. https://doi.org/10.1016/j.jpsychires.2013.10.017 PMID: 24290898

36. Lee H, Lin M chin A, Kornblum HI, Papazian DM, Nelson SF. Exome sequencing identifies de novo gain of function missense mutation in KCND2 in identical twins with autism and seizures that slows potassium channel inactivation. Hum Mol Genet. 2014; https://doi.org/10.1093/hmg/ddu056 PMID: 24501278

37. Guilherme R, Drunat S, Delezoide AL, Oury JF, Luton D. Zygosity and chorionicity in triplet pregnancies: New data. Hum Reprod. 2009; 24: 100–105. https://doi.org/10.1093/humrep/den364 PMID: 18945712

38. Córdova-Palomera A, Alemany S, Fatjó-Vilas M, Goldberg X, Leza JC, González-Pinto A, et al. Birth Weight, Working Memory and Epigenetic Signatures in *IGF2* and Related Genes: A MZ Twin Study. PLoS One. Public Library of Science; 2014; 9: e103639. Available: http://dx.doi.org/10.1371/journal.pone.0103639 PMID: 25171170

39. First MB, Spitzer RL, Gibbon M, Williams JBW. Structured Clinical Interview for DSM-IV Axis I Disorders, Clinician Version (SCID-CV). Washington, D.C.: American Psychiatric Press; 1996.
40. Klengel T, Pape J, Binder EB, Mehta D. The role of DNA methylation in stress-related psychiatric disorders. Neuropharmacology. 2014; https://doi.org/10.1016/j.neuropharm.2014.01.013 PMID: 24452011
41. Mosing MA, Gordon SD, Medland SE, Statham DJ, Nelson EC, Heath AC, et al. Genetic and environmental influences on the co-morbidity between depression, panic disorder, agoraphobia, and social phobia: a twin study. Depress Anxiety. 2009; https://doi.org/10.1002/da.20611 PMID: 19750555
42. Ressler KJ, Mayberg HS. Targeting abnormal neural circuits in mood and anxiety disorders: From the laboratory to the clinic. Nature Neuroscience. 2007. https://doi.org/10.1038/nn1944 PMID: 17726478
43. Wittchen H-U, Kessler RC, Beesdo K, Krause P, Höfler M, Hoyer J. Generalized anxiety and depression in primary care: prevalence, recognition, and management. J Clin Psychiatry. 2002;
44. Zbozinek TD, Rose RD, Wolitzky-Taylor KB, Sherbourne C, Sullivan G, Stein MB, et al. Diagnostic overlap of generalized anxiety disorder and major depressive disorder in a primary care sample. Depress Anxiety. 2012; https://doi.org/10.1002/da.22026 PMID: 23184657
45. Palma-Gudiel H, Fañanás L. An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR. Neurosci Biobehav Rev. 2017; 72: 190–209. https://doi.org/10.1016/j.neubiorev.2016.11.011 PMID: 27880876
46. Derogatis LR, Melisaratos N. brief symptom inventory: an introduction report. A J Res Psychiatry Allied Sci. 1983;
47. Ruipérez MÁ, Ibáñez MI, Lorente E, Moro M, Ortet G. Psychometric Properties of the Spanish Version of the BSI: Contributions to the Relationship between Personality and Psychopathology. Eur J Psychol Assess. 2001; 17: 241–250. https://doi.org/10.1027//1015-5759.17.3.241
48. Lynn R, Meisenberg G. National IQs calculated and validated for 108 nations. Intelligence. 2010. https://doi.org/10.1016/j.intell.2010.04.007
49. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011; 98: 288–295. https://doi.org/10.1016/j.ygeno.2011.07.007 PMID: 21839163
50. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics. 2011; 6: 692–702. https://doi.org/10.4161/epi.6.6.16196 PMID: 21593595
51. Houseman E, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012; 13: 86. https://doi.org/10.1186/1471-2105-13-86 PMID: 22568884
52. Yang I V., Pedersen BS, Liu A, O'Connor GT, Teach SJ, Kattan M, et al. DNA methylation and childhood asthma in the inner city. J Allergy Clin Immunol. 2015; https://doi.org/10.1016/j.jaci.2015.01.025 PMID: 25769910
53. Box GEP. Non-Normality and Tests on Variances. Biometrika. [Oxford University Press, Biometrika Trust]; 1953; 40: 318–335. https://doi.org/10.2307/2333350
54. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. JRStatSocSerB StatMethodol. 1995;
55. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci. 2003; https://doi.org/10.1073/pnas.1530509100 PMID: 12883005
56. Hannon E, Lunnon K, Schalkwyk L, Mill J. Interindividual methylomic variation across blood, cortex, and cerebellum: Implications for epigenetic studies of neurological and neuropsychiatric phenotypes. Epigenetics. 2015; 10: 1024–1032. https://doi.org/10.1080/15592294.2015.1100786 PMID: 26457534
57. Dabney A, Storey JD. Q-value estimation for false discovery rate control. 2014.
58. Gogarten SM, Bhangale T, Conomos MP, Laurie CA, McHugh CP, Painter I, et al. GWASTools: an R/Bioconductor package for quality control and analysis of genome-wide association studies. Bioinformatics. 2012; https://doi.org/10.1093/bioinformatics/bts610 PMID: 23052040
59. R Development Core Team. R: A Language and Environment for Statistical Computing [Internet]. Team RDC, editor. R Foundation for Statistical Computing. R Foundation for Statistical Computing; 2011. p. 409. https://doi.org/10.1007/978-3-540-74686-7
60. Wickham H. ggplot2:Elegant Graphics for Data Analysis. Media. 2009. https://doi.org/10.1007/978-0-387-98141-3
61. Binder AM, LaRocca J, Lesseur C, Marsit CJ, Michels KB. Epigenome-wide and transcriptome-wide analyses reveal gestational diabetes is associated with alterations in the human leukocyte antigen complex. Clin Epigenetics. Clinical Epigenetics; 2015; 7: 1–12. https://doi.org/10.1186/s13148-014-0036-2

62. Wang G-Z, Belgard TG, Mao D, Chen L, Berto S, Preuss TM, et al. Correspondence between Resting-State Activity and Brain Gene Expression. Neuron. 2015; https://doi.org/10.1016/j.neuron.2015.10.022 PMID: 26590343

63. Barua S, Kuizon S, Chadman KK, Flory MJ, Brown WT, Junaid MA. Single-base resolution of mouse offspring brain methylome reveals epigenome modifications caused by gestational folic acid. Epigenetics and Chromatin. 2014; https://doi.org/10.1186/1756-8935-7-3 PMID: 24484737

64. Caglayan AO, Comu S, Baranoski JF, Parman Y, Kaymakçalan H, Akgumus GT, et al. NGLY1 mutation causes neuromotor impairment, intellectual disability, and neuropathy. Eur J Med Genet. 2015; https://doi.org/10.1016/j.ejmg.2014.08.008 PMID: 25220016

65. Suchankova P, Nilsson S, von der Pahlen B, Santtila P, Sandnabba K, Johansson A, et al. Genetic variation of the growth hormone secretagogue receptor gene is associated with alcohol use disorders identification test scores and smoking. Addict Biol. 2015; https://doi.org/10.1111/adb.12277 PMID: 26059200

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Epigenetic outlier profiles in depression: A genome-wide DNA methylation analysis of monozygotic twins" included the following tasks:

- Participation in the conception and design of the study
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

Prenatal suffering is associated with epigenetic age deceleration at birth and hypomethylation at the hypoxia-responsive *EP300* gene.

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Prenatal suffering is associated with epigenetic age deceleration at birth and hypomethylation at the hypoxia-responsive *EP300* gene

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Abstract

Background: Obstetric complications have long been retrospectively associated with a wide range of short- and long-term disorders, including neurodevelopmental disorders such as schizophrenia. However, prospective studies assessing fetal well-being during pregnancy exclusively focus on perinatal outcomes. Thus, hemodynamic variables assessed during routine ultrasounds in a sample of monozygotic monochorionic twins (n = 60) enriched for several prenatal complications were analyzed with regard to (i) epigenetic age acceleration, and (ii) DNA methylation at genes included in the polygenic risk score for schizophrenia, and highly expressed in placental tissue.

Results: Decreased cerebroplacental ratio (CPR) measured during the third trimester was associated with epigenetic age deceleration ($\beta = 0.21$, $t = 3.362$, $p = 0.002$). Methylation at cg06793497 (*EP300* gene) was associated with CPR ($r = 0.41$, $p = 0.001$, $q = 0.12$). This association was reinforced by means of an intrapair analysis in monozygotic twins discordant for prenatal suffering ($r = 0.64$, $p < 0.001$).

Conclusions: Prenatal suffering during the third trimester of pregnancy is associated with both (i) developmental immaturity in terms of epigenetic age, and (ii) decreased CpG-specific methylation in a gene involved in hypoxia response.

Keywords: DNA methylation; obstetric complications; epigenetic clock; monozygotic twins; hypoxia

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Background

Prenatal environment constitutes the first modulating agent the developing fetus encounters as it progresses through gestation. The tremendous impact of any environmental threat occurring during this period for both short- and long-term consequences is now widely accepted and well-known as the Developmental Origins of Health and Disease (DOHaD) hypothesis [1]. Also known as the theory of fetal programming, the embedding of early life and its ability to exert long-term effects in late-life is thought to rely on epigenetic mechanisms [2].

Recently, several DNA methylation-based epigenetic clocks have been developed in order to predict chronological age with high accuracy [3, 4]; afterwards, Knight and colleagues developed a new predictor specifically aimed to predict gestational age (GA) in perinatal samples [5]. Although epigenetic and chronological age robustly show high correlation across studies, the difference between both variables allows the estimation of the so-called age acceleration (i.e. when epigenetic age is higher than chronological age).

On the one hand, epigenetic age acceleration in adult subjects has been associated with cumulative lifetime stress, lifestyle and all-cause mortality, among others, suggesting its utility as a better predictor for life expectancy than chronological age itself [6-8]. On the other hand, epigenetic GA deceleration (i.e. when chronological age is higher than epigenetic age), as measured in cord blood, has been described in newborns born to women with low socioeconomic status, Sjögren syndrome, insulin-treated gestational diabetes mellitus, and experiencing antenatal depressive symptoms [5,9,10]. Such findings suggest that newborns exposed to prenatal stressors are born in an immature state independently of their chronological GA. In this regard, boys – but not girls – who exhibited lower epigenetic GA at birth exhibited higher internalizing problems at follow-up (mean age 3.7 years), suggesting they are born with a developmental disadvantage [10].

Nevertheless, there is a dearth of studies examining the putative relationship between clinical measures routinely acquired during trimestral pregnancy ultrasounds and epigenetic GA acceleration. In this regard, the cerebroplacental ratio (CPR) has been reported to predict lower GA, lower birthweight and an increased rate of newborn intensive care unit admissions, among other detrimental perinatal outcomes [11,12]. Briefly, CPR is calculated by dividing the middle cerebral artery (MCA) pulsatility index (PI) by the umbilical artery (UA) PI [13]. The pulsatility index is a Doppler ultrasound measure reflecting vascular impedance or resistance, i.e. decreased blood flow. Specifically, fetal brain blood supply is known to increase in front of hypoxic stimuli thus decreasing PI in the MCA [14]; while placental insufficiency decreases umbilical blood flow hence increasing UA PI, and has been associated with both short- and long-term detrimental outcomes, including increased cardiovascular risk and deficits in cognition [15,16]. Consequently, a decreased CPR reflects the combination of both alterations and is an indicator of fetal suffering [11].

Obstetric complications (OCs) constitute one of the risk factors more reliably associated with psychopathology, particularly with neurodevelopmental disorders; specifically, the putative association between OCs and schizophrenia has been debated since the

70s [17-19]. In this regard, a recent umbrella review evaluating all published meta-analysis regarding risk factors and biomarkers for schizophrenia spectrum disorders revealed a history of OCs to significantly increase the risk for developing the disorder with an OR of 2 [20]. Furthermore, exposure to severe OCs together with increased genetic vulnerability, as measured with the polygenic risk score (PRS) for schizophrenia, interact to increase the risk to suffer the disorder up to an OR of 8.36 [21]. Since CPR is a robust indicator of prenatal stress and a predictor of perinatal and long-term morbidity, DNA methylation analysis of genes included in the PRS for schizophrenia could shed light on the epigenetic mechanisms mediating the interaction between OCs and neurodevelopmental disorders.

Results

GA estimation using Knight's epigenetic clock

The mean GA at birth of this cohort was 35.3 weeks (range = 31.7 – 37.1) and the mean DNA methylation GA at birth was 35 weeks (range = 31.4 – 37.7). To validate the epigenetic clock predictor in our sample, DNA methylation-based GA was tested for correlation with chronological GA ($r = 0.76$, $p = 1.68 \times 10^{-12}$; Fig. 1). The average absolute difference between epigenetic GA and chronological GA—hereinafter referred as ΔGA—was 0.9 weeks (range = 0.03 – 4.02), *i.e.* 6.3 days. There was a significant negative correlation between ΔGA and chronological GA, according to previous studies ($r = -0.47$; $p < 0.001$).

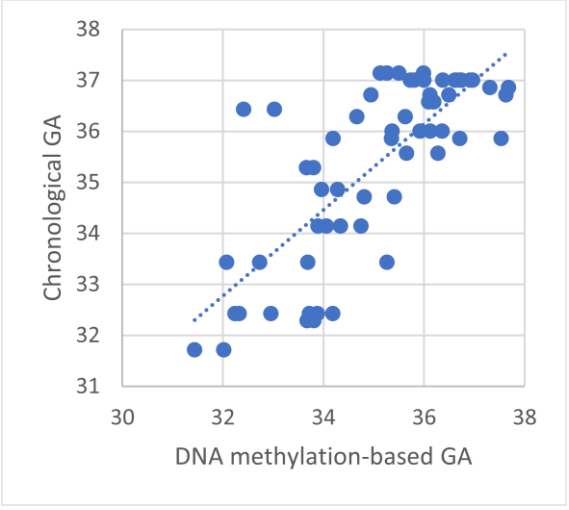

Figure 1. Correlation between chronological GA and epigenetic GA. Chronological GA was clinically assessed as a function of last menstrual period and epigenetic age was calculated based on DNA methylation-based Knight's clock. Both GA estimations were significantly correlated ($r = 0.76$; $p = 1.68 \times 10^{-12}$).

Association between ΔGA and CPR

ΔGA was tested for associations with CPR measured during the third trimester (mean = 33.8 weeks, range = 28.3 – 36.4), a few days before childbirth (median = 6.5 days). CPR was significantly associated with ΔGA (β = 0.21, t = 3.362, p = 0.002) when adjusting for sex, birthweight, diagnostic of either TTTS or sIUGR, surgery time interval (when laser fetoscopy had been applied) and gestational age at ultrasound as covariates. Figure 2 shows the positive association between 3rd trimester CPR and ΔGA.

Epigenetic exploration of placental PRS for schizophrenia with regard to CPR

Following the approach developed by Ursini and collaborators (2018), association between CPR and DNA methylation was tested in all CpG sites included in the DNA methylation array located within genes of the PRS for schizophrenia expressed in placental tissue (placental PRS) [21]. There were 1,466 CpG sites annotated to placental PRS genes out of 866,091 CpG sites included in the array. After FDR correction for multiple testing, methylation at one single CpG site, cg06793497, was significantly associated with CPR (r = 0.41, p = 0.001, q = 0.12; Fig. 3a).

To further explore the association between cg06793497 methylation and CPR, it was analyzed in a monozygotic twin intrapair design. Thus, intrapair differences for both measures were calculated for all twin pairs

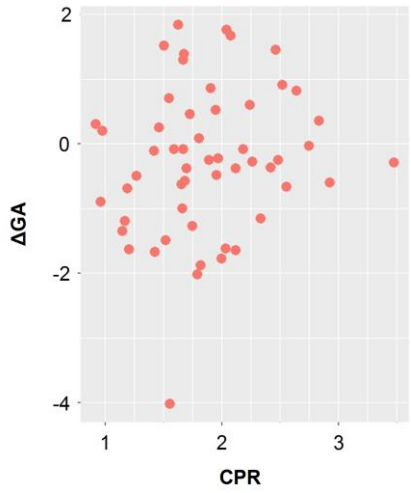

Figure 2. Association between epigenetic age acceleration and cerebroplacental ratio measured during the third trimester. Epigenetic age delta (ΔGA) corresponds to estimated epigenetic age minus chronological age. Thus, ΔGA positive values reflect epigenetic age acceleration while negative values point out the presence of epigenetic age deceleration. The cerebroplacental ratio (CPR) is calculated as the ration between the pulsatility indexes of middle cerebral and umbilical arteries. Thus, lower CPR values reflect a combination of placental vascular resistance and hypoxia. Both variables were significantly correlated when adjusting for sex, chronological gestational age, birthweight, and gestational age at ultrasound.

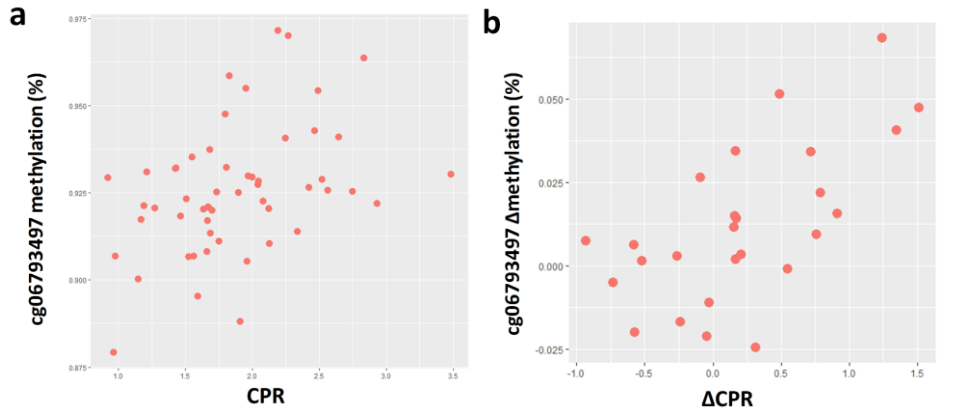

Figure 3. Schizophrenia PRS methylation exploration with regard to cerebroplacental ratio measured during the third trimester. (A) Methylation at cg06793497 (*EP300* gene) was significantly associated with CPR in the whole sample (n = 54). **(B)** Intrapair methylation difference at cg06793497 was significantly associated with intrapair CPR difference (n = 26).

of the sample (n = 30). Four observations were removed from the analysis due to missingness for any of the variables in one of the co-twins of a pair. The intrapair twin design further allows controlling for chronological GA, sex and timing of the Doppler ultrasound, since these variables are shared by co-twins of a pair. In this regard, intrapair differences in cg06793497 methylation and CPR, measured during the third trimester, were significantly correlated ($r = 0.64$, $p < 0.001$; Fig. 3b). The association between both variables remained significant after adjusting for cell type count intrapair differences ($\beta = 17.6$, $t = 3.924$, $p = 0.001$).

Discussion

This is the first study to date analyzing the epigenetic age in association with an obstetric marker of prenatal risk. Firstly, we describe the significant association between CPR measured during the third trimester of pregnancy with epigenetic age acceleration. Specifically, subjects exhibiting decreased CPR—exposed to prenatal suffering—were born with decelerated epigenetic age, i.e. prenatally stressed subjects were born immature adjusting for their gestational age at birth. Additionally, methylomic exploration of schizophrenia PRS genes known to be expressed in placenta revealed the association between CPR and cg06793497 methylation.

Developmental deficits and developmental delays have been previously described in children who would later develop schizophrenia [22]; although such prodromal symptoms were in accordance with the neurodevelopmental hypothesis for schizophrenia, biological mechanisms mediating these effects remain largely unknown. Epigenetic immaturity in response to prenatal stress could be contributing to this developmental delay. Interestingly, epigenetic age deceleration has been previously described in association with maternal pathologies during pregnancy suggesting it could be a robust biomarker of prenatal suffering [9,10]. It is worth noting CPR was measured a few days

prior to childbirth; thus, it can be used as a surrogate marker of prenatal suffering experienced at the end of the pregnancy, *i.e.* as a marker of perinatal risk.

Integration of the schizophrenia PRS [23] with obstetric and placental information [21], allowed the identification of *E1A binding protein p300* (*EP300*) gene CpG-specific methylation as a putative marker of exposure to prenatal stress. Interestingly, *EP300* gene encodes a histone acetyltransferase (HAT) involved in several cell pathways such as cell proliferation and differentiation. Mutations at EP300 gene have been described to cause Rubinstein-Taybi syndrome, a rare autosomal dominant neurodevelopmental disorder characterized by intellectual disability, psychomotor and language delay and facial dysmorphisms [24].

Remarkably, *EP300* has been identified as a co-activator of the hypoxia-inducible factor 1 alpha (*HIF1A*). In this regard, hypoxic conditions stimulate *EP300* expression, which has a neuroprotective role [25]. Accordingly, genetic variability at *EP300* gene has been associated with human adaptations to high altitude regions, e.g. the Tibet [26]. Likewise, pre- and peri- natal hypoxia have been associated with schizophrenia spectrum disorders, particularly by decreasing hippocampal volume [27;28]; complementarily, a decreased or impaired response to hypoxia via neurotrophic factors has also been implicated in the etiology of schizophrenia [29]. Overall, these findings point to the existence of a GxE interaction between genetic vulnerability and exposure to prenatal hypoxia, as already highlighted by Ursini and collaborators [21]. In this framework, *EP300* methylation could be one of the mediators of such interaction.

A number of limitations of the present study should be noted. First, the moderate sample size (n = 60) limits the statistical power of the analysis; however, smaller sample sizes (n = 22 MZ twin pairs) have been described to be sufficient to identify methylation differences of 6% with >80% power [30]. Moreover, a lenient significance threshold after correction multiple testing was used; however, previous epigenetic studies have described FDR values between 5 and 20% as markers of medium-confidence sites [31]. Finally, MZ twin pregnancies are characterized by lower gestational ages at birth than singleton pregnancies; besides, obstetric scales commonly used in psychiatric studies include twin pregnancies as an obstetric complication. Thus, findings derived from the present design might not be generalizable to the general population.

Conclusions

Further studies are needed to test the time stability of the hereby identified methylation signature. It will be equally relevant to explore neurobehavioral correlates of EP300 methylation during early childhood along with its putative association with neurodevelopmental outcomes, including psychosis liability. Additionally, a longitudinal follow-up is required to test the role of postnatal environment in these phenotypes since both epigenetic age deceleration and CpG-specific differential methylation in association with CPR could return to basal levels after birth. Finally, genetic exploration of these subjects regarding schizophrenia PRS will be instrumental for the study of GxE interactions and genetic liability for an impaired hypoxia response.

Methods

Study Population

This was a prospective study including fetal pairs from monochorionic diamniotic twin pregnancies attended at Hospital Clínic de Barcelona (Spain) during a two-year recruitment period. The study protocol was approved by the hospital ethics committee and all patients provided written informed consent.

Fetal cord blood was collected at birth from 32 monochorionic pregnancies (n = 64 samples).

Maternal age and pre-pregnancy BMI were retrieved from hospital records. Gestational age was calculated based on last menstrual period. The sample was enriched for two monochorionic-specific severe obstetric complications: twin-to-twin transfusion syndrome (TTTS; n = 8) and selective intrauterine growth restriction (sIUGR; n = 9). Seven out of 8 TTTS cases were treated upon detection by means of laser fetoscopy [32].

Fetal Ultrasound Assessment

Ultrasound assessment was performed on a Voluson Expert 8 (General Electrical Medical Systems, Milwaukee, WI, USA) or a Siemens Sonoline Antares (Siemens Medical Systems, Erlangen, Germany) with 8- to 4-MHz or 6- to 4- MHz curved array probes, respectively. All fetuses underwent detailed ultrasound evaluation including fetal anatomy and Doppler measurements such as umbilical artery pulsatility index (PI), middle cerebral artery PI and ductus venosus PI. All Doppler evaluations were acquired at a normal fetal heart rate (FHR) in the absence of fetal body/respiratory movements and at an angle of insonation as close to 0° as possible (but always < 15°), and the mechanical and thermal indices were maintained below 1. CPR was calculated as the ratio between middle cerebral artery PI and umbilical artery PI, according to previous studies [11].

DNA methylation

Genomic DNA was extracted from fetal cord blood using QIAamp DNA Mini Kit (Qiagen). DNA quality and quantity were assessed by NanoDrop One (Thermo Scientific). Genomic DNA was bisulfite converted using the Zymo EZ-96 DNA Methylation Kit (Zymo Research). Genome-wide DNA methylation levels were assessed over 850,000 CpG sites by means of the Infinium MethylationEPIC BeadChip Kit (Illumina Inc., CA, USA) according to the manufacturer's protocol. Pre-processing and normalization were performed using the Bioconductor minfi package [33]. CpG probes containing common SNPs were discarded. All probes mapping to the X and Y chromosomes were also removed. All samples (n = 64) were run in the same plate.

Absence of maternal contamination was confirmed after retrieving DNA methylation values at 10 CpG sites previously described to identify sample contamination by maternal blood during sample collection [34]. None of the samples assayed exhibited DNA methylation values above the threshold at 5 or more of those CpG sites (see Supplementary material for specific methylation values). Two samples (from the same twin-pair) were excluded from further analyses due to lack of monozygosity as assessed by 59 SNPs included in the array. One of the samples was removed from analysis due to insufficient DNA concentration, the co-twin sample was also excluded from further analysis.

Statistical analyses

All statistical analyses were conducted in R version 3.5.0 [35]. DNA methylation-based GA prediction was performed using the R code and statistical pipeline developed by Knight, based on the methylation profile of 148 CpG sites [5]; this predictor was developed using 15 Illumina DNA methylation datasets (n = 1434 neonates). Following Simpkin et al. recommendations, the Knight clock was preferred for our analysis as it was developed and tested in preterm infants' datasets such as our monozygotic twin population, characterized by a mean gestational age at birth of 35.3 weeks [36]. The EPIC array lacks 6 of the CpG sites originally included in the Knight clock, these values were inputed manually as non-available. Interestingly, DNA methylation-based age estimation relying on EPIC array data has already been described to accurately predict age despite the lack of several CpG sites originally included in Horvath's and Hannum's clocks [37].

Gestational age acceleration (ΔGA) was calculated as the absolute difference between epigenetic GA and chronological GA. Since ΔGA was associated with chronological GA ($r = -0.47$; $p < 0.001$), the latter was included as a covariate in all statistical models; this association has been already reported in prior studies exploring epigenetic-based GA estimations at birth [5,9,10].

Cell counts of $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, granulocytes, monocytes, and nucleated red blood cells (nRBCs) were estimated using the R code and statistical pipeline developed by Houseman [38].

A multiple linear regression model was built to analyze the correlation between ΔGA and CPR. Fetal sex, birthweight, diagnostic of either TTTS or sIUGR (binary variable), post-surgery interval (in TTTS cases where laser fetoscopy had been applied) and gestational age at ultrasound were included as independent variables in the model as they are known to influence either DNA methylation (from which ΔGA is calculated) or CPR. This analysis was not adjusted for cell types' proportions since the gestational age estimation has been developed in cord blood. This analysis was conducted in the total MZ twin sample (n = 60).

DNA methylation at CpG sites annotated to the 43 genes of the Placental PRS1 as described by Ursini et al. [21] were retrieved to test their association with CPR. A second multiple linear regression model was then designed to explore putative effects of CPR upon methylation of PRS genes, testing 1,466 associations. The aforementioned confounding variables along with cell types proportions ($CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, granulocytes, monocytes, and nRBCs) were included as covariates, as they are known to affect methylation values. False discovery rate (FDR) correction for multiple testing was applied, considering q values under 20% to be indicative of medium-confidence probes following prior studies [31].

A twin-based approach previously developed in our group [39] was also applied to refine the association between cg06793497 methylation and CPR. Briefly, intrapair differences for both variables of interest were computed for each twin pair; afterwards, a regression model was fitted with estimated intrapair cg06793497 methylation (Δmethylation) and intrapair CPR (ΔCPR). This last model was not adjusted for either sex or chronological gestational age since

both variables are identical for both twins of a pair.

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Ethics approval and consent to participate

Written informed consent was obtained from all participants after a detailed description of the study aims and design, as approved by the Bioethics Committee of the University of Barcelona. All procedures contributing to this work were performed in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Competing interests

The authors declare that they have no competing interests.

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References

1. Graignic-Philippe R, Dayan J, Chokron S, Jacquet AY, Tordjman S. Effects of prenatal stress on fetal and child development: A critical literature review. Neurosci Biobehav Rev. 2014;43:137–62.
2. Tobi EW, Goeman JJ, Monajemi R, Gu H, Putter H, Zhang Y, et al. DNA methylation signatures link prenatal famine exposure to growth and metabolism. Nat Commun. 2014;5:5592.
3. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013;14:R115.
4. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda SV, et al. Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. Mol Cell. 2013;49:359–67.
5. Knight AK, Craig JM, Theda C, Bækvad-Hansen M, Bybjerg-Grauholm J, Hansen CS, et al. An epigenetic clock for gestational age at birth based on blood methylation data. Genome Biol. Genome Biology; 2016;17:1–11.
6. Zannas AS, Arloth J, Carrillo-Roa T, Iurato S, Röh S, Ressler KJ, et al. Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. Genome Biol. Genome Biology; 2015;16:1–12.
7. Chen B, Marioni RE, Bressler J, Fornage M, Studenski S, Vandiver AR, et al. DNA methylation-based measures of biological age: meta-analysis predicting time to death. Aging (Albany NY). 2016;8:1–22.
8. Declerck K, Vanden Berghe W. Back to the future: Epigenetic clock plasticity towards healthy aging. Mech Ageing Dev. 2018

9. Girchenko P, Lahti J, Czamara D, Knight AK, Jones MJ, Suarez A, et al. Associations between maternal risk factors of adverse pregnancy and birth outcomes and the offspring epigenetic clock of gestational age at birth. Clin Epigenetics. Clinical Epigenetics; 2017;9:1–14.
10. Suarez A, Lahti J, Czamara D, Lahti-Pulkkinen M, Knight AK, Girchenko P, et al. The Epigenetic Clock at Birth: Associations With Maternal Antenatal Depression and Child Psychiatric Problems. J Am Acad Child Adolesc Psychiatry. 2018;57:321–8.e2.
11. DeVore GR. The importance of the cerebroplacental ratio in the evaluation of fetal well-being in SGA and AGA fetuses. Am J Obstet Gynecol. 2015;213:5–15.
12. Morales-Roselló J, Khalil A, Morlando M, Papageorghiou A, Bhide A, Thilaganathan B. Changes in fetal Doppler indices as a marker of failure to reach growth potential at term. Ultrasound Obstet Gynecol. 2014;43:303–10.
13. Bahado-Singh RO, Kovanci E, Jeffres A, Oz U, Deren O, Copel J, et al. The Doppler cerebroplacental ratio and perinatal outcome in intrauterine growth restriction. Am J Obstet Gynecol. 1999;180:750–6.
14. Peeters LLH, Sheldon RE, Jones Jr. MD, Makowski EL, Meschia G. Blood flow to fetal organs as a function of arterial oxygen content. Am J Obstet Gynecol. 1979;135:637–46.
15. Mone F, Thompson A, Stewart MC, Ong S, Shields MD. Fetal umbilical artery Doppler pulsatility index as a predictor of cardiovascular risk factors in children – a long-term follow up study. J Matern Neonatal Med. 2014;27:1633–6.
16. Mone F, McConnell B, Thompson A, Segurado R, Hepper P, Stewart MC, et al. Fetal umbilical artery Doppler pulsatility index and childhood neurocognitive outcome at 12 years. BMJ Open. 2016;6.
17. Gottesman II, Shields J. A Critical Review of Recent Adoption, Twin, and Family Studies of Schizophrenia: Behavioral Genetics Perspectives*. Schizophr Bull. 1976;2:360–401.
18. Cannon M, Ph D, Jones PB, Ph D, Murray RM, Sc D, et al. Obstetric Complications and Schizophrenia : Historical and Meta-Analytic Review. Am J Psychiatry. 2002;159:1080–92.
19. Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, et al. Early life programming and neurodevelopmental disorders. Biol Psychiatry. 2010;68:314–9.
20. Belbasis L, Köhler CA, Stefanis N, Stubbs B, Os J, Vieta E, et al. Risk factors and peripheral biomarkers for schizophrenia spectrum disorders: an umbrella review of meta-analyses. Acta Psychiatr Scand [Internet]. John Wiley & Sons, Ltd (10.1111); 2017;137:88–97.
21. Ursini G, Punzi G, Chen Q, Marenco S, Robinson JF, Porcelli A, et al. Convergence of placenta biology and genetic risk for schizophrenia. Nat Med. 2018;1–10.
22. Reichenberg A, Caspi A, Harrington H, Houts R, Keefe RSE, Murray RM, et al. Static and Dynamic Cognitive Deficits in Childhood Preceding Adult Schizophrenia: A 30-Year Study. Am J Psychiatry. 2010;167:160–9.
23. Schizophrenia Working Group of the Psychiatric Genomics Consortium. Biological insights from 108 schizophrenia-associated genetic loci. Nature. 2014;511:421–7.
24. López M, García-Oguiza A, Armstrong J, García-Cobaleda I, García-Miñaur S, Santos-Simarro F, et al. Rubinstein-Taybi 2 associated to novel EP300 mutations: deepening the clinical and genetic spectrum. BMC Med Genet. 2018;19:36.
25. Tan XL, Zhai Y, Gao WX, Fan YM, Liu FY, Huang QY, et al. p300 expression is induced by oxygen deficiency and protects neuron cells from damage. Brain Res. 2009;1254:1–9.
26. Peng Y, Yang Z, Zhang H, Cui C, Qi X, Luo X, et al. Genetic Variations in Tibetan Populations and High-Altitude Adaptation at the Himalayas. Mol Biol Evol [Internet]. 2011;28:1075–81.
27. van Erp TGM, Saleh PA, Rosso IM, Huttunen M, Lönnqvist J, Pirkola T, et al. Contributions of Genetic Risk and Fetal Hypoxia to Hippocampal Volume in Patients With Schizophrenia or Schizoaffective Disorder, Their Unaffected Siblings, and Healthy Unrelated Volunteers. Am J Psychiatry. 2002;159:1514–20.
28. Cannon TD, van Erp TGM, Rosso IM, Huttunen M, Lönnqvist J, Pirkola T, et al. Fetal hypoxia and structural brain abnormalities in schizophrenic patients, their siblings, and controls. Arch Gen Psychiatry. 2002;59:35–41.

29. Cannon TD, Yolken R, Buka S, Torrey EF. Decreased Neurotrophic Response to Birth Hypoxia in the Etiology of Schizophrenia. Biol Psychiatry. 2008;64:797–802.
30. Dempster EL, Pidsley R, Schalkwyk LC, Owens S, Georgiades A, Kane F, et al. Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder. Hum Mol Genet. 2011;20:4786–96.
31. Essex MJ, Thomas Boyce W, Hertzman C, Lam LL, Armstrong JM, Neumann SMA, et al. Epigenetic Vestiges of Early Developmental Adversity: Childhood Stress Exposure and DNA Methylation in Adolescence. Child Dev. 2013;84:58–75.
32. Sago H, Ishii K, Sugibayashi R, Ozawa K, Sumie M, Wada S. Fetoscopic laser photocoagulation for twin–twin transfusion syndrome. J Obstet Gynaecol Res. 2018;44:831–9.
33. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30:1363–9.
34. Morin AM, Gatev E, McEwen LM, MacIsaac JL, Lin DTS, Koen N, et al. Maternal blood contamination of collected cord blood can be identified using DNA methylation at three CpGs. Clin Epigenetics. 2017;9:75.
35. R Development Core Team. R: A Language and Environment for Statistical Computing [Internet]. Team RDC, editor. R Found. Stat. Comput. R Foundation for Statistical Computing; 2011. p. 409. Available from: http://www.r-project.org
36. Simpkin AJ, Suderman M, Howe LD. Epigenetic clocks for gestational age: Statistical and study design considerations. Clin Epigenetics. Clinical Epigenetics; 2017;9:1–2.
37. Mcewen LM, Jones MJ, Tse D, Lin S, Edgar RD, Husquin LT, et al. Systematic evaluation of DNA methylation age estimation with common preprocessing methods and the Infinium MethylationEPIC BeadChip array. Clin Epigenetics. Clinical Epigenetics; 2018;10:123.
38. Houseman E, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86.
39. Córdova-Palomera A, Fatjó-Vilas M, Palma-Gudiel H, Blasco-Fontecilla H, Kebir O, Fañanás L. Further evidence of DEPDC7 DNA hypomethylation in depression: A study in adult twins. Eur Psychiatry. 2015;30:715–8.

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, Full Professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology, University of Barcelona, and supervisor of the present doctoral thesis by Helena Palma Gudiel, hereby certifies that the participation of the PhD applicant in the article "Prenatal suffering is associated with epigenetic age deceleration at birth and hypomethylation at the hypoxia-responsive EP300 gene" included the following tasks:

- Participation in the conception and design of the study
- Statistical analysis
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Dr. Lourdes Fañanás

Barcelona, December 13th 2018

GLOBAL SUMMARY OF RESULTS

The main hypothesis was tested throughout the seven independent studies mentioned above.

Regarding the first specific hypothesis, DNA methylation at *NR3C1* and *SLC6A4* genes contributed to explain the risk for anxious-depressive disorders, as revealed by the first five publications presented in this dissertation.

I. A systematic review on *NR3C1* methylation unveiled a gap in the scientific literature with regard to non-coding alternative first exons other than exon 1_F. This work also showed *NR3C1* methylation to be robustly associated with early exposure to stress while more inconsistent findings were found with regard to psychopathology.

II. A meta-analysis on the effects of maternal psychosocial stress during pregnancy implied the existence of intergenerational effects mediated by *NR3C1* exon 1_F methylation. The specific CpG probe identified as differentially methylated in newborns exposed to maternal stress was homologous to one of the CpG sites previously described in a rat model.

III. Methylation at exon 1_D, in a biologically relevant region (glucocorticoid responsive element), was associated with both (i) the familial component of anxious-depressive disorders, and (ii) lower hippocampal connectivity within the emotional network.

IV. A systematic review on *SLC6A4* methylation exposed there is suggestive evidence favoring the existence of a GxE interaction between early life stress and 5-HTTLPR in increasing psychopathological liability by means of *SLC6A4* methylation. Moreover, *SLC6A4* methylation has been found to influence SERT expression in several independent studies.

V. Exploration of *SLC6A4* shore methylation revealed sex-differential *SLC6A4* methylation signatures to be associated with somatization symptoms, such that women exhibit higher levels of both variables when compared to men, according to prior clinical evidence.

Regarding the second specific hypothesis, epigenetic stochasticity was described to be involved in anxious-depressive vulnerability.

VI. DNA methylation outliers were found exclusively in some subjects of the twin pairs discordant for anxious-depressive disorders, with an enrichment on genes previously associated with neuropsychiatric disorders.

Finally, the newborn epigenome measured in cord blood exhibited changes related to prenatal suffering.

VII. An exploratory study on an independent sample of monozygotic monochorionic twins exposed to severe prenatal stress highlighted prenatal suffering as a modulator of the epigenetic landscape during intrauterine development. Prenatally stressed subjects were born in an immature state as reflected by epigenetic age deceleration. Furthermore, *EP300* gene CpG-specific methylation was also associated with prenatal stress. Remarkably, *EP300* gene is involved in response to hypoxia and its genetic variants have been identified in the largest schizophrenia GWAS to date.

DISCUSSION

Regarding *NR3C1* methylation and its association with exposure to stress, psychopathological liability and abnormal brain connectivity (manuscripts 1, 2 and 3)

After the seminal study in rats revealing the ability of maternal postnatal rearing behavior to epigenetically program the offspring stress response (Weaver et al., 2004), dozens of projects focusing on human *NR3C1* methylation analysis were developed. However, the complexity of *NR3C1* gene structure and expression were neglected in most of the studies. As introduced in section 2.2.3, a CpG island in the *NR3C1* promoter region includes 303 CpG sites distributed across seven alternative non-coding exons. There is no study assessing DNA methylation through the whole CpG island, i.e. including all CpG sites; authors rather focus in smaller regions with a clear preference for 1_F exon (Palma-Gudiel et al., 2015b). This bias originates from the seminal observation in rats of differential methylation at *Nr3c1* exon 1_7 in association with early stress, given that human exon 1_F is homologous to 1_7 in rats. Furthermore, CpG sites within exon 1_F are characterized by homogenous hypomethylation; thus, most of the studies focusing on this region reported methylation differences across diagnostic groups lower than 5%, which are difficult to interpret from a biological point of view.

Nevertheless, a modest meta-analysis of all published studies specifically analyzing *NR3C1* 1_F methylation in association with prenatal stress (n = 7) revealed a significant cross-study effect in a specific CpG site very close to an NGFI binding site (Palma-Gudiel et al., 2015a). Although the effect size was limited (0.14) and the confidence interval was close to zero (0.05 – 0.23), the combined effects of almost one thousand dyads revealed a significant association between maternal exposure to psychosocial stress during pregnancy and DNA methylation at a specific CpG site located at the exon 1_F in their offspring. These results support the relevance of maternal experiences for the future HPA axis functioning and stress reactivity of their children.

In the light of controversial associations between *NR3C1* methylation and psychopathology, exon 1_D was selected for epigenetic analysis in a sample of monozygotic twins from the general population informative for anxious-depressive disorders as diagnosed following DSM-IV-TR criteria. In

order to compare our results with the wider body of evidence regarding *NR3C1* methylation, exon 1_F was also explored. Unsurprisingly, we did not find any significant association between 1_F methylation and anxious-depressive psychopathology. Instead, 1_D findings revealed the role of familial factors—either genetic or due to common environment—in the modulation of DNA methylation at this region and its dialogue with hippocampal connectivity (Palma-Gudiel et al., 2018). As discussed in sections 2.2.3 and 3.7, hippocampal reductions have been described in association to excessive glucocorticoid exposure; additionally, exposure to low maternal rearing behavior was associated with decreased levels of GR expression in rat hippocampus. Thus, the association between human *NR3C1* exon 1_D increased methylation and lower hippocampal connectivity suggests epigenetic modifications to mediate the impact of familial liability to anxious-depressive disorders via altered brain wiring. Furthermore, these results added to preliminary exploratory evidence of the role of prenatal environment in the modulation of exon 1_D methylation in humans (Hompes et al., 2013).

Regarding *SLC6A4* methylation and its association with exposure to stress, psychopathological liability and sex-biased presentation of psychiatric symptoms (manuscripts 4 and 5)

Likewise, *SLC6A4* methylation has received similar attention from the scientific community although it does not build upon animal models but on the relevance of SERT in human response to antidepressants and stress. Thus, a region preferentially assessed by researchers does not exist in the *SLC6A4* gene (Palma-Gudiel and Fañanás, 2017).

Following the exon 1_D approach, we focused in a CpG island shore region, from now on *SLC6A4* shore methylation, that had been scarcely studied in relation to prenatal stress and bipolar disorder (Dukal et al., 2015; Sugawara et al., 2011). The reported association between *SLC6A4* methylation and somatization symptoms was revealing in many ways (Palma-Gudiel et al., 2019). On the one hand, it adds to the evidence that shore regions might be more informative than CpG islands about psychopathological phenotypes. On the other hand, DNA methylation is not associated with either nosological entities or more lenient categorizations including a range of anxious-depressive disorders; instead, it correlated to symptoms of the somatic dimension.

More importantly, the main modulator of methylation in this region is sex. As opposed to *NR3C1* (despite the well described differences in HPA axis functioning between men and women), *SLC6A4* shore methylation has been repeatedly shown to be higher in females when compared to males. This moderator effect of sex has been described in cord blood samples—obtained at birth—, adult peripheral blood and postmortem brain samples, highlighting the temporal stability and cross-tissue nature of this sex-based differential methylation mark. Thus, the association between methylation at a sex-controlled region and somatization contributes to the sex-biased presentation of these psychiatric symptoms and is congruent with epidemiological data. Hence, these unprecedented findings pave the way for further studies focusing on DNA methylation and its putative role in mediating sex-biased phenotypes. Interestingly, differential serotonergic functioning in females had been already suggested following animal findings in which estrogen administration resulted in a decrease of brain SERT expression (Pecins-Thompson et al., 1998).

Although DNA methylation was first proposed as a putative mediator of gene—environment interactions, studies assessing *SLC6A4* methylation and 5-HTTLPR hardly provide any evidence of such an epigenetic mediated interaction (Palma-Gudiel and Fañanás, 2017). 5-HTTLPR associations with either treatment response or psychopathological liability along with GxE models of stress exposure have been largely controversial (de Vries et al., 2016). Thus, current inability to detect robust GxE interactions involving SERT could be due to an excessive focus on 5-HTTLPR, neglecting other *SLC6A4* genetic variants, or poor targeting of CpG sites (Murphy et al., 2013).

Regarding the contribution of epigenetic stochasticity in the etiology of psychopathological manifestations (manuscript 6)

The identification of methylation outliers in MZ twin pairs discordant for anxious-depressive disorders builds upon a growing body of evidence regarding epigenetic stochasticity in complex disorders and traits other than cancer (Córdova-Palomera et al., 2018). This methodology bears the potential to identify novel molecular pathways that could contribute to the etiology of psychiatric phenotypes in only a subgroup of patients. These targets cannot be identified by means of classical GWAS approaches since

these differences are not generalizable to a high proportion of affected subjects. Despite the divergent statistical management of data, epigenome-wide DNA methylation of affected and non-affected subjects is required to develop this stochastic model. The acknowledgment of stochasticity as another player in the emergence of mental health issues is essential for the understanding of their heterogeneity in terms of clinical presentation, prognostic, comorbidities, and treatment response, among others. Chorionicity might be underlying the different number of methylation outliers detected in the available MZ twin pairs discordant for anxious-depressive disorders. Unfortunately, detailed obstetric information including chorionicity was not available with optimal reliability for this sample of adult twins and this hypothesis could not be tested.

Conceptually, methylation outliers could operate like copy number variants (CNVs) of very low frequency in the population but outstanding penetrance (for more details see **Figure 6**, in section 2.1 of the Introduction). As opposed to reported findings in both *NR3C1* and *SLC6A4* genes, methylation outliers identified in our sample of MZ twins discordant for anxious-depressive disorders ranged from 14.2 to 39.9% absolute methylation difference between co-twins of a pair. Reported differences are several-fold higher than those described in candidate gene approaches. The magnitude of such changes might indicate a greater functional effect on gene expression.

Regarding epigenetic embedding of severe prenatal suffering already detectable at birth (manuscript 7)

Finally, an independent MZ monochorionic twin sample enriched for subjects exposed to severe prenatal complications—namely, TTTS and sIUGR—allowed us to explore the epigenetic landscape at birth in response to perinatal stress. Although epigenetic age acceleration has been repeatedly associated with both exposure to stress and a wide range of deleterious outcomes, research focusing on epigenetic aging in the context of prenatal stress has robustly revealed this association to be in the opposite direction; i.e. exposure to prenatal suffering is associated with epigenetic age deceleration instead of acceleration. Thus, exposure to stress during prenatal stages would render the fetus developmentally immature. This development

delay or deficit is in line with the neurodevelopmental hypothesis for schizophrenia.

Accordingly, *EP300* gene CpG-specific methylation was found to be associated with prenatal suffering. Remarkably, this histone acetyltransferase has been previously identified in hypoxia response and several *EP300* mutations are known to cause several forms of Rubinstein-Taybi syndrome, a rare congenital disease characterized by facial dysmorphisms, psychomotor and language delays, and intellectual disability, among other symptoms; most of these symptoms are also characteristic of ASD and schizophrenia. On the other hand, fetal hypoxia has been associated with brain volume abnormalities in several regions; remarkably, this effect seems to rely in particular genetic liability suggesting the involvement of a GxE interaction. Thus, *EP300* methylation emerges as a putative mediator of hypoxia-conferred risk to neurodevelopmental disorders, and particularly schizophrenia. These findings highlight the need for multidisciplinary approaches; the joint work of obstetricians, psychiatrists, psychologists and biologists is required for disentangling the role of prenatal environment in the etiology of neurodevelopmental disorders such as autism spectrum disorders or schizophrenia.

Further studies are needed to understand the nature of these associations. Specifically, a longitudinal follow-up would allow (i) the analysis of temporal stability of epigenetic marks identified, (ii) the study of postnatal factors, such as attachment or parenting styles, in modulating the DNA methylation patterns derived from exposure to prenatal stress, and (iii) the identification of neurobehavioral, cognitive and clinical long-term outcomes of exposure to stress, in relation with identified epigenetic marks. Furthermore, an independent sample is required to test the replicability of our reported findings. Finally, a GxE interaction between schizophrenia PRS and exposure to prenatal stress could be tested in the currently recruited sample in the near future.

Overall limitations of the presented work

As introduced in section 3.9, DNA methylation studies entail a number of limitations to be cautious about. Although the focus of this dissertation is to understand the (epigenetic) nature of psychopathological pheno-

types, all novel findings reported herein are based on DNA methylation assessments in peripheral samples, namely peripheral blood and cord blood. The field of psychiatric genetics has been constrained by the unavailability of brain tissue, with the exception of postmortem samples. Nevertheless, the later do not allow complementary assessments such as direct interviews, including state measures as collected by psychometric instruments such as the Brief Symptom Inventory (BSI). Thus, most of the findings in the field rely on either peripheral blood or saliva samples. This issue has been addressed by four different strategies.

Regarding the association between *NR3C1* exon 1_D methylation and familial liability to anxious-depressive disorders (Palma-Gudiel et al., 2018), the study of peripheral blood is justified by HPA axis functioning and regulation. Although the psychiatric literature has often focused on the central effects of hypercortisolism and glucocorticoid resistance, HPA activation leads to the release of cortisol from the adrenal glands intro the bloodstream (for further details, see section 2.2.3); thus, cortisol is distributed systemically. One of its targets is the immune system, where it regulates the immune system. In this regard, overactivation of the innate immune system has been described in several psychiatric disorders. Peripheral blood *NR3C1* methylation patterns leading to changes in its expression and, potentially, to glucocorticoid resistance, would have a clinically measurable outcome involved in the etiology of psychopathological manifestations. Thus, peripheral methylation of genes acting at a peripheral level is meaningful on its own.

Since *SLC6A4* gene, encoding the serotonin transporter, has a clear role in neurotransmission, biological relevance of peripheral *SLC6A4* methylation for psychological symptoms such as somatization could be questionable. That is why we complemented our findings by exploring putative sex-based differences in postmortem brain *SLC6A4* methylation (Palma-Gudiel et al., 2019). Additionally, the same sex-biased pattern was also found in *SLC6A4* methylation as measured in cord blood revealing the stability of this mark. These analyses only allow us to suggest *SLC6A4* shore is differentially methylated according to sex. However, the association between *SLC6A4* methylation and somatization symptoms is most likely to rely on this cross-tissue correlation rather than to be artefactual.

As to methylation outliers identified in monozygotic twins discordant for anxious-depressive psychopathology, they were further validated by means of an online tool providing brain-blood correlations across CpG sites included in methylomic commercial arrays.

Finally, peripheral epigenetic signatures of prenatal suffering rely on hemodynamic surrogates of prenatal stress. Thus, cord blood derived epigenetic patterns must be informative of stress adaptations. Furthermore, epigenetic age is known to reflect biological aging irrespective of the tissue used for analysis. Conversely, the *EP300* gene is highly expressed in the bone marrow, consistent with its role in response to hypoxia, hinting the biological role of its methylation in cord blood.

Two reviews and one meta-analysis have been included in the present dissertation due to their relevance for the subsequent original research developed (Palma-Gudiel and Fañanás, 2017; Palma-Gudiel et al., 2015a, 2015b); nevertheless, limitations of such works are mainly limitations of the papers reviewed and thus they will not be discussed herein.

Strengths of the present work

As revealed by the first systematic review (Palma-Gudiel et al., 2015b), although numerous articles assessing *NR3C1* methylation have been published, there is a clear scarcity of works integrating epigenetics, psychiatry and neuroimaging findings. Thus, brain functional connectivity (specifically, hippocampal connectivity within the emotional network) was analyzed with regard to *NR3C1* methylation for the first time (Palma-Gudiel et al., 2018).

Likewise, *SLC6A4* methylation has been assessed in myriad approaches demonstrating its relevance as a biomarker of early stress exposure and its involvement in depression and other psychiatric conditions (Palma-Gudiel and Fañanás, 2017). Nevertheless, no published study to date had previously assessed *SLC6A4* methylation in association with somatization symptoms (Palma-Gudiel et al., 2019).

Although evidence of epigenetic outliers had been already reported in a number of complex phenotypes including depression, our MZ twin approach is the first to verify the CpG sites identified in discordant twin pairs

with the study of healthy and concordant twin pairs, suggesting the reported findings to be associated with observed phenotypic divergence between genetically identical individuals (Córdova-Palomera et al., 2018).

Finally, despite the recognition of the neurodevelopmental hypothesis of schizophrenia, it was entirely build upon retrospective assessments of clinical records and animal models without any attempt to prospectively analyze human prenatal trajectories and epigenomic signatures in candidate gens of risk for mental disorders. Accordingly, our findings revealed association between prenatal suffering and both (i) epigenetic age, and (ii) *EP300* methylation in the first epigenetic exploration of the cerebroplacental ratio, a validated obstetric marker of prenatal stress (Palma-Gudiel et al., submitted).

CONCLUSIONS

Our understanding of epigenetic mechanisms, and more specifically DNA methylation, in the comprehension of complex human characters and its environmental modulation has only just begun. Hundreds of papers deepening in the nature of these modifications are currently published each year, in an increasing fashion; nevertheless, translation and applicability of reported findings is still very low. The epigenetic field was born in the context of cancer studies and continues to develop and provide research breakthroughs in carcinogenesis. Epigenetic approaches in the context of psychiatry are way more recent. The epigenetic exploration of psychiatric phenotypes is largely constrained due to the nature of psychopathological manifestations, i.e. their heterogeneity, the diagnostic overlap, their unpredictable course and the fact that mental health can only be understood from a dimensional, dynamic and probably biographic point of view.

The findings reported in the seven publications included in the present dissertation are a good representation of both promises and pitfalls of psychiatric epigenetics. DNA methylation is most certainly involved in the pathophysiology of anxiety and depression. Excess circulating cortisol—due to either chronic stress or glucocorticoid resistance, which are commonly associated—during developmental periods is a great candidate for the establishment of long-term epigenetic marks near glucocorticoid responsive elements. Theories and evidence of fetal programming might only be feasible in the context of DNA methylation and other epigenetic mechanisms, analyzed in follow-up studies.

Monozygotic twin studies are instrumental in order to understand when and why DNA methylation abnormal patterns are established. Epigenetic patterns rely on genetic variability and controversy regarding transgenerational and intergenerational effects of stress suggest they might be heritable. The only human design that allows the disentangling of genuinely acquired epigenetic signatures from inherited patterns is the study of monozygotic twins. Beyond cross-sectional and retrospective approaches, the longitudinal and prospective follow-up of newborns exposed to prenatal stress offers a quasi-experimental study design that will continue to shed light on the origins of psychiatric liability.

Specific conclusions derived from studies included in the present dissertation are developed below:

1) *NR3C1* methylation is reliably associated with exposure to early life stress. However, biased approximations and methodological heterogeneity prompt the scientific community to widen their scopes and explore widely unknown regions of the *NR3C1* promoter.
2) Association between maternal psychosocial stress experienced during pregnancy and newborn *NR3C1* methylation revealed the existence of intergenerational effects that might be involved in abnormal or dysregulated newborn stress response and subsequent psychiatric liability.
3) *NR3C1* exon 1_D increased methylation was found associated with the familial component of anxious-depressive disorders suggesting it might arise during developmental prenatal shared environment between monozygotic twin pairs. Furthermore, this epigenetic signature was also associated with lower hippocampal connectivity within the emotional network suggesting downstream effects of epigenetic modifications might be mediated by abnormal brain wiring.
4) *SLC6A4* methylation is reliably associated with exposure to early life stress. Furthermore, there is moderate evidence suggesting it might mediate the so far controversial GxE interaction between 5-HTTLPR and depression.
5) The interaction between *SLC6A4* methylation, sex and somatization symptoms suggests hormone-based DNA methylation modifications to underlie the epidemiologically described increased prevalence of this psychiatric dimension in women when compared to men.
6) Identification of DNA methylation outliers only in monozygotic twin pairs discordant for anxious-depressive disorders highlights the role of stochastic epimutations in the etiology of complex psychiatric phenotypes.
7) Exposure to severe prenatal suffering is embedded via epigenetic mechanisms as reflected by both epigenetic age deceleration and *EP300* decreased methylation at birth in exposed monochorionic twins. These findings highlight the epigenetic landscape as a biomarker of prenatal exposures and as a putative predictor or delayed neurodevelopmental trajectories, of great interest in psychiatric disorders.

REFERENCES

Alemany, S., Arias, B., Aguilera, M., Villa, H., Moya, J., Ibáñez, M.I., Vossen, H., Gastó, C., Ortet, G., Fañanás, L., 2011. Childhood abuse, the BDNF-Val66Met polymorphism and adult psychotic-like experiences. Br. J. Psychiatry 199, 38–42.

Allen, A.P., Kennedy, P.J., Cryan, J.F., Dinan, T.G., Clarke, G., 2014. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. Neurosci. Biobehav. Rev. 38, 94–124. doi:10.1016/j.neubiorev.2013.11.005

Anda, R.F., Felitti, V.J., Bremner, J.D., Walker, J.D., Whitfield, C., Perry, B.D., Dube, S.R., Giles, W.H., 2006. The enduring effects of abuse and related adverse experiences in childhood: A convergence of evidence from neurobiology and epidemiology. Eur. Arch. Psychiatry Clin. Neurosci. 256, 174–186. doi:10.1007/s00406-005-0624-4

Andrews, P.W., Bharwani, A., Lee, K.R., Fox, M., Thomson, J.A., 2015. Is serotonin an upper or a downer? The evolution of the serotonergic system and its role in depression and the antidepressant response. Neurosci. Biobehav. Rev. 51, 164–188. doi:https://doi.org/10.1016/j.neubiorev.2015.01.018

Anney, R.J.L., Ripke, S., Anttila, V., Grove, J., Holmans, P., Huang, H., Klei, L., Lee, P.H., Medland, S.E., Neale, B., Robinson, E., Weiss, L.A., Zwaigenbaum, L., Yu, T.W., Wittemeyer, K., Willsey, A.J., Wijsman, E.M., Werge, T., Wassink, T.H., Waltes, R., Walsh, C.A., Wallace, S., Vorstman, J.A.S., Vieland, V.J., Vicente, A.M., vanEngeland, H., Tsang, K., Thompson, A.P., Szatmari, P., Svantesson, O., Steinberg, S., Stefansson, K., Stefansson, H., State, M.W., Soorya, L., Silagadze, T., Scherer, S.W., Schellenberg, G.D., Sandin, S., Sanders, S.J., Saemundsen, E., Rouleau, G.A., Rogé, B., Roeder, K., Roberts, W., Reichert, J., Reichenberg, A., Rehnström, K., Regan, R., Poustka, F., Poultney, C.S., Piven, J., Pinto, D., Pericak-Vance, M.A., Pejovic-Milovancevic, M., Pedersen, M.G., Pedersen, C.B., Paterson, A.D., Parr, J.R., Pagnamenta, A.T., Oliveira, G., Nurnberger, J.I., Nordentoft, M., Murtha, M.T., Mouga, S., Mortensen, P.B., Mors, O., Morrow, E.M., Moreno-De-Luca, D., Monaco, A.P., Minshew, N., Merikangas, A., McMahon, W.M., McGrew, S.G., Mattheisen, M., Martsenkovsky, I., Martin, D.M., Mane, S.M., Magnusson, P., Magalhaes, T., Maestrini, E., Lowe, J.K., Lord, C., Levitt, P., Martin, C.L., Ledbetter, D.H., Leboyer, M., LeCouteur, A.S., Ladd-Acosta, C., Kolevzon, A., Klauck, S.M., Jacob, S., Iliadou, B., Hultman, C.M., Hougaard, D.M., Hertz-Picciotto, I., Hendren, R., Hansen, C.S., Haines, J.L., Guter, S.J., Grice, D.E., Green, J.M., Green, A., Goldberg, A.P., Gillberg, C., Gilbert, J., Gallagher, L., Freitag, C.M., Fombonne, E., Folstein, S.E., Fernandez, B., Fallin, M.D., Ercan-Sencicek, A.G., Ennis, S., Duque, F., Duketis, E., Delorme, R., DeRubeis, S., DeJonge, M. V, Dawson, G., Cuccaro, M.L., Correia, C.T., Conroy, J., Conceição, I.C., Chiocchetti, A.G., Celestino-Soper, P.B.S., Casey, J., Cantor, R.M., Café, C., Bybjerg-Grauholm, J., Brennan, S., Bourgeron, T., Bolton, P.F., Bölte, S., Bolshakova, N., Betancur, C., Bernier, R., Beaudet, A.L., Battaglia, A., Bal, V.H., Baird, G., Bailey, A.J., Bækvad-Hansen, M., Bader, J.S.,

Bacchelli, E., Anagnostou, E., Amaral, D., Almeida, J., Børglum, A.D., Buxbaum, J.D., Chakravarti, A., Cook, E.H., Coon, H., Geschwind, D.H., Gill, M., Hallmayer, J., Palotie, A., Santangelo, S., Sutcliffe, J.S., Arking, D.E., Devlin, B., Daly, M.J., Consortium, T.A.S.D.W.G. of T.P.G., 2017. Meta-analysis of GWAS of over 16,000 individuals with autism spectrum disorder highlights a novel locus at 10q24.32 and a significant overlap with schizophrenia. Mol. Autism 8, 21. doi:10.1186/s13229-017-0137-9

Anxiety Disorders, 2013. , in: Diagnostic and Statistical Manual of Mental Disorders, DSM Library. American Psychiatric Association. doi:doi:10.1176/appi.books.9780890425596.dsm05

Arias, B., Catalán, R., Gastó, C., Gutiérrez, B., Fañanás, L., 2003. 5-HTTLPR polymorphism of the serotonin transporter gene predicts non-remission in major depression patients treated with citalopram in a 12-weeks follow up study. J. Clin. Psychopharmacol. 23, 563–567.

Barker, D.J., 1998. In utero programming of chronic disease. Clin. Sci. 95, 115–28. doi:10.1042/cs0950115

Baxter, A.J., Scott, K.M., Vos, T., Whiteford, H.A., 2013. Global prevalence of anxiety disorders: a systematic review and meta-regression. Psychol. Med. 43, 897–910. doi:DOI: 10.1017/S003329171200147X

Bayer, T.A., Falkai, P., Maier, W., 1999. Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the “Two hit hypothesis.” J. Psychiatr. Res. 33, 543–548. doi:https://doi.org/10.1016/S0022-3956(99)00039-4

Bebbington, M., 2010. Twin-to-twin transfusion syndrome: current understanding of pathophysiology, in-utero therapy and impact for future development. Semin. Fetal Neonatal Med. 15, 15–20. doi:10.1016/j.siny.2009.05.001

Belbasis, L., Köhler, C.A., Stefanis, N., Stubbs, B., van Os, J., Vieta, E., Seeman, M. V., Arango, C., Carvalho, A.F., Evangelou, E., 2018. Risk factors and peripheral biomarkers for schizophrenia spectrum disorders: an umbrella review of meta-analyses. Acta Psychiatr. Scand. 137, 88–97. doi:10.1111/acps.12847

Belbasis, L., Savvidou, M.D., Kanu, C., Evangelou, E., Tzoulaki, I., 2016. Birth weight in relation to health and disease in later life: an umbrella review of systematic reviews and meta-analyses. BMC Med. 14, 147. doi:10.1186/s12916-016-0692-5

Binder, E.B., Bradley, R.G., Liu, W., Epstein, M.P., Deveau, T.C., Mercer, K.B., Tang, Y., Gillespie, C.F., Heim, C.M., Nemeroff, C.B., Schwartz, A.C., Cubells, J.F., Ressler, K.J., 2008. Association of fkbp5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA 299, 1291–1305.

Blickstein, I., Keith, L.G., 2007. On the Possible Cause of Monozygotic Twinning: Lessons From the 9-Banded Armadillo and From Assisted Reproduction. Twin Res. Hum. Genet. 10, 394–399. doi:DOI: 10.1375/twin.10.2.394

Borenstein, J., 2018. Preventing Mental Illness. New York Times A26.

Bowers, M.E., Yehuda, R., 2016. Intergenerational Transmission of Stress in

Humans. Neuropsychopharmacology 41, 232–44. doi:10.1038/npp.2015.247
Bowers, M.E., Yehuda, R., 2015. Intergenerational Transmission of Stress in Humans. Neuropsychopharmacol. Rev. 41, 232–244. doi:10.1038/npp.2015.247
Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M., Meaney, M.J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. 95, 5335 LP – 5340.
Cannon, M., Ph, D., Jones, P.B., Ph, D., Murray, R.M., Sc, D., Psych, F.R.C., 2002. Obstetric Complications and Schizophrenia : Historical and Meta-Analytic Review. Am. J. Psychiatry 159, 1080–1092.
Capuron, L., Miller, A.H., 2004. Cytokines and psychopathology: lessons from interferon-alpha. Biol. Psychiatry 56, 819–24. doi:10.1016/j.biopsych.2004.02.009
Carvalho, L. a, Bergink, V., Sumaski, L., Wijkhuijs, J., Hoogendijk, W.J., Birkenhager, T.K., Drexhage, H. a, 2014. Inflammatory activation is associated with a reduced glucocorticoid receptor alpha/beta expression ratio in monocytes of inpatients with melancholic major depressive disorder. Transl. Psychiatry 4, e344.
Caspi, A., McClay, J., Moffitt, T.E., Mill, J., Martin, J., Craig, I.W., Taylor, A., Poulton, R., 2002. Role of Genotype in the Cycle of Violence in Maltreated Children. Science (80-.). 297, 851 LP – 854.
Caspi, A., Moffitt, T.E., 2018. All for One and One for All: Mental Disorders in One Dimension. Am. J. Psychiatry 175, 831–844. doi:10.1176/appi.ajp.2018.17121383
Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., Mcclay, J., Mill, J., Martin, J., Braithwaite, A., Poulton, R., 2003. Influence of Life Stress on Depression : Moderation by a Polymorphism in the 5-HTT Gene. Science (80-.). 301, 386–389.
Chalouhi, G.E., Stirnemann, J.J., Salomon, L.J., Essaoui, M., Quibel, T., Ville, Y., 2010. Specific complications of monochorionic twin pregnancies: Twin-twin transfusion syndrome and twin reversed arterial perfusion sequence. Semin. Fetal Neonatal Med. 15, 349–356. doi:10.1016/j.siny.2010.09.003
Chen, B., Marioni, R.E., Bressler, J., Fornage, M., Studenski, S., Vandiver, A.R., Tanaka, T., Kiel, D.P., Liang, L., Vokonas, P., Lunetta, K.L., Murabito, J.M., Bandinelli, S., Dena, G., Melzer, D., Nalls, M., Pilling, L.C., Price, T.R., Andrew, B., Gieger, C., Holle, R., Kretschmer, A., Kronenberg, F., Visscher, P.M., Shah, S., Wray, N.R., Mcrae, A.F., Levine, M.E., Lu, A.T., Tsao, P.S., Hou, L., Manson, J.E., Levy, D., Baccarelli, A., Meurs, J. Van, Bell, J.T., Chen, B.H., Marioni, R.E., Colicino, E., Peters, M.J., Ward, C.K., Tsai, P.C., Roetker, N.S., Just, A.C., Demerath, E.W., 2016. DNA methylation-based measures of biological age : meta-analysis predicting time to death. Aging (Albany. NY). 8, 1–22. doi:10.18632/aging.101020

Chen, Q., Yan, W., Duan, E., 2016. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. Nat. Rev. Genet. 17, 733.

Choi, K.W., Sikkema, K.J., Vythilingum, B., Geerts, L., Faure, S.C., Watt, M.H., Roos, A., Stein, D.J., 2017. Maternal childhood trauma, postpartum depression, and infant outcomes: Avoidant affective processing as a potential mechanism. J. Affect. Disord. 211, 107–115. doi:https://doi.org/10.1016/j.jad.2017.01.004

Cipriani, A., Furukawa, T.A., Salanti, G., Chaimani, A., Atkinson, L.Z., Ogawa, Y., Leucht, S., Ruhe, H.G., Turner, E.H., Higgins, J.P.T., Egger, M., Takeshima, N., Hayasaka, Y., Imai, H., Shinohara, K., Tajika, A., Ioannidis, J.P.A., Geddes, J.R., 2018. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. Lancet 391, 1357–1366. doi:10.1016/S0140-6736(17)32802-7

Clifton, V.L., 2010. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta 31 Suppl, S33–9. doi:10.1016/j.placenta.2009.11.010

Clouston, T.S., 1891. The Neuroses of Development: being the Morison lectures for 1890. Oliver and Boyd, Edinburgh.

Cohen, S., Janicki-Deverts, D., Doyle, W.J., Miller, G.E., Frank, E., Rabin, B.S., Turner, R.B., 2012. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proc. Natl. Acad. Sci. U. S. A. 109, 5995–9. doi:10.1073/pnas.1118355109

Compton, W.M., Conway, K.P., Stinson, F.S., Grant, B.F., 2006. Changes in the Prevalence of Major Depression and Comorbid Substance Use Disorders in the United States Between 1991–1992 and 2001–2002. Am. J. Psychiatry 163, 2141–2147. doi:10.1176/ajp.2006.163.12.2141

Córdova-Palomera, A., Goldberg, X., Alemany, S., Nenadic, I., Gastó, C., Fañanás, L., 2014. Letter to the Editor: Low birth weight and adult depression: eliciting their association. Psychol. Med. 44, 1117–1119.

Córdova-Palomera, A., Palma-Gudiel, H., Forés-Martos, J., Tabarés-Seisdedos, R., Fañanás, L., 2018. Epigenetic outlier profiles in depression: A genome-wide DNA methylation analysis of monozygotic twins. PLoS One 13, e0207754.

Courtet, P., Olié, E., 2012. Circadian dimension and severity of depression. Eur. Neuropsychopharmacol. 22, S476–S481. doi:https://doi.org/10.1016/j.euroneuro.2012.07.009

Coutinho Nunes, F., Domingues, A.P., Vide Tavares, M., Belo, A., Ferreira, C., Fonseca, E., Moura, P., 2016. Monochorionic versus dichorionic twins: Are obstetric outcomes always different? J. Obstet. Gynaecol. (Lahore). 36, 598–601. doi:10.3109/01443615.2015.1116501

Craske, M.G., Stein, M.B., 2016. Anxiety. Lancet 388, 3048–3059. doi:10.1016/S0140-6736(16)30381-6

Crick, F., 1970. Central Dogma of Molecular Biology. Nature 227, 561–563.

Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013. Identification

of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet 381, 1371–1379.

Crow, T.J., 1997. Is schizophrenia the price that Homo sapiens pays for language? Schizophr. Res. 28, 127–141. doi:https://doi.org/10.1016/S0920-9964(97)00110-2

D'Antonio, F., Khalil, A., Dias, T., Thilaganathan, B., 2012. Early fetal loss in monochorionic and dichorionic twin pregnancies: analysis of the Southwest Thames Obstetric Research Collaborative (STORK) multiple pregnancy cohort. Ultrasound Obstet. Gynecol. 41, 632–636. doi:10.1002/uog.12363

Dantzer, R., Kelley, K.W., 2007. Twenty years of research on cytokine-induced sickness behavior. Brain. Behav. Immun. 21, 153–60. doi:10.1016/j.bbi.2006.09.006

de Vries, Y.A., Roest, A.M., Franzen, M., Munafò, M.R., Bastiaansen, J.A., 2016. Citation bias and selective focus on positive findings in the literature on the serotonin transporter gene (5-HTTLPR), life stress and depression. Psychol. Med. 46, 2971–2979. doi:10.1017/S0033291716002178

Deckert, J., Weber, H., Villmann, C., Lonsdorf, T.B., Richter, J., Andreatta, M., Arias-Vasquez, A., Hommers, L., Kent, L., Schartner, C., Cichon, S., Wolf, C., Schaefer, N., von Collenberg, C.R., Wachter, B., Blum, R., Schümann, D., Scharfenort, R., Schumacher, J., Forstner, A.J., Baumann, C., Schiele, M.A., Notzon, S., Zwanzger, P., Janzing, J.G.E., Galesloot, T., Kiemeney, L.A., Gajewska, A., Glotzbach-Schoon, E., Mühlberger, A., Alpers, G., Fydrich, T., Fehm, L., Gerlach, A.L., Kircher, T., Lang, T., Ströhle, A., Arolt, V., Wittchen, H.-U., Kalisch, R., Büchel, C., Hamm, A., Nöthen, M.M., Romanos, M., Domschke, K., Pauli, P., Reif, A., 2017. GLRB allelic variation associated with agoraphobic cognitions, increased startle response and fear network activation: a potential neurogenetic pathway to panic disorder. Mol. Psychiatry 22, 1431.

DeVore, G.R., 2015. The importance of the cerebroplacental ratio in the evaluation of fetal well-being in SGA and AGA fetuses. Am. J. Obstet. Gynecol. 213, 5–15. doi:10.1016/j.ajog.2015.05.024

Doherty, J.L., Owen, M.J., 2014. Genomic insights into the overlap between psychiatric disorders: implications for research and clinical practice. Genome Med. 6, 29. doi:10.1186/gm546

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., Lanctôt, K.L., 2010. A meta-analysis of cytokines in major depression. Biol. Psychiatry 67, 446–57. doi:10.1016/j.biopsych.2009.09.033

Dukal, H., Frank, J., Lang, M., Treutlein, J., Gilles, M., Wolf, I.A., Krumm, B., Massart, R., Szyf, M., Laucht, M., Deuschle, M., Rietschel, M., Witt, S.H., 2015. New-born females show higher stress- and genotype-independent methylation of SLC6A4 than males. Borderline Personal. Disord. Emot. dysregulation 2, 8. doi:10.1186/s40479-015-0029-6

Dunbar, R.I.M., 2009. Darwin and the ghost of Phineas Gage: Neuro-evolution and the social brain. Cortex 45, 1119–1125. doi:10.1016/j.cortex.2009.05.005

Edwards, J.R., Yarychkivska, O., Boulard, M., Bestor, T.H., 2017. DNA methylation and DNA methyltransferases. Epigenetics Chromatin 10, 23. doi:10.1186/s13072-017-0130-8

Eisenberger, N.I., Taylor, S.E., Gable, S.L., Hilmert, C.J., Lieberman, M.D., 2007. Neural pathways link social support to attenuated neuroendocrine stress responses. Neuroimage 35, 1601–1612. doi:https://doi.org/10.1016/j.neuroimage.2007.01.038

Ekelund, C.K., Skibsted, L., Søgaard, K., Main, K.M., Dziegiel, M.H., Schwartz, M., Moeller, N., Roos, L., Tabor, A., 2008. Dizygotic monochorionic twin pregnancy conceived following intracytoplasmic sperm injection treatment and complicated by twin-twin transfusion syndrome and blood chimerism. Ultrasound Obstet. Gynecol. 32, 832–834. doi:10.1002/uog.6221

Estes, M.L., McAllister, A.K., 2016. Maternal immune activation: Implications for neuropsychiatric disorders. Science (80-.). 353, 772 LP – 777.

Falconer, D., Mackay, T.F.C., 1996. Introduction to Quantitative Genetics, 4th ed. Harlow.

Fañanás, L., Bertranpetit, J., 1995. Reproductive rates in families of schizophrenic patients in a case-control study. Acta Psychiatr. Scand. 91, 202–204. doi:10.1111/j.1600-0447.1995.tb09767.x

Farrell, M.S., Werge, T., Sklar, P., Owen, M.J., Ophoff, R.A., O'Donovan, M.C., Corvin, A., Cichon, S., Sullivan, P.F., 2015. Evaluating historical candidate genes for schizophrenia. Mol. Psychiatry 20, 555.

Fava, M., Freeman, M.P., Flynn, M., Judge, H., Hoeppner, B.B., Cusin, C., Ionescu, D.F., Mathew, S.J., Chang, L.C., Iosifescu, D. V, Murrough, J., Debattista, C., Schatzberg, A.F., Trivedi, M.H., Jha, M.K., Sanacora, G., Wilkinson, S.T., Papakostas, G.I., 2018. Double-blind, placebo-controlled, dose-ranging trial of intravenous ketamine as adjunctive therapy in treatment-resistant depression (TRD). Mol. Psychiatry. doi:10.1038/s41380-018-0256-5

Fava, M., Rankin, M.A., Wright, E.C., Alpert, J.E., Nierenberg, A.A., Pava, J., Rosenbaum, J.F., 2000. Anxiety disorders in major depression. Compr. Psychiatry 41, 97–102. doi:https://doi.org/10.1016/S0010-440X(00)90140-8

Feinberg, A.P., Irizarry, R.A., 2010. Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. Proc. Natl. Acad. Sci. 107, 1757 LP – 1764.

Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suñer, D., Cigudosa, J.C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T.D., Wu, Y.-Z., Plass, C., Esteller, M., 2005. Epigenetic differences arise during the lifetime of monozygotic twins. Proc. Natl. Acad. Sci. United States Am. 102 , 10604–10609. doi:10.1073/pnas.0500398102

Francis, D., Diorio, J., Liu, D., Meaney, M.J., 1999. Nongenomic Transmission Across Generations of Maternal Behavior and Stress Responses in the Rat. Science (80-.). 286, 1155 LP – 1158.

Friedrich, M., 2017. Depression is the leading cause of disability around the world. JAMA 317, 1517.

Gaunt, T.R., Shihab, H.A., Hemani, G., Min, J.L., Woodward, G., Lyttleton, O., Zheng, J., Duggirala, A., McArdle, W.L., Ho, K., Ring, S.M., Evans, D.M., Davey Smith, G., Relton, C.L., 2016. Systematic identification of genetic influences on methylation across the human life course. Genome Biol. 17, 61. doi:10.1186/s13059-016-0926-z

Gentilini, D., Garagnani, P., Pisoni, S., Bacalini, M.G., Calzari, L., Mari, D., Vitale, G., Franceschi, C., Di Blasio, A.M., 2015. Stochastic epigenetic mutations (DNA methylation) increase exponentially in human aging and correlate with X chromosome inactivation skewing in females. Aging (Albany. NY). 7, 568–578. doi:10.18632/aging.100792

Giovanoli, S., Engler, H., Engler, A., Richetto, J., Voget, M., Willi, R., Winter, C., Riva, M.A., Mortensen, P.B., Feldon, J., Schedlowski, M., Meyer, U., 2013. Stress in Puberty Unmasks Latent Neuropathological Consequences of Prenatal Immune Activation in Mice. Science (80-.). 339, 1095 LP – 1099.

Glover, V., Hill, J., 2012. Sex differences in the programming effects of prenatal stress on psychopathology and stress responses: an evolutionary perspective. Physiol. Behav. 106, 736–40. doi:10.1016/j.physbeh.2012.02.011

Goldney, R.D., Eckert, K.A., Hawthorne, G., Taylor, A.W., 2010. Changes in the Prevalence of Major Depression in an Australian Community Sample Between 1998 and 2008. Aust. New Zeal. J. Psychiatry 44, 901–910. doi:10.3109/00048674.2010.490520

Goodkind, M., SB, E., DJ, O., al, et, 2015. Identification of a common neurobiological substrate for mental illness. JAMA Psychiatry 72, 305–315.

Gordon, L., Joo, J.E., Powell, J.E., Ollikainen, M., Novakovic, B., Li, X., Andronikos, R., Cruickshank, M.N., Conneely, K.N., Smith, A.K., Alisch, R.S., Morley, R., Visscher, P.M., Craig, J.M., Saffery, R., 2012. Neonatal DNA methylation profile in human twins is specified by a complex interplay between intrauterine environmental and genetic factors, subject to tissue-specific influence. Genome Res. 22 , 1395–1406. doi:10.1101/gr.136598.111

Gunnewiek, T.M.K., Homberg, J.R., Kozicz, T., 2018. Modulation of glucocorticoids by the serotonin transporter polymorphism: A narrative review. Neurosci. Biobehav. Rev. 92, 338–349. doi:https://doi.org/10.1016/j.neubiorev.2018.06.022

Guo, J.U., Su, Y., Shin, J.H., Shin, J., Li, H., Xie, B., Zhong, C., Hu, S., Le, T., Fan, G., Zhu, H., Chang, Q., Gao, Y., Ming, G., 2013. Distribution , recognition and regulation of non-CpG methylation in the adult mammalian brain. Nat. Publ. Gr. 17, 215–222. doi:10.1038/nn.3607

Hackmon, R., Jormark, S., Cheng, V., Green, C.O., Divon, M.Y., 2009. Monochorionic dizygotic twins in a spontaneous pregnancy: a rare case report. J. Matern. Neonatal Med. 22, 708–710. doi:10.1080/14767050902763159

Hales, C.N., Barker, D.J.P., 1992. Type 2 (non-insulin-dependent) diabetes mellitus:

the thrifty phenotype hypothesis. Diabetologia 35, 595–601. doi:10.1007/BF00400248

Hannon, E., Dempster, E., Viana, J., Burrage, J., Smith, A.R., Macdonald, R., St Clair, D., Mustard, C., Breen, G., Therman, S., Kaprio, J., Toulopoulou, T., Pol, H.E.H., Bohlken, M.M., Kahn, R.S., Nenadic, I., Hultman, C.M., Murray, R.M., Collier, D.A., Bass, N., Gurling, H., McQuillin, A., Schalkwyk, L., Mill, J., 2016. An integrated genetic-epigenetic analysis of schizophrenia: evidence for co-localization of genetic associations and differential DNA methylation. Genome Biol. 17, 176. doi:10.1186/s13059-016-1041-x

Hannon, E., Gorrie-Stone, T.J., Smart, M.C., Burrage, J., Hughes, A., Bao, Y., Kumari, M., Schalkwyk, L.C., Mill, J., 2018a. Leveraging DNA-Methylation Quantitative-Trait Loci to Characterize the Relationship between Methylomic Variation, Gene Expression, and Complex Traits. Am. J. Hum. Genet. 103, 654–665. doi:10.1016/j.ajhg.2018.09.007

Hannon, E., Knox, O., Sugden, K., Burrage, J., Wong, C.C.Y., Belsky, D.W., Corcoran, D.L., Arseneault, L., Moffitt, T.E., Caspi, A., Mill, J., 2018b. Characterizing genetic and environmental influences on variable DNA methylation using monozygotic and dizygotic twins. PLOS Genet. 14, e1007544.

Hannon, E., Lunnon, K., Schalkwyk, L., Mill, J., 2015. Interindividual methylomic variation across blood, cortex, and cerebellum: Implications for epigenetic studies of neurological and neuropsychiatric phenotypes. Epigenetics 10, 1024–1032. doi:10.1080/15592294.2015.1100786

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S.V., Klotzle, B., Bibikova, M., Fan, J.B., Gao, Y., Deconde, R., Chen, M., Rajapakse, I., Friend, S., Ideker, T., Zhang, K., 2013. Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. Mol. Cell 49, 359–367. doi:10.1016/j.molcel.2012.10.016

Hansen, K.D., Timp, W., Bravo, H.C., Sabunciyan, S., Langmead, B., McDonald, O.G., Wen, B., Wu, H., Liu, Y., Diep, D., Briem, E., Zhang, K., Irizarry, R.A., Feinberg, A.P., 2011. Increased methylation variation in epigenetic domains across cancer types. Nat. Genet. doi:10.1038/ng.865

Hanson, J.L., Nacewicz, B.M., Sutterer, M.J., Cayo, A.A., Schaefer, S.M., Rudolph, K.D., Shirtcliff, E.A., Pollak, S.D., Davidson, R.J., 2015. Behavioral Problems After Early Life Stress: Contributions of the Hippocampus and Amygdala. Biol. Psychiatry 77, 314–323. doi:https://doi.org/10.1016/j.biopsych.2014.04.020

Hay, M., Thomas, D.W., Craighead, J.L., Economides, C., Rosenthal, J., 2014. Clinical development success rates for investigational drugs. Nat. Biotechnol. 32, 40.

Hettema, J.M., Aggen, S.H., Kubarych, T.S., Neale, M.C., Kendler, K.S., 2015. Identification and validation of mixed anxiety–depression. Psychol. Med. 45, 3075–3084. doi:DOI: 10.1017/S0033291715001038

Holsboer, F., 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23, 477–501. doi:10.1016/S0893-133X(00)00159-7

Holt-Lunstad, J., Smith, T.B., Baker, M., Harris, T., Stephenson, D., 2015. Loneliness and Social Isolation as Risk Factors for Mortality: A Meta-Analytic Review. Perspect. Psychol. Sci. 10, 227–237. doi:10.1177/1745691614568352

Hompes, T., Izzi, B., Gellens, E., Morreels, M., Fieuws, S., Pexsters, A., Schops, G., Dom, M., Van Bree, R., Freson, K., Verhaeghe, J., Spitz, B., Demyttenaere, K., Glover, V., Van den Bergh, B., Allegaert, K., Claes, S., 2013. Investigating the influence of maternal cortisol and emotional state during pregnancy on the DNA methylation status of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood. J. Psychiatr. Res. 47, 880–891. doi:10.1016/j.jpsychires.2013.03.009

Horvath, S., 2013. DNA methylation age of human tissues and cell types. Genome Biol. 14, R115. doi:10.1186/gb-2013-14-10-r115

Houseman, E., Accomando, W.P., Koestler, D.C., Christensen, B.C., Marsit, C.J., Nelson, H.H., Wiencke, J.K., Kelsey, K.T., 2012. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 13, 86. doi:10.1186/1471-2105-13-86

Howard, D.M., Adams, M.J., Shirali, M., Clarke, T.-K., Marioni, R.E., Davies, G., Coleman, J.R.I., Alloza, C., Shen, X., Barbu, M.C., Wigmore, E.M., Gibson, J., Agee, M., Alipanahi, B., Auton, A., Bell, R.K., Bryc, K., Elson, S.L., Fontanillas, P., Furlotte, N.A., Hinds, D.A., Huber, K.E., Kleinman, A., Litterman, N.K., McCreight, J.C., McIntyre, M.H., Mountain, J.L., Noblin, E.S., Northover, C.A.M., Pitts, S.J., Sathirapongsasuti, J.F., Sazonova, O. V, Shelton, J.F., Shringarpure, S., Tian, C., Tung, J.Y., Vacic, V., Wilson, C.H., Hagenaars, S.P., Lewis, C.M., Ward, J., Smith, D.J., Sullivan, P.F., Haley, C.S., Breen, G., Deary, I.J., McIntosh, A.M., Team, 23andMe Research, 2018. Genome-wide association study of depression phenotypes in UK Biobank identifies variants in excitatory synaptic pathways. Nat. Commun. 9, 1470. doi:10.1038/s41467-018-03819-3

Hyde, C.L., Nagle, M.W., Tian, C., Chen, X., Paciga, S.A., Wendland, J.R., Tung, J.Y., Hinds, D.A., Perlis, R.H., Winslow, A.R., 2016. Identification of 15 genetic loci associated with risk of major depression in individuals of European descent. Nat. Genet. 48, 1031–1036. doi:10.1038/ng.3623

Inoshita, M., Numata, S., Tajima, A., Kinoshita, M., Umehara, H., Yamamori, H., Hashimoto, R., Imoto, I., Ohmori, T., 2015. Sex differences of leukocytes DNA methylation adjusted for estimated cellular proportions. Biol. Sex Differ. 6, 11. doi:10.1186/s13293-015-0029-7

International Human Genome Sequencing Consortium, 2004. Finishing the euchromatic sequence of the human genome. Nature 431, 931–945.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33 Suppl, 245–54. doi:10.1038/ng1089

Jiang, X., Wang, J., Luo, T., Li, Q., 2009. Impaired hypothalamic-pituitary-adrenal axis and its feedback regulation in serotonin transporter knockout mice.

Psychoneuroendocrinology 34, 317–331. doi:https://doi.org/10.1016/j.psyneuen.2008.09.011
Jones, P.A., 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 13. doi:10.1038/nrg3230
Josse, J., Kaiser, A.D., Kornberg, A., 1961. Enzymatic synthesis of deoxyribonucleic acid. VIII. Frequencies of nearest neighbor base sequences in deoxyribonucleic acid. J. Biol. Chem. 236, 864–875.
Jylhävä, J., Pedersen, N.L., Hägg, S., 2017. Biological Age Predictors. EBioMedicine 21, 29–36. doi:https://doi.org/10.1016/j.ebiom.2017.03.046
Kaminsky, Z.A., Tang, T., Wang, S.-C., Ptak, C., Oh, G.H.T., Wong, A.H.C., Feldcamp, L.A., Virtanen, C., Halfvarson, J., Tysk, C., McRae, A.F., Visscher, P.M., Montgomery, G.W., Gottesman, I.I., Martin, N.G., Petronis, A., 2009. DNA methylation profiles in monozygotic and dizygotic twins. Nat. Genet. 41, 240.
Kessler, R.C., Berglund, P., Demler, O., Jin, R., KR, M., EE, W., 2005a. Lifetime prevalence and age-of-onset distributions of dsm-iv disorders in the national comorbidity survey replication. Arch. Gen. Psychiatry 62, 593–602.
Kessler, R.C., Bromet, E.J., 2013. The epidemiology of depression across cultures. Annu. Rev. Public Health 34, 119–38.
Kessler, R.C., Chiu, W., Demler, O., EE, W., 2005b. Prevalence, severity, and comorbidity of 12-month dsm-iv disorders in the national comorbidity survey replication. Arch. Gen. Psychiatry 62, 617–627.
Kessler, R.C., Petukhova, M., Sampson, N.A., Zaslavsky, A.M., Wittchen, H.-U., 2012. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. Int. J. methods Psychiatr. reserach 21, 169–184.
Kessler, R.C., Ruscio, A.M., Shear, K., Wittchen, H.-U., 2010. Epidemiology of Anxiety Disorders, in: Stein, M.B., Steckler, T. (Eds.), Behavioral Neurobiology of Anxiety and Its Treatment. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 21–35. doi:10.1007/7854_2009_9
Khalek, N., Johnson, M.P., Bebbington, M.W., 2013. Fetoscopic laser therapy for twin-to-twin transfusion syndrome. Semin. Pediatr. Surg. 22, 18–23. doi:https://doi.org/10.1053/j.sempedsurg.2012.10.004
Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16, 33–41. doi:10.1038/nn.3275
Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., Lehner, B., 2017. Transgenerational transmission of environmental information in C. elegans. Science (80-.). 356, 320 LP – 323.
Knight, A.K., Craig, J.M., Theda, C., Bækvad-Hansen, M., Bybjerg-Grauholm, J., Hansen, C.S., Hollegaard, M. V., Hougaard, D.M., Mortensen, P.B., Weinsheimer, S.M., Werge, T.M., Brennan, P.A., Cubells, J.F., Newport, D.J.,

Stowe, Z.N., Cheong, J.L.Y., Dalach, P., Doyle, L.W., Loke, Y.J., Baccarelli, A.A., Just, A.C., Wright, R.O., Téllez-Rojo, M.M., Svensson, K., Trevisi, L., Kennedy, E.M., Binder, E.B., Iurato, S., Czamara, D., Räikkönen, K., Lahti, J.M.T., Pesonen, A.K., Kajantie, E., Villa, P.M., Laivuori, H., Hämäläinen, E., Park, H.J., Bailey, L.B., Parets, S.E., Kilaru, V., Menon, R., Horvath, S., Bush, N.R., LeWinn, K.Z., Tylavsky, F.A., Conneely, K.N., Smith, A.K., 2016. An epigenetic clock for gestational age at birth based on blood methylation data. Genome Biol. 17, 1–11. doi:10.1186/s13059-016-1068-z

Koopman, M., El Aidy, S., consortium, Mid., 2017. Depressed gut? The microbiota-diet-inflammation trialogue in depression. Curr. Opin. Psychiatry 30.

Kuehner, C., 2017. Why is depression more common among women than among men? The Lancet Psychiatry 4, 146–158. doi:10.1016/S2215-0366(16)30263-2

Kuehner, C., 2003. Gender differences in unipolar depression: an update of epidemiological findings and possible explanations. Acta Psychiatr. Scand. 108, 163–174. doi:10.1034/j.1600-0447.2003.00204.x

Kurdyukov, S., Bullock, M., 2016. DNA Methylation Analysis: Choosing the Right Method. Biology (Basel). 5. doi:10.3390/biology5010003

Lacroix, A., Feelders, R.A., Stratakis, C.A., Nieman, L.K., 2015. Cushing's syndrome. Lancet 386, 913–927. doi:10.1016/S0140-6736(14)61375-1

Lahti, J., Räikkönen, K., Bruce, S., Heinonen, K., Pesonen, A.-K., Rautanen, A., Wahlbeck, K., Kere, J., Kajantie, E., Eriksson, J.G., 2011. Glucocorticoid receptor gene haplotype predicts increased risk of hospital admission for depressive disorders in the Helsinki birth cohort study. J. Psychiatr. Res. 45, 1160–1164. doi:https://doi.org/10.1016/j.jpsychires.2011.03.008

Lamers, F., van Oppen, P., Comijs, H.C., Smit, J.H., Spinhoven, P., van Balkom, A.J.L.M., Nolen, W.A., Zitman, F.G., Beekman, A.T.F., Penninx, B.W.J.H., 2011. Comorbidity patterns of anxiety and depressive disorders in a large cohort study: the Netherlands Study of Depression and Anxiety (NESDA). J. Clin. Psychiatry 72, 341–348. doi:10.4088/JCP.10m06176blu

Lanfumey, L., Mongeau, R., Cohen-Salmon, C., Hamon, M., 2008. Corticosteroid–serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neurosci. Biobehav. Rev. 32, 1174–1184. doi:https://doi.org/10.1016/j.neubiorev.2008.04.006

Leistner, C., Menke, A., 2018. How to measure glucocorticoid receptor's sensitivity in patients with stress-related psychiatric disorders. Psychoneuroendocrinology 91, 235–260. doi:https://doi.org/10.1016/j.psyneuen.2018.01.023

Lewis, S.W., Murray, R.M., 1987. Obstetric complications, neurodevelopmental deviance, and risk of schizophrenia. J. Psychiatr. Res. 21, 413–421. doi:https://doi.org/10.1016/0022-3956(87)90088-4

Li, Z., Chen, J., Yu, H., He, L., Xu, Y., Zhang, D., Yi, Q., Li, C., Li, X., Shen, J., Song, Z., Ji, W., Wang, M., Zhou, J., Chen, B., Liu, Y., Wang, J., Wang, P., Yang, P., Wang, Q., Feng, G., Liu, B., Sun, W., Li, B., He, G., Li, W., Wan, C., Xu, Q., Li, W., Wen,

Z., Liu, K., Huang, F., Ji, J., Ripke, S., Yue, W., Sullivan, P.F., O'Donovan, M.C., Shi, Y., 2017. Genome-wide association analysis identifies 30 new susceptibility loci for schizophrenia. Nat. Genet. 49, 1576–1583. doi:10.1038/ng.3973

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., Meaney, M.J., 1997. Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress. Science (80-.). 277, 1659 LP – 1662.

MacCabe, J.H., Koupil, I., Leon, D.A., 2009. Lifetime reproductive output over two generations in patients with psychosis and their unaffected siblings: the Uppsala 1915–1929 Birth Cohort Multigenerational Study. Psychol. Med. 39, 1667–1676. doi:DOI: 10.1017/S0033291709005431

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J., McCarthy, M.I., Ramos, E.M., Cardon, L.R., Chakravarti, A., Cho, J.H., Guttmacher, A.E., Kong, A., Kruglyak, L., Mardis, E., Rotimi, C.N., Slatkin, M., Valle, D., Whittemore, A.S., Boehnke, M., Clark, A.G., Eichler, E.E., Gibson, G., Haines, J.L., Mackay, T.F.C., McCarroll, S.A., Visscher, P.M., 2009. Finding the missing heritability of complex diseases. Nature 461, 747.

Mathers, C.D., Loncar, D., 2006. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med. 3, 2011–2030.

Mayer, E. a, 2011. Gut feelings: the emerging biology of gut-brain communication. Nat. Rev. Neurosci. 12, 453–66. doi:10.1038/nrn3071

McGrath, J., Saha, S., Chant, D., Welham, J., 2008. Schizophrenia: A Concise Overview of Incidence, Prevalence, and Mortality. Epidemiol. Rev. 30, 67–76. doi:10.1093/epirev/mxn001

Melo, A., Aguiar, R., Amorim, M., al, et, 2016. Congenital zika virus infection: Beyond neonatal microcephaly. JAMA Neurol. 73, 1407–1416.

Meyer, U., Feldon, J., 2012. To poly(I:C) or not to poly(I:C): Advancing preclinical schizophrenia research through the use of prenatal immune activation models. Neuropharmacology 62, 1308–1321. doi:https://doi.org/10.1016/j.neuropharm.2011.01.009

Miura, K., Niikawa, N., 2004. Do monochorionic dizygotic twins increase after pregnancy by assisted reproductive technology? J. Hum. Genet. 50, 1.

Mojtabai, R., 2011. National Trends in Mental Health Disability, 1997–2009. Am. J. Public Health 101, 2156–2163. doi:10.2105/AJPH.2011.300258

Mokhtari, M., Arfken, C., Boutros, N., 2013. The DEX/CRH test for major depression: A potentially useful diagnostic test. Psychiatry Res. 208, 131–139. doi:https://doi.org/10.1016/j.psychres.2012.09.032

Munafò, M.R., Brown, S.M., Hariri, A.R., 2008. Serotonin Transporter (5-HTTLPR) Genotype and Amygdala Activation: A Meta-Analysis. Biol. Psychiatry 63, 852–857. doi:10.1016/j.biopsych.2007.08.016

Murphy, D.L., Maile, M.S., Vogt, N.M., 2013. 5HTTLPR: White Knight or Dark Blight? ACS Chem. Neurosci. 4, 13–15.

Murray, R.M., 2010. Is schizophrenia a developmental disorder? J. R. Army Med. Corps 156, 150–153. doi:10.1038/eye.2010.181

Nanni, V., Uher, R., Danese, A., 2012. Childhood maltreatment predicts unfavorable course of illness and treatment outcome in depression: A meta-analysis. Am. J. Psychiatry 169, 141–151. doi:10.1176.appi.ajp.2011.11020335

O'Donnell, K.J., Bugge, A., Freeman, L., Khalife, N., Connor, T.G.O., Glover, V., 2012. Maternal prenatal anxiety and downregulation of placental 11 b -HSD2. Psychoneuroendocrinology 37, 818–826. doi:10.1016/j.psyneuen.2011.09.014

Oakley, R.H., Jewell, C.M., Yudt, M.R., Bofetiado, D.M., Cidlowski, J.A., 1999. The Dominant Negative Activity of the Human Glucocorticoid Receptor β Isoform: Specifity and mechanisms of action. J. Biol. Chem. 274, 27857–27866. doi:10.1074/jbc.274.39.27857

Oh, G., Wang, S.C., Pal, M., Chen, Z.F., Khare, T., Tochigi, M., Ng, C., Yang, Y.A., Kwan, A., Kaminsky, Z.A., Mill, J., Gunasinghe, C., Tackett, J.L., Gottesman, I.I., Willemsen, G., De Geus, E.J.C., Vink, J.M., Slagboom, P.E., Wray, N.R., Heath, A.C., Montgomery, G.W., Turecki, G., Martin, N.G., Boomsma, D.I., McGuffin, P., Kustra, R., Petronis, A., 2015. DNA modification study of major depressive disorder: Beyond locus-by-locus comparisons. Biol. Psychiatry. doi:10.1016/j.biopsych.2014.06.016

Palma-Gudiel, H., Córdova-Palomera, A., Eixarch, E., Deuschle, M., Fañanás, L., 2015a. Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: A meta-analysis. Epigenetics 10, 893–902. doi:10.1080/15592294.2015.1088630

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J.C., Fañanás, L., 2015b. Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review. Neurosci. Biobehav. Rev. 55, 520–535. doi:10.1016/j.neubiorev.2015.05.016

Palma-Gudiel, H., Córdova-Palomera, A., Tornador, C., Falcón, C., Bargalló, N., Deco, G., Fañanás, L., 2018. Increased methylation at an unexplored glucocorticoid responsive element within exon 1 D of NR3C1 gene is related to anxious-depressive disorders and decreased hippocampal connectivity. Eur. Neuropsychopharmacol. 28, 579–588. doi:10.1016/j.euroneuro.2018.03.015

Palma-Gudiel, H., Fañanás, L., 2017. An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR. Neurosci. Biobehav. Rev. 72, 190–209. doi:10.1016/j.neubiorev.2016.11.011

Palma-Gudiel, H., Peralta, V., Deuschle, M., Navarro, V., Fañanás, L., 2019. Epigenetics-by-sex interaction for somatization conferred by methylation at the promoter region of SLC6A4 gene. Prog. Neuropsychopharmacol. Biol. Psychiatry 89, 125–131. doi:10.1016/j.pnpbp.2018.09.002

Pariante, C.M., 2017. Why are depressed patients inflamed? A reflection on 20 years of research on depression, glucocorticoid resistance and inflammation. Eur. Neuropsychopharmacol. 554–559. doi:10.1016/j.euroneuro.2017.04.001

Patel, V., Saxena, S., Lund, C., Thornicroft, G., Baingana, F., Bolton, P., Chisholm, D., Collins, P.Y., Cooper, J.L., Eaton, J., Herrman, H., Herzallah, M.M., Huang, Y., Jordans, M.J.D., Kleinman, A., Medina-Mora, M.E., Morgan, E., Niaz, U., Omigbodun, O., Prince, M., Rahman, A., Saraceno, B., Sarkar, B.K., De Silva, M., Singh, I., Stein, D.J., Sunkel, C., UnÜtzer, Jü., 2018. The Lancet Commission on global mental health and sustainable development. Lancet 392, 1553–1598. doi:10.1016/S0140-6736(18)31612-X

Paus, T., Keshavan, M., Giedd, J.N., 2008. Why do many psychiatric disorders emerge during adolescence? Nat. Rev. Neurosci. 9, 947.

Pecins-Thompson, M., Brown, N.A., Bethea, C.L., 1998. Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. Mol. brain Res. 53, 120–9.

Peralta, V., Goldberg, X., Ribeiro, M., Sanchez-Torres, A.M., Fañanás, L., Cuesta, M.J., 2016. Familiality of Psychotic Disorders: A Polynosologic Study in Multiplex Families. Schizophr. Bull. 42, 975–983.

Plieger, T., Felten, A., Splittgerber, H., Duke, É., Reuter, M., 2018. The role of genetic variation in the glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2) in the association between cortisol response and cognition under acute stress. Psychoneuroendocrinology 87, 173–180. doi:https://doi.org/10.1016/j.psyneuen.2017.10.020

Polderman, T.J.C., Benyamin, B., de Leeuw, C.A., Sullivan, P.F., van Bochoven, A., Visscher, P.M., Posthuma, D., 2015. Meta-analysis of the heritability of human traits based on fifty years of twin studies. Nat. Genet. 47, 702.

Raison, C.L., Miller, A.H., 2017. Pathogen – Host Defense in the Evolution of Depression: Insights into Epidemiology, Genetics, Bioregional Differences and Female Preponderance. Neuropsychopharmacology 42, 5–27. doi:10.1038/npp.2016.194

Raznahan, A., Greenstein, D., Lee, N.R., Clasen, L.S., Giedd, J.N., 2012. Prenatal growth in humans and postnatal brain maturation into late adolescence. Proc. Natl. Acad. Sci. 109, 11366 LP – 11371. doi:10.1073/pnas.1203350109

Reichenberg, A., Caspi, A., Harrington, H., Houts, R., Keefe, R.S.E., Murray, R.M., Poulton, R., Moffitt, T.E., 2010. Static and Dynamic Cognitive Deficits in Childhood Preceding Adult Schizophrenia: A 30-Year Study. Am. J. Psychiatry 167, 160–169. doi:10.1176/appi.ajp.2009.09040574

Ressler, K.J., Mayberg, H.S., 2007. Targeting abnormal neural circuits in mood and anxiety disorders: From the laboratory to the clinic. Nat. Neurosci. doi:10.1038/nn1944

Riem, M.M.E., Alink, L.R.A., Out, D., Van Ijzendoorn, M.H., Bakermans-Kranenburg, M.J., 2015. Beating the brain about abuse: Empirical and meta-analytic studies of the association between maltreatment and hippocampal volume across childhood and adolescence. Dev. Psychopathol. 27, 507–520. doi:DOI: 10.1017/S0954579415000127

Riggs, A., 1975. X inactivation, differentiation, and DNA methylation. Cytogenet. Cell

Genet. 14, 9–25.
Robinson, R., Lahti-Pulkkinen, M., Heinonen, K., Reynolds, R.M., Räikkönen, K., 2018. Fetal programming of neuropsychiatric disorders by maternal pregnancy depression: a systematic mini review. Pediatr. Res. doi:10.1038/s41390-018-0173-y
Romanoski, C.E., Glass, C.K., Stunnenberg, H.G., Wilson, L., Almouzni, G., 2015. Roadmap for regulation. Nature 518, 314.
Rossi, A.C., D'Addario, V., 2008. Laser therapy and serial amnioreduction as treatment for twin-twin transfusion syndrome: a metaanalysis and review of literature. Am. J. Obstet. Gynecol. 198, 147–152. doi:10.1016/j.ajog.2007.09.043
Roy, A., Gorodetsky, E., Yuan, Q., Goldman, D., Enoch, M.-A., 2010. Interaction of FKBP5, a Stress-Related Gene, with Childhood Trauma Increases the Risk for Attempting Suicide. Neuropsychopharmacology 35, 1674.
Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014. Biological insights from 108 schizophrenia-associated genetic loci. Nature 511, 421–427.
Serpeloni, F., Radtke, K., de Assis, S.G., Henning, F., Nätt, D., Elbert, T., 2017. Grandmaternal stress during pregnancy and DNA methylation of the third generation: an epigenome-wide association study. Transl. Psychiatry 7, e1202.
Simonti, C.N., Vernot, B., Bastarache, L., Bottinger, E., Carrell, D.S., Chisholm, R.L., Crosslin, D.R., Hebbring, S.J., Jarvik, G.P., Kullo, I.J., Li, R., Pathak, J., 2016. The phenotypic legacy of admixture between modern humans and Neandertals. Science (80-.). 351, 737–742.
Srinivasan, S., Bettella, F., Mattingsdal, M., Wang, Y., Witoelar, A., Schork, A.J., Thompson, W.K., Zuber, V., Schizophrenia Working Group of the Psychiatric Genomics Consortium, Winsvold, B.S., Zwart, J., Collier, D.A., Desikan, R.S., Melle, I., Werge, T., Dale, A.M., Djurovic, S., Andreassen, O.A., 2016. Genetic Markers of Human Evolution Are Enriched in Schizophrenia. Biol. Psychiatry 80, 284–292. doi:10.1016/j.biopsych.2015.10.009
Steinman, G., 2000. The mechanism initiating and controlling monozygotic twinning in humans remains to be elucidated. Twin Res.
Sugawara, H., Iwamoto, K., Bundo, M., Ueda, J., Miyauchi, T., Komori, a, Kazuno, a, Adati, N., Kusumi, I., Okazaki, Y., Ishigooka, J., Kojima, T., Kato, T., 2011. Hypermethylation of serotonin transporter gene in bipolar disorder detected by epigenome analysis of discordant monozygotic twins. Transl. Psychiatry 1, e24. doi:10.1038/tp.2011.26
Sullivan, P.F., Daly, M.J., O'Donovan, M., 2012. Genetic architectures of psychiatric disorders: the emerging picture and its implications. Nat. Rev. Genet. 13, 537–51. doi:10.1038/nrg3240
Sullivan, P.F., Neale, M.C., Ph, D., Kendler, K.S., 2000. Genetic Epidemiology of Major Depression : Review and Meta-Analysis. Am. J. Psychiatry 1552–1562.
Swartz, J.R., Knodt, A.R., Radtke, S.R., Hariri, A.R., 2015. A Neural Biomarker of

Psychological Vulnerability to Future Life Stress. Neuron 85, 505–511. doi:10.1016/j.neuron.2014.12.055

Szczepankiewicz, A., Leszczyńska-Rodziewicz, A., Pawlak, J., Rajewska-Rager, A., Dmitrzak-Weglarz, M., Wilkosc, M., Skibinska, M., Hauser, J., 2011. Glucocorticoid receptor polymorphism is associated with major depression and predominance of depression in the course of bipolar disorder. J. Affect. Disord. 134, 138–144. doi:https://doi.org/10.1016/j.jad.2011.06.020

Szyf, M., 2013. Lamarck revisited: epigenetic inheritance of ancestral odor fear conditioning. Nat Neurosci 17, 2–4. doi:10.1038/nn.3603

Taylor, M.J., Sen, S., Bhagwagar, Z., 2010. Antidepressant Response and the Serotonin Transporter Gene-Linked Polymorphic Region. Biol. Psychiatry 68, 536–543. doi:10.1016/j.biopsych.2010.04.034

Teicher, M.H., Samson, J.A., 2013. Childhood Maltreatment and Psychopathology: A Case for Ecophenotypic Variants as Clinically and Neurobiologically Distinct Subtypes. Am. J. Psychiatry 170, 1114–1133. doi:10.1176/appi.ajp.2013.12070957

Teicher, M.H., Samson, J.A., Anderson, C.M., Ohashi, K., 2016. The effects of childhood maltreatment on brain structure, function and connectivity. Nat. Rev. Neurosci. 17, 652.

The International Human Genome Mapping Consortium, McPherson, J.D., Marra, M., Hillier, L., Waterston, R.H., Chinwalla, A., Wallis, J., Sekhon, M., Wylie, K., Mardis, E.R., Wilson, R.K., Fulton, R., Kucaba, T.A., Wagner-McPherson, C., Barbazuk, W.B., Gregory, S.G., Humphray, S.J., French, L., Evans, R.S., Bethel, G., Whittaker, A., Holden, J.L., McCann, O.T., Dunham, A., Soderlund, C., Scott, C.E., Bentley, D.R., Schuler, G., Chen, H.-C., Jang, W., Green, E.D., Idol, J.R., Maduro, V.V.B., Montgomery, K.T., Lee, E., Miller, A., Emerling, S., Kucherlapati, R., Gibbs, R., Scherer, S., Gorrell, J.H., Sodergren, E., Clerc-Blankenburg, K., Tabor, P., Naylor, S., Garcia, D., de Jong, P.J., Catanese, J.J., Nowak, N., Osoegawa, K., Qin, S., Rowen, L., Madan, A., Dors, M., Hood, L., Trask, B., Friedman, C., Massa, H., Cheung, V.G., Kirsch, I.R., Reid, T., Yonescu, R., Weissenbach, J., Bruls, T., Heilig, R., Branscomb, E., Olsen, A., Doggett, N., Cheng, J.-F., Hawkins, T., Myers, R.M., Shang, J., Ramirez, L., Schmutz, J., Velasquez, O., Dixon, K., Stone, N.E., Cox, D.R., Haussler, D., Kent, W.J., Furey, T., Rogic, S., Kennedy, S., Jones, S., Rosenthal, A., Wen, G., Schilhabel, M., Gloeckner, G., Nyakatura, G., Siebert, R., Schlegelberger, B., Korenberg, J., Chen, X.-N., Fujiyama, A., Hattori, M., Toyoda, A., Yada, T., Park, H.-S., Sakaki, Y., Shimizu, N., Asakawa, S., Kawasaki, K., Sasaki, T., Shintani, A., Shimizu, A., Shibuya, K., Kudoh, J., Minoshima, S., Ramser, J., Seranski, P., Hoff, C., Poustka, A., Reinhardt, R., Lehrach, H., 2001. A physical map of the human genome. Nature 409, 934.

Turner, J.D., Muller, C.P., 2005. Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. J. Mol. Endocrinol. 35, 283–92.

Ursini, G., Punzi, G., Chen, Q., Marenco, S., Robinson, J.F., Porcelli, A., Hamilton, E.G., Mitjans, M., Maddalena, G., Begemann, M., Seidel, J., Yanamori, H., Jaffe, A.E., Berman, K.F., Egan, M.F., Straub, R.E., Colantuoni, C., Blasi, G., Hashimoto, R., Rujescu, D., Ehrenreich, H., Bertolino, A., Weinberger, D.R., 2018. Convergence of placenta biology and genetic risk for schizophrenia. Nat. Med. 1–10. doi:10.1038/s41591-018-0021-y

Valsky, D. V., Eixarch, E., Martinez, J.M., Crispi, F., Gratacós, E., 2010. Selective intrauterine growth restriction in monochorionic twins: Pathophysiology, diagnostic approach and management dilemmas. Semin. Fetal Neonatal Med. 15, 342–348. doi:10.1016/j.siny.2010.07.002

Van Baak, T.E., Coarfa, C., Dugué, P.-A., Fiorito, G., Laritsky, E., Baker, M.S., Kessler, N.J., Dong, J., Duryea, J.D., Silver, M.J., Saffari, A., Prentice, A.M., Moore, S.E., Ghantous, A., Routledge, M.N., Gong, Y.Y., Herceg, Z., Vineis, P., Severi, G., Hopper, J.L., Southey, M.C., Giles, G.G., Milne, R.L., Waterland, R.A., 2018. Epigenetic supersimilarity of monozygotic twin pairs. Genome Biol. 19, 2. doi:10.1186/s13059-017-1374-0

van Dongen, J., Boomsma, D.I., 2013. The evolutionary paradox and the missing heritability of schizophrenia. Am. J. Med. Genet. Part B Neuropsychiatr. Genet. 162, 122–136. doi:10.1002/ajmg.b.32135

van Rossum, E.F.C., Binder, E.B., Majer, M., Koper, J.W., Ising, M., Modell, S., Salyakina, D., Lamberts, S.W.J., Holsboer, F., 2006. Polymorphisms of the glucocorticoid receptor gene and major depression. Biol. Psychiatry 59, 681–8.

Vos, T., Barber, R.M., Bell, B., Bertozzi-Villa, A., Biryukov, S., Bolliger, I., Charlson, F., Davis, A., Degenhardt, L., Dicker, D., Duan, L., Erskine, H., Feigin, V.L., Ferrari, A.J., Fitzmaurice, C., Fleming, T., Graetz, N., Guinovart, C., Haagsma, J., Hansen, G.M., Hanson, S.W., Heuton, K.R., Higashi, H., Kassebaum, N., Kyu, H., Laurie, E., Liang, X., Lofgren, K., Lozano, R., MacIntyre, M.F., Moradi-Lakeh, M., Naghavi, M., Nguyen, G., Odell, S., Ortblad, K., Roberts, D.A., Roth, G.A., Sandar, L., Serina, P.T., Stanaway, J.D., Steiner, C., Thomas, B., Vollset, S.E., Whiteford, H., Wolock, T.M., Ye, P., Zhou, M., Ãvila, M.A., Aasvang, G.M., Abbafati, C., Ozgoren, A.A., Abd-Allah, F., Aziz, M.I.A., Abera, S.F., Aboyans, V., Abraham, J.P., Abraham, B., Abubakar, I., Abu-Raddad, L.J., Abu-Rmeileh, N.M.E., Aburto, T.C., Achoki, T., Ackerman, I.N., Adelekan, A., Ademi, Z., Adou, A.K., Adsuar, J.C., Arnlov, J., Agardh, E.E., Al Khabouri, M.J., Alam, S.S., Alasfoor, D., Albittar, M.I., Alegretti, M.A., Aleman, A. V, Alemu, Z.A., Alfonso-Cristancho, R., Alhabib, S., Ali, R., Alla, F., Allebeck, P., Allen, P.J., AlMazroa, M.A., Alsharif, U., Alvarez, E., Alvis-Guzman, N., Ameli, O., Amini, H., Ammar, W., Anderson, B.O., Anderson, H.R., Antonio, C.A.T., Anwari, P., Apfel, H., Arsenijevic, V.S.A., Artaman, A., Asghar, R.J., Assadi, R., Atkins, L.S., Atkinson, C., Badawi, A., Bahit, M.C., Bakfalouni, T., Balakrishnan, K., Balalla, S., Banerjee, A., Barker-Collo, S.L., Barquera, S., Barregard, L., Barrero, L.H., Basu, S., Basu, A., Baxter, A., Beardsley, J., Bedi, N., Beghi, E., Bekele, T., Bell, M.L.,

Benjet, C., Bennett, D.A., Bensenor, I.M., Benzian, H., Bernabe, E., Beyene, T.J., Bhala, N., Bhalla, A., Bhutta, Z., Bienhoff, K., Bikbov, B., Abdulhak, A. Bin, Blore, J.D., Blyth, F.M., Bohensky, M.A., Basara, B.B., Borges, G., Bornstein, N.M., Bose, D., Boufous, S., Bourne, R.R., Boyers, L.N., Brainin, M., Brauer, M., Brayne, C.E.G., Brazinova, A., Breitborde, N.J.K., Brenner, H., Briggs, A.D.M., Brooks, P.M., Brown, J., Brugha, T.S., Buchbinder, R., Buckle, G.C., Bukhman, G., Bulloch, A.G., Burch, M., Burnett, R., Cardenas, R., Cabral, N.L., Nonato, I.R.C., Campuzano, J.C., Carapetis, J.R., Carpenter, D.O., Caso, V., Castaneda-Orjuela, C.A., Catala-Lopez, F., Chadha, V.K., Chang, J.-C., Chen, H., Chen, W., Chiang, P.P., Chimed-Ochir, O., Chowdhury, R., Christensen, H., Christophi, C.A., Chugh, S.S., Cirillo, M., Coggeshall, M., Cohen, A., Colistro, V., Colquhoun, S.M., Contreras, A.G., Cooper, L.T., Cooper, C., Cooperrider, K., Coresh, J., Cortinovis, M., Criqui, M.H., Crump, J.A., Cuevas-Nasu, L., Dandona, R., Dandona, L., Dansereau, E., Dantes, H.G., Dargan, P.I., Davey, G., Davitoiu, D. V, Dayama, A., De la Cruz-Gongora, V., de la Vega, S.F., De Leo, D., del Pozo-Cruz, B., Dellavalle, R.P., Deribe, K., Derrett, S., Des Jarlais, D.C., Dessalegn, M., DeVeber, G.A., Dharmaratne, S.D., Diaz-Torne, C., Ding, E.L., Dokova, K., Dorsey, E.R., Driscoll, T.R., Duber, H., Durrani, A.M., Edmond, K.M., Ellenbogen, R.G., Endres, M., Ermakov, S.P., Eshrati, B., Esteghamati, A., Estep, K., Fahimi, S., Farzadfar, F., Fay, D.F.J., Felson, D.T., Fereshtehnejad, S.-M., Fernandes, J.G., Ferri, C.P., Flaxman, A., Foigt, N., Foreman, K.J., Fowkes, F.G.R., Franklin, R.C., Furst, T., Futran, N.D., Gabbe, B.J., Gankpe, F.G., Garcia-Guerra, F.A., Geleijnse, J.M., Gessner, B.D., Gibney, K.B., Gillum, R.F., Ginawi, I.A., Giroud, M., Giussani, G., Goenka, S., Goginashvili, K., Gona, P., de Cosio, T.G., Gosselin, R.A., Gotay, C.C., Goto, A., Gouda, H.N., Guerrant, R. l, Gugnani, H.C., Gunnell, D., Gupta, R., Gupta, R., Gutierrez, R.A., Hafezi-Nejad, N., Hagan, H., Halasa, Y., Hamadeh, R.R., Hamavid, H., Hammami, M., Hankey, G.J., Hao, Y., Harb, H.L., Haro, J.M., Havmoeller, R., Hay, R.J., Hay, S., Hedayati, M.T., Pi, I.B.H., Heydarpour, P., Hijar, M., Hoek, H.W., Hoffman, H.J., Hornberger, J.C., Hosgood, H.D., Hossain, M., Hotez, P.J., Hoy, D.G., Hsairi, M., Hu, H., Hu, G., Huang, J.J., Huang, C., Huiart, L., Husseini, A., Iannarone, M., Iburg, K.M., Innos, K., Inoue, M., Jacobsen, K.H., Jassal, S.K., Jeemon, P., Jensen, P.N., Jha, V., Jiang, G., Jiang, Y., Jonas, J.B., Joseph, J., Juel, K., Kan, H., Karch, A., Karimkhani, C., Karthikeyan, G., Katz, R., Kaul, A., Kawakami, N., Kazi, D.S., Kemp, A.H., Kengne, A.P., Khader, Y.S., Khalifa, S.E.A.H., Khan, E.A., Khan, G., Khang, Y.-H., Khonelidze, I., Kieling, C., Kim, D., Kim, S., Kimokoti, R.W., Kinfu, Y., Kinge, J.M., Kissela, B.M., Kivipelto, M., Knibbs, L., Knudsen, A.K., Kokubo, Y., Kosen, S., Kramer, A., Kravchenko, M., Krishnamurthi, R. V, Krishnaswami, S., Defo, B.K., Bicer, B.K., Kuipers, E.J., Kulkarni, V.S., Kumar, K., Kumar, G.A., Kwan, G.F., Lai, T., Lalloo, R., Lam, H., Lan, Q., Lansingh, V.C., Larson, H., Larsson, A., Lawrynowicz, A.E.B., Leasher, J.L., Lee, J.-T., Leigh, J., Leung, R., Levi, M., Li, B., Li, Y., Li, Y., Liang, J., Lim, S., Lin, H.-H., Lind, M., Lindsay, M.P., Lipshultz, S.E., Liu, S., Lloyd, B.K., Ohno, S.L., Logroscino, G.,

Looker, K.J., Lopez, A.D., Lopez-Olmedo, N., Lortet-Tieulent, J., Lotufo, P.A., Low, N., Lucas, R.M., Lunevicius, R., Lyons, R.A., Ma, J., Ma, S., Mackay, M.T., Majdan, M., Malekzadeh, R., Mapoma, C.C., Marcenes, W., March, L.M., Margono, C., Marks, G.B., Marzan, M.B., Masci, J.R., Mason-Jones, A.J., Matzopoulos, R.G., Mayosi, B.M., Mazorodze, T.T., McGill, N.W., McGrath, J.J., McKee, M., McLain, A., McMahon, B.J., Meaney, P.A., Mehndiratta, M.M., Mejia-Rodriguez, F., Mekonnen, W., Melaku, Y.A., Meltzer, M., Memish, Z.A., Mensah, G., Meretoja, A., Mhimbira, F.A., Micha, R., Miller, T.R., Mills, E.J., Mitchell, P.B., Mock, C.N., Moffitt, T.E., Ibrahim, N.M., Mohammad, K.A., Mokdad, A.H., Mola, G.L., Monasta, L., Montico, M., Montine, T.J., Moore, A.R., Moran, A.E., Morawska, L., Mori, R., Moschandreas, J., Moturi, W.N., Moyer, M., Mozaffarian, D., Mueller, U.O., Mukaigawara, M., Murdoch, M.E., Murray, J., Murthy, K.S., Naghavi, P., Nahas, Z., Naheed, A., Naidoo, K.S., Naldi, L., Nand, D., Nangia, V., Narayan, K.M.V., Nash, D., Nejjari, C., Neupane, S.P., Newman, L.M., Newton, C.R., Ng, M., Ngalesoni, F.N., Nhung, N.T., Nisar, M.I., Nolte, S., Norheim, O.F., Norman, R.E., Norrving, B., Nyakarahuka, L., Oh, I.H., Ohkubo, T., Omer, S.B., Opio, J.N., Ortiz, A., Pandian, J.D., Panelo, C.I.A., Papachristou, C., Park, E.-K., Parry, C.D., Caicedo, A.J.P., Patten, S.B., Paul, V.K., Pavlin, B.I., Pearce, N., Pedraza, L.S., Pellegrini, C.A., Pereira, D.M., Perez-Ruiz, F.P., Perico, N., Pervaiz, A., Pesudovs, K., Peterson, C.B., Petzold, M., Phillips, M.R., Phillips, D., Phillips, B., Piel, F.B., Plass, D., Poenaru, D., Polanczyk, G. V, Polinder, S., Pope, C.A., Popova, S., Poulton, R.G., Pourmalek, F., Prabhakaran, D., Prasad, N.M., Qato, D., Quistberg, D.A., Rafay, A., Rahimi, K., Rahimi-Movaghar, V., Rahman, S. ur, Raju, M., Rakovac, I., Rana, S.M., Razavi, H., Refaat, A., Rehm, J., Remuzzi, G., Resnikoff, S., Ribeiro, A.L., Riccio, P.M., Richardson, L., Richardus, J.H., Riederer, A.M., Robinson, M., Roca, A., Rodriguez, A., Rojas-Rueda, D., Ronfani, L., Rothenbacher, D., Roy, N., Ruhago, G.M., Sabin, N., Sacco, R.L., Ksoreide, K., Saha, S., Sahathevan, R., Sahraian, M.A., Sampson, U., Sanabria, J.R., Sanchez-Riera, L., Santos, I.S., Satpathy, M., Saunders, J.E., Sawhney, M., Saylan, M.I., Scarborough, P., Schoettker, B., Schneider, I.J.C., Schwebel, D.C., Scott, J.G., Seedat, S., Sepanlou, S.G., Serdar, B., Servan-Mori, E.E., Shackelford, K., Shaheen, A., Shahraz, S., Levy, T.S., Shangguan, S., She, J., Sheikhbahaei, S., Shepard, D.S., Shi, P., Shibuya, K., Shinohara, Y., Shiri, R., Shishani, K., Shiue, I., Shrime, M.G., Sigfusdottir, I.D., Silberberg, D.H., Simard, E.P., Sindi, S., Singh, J.A., Singh, L., Skirbekk, V., Sliwa, K., Soljak, M., Soneji, S., Soshnikov, S.S., Speyer, P., Sposato, L.A., Sreeramareddy, C.T., Stoeckl, H., Stathopoulou, V.K., Steckling, N., Stein, M.B., Stein, D.J., Steiner, T.J., Stewart, A., Stork, E., Stovner, L.J., Stroumpoulis, K., Sturua, L., Sunguya, B.F., Swaroop, M., Sykes, B.L., Tabb, K.M., Takahashi, K., Tan, F., Tandon, N., Tanne, D., Tanner, M., Tavakkoli, M., Taylor, H.R., Te Ao, B.J., Temesgen, A.M., Have, M. Ten, Tenkorang, E.Y., Terkawi, A.S., Theadom, A.M., Thomas, E., Thorne-Lyman, A.L., Thrift, A.G., Tleyjeh, I.M., Tonelli, M., Topouzis, F., Towbin, J.A., Toyoshima, H., Traebert, J., Tran, B.X., Trasande, L.,

Trillini, M., Truelsen, T., Trujillo, U., Tsilimbaris, M., Tuzcu, E.M., Ukwaja, K.N., Undurraga, E.A., Uzun, S.B., van Brakel, W.H., van de Vijver, S., Dingenen, R. Van, van Gool, C.H., Varakin, Y.Y., Vasankari, T.J., Vavilala, M.S., Veerman, L.J., Velasquez-Melendez, G., Venketasubramanian, N., Vijayakumar, L., Villalpando, S., Violante, F.S., Vlassov, V. V, Waller, S., Wallin, M.T., Wan, X., Wang, L., Wang, J., Wang, Y., Warouw, T.S., Weichenthal, S., Weiderpass, E., Weintraub, R.G., Werdecker, A., Wessells, K.R.R., Westerman, R., Wilkinson, J.D., Williams, H.C., Williams, T.N., Woldeyohannes, S.M., Wolfe, C.D.A., Wong, J.Q., Wong, H., Woolf, A.D., Wright, J.L., Wurtz, B., Xu, G., Yang, G., Yano, Y., Yenesew, M.A., Yentur, G.K., Yip, P., Yonemoto, N., Yoon, S.-J., Younis, M., Yu, C., Kim, K.Y., Zaki, M.E.S., Zhang, Y., Zhao, Z., Zhao, Y., Zhu, J., Zonies, D., Zunt, J.R., Salomon, J.A., Murray, C.J.L., 2015. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 386, 743–800. doi:10.1016/S0140-6736(15)60692-4

Waddington, C.H., 2012. The Epigenotype. Int. J. Epidemiol. 41, 10–13.

Weaver, I.C.G., Cervoni, N., Champagne, F. a, D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–54.

Weinberger, D.R., 1996. On the Plausibility of "The Neurodevelopmental Hypothesis" of Schizophrenia. Neuropsychopharmacology 14, 1S–11S.

Welberg, L. a M., Thrivikraman, K. V, Plotsky, P.M., 2005. Chronic maternal stress inhibits the capacity to up-regulate placental 11beta-hydroxysteroid dehydrogenase type 2 activity. J. Endocrinol. 186, R7–R12. doi:10.1677/joe.1.06374

Wen, Z., Song, H., Ming, G., 2017. How does Zika virus cause microcephaly? Genes Dev. 31 , 849–861. doi:10.1101/gad.298216.117

Wojcik, W., Lee, W., Colman, I., Hardy, R., Hotopf, M., 2013. Foetal origins of depression? A systematic review and meta-analysis of low birth weight and later depression. Psychol. Med. 43, 1–12. doi:DOI: 10.1017/S0033291712000682

Wolf, E.J., Logue, M.W., Morrison, F.G., Wilcox, E.S., Stone, A., Schichman, S.A., McGlinchey, R.E., Milberg, W.P., Miller, M.W., 2018. Posttraumatic psychopathology and the pace of the epigenetic clock: a longitudinal investigation. Psychol. Med. 1–10. doi:DOI: 10.1017/S0033291718001411

World Health Organization, 2017. Depression and other common mental disorders, global health estimates.

Wray, N.R., Ripke, S., Mattheisen, M., Trzaskowski, M., Byrne, E.M., Abdellaoui, A., Adams, M.J., Agerbo, E., Air, T.M., Andlauer, T.M.F., Bacanu, S.-A., Bækvad-Hansen, M., Beekman, A.F.T., Bigdeli, T.B., Binder, E.B., Blackwood, D.R.H., Bryois, J., Buttenschøn, H.N., Bybjerg-Grauholm, J., Cai, N., Castelao, E., Christensen, J.H., Clarke, T.-K., Coleman, J.I.R., Colodro-Conde, L., Couvy-

Duchesne, B., Craddock, N., Crawford, G.E., Crowley, C.A., Dashti, H.S., Davies, G., Deary, I.J., Degenhardt, F., Derks, E.M., Direk, N., Dolan, C. V, Dunn, E.C., Eley, T.C., Eriksson, N., Escott-Price, V., Kiadeh, F.H.F., Finucane, H.K., Forstner, A.J., Frank, J., Gaspar, H.A., Gill, M., Giusti-Rodríguez, P., Goes, F.S., Gordon, S.D., Grove, J., Hall, L.S., Hannon, E., Hansen, C.S., Hansen, T.F., Herms, S., Hickie, I.B., Hoffmann, P., Homuth, G., Horn, C., Hottenga, J.-J., Hougaard, D.M., Hu, M., Hyde, C.L., Ising, M., Jansen, R., Jin, F., Jorgenson, E., Knowles, J.A., Kohane, I.S., Kraft, J., Kretzschmar, W.W., Krogh, J., Kutalik, Z., Lane, J.M., Li, Y., Li, Y., Lind, P.A., Liu, X., Lu, L., MacIntyre, D.J., MacKinnon, D.F., Maier, R.M., Maier, W., Marchini, J., Mbarek, H., McGrath, P., McGuffin, P., Medland, S.E., Mehta, D., Middeldorp, C.M., Mihailov, E., Milaneschi, Y., Milani, L., Mill, J., Mondimore, F.M., Montgomery, G.W., Mostafavi, S., Mullins, N., Nauck, M., Ng, B., Nivard, M.G., Nyholt, D.R., O'Reilly, P.F., Oskarsson, H., Owen, M.J., Painter, J.N., Pedersen, C.B., Pedersen, M.G., Peterson, R.E., Pettersson, E., Peyrot, W.J., Pistis, G., Posthuma, D., Purcell, S.M., Quiroz, J.A., Qvist, P., Rice, J.P., Riley, B.P., Rivera, M., Saeed Mirza, S., Saxena, R., Schoevers, R., Schulte, E.C., Shen, L., Shi, J., Shyn, S.I., Sigurdsson, E., Sinnamon, G.B.C., Smit, J.H., Smith, D.J., Stefansson, H., Steinberg, S., Stockmeier, C.A., Streit, F., Strohmaier, J., Tansey, K.E., Teismann, H., Teumer, A., Thompson, W., Thomson, P.A., Thorgeirsson, T.E., Tian, C., Traylor, M., Treutlein, J., Trubetskoy, V., Uitterlinden, A.G., Umbricht, D., Van der Auwera, S., van Hemert, A.M., Viktorin, A., Visscher, P.M., Wang, Y., Webb, B.T., Weinsheimer, S.M., Wellmann, J., Willemsen, G., Witt, S.H., Wu, Y., Xi, H.S., Yang, J., Zhang, F., Arolt, V., Baune, B.T., Berger, K., Boomsma, D.I., Cichon, S., Dannlowski, U., de Geus, E.C.J., DePaulo, J.R., Domenici, E., Domschke, K., Esko, T., Grabe, H.J., Hamilton, S.P., Hayward, C., Heath, A.C., Hinds, D.A., Kendler, K.S., Kloiber, S., Lewis, G., Li, Q.S., Lucae, S., Madden, P.F.A., Magnusson, P.K., Martin, N.G., McIntosh, A.M., Metspalu, A., Mors, O., Mortensen, P.B., Müller-Myhsok, B., Nordentoft, M., Nöthen, M.M., O'Donovan, M.C., Paciga, S.A., Pedersen, N.L., Penninx, B.W.J.H., Perlis, R.H., Porteous, D.J., Potash, J.B., Preisig, M., Rietschel, M., Schaefer, C., Schulze, T.G., Smoller, J.W., Stefansson, K., Tiemeier, H., Uher, R., Völzke, H., Weissman, M.M., Werge, T., Winslow, A.R., Lewis, C.M., Levinson, D.F., Breen, G., Børglum, A.D., Sullivan, P.F., eQTLGen, 23andMe, Consortium, the M.D.D.W.G. of the P.G., 2018. Genome-wide association analyses identify 44 risk variants and refine the genetic architecture of major depression. Nat. Genet. 50, 668–681. doi:10.1038/s41588-018-0090-3

Wu, X., Zhang, Y., 2017. TET-mediated active DNA demethylation: mechanism, function and beyond. Nat. Rev. Genet. 18, 517.

Xu, H., Wang, F., Liu, Y., Yu, Y., Gelernter, J., Zhang, H., 2014. Sex-biased methylome and transcriptome in human prefrontal cortex. Hum. Mol. Genet. 23, 1260–1270.

Yehuda, R., Daskalakis, N.P., Bierer, L.M., Bader, H.N., Klengel, T., Holsboer, F.,

Binder, E.B., 2016. Holocaust Exposure Induced Intergenerational Effects on FKBP5 Methylation. Biol. Psychiatry 80, 372–380. doi:10.1016/j.biopsych.2015.08.005

Zannas, A.S., Arloth, J., Carrillo-Roa, T., Iurato, S., Röh, S., Ressler, K.J., Nemeroff, C.B., Smith, A.K., Bradley, B., Heim, C., Menke, A., Lange, J.F., Brückl, T., Ising, M., Wray, N.R., Erhardt, A., Binder, E.B., Mehta, D., 2015. Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. Genome Biol. 16, 1–12. doi:10.1186/s13059-015-0828-5

Zimmerman, M., Martinez, J., Attiullah, N., Friedman, M., Toba, C., Boerescu, D.A., 2012. Why do some depressed outpatients who are not in remission according to the Hamilton Depression Rating Scale nonetheless consider themselves to be in remission? Depress. Anxiety 29, 891–895. doi:10.1002/da.21987

Zimmermann, P., Brückl, T., Nocon, A., Pfister, H., Binder, E.B., Uhr, M., Lieb, R., Moffitt, T.E., Caspi, A., Holsboer, F., Ising, M., 2011. Interaction of FKBP5 Gene Variants and Adverse Life Events in Predicting Depression Onset: Results From a 10-Year Prospective Community Study. Am. J. Psychiatry 168, 1107–1116. doi:10.1176/appi.ajp.2011.10111577

Zobel, A., Jessen, F., von Widdern, O., Schuhmacher, A., Höfels, S., Metten, M., Rietschel, M., Scheef, L., Block, W., Becker, T., Schild, H.H., Maier, W., Schwab, S.G., 2008. Unipolar depression and hippocampal volume: impact of DNA sequence variants of the glucocorticoid receptor gene. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 147B, 836–43. doi:10.1002/ajmg.b.30709

Zorn, J. V, Schür, R.R., Boks, M.P., Kahn, R.S., Joëls, M., Vinkers, C.H., 2017. Cortisol stress reactivity across psychiatric disorders: A systematic review and meta-analysis. Psychoneuroendocrinology 77, 25–36. doi:https://doi.org/10.1016/j.psyneuen.2016.11.036

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