Genomic impact of environmental risk factors for psychosis

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De genomische impact van risico factoren voor psychose

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction and outline of thesis

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Environmental factors contribute to vulnerability of psychosis

Approximately 3% of the population world-wide suffers from psychotic disorders, such as schizophrenia and bipolar disorder. These psychotic disorders comprise a heterogeneous group of patients and the etiology of these disorders remains largely unknown. The estimated heritability of schizophrenia is approximately 65 percent (Lichtenstein *et al*, 2009; Wray and Gottesman, 2012) and of bipolar disorders 58 percent (Song *et al*, 2015). Due to the high heritability of psychosis, earlier studies mainly focused on identifying specific genes that contribute to the etiology of these disorders. Though successfully identified genetic factors substantially contribute to our understanding of the etiology of these two disorders, it cannot fully explain the disease process and progression. Despite advances due to large scale genomic studies, epidemiological studies show that multiple environmental exposures are also strongly and consistently associated with psychosis (Marconi et al. 2016; Varese et al. 2012). Clearly environmental exposures also play a prominent role in the vulnerability to develop psychosis.

For decades a dominant hypothesis in the field is that detrimental environmental factors impact on neurodevelopment, and subsequently lead to the onset of psychotic diseases. This neurodevelopmental hypothesis led towards awareness of factors that can affect early neurodevelopment during pregnancy (Vohr *et al*, 2017), such as nutritional deficiencies, maternal infections etc. However, as the brain continues to develop postnatally, environmental factors, such as childhood abuse, can also contribute to developing psychosis later in life (John *et al*, 2017; Krebs *et al*, 2017). Moreover, during adolescence, exposure to cannabis is associated with mental illness in general and schizophrenia in particular (Galler *et al*, 2017).

Exposure to detrimental environmental factors, such as those described above, clearly increase the risk of psychosis. However, not all persons who are exposed to an adverse environment will develop psychosis. Therefore, it is plausible that individual genetic factors also contribute to the vulnerability to the diseases. These individual genetic factors interplay with the environmental exposures, resulting in a gene–environment interaction.

The candidate gene approach and genome-wide studies of gene environment interactions

To determine the gene-environment interaction involved in the etiology of psychosis, researchers initially focused on family history of severe mental disorders. For example, it is clear from the Genetic Risk and Outcome in Psychosis (GROUP) study that individuals from a family with a history of psychotic illnesses are more sensitive to develop psychosis upon cannabis use (Van Winkel *et al*, 2011). However, since the family history of mental disorders is not always known, this result could also reflect a gene-environment correlation or interaction between multiple genetic factors (Han *et al*, 2016). Researchers later included unrelated individuals to limit this source of bias (Sullivan *et al*, 2018). Based on prior knowledge, the interactions of candidate genes that were identified to be involved in psychotic disorders with detrimental environment factors have been studied. This approach successfully identified environments and genes that influence the risk of psychosis together. For example, *FKBP5* gene A-allele carriers are more vulnerable to psychosis-inducing effects of childhood trauma (Collip *et al*, 2013), and this outcome has been successfully replicated in another study (Alemany *et al*, 2016). Moreover, by screening polymorphisms from functionally defined genes, a SNP in *AKT1* was found that is linked to the vulnerability of the psychosis-inducing properties of cannabis (Van Winkel *et al*, 2011), and this result has been replicated by two other groups (Di Forti *et al*, 2012; Morgan *et al*, 2016).

The candidate genes approach clearly helps to assess the interaction of environmental factors with these genes in relation to an increase in the sensitivity to develop psychosis. However, to successfully determine the gene and environment interaction leading to psychosis, it is essential to carefully select a specific environmental factor in combination with a specific candidate gene, based on prior knowledge. This is not always successful as is exemplified in the case of studies of the vulnerability to develop psychosis when using cannabis by valine158 allele carriers in the catechol-O-methyltransferase (*COMT*) gene. The data led to contradicting results (Caspi *et al*, 2005; Zammit *et al*, 2011). Thus, replicating the candidate gene approach does not always lead to the same outcome, moreover, it rules out identification of other genes that can be involved in the interaction with environmental factors. Therefore, more comprehensive genome-wide search strategy, i.e. Genome–Wide Environment Interaction studies (GWEIS), is preferred to the hypothesis-driven method. However, until now, there is no GWEIS study which focuses on cannabis as the environmental factor that increases the vulnerability to psychotic experiences. In chapter 3 of this thesis we describe the first GWEIS study in the effect of cannabis use on developing psychosis.

Epigenetic modifications as a mechanism in gene environment interactions

One of the potential mechanisms involved in the interplay between environmental factors and the genome are chemical modifications of the DNA at epigenetic level. Epigenetics focuses on changes in gene function that are heritable but do not entail a change in DNA sequence (Waddington, 1942). Epigenetic mechanisms instruct the cell to interpret the signals from both internal (genetic) and external (environmental) and then adjust the access to DNA accordingly. The most studied epigenetic mechanism is DNA methylation which means adding methyl tags on DNA. This methylation will influence the function of DNA without changing the sequence of the DNA. The methyl tags are mostly added at cytosines in CpG islands, an area with abundant CG nucleotides (definition of a CpG island: the CpG content is higher than 50% and the [number of CpG/number of (C*G)] higher than 60% within a 200 base pair windows) (Gardiner-Garden and Frommer, 1987). When the high methylation occurs in a promotor region of a gene, it prevents the transcription of that gene and therefore usually leads to gene silencing. However, a recent study reported that the relation between DNA methylation and gene transcription can both lead to silencing as well as to activating gene expression (van Eijk *et al*, 2012). Once the methyl tags are fixed onto the genome, the epigenetic modifications are chemically stable during cell division by DNA methyltransferases (DNMT) recognizing hemi-methylated sites and adding methyl tags onto the other hemi-nonmethylated sites, thus maintain the methylation status (Trerotola *et al*, 2015). Thereby the epigenetic information will be inherited by the daughter cells and last for multiple generations (Kular and Kular, 2018).

Given that epigenetic modifications are sensitive to environment, especially during early development (Iurlaro *et al*, 2017), studies of epigenetic programming on adverse early life experiences have the potential to improve our understanding of the etiology of psychiatric disorders. One early example of the potential of this approach is the study of epigenetic programming of genes involved in the hypothalamic–pituitary–adrenal (HPA) axis by childhood adversity (McGowan, 2013). Both rodent and human studies showed that the epigenetic status of the glucocorticoid receptor (GR) gene *NR3C1* was associated with the levels of *NR3C1* transcription, and vulnerability to stress (Bockmühl *et al*, 2015; Radtke *et al*, 2015; Shields *et al*, 2016; Watkeys *et al*, 2018). Since the negative feedback of HPA axis is impaired due to DNA methylation of the GR gene, the HPA associated cortisol stress reactivity is subsequently increased. This is also supported by the finding of epigenetic alterations of the intracellular GR regulator *FKBP5* gene in response to stress (Zannas *et al*, 2016). Previous study found that *KITLG* methylation is a mediator between childhood adversity and blunted cortisol reactivity, whether this association is also present in psychosis is still not clear. Apart from early life adverse stimuli, in utero the nutritional status is also critical for neurodevelopment. Epigenetic dysregulation during this dynamic stage thus could be responsible for pathogenesis of neurodevelopmental disorders, including psychiatric ones. Up till now there is a lack of studies that focus on detrimental early environmental factor, such as studying the effect of maternal malnutrition on the neuro-epigenome of the child. Therefore, our studies described in this thesis will focus on DNA methylation regarding childhood adversity and nutritional deprivation (chapter 4 and 5).

Functional in vitro studies of gene-environment interaction

The limited replication success of candidate gene studies and the emerging insight that functional studies whereby the impact of environmental exposures and changes in gene function are biological relevant has called for the use of alternative systems. Cell studies may provide additional mechanistic insights as environmental exposure can be precisely controlled in a cell culture dish. In an *in vitro* approach we can mimic environmental factors and focus on complex gene pathways.

Several studies have applied this approach to test the functional consequences of a genetic or environmental change in an *in vitro* model after cross-sectional study. This provides strong evidence for GWAS-based gene-environment interaction findings and helps to further explore functional role of the identified genes (Cattaneo *et al*, 2018). A recent GWAS study identified SNPs in three genes (*FoxO1*, *A2M*, and *TGF-β1*) that were correlated to depression after childhood traumatic experience. This study was followed by *in vitro* experiments with exposure of cortisol to mimic stress in a human hippocampal progenitor cell line, to further confirm the role of the identified genes in stress responsivity (Cattaneo *et al*, 2018).

Combining GWEIS with *in vitro* studies strengthens the findings of gene-environment interactions. Therefore, the approach of the studies in this thesis is first to conduct a genome-wide analysis in a cross-sectional study to identify the genomic factors that interact with environmental factors (cannabis use, nutritional deprivation) and subsequently to perform studies *in vitro* to verify the cross-sectional study and further explore the functional relevance of the identified genes.

Three main environmental determinants of psychosis

Cannabis as an environmental determinant

The number of cannabis users is still increasing, rising to 183 million individuals in 2015 world-wide (United Nations Office on Drugs and Crime, 2017). This has raised concern about potentially detrimental effects of cannabis on human health. Actually, mounting epidemiological evidence already shows that cannabis use has detrimental effects on mental health (Gage *et al*, 2016; Marconi *et al*, 2016), especially for those who start using in their early teens (Arseneault *et al*, 2002). Not only cannabis can increase the risk of psychotic like experiences, but it can also increase the risk to develop psychiatric disorders, such as schizophrenia (Kelley *et al*, 2016). However, some argue that people with a genetic predisposition for schizophrenia may be slightly more likely to use a range of drugs, therefore the vulnerability to suffer psychotic experiences can also be intrinsically genetically determined (Ksir and Hart, 2016) and not environmentally. Recent studies, nevertheless, strongly support cannabis initiation on risk of schizophrenia, and the genetic predisposition for schizophrenia could not explain the more than 5% of cannabis use in the general population or patients (Gage *et al*, 2017; Vaucher *et al*, 2017)

Though it is clear that cannabis use can increase the risk to develop psychotic disorders, the underlying mechanism is still obscure. What we know is that the main psychoactive constituent of the cannabis plant is Δ9- tetrahydrocannabinol (THC) (Wachtel *et al*, 2002). This compound has higher affinity to the endocannabinoid receptors-CB1R than endocannabinoids (2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (AEA)), and thus it competitively binds to endocannabinoids receptors in the central nervous systems (CNS) (Bloomfield *et al*, 2016). Once the endocannabinoid system loses the balance by external cannabinoids, the signaling of dopaminergic neurons which are modulated by endocannabinoid system will be disrupted. The genes involved in the dopamine system are therefore, the most widely studied candidate genes of cannabis induced psychosis. Indeed, previous candidate gene studies showed involvement of *DRD2* (Luykx *et al*, 2017), *COMT* (Caspi *et al*, 2005) and *AKT1* (Morgan *et al*, 2016; Van Winkel *et al*, 2011), and found that SNPs in these genes increases the risk of psychotic illness in cannabis users. These genes are involved in post synaptic dopamine signaling and thus contribute to the idea that chronic cannabis use induces postsynaptic super-sensitivity in the face of low striatal dopamine levels (Murray et al., 2014).

However, the results of candidate gene studies are not always consistent. *COMT* for example, study reported that cannabis users with single nucleotide polymorphism in this gene increase the risk to psychosis (Caspi *et al*, 2005). Whereas this finding was either corroborated (Henquet *et al*, 2009) or refuted (Zammit *et al*, 2011) by other studies. The a priori selection of genes of interests prevents the discovery of new genes/pathways that could be involved in cannabis induced psychosis. In order to maximize the chance to identify genes that contribute to the vulnerability of developing psychosis when exposed to cannabis in a non-bias way, *i.e*. a genome-wide approach in cannabis users, this can help to understand how this environmental determinant impact on genetics and increases the vulnerability of psychosis. Until now, no such unbiased genome-wide approach has been done with a focus on cannabis as an environmental determinant for psychosis. Therefore, in this thesis we perform a genome-wide approach to determine which genomic factors interact with heavy cannabis use and lead to a high vulnerability to develop psychosis. To further strength and explore our identified genes from cross sectional discoveries, we subsequently conducted *in vitro* studies.

Nutritional deprivation as an environmental determinant

Maternal famine can lead to a two times higher chance to develop schizophrenia of the offspring, which is a worrying perspective for a whole generation of children born after the three-year great Chinese famine (1959-1961) (St Clair *et al*, 2005). Apart from the great Chinese famine, the other most well-known famine is the Dutch hunger winter during the Second World War (1944-1945). Both cohorts reported significant increases in multiple chronic pathologies ranging from autism to schizophrenia (St Clair *et al*, 2005), from autoimmune disease to serious infectious disease (Han *et al*, 2016), and from obesity to diabetes (Meng *et al*, 2017). And both famines provide a natural experimental setting for research on the long-term effects of nutritional deprivation on human development.

The fetal stage is a critical stage in human brain development, as CNS structures are formed in the first trimester of pregnancy. During this stage the fetus obtains various nutrients from the mother through the placenta and the umbilical cord. As a consequence, the growth and development of the fetus depends on its mother's nutritional status. As a regulatory factor, nutrition affects gene expression with various mechanisms at different levels, including epigenetic modification, more specifically DNA methylation. During the fetal stage the gene expression is extremely dynamic and vulnerable to exogenous factors, therefore dysregulation of these precise and coordinated gene expression patterns through epigenetic mechanisms due to malnutrition may have a vital role in the pathogenesis of neurodevelopmental disorders and can eventually lead to disease state.

A genome-wide DNA methylation study could provide a specific DNA methylation pattern induced by famine. The evidence from a genome-wide DNA methylation study in offspring of mothers that faced the Dutch hunger winter showed that specific DNA methylation patterns of prenatal malnutrition are related to growth and metabolism (Tobi *et al*, 2014). Another example of such an epigenetically regulated genomic locus due to famine is insulin-like growth factor II (*IGF2*), the key factor in human growth and development. Prenatal exposure to famine, as studied in the Dutch hunger cohort, was associated with persistent differences in methylation of the *IGF2* locus (Heijmans *et al*, 2008).

However, up till now only one study has been performed in the Chinese famine cohort (Boks et al., 2018). Therefore, in this thesis we study changes in the epigenome due to malnutrition in the Chinese famine cohort. We selected individuals that were exposed to hunger within the first three months of gestation based on birth date between January 1960 and September 1961. Furthermore, a study of an *in vitro* nutritional deprivation model was conducted. By combing the analyses from two studies, we focused on providing more persuasive arguments on which epigenome factors were involved in nutritional deprivation.

Childhood adversity as an environmental determinant

Early life stress, such as childhood adversity, is a well-established major risk factor for developing psychiatric disorders, such as bipolar disorders, later in life (Shields *et al*, 2016; Varese *et al*, 2012). Childhood adversity usually occurs before the age of 16 and this term comprises a wide range of adversities, such as physical, emotional and sexual abuse, household poverty, separation from a parent, and neglect. It was observed that mothers with a history of childhood trauma also have a profound impact on their offspring (Plant *et al*, 2017), especially in regard to general cognitive abilities, memory, and executive functions.

The biological mechanisms linking childhood adversity and vulnerability to psychosis include dysregulation of the HPA axis and the dopamine systems (Morgan and Gayer-Anderson, 2016). The evidence of the involvement of the HPA axis is the strongest, as HPA axis dysregulation compellingly leads to bipolar disorder (Belvederi Murri *et al*, 2016), and HPA axis is one of the main biological systems involved in the response to stress (Smith and Vale, 2006). For example, a candidate gene study found that a polymorphism in the mineralocorticoid receptor (MR) gene, which is an important regulator of the HPA axis and a prime target for corticosteroids, is moderately involved in the etiology of depression following childhood maltreatment (Vinkers *et al*, 2015). Moreover, other candidate studies found DNA methylation is involved in stress-related HPA dysregulation. For example, childhood trauma is associated with DNA methylation of intracellular glucocorticoid receptor (GR) gene *NR3C1* and also its regulator *FKBP5* gene. Both of them are key players in the HPA axis function and associated cortisol stress reactivity.

Others investigated the role of DNA methylation in bipolar disorder (Fries *et al*, 2016). Genome-wide methylation as well as methylation of several specific candidate genes known to link to bipolar disorders have been extensively investigated for the past years. These studies suggest that DNA methylation plays an important role in the dysregulation of gene expression in bipolar disorder. However, the link between childhood adversity and bipolar disorder though DNA methylation mechanisms has not been widely studied, except for one candidate gene study linking childhood trauma to bipolar disease via DNA methylation of loci in the *5HT3AR* gene (Perroud *et al*, 2016).

A recent genome-wide DNA methylation study from our group identified a locus in the *KITLG* gene (cg27512205) of which the methylation level was positively associated with childhood trauma and served as a mediator between childhood adversity and blunted cortisol stress reactivity in healthy controls (Houtepen *et al*, 2016). The identification of such an epigenetic mark leads us further to explore in this thesis whether such an association between childhood adversity and *KITLG* methylation is also present in bipolar disorders.

Aim of this thesis

Considering the need to understand how the environment impacts on psychosis risk and what the underlying mechanisms are, we here combine epidemiological genetic studies for gene discovery with cellular studies that further interrogate the functional meaning of the identified genes. The aim of this thesis is to define which (epi)genomic determinants lead to neuropsychological dysfunctions and psychiatric disorders in combination with selected detrimental environmental factors (cannabis use, maternal malnutrition, and childhood adversity). The general approach of the studies in this thesis is firstly to identify the involved genes using (epi)genome-wide approach and subsequently, to investigate *in vitro* the potential functional pathway and to provide additional mechanistic evidence.

Main research questions

- Which genes confer the increased risk to psychosis on those that use cannabis?
- Which genes are epigenetically changed by in utero famine exposure?
- What is the role of genes epigenetically changed due to childhood adversity in the etiology of bipolar disorders?

Hypothesis

We hypothesize that the interaction between gene and environment is not only on gene transcription level, but also on the epigenetic level.

Outline of the thesis

This thesis addresses the effects of three main environmental factors on epigenetic and translational regulation of genes that are involved in psychiatry vulnerabilities. The first part (chapter 2 and 3) focuses on cannabis use as a detrimental environment factor of psychiatric symptoms. Both studies used unbiased genome-wide approaches followed by an *in vitro* study. The second part (chapter 4) discusses the epigenetic impact of nutritional deprivation on gene regulation by applying an epigenome-wide approach. The third part (chapter 5) targets on childhood adversity in the pathway of psychiatry pathogenesis by interacting with *KITLG* methylation.

Chapter 1 provides a general Introduction and outline of the thesis

Chapter 2 focuses on cannabis use as an environmental factor that decreases neuropsychological function and increases the risk of psychosis. By examining genome-wide gene expression profiles and *in vitro* validation, we provide close insight into molecular mechanisms driving the effects that are associated with cannabis use and neuropsychological function.

Chapter 3 focuses on interaction between the *P2RX7* gene polymorphism and cannabis use and the effect of this interaction on mental health. First, we performed a Genome-Wide Environment Interaction Study (GWEIS) that aimed to provide information on the genes involved in cannabis use as a risk factor of psychosis. In a subsequent *in vitro* model, we exposed monocytes to cannabinoids and we studied the effect on the P2X7 receptor in order to further validate our cross-sectional finding. In both chapter 2 and 3, we define cannabis non-users as lifetime cannabis exposure of less than six times and heavy cannabis users as a current expenditure for personal cannabis use which exceeds €10 weekly.

Chapter 4 By performing a genome-wide DNA methylation analysis on blood samples, we investigated the potential altered epigenetic regulation due to early life famine exposure. To provide more solid epigenetic evidence for the effects of early life famine exposure, a genome-wide DNA methylation analysis on fibroblasts from a famine-mimic *in vitro* study was performed. The overlap of genetic candidates provides further insight into the epigenetic changes.

Chapter 5 studies the transcriptional regulation of the *KITLG* gene in blood of psychosis patients to investigate the role of epigenetic regulation of this gene involved in the childhood adversity, in the pathogenesis of bipolar disorder.

Chapter 6 provides a summary and general discussion of the main findings of this thesis.

Chapter 7 is a summary of this thesis in Dutch.

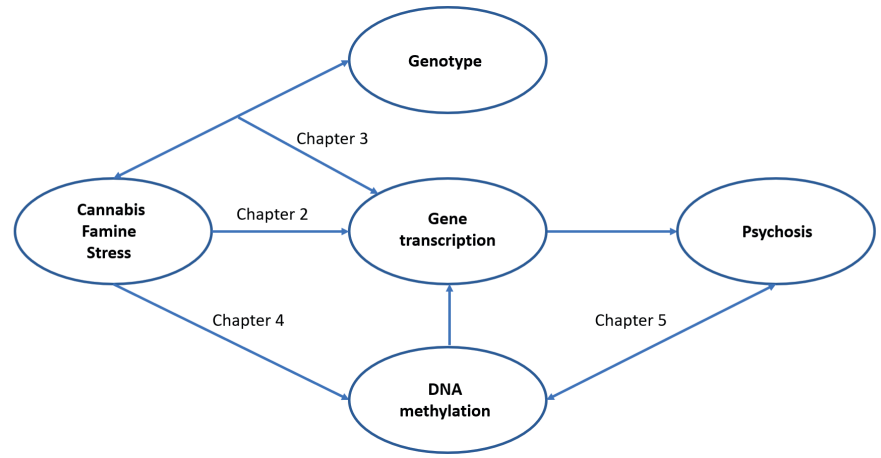

Figure 1. Overview of the main subject discussed in the thesis and the connection between them.

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Part I

Cannabis use as a detrimental environmental factor

Chapter 2

Liprin alfa 2 gene expression is increased by cannabis use and associated with neuropsychological function

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Abstract

The relation of heavy cannabis use with decreased neuropsychological function has frequently been described but the underlying biological mechanisms are still largely unknown. This study investigates the relation of cannabis use with genome wide gene expression and subsequently examines the relations with neuropsychological function. Genome-wide gene expression in whole blood was compared between heavy cannabis users (N=90) and cannabis naïve participants (N=100) that were matched for psychotic like experiences. The results were validated using quantitative real-time PCR. Psychotic like experiences were assessed using the Comprehensive Assessment of Psychotic Experiences (CAPE). Neuropsychological function was estimated using four subtasks of the Wechsler Adult Intelligence Scale (WAIS). Subsequent *in vitro* studies in monocytes and a neuroblastoma cell line investigated expression changes in response to two major psychotropic components of cannabis; tetrahydrocannabinol (THC) and cannabidiol (CBD). mRNA expression of Protein Tyrosine Phosphatase Receptor Type F Polypeptide-Interacting-Protein Alpha-2 (*PPFIA2*) was significantly higher in cannabis users (LogFold Change 0.17) and confirmed by qPCR analysis. *PPFIA2* expression level was negatively correlated with estimated intelligence (B=-22.9, p=0.002) also in the 100 non-users (B=-28.5, p=0.037). In vitro exposure of monocytes to CBD led to significant increase in *PPFIA2* expression. However, exposure of monocytes to THC and neuroblastoma cells to THC or CBD did not change *PPFIA2* expression. Change in *PPFIA2* gene expression in response to cannabinoids is a putative mechanism by which cannabis could influence neuropsychological functions. The findings warrant further exploration of the role of *PPFIA2* in cannabis induced changes of neuropsychological function, particularly in relation to CBD.

1. Introduction

Global cannabis consumption is rising and has increased to 182.5 million users in 2016 (Bussink et al., 2016). This has raised concern about potentially detrimental effects of cannabis on mental health and neuropsychological function. Compelling evidence suggests that cannabis increases the risk to psychosis (Vaucher et al., 2017) and psychotic like experiences (Van Gastel et al., 2013). Also neuropsychological function may be affected by cannabis use (Broyd et al., 2016; Grant et al., 2003; Schnakenberg Martin et al., 2016). Acute cannabis use impairs neuropsychological functioning (Morrison et al., 2009), especially short-term episodic and working memory (Ranganathan and D'Souza, 2006) and persistent cannabis use from adolescent age onwards is associated with a decline in intelligence (Castellanos-Ryan et al., 2016; Meier et al., 2012) although the role of potential confounding factors, particularly social economic status is debated (Meier et al., 2017; Moffitt et al., 2013). The mechanisms underlying the relationship between cannabis use, psychotic experiences and decreased neuropsychological function have remained largely unknown.

One of the most prevailing hypothesis on the relation between cannabis and neuropsychological function involves the dopamine system (Bloomfield et al., 2016; Freund et al., 2003). It is thought that one of the most active neuropsychiatric compounds, tetrahydrocannabinol (THC) (Dinis-Oliveira, 2016; Wachtel et al., 2002), dysregulates the dopamine system via the endocannabinoid type 1 receptor (CB1R). Acute THC consumption increases dopamine release (Bloom and Dewey, 1978; Bossong et al., 2015, 2009; Howes and Osgood, 1974; Stokes et al., 2010), whereas long term THC consumption blunts dopamine synthesis and release capacity (Freund et al., 2003; van de Giessen et al., 2016). Since the dopamine system is closely related to neuropsychological function (Hélie et al., 2012; Nakajima et al., 2013), dysregulation by cannabis is thought to contribute to this impairment in cannabis users. An alternative hypothesis comes from neuroimaging studies that suggest that white matter changes in cannabis users (Jakabek et al., 2016; Weinstein et al., 2011), may account for reduced neuropsychological functioning (Filley and Fields, 2016; O'Muircheartaigh et al., 2014; Ohtani et al., 2017).

However, evidence so far has been inconclusive and further genetic studies may improve our understanding of the effects of cannabis in the brain. A range of studies identified the potential role of a few candidate genes that likely play a role in the mental health effects of cannabis including AKT Serine/Threonine Kinase 1 (*AKT1*) (Boks, 2012; Bossong et al., 2015; Di Forti et al., 2012), Catecholamine O-Methyl

transferase (*COMT*) (Caspi et al., 2005; Henquet et al., 2009, 2006; Nieman et al., 2016), and the gene that codes for cannabis receptors, cannabinoid receptor type 1 (*CNR1*) (D'Addario et al., 2017; Suarez-Pinilla et al., 2015). However, such a priori selection of specific genes limits the ability to uncover new leads in understanding the effects of cannabis on the brain whereas genome wide studies may facilitate our understanding of the role of cannabis in neuropsychological function. Considering the scarceness of post-mortem human brain samples of cannabis users this study reverts to whole blood of cannabis users and cannabis naïve controls. In order to deal with the potential role of psychotic like experiences, cannabis users and non-users were matched for the level of psychotic like experiences. We examined gene expression rather than the underlying genetic variants in order to directly investigate the biological response of cells to the cannabinoids as a way of informing on the biological mechanisms that are involved in cannabis use, and not genetic vulnerability. The causality of the identified relation between cannabis and gene expression was subsequently examined in *in vitro* experiments. To provide insight in potential tissue types differences in the responses to cannabinoids both blood cells and neuronal cell lines were used.

2. Experimental procedures

2.1. Recruitment

The recruitment details is described somewhere else (Schubart et al., 2011a). In short, participants were recruited by a website survey (www.cannabisquest.nl) which launched in 2006 targeted at Dutch young adults and adolescents (18-25 years). The online assessment included several verification questions to protect against random answers, and only complete forms filled by participants were subsequently included. From a cohort of 1259 participants, 192 participants with absent or extreme cannabis exposure and high or low scores (top or bottom 20%) for psychotic experiences measured by the Community Assessment of Psychic Experiences (CAPE) (Konings et al., 2006) Neuropsychological function (Intelligence Quotient, IQ) of the participants was measured with four subtests of the WAIS: symbol substitution, math, block design, and information processing. The grandparents of all individuals were all born in the Netherlands to limit genetic heterogeneity. All participants gave written informed consent. The online survey in the current study is to recruit participant and collect basic information in an effective way. All participants were comprehensively assessed during a hospital visit. This study was approved by Medical Ethical Committee Utrecht.

2.2 Cannabis exposure

In the Netherlands THC-concentration and market value of cannabis are highly correlated (Schubart et al., 2011b), therefore weekly amount of Euro's spent on cannabis was used to measure the exposure to THC. Participants that spend more than €10 per week were considered heavy cannabis users and lifetime cannabis naïve participants were considered non-users. Urine samples were obtained to verify their report on recent cannabis use.

2.3. Whole blood gene expression analysis

Whole blood samples were obtained by venepuncture from all participants at the time of assessment. RNA was isolated and purified from whole blood using the PAXgene extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total mRNA was quantified using Quant-itTM Ribogreen assay (Invitrogen, California, USA). RNA samples were prepared with the Illumina TotalPrep kit (Invitrogen, California, USA) amplification and labelling protocol. The samples were randomly distributed over the different arrays to prevent batch effects. Genome-wide RNA expression profiling was obtained with a HumanHT-12 v3 Beadchip (Illumina, California, USA) that includes over 48,000 probes. Array hybridization was done using 750 ng of amplified and biotinylated labelled cRNA using Illumina's standard protocol at the UCLA Neuroscience Genomics Core facility.

2.4. Expression data preprocessing

GenomeStudio software version 3.2 was used to extract raw data and generate background-corrected gene expression data. Background correction was performed by subtracting the average value of negative control beads present on the array. Further pre-processing was done using the Lumi package in R. A variance stabilizing transformation (VST) and robust spline normalization (RSN) were applied to the data according to the Lumi procedure (Du et al., n.d.). Genes were then filtered based on detection values. Expression probes had to reach the detection p-value threshold <0.01 in at least one sample. Array quality and outlier detection was performed by assessing quality statistics and plots before and after transformation and normalization.

2.5. Bead chip array expression data analysis

Expression values were taken as dependent variables and tested for association with cannabis status as the independent variable using the Limma software package (Smith, 2005). Cigarette smoking (present or absent), the use of other drugs besides cannabis (present or absent), age, gender and psychotic experiences were taken along as covariates. Significance threshold was set at a False-Discovery-Rate (FDR) corrected $P < 0.05$.

2.6. Quantitative Real time PCR data analysis

Expression levels of the genome wide significant transcripts was analysed by Quantitative Real Time PCR (qPCR) (Applied Biosystems, California, USA). Absolute quantities were obtained by running all samples in quadruplicates. First-strand cDNA was synthesized according to the manufacturer's instructions. Quantitative PCR was carried out using a TaqMan assay from Applied Biosystems. The following TaqMan gene expression assays were used: *PPFIA2* (Hs01548846), *PPFIA2* (Hs01548855), *PPFIA2* (Hs01548860). *GUSB* gene was selected as reference gene and Ct values were normalized against *GUSB* expression. Log-fold changes were calculated using a and delta-delta method and significance analysed using the non-parametric Mann-Whitney test.

2.7. cis expression Quantitative trait loci (eQTL)

Whole-genome Single Nucleotide polymorphisms (SNP) data of all included subjects were available from a previous study. Expression quantitative trait loci (eQTL) analysis was performed to identify SNP's that regulate mRNA expression levels of the identified loci. Genetic association were calculated using PLINK (Purcell et al., 2007), with a linear regression analysis, using gene expression as a quantitative trait. The p-value significance threshold was set for a Bonferroni correction for the number of SNP's in *cis* (within 100 base pairs of the gene).

2.8. Regulation of PPFIA2 after in vitro cannabinoid exposure

2.8.1. Cannabis exposure in monocytes

Changes in *PPFIA2* expression in response to cannabis were investigated in blood cells. Baseline expression in monocytes (N=5) relative to reference genes Ribosomal protein S28 (*RPS28*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was measured in monocytes. Based on a relative expression of 8.20 (arbitrary units (AU)) monocytes were identified as a suitable cell type. Therefore, monocytes from 12 donors were isolated with CD14 microbeads (MACS sorting) (Miltenyl Biotec, Bergisch Gladbach, Germany) from anonymous buffy coat blood donors. Cells were plated in 96 well plate with a density of 100,000 cells/ well in 100ul RPMI 1640 (Life Technology, CA, USA) medium with 10% fetal bovine serum (FBS) (Life Technology, CA, USA). After inspection of dose-response curves for 1, 10 and 100 uM of CBD and THC experiments were conducted using 10uM based on the fact that no responses were obtained for 1 uM and at the dose of 100uM most of the cells died. Monocytes from 12 donors were treated with 10 uM of CBD (Sigma-Aldrich, Missouri, USA) and 10 uM THC (Farmalyse, Zaandam, NL). Since ethanol was used to dissolve both THC and CBD, it was used as control condition. After 6 hours, medium was removed and Phosphate-buffered saline (PBS) was used to wash the cells. After TRIzol (Invitrogen, California, USA) addition for RNA isolation the cells were stored at a temperature of -80 °C until cDNA synthesis.

2.8.2. Cannabis exposure in neuroblastoma cell lines

PPFIA2 is expressed in neurons and oligodendrocytes and plays important functions in neuronal development and function (Zhang et al., 2014). We therefore assessed whether the expression of *PPFIA2* in the neuroblastoma cell line SH-SY5Y is affected by exposure to cannabinoids despite the low baseline level of expression that we found in this cell line. A human neuroblastoma cell line, SH-SY5Y was used as an in-vitro model of neuronal response to cannabinoids even though the priory suitability was not clear. We found that the baseline *PPFIA2* mRNA expression level in SH-SY5Y cell line (normalized to our reference genes) was low (0.51 AU). Nevertheless, cells were plated in 96-well plate with a density of 70,000 cells/well in DMEM high glucose medium (Life Technology, CA, USA) with 10% of FBS. Dose finding for THC and CBD stimulation of neuroblastoma cell line identified a similar optimal concentration of 10 uM as in the monocytes. Ethanol was used as control condition and the protocol was the same as the monocytes (6 hours stimulation, PBS washout, TRIzol for RNA isolation, storage at -80 °C until cDNA synthesis).

2.8.3. RNA isolation and qPCR

RNA was isolated using the TRIzol method. Complementary DNA (cDNA) was synthesized by using the Reverse Transcription Kit (Qiagen, Hilden, Germany. Quantitative polymerase chain reaction (qPCR) was performed to quantify mRNA expression level as described before (Melief et al., 2016). Ribosomal protein S28 (*RPS28*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were selected as reference genes. The following primers were used: *PPFIA2* forward (5'- GGCCCAGTGTCCGGTTTTTC -3') and reverse (5'- GGTAACCCCAAGCTGGGAAG -3'), *RPS28* forward (5'- GACACGAGCCGATCCATCATC -3') and reverse (5'- TGACTCCAAAAGGGTGAGCAC -3'), and *GAPDH* forward (5'- TGCACCACCAACTGCTTAGC -3') and reverse (5'- GGCATGGACTGTGGTCATGA -3'). The response to different cannabinoids in gene expression was calculated as fold change relative to its ethanol exposed control.

2.9. Statistical analyses of qPCR PPFIA2 expression data and estimated IQ

Linear regression was used to analyse the association of *PPFIA2* expression as outcome with cannabis use as main indicator. Age, gender, smoking, other drug consumption (yes/no) and psychotic experiences were added as covariate. A separate linear regression model was used to analyse the relation of *PPFIA2* expression with an estimate of IQ, using IQ as outcome and cannabis use as well as *PPFIA2* expression as indicators and age, gender, smoking, other drug consumption and psychotic experiences were added as covariate. Multivariate Analysis Of Covariance (MANCOVA) was used to analyse the relation of *PPFIA2* expression with 4 subtests of the Wechsler Adult Intelligence Scale (WAIS) simultaneously (Hijman et al., 2003). Age, gender,

smoking, drug consumption, psychotic experiences and cannabis use were added as covariates in the model. *PPFIA2* expression differences after cannabinoids stimulation in monocytes and neuroblastoma cells were analysed using the nonparametric Wilcoxon paired test. P-values smaller than 0.05 were considered to be statistically significant.

3. Results

3.1. Sample

Data on a total of 100 cannabis naïve subjects and 90 heavy cannabis users was analysed after exclusion of two participants with failed expression data due to technical outliers. No significant of psychotic experiences between the two groups. Whereas the IQ score in the heavy cannabis users is significantly lower than cannabis naïve participants (IQ heavy cannabis users=101.33, IQ cannabis naïve participants=113.19; B=-11.86, p<0.001). **Table 1** presents the sample characteristics.

Table 1. Sample characteristics

	Total group n	Cannabis naïve 190	Heavy Cannabis users 100	BB, p-value 90
Mean age (sd)	23.1 (2.0)	22.8 (1.8)	23.3 (2.2)	B=0.533, p=0.07
Gender (%male)	59.5	39.6	81.1	B=0.411, p<0.001
Other drugs	52 (27%)	0 (0%)	52 (58%)	B=5.778e-01, p<0.001
Cigarette smoking	80 (42%)	8 (8%)	71 (79%)	B=0.709, p<0.001
Alcohol use lifetime	170 (89%)	90 (90%)	80 (89%)	B=-0.011, p=0.805
Medication use	46 (24%)	26 (26%)	20 (22%)	B=-0.038, p=0.550
Psychotic experiences	93 (49%)	50 (50%)	43 (48%)	X=5.9, p=0.864
IQ score (sd)	107.69 (13.99)	113.19 (13.31)	101.33 (11.98)	B=-11.860, p<0.001

Psychotic experiences measured by the Community Assessment of Psychic Experiences (CAPE). IQ score of the participants was calculated with measurement of four subtests of the WAIS: symbol substitution, math, block design, and information processing.

3.2. Whole genome expression analysis

After filtering and quality control, 20,765 probes (42.5%) remained for further analysis. Linear regression analysis yielded 2,131 probes with a nominal significance between users and non-users. Supplementary **Table 1** gives the full results of the nominal significant probes. After FDR-correction at the 0.05 level, the expression of two transcripts, Protein Tyrosine Phosphatase Receptor Type F Polypeptide-Interacting Protein Alpha-2 (*PPFIA2*) (probe ID: ILMN_1803318) (LogFold Change 0.17, p= $3.27x10^{-08}$, FDR adjusted p=0.038) and C-X3-C Motif Chemokine Receptor 1 (*CX3CR1*) (probe ID: ILMN_1745788) (LogFold Change -0.42, p= $3.59 x10^{-08}$, FDR adjusted p= 0.038) were significantly associated with cannabis use, see **Table 2**.

3.3. qPCR validation

Due to depletion of RNA quantity, only 65 heavy users and 78 samples from cannabis naïve participants were available for qPCR validation. These participants did not differ from the original sample with respect to the distribution of covariates. We confirmed the upregulation of one of the *PPFIA2* transcript (log Fold Change 1.40, p= 0.046), the other *PPFIA2* transcripts were undetectable. The downregulation of *CX3CR1* could not be validated (Fold Change 1.04, p= 0.516), see **Table 2**.

Table 2. Gene transcripts with genome wide association to cannabis and validation

Gene	Transcript	Probe_ID	Array Fold Change	Array p-value	FDR p-value	qPCR Fold Change	qPCR p-value
PPFIA2	ILMN_7280	ILMN_1803318	3.2	$3.27 x10^{-08}$	0.04	1.4	0.05
CX3CR1	ILMN_8593	ILMN_1745788	0.74	$3,59 x10^{-08}$	0.04	1.0	0.50

3.4. cis expression quantitative trait loci (eQTL)

The cis eQTL analysis of 262 SNP's in the *PPFIA2* gene region from 190 participants shows no significant association with the expression of the *PPFIA2* gene at a Bonferroni multiple testing threshold of $1.9 x 10^{-04}$. In the GTEx database also no significant eQTLs are reported in blood, but in the hippocampus an eQTL SNP is identified; rs35137985 (GRCh38, Chr12:80919319) that is located in an intron of the LIN7A gene that is associated with learning disabilities (Matsumoto et al., 2014).

3.5. PPFIA2 expression and estimated IQ

Neuropsychological function (IQ score) was calculated with four subtests measurement of the WAIS: symbol substitution, math, block design, and information processing. A significant negative association between IQ estimate and *PPFIA2* expression was present in the entire group (unadjusted for cannabis use) (B=-22.95, p=0.002), and in the non-cannabis using group (N=100, B=-28.5, p=0.04) but not in the entire group while adjusting for cannabis use (**Figure 1**). MANOVA indicated significant differences in test profiles associated with *PPFIA2* expression (F(171,4)=3.9, p=0.004) over and above the role of cannabis use (F(171,4)=7.2, p<0.001).

3.6. Regulation of PPFIA2 gene expression in response to cannabinoids

The response of monocytes to cannabinoids showed a large variability in *PPFIA2* expression: Eight of 12 donors (66.7%) showed increase *PPFIA2* expression in response to CBD 10uM (median fold change = 2.92, Wilcoxon paired rank test, p=0.04). One expression level of *PPFIA2* in CBD exposed monocytes was an outlier (3 standard deviations from the mean) and censoring this observation reduced the significance to (p=0.075). Gene expression in monocytes after THC stimulation, showed an increase in 6 of 9 (66.7%), (median fold change = 1.6, Wilcoxon paired rank test, p=0.26) (**Figure 2**). *PPFIA2* gene expression in SH-SY5Y slightly increased after 6 hours stimulation by CBD (mean fold change=1.56) and THC (mean fold change=1.37) but the differences did not reach statistical significance (**Figure 3**).

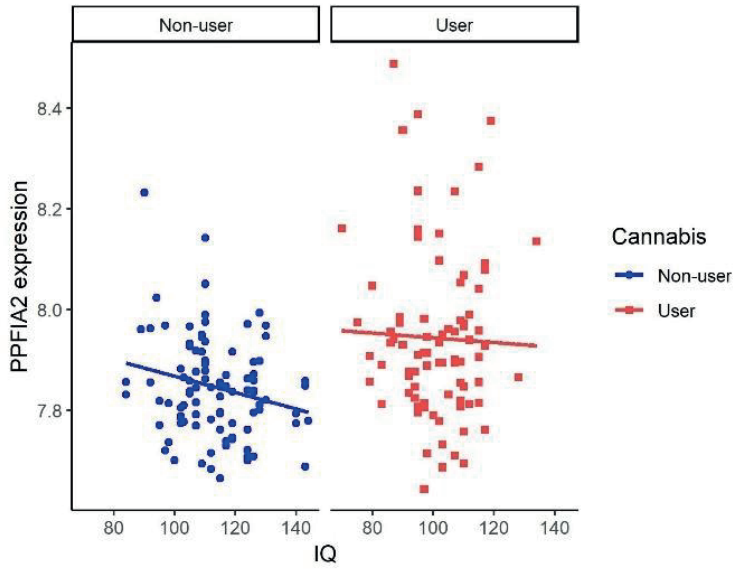

Figure 1. The association between PPFIA2 expression and IQ in cannabis non-users and heavy cannabis users. The y-axis is the PPFIA2 expression level after adjust age, gender, smoking, other drug consumption and psychotic experiences. The x-axis is the IQ score. Significant negative association between IQ estimate and PPFIA2 expression was present in the non-cannabis using group (N=100, B=-28.5, p=0.04) but not in the heavy-cannabis using group.

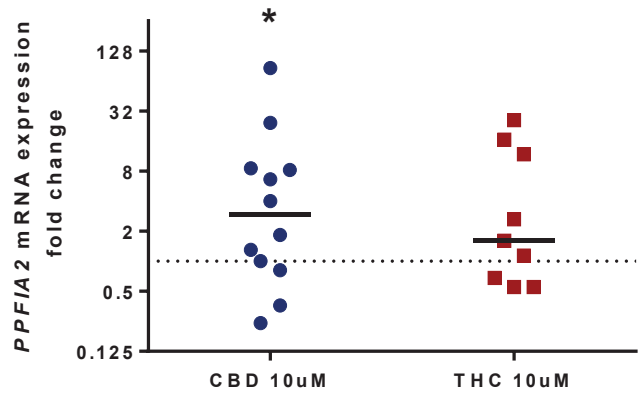

Figure 2. *PPFIA2* expression fold change after cannabinoids stimulation in monocytes. The horizontal bar represents the median fold change. Monocytes were treated with 10 uM of cannabidiol (CBD) (N=12), or THC for 6 hours. *PPFIA2* expression fold change was calculated by cannabinoid stimulation divided by their ethanol control. Three data points in the THC group that were undetectable were omitted. * p<0.05

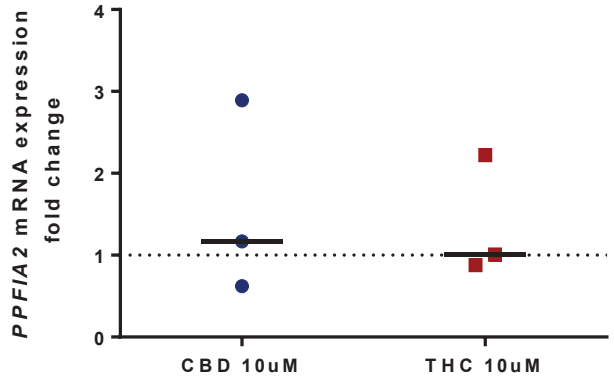

Figure 3. *PPFIA2* expression fold change after cannabinoids stimulation in SH-SY5Y. The horizontal bar represents the median fold change. SH-SY5Y cells were treated with 10 uM of CBD or THC for 6 hours. *PPFIA2* expression fold change was calculated by cannabinoid stimulation divided by ethanol control. Plotted values are based on triplicates.

4. Discussion

Comparison of gene expression in whole blood of heavy cannabis users (N=90) and cannabis naïve participants (N=100), showed that *PPFIA2* and *CX3CR1* expression were significantly higher in heavy cannabis users. Upregulated expression of *PPFIA2* in heavy cannabis users was confirmed with qPCR. *PPFIA2* expression was also significantly negatively related with estimated IQ in the cannabis abstinent group. Supportive evidence for a direct relation between cannabinoid and *PPFIA2* expression comes from data from monocytes that also show *PPFIA2* upregulation in response to cannabinoid stimulation, particularly cannabidiol (CBD).

The identified gene: Protein Tyrosine Phosphatase Receptor Type F Polypeptide-Interacting Protein Alpha-2 (*PPFIA2*) encodes the liprin-α-2 protein. It is highly expressed in neurons and oligodendrocytes (Zhang et al., 2014). Important functions of liprin-α proteins in neuronal cells are suggested by its localization in mossy fiber endings in the CA3 region of the hippocampus, in synapses, axons, dendrites and the cell body (S A Spangler et al., 2011). A role of liprin-α-2 in axon guidance function was suggested by reports that liprin-α-2 contributes to the normal density and growth of excitatory synapses and dendritic spines (Baran and Jin, 2002; Dunah et al., 2005; Pulido et al., 1995; Serra-Pagès et al., 1998) in hippocampal neurons. In the synapse active zone in hippocampal neurons Liprin-α proteins have a role in organizing pre and post synaptic vesicle preparation and neurotransmitter function (Ko et al., 2003; Olsen et al., 2005; Owald et al., 2012; Patel et al., 2006). Liprin-alpha-2 is one of the predominant Liprin isoforms in the hippocampus (Samantha A Spangler et al., 2011; Zürner et al., 2011) where it is likely involved in dendrite development (Spangler and Hoogenraad, 2007) long term depression (LTD) (Dickinson et al., 2009) and neuropsychological function (Kolkman et al., 2004). By forming a liprin-α-GRIP-GluA2 complex, liprin- α interacts with muscarinic acetylcholine receptors (mAChR) and plays a role in mAChR dependent LTD (mAChR-LTD) in hippocampus (Dickinson et al., 2009).

Interestingly, previous study discovered that activation of mAChR could enhance the release of endogenous cannabinoids in the hippocampus (Kim et al., 2002). Such increased endocannabinoids could trigger LTD and mediated the release of neurotransmitters in the hippocampal inhibitory synapses (Chevaleyre and Castillo, 2003; Kano et al., 2009). However, when the exogenous cannabinoids was treated, THC for examples, it can block the endocannabinoid-mediated LTD in the hippocampus (Mato et al., 2004) and lead to disruption of GABA and glutamate neurotransmitter release (Bossong and Niesink, 2010). Therefore, heavy cannabis consumption could disrupt the balance of endocannabinoids system and might further influence the liprin- α level by involving mAChR-LTD.

The result from the current study did not directly support dopamine system (Bloomfield et al., 2016; Freund et al., 2003) and white matter changes (Jakabek et al., 2016; van de Giessen et al., 2016) are the most prevailing hypothesis on the cannabis and neuropsychological function. Whereas the current study took hypothesis-free approach and found *PPFIA2* highly expressed in the heavy cannabis users. This highlighted the advantage of genome-wide approach which could help to discover new potential pathway to understand the role of cannabis in neuropsychological function.

To overcome the limitation of our cross-sectional discovery in blood RNA, in vitro experimental studies in monocytes and a neuroblastoma cell line were conducted to support a causal relationship between cannabis and *PPFIA2* expression. In monocytes, *PPFIA2* expression increased after exposure to cannabinoids, particularly in response to CBD. Such a relation in an experiment whereby the unexposed cells from the same donor are used as reference gives strong support for longitudinal changes in response to these cannabinoids. We do not know how other blood cell types respond to cannabinoids. However, the results from exposing the neuroblastoma cell line to cannabinoids did not show a robust increase in *PPFIA2* expression and this demonstrates that large differences between tissue types exist. Several explanations may account for the differences that were observed between monocytes and the neuroblastoma cell line. One reason may be the differences in baseline expression. The expression of *PPFIA2* is quite low in the SH-SY5Y cell line. *PPFIA2* expression in SH-SY5Y was less than 10 percent compared to monocytes (0.50 versus 8.20 AU respectively). Such a low expression in SH-SY5Y may lead to a relatively low sensitivity to cannabinoids. Further research is required to investigate whether other cells are also involved in the response to cannabinoids, particularly hippocampal cell lines.

The finding that particularly CBD lead to increased *PPFIA2* expression is noteworthy, since CBD is generally viewed as the less toxic of the cannabinoids and is under study for efficacy in treatment of anxiety and potentially psychosis (Schubart et al., 2011a; Schubart et al., 2014). Our finding suggests that particularly CBD may play a role in the adverse effects of cannabis, but since the observations are based on work in monocytes only, further studies are required. A finding that was not further investigated in this study is the result of the genome wide gene expression profiling that suggested that cannabis use leads to downregulation of *CX3CR1* in whole blood. *CX3CR1* is the receptor of neuron-produced Fractalkine (*CX3CL1*), exclusively expressed by microglia in the central nerve system. The interaction between *CX3CL1* and *CX3CR1* constitutes the neuron-microglial signalling system (Ransohoff and Khoury, 2016) and deficiency either of them could increase the production of pro-

inflammatory molecules (Sheridan and Murphy, 2013). Though this finding was not validated with later qPCR validation, considering the limited power of our qPCR validation analysis due to loss of 47 subjects and its role in the central nerve systems, *CX3CR1* remains a potential candidate for further study.

This is the first human genome wide gene expression study to identify genes that may be involved in a cognitive effect of cannabis smoking. Strongpoints are the exclusive inclusion of heavy cannabis users or cannabis naïve individuals to maximize the potential contrast in gene expression changes (Boks et al., 2007) and matching for psychotic like experiences to remove the influence of psychotic experiences from the study. Previous studies on cannabis induced gene-expression investigated preselected genes of interest, thus limiting the ability to discover new genes that are involved in the biological pathway of the cannabis influence. A limitation of this study is the self-report method. Though we applied the urine screen to control the self-report quality and inform the participant do not use cannabis on the day of blood draw. Whereas, the screen could not effectively distinguish anywhere in the past few days and the same day of the cannabis use. Therefore, there was no way to effectively validate the report. Another limitation of this study is the crude measure of neuropsychological function. These four subtests of the WAIS can reliably estimate IQ but do not provide a comprehensive overview of cognitive function. Another limitation of this study is the potential presence of residual confounding including smoking and social economic status. This limitation is inherent to the cross-sectional discovery. The current study does not aim to resolve the debate on the relation between cannabis use and neuropsychological functions. Analysis of estimated IQ is merely used to identify a putative role of the identified cannabis associate gene transcript. The data presented from the follow up in vitro studies that show expression changes in response to cannabinoids are an essential step to tie CBD exposure to the *PPFIA2* changes and increases the level of evidence. Questions that remain are how neurons (and particularly hippocampal neurons) respond to cannabinoids and how neuropsychological function is altered in human experimental studies of cannabidiol.

Overall the upregulation of *PPFIA2* in response to cannabidiol and the correlation with estimated IQ suggest a role of this gene in the pathways underlying the adverse effects of cannabis on neuropsychological function.

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Conflict of Interest

The authors have no potential conflict of interest to report.

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Supplemental Table 1 provides the full results of the nominal significant probes.

Supplemental Table 1 is available at request.

Chapter 3

A genetic variant in the P2X purinoceptor 7 gene (P2RX7) moderates the effect of cannabinoids on psychosis risk

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Addiction
In preparation

Abstract

Aims
Despite mounting evidence of the relation between cannabis use and psychosis, the underlying biological pathways are not fully understood. The current study aims to identify biological mechanisms that confer the increased risk for psychosis in those who use cannabis.

Participants and Design
In a sample of 1262 non-psychiatric Dutch individuals, we conducted a genome-wide environment-interaction study (GWEIS) to investigate interactions between cannabis use and genotype with psychotic experiences as outcome.

Setting
A selected population sample from the Netherlands.

Measurement
Psychotic experiences were measured using the Community Assessment of Psychic Experiences (CAPE). The sample was enriched for those at the extremes of cannabis use and having psychotic experiences to increase power. Genotyping was performed using the Illumina platform and the significant findings were validated using Taqman PCR. To further investigate how cannabis exposure may interact with the identified genetic variant, we performed an *in vitro* study in which monocytes were exposed to tetrahydrocannabinol (THC) and cannabidiol (CBD).

Findings
We found that a SNP in the *P2RX7* gene (rs7958311) was associated with an increased risk ($p=1.10 \times 10^{-7}$) for a high level of psychotic experiences in heavy cannabis users. *In vitro* exposure of monocytes to THC and CBD showed that cannabinoids can modulate the function of the P2X7 receptor, resulting in a decreased release of IL-1β induced by ATP, and that this modulation is dependent on the identified genetic variant. *P2RX7* receptor polymorphisms have been associated with psychiatric disorders before, and the P2X7 receptor is involved in several pathways that are relevant for the aetiology of psychosis.

Conclusions
Our data point to a role of the *P2RX7* gene in vulnerability to develop psychotic symptoms when using cannabis and unveils to new potential pathways in the relation between cannabis and psychosis.

Introduction

Compelling evidence shows that cannabis is associated with psychosis but the causality of this relation remains subject of fierce debate (Marconi *et al*, 2016; Murray *et al*, 2017). In epidemiological studies, cannabis use has consistently been associated with a higher rate of psychotic disorders and psychotic symptoms in the general population (Van Gastel *et al*, 2013). Cannabis use significantly decreases the age of the first psychotic episode, especially in schizophrenia (Large *et al*, 2011) and increases the number and severity of psychotic exacerbations (Manrique-Garcia *et al*, 2014). However, it remains possible that this association is not causal and vulnerable individuals are more prone to use cannabis (Van Gastel *et al*, 2013). In concordance with this possibility some studies suggest that genetic predisposition to schizophrenia is associated with higher levels of cannabis use (Power *et al*, 2014; Verweij *et al*, 2017). However, most recently, sophisticated genetic studies using mendelian randomization do suggest a causal relationship (Gage *et al*, 2017; Vaucher *et al*, 2017).

Whatever the nature of the relation between cannabis use and psychosis is, the majority of cannabis users do not develop a psychotic disorder (Van Gastel *et al*, 2013). It is therefore plausible that the association between cannabis use and psychotic disorders depends on individual biological vulnerability that might be genetically determined. In support of such a genetic component, several family studies reveal that the mental health impact of cannabis exposure is higher in first grade relatives of schizophrenia patients (McGuire *et al*, 1995). Evidence for specific gene environment interactions is provided by several candidate gene-environment studies. Caspi et al (Caspi *et al*, 2005) reported that a single nucleotide polymorphism (SNP) in the catecholamine transferase (*COMT*) gene, increased the risk to psychotic disorders in cannabis users. This finding was corroborated (Henquet *et al*, 2009) as well as refuted (Wigman *et al*, 2011; Zammit *et al*, 2011) by other studies. Another candidate gene study provided evidence that a SNP in the *AKT1* gene increases the risk of psychosis in cannabis users (van Winkel *et al*, 2011), followed by independent replications (Boks, 2012; Di Forti *et al*, 2012).

These candidate gene studies have produced relevant hypotheses for the aetiology of psychotic disorders. However, candidate gene studies are unlikely to provide the whole story (Ioannidis *et al*, 2001) and genome wide association studies (GWAS) provide the possibility to screen the genome without *a priori* selection.

The aim of the present study was to discover genetic variants that are associated with the increased risk to psychotic symptoms in cannabis users. We therefore first conducted a genome wide gene finding study using a previously described extreme sampling method (Boks *et al*, 2007) from a population sample of individuals enriched for extreme cannabis users and extreme high or low scores of psychotic experiences. In order to provide supportive information on the functional relevance of the genetic findings we subsequently conducted *in vitro* experiments in which the effect of cannabinoids on the function of the identified gene was investigated.

Methods

Utrecht Cannabis Cohort (CannabisQuest)

Participants were recruited using a project website launched in 2006 targeted at Dutch young adults and adolescents from 18 to 25 years (Schubart *et al*, 2011a). Strategies to generate traffic on the project website included collaboration with over a hundred colleges, universities, and youth centres, as well as the use of online commercial advertisement products (i.e. banners and text links) (Schubart *et al*, 2011a). The chance to win an Apple iPod™ or a Nintendo Wii™ was used as an incentive. Double entries were prevented by exclusion of subjects with an identical e-mail address, surname, and date of birth. Anonymous submission of data was not possible. The online assessment included verification questions to protect against random answers, and participants failing to correctly complete the verification questions were subsequently excluded. From the online data (N = 17,698), 1259 participants were included for subsequent genetic assessment in two waves. Since THC-concentration and market value of cannabis are highly correlated in the Netherlands (Schubart *et al*, 2011b), we used the weekly amount of euros spent on cannabis as a proxy measure of exposure to THC. We gather this information by online questionnaires to assess the amount in euros (€) that individuals spent on cannabis per week. The THC exposure was categorized into five classes: cannabis naïve, cannabis use equivalent of less than €3 per week, €3 to €10 per week, €10 to €25 per week and more than €25 per week. THC-concentration and market value of cannabis are highly correlated in the Netherlands (Schubart *et al*, 2011b), the weekly amount of euros spent on cannabis as a proxy measure of exposure to THC. A urine drugs screen was performed to confirm recent cannabis use or abstinence.

In order to increase power for gene × environment interactions (Boks *et al*, 2007), we mixed an unselected sample of 719 individuals from the population with a sample of 540 participants selected for belonged to the top or bottom quintile of total scores of psychotic experiences as measured by the Community Assessment of Psychic

Experiences (CAPE) score that were either non-users (defined by a lifetime cannabis exposure frequency less than 6 times) or were heavy cannabis users (i.e. current expenditure for personal cannabis use exceeded €10 weekly).

To prevent population stratification, we confined to inclusion of individuals with four grandparents born in The Netherlands. In subjects with a high CAPE and high exposure mental health was ascertained with the validated Dutch version of either the Structured Clinical Interview (SCID) (Spitzer *et al*, 1992) for the other participants the MINI International Neuropsychiatric Interview was used (Sheehan *et al*, 1998). No participants with a history of any psychotic disorder were included. The possible concomitant use of recreational drugs was assessed with the substance abuse module of the Composite International Diagnostic Interview (CIDI) (Robins *et al*, 1988). The study was approved by the Ethical Review Board of the University Medical Center Utrecht and all participants gave written informed consent.

Online assessments

Participants provided their age, educational level, contact details, and the country of birth of their grandparents. As a measure of psychometric psychosis vulnerability, an online version of the CAPE (Konings *et al*, 2006) was used. The questionnaire has discriminative validity for the different symptom dimensions in individuals from the general population and is considered a valid instrument for detecting individuals at high risk for psychosis (Hanssen *et al*, 2003; Konings *et al*, 2006; Mossaheb *et al*, 2012; Stefanis *et al*, 2002). The use of an online version of the CAPE for this purpose was validated and is described in detail elsewhere (Vleeschouwer *et al*, 2014).

Genotyping

DNA was extracted from whole blood of two 10ml EDTA tubes obtained using vena venipuncture. For logistic reasons, genotype data for subjects of Dutch ancestry was generated on two different array platforms; 576 individuals on Illumina® HumanOmniExpress (733,202 SNPs), 768 individuals on the Illumina® Human610-Quad Beadchip (620,901 SNPs) and 34 individuals on the Illumina® HumanHap550 array (550,000 SNPs). For validation of the genetic findings, we genotyped 1211 samples of which DNA was not exhausted from the original discovery cohort using Taqman probes for *P2RX7* rs7958311 (GGTTCCATCACTGCC[A/G]TCCCAAATACAGTT).

Pre-processing genotype data, quality control and imputation

For each SNP platform, quality control procedures were initially performed separately using PLINK (Purcell *et al*, 2007). Participants were excluded based on gender errors and on >5% missing genotypes. We used linkage disequilibrium (LD) based SNP

pruning to select the most informative SNPs (R^2<0.2), only for the subsequent quality control step. Datasets were merged with Hapmap Phase 3 to check ethnicity. Ethnic outliers were detected by visual inspection. After these QC procedures on subjects, we performed quality control on SNPs. All SNPs were filtered on missingness (>2%), Minor Allele Frequency (MAF) > 5% and Hardy Weinberg (p>1e^{-6}) before merging the three datasets. We imputed the merged dataset with Hapmap3 release 24 using Beagle (Browning and Browning, 2009). SNPs with an imputation score > 0.8 and SNPs that were present originally in all of the datasets were extracted.

Monocyte isolation

To determine the impact of cannabinoids on the function of P2X7 we analyzed a key function of this receptor *in vitro*: the release of IL-1β from activated monocytes after binding adenosine triphosphate (ATP) (Wewers and Sarkar, 2009). We used monocytes for this study, since these cells can be easily isolated from blood and express P2X7, as well as cannabinoid receptors CB1, CB2, and GPR18 as confirmed by qPCR (data not shown). We used peripheral blood samples of 42 individuals obtained from Sanquin (www.sanquin.nl) and the University Medical Center Utrecht. All donors provided informed consent approved by the local institutional review boards prior to participation in the study. Monocytes were isolated by density gradient separation using Ficoll (Ficoll Paque plus, GE Healthcare, Sweden), followed by magnetic isolation using CD14-microbeads according to manufacturer's protocol (Miltenyi Biotech, Germany).

P2RX7 genotyping

To determine *P2RX7* genotype of the monocyte donors we extracted genomic DNA using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Germany). A PCR was performed to amplify the target DNA fragment contain SNP rs7958311 using the following primers: Forward CTTCAGGGCGGAATAATGGG; Reverse TTGGAGTTACCTGAAGTTGTAG. Sanger sequencing was subsequently used to determine *P2RX7* genotype by sequencing the PCR products using the BigDye sequencing kit (BigDye Teminator version 3.1, Applied Biosystems, USA), a 3730xl Capillary sequencer (Applied Biosystems, USA) and analysis with ChromasLite 2.1.1 software.

P2X7 function in vitro

1x10^5 monocytes were plated in 96 well plates and stimulated with 10 ng/ml lipopolysaccharide (LPS) from E. Coli (Sigma, USA) for four hours. Monocytes were then incubated with different concentrations of CBD (Sigma, USA), THC (Farmalyse, the Netherlands), or their ethanol carrier as control for 10 minutes, followed by different concentrations of ATP (Sigma, USA) for one hour. Thereafter, the cells were

centrifuged, the supernatant was harvested and the concentration of IL-1β was quantified using a commercial IL-1β enzyme-linked immunosorbent assay (ELISA) kit (Ready-SET-go, eBioscience, USA), according to the manufacturer's protocol.

Statistical Analysis

To calculate the effect of gene by environment interaction on CAPE scores, we performed a logistic regression, using genotype, cannabis use, a GxE interaction term and gender as predictors. Dichotomized CAPE scores (by median split) were used as the dependent variable. All genome-wide association calculations were performed using the open-source whole genome association analysis toolset PLINK (Purcell *et al*, 2007). To analyze the functional interaction between cannabinoids and P2X7 genotype *in vitro,* we compared the effect of cannabinoids on the ATP response between different genotypes. Due to the low minor allele frequency rate of the rs7968311 only four homozygote variants (AA) were present in the 42 monocyte donors. We therefore decided to compare the impact of cannabinoids on P2X7 function between A and non-A carriers using a mixed model for repeated measures (MMRM).

Results

Sample description

The mean age of the included participants was 20.5 years. The mean CAPE score in the total sample was 67.3. For further details on characteristics of the sample, see **Table 1**.

Table 1: Characteristics of the subjects used in this GxE study

Characteristic	All (N=1259)	Cannabis users (N=401)	Non cannabis users (N=858)	Statistics
Age mean in years (sd)	20.5 (2.5)	20.7 (2.5)	20.4 (2.5)	T= 2.1, P= 0.04
Male %	47%	73%	35%	X= 157.3, P< 0.001
Psychotic experiences	67.3 (13.9)	70.5 (15.5)	65.9 (12.8)	T= 5.6, P< 0.001

T, t statistic; P, p-value.

Pre-processing, quality control and imputation

Linkage disequilibrium (LD) based SNP pruning resulted in ~78k SNPs for the sets to assess heterozygosity (F<3SD), homozygosity (F>3SD) and relatedness by pairwise IBD values (pihat >0.1). Subject quality control resulted in the exclusion of 101 individuals. Four duplicates and three related sample-pairs were detected in

the merged datasets (according to criteria described in methods section) and one outlier after clustering the merged dataset. Imputation resulted in 2,504,766 SNPs for analysis.

GWEIS analysis

Figure 1 shows a Manhattan plot of p-values for the G x E term. **Table 2** lists the top 10 results of the G x E logistic regression analysis for a high CAPE score as a binary trait. We found that a variant in the *P2RX7* receptor gene (rs7958311, Chr12:121167552) was ranked first of SNPs associated with an increased risk (OR: 1.746, p: 1.10E-07) for experiencing psychotic symptoms in heavy cannabis users. The identified SNP was imputed (imputation score of 0.885), has a minor allele frequency (MAF) of 0.289 and is a non-synonymous coding SNP (835A>G) that results in an amino acid change from histidine to arginine (His270>Arg) in an extracellular subunit of P2X7 transmembrane receptor. **Table 3** shows the odds ratios (adjusted for gender) for A/G or A/A carriers compared with G/G carriers depending on cannabis use status, showing an increased risk for psychotic experiences in A carriers of the rs7958311 SNP. Interestingly, **Table 3** also shows a trend towards a decreased risk to psychotic experiences in the group that does not use cannabis. **Figure 2** shows the Q-Q plot for the interaction in the imputed data set (2.5M SNP's), λ= 0.993. Six SNP's that are in LD in the CADM2 gene (p: 1.05E-06), rank second in lowest p-values in our G x E interaction association analysis. We genotyped 1211 samples from the original discovery to validate the imputation. Interaction between cannabis and P2X7 remained significant (B=0.936, t=4.420, p=9.86 x10^{-6}).

Table 2. Top 10 genome-wide GxE association results.

CHR	SNP	BP	A1	MAF	OR	T	P	GENE
12	rs7958311	120089738	A	0.2889	1.746	5.309	1.10E-07	*P2RX7*
3	rs1003984	85952889	T	0.2587	1.981	4.881	1.05E-06	*CADM2*
3	rs1003985	85952993	A	0.2170	1.981	4.881	1.05E-06	*CADM2*
3	rs1003986	85953053	T	0.2582	1.981	4.881	1.05E-06	*CADM2*
3	rs12487728	85953621	T	0.2212	1.981	4.881	1.05E-06	*CADM2*
3	rs9968137	85953797	G	0.2995	1.981	4.881	1.05E-06	*CADM2*
3	rs12488483	85954040	A	0.2212	1.981	4.881	1.05E-06	*CADM2*
10	rs4342983	115440504	C	0.0421	2.031	4.76	1.93E-06	*CASP7*
6	rs17710848	153108495	A	0.0696	2.469	4.696	2.65E-06	Not annotated
10	rs3121454	115365937	C	0.0481	2.029	4.645	3.39E-06	*NRAP*

CHR, Chromosome; SNP, Single Nucleotide Polymorphism; A1, Allele; MAF, minor allele frequency; OR, Odds Ratio; T, t statistic; P, p-value.

Table 3. Odds Ratio adjusted for gender for A/G or A/A carriers compared with G/G wildtype depending on cannabis use status.

	NO CANNABIS			HEAVY CANNABIS USERS		
Genotype	**adjusted OR**	**95% CI**	**P**	**adjusted OR**	**LO 95% CI**	**P**
P2RX7 (GG)	1	-	-	1	-	-
P2RX7 (GA)	0.622	0.47-0.83	0.001	2.347	1.54-3.60	0.000
P2RX7 (AA)	0.478	0.27-0.85	0.011	3.693	1.50 -9.11	0.005

OR, Odds Ratio; CI, Confidence Intervals; P, p-value.

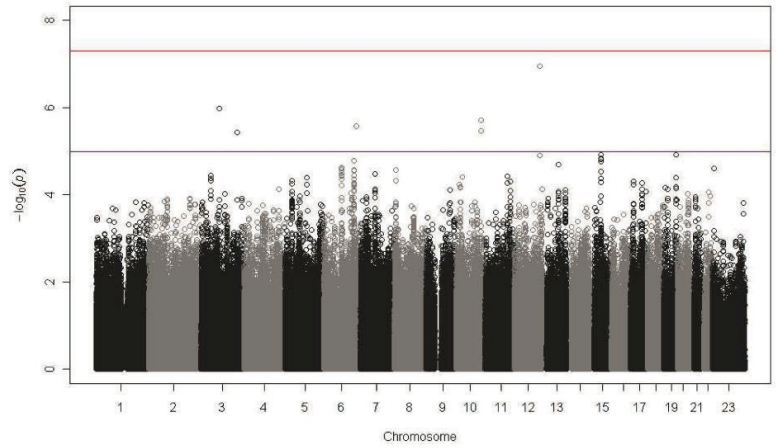

Figure 1. Manhattan plot for interaction p-values

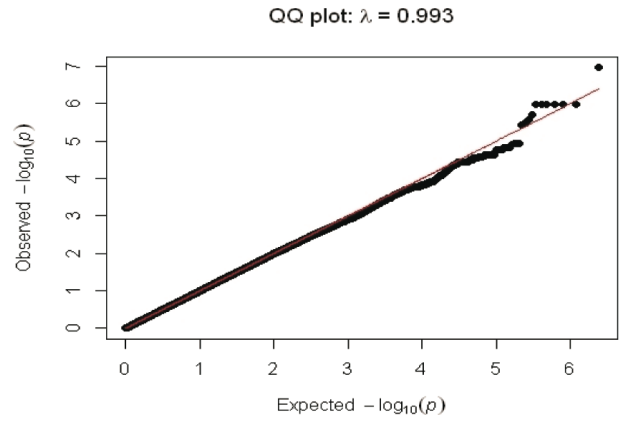

Figure 2. QQ plot for interaction term in the imputed set (2.5M) using a full model

The interaction between P2RX7 and cannabinoids in vitro

The P2X purinoceptor 7 (P2X7) protein is a purinergic receptor. Triggering of the receptor with low concentration of adenosine triphosphate (ATP) leads to formation of a cation-specific channel, whereas a higher concentration of ATP results in the formation of a non-selective large pore in the cell membrane (Li *et al*, 2005). This receptor is ubiquitously expressed, but most highly expressed in immune cells. ATP activation of P2X7 results in a variety of downstream effects, including the secretion of IL1-related cytokines by immune cells. To validate that cannabis use may impact on psychotic symptoms by interacting with the P2X7 receptor, we therefore analyzed whether the cannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD) affect the secretion of IL-1β after triggering LPS-activated monocytes with ATP. As described before, we found that ATP induces a concentration dependent secretion of IL-1β (**Figure 3**A, B). Treating the cells with 10 µM cannabidiol before the cells were exposed to ATP resulted in a decrease of the concentration dependent IL-1β release in all donors (**Figure 3**A). THC and lower concentrations of cannabidiol pretreatment resulted in a decrease of P2X7-induced Il-β release in some, but not all donors (**Figure 3**B, supplementary Figure 1). Next, we investigated the impact of cannabinoids on P2X7 functioning in relation to the associated SNP. We performed the same experiment using monocytes from 42 donors that were genotyped for *P2RX7* rs7958311 (**Figure 3**C, D). Mixed model repeated measure (MMRM) analysis showed that the inhibition of the ATP response by cannabinoids was stronger in A allele carriers than non-A allele carriers: For CBD: t=4.27, p<0.001, and for THC: t=2.93, p=0.006.

Discussion

We performed a genome wide environment interaction study (GWEIS) in a sample of 1261 participants, enriched for individuals at the extremes of psychotic experiences and cannabis use. We found a SNP (rs7958311, 12: 121167552) in the P2RX7 gene that moderates the relation between cannabis use and psychotic experiences at borderline significance level (p=1.10 x 10-7). The relevance of this genetic variant was supported by data from cultured monocytes that showed that cannabinoids can modulate the function of the P2X7 receptor and that this modulation is dependent on the identified genetic variant.

rs7958311 SNP

The *P2RX7* gene is a highly polymorphic gene located on chromosome 12 at 12q24.31 and encodes for the P2X7 receptor. The identified SNP, rs7958311, is common and has a minor allele frequency (MAF) of 0.289. It is a non-synonymous coding SNP (835A>G) that results in an amino acid change from histidine to arginine (His270>Arg) in an extracellular subunit of this transmembrane receptor. Two previous studies have shown that the arginine variant of this SNP leads to a gain of function, measured as an increased P2X7-dependent uptake of ethidium or a fluorescent dye (Sorge *et al*, 2012; Stokes *et al*, 2010) of which one study identified a role in chronic pain of this variant (Sorge *et al*, 2012). Another study did not find an alteration of P2X7 function for this SNP (Sun *et al*, 2010).

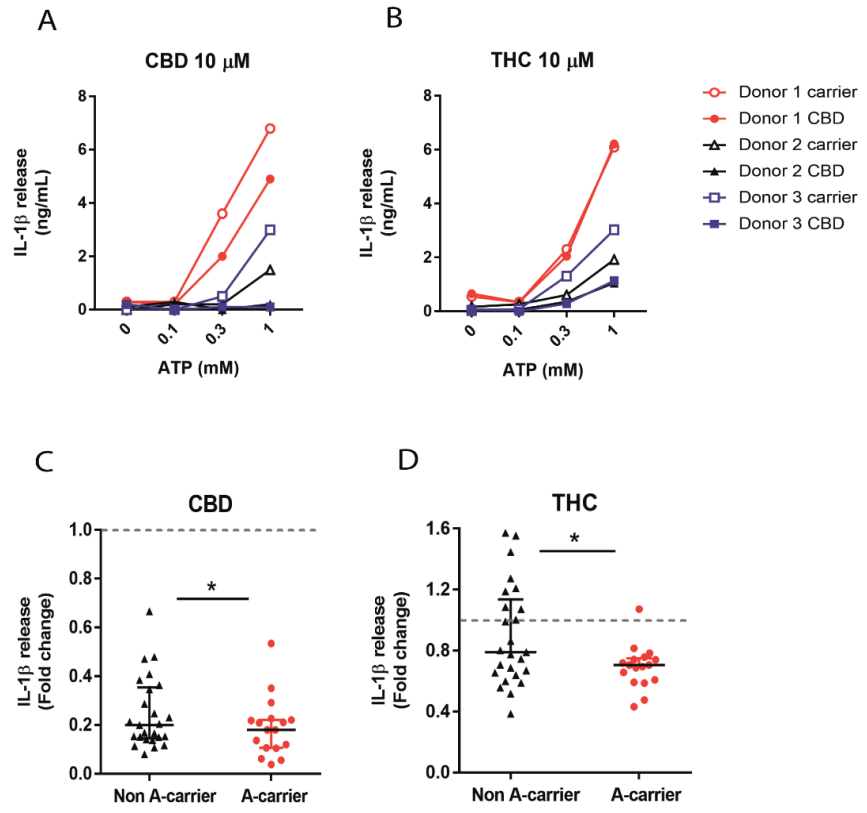

Figure 3. The impact of the cannabinoids THC and CBD on P2X7 function in vitro and the role of SNP rs7958311 genotype.

LPS-stimulated monocytes were exposed to CBD, THC (both 10 µM) or the ethanol carrier as control. The cells were subsequently stimulated with different concentrations of ATP to measure the release of IL-1β: one of the key inflammatory functions of the P2X7 receptor. A,B) A dose response curve of IL-1β release to ATP is presented for CBD 10µM (A) and THC 10µM (B) in three different healthy control donors. C,D) The impact of rs7958311 genotype on the effect of cannabinoids on P2X7 function is shown comparing A-carriers (AA or GA; N=17) and non A-carriers (GG; N=25). The fold change is depicted, representing [IL-1β release with cannabinoids]/[IL-1β release with carrier control]. Grey dashed line represents no change in IL-1β release. The differences between genotype groups were measured by a mixed model for repeated measures. * = significantly different.

P2RX7 and psychiatric disorders

Variation in the *P2RX7* gene has previously been associated with an increased risk for several psychiatric disorders. Linkage scans in bipolar disorder (McQuillin *et al*, 2009) firstly implied P2X7 as a candidate gene (Barden *et al*, 2006). The 12q24 area, in which the *P2RX7* gene is located, was also shown to be linked to unipolar depression in two genome wide linkage scans (Abkevich *et al*, 2003; McGuffin *et al*, 2005) Several follow up studies corroborated these findings and a recent meta-analysis showed that one particular SNP in the *P2RX7* gene (rs2230912; Gln460Arg) is associated with both bipolar disorder and unipolar depression (Czamara *et al*, 2017). The 12q24 region has also been implied in schizophrenia (Bailer *et al*, 2000, 2002), but in the single candidate gene study on *P2RX7* and schizophrenia, no association was found for nine different *P2RX7* SNP's. The most recent GWAS meta-analysis on schizophrenia did not show an association with SNP's in the *P2RX7* gene either (Ripke *et al*, 2011; Steinberg *et al*, 2011). Given the complex etiology of schizophrenia and the fact that we found a bidirectional effect of *P2RX7* genotype in cannabis users and non-users, it is possible that this SNP is contributing to the pathogenesis of psychotic disorders in cannabis users, but not in non-users. However, in a previous G x E candidate gene study on cannabis use and schizophrenia outcome another *P2RX7* SNP (rs2230912), was investigated but did not show any evidence for G x E interaction (Van Winkel *et al*, 2011). In the current study, this SNP was nominally significant (p= 0.0112). Given that the rs2230912 SNP is in linkage disequilibrium (LD) with the SNP identified in the current study (rs7958311) (Fuller *et al*, 2009; Stokes *et al*, 2010), these results suggest that *P2RX7* may only be involved in causing psychotic experiences, but not clinical psychosis.

Hypotheses on P2X7 – cannabis interaction

The P2X7 receptor is present in all tissues, including the central nervous system, and is predominantly expressed in immune cells, such as microglia cells, but also found on astrocytes and neurons (BioGPS gene expression database). Several downstream effects have been described for the activation of P2X7 by ATP, including secretion of cytokines, formation of reactive oxygen species, apoptosis, proliferation, the regulation of neurotransmitter release and long term potentiation (LTP) (Sperlágh *et al*, 2006). An altered function of P2X7 may therefore impact on neuroinflammation, but also on neurodevelopment and neuronal functioning, processes that all have been linked to the pathogenesis of psychotic disorders. In our *in vitro* experiments, we found that cannabinoids decreased ATP-dependent IL-1β release in monocytes and that this decrease of function is more pronounced in A carriers of the rs7958311 SNP. We therefore hypothesize that decreased function of P2X7 by cannabinoids may be involved in the development of psychotic symptoms by affecting one of the several important functions of P2X7 in the brain, including neurotransmitter release, synaptic plasticity and inflammatory processes. An alternative hypothesis is formed by studies on the endocannabinoid system. Endocannabinoids bind to the same receptors as cannabinoids, such as CB1 and CB2, and play a role in regulating neuroinflammation, neuroprotection, and neurotransmission. Interestingly, it has been shown that triggering of the P2X7 receptor by ATP modulates the production of endocannabinoids in microglia (Lu *et al*, 2012) and astrocytes (Gao *et al*, 2017). These studies indicate that the purinergic and endocannabinoid system closely interact and that rs7958311 variant may therefore modulate the vulnerability to cannabinoids at several levels.

CADM2 gene identified from this study

Although we focused in this study on validation of the most significant GxE association P2X7, our genome-wide analysis also identified six SNPs in the CADM2 gene. CADM2 codes for the protein synaptic cell adhesion molecule 1, which belongs to the immunoglobulin (Ig) superfamily. Interestingly, previous study found that variants in this gene were associated with autism disorders (Casey *et al*, 2012), cognitive functioning (Davies *et al*, 2016; Ibrahim-Verbaas *et al*, 2016) and alcohol consumption (Clarke *et al*, 2017). Large genome-wide association studies have also identified strong associations of this gene with lifetime cannabis users (Stringer *et al*, 2016) which strengthen our genome-wide discovery.

Strengths and limitations

Strongpoints of the study is the sampling from a homogeneous population of Dutch adolescents enriched with individuals with extreme psychosis vulnerability and extreme exposures to cannabis in order to increased power andvalidation of the functional consequence of the identified G x E interaction in cell models. Nevertheless, also a number of limitations have to be considered when interpreting the presented data. One limitation is the use of a dichotomized outcome measure (CAPE score) instead of treating the CAPE as a quantitative trait. Due to our extreme phenotype sampling method, the distribution of the CAPE scores was not normal. Particularly when performing regression analysis with a G x E interaction term, the risk of heteroscedasticity is much higher, given the increased variation due to small number of subjects per group when combining SNP and environmental exposure data. In our sample, this resulted in inflation of type I error reflected by a high lambda. Robust error correction did not resolve this issue. We therefore decided to dichotomize the dependent (cape score) and performed a binary trait analysis. Another limitation is the fact that the discovery was performed using an imputed SNP call (R2:0.885) and PCR validation yielded a lower significance level under strict genome wide threshold. Therefore, the conformation we provided using a measured SNP call was essential. We also reiterate that this study does not per se investigate schizophrenia vulnerability. The CAPE questionnaire is a well validated instrument to measure psychotic experiences as reflection of psychosis vulnerability, however the majority of participants that have high scores on the CAPE questionnaire have not and will never develop a psychotic disorder. Another potential limitation is that we relied on self-report data of cannabis use. However, we measured the

presence of cannabis metabolites in urine in the majority of our sample and there is no clear reason to assume that the over- or underreporting is over-represented in subjects with high or low cape scores. Nor is there a reason to assume that the distribution of inaccurate measurement of cannabis exposure is unequal and has led to bias. A final limitation pertains the fact that using a selected sample of participants is useful for increasing power but limits the generalizability of the findings.

Overall, we identified a putative role for the P2X7 receptor in the biological mechanisms underlying the relation between cannabis and psychosis. These results warrant further study on the role of P2X7 and its crosstalk with cannabinoids and the endocannabinoid system in psychosis using cell and animal models. Considering the recent interest in treatment of neuroinflammation through P2X7 antagonists and the emergence of new P2X7 antagonists (Pevarello *et al*, 2017) such approaches seem highly relevant.

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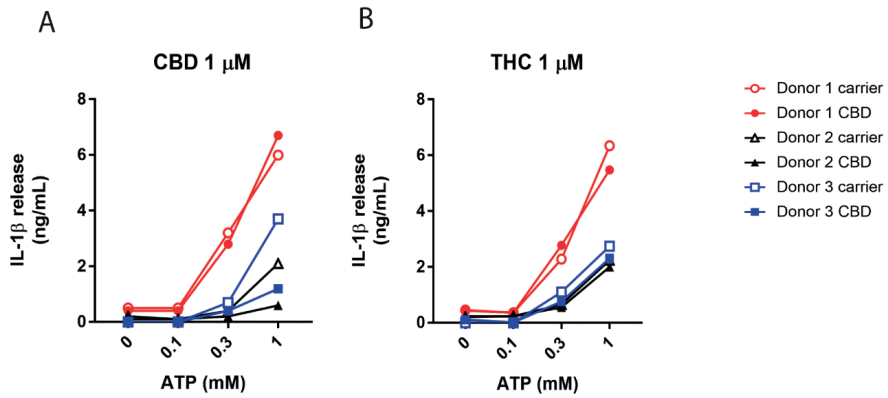

Supplementary figure 1. The impact of the cannabinoids THC and CBD on P2X7 function *in vitro*. LPS-stimulated monocytes were exposed to CBD, THC (both 1 μM) or the ethanol carrier as control. The cells were subsequently stimulated with different concentrations of ATP to measure the release of IL-1β: one of the key inflammatory functions of the P2X7 receptor. A,B) A dose response curve of IL-1β release to ATP is presented for CBD 1μM (A) and THC 1μM (B) in three different healthy control donors.

Part II

Famine exposure as a detrimental environmental factor

Chapter 4

DNA methylation changes related to nutritional deprivation

A genome-wide analysis of population and in vitro data

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Clinical Epigenetics
In revision

Abstract

Background

DNA methylation has recently been identified as a mediator between *in utero* famine exposure and a range of metabolic and psychiatric traits. However, genome-wide analyses are scarce and cross-sectional analyses are hampered by many potential confounding factors. Moreover, causal relations are hard to identify due to lack of controlled experimental designs. In the current study we therefore combined a comprehensive assessment of genome-wide DNA methylation differences in people exposed to the great Chinese famine *in utero* with an *in vitro* study in which we deprived fibroblasts of nutrition.

Methods

We compared whole blood DNA methylation differences between 25 individuals *in utero* exposed to famine and 54 healthy control individuals using the HumanMethylation450 platform. *In vitro* we analyzed DNA methylation changes in 10 fibroblasts cultures that were nutritional deprived for 72 hours by withholding fetal bovine serum.

Results

We identified three differentially methylated regions (DMRs) in four genes (*ENO2*, *ZNF226*, *CCDC51*, and *TMA7*) that were related to famine exposure in both analyses. Pathway analysis with data from both Chinese famine samples and fibroblasts highlighted nervous system and neurogenesis pathways as the most affected by nutritional deprivation.

Conclusions

The combination of cross-sectional and experimental data provides indications that biological adaptation to famine lead to DNA methylation changes in genes involved in the central nervous system.

Background

DNA methylation is one of the epigenetic mechanisms that plays an important role in the cellular responses to detrimental environmental influences that are involved in the etiology of many diseases (Schübeler, 2015). Studies show that early life exposure to nutritional deprivation is associated with stable DNA methylation differences (Cho *et al*, 2015; Heijmans *et al*, 2008). Nutritional deprivation, especially *in utero* and early in life has detrimental effects on human development and significantly increases the risk of multiple chronic diseases later in life (Finer *et al*, 2016; Heijmans *et al*, 2008; Huang *et al*, 2010; Tobi *et al*, 2018).

A seminal example of the impact of *in utero* exposure to nutritional deprivation is the cohort study on offspring from mothers that were pregnant during the Dutch hunger winter during the second world war, which was intense, well-documented but with brief duration (Lumey *et al*, 2007). This study identified persistent differential methylation of the insulin-like growth factor II (IGF2), as a key human growth and development factor involved in the response to famine *in utero* (Heijmans *et al*, 2008). Subsequent studies of this cohort identified DNA methylation changes as mediators of the association between maternal famine and metabolic disease in adulthood (Tobi *et al*, 2014, 2018). Other epigenetic differences associated with famine exposure *in utero* have been related to schizophrenia (Boks *et al*, 2018b) and type 2 diabetes (Vaiserman, 2017).

While the Dutch Famine is the most extensively studied famine in the literature, the Chinese great famine (1959-1961) was one of the largest famines recorded around the world and had more severe consequences resulting in an estimated 30 million deaths (Ashton *et al*, 1984). The offspring of those mothers who suffered famine were shorter in length (Huang *et al*, 2010), had worse midlife health (Fan and Qian, 2015), and had a higher rate of chronic diseases (Li and Lumey, 2017; Sun *et al*, 2018). Studies also showed a two-fold increased risk to develop schizophrenia among offspring conceived at the height of famine (St Clair *et al*, 2005; Xu *et al*, 2009). However, only one genome-wide DNA methylation study is reported in Chinese famine population (Boks *et al*, 2018a). To further understand the impact of maternal famine on DNA methylation changes in offspring, we compared genome-wide DNA methylation from whole blood of Chinese participants exposed to famine in the first trimester to unexposed controls from the same populations.

Since a cross-sectional population-based study is subject to residual confounding and does not allow the examination of the direct effect of nutritional deprivation, we subsequently performed an *in vitro* study of human fibroblasts before and after exposure to nutritional deprivation. By combining the result of a genome-wide methylation approach of both studies, we aim to provide an unbiased investigation of DNA methylation changes induced by nutritional deprivation.

Methods

Chinese famine sample

The sample of Chinese famine is part of our previous study and has been described in more detail elsewhere (Boks *et al*, 2018b). In short, volunteers were recruited in the northern province of Jilin, China. Considering the almost complete penetration of famine during January 1960 and September 1961, it is assumed that those born during that period will have been exposed. A total of 79 healthy participants were included of which 25 were exposed to famine during the first three months *in utero*. All participants provided written informed consent. **Table 1** gives the full details of the participants.

Fibroblast in vitro study

The description of in vitro fibroblasts experiment in more detail before (Boks *et al*, 2018b). In short, fibroblasts were obtained by skin biopsies from five healthy participants of Dutch descent, of which one was male and four were female (mean age=38.4 year, sd=7.0). See **Table 1**. All participants provided written informed consent. Fibroblasts were plated in two T25 flasks in Minimum Essential Medium (MEM) (Gibco®) with 15% fetal bovine serum (FBS)(Gibco®), 1% Penicillin Streptomycin PenStrep (Gibco®), and in an atmosphere of 95% atmospheric air, 5% CO2, at 37 ˚C (normal conditions). After reaching 70- 80% confluence, the supernatant was removed and the cells were washed three times with phosphate buffered saline (PBS) (BioWhittaker® Reagents, Lonza). Next, one of the T25 flask from each donor was cultured in the non-famine condition with Minimum Essential Medium (MEM) (Gibco®) supported with 15% FBS, while the other T25 flask was cultured in only Minimum Essential Medium (MEM) as famine condition. After 72 hours cells were harvested from each flask and stored as cell pellet for DNA isolation.

Table 1. Summary of characteristics of the Chinese famine samples.

	Unexposed	Exposed to maternal famine
N	54	25
Age (sd)	46.8 (1.0)	50.3 (0.5)
Male N (%)	21 (39%)	10 (40%)

DNA processing

DNA from Chinese famine samples was extracted from whole blood using the Gentra Puregene Kit (Qiagen, Valencia, CA, USA). Fibroblasts cell pellets were used for DNA isolation according to manufacturer's instruction (Qiagen, Hilden, Germany). The DNA concentration and quality was examined using Nano drop (Thermo Fisher Scientific, Massachusetts, USA). Bisulfite conversion of each DNA sample was conducted according to the manufacturer's instructions of the Zymo EZ DNA MethylationTM kit (Zymo, Irvine, CA, USA). Single stranded bisulfited DNA was quantified and qualified with the NanoDrop.

Genome wide analysis of DNA methylation

One hundred and fifty nanograms of bisulfite converted DNA from Chinese famine study was used to quantify genome-wide patterns of DNA methylation using the Illumina Infinium HumanMethylation450 BeadChip. Genome-wide DNA methylation levels of fibroblasts were obtained using Illumina HumanMethylation EPIC BeadChip arrays. For the Chinese famine samples, intensity read outs, beta and M-value calculation and cell-type proportion estimates were obtained using the minfi package (version 1.10.2) in Bioconductor (Aryee *et al*, 2014). Probes were excluded based on a bead count less than three (n=279 probes) or a detection p-value larger than 0.001 in at least 5% of the samples (n=2,125 probes). Non-autosomal or cross hybridizing probes were discarded as were loci with SNPs of Minor Allele Frequency larger than 1 percent within 1 base pairs of the primer (Barfield *et al*, 2014). None of the blood samples had over 1% of failed probes. All 79 DNA samples survived quality control (Chen *et al*, 2013) and 397,985 loci were left in the dataset for further analysis. The minfi package for normalization includes: Cell-type composition estimates including B cells, CD8 T cells, CD4 T cells, natural killer cells, monocytes and granulocytes (another well-known potential confounder in whole blood samples) by Houseman algorithm (Houseman *et al*, 2012); the first two DNA methylation-based ancestry principal components (Barfield *et al*, 2014) and batch effect.

The quality control for fibroblasts was performed in a similar workflow as the Chinese famine samples but adjusted to the newer EPIC methylation beadchip. The dataset was pre-processed in R version 3.3.1 with the meffil package (Min *et al*, 2017) using functional normalization (Fortin *et al*, 2014) to reduce the non-biological differences between

probes. To account for technical batch variables pre-processing was performed in a larger dataset (n= 80), including DNA samples of other studies that included brain and blood DNA. However, normalization was conducted for the fibroblast samples only. Samples were removed if the reported sex did not match the methylation-predicted sex (n=0), the sample was an outlier on mean methylated and unmethylated channels (n=0). Probes were removed if they failed quality control (a detection P-value >0.01 for >10% of samples (n= 4610) or a bead count < 3 for >10% of samples (n= 68)), were nonspecific (Chen *et al*, 2013) or were one of the SNP probes included on the array for quality control purposes. All 10 fibroblast DNA samples survived quality control and 862,160 probes were left in the dataset for further analysis.

For both Chinese sample and fibroblast samples, the level (percentage) of methylation is expressed as a β-value, ranging from 0 (unmethylated cytosine) to 1 (completely methylated cytosine), but analyses were performed using M-values (log2 of beta values), for better statistical validity (Du *et al*, 2010). To examine the overlap between the results of the two datasets, DMR and pathway analyses were performed for the 397,985 CpGs that were present on the EPIC as well as the 450k arrays.

Pathway analysis

We performed gene set enrichment analysis (GSEA) for the nominal significant CpGs that overlapped from Chinese famine and fibroblasts samples. SetRank tool was chosen in the current study for GSEA analysis since it could eliminate many false positive hits (Simillion *et al*, 2017), especially those biased toward neuronal pathways as these genes are much more abundant and larger in size. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Wikipathways and Reactome pathway database are included in the SetRank tool.

Statistical analyses

Statistical analyses were carried out using R (R Core Team, 2014). Analysis of the association of DNA methylation with famine in the Chinese famine samples was performed using linear regression with DNA methylation as dependent and famine, age, gender, cell-type proportion estimates based on the Houseman algorithm (Houseman *et al*, 2012) as well as the first two DNA methylation-based ancestry principal components as indicators(Barfield *et al*, 2014). In addition, similar as previously we adjusted for the effects of smoking by deriving a proxy for smoking based on methylation levels of CpGs that were previously associated with smoking (Hannon *et al*, 2016). For the fibroblasts experiment, methylation changes under the famine condition was assed using wilcoxon paired rank test. The QQ plots were inspected to assess type I error inflation and power (addition file 1). DMRcate (version 1.4.2) was used to identify differentially methylated regions (DMRs) with all the CpGs nominal significantly associated to famine from both Chinese famine and fibroblasts samples (p<0.01) (Peters *et al*, 2015). Only DMRs with the same direction of effect (hyper- or hypomethylation) in both samples were considered overlapping.

Results

Identification of differentially methylated regions (DMRs)

Analysis of single CpG methylation did not identify significant differences after adjustment for multiple testing due to insufficient power. The QQ plot indicated the analysis was underpowered to detect genome-wide differentially methylated probes (Supplemental file 1 shows the QQ plots). Supplemental file 2 provides the information and test statistics of the nominally associated loci (18871 for the Chinese famine and 56375 for the fibroblast experiment). Analysis of DMRs in the Chinese famine cohort identified 613 different methylated regions (DMRs) and 1080 DMRs in fibroblasts samples. Among these significant DMRs, three DMRs were similarly associated (significant and same direction of effect) in both samples. The three replicated DMRs are all hypomethylated in relation to famine exposure and highlight four gene promoters. DMR1: enolase 2 (*ENO2*) (cg08003732, cg13334990, cg18912645, cg19720347). DMR2: zinc finger protein 226 (*ZNF226*) (cg19331658, cg03559973, cg19836894, cg19599862, cg03573702). DMR3 is related to 2 gene promoters: coiled-coil domain containing 51 (*CCDC51*) and translation machinery associated 7 homolog (TMA7) (cg00329014, cg06625258, cg07744328, cg01538982, cg24981564, cg12370248, cg07095599, cg11196693, cg03629318, cg15853329, cg21856689, cg26094714, cg25858682). Study from Hannon et al. (Hannon *et al*, 2015) were used to compare the methylation profile in the blood with brain. The result showed that cg08003732 and cg13334990 loci in *ENO2* gene were all significantly correlated with 4 brain regions [prefrontal cortex (PFC), entorhinal cortex (EC), superior temporal gyrus (STG), and cerebellum (CER)]. Other loci significantly corrected brain regions are: cg19331658 in *ZNF226* and cg26094714 in *CCD51/TMA7* were corrected with PFC; cg18912645 in *ENO2*, cg12370248 and cg15853329 in *CCD51/TMA7* were corrected with EC; cg19720347 in *ENO2* is corrected with CER. **Table 2** shows the characteristics of the DMRs consistently associated to famine in both experiments.

Table 2. Three DMRs consistently associated with famine in both experiments (Chinese famine samples and fibroblasts samples)

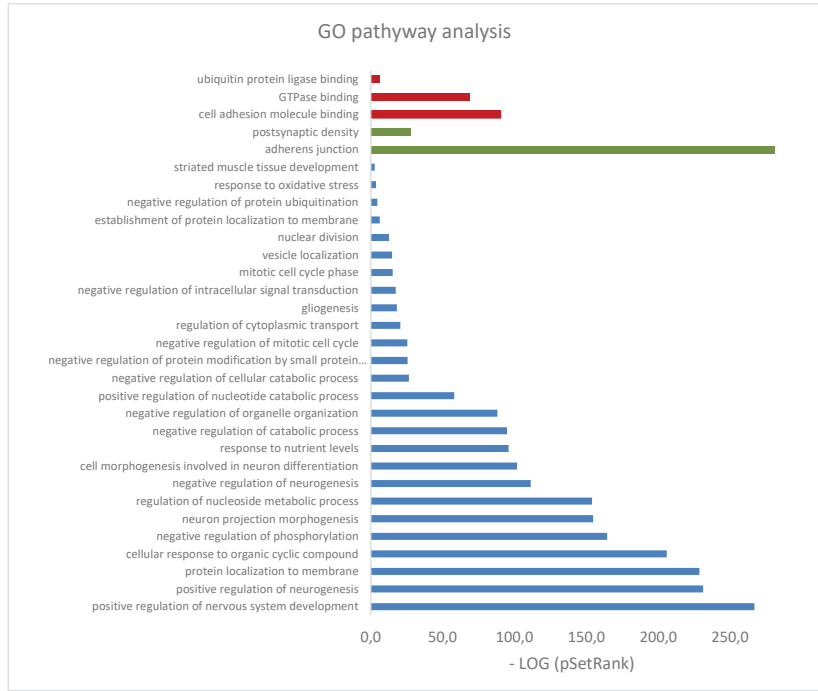
DMRs	Gene promoters	CHR	Region (hg19)	CpG numbers	β value_ Chinese	p value_ Chinese	β value_ Fibroblasts	p value_ Fibroblasts
DMR1	*ENO2*	chr12	7023752 - 7024121	4	-0.0243	1.19E-04	-0.1523	7.42E-04
DMR2	*ZNF226*	chr19	44669146 - 44669354	5	-0.0636	9.21E-03	-0.3155	1.07E-03
DMR3	*CCDC51* *TMA7*	chr3	48481268 - 48481793	13	-0.0290	7.87E-04	-0.2318	1.25E-06

DMR differentially methylated regions; CHR chromosome; hg19 human genome version 19 The first column of the table shows DMR identifier and followed by the gene name which belongs to the DMR. The chromosome of the gene is provided and followed by the more precise region in hg19 (human genome version 19). The number of significant CpGs response to nutrition deprivation in both studies is presented and β value and p value of DMRs in both studies are also presented. β value in each study refers to the mean β values of identified CpG in each DMR.

Pathway-analysis of identified CpG loci

The number of overlapping CpGs identified from both Chinese famine and fibroblasts is 2706. **Figure 1** show the significant pathways that are associated with all the identified CpGs based on GO, KEGG, Wikipathways, and Reactome pathway databases. GO pathway analysis highlighted three significant molecular function pathways, among which cell adhesion molecule binding is mostly prevalent. Adherens junction is most relevant regarding to the cellular components. In addition, we found that the famine condition influenced a wide range of biological processes, among which neuronal systems are most strongly implied. For example, pathways in nervous system development, both positive and negative neurogenesis and neuron projection morphogenesis are highly involved. The pathway analysis from significant Reactome and WikiPathways analysis showed that DNA damage response and signalling by nerve growth factor (NGF) are mostly involved by nutritional deprivation.

A



B

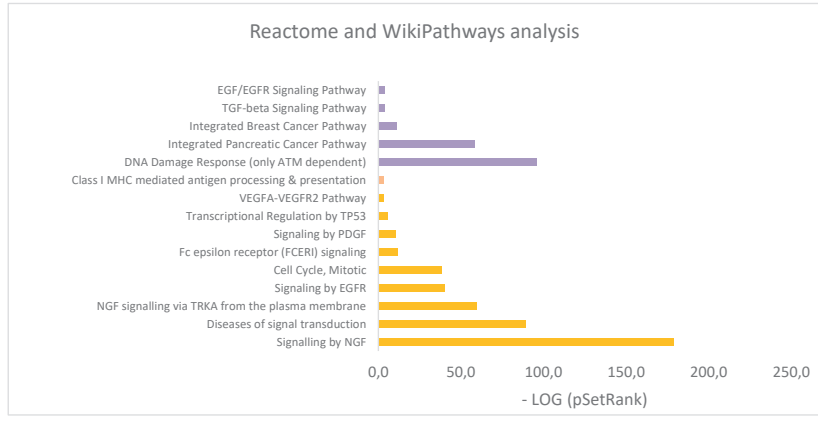


Figure 1. Significant pathways analysis based on different databases.
A Significant pathways from GO analysis. Pathways in red represent molecular functions, in green represent cellular components and in blue represent biological processes. X-axis displays the minus log p-value of the association with the SetRank value of the gene set. **B** Significant pathways analysis from Reactome and WikiPathways. Reactome pathway in purple and WikiPathways in orange. X-axis displays the minus log p-value of association with the SetRank value of the gene set.

Discussion

This is the first study that combines genome-wide DNA methylation analysis of famine exposure with an *in vitro* study of nutritional deprivation to explore the effect of famine on DNA methylation. The results highlight several gene promoters that are differentially methylated due to nutritional deprivation. Further pathway analysis show that the nervous system development and signaling by nerve growth factor (NGF) are sensitive to nutritional deprivation.

Analysis of the overlapping DMRs from Chinese famine samples and *in vitro* fibroblast samples identified three DMRs in four gene promoters (*ENO2*, *ZNF226*, *CCDC51* and *TMA7*) that are consistently hypomethylated in relation to nutrition deprivation in both Chinese famine and fibroblasts *in vitro* samples (**Table 2**). The fact that famine is consistently linked to hypomethylation and no occurrences of hypermethylation were identified suggests reduced methylation efficacy, for instance due to the limited production of the methyl donor S-Adenosyl methionine (SAMe) which is dependent on nutrients such as folate, vitamin B1, B6, and B12. Genes identified in the current study have a wide range of functions but considering the largest effect size and relatively high levels of methylation, involvement of the gene *ENO2* is one of the most interesting findings. *ENO2* is abundantly expressed neurons and peripheral neuroendocrine tissue (Craig *et al*, 1990) and often used as neuron-specific reference genes (Gatta *et al*, 2017; Guidotti *et al*, 2000; Teocchi *et al*, 2013). Functional studies showed that *ENO2* promotes cell proliferation, glycolysis, and glucocorticoid-resistance (Liu *et al*, 2018), and silencing of this gene was found to inhibit the growth of glioblastoma cells (Muller *et al*, 2012). Consistently, *ENO2* serves as a biochemical marker for tumors derived from neuronal and peripheral neuroendocrine tissues (Liu *et al*, 2018). Furthermore, *ENO2* is found to be higher expressed in the brain of schizophrenia (SCZ) patient as compared to controls and may affect glucose metabolism in SCZ patients (Martins-de-Souza *et al*, 2009). Moreover a recent study found *ENO2* hypermethylation in autism alongside with decreased transcription and translation of this gene (Wang *et al*, 2014). A look-up in BECon (Edgar *et al*, 2017), an online database to compare the methylation pattern between brain and blood, suggests that part of the DMR in *ENO2* (cg08003732) has a similar DNA methylation pattern in blood and brain tissue.

Interpretation of the involvement of zinc-finger protein gene *ZNF226* is less straightforward as not much is currently known about this specific gene. Zinc-finger proteins have a broad range of molecular functions and they are widely targeted for aberrant DNA hypermethylation during toxicant-induced malignant transformation (Severson *et al*, 2013), and as a driver of detrimental environment factor associated carcinogenesis, leading to suggestions of their suitability for cancer prevention (Rao *et al*, 2017). The third DMR identified, *CCDC51* is a protein coding gene, which is present in endosomes (Gosney *et al*, 2018). This gene is involved in several signaling pathways, such as B cell receptor activation (Schrader *et al*, 2016), micronucleus formation regulation (McIntyre *et al*, 2016), cellular senescence (Lopez *et al*, 2017), liver-specific microRNA binding (Fan *et al*, 2015), and tumor suppressor activity (D'Agostino *et al*, 2018); as well as kidney disease (Schmidts *et al*, 2013). Mouse *Ccdc51* gene is the target gene of miR-672-5p, which is highly expressed after steroid-induced osteonecrosis (Li *et al*, 2016). Considering that nutritional deprivation could potentially disturb steroid levels, the current finding of *CCDC51* hypomethylation raises the possibility of a relation between famine and steroid imbalance. The final DMR gene *TMA7* codes for the TMA7 protein and deletion of this gene is consistent with loss of proteins involved in ribosome biogenesis (Fleischer *et al*, 2006). Though the current finding is based on blood and fibroblasts, the database from Hannon et al shows that methylation of 4 identified loci are corrected with prefrontal cortex; 5 are corrected with entorhinal cortex; 2 are corrected with superior temporal gurus and 3 are corrected with cerebellum. This implies that blood methylation profile in some loci may serve as a proxy for methylation in these brain areas.

In the previous genome wide methylation study of the Dutch hunger winter 181 genes were identified through reduced representation bisulfite sequencing (RRBS) and a further 6 genes were verified in mass spectrometry-based EpiTYPER assay (Tobi *et al*, 2014). Later in a Bangladesh famine cohort, 7 epialleles were identified (Finer *et al*, 2016). Although the DMRs from these previous studies do not overlap with our DMRs, the DMRs are near genes from the same pathway. For example, *ZNF251* and *CCDC57* were identified in the Dutch hunger cohort, whereas in our study *ZNF226* and *CCDC51* are found differentially methylated. The different genetic background of the three famine cohort studies could be one of the explanations of these differences since the vulnerability to environmental factors could be inherent genetically (Schoenrock *et al*, 2017). Another explanation for the diverging results could be that although all three populations suffered from famine, the remaining food consumption pattern probably was quite different between countries. Differences in dietary nutrient intake could eventually lead to different patterns of malnutrition, and eventually lead to different outcomes.

The pathways most commonly related to malnutrition exposure are in the nervous system and neurogenesis. Specifically, positive regulation of nervous system development in the GO pathway analysis (blue in **Figure 1**); and nerve growth factor

(NGF) signaling in the WikiPathways analysis (orange in **Figure 1**). This points to the high relevance of epigenetic adaptations to famine for the brain (Delgado-Morales *et al*, 2017) (even though current study did not analyze brain). Impact of famine on the brain has been shown in rodent studies, that showed large epigenetic changes in the hippocampus in off springs of nutritional deprived rats (Xu *et al*, 2014).

Performing DNA methylation analysis on fibroblasts in addition to whole blood increases the diversity of the tissue types and strongly reduces the risk that residual confounding factors are driving the results. Fibroblasts provide a different tissue type and using longitudinal analysis within the same participants poses the opportunity to directly relate DNA methylation changes to famine. The replicating DMRs from fibroblasts and blood therefore provide compelling evidence that these are relevant genes that are involved in the response to malnutrition.

Some limitations should be considered when interpreting the current study. Other potential confounding factors such as blood pressure, diet etc. may also need to be considered, whereas based on the information of the current study, these potential factors were not able to be analyzed. The sample size of the Chinese famine cohort is relatively small and therefore power is limited. Also, the two tissue types were used both the blood and fibroblasts methylation may still not represent the situation in the developing brain. Finally, the genetic background from this study limits our conclusion on malnutrition response to Chinese and Dutch ancestry and may not represent other ethnic groups.

Conclusions

Using an unbiased genome-wide approach, the current study examined the association between DNA methylation and severe nutritional deprivation in two unique samples separately (Chinese famine and *in vitro* fibroblasts), and lead to the identification of DMRs that were consistently hypomethylated in both samples. The three DMRs in the four genes promoters *ENO2*, *ZNF226*, *CCDC51* and *TMA7*, and the involvement of the nervous system development and signaling by nerve growth factor (NGF) that are suggested by pathway analyses can provide new leads to understand the pathways from nutrition deprivation to disease.

Ethics approval and consent to participate

Informed consent was obtained for all participants.

Consent for publication

The manuscript does not contain any individual person's data in any form.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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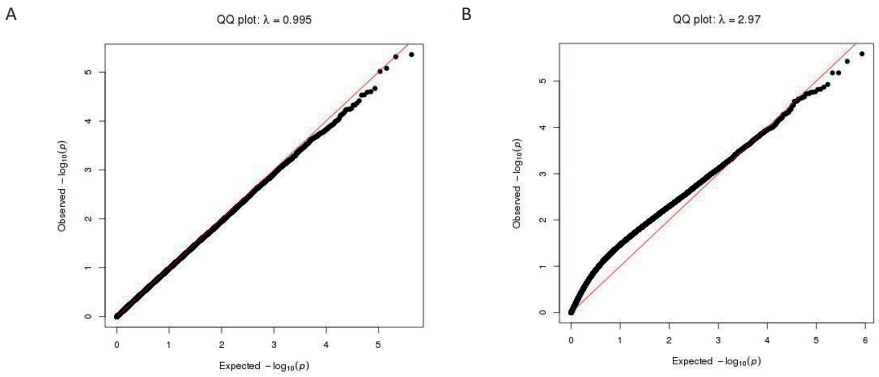
Supplemental Figure



Supplement Figure 1. QQplot of the p-value distribution for the famine status and DNA methylation in the Chinese famine (A) and fibroblasts (B) samples.
The examination of the QQplot indicated the analysis was underpowered to detect genome-wide differentially methylated probes.

Supplemental file 2 provides the information and test statistics of the nominally associated loci (18871 for the Chinese famine and 56375 for the fibroblast experiment).

Supplementary file 2 is available at request.

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Part III

Childhood adversity as a detrimental environmental factor

Chapter 5

Childhood adversity is associated with increased KITLG methylation in healthy individuals but not in bipolar disorder patients

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Abstract

Backgrounds

Childhood adversity increases the risk of a range of mental disorders including bipolar disorder, but the underlying mechanisms are still unknown. Previous studies identified DNA methylation levels at the cg27512205 locus on the KIT Ligand (*KITLG*) gene as a mediator between childhood adversity and stress responsivity. This raises the question whether this locus also plays a role in stress related disorders such as bipolar disorder. Therefore, the current study aims to compare the level of *KITLG* (cg27512205) methylation between bipolar patients and healthy individuals and its relation to childhood adversity.

Methods

KITLG (cg27512205) methylation was measured in 50 bipolar disorder patients and 91 healthy control participants using the HumanMethylation450K BeadChip platform. Childhood adversity in each individual was assessed using the Childhood Trauma Questionnaire. Analyses of the association of *KITLG* methylation with bipolar disorder, the association of childhood adversity with bipolar disorder as well as the association of *KITLG* methylation with childhood adversity in bipolar patients and controls were conducted using linear regression with age, gender, childhood adversity, smoking and cell-type composition estimates as covariates.

Results

KITLG (cg27512205) methylation level was significantly lower in bipolar disorder patients (β= -0.351, t = -6.316 $p < 0.001$). Childhood adversity levels were significantly higher in the bipolar disorder group (β= 4.903, t = 2.99, p = 0.003). In the bipolar disorder patients *KITLG* methylation was not associated with childhood adversity (β= 0.004, t = 1.039, p = 0.304) in contrast to the healthy controls (β= 0.012, t = 3.15, p = 0.002).

Conclusions

KITLG methylation was lower in bipolar disorder despite high levels of childhood adversity, whereas childhood adversity was associated with higher *KITLG* methylation in healthy controls. In addition to lower methylation at this locus there is an indication that failure to adjust *KITLG* methylation to high levels of childhood adversity is a risk factor for bipolar disorder.

Introduction

Bipolar disorder is a severe psychiatric disorder characterized by mood episodes ranging from mania to severe depression (Craddock and Sklar, 2013). The life time prevalence of bipolar disorder is 0.5-1.5% in the general population and 5-10% for first degree relatives (Craddock and Jones, 1999). Although the pathogenesis of bipolar disorder is not well understood, both genetic and environment factors are involved.

One major detrimental environmental factor for developing mental disorders including bipolar disorder later in life is childhood adversity (Aas *et al*, 2016; Varese *et al*, 2012). Childhood adversity encompasses a wild range of adversities before the age of 16, such as physical, emotional and sexual abuse, household poverty, separation from a parent and neglect. Previous studies found that children with childhood adversity have a high risk to develop bipolar disorder (Mesman *et al*, 2013). However, how childhood adversity contributes to the development of bipolar disorder is still largely unknown.

Recent studies highlight the role of DNA methylation in the pathway of childhood adversity to bipolar disorder (Fries *et al*, 2016). DNA methylation is one of the epigenetic mechanisms that can modulate gene expression in response to the environment might account for part of the risk to bipolar disorder (Ludwig and Dwivedi, 2016). Childhood adversity as a detrimental environmental factor could therefore, contribute to DNA methylation differences in key pathways involved in bipolar disorder. In our previous genome-wide DNA methylation analysis, KIT Ligand (*KITLG*) (cg27512205) methylation was positively associated with childhood trauma and served as a mediator between childhood trauma and blunted cortisol stress reactivity in healthy controls (Houtepen *et al*, 2016b). Since impaired cortisol stress reactivity is associated with bipolar disorder (Belvederi Murri *et al*, 2016; Zak *et al*, 2018), this could imply an association between *KITLG* methylation with bipolar disorder. Moreover, bipolar disorder patients also report higher levels of childhood adversity (Kefeli *et al*, 2018), which may lead to higher *KITLG* methylation if the previous findings in healthy controls were to be extrapolated to bipolar disorder patients.

Therefore, the current study hypothesizes the presence of higher *KITLG* methylation in bipolar disorder patients as compared to healthy controls in agreement with expected higher level of childhood adversity. To examine this hypothesis, we investigate the relationship between *KITLG* (cg27512205) methylation level in a case-control sample of bipolar disorder patients and healthy controls and the relation to childhood adversity.

Materials and Methods

Study population

Sample recruitment has been previously described (Houtepen *et al*, 2016a, 2016b). In short, 50 bipolar patients and 91 control participants were included at the University Medical Center Utrecht (UMCU). All participants had three or more Dutch grandparents. All participants provided informed consent prior to the inclusion of the study, and the study was approved by the Medical Ethics Committee of the UMCU and performed according to the ICH guidelines for Good Clinical Practice and the latest amendments of the Declaration of Helsinki. All the blood samples from the participants were drawn in the morning before 12AM. None of the healthy controls were taking any prescription medication at the time of testing nor did any of the participants ever participate in stress-related research before. To verify drug use, first self-report of current use of psychoactive substances was obtained followed by checking with urine multi-drug screening device (InstantView). If participants smoked daily, they were defined as a smoker. Confirmation of the absence of any mental or physical disorder in the healthy controls was obtained by an independent rater in an interview according to the Mini-International Neuropsychiatric Interview (MINI) plus criteria (Sheehan *et al*, 1998). For bipolar disorder participants only, the Structured Clinical Interview for DSM-IV (SCID) was used to diagnose the clinical characteristics, including mood and psychotic symptoms, number of manic and depressive episodes, comorbid psychiatric diagnosis and age of disease onset (First, M. B., Spitzer, R.L, Gibbon M., and Williams, 2002). Euthymia in the bipolar disorder patients was established using the Inventory for Depressive Symptoms - Self Report (IDS-SR) (Rush *et al*, 2000) and manic symptoms were assessed using the Altman Self-Rating Mania Scale (ASRM) (Altman *et al*, 1997). All patients were on a stable (at least 1 month) medication dose. The sample characteristics are provided in **Table 1**.

Childhood adversity

Childhood adversity was measured using the short version of the Childhood Trauma Questionnaire (CTQ) (Bernstein *et al*, 2003). The Dutch translation of CTQ and validity of the 25 clinical CTQ items has been demonstrated in clinical and population samples (Bernstein *et al*, 2003; Thombs *et al*, 2009). One translation item "I believe I was molested" was excluded since this translation was found to be an invalid indicator of childhood sexual abuse in a previous validation study (Thombs *et al*, 2009). We calculate the sum score of all individual abuse questions to generate a continuous outcome.

DNA methylation analyses

DNA methylation level of *KITLG* (cg27512205) was extracted from previously described Illumina Infinium HumanMethylation450K BeadChip data (Houtepen *et al*, 2016a). In short, DNA was obtained from blood using a commercial kit (Qiagen, CA, USA). The DNA concentration and integrity were assessed by riboGreen and BioAnalyser respectively. Bisulfite conversion was performed by using Zymo Kit (ZYMO Research, CA, USA). Samples were distributed on different chips based on gender and age to reduce batch effects. To remove further systematic differences, the samples were normalized using Beta MIxture Quantile dilation (BMIQ) and batch effects of sentrix array and position were removed with the Combat procedure from the sva package (Leek *et al*, 2016). Intensity and quality parameters were obtained from genome studio software. X chromosome, Y chromosome and nonspecific binding probes were removed (Chen *et al*, 2013). Based on literature (Schalkwyk *et al*, 2013), probes were excluded based on a detection P value >0.001 and bead count <5 in 5% of the samples. In addition, probes with SNPs of minor allele frequency >5% within 10 base pairs of the primer were excluded after constructing ancestry estimates as proposed by Barfield et al. (Barfield *et al*, 2014). 385,882 DNA methylation probes survived quality control, including the *KITLG* cg27512205 probe. All samples were included as none of the samples had more than 1% of probes failed. Cell-type composition estimates were derived using the Houseman procedure(Houseman *et al*, 2012). Methylation analyses were carried out using M-values (log2 ratio of methylation probe intensity) for better statistical validity (Du *et al*, 2010), but beta values of methylation were used for graphical display.

Statistical analysis

Quality control of DNA methylation was conducted with R version 3.1.2 (R Core Team, 2014). Other statistical analyses were performed using SPSS Statistics 23.0. Analysis of the association of *KITLG* (cg27512205) methylation with bipolar disorder was done using linear regression with *KITLG* methylation as dependent and diagnosis as the main determinant. Age, gender, childhood adversity, smoking and six different cell-type composition estimates (B cells, CD8 T cells, CD4 T cells, natural killer cells, monocytes and granulocytes) were included as covariates since they have a potential impact on DNA methylation (Boks *et al*, 2009). Differences in childhood adversity between patients and controls were examined using linear regression, in a separate model. This relation was analyzed while adjusted for age, gender and smoking status. The association between *KITLG* methylation and childhood adversity was analyzed by linear regression model in control and bipolar patients separately. Age, gender and smoking were included as covariates.

Results

Baseline characteristics of bipolar cohort

A summary of the sample characteristics of bipolar disorder cohort is provided in **Table 1**. In the bipolar disorder group, 46 participants were diagnosed with bipolar disorder I type and 4 with bipolar disorder II type. The mean age of participants in the control group was significantly lower than in the bipolar disorder (BD) group (control = 33.5, BD = 43.52, $p < 0.001$). The proportion of smokers was significantly higher in the BD group (control = 12.1%, BD = 36%, P = 0.001), but no relation was present between *KITLG* methylation and smoking status ($\beta = 0.001$, $t = 0.099$, $p = 0.922$). Childhood trauma score was significantly higher in bipolar group than in controls ($\beta = 4.903$, $t = 2.990$, $p = 0.003$; model fit: $F = 8.940$, $p = 0.003$, $R^2 = 0.060$), but these differences were attenuated after adjustment for age gender and smoking($\beta = 3.043$, $t = 1.817$, $p = 0.071$; model fit: $F = 8.498$, $p < 0.001$, $R^2 = 0.200$). In the bipolar disorder group, comorbid psychiatric diagnosis were: Anxiety disorder Not Otherwise Specified (NOS) (n = 1), Generalized anxiety disorder (n = 2), Panic disorders (n = 4); Agoraphobia without history of panic disorder (n = 1), Specific phobia (n = 2), Obsessive-compulsive disorder (n = 2), Posttraumatic stress disorder (n = 1). Considering to the low frequency of the comorbid psychiatric diagnosis in the bipolar disorder group, we do not specifically exam the association of each comorbid psychiatric diagnosis with *KITLG* methylation level.

Table 1. Sample characteristics (n=141)

Variable n (%) or mean (range)	Control	Bipolar disorder	p
Number, n	91	50	
Age, years; mean (sd)	33.50 (15.68)	43.52 (12.83)	<0.001
Female sex, n (%)	44 (48.4%)	25 (50%)	0.853
Smoking, n (%)	11 (12.1%)	18 (36%)	0.001
Age at onset, years; mean (sd)	None	26.37 (11.45)	
Number of episodes; mean (sd)	None	6.39 (5.12)	
Childhood trauma score (mean, sd)	31.77 (8.37)	36.56 (10.28)	0.004
Bipolar disorder group			
Bipolar I, n	None	46	
Bipolar II, n	None	4	
Bipolar disorder Not Otherwise Specified (NOS), n	None	0	

KITLG methylation analyses

KITLG methylation level was significantly lower in bipolar disorder patients compared to the healthy controls (mean control = 0.185, mean bipolar = 0.139) ($\beta = -0.351$, $t = -6.316$ $p < 0.001$; model fit: $F = 18.56$, $p < 0.001$, $R^2 = 0.407$) after adjustment for age, gender, childhood adversity, smoking and cell types. **Figure 1** shows the adjusted individual levels of *KITLG* methylation per diagnostic group. No association of medication (mood stabilizer, antidepressant and antipsychotics) with *KITLG* methylation was present in the bipolar disorder group: Mood-stabilizers ($\beta = 0.008$, $t = 1.153$, $p = 0.255$); antidepressants ($\beta = 0.006$, $t = 0.732$, $p = 0.468$) and antipsychotics ($\beta = -0.008$, $t = -1.279$, $p = 0.208$), (Model fit: $F = 0.937$, $p = 0.488$, $R2 = 0.135$).

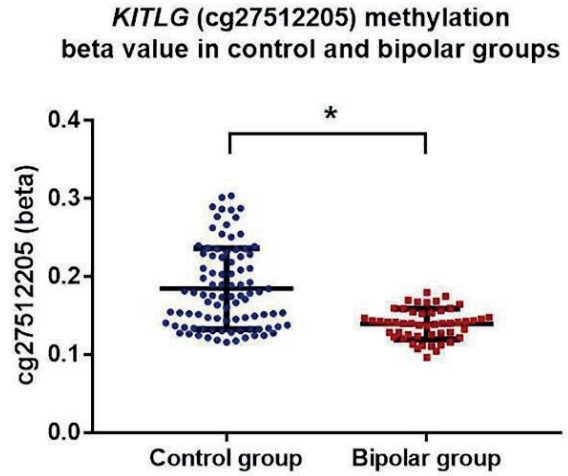

Figure 1. KITLG (cg27512205) methylation (beta value) in healthy controls (blue dots) and bipolar disorder patients (red square). Black bar on each column shows the standard deviation of beta value of KITLG methylation in each group. Mean methylation level of KITLG is significantly lower in the bipolar group (*p<0.001).

Association between childhood adversity and KITLG methylation

Figure 2 shows the association of *KITLG* (cg26512205) methylation level (beta value) with childhood adversity in both healthy controls and bipolar disorder patients. There was no significant association between *KITLG* methylation and childhood adversity in the bipolar disorder patients ($\beta = 0.004$, $t = 1.039$, $p = 0.304$), whereas there was a significant positive association between childhood adversity and *KITLG* methylation associated in the healthy individuals ($\beta = 0.012$, $p = 0.002$; model fit: $F = 23.11$, $p < 0.001$, $R^2 = 0.444$).

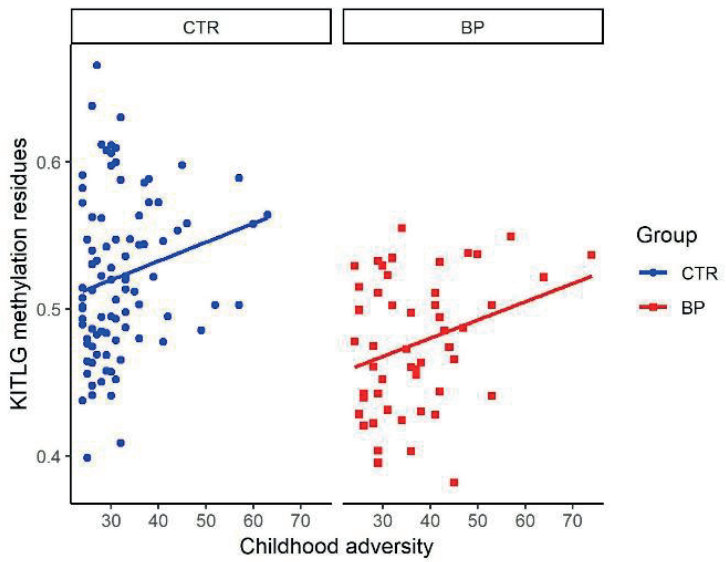

Figure 2. Association of KITLG (cg26512205) methylation level with childhood adversity in both healthy controls (blue dots) and bipolar disorder patients (red square). Y-axis is the beta value of KITLG (cg26512205) methylation level after adjustment for cell type composition, age, gender and smoking. X-axis is the childhood adversity score. Significant positive association between childhood adversity and KITLG methylation was present in the healthy individuals (β= 0.012, p= 0.002). No significant association between KITLG methylation and childhood adversity in the bipolar disorder patients was present (β = 0.004, t = 1.039, p = 0.304).

Discussion

Here, we follow up the previously reported association of *KITLG* methylation with childhood adversity and stress reactivity by exploring the relationship between *KITLG* DNA methylation levels at the locus cg27512205 and bipolar disorder. To our knowledge, this is the first study to report the association of *KITLG* methylation with bipolar disorder. We found lower DNA methylation levels at this stress related gene in bipolar disorder patients (n=50) than in healthy controls (n=91). In contrast to the positive association between childhood adversity with *KITLG* methylation in controls, we did not observe such an association in bipolar disorder patients. These findings suggest that failure to increase *KITLG* methylation in response to childhood adversity may constitute a risk factor for bipolar disorder.

Previously, we already reported of the positive association between *KITLG* methylation and childhood adversity in healthy controls (Houtepen *et al*, 2016b). It is this finding that led to the expectation of *KITLG* hypermethylation among bipolar disorder patients exposed to higher levels of childhood adversity. However, the current study found *KITLG* hypomethylation in bipolar disorder patients and no relationship between childhood adversity and *KITLG* methylation in this group. This finding is consistent with a model whereby *KITLG* hypermethylation after childhood adversity is adaptive and failure to adapt is a characteristic of bipolar disorder patients. However, visual inspection of the relations between childhood adversity and *KITLG* methylation (**Figure 2**) points to systematic lower *KITLG* methylation in bipolar disorder.

Although unexpected, these findings are consistent with other recent reports that the protein coded by *KITLG* gene, known as stem cell factor (SCF), is significantly higher in children of bipolar disorder patients who develop mood disorder later in life (Snijders *et al*, 2017). These higher levels of the *KITLG* protein SCF before disease onset are consistent with less repression on gene expression and transcription (Schübeler, 2015) and DNA hypomethylation at this locus. The specific *KITLG* locus (cg27512205, chr12: 88579621) that we focused on in the current study, is located in a H3K27ac-enriched region as well as on the 5' end of a CpG island near the *KITLG* gene. Mechanistically, DNA hypomethylation in the H3K27ac-enriched region is associated with a more open chromatin structure which indicates active gene transcription (Vermunt *et al*, 2014; Yang *et al*, 2014). Moreover, DNA methylation differences frequently occur in CpG island shores and subsequently affect gene transcription and expression (Irizarry *et al*, 2009). These two co-occurrences suggest that *KITLG* hypomethylation at this CpG locus could indeed alter gene transcription and SCF levels. Another factor that could influence gene transcription level are genetic variants. For instance, gene polymorphism of *FKBP5*, an important functional regulator of the glucocorticoid receptor (GR), can mediated gene–childhood trauma interactions through DNA methylation level (Klengel *et al*, 2013) and similarly genetic variants modify the methylation response to maternal famine (Boks *et al*, 2018). The *KITLG* locus in the current study contains just one genetic variant with no functional relevance for expression and therefore no indication of a role in genetic regulation is currently available.

A putative link between *KITLG* function and bipolar disorder is that the ligand of the C-kit receptor (SCF), is involved in hematopoiesis (Su *et al*, 2013), neurogenesis and neuroprotection (Zhao *et al*, 2007) and induces glucocorticoid receptor gene (*NR3C1*) expression in response to stress induced erythropoiesis (Varricchio *et al*, 2012). This implies a positive regulation of *KITLG* gene to *NR3C1* expression, a key gene in the

stress response (Palma-Gudiel *et al*, 2015; Schur *et al*, 2018), that in term plays a role in bipolar disorder (Belvederi Murri *et al*, 2016) and the response to trauma (Light *et al*, 2018; Perroud *et al*, 2011; Smart *et al*, 2015). Though the current finding is based on blood, the database from Hannon et al shows that methylation of this specific *KITLG* locus (cg27512205) in the blood is significantly correlated with prefrontal cortex and superior temporal gyrus in the brain (Hannon *et al*, 2015). This implies that blood *KITLG* methylation may serve as a proxy for *KITLG* methylation in these brain areas.

Some limitations need to be considered when interpreting these results. First, the focus on one specific locus (cg27512205) based on our previous work, could potentially neglect DNA methylation at other genes that play a role in bipolar disorder. Using available Illumina Infinium HumanMethylation450K BeadChip data, an unbiased genome-wide DNA methylation analysis to investigate the interaction between bipolar disorder and childhood adversity may further our understanding of epigenetic difference related to childhood adversity and bipolar disorder. Second, though for some epigenetic loci blood may provide a reasonable proxy based on concordances in methylation patters between blood and brain (Davies *et al*, 2012; Horvath *et al*, 2012), it is a limitation considering that bipolar disorder is a psychiatry disorder residing largely in the brain. Another limitation of the study is that the Illumina 450k BeadChip cannot distinguish between 5-Methylcytosine and 5-Hydroxymethylcytosin.

In conclusion, this study shows that *KITLG* methylation level is significantly lower in bipolar disorder despite relatively high childhood adversity exposure in bipolar disorder patients. This suggests a failure to adjust this epigenetic mark in response to childhood adversity in those vulnerable to bipolar disorder.

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Chapter 6

General Discussion

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The research performed in this thesis focused on three detrimental environmental factors associated with either gene transcription or DNA methylation or that showed interaction with genotype in an etiological pathway to psychosis. In this chapter, a summary of each study is provided followed by a general discussion.

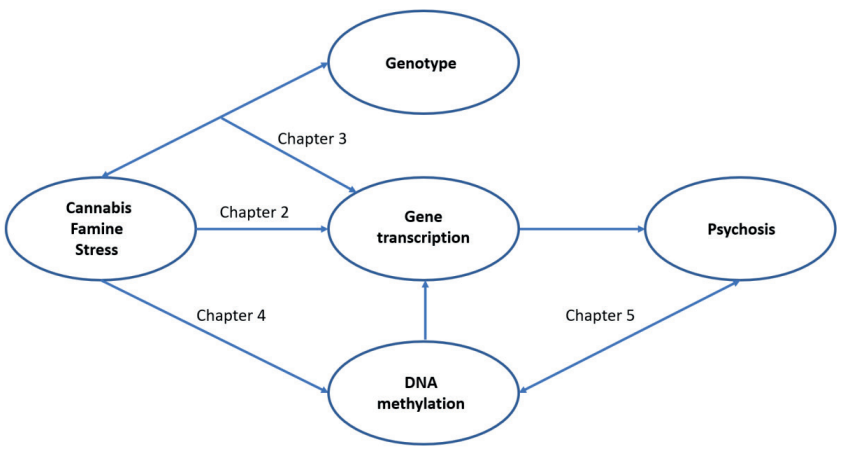

Figure 1. Overview of the main subject discussed in the thesis and the connection between them.

Summary

Change in *PPFIA2* gene expression in response to cannabinoids is a putative mechanism by which cannabis could influence neuropsychological functions

The genome-wide gene expression analysis in whole blood RNA of heavy cannabis users and cannabis naïve participants showed that *PPFIA2* expression is significantly higher in heavy cannabis users (chapter 2). This result was further confirmed by an *in vitro* model, where *PPFIA2* expression was significantly increased after a cannabinoid challenge in monocytes. Apart from higher *PPFIA2* expression, heavy cannabis users also showed significantly lower cognitive performance as measured with the Wechsler Adult Intelligence Scale (WAIS). Further exploration of association between *PPFIA2* expression and neuropsychological function showed a negative relation of *PPFIA2* expression with neuropsychological function in all participants, including those without cannabis use. These results indicate *PPFIA2* could be a potential link between cannabis use and neuropsychological functions.

Genome-wide environment-interaction study (GWEIS) on cannabis use determined P2X7 receptor polymorphism could mediate risk of psychosis

Although the cannabis use has been recently suggested as a casual factor for schizophrenia, the majority of cannabis users do not develop psychosis. Therefore, the individual genetic background is likely to modify the effects of cannabis. In chapter 3 we reported that the P2X7 receptor polymorphism (rs7958311) moderates the cannabis mediated risk of psychosis: heavy cannabis users with the A allele of the *P2RX7* gene have a significant higher risk to psychotic experiences. Further *in vitro* exploration showed that the genetic variant of *P2RX7* gene (A allele) indeed could influence the P2X7 receptor function by reducing ATP induced IL-1β release under challenge of cannabinoids. Considering the abundant expression of *P2RX7* in immune cells in the CNS and its potential impact on neuroinflammation and neurodevelopment the altered P2X7 receptor function could therefore be linked to the pathogenesis of psychotic disorders by means of immune regulation of neurotransmitter release and long-term potentiation linked to pathogenesis of psychotic disorders.

Prenatal nutritional deprivation is associated with DNA methylation in nervous system

Nutritional deprivation as a detrimental environmental factor that changes the epigenome was explored in chapter 4. We combined the analysis from a cross-sectional study (Chinese famine sample) with an *in vitro* study (*in vitro* nutritional deprivation of fibroblasts). Four genes (*ENO2*, *ZNF226*, *CCDC51* and *TMA7*) in three differentially methylated regions (DMRs) were identified that are associated with famine exposure. Among these identified genes *ENO2* is one of the most interesting, since it is highly specific for neurons and is found to be associated with schizophrenia (SCZ) and bipolar disorders. With previous reports of two-fold increase of schizophrenia among offspring who had prenatal famine, *ENO2* gene could be a new link between nutritional deprivation and psychosis. Further pathway analysis from both samples showed nervous system and neurogenesis are most widely and strongly associated with nutritional deprivation. These findings provide strong indications of the involvement of DNA methylation changes in the biological adaptation to famine.

Childhood adversity is associated with increased KITLG methylation in healthy individuals but not in bipolar disorder patients

Childhood adversity as a detrimental environmental factor was interrogated in both healthy individuals and bipolar disorder patients in chapter 5. We found that childhood adversity is positively associated with *KITLG* methylation in healthy individuals. However, there is much weaker association in bipolar disorders. Though bipolar disorder patients experienced higher childhood adversity, they displayed significantly lower *KITLG* methylation.

General approach of this thesis

In this thesis we tried to understand the effects of detrimental environmental factors for psychosis in relation with their genomic impacts. The environmental factors on which we focused in this thesis are: cannabis use, nutritional deprivation, and childhood adversity. These three detrimental factors occur in different development stages of an individual and have profound psychiatric effects later in life: Prenatal maternal famine is associated with a two-fold increased risk of schizophrenia in offspring (Brown and Susser, 2008; St Clair *et al*, 2005); childhood adversity is associated with an increased risk of developing bipolar disorder (Mesman *et al*, 2013); heavy cannabis use is linked with higher chance of developing schizophrenia (Vaucher *et al*, 2017). However, not all the individuals who are exposed to detrimental environmental factors eventually develop psychosis. This led us to interrogate whether individual genetic factors modify the vulnerability for the diseases.

In chapter 2 and 3, we performed genome-wide analysis in cross-sectional studies to examine which genomic factor is involved in heavy cannabis associated neuropsychological function and psychotic vulnerability, respectively. In chapter 4, a genome-wide methylation study was performed to explore the association between maternal famine and DNA methylation. This genome-wide strategy is hypothesis-free, and not based on previous knowledge of which genomic factors are involved. Therefore, the genome-wide method can uncover new genetic leads in understanding the effects of cannabis on psychotic vulnerability. With this approach, we identified a higher *PPFIA2* gene expression in heavy cannabis users and found that the transcript levels related to lower neuropsychological function. We identified that a P2X7 receptor polymorphism could mediate the risk of psychosis; and we provided evidence of epigenetic alterations in nervous system genes in response to nutritional deprivation.

However, these cross-sectional studies also have several important drawbacks: Firstly, the confounding effects of characteristics such as age, gender, smoking and other detrimental environmental factors could potentially affect the results. In the cross-sectional studies in this thesis we reduced their influence by either matching the samples with confounding factors, or we adjusted for these confounding factors when performing the analysis. In this way we could minimize the effects of these interferences and we could determine the real signal. Secondly, a challenge was the source of the biomaterials, as it is impossible to get CNS biopsies from the living donors, blood is the most frequently used proxy in cross-sectional studies. However, these samples contain many different cell types, and it is not clear from our data which cell type contributed to the differences we found in blood. In chapter 4 and 5 the cell-type proportion was estimated using either the Houseman algorithm (Houseman *et al*, 2012) to minimize the effect from different cell types. Thirdly, another disadvantage of cross-sectional studies is that they cannot confirm the causal effects of detrimental environmental factors. The *in vitro* approach was implemented not only to rule out the confounding effects from cross-sectional studies, but also to target a specific cell type or to focus on one complex gene pathway. Therefore, the studies in chapter 2 and 3 combined epidemiological genetic studies for gene discovery with cellular studies that further interrogate the functional meaning of the identified genes. In chapter 4, we combined the genome-wide DNA methylation data from both cross-sectional study and an *in vitro* study, and use the overlapping differentially methylated regions (DMRs) to identify the key pathways of nutritional deprivation. Finally, another limitation of current genome-wide cross-section studies is the relatively small sample size which decreased the statistical power. To maximize the power, in chapter 2 and 3, we performed an extreme sampling method by including either cannabis naive

or heavy cannabis users. Moreover, in chapter 3, samples were also polarized by top or bottom quintile of total scores of psychotic experiences as measured by the CAPE score.

Taken together, by combining unbiased genome-wide approach and subsequent specific *in vitro* studies the current thesis provides new insights in how detrimental environmental factors interplay with genomic factors and may contribute to psychosis.

Interrelation of genomic measures (genotype, epigenetics and transcriptome)

The current thesis describes genome-wide data for gene expression, genotype, and DNA methylation and their interaction with detrimental environmental factors. Earlier studies have shown that these genome factors also display an inter-relationship on a genome-wide scale (Roadmap Epigenomics Consortium *et al*, 2015). The correlation between SNPs and gene expression is represented as expression quantitative trait loci (eQTLs), the correlation between SNPs and methylation is represented as methylation quantitative trait loci (mQTLs), and correlations between gene expression and methylation is represented as expression quantitative trait methylations (eQTMs). These inter-relations of genomic factors could potentially link with diseases (Grundberg *et al*, 2013).

As a reflection of the importance of the relation between genomic layers, recent study found widespread eQTLs in the transcriptome of the frontal cortex of schizophrenia individuals, many of these transcriptomes overlap with previously identified 108 schizophrenia risk loci (Jaffe *et al*, 2018). The eQTLs pattern showed that 48.1% of the genetic risk loci in schizophrenia were associated with nearby gene expression. This provided a strong eQTL link between genotype and phenotype and helped to annotate the putative biological relevance of disease associated loci. In chapter 2, we analyzed cis-eQTLs after identified higher expression of *PPFIA2* gene in the heavy user cannabis group, to examine whether 272 SNPs around the gene were associated with *PPFIA2* expression level. The result showed no such association of eQTLs around *PPFAI2*, thus strengthen our finding that it was cannabis use instead of the genetic variation that accounted for the differentially expression of *PPFIA2*. In chapter 3 we examined *P2RX7* expression level in rs7958311 A and G carriers, and we did not find an association between genotype and gene expression level. However, the P2X7 function differed between the two genotypes, implying that the interaction between the genetic variant with P2X7 function may occur through another mechanisms, such as allosteric alterations in the receptor.

The mQTLs of many risk variants across the catalog of GWAS-associated loci within psychosis has been recently studied (Hannon *et al*, 2015, 2017, 2018; Jaffe *et al*, 2015). mQTLs were significantly enriched for risk variants across the catalog of schizophrenia GWAS-associated loci (Jaffe *et al*, 2015). It suggested that DNA methylation levels are a more proximal read out of genetic variation than the gene expression levels, since 59.6% of genome-wide significant genetic loci for schizophrenia risk were associated with local DNA methylation levels, whereas only 16.7% of the GWAS-positive loci are eQTLs across the human brain (Ripke *et al*, 2014). Moreover, the mQTL analysis from the Autism spectrum disorders (ASD) cohort showed that a polygenic risk score was associated with an increase in DNA methylation, especially in two loci which were identified in a previous ASD GWAS study (Hannon *et al*, 2018). Though the casual relation between these two genomic factors is still not clear, it highlights the utility of polygenic risk scores for identifying molecular pathways associated with etiological variation.

By matching DNA methylation from prefrontal cortex to gene expression data from the same donor, Jaffe *et al*. found that many of these DNA methylation changes were associated with nearby gene expression levels (Jaffe *et al*, 2015), however, the relationship between DNA methylation and gene expression (eQTMs) is still not fully clear. The gene expression may be functionally regulated by methylation, or methylation is a reflection or an independent mark of gene expression levels (Gutierrez-Arcelus *et al*, 2013). Furthermore, even if DNA methylation plays an active role in changing gene expression, it depends on the location of the DNA methylation on the genome whether this active role will lead to an increase or decrease in gene expression. Generally, DNA methylation in the gene promoter region is thought to repress gene expression, whereas methylation in the gene coding region is associated with active gene expression (Jones, 2012). When the DNA methylation occurs, the added methyl group is makes it psychically difficult for a transcription factor to bind to a gene, thus it will reduce the accessibility of DNA to the transcriptional machinery. Genome-wide studies indicated that irrespective the location of methylation, the relation between DNA methylation and gene expression can be both negative and positive (van Eijk *et al*, 2012; Wagner *et al*, 2014). Though the mechanism of position association between DNA methylation and gene expression is still largely unknow, studies suggested that when DNA methylation occur at CTCF-binding site, it promotes RNA polymerase II pausing, thus regulating alternative splicing and increase the gene transcription (Shukla *et al*, 2011; Yu *et al*, 2013). In chapter 5, we observed lower *KITLG* (cg27512205) methylation in bipolar disorder. This locus is located in a H3K27ac-enriched region as well as on the north shore of a CpG island near the *KITLG* gene. DNA methylation differences frequently occur in CpG island shores (Irizarry *et al*, 2009),

furthermore, DNA hypomethylation in the H3K27ac-enriched region is associated with a more open chromatin structure which indicates active gene transcription (Vermunt *et al*, 2014; Yang *et al*, 2014). These two co-occurrences suggest that *KITLG* hypomethylation at this CpG locus could indeed alter gene transcription. Study from *Snijders* et al. found an increased level of protein coded by *KITLG* gene, known as stem cell factor (SCF), in the serum of offspring of bipolar disorder patients (Snijders *et al*, 2017). This indicates that *KITLG* hypomethylation in bipolar disorders is associated with an increased *KITLG* transcription and translation.

Is there one final-common pathway for environmental risk factors associated psychosis?

The estimated heritability of schizophrenia and bipolar disorder is around 65 and 58 percent, respectively (Lichtenstein *et al*, 2009; Song *et al*, 2015; Wray and Gottesman, 2012). Genetic variants discovered to date, however, cannot fully explained the high estimated heritability. This implies that other mechanisms such as gene–environment interactions and epigenetic mechanisms may account. The schizophrenia GWAS identified the C4 gene of the major histocompatibility complex (MHC) as a robust genetic risk locus (Stefansson *et al*, 2009). The MHC locus on chromosome 6 is involved in the immune response and the C4 gene is a well-established member of the complement system, which is suggested to be involved in synaptic pruning (Sekar *et al*, 2016). This further led researchers to focus on the involvement of inflammatory pathways in schizophrenia (van Kesteren *et al*, 2017).

The link between an immune response dysregulation and schizophrenia was described in epidemiological studies, in postmortem brains, and at peripheral inflammatory level. Except for some contradictory findings, there was a consistent result from peripheral inflammatory markers in schizophrenia showing elevated cytokine concentrations, both as a state- and trait-marker. These cytokines include IL-1β (Fillman *et al*, 2013), interleukin 6 (IL-6), tumor necrosis factor α (TNFα), soluble IL-2 receptor (sIL-2R), and IL-1 receptor antagonist (IL-1RA) in schizophrenia and bipolar subjects (Goldsmith *et al*, 2016). These cytokines are all modulated by the nuclear factor kappa B (NF-κB) signaling pathway and this pathway is identified as a potential therapeutic targeting for psychiatry disorders (Altinoz *et al*, 2016; Datta-Mitra *et al*, 2015). In this thesis we observed immune related genomic factors that are involved in the impact of detrimental environments on psychotic vulnerability. The *P2RX7* genetic variant we identified in chapter 3 is predominantly expressed in immune cells, both peripheral and in the CNS. The activation of P2X7 receptor could induce IL-1β release, whereas the amount of IL-1β release could different due to the different sensitivity to cannabinoids on P2RX7 genetic variants. Therefore, one of our hypotheses is that cannabis could decrease the function of P2X7 receptor in the brain, by disrupting the inflammatory processes and thus contributing to the development of psychotic symptoms. The other hypothesis is based on the endocannabinoids system. THC more competitively binds to the cannabinoid receptors than endocannabinoids, and this disturbs the endocannabinoids balance. The imbalanced endocannabinoids due to heavy cannabis use could lead to a dysregulated neuroinflammation, neuroprotection and neurotransmission, as the production of endocannabinoids were found to be triggered by ATP-induced P2X7 receptor in both microglia (Lu *et al*, 2012) and astrocytes (Gao *et al*, 2017), the altered function of P2X7 receptor due to the rs7958311 variant may therefore modulate the vulnerability to cannabinoids at several levels. Another example that detrimental environmental factors have an impact on psychosis involving the immune system is from chapter 5, in which we show that *KITLG* methylation is associated with childhood adversity. *KITLG* encodes stem cell factor (SCF), which is an immune growth factor that plays an important role in a proper development of the immune system. SCF crosses the blood-brain barrier and influences proliferation and migration of neural progenitor cells (Zhao *et al*, 2007). The lower *KITLG* methylation we observed in blood cells of bipolar disorders implied that an SCF related immune mechanism is involved in bipolar disorder.

It seems that cannabis and childhood adversity could influence immune epigenomic or genomic factors and this change of the immune system could contribute to the vulnerability of psychosis. However, how these immune signaling pathways lead to final disease state remains an open question for the field.

Additional methodological remarks

Psychosis like experiences as a model for psychotic vulnerability

In chapter 2 and chapter 3, the outcome measure of psychotic vulnerability is based on self-reported subclinical psychiatric symptoms measured by the Community Assessment of Psychic Experiences (CAPE) questionnaire. CAPE has been proved as a valid, simple and cost-effective instrument for detecting individuals at psychotic vulnerability (Konings *et al*, 2006; Mossaheb *et al*, 2012) and has been widely used (Baryshnikov *et al*, 2018; Catalan *et al*, 2017; Papanastasiou *et al*, 2018). The subclinical psychiatric symptoms in CAPE are measured in three dimensions: positive symptoms (psychosis), negative symptoms (deficits in emotional response and cognition) and depression symptoms. One of the advantages of this approach is that it allows us to understand the influence of cannabis use on psychosis vulnerability in the general population with limited influence of potential confounding factors in patient populations such as genetic other genetic vulnerabilities).

Cannabis measures

The cannabis consumption measurement in chapter 2 and chapter 3 were represented by the amount of money spent on cannabis. The main psychoactive component of cannabis is THC. Since the concentration of THC significantly varies between different cannabis products, the frequency of cannabis use is not the most accurate way to quantify the THC exposure. In the Netherlands, a study showed that the THC concentration is highly correlated with the amount of money spent on cannabis (Niesink and Rigter, 2009), with price ranging from 4.30 euro for one gram of imported marijuana with an average THC percentage of 5.5% to 15 euro per gram of Dutch hashish with an average THC concentration of 33.3%. We therefore assessed the amount of euros (€) spent on cannabis per week in the last month, as a proxy measure of exposure to THC. To further confirm the recent cannabis use, urine samples were obtained to verify their report.

Extreme sampling

In both chapter 2 and chapter 3, we applied the extreme sampling method (Boks *et al*, 2007) to maximize the power of the genome-wide gene finding approach. In chapter 2, heavy cannabis users or cannabis naïve individuals were included and matched for psychotic like experiences to remove the influence of psychotic experiences from the study. In chapter 3, we performed a gene-environment interaction study to interrogate which genetic factor is interacted with cannabis use. Regarding to the statistical power, gene-environment interactions study requires a larger sample size than those necessary to detect genetic or environmental factors in isolation. This could be explained by the fact that risks are relatively small in an unselected epidemiological sample, leading to insufficient numbers of case subjects (high CAPE score in our case) that also have been exposed to the detrimental environmental factors (heavy cannabis use in our case). Therefore, in chapter 3, participants were not only selected by cannabis use, but also polarized by top or bottom quintile of total scores of psychotic experiences as measured by the CAPE score. By applying this extreme sampling method, we could increase power to detect how genetic variation on the SNP level impacts on the cannabis associated risk for psychosis.

CBD and THC in the current studies

Cannabis consists of a wide range of chemical compounds with more than 100 kinds of cannabinoids, including Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (ElSohly *et al*, 2017). Among these, THC is the most widely studied phytocannabinoid since it is identified as the most psychoactive compound in cannabis (Grunfeld and Edery, 1969; Mechoulam *et al*, 1970). CBD, however, does not appear to have a psychoactive effect as THC does. In contract, one study found that a higher concentration of CBD in cannabis is associated with fewer psychotic experiences (Schubart *et al*, 2011) and it also used for medicinal purposes (Izzo *et al*, 2009; Pertwee, 2008). Later, a study also focused on the cognitive effect induced by different ratios of THC and CBD combination in humans. The higher CBD/THC ratio in cannabis was found to be associated with a better recognition recall (Morgan *et al*, 2010, 2012).

The measurement of cannabis use in the cross-sectional study in chapter 2 and chapter 3 relied on self-report on cannabis use in combination with a urine check. However, the exact amount of THC and CBD consumption was not available in these two studies. To explore which cannabinoid has a more profound effect on gene transcription in chapter 2 and on interaction with *P2RX7* genotype in chapter 3, we subsequently performed *in vitro* study by stimulating cells with THC and CBD separately. The result from chapter 2 showed that CBD could significantly increase *PPFAI2* transcription in monocytes, whereas THC could not. In chapter 3, we observed a significant decrease of IL-1 β release in monocytes with *P2RX7* non-A carriers after CBD stimulation, whereas the response to THC was not profound as with the CBD challenge. These results suggest that CBD overall has a more significant effect on monocytes compare with THC.

DNA methylation measurement

In the context of DNA methylation, it is necessary to consider the timepoint of observation on DNA methylation. In the chapter 4 and chapter 5 we explored two different environmental factors: nutritional deprivation and childhood adversity. These two detrimental environments occur in prenatal stage and childhood stage, respectively, whereas the measurement of genome-wide DNA methylation was performed at adult age. Therefore, the following questions are relevant: do the current DNA methylation results efficiently reflect the impact of detrimental environmental factors? Do the DNA methylation changes, due to the harmful environments during prenatal and childhood period, last until adulthood? Candidate gene research could help to illuminate whether DNA methylation due to environment factors could last long. For instance, human GR promoter methylation in offspring was positively associated with mothers' intimate partner violence experience during their pregnancy, and this difference could last until 10–19 years after birth (Radtke *et al*, 2011). This supports the hypothesis that epigenetic regulation of early adversity can mediate long term effects. Studies also showed changes in DNA methylation in the brain during development and learning (Lister *et al*, 2013; Shulha *et al*, 2013), in addition other studies showed that the DNA methylation level gradually decreases over time (Van Dongen *et al*, 2016; Gaunt *et al*, 2016). These studies show the variability of DNA methylation. Though the epigenetic marks slowly degraded overtime, the dramatic

change due to detrimental environmental factors was still distinguishable. Therefore, the epigenetic measurement from adolescents or later stage, may still represent the detrimental environmental impacts during early development.

Future perspectives

Increase sample size and ethnical diversity

Studies on the association of multiple detrimental environmental factors with psychosis may lead to a better understanding of the etiology of psychosis. Hypothesis free approaches of genome-wide technology uncovered numerous individual genetic factors that have different sensitivity to the detrimental environments. However, the limitation of this approach is the demand of a large sample size to get enough statistical power. Considering this demand collaboration between institutes could help to secure large samples and facilitate future GWEIS investigation. Furthermore, most of the genome-wide studies were carried out based on ethnically homogenous samples. This limits the generalizability to other ethnicities. For example, the polygenetic score for schizophrenia generated from European samples could only explain 1/9th of the proportion of variance of Africans (Vassos *et al*, 2017). The genome-wide DNA methylation analysis of maternal famine in Chinese (chapter 5), Dutch, and Bangladesh samples also showed different DMRs. One of the potential explanations is that the genomic vulnerability to maternal famine is different due to ethnical background, and the sample size of the studies are hardly satisfying with respect to statistical power to detect true signal, thus leading to different results among the three studies. To uncover a common genomic influence of a certain environment factor, future gene-environment investigations should cover a broader range of ethnically diverse samples and increase the sample size. Therefore, large-scale collaborations and data sharing mechanisms are recommended.

Advanced in vitro models

Psychotic disorders are residing largely in the brain, but in our studies we mainly made use of blood samples of living subjects since human brain tissue is scarce. Studies can also use postmortem brain tissues or blood as brain proxy to study psychosis. The *in vitro* studies which assist to understand the underlying mechanism also frequently use peripheral tissue or cell lines to mimic a situation in the brain. Although *in vitro* study with neural cell lines partially compensate for the tissue differences, it raises the additional concerns that their gene expression and cell behavior differ considerably from that of primary neurons and glia. Therefore, a better model is needed for replication of genetic studies or mechanism exploration.

The ability to develop induced pluripotent stem cells (iPSCs) from adult somatic cells (Takahashi and Yamanaka, 2006) and differentiate them into neurons provides the opportunity to use cell cultures which are more similar to the human brain (O'Shea and McInnis, 2016). When the goal is to study genetic background of psychosis, induced neurons or glia could provide from patients may pose very useful starting material. A recent developed brain organoid model provides new opportunities to study brain disease (Lancaster *et al*, 2013). It provides three-dimensional neural tissues which derived from the self-organization of pluripotent stem cells, and they recapitulate the developmental process of the human brain. In future study, GWEIS could be performed on iPSCs derived glia or neuron or organoids to interrogate the interaction of individual genetic factors with detrimental environmental factors during development stage. Or use psychiatric patient-derived iPSCs and organoids to examine the individual generic reaction on detrimental environmental factors. One of the concerns of iPSCs and organoids is their epigenetic signature. Recent study on iPCSs and brain organoids suggested an overall similarity of DNA methylation signature as of human primary cells and human embryonic stem cells (hESCs)-derived neurons (de Boni *et al*, 2018; Luo *et al*, 2016).

Conclusion

Studies in this thesis identified several genomic factors that response to three detrimental environmental factors in the concept of vulnerability to psychosis. By combining epidemiology and *in vitro* strategies, this thesis not only explored the genomic regulation in the population, but also biological mechanisms underlying the involvement of these genes. Overall these studies shed new light on how nutritional deprivation, childhood adversity and cannabis use impact on the human genome and are related to vulnerability to psychotic symptoms.

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Chapter 7

Nederlandse samenvatting

In deze thesis worden de resultaten van het onderzoek naar drie verschillende externe invloeden op de ontwikkeling van psychose beschreven. De studie bestudeert de relatie tussen een psychotisch ziektebeeld en gentranscriptie en DNA-methylatie. Dit hoofdstuk bevat een samenvatting van elk onderdeel van de studie gevolgd door een algemene discussie.

Verandering in *PPFIA2* genexpressie als reactie op cannabinoïden is een mogelijk mechanisme waar via cannabis invloed heeft op neuropsychologische functies.
De analyse van genoombrede genexpressie van RNA uit compleet bloed van zware canabis gebruikers en naïeve deelnemers liet zien dat *PPFIA2*-expressie significant hoger is in gebruikers van cannabis (**Hoofdstuk 2**). De resultaten werden bevestigd door middel van een *in vitro* model. Hierbij werden monocyten blootgesteld aan cannabinoïden, met als resultaat dat *PPFIA2*-expressie toe nam.

Naast de verhoogde *PPFIA2*-expressie is er ook een significant lagere cognitieve prestatie waargenomen in zware cannabis gebruikers. Dit werd gemeten aan de hand van de Wechsler Adult Intelligence Scale (WAIS). Verdere analyse wees uit dat er een tegenovergesteld verband is tussen *PPFIA2*-expressie en neuropsychologische functies in alle deelnemers, inclusief naïeve deelnemers. Deze resultaten tonen een potentieel verband tussen *PPFIA2*-expressie, cannabis gebruik en neuropsychologische functie.

Genoomwijde interactiestudie (GWEIS) naar de invloed van genen die van invloed zijn op het ontwikkelen psychose onder invloed van cannabis en P2X7-receptor polymorfisme
De meeste cannabisgebruikers ontwikkelen geen psychose, ondanks de vastgestelde relatie tussen cannabisgebruik en de ontwikkeling van schizofrenie. Daarom speelt de individuele genetische achtergrond van cannabisgebruikers een waarschijnlijke rol in de ontwikkeling van psychoses. In **hoofdstuk 3** tonen we aan dat P2X7-receptor polymorfisme (rs7958311) het risico verhoogd op het ontwikkelen van psychose bij cannabisgebruik. Zware cannabisgebruikers met allel A van *P2RX7* hebben een significant hogere kans op psychotische ervaringen. Verder *in vitro* onderzoek liet zien dat het bezitten van allel A van *P2RX7* inderdaad van invloed is op de functie van de receptor, waarbij vermindering van ATP-gedreven IL-1β uitscheiding na blootstelling aan cannabinoïden wordt waargenomen. Immuuncellen in het centraal zenuwstelsel hebben een hoge expressie van *P2RX7* waar het een grote invloed heeft op neuro-inflammatie en neuro-ontwikkeling. P2X7-receptor polymorfisme kan dus gerelateerd worden aan de pathogenese van psychotische stoornissen door middel van verandering in immuunregulatie van neurotransmitter uitscheiding.

Prenataal voedingstekort is geassocieerd met DNA-methylatie in het zenuwstelsel
In **hoofdstuk 4** onderzoeken we het effect van voedingsdeprivatie tijdens de zwangerschap op veranderingen in het epigenoom van de infant. Onze analyse combineert data van een cross-sectioneel onderzoek (Chinese hongersnood 1859) en een *in vitro* studie (voedingsdeprivatie van fibroblasten). In totaal werden er 4 genen (*ENO2*, *ZNF226*, *CCDC51* and *TMA7*) gevonden die zich in drie unieke gemethyleerde regio's (DRM) bevinden die geassocieerd worden met voedingsdeprivatie. Het meest interessante gen is *ENO2*, omdat dit gen neuron-specifiek is en wordt geassocieerd met schizofrenie en bipolaire stoornissen. Voorgaande onderzoeken hebben uitgewezen dat er tweevoud hogere kans op schizofrenie is bij nakomelingen als er sprake was van prenataal voedingstekort. *ENO2* kan een nieuwe link zijn tussen voedingsdeprivatie en de ontwikkeling van psychose. Verdere analyse laat zien dat het zenuwstelsel en neurogenesis een brede en sterke associatie vertonen met voedingsdeprivatie, waarbij veranderingen in DNA-methylatie ontstaan als reactie op voedingsdeprivatie.

Jeugdtrauma is geassocieerd met verhoogd *KITLG* methylatie in gezonde personen maar niet in patiënten met bipolaire stoornis
In **hoofdstuk 5** onderzoeken we het effect van jeugdtrauma als een externe factor op methylatie van het gen *KITLG* in personen met of zonder een bipolaire stoornis. Onze data laat zien dat DNA-methylatie van *KITLG* positief gecorreleerd wordt met jeugdtrauma maar toch minder gemethyleerd is in personen met een bipolaire stoornis. Alhoewel jeugdtrauma vaker voorkomt bij mensen met bipolaire stoornissen.

Chapter 8

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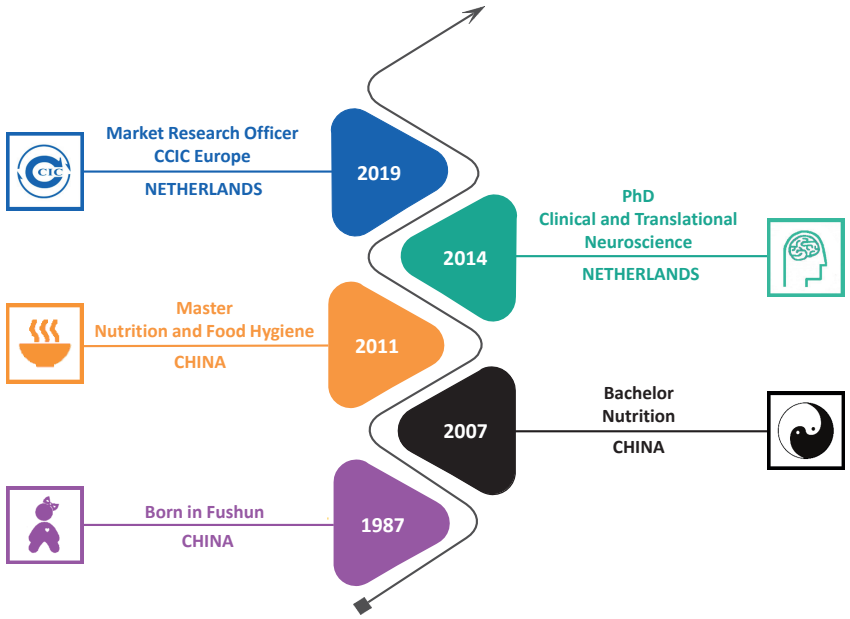
Chapter 9

Curriculum Vitae

Yujie He was born in Fushun City, China on 12th September 1987. In 2007 she finished her high school at Qingdao City, China and started studying Nutrition at the Shandong University of Traditional Chinese Medicine (China). She obtained her bachelor degree in 2011 and started with her research Master in Nutrition and Food Hygiene at the Harbin Medical University (China) under the supervision of prof Changhao Sun. After she gained her master degree she continued her PhD journey in the year of 2014 in the University Medical Center Utrecht (UMC Utrecht, the Netherlands), under the supervision of dr. Marco P. Boks, dr. Lot D. de Witte, prof. Elly M. Hol and prof. dr. René S. Kahn. Her PhD research mainly focused on genomic impact of environmental risk factors for psychosis. On 1st January 2019 Yujie started working as a market research officer in China Inspection & Certification Group Europe in Rotterdam.



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