Aberrant TNF and Notch signaling pathways in schizophrenia and bipolar disorder

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2 LIST OF PUBLICATIONS

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3 ABBREVIATIONS

5-HT – Serotonin

ADAM17 – A disintegrin and metalloproease-17

ALS – Amyotrophic lateral sclerosis

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB – Blood brain barrier

BD – Bipolar disorder

CD40L – Cluster of differentiation 40 ligand

CDSS – Calgary Depression Scale for Schizophrenia

CNS – Central nervous system

CRP – C-reactive protein

CVLT – California verbal learning test

DLPFC – Dorsolateral prefrontal cortex

DRD2 – Dopamine 2 receptors

DSM-IV – Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition

GABA – Gamma-aminobutyric acid

GAF – Global Assessment of Functioning

GWAS – Genome Wide Association Study

HC – Healthy controls

IDS – Inventory of Depressive Symptoms

IL1Ra – Interleukin 1 receptor antagonist

IL6 – Interleukin 6

LSD – Lysergic acid diethylamide

LTD – Long-term depression

LTP – Long-term potentiation

MHC – Major histocompatibility complex

MS – Multiple sclerosis

NMDA – N-methyl-D-aspartate

NORMENT – Center for Mental Health Research

OPG – Osteoprotegerin

PGC - Psychiatric Genomics Consortium

PANSS – Positive and negative symptom scale

qPCR – Quantitative polymerase chain reaction

SCZ – Schizophrenia

TNF – Tumor necrosis factor

TNFR1 and TNFR2 – Tumor necrosis factor receptor 1 and 2 respectively

TOP-study – Thematically Organized Psychosis Research Study

vWF – von Willebrant factor

YMRS – Young Mania Rating Scale

4 SUMMARY

The role of the immune system is increasingly recognized in severe mental disorders. Altered TNF-pathway cytokines are a consistent finding, however, this pathway is complex and its role in schizophrenia (SCZ) and bipolar disorder (BD) requires further elucidation. Other immune related pathways that have been implicated in the pathophysiology of severe mental disorders include the Notch signaling pathway. This pathway fine-tunes the immune system and is involved in cell fate determination, cell differentiation and in the maintenance of adult brain homeostasis, making it a highly relevant candidate for further investigation.

This thesis aimed at increasing our knowledge of the immune mechanisms involved in severe mental disorders. In the first study we examined the relationship between systemic immune markers, verbal memory performance and hippocampal subfield volumes. In the second study we investigated the TNF pathway by measuring differences in its activity between patients and controls both in peripheral blood and in the brain. Further, we examine whether TNF pathway activity would be associated with performance on working memory tasks, and whether psychotropic drugs and clinical symptoms would be associated with TNF pathway proteins and mRNA levels. In our final study we characterized the Notch signaling pathway in patients with severe mental disorders and in healthy controls (HC) and explored associations between psychotropic drugs and Notch signaling components.

These studies were carried out at the Norwegian Center for Mental Health Research (NORMENT), and we collaborated with the Lieber Institute in the USA for our TNF pathway analysis study. We included large samples (*n* up to 1436) which provided well-powered statistical analyses of data from multidisciplinary methods including brain imaging, cognitive testing, as well as measurement of clinical symptoms. We assessed plasma proteins, TNF and Notch pathway-related gene expression in whole blood and TNF pathway-related gene expression in post-mortem brain tissue.

We found a modest but significant association between increased plasma levels of soluble TNFR1 and decreased performance on verbal memory tests, and a similar relationship between TNF and working memory. Further, we determined that patients had increased TNF pathway cytokines and increased TNF/TNFR ratio in the plasma while systemic *TNF* mRNA levels were decreased compared to HC. We also showed that increased *ADAM17* mRNA levels were associated with BD. Our Notch pathway analysis revealed significant differences in Notch signaling between patients and controls, and our results suggest potential attenuated Notch canonical signaling in SCZ, and to a

lesser extent, in BD. Finally, we observed that lithium is associated with increased TNF pathway activity, and that lithium use is also associated with *RBPJ* and *ADAM17* expression.

This thesis highlights the role of aberrant TNF and Notch signaling pathways in severe mental disorders. We show that alterations in the TNF pathway are associated with slightly impaired cognitive function (such as verbal memory and working memory). Our results support other observations of a low grade pro-inflammatory state in SCZ and BD, and through measuring the TNF/TNFR ratio we also demonstrate that patients have increased systemic TNF pathway activity compared to HC. It is unknown whether this imbalance is the result of a primary immune dysfunction or is secondary to co-morbidity, and the mechanisms and cellular sources will have to be further evaluated. The Notch signaling pathway is complex and not well understood at present. We found distinct differences in Notch signaling pathway-related gene expression which further implicates a dysregulation of Notch signaling in severe mental disorders. This pathway may provide novel therapeutic targets for future drug development as Notch fine-tunes the immune system and is involved in governing adult brain homeostasis.

The findings in this thesis support an imbalance in the TNF pathway, and suggest that drugs targeting the TNF pathway may have a role in the treatment of SCZ and BD. Future studies should aim at identifying subgroups within SCZ and BD that are associated with immune dysregulation. Further, it may be relevant to investigate the expression pattern of the Notch signaling pathway in post-mortem brain tissue to determine whether attenuated Notch signaling is also present in the brain. Finally, lithium is an effective psychotropic drug although it has significant side effects. We have identified potential gene targets for lithium and the mechanisms for this regulation could be further investigated *in vitro* and in experimental studies.

5 INTRODUCTION

Schizophrenia (SCZ) and bipolar disorder (BD) are severe mental disorders that have been recognized for centuries yet the mechanisms underlying these disorders remain elusive and their treatment unsatisfactory. Patients with SCZ and BD die 10-20 years earlier than the general population and suicide rates remain high (Crump et al., 2013a; Gomez-Duran et al., 2012; Latalova et al., 2014; Laursen et al., 2012). They also rank among the leading causes of worldwide disability further supporting the need for better treatment (Chong et al., 2016; Ferrari et al., 2016). Although the pathophysiology of both disorders is largely unknown, emerging evidence points to a complex interplay between genetic vulnerability and environmental factors (Davis et al., 2016). A role for the immune system in disease mechanisms underlying severe mental disorders is increasingly recognized and may reveal novel therapeutic targets.

This thesis is dedicated to deepening our understanding of the pathological processes involved in SCZ and BD with an emphasis on immune pathways, and is the continuation of the works of the Translational Psychiatry Group at the Norwegian Centre for Mental Disorders Research (NORMENT), Oslo.

In the introduction section I will give an overview of the clinical features, etiological hypotheses and present treatment options for severe mental disorders. I will then introduce the immune system, its relation to the brain, and finally, focus specifically on the hippocampus before identifying knowledge gaps.

Psychotic disorders

SCZ and BD are often studied together as they share common symptoms and there is considerable genetic overlap between the two disorders (Cardno and Owen, 2014; Tesli et al., 2014). SCZ has an overweight of psychotic symptoms (*e.g.* delusions and hallucinations), while BD is dominated by the presence of affective symptoms (*i.e.* depressed and elevated mood), but affective and psychotic symptoms are common in both disorders. Patients with SCZ often experience changes in affect, and depressive symptoms are common during the course of SCZ. In bipolar depression and mania patients may become psychotic with delusions and hallucinations that are frequently observed in SCZ. In the following sections I will describe SCZ and bipolar spectrum disorders in detail.

5.1 Schizophrenia spectrum disorders

SCZ spectrum disorders include the psychotic disorders SCZ, schizophreniform disorder, brief psychotic disorder, schizoaffective disorder, delusional disorders and psychosis not otherwise specified. The following section will describe the clinical characteristics, etiology and treatment of SCZ spectrum disorders with a focus on SCZ.

5.1.1 Clinical characteristics

Schizophrenia, Schizophreniform disorder and Brief psychotic disorder

Our concept of SCZ continually changes due to progress in research (clinical, including symptoms, biological as well as psychological). As a result, how we define SCZ today differs from how it was first described in the early 19th century (Dollfus and Lyne, 2016). SCZ is primarily a psychotic disorder, and in the present thesis it is defined according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV).

The symptoms associated with SCZ are systematized into 5 main groups in DSM-IV: delusions (*e.g.* paranoid, grandiose), hallucinations (*e.g.* auditory, visual and tactile), disorganized speech, disorganized behavior and negative symptoms (such as loss of speech, affective flattening and avolition). Patients experiencing symptoms from two out of the five groups (Criterion A), with the symptoms lasting for a period of one month with signs of the disorder for minimum 6 months meet the criteria for SCZ. Criterion A is also met if patients present with bizarre delusions alone or if they hear commentating/conversing voices (so called Schneiderian first-rank symptoms (Silverstein and Harrow, 1981)). In addition, these symptoms must interfere with everyday life during a significant portion of the illness leading to loss of function. The symptoms and loss of function must not be due to a medical condition or substance abuse (APA, 2000). Recently there has been a change in the definition of SCZ with the publication of the latest diagnostic manual, DSM-5. In DSM-5 the presence of Schneiderian first-rank symptoms alone is not sufficient to satisfy Criterion A, and patients are required two out of five groups to meet Criterion A independent of the quality of symptoms (APA, 2013).

When all criteria for SCZ are met, but the duration is limited to less than 6 months, the disorder is termed *schizophreniform disorder*. When symptoms are only present for less than one month, the diagnosis of *brief psychotic disorder* is given.

Prevalence and Course

The prevalence of SCZ has generally been accepted to have a uniform lifetime morbid risk of approximately 1% across geography and gender. However, meta-analysis of prevalence data point to a significant variation, and the median lifetime morbid risk for SCZ is 0.7% (McGrath et al., 2008). The age at onset is typically late adolescent – early adulthood, between the ages of 15 and 25. The course of the disorder varies. The recovery rate for SCZ is approximately 14% in the course of 10 years, where recovery includes both clinical and functional aspects, and recovery is sustained for over 2 years (Jaaskelainen et al., 2013). Between 20% to 30% of patients with SCZ do not respond to treatment with conventional antipsychotics (Elkis, 2007). Suicide rates in patients with SCZ range between 3% to 6% with the steepest increase in suicide risk occurring during the first few years after contact with mental health services (Laursen et al., 2014). SCZ is also associated with significant somatic comorbidity such as cardiovascular diseases and chronic obstructive pulmonary disease, which further increases mortality rates (Olfson et al., 2015).

Associated features

There are no radiological, laboratory or psychometric findings at present that are specific to SCZ. Nevertheless, neuroimaging and neuropsychological studies have revealed several differences between SCZ patients and HC.

Brain Imaging

Among the most consistent brain morphological findings are cortical thinning in the prefrontal cortex and atrophy of the temporal lobe, especially the hippocampus (Moberget et al., 2017). Despite established cortical thinning in SCZ, several cell counting techniques have not been able to demonstrate neuronal loss in the cortex or in the hippocampus, and the authors of a recent meta-analysis propose that volume loss may be attributed to dendritic pathology and reduced cortical connectivity (Coyle et al., 2016). Further, hippocampal volume of first-episode and chronic patients did not differ in another recent meta-analysis, which supports neurodevelopmental disturbances in SCZ (Adriano et al., 2012; Moberget et al., 2017; van Erp et al., 2016).

Cognition

Cognitive impairment in SCZ is general and includes the following domains that have been replicated in several studies: impaired verbal learning and memory, visual learning and memory, working memory, attention/vigilance, processing speed, reasoning and problem solving as well as social cognition (Schulz and Murray, 2016). Longitudinal studies show that the cognitive

impairment remains relatively stable for all domains apart from verbal memory, which shows a decline in the patient group relative to HC. This relative decline, however, may be partly due to an improvement in the control group whereas SCZ patients show no improvement (Heilbronner et al., 2016). Of interest, people with ultra-high-risk of developing SCZ and young people with family history of SCZ show significantly impaired cognitive functions compared to HC, which also supports neurodevelopmental alterations in SCZ (Bora et al., 2014).

Delusional disorders

Delusional disorders differ from SCZ in that the clinical picture is dominated by delusions and the disorder does not interfere with day to day life apart from the impact of the delusion or its ramifications. The age at onset is typically later than in SCZ, mid or late adulthood, but delusional disorders can also occur in younger age groups. Treatment is mainly antipsychotics, and delusional disorders have a moderate outcome (Mews and Quante, 2013).

Schizoaffective disorder

In schizoaffective disorder the symptoms patients present with meet criterion A of SCZ, however, the psychotic symptoms also overlap with affective episodes and the duration of affective episodes is present during a significant proportion of the illness. Thus during the course of the illness there is a period of an affective episode and a psychotic episode which overlap for the minimum of two weeks. The age of onset is typically early adulthood and long-term outcomes are generally better than for SCZ (Alphs et al., 2016).

Psychotic disorder not otherwise specified

Psychotic disorder not otherwise specified is a diagnostic category where clinicians observe distinct psychotic symptoms, however the criteria are not met for a specific disorder due to lack of information or comorbid illnesses that disturb the clinical picture (*e.g.* use of illicit drugs). With the accumulation of information over time a more precise diagnosis is made later in the course of the illness.

5.1.2 Etiology and pathophysiology

Decades of research have led to the postulation of numerous hypotheses regarding the pathophysiology of SCZ, and the most acknowledged hypotheses will be described below. Hypotheses and findings relating to the role of the immune system are detailed in section 5.4.

5.1.2.1 Diathesis-stress hypothesis

It is now generally accepted that SCZ and related psychotic disorders are neither purely genetic nor exclusively a consequence of environmental factors. They are a combination of intrinsic vulnerability and external stressors (gene – environment interaction) (Misiak et al., 2017c).

Genetic risk factors

Genetic epidemiological studies using twin and adoption studies suggest that SCZ is highly heritable with heritability estimates ranging up to 80% (Sullivan et al., 2003). Extensive genetic research during the past decades has identified common and rare genomic variants associated with SCZ (Owen et al., 2016).

The most recently published large-scale SCZ genome wide association study (GWAS) by the Psychiatric Genomics Consortium (PGC) was carried out in 2014 and investigated over 35,000 cases and over 110,000 controls. They identified 128 common variants and 108 independent loci associated with SCZ (PGC-SCZ, 2014). Findings from GWAS studies implicate the immune system in the pathophysiology of SCZ through the strong association peak in the extended major histocompatibility complex of chromosome 6 (van de Leemput et al., 2016). Other notable single nucleotide polymorphism associations relevant to the etiology and treatment of SCZ from GWAS studies include *DRD2* (dopamine receptor 2 gene), *CACNA1C* and other calcium channel subunits (Devor et al., 2017), as well as multiple genes involved in glutamatergic signaling and synaptic plasticity (PGC-SCZ, 2014). The individual effect sizes of common variants is very small (Birnbaum and Weinberger, 2017). Thus GWASs confirm a substantial polygenetic component to the risk of SCZ.

Rare genomic variants occurring through point mutations and chromosomic aberrations (copy number variations, CNVs) have also been associated with SCZ. The largest genome-wide analysis of CNVs using the PGC SCZ CNV data set identified 8 loci for CNVs that were associated with SCZ. These rare variants were carried by a small fraction (1.4%) of SCZ patients in the PGC sample. Genes identified within these loci encode proteins that are involved in synaptic plasticity and glutamatergic signaling supporting the GWASs' findings of common genomic variants

(Marshall et al., 2017; Purcell et al., 2014). It is now well established that increasing paternal age leads to elevated risk of SCZ due to the increasing rate of *de novo* mutations with age in the sperm (Kong et al., 2012). Epigenetic alterations may also be involved in SCZ (de Kluiver et al., 2017) in addition to common and rare genomic variants. A role for epigenetics in major psychiatric illnesses has gained increasing attention in recent years. Epigenetics is the study of changes in gene expression that are caused most commonly by methylation of the DNA. Nevertheless, these epigenetic changes do not actually alter the sequence of nucleotides. Indeed, a recent study by Jaffe et al (Jaffe et al., 2016) found that the 108 loci associated with SCZ through GWAS were enriched for epigenetic changes associated with fetal life.

Environmental risk factors

During the past century several environmental risk factors have been linked to SCZ mainly through epidemiological studies. Being born during the winter or spring slightly increases the risk of developing SCZ. This is evidenced by the 5-8% increase in birth rates of SCZ patients during winter and spring compared to the general population worldwide (Suvisaari et al., 2001). The reason for this remains unclear. However several theories have been suggested. These include: procreational habits, increased premature birth in mothers with SCZ and viral and bacterial infections (Suvisaari et al., 2001). Living in an urban environment (urbanicity), is also associated with increased risk for SCZ in high-income Nordic and northern European countries, and may be due to increased stress or increased exposure to viral and bacterial epidemics (Brown, 2011; van Os et al., 2004). However, recent studies indicate that urbanicity does not seem to increase the prevalence of SCZ in low- and middle-income countries, which brings into question the risk factors associated with urbanicity (infections, pollution, drugs and migration) (Plana-Ripoll et al., 2018). Ethnic origin and immigrant status are important risk factors and may be related to epigenetic changes and vitamin D deficiency (Chiang et al., 2016; Dealberto, 2010). The effect of childhood trauma on developing a psychotic disorder has received increased focus the past years, and several studies imply that this subgroup of patients with SCZ might have a more severe clinical manifestation and poor prognosis of functional outcome (Misiak et al., 2017a). Prenatal and perinatal adversities including infections (Brown, 2011), prenatal stress (Negron-Oyarzo et al., 2016), maternal malnutrition (Morgese and Trabace, 2016) and maternal metabolic disease (including obesity) (Rivera et al., 2015), low birth weight as well as birth complications such as hypoxia during birth also increase the risk of developing SCZ (Faa et al., 2016).

5.1.2.2 Dopamine hypothesis

The original dopamine hypothesis for SCZ dates back to the discovery of dopamine by Arvid Carlsson almost 60 years ago, and states that hyperactive dopamine transmission is responsible for the symptoms seen in SCZ (Iversen and Iversen, 2007). This was underpinned by the observations that amphetamines and other psychostimulant drugs could induce psychotic symptoms, and later the findings that clinical effectiveness of antipsychotics was dependent on their affinity to dopamine receptors (Seeman et al., 1976). The dopamine hypothesis has been brought into question and there is consensus that the dopamine hypothesis alone does not explain all the aspects of psychotic disorders (Moncrieff, 2009).

Dopamine is a neurotransmitter that is produced in ten nuclei within the brain. The most studied dopamine producing nuclei are in the midbrain namely the substantia nigra and the ventral tegmental area (Tritsch and Sabatini, 2012). There are four main dopamine pathways that are significant in the treatment of psychotic symptoms with antipsychotics both related to effect and side effects. (i) The mesocortical pathway transmits dopamine from the midbrain ventral tegmental area to the prefrontal cortex and can modulate cognitive processes, (ii) the mesolimbic pathway also projects from the midbrain ventral tegmental area but to the limbic system (ventral striatum) and is part of the reward system, (iii) the nigrostriatal pathway sends dopamine from the midbrain to the basal ganglia (dorsal striatum) where it influences motor function, (iv) and the tuberoinfundibular pathway transmits dopamine from the hypothalamus to the hypophysis where it controls the release of prolactin (Ledonne and Mercuri, 2017; Lieberman, 2004).

5.1.2.3 Serotonin hypothesis

Serotonin (5-HT) has also been implicated in the pathogenesis of SCZ through the observation that hallucinogenic drugs produce SCZ-like symptoms. Later, it was discovered that hallucinogens such as lysergic acid diethylamide (LSD) act on serotonin receptors, and serotonin and LSD show similar chemical structure (Halberstadt and Geyer, 2013). Interestingly, atypical antipsychotics such as olanzapine have an antagonistic effect on 5-HT_{2A} receptors, and this interaction may contribute to its efficacy (Halberstadt and Geyer, 2013). Nevertheless, there is little direct evidence linking a serotoninergic dysfunction to SCZ at present (Yang and Tsai, 2017).

5.1.2.4 Glutamate and kynurenic acid hypothesis

Glutamate is an abundant excitatory neurotransmitter in the brain that acts on three families of ionotropic receptors [*i.e.* N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite] and three groups of metabotropic receptors (Meldrum, 2000). It is involved in neurodevelopmental and neurodegenerative processes. Early investigations showed that patients with SCZ had lower glutamate levels in their cerebrospinal fluid compared to HC, which implicated glutamate in the pathophysiology of SCZ. Later, this hypothesis was strengthened by the observations that NMDA antagonists, *i.e.* phencyclidine and ketamine, can produce psychotic symptoms (Yang and Tsai, 2017). Due to the psychotomimetic properties of ketamine, where it can induce positive, negative and cognitive symptoms, it is used to model SCZ (Frohlich and Van Horn, 2014).

Kynurenic acid is a naturally occurring astrocyte-derived NMDA receptor antagonist, and patients with SCZ have been found to have elevated levels of kynurenic acid in the cerebrospinal fluid and postmortem prefrontal cortex (Erhardt et al., 2017). Kynurenic acid and quinolinic acid are two end-products of the tryptophan pathway, where the latter is an NMDA receptor agonist and induces apoptosis and neurodegeneration (Schwarcz and Stone, 2017). The kynurenic acid hypothesis of SCZ suggests that increased concentrations of kynurenic acid disturb glutamatergic, cholinergic and indirectly dopaminergic signaling, thus resulting in psychotic symptoms (Erhardt et al., 2017).

5.1.2.5 Gamma-aminobutyric acid hypothesis

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter. Postmortem findings in brains of SCZ patients include reductions in GABA pathway related enzymes and aberrant GABAergic interneurons in the prefrontal cortex (Tse et al., 2015). The GABA hypothesis proposes that GABAergic dysfunction in the frontal lobes could contribute to the cognitive disturbances observed in SCZ such as working memory and attention (Tse et al., 2015).

5.1.2.6 Neuronal network dysfunction hypothesis

The neuronal network dysfunction hypothesis is a comprehensive model of SCZ pathophysiology that integrates the independent neurotransmitter pathway hypotheses (glutamatergic, dopaminergic and calcium homeostasis). It is deduced from the results of a recent re-analysis of GWAS studies, and suggests that neuronal excitability is involved in SCZ (Devor et al., 2017).

5.1.3 Treatment

As the underlying pathophysiology of SCZ is yet largely unknown, we do not have medication or other treatment options that as of today are targeting the cause of SCZ. Treatment is thus symptomatic and is aimed at reducing psychotic symptoms such as hallucinations, delusions, negative symptoms and cognitive symptoms. Treatment options include medication and psychosocial interventions such as cognitive therapy and community-based psychosocial interventions (Asher et al., 2017; Owen et al., 2016).

At present, pharmacological treatment is restricted to the use of antipsychotic drugs and heavily relies upon the dopamine hypothesis where drug efficacy is associated with the drugs ability to block the dopamine 2 receptor (DRD2). We distinguish between first generation and second generation antipsychotics depending on the drugs ability to bind DRD2 receptors and affect the serotoninergic system (Amato et al., 2017; Sangani and Saadabadi, 2017). Antipsychotics can effectively treat positive symptoms in a large portion of patients leading to symptom reduction or, in a smaller portion of patients, recovery (Goff et al., 2017). However, the cognitive deficits and negative symptoms that predict long-term outcome do not show the same response rate and their treatment remains unsatisfactory (Muller, 2017). Further, between 20% to 30% of patients with SCZ do not respond to treatment with conventional antipsychotics (Elkis, 2007).

In addition to antipsychotic medication, extensive research is directed at identifying novel drugs that may have a role in severe mental disorders. These include drugs that are aimed at reducing glutamate release early in the course of SCZ (Caraci et al., 2017); treatment with cannabidiol, which has been found to alleviate the signs and symptoms of SCZ in preclinical trials (Seeman, 2016); and non-steroid anti-inflammatory drugs like cyclooxigenase-2 inhibitors. The cyclooxigenase-2 inhibitor celecoxib may alleviate psychotic symptoms in first episode psychosis as add-on treatment to antipsychotics (risperidone and amisulpride), however, its role in chronic SCZ is uncertain (Muller, 2017). Inhibition of kynurenine aminotransferases leads to reduction of kynurenic acid, and may improve cognitive function and reduce psychotic symptoms (Erhardt et al., 2017).

As treatment is unsatisfactory at present for a great proportion of patients, significant research is directed at identifying novel therapeutic interventions for the treatment of SCZ.

5.2 Bipolar spectrum disorders

Historically, BD was categorized as a psychotic disorder in DSM-I (1952) showing closer resemblance to the Kraepelinian understanding of "manic-depressive insanity" (Mason et al., 2016). Today, BD is considered primarily an affective disorder where psychotic symptoms can be present both during depressive and manic episodes. In this thesis, BD is defined according to the DSM-IV criteria.

5.2.1 Clinical characteristics

In BD, patients have periods with either elevated mood, depressed mood or both mood episodes co-occurring. To qualify for a mood (or an affective) episode, patients need to experience additional symptoms to elevated/depressed mood for a certain duration of time. Thus a major depressive episode is defined by the presence of depressed mood and/or loss of interest, in addition to minimum 3-4 of the following symptoms: significant weight loss or decreased appetite/weight gain or increased appetite, insomnia/hypersomnia, psychomotor agitation/retardation, fatigue, feelings of worthlessness/excessive guilt, diminished ability to concentrate/indecisiveness and suicidal ideation. The symptoms must be present most of the day, almost every day for a minimum of 2 weeks. During a manic or hypomanic episode, patients present with elevated or irritable mood, and in addition have minimum 3 of the following (4 if mood is irritable and not elevated): Inflated self-esteem/grandiosity, decreased need for sleep, talkativeness/pressure to keep talking, flight of ideas, distractibility, increase in goal-directed activity/psychomotor agitation and excessive involvement in activities that have a potential for painful consequences. The difference between manic and a hypomanic episodes is (i) the difference in the duration of symptoms (1 week, or any duration if hospitalization is necessary for mania, and minimum 4 days for hypomania), (ii) the presence of psychotic symptoms (occurring only in mania), (iii) the need for hospitalization (only in mania) and other signs of loss of function (APA, 2000; Grande et al., 2016).

The distinguishing feature between bipolar type I (BD-I) and type II (BD-II) disorder is the presence of at least one manic episode in bipolar I disorder, whereas patients with BD-II have exclusively hypomanic episodes in addition to depressive episodes. BD-I patients may also have depressive episodes but it is not a requirement to meet the diagnosis. The severity of depressive episodes may be equal in both disorders.

Prevalence and Course

The prevalence of BD spectrum disorders is 2.4% worldwide (0.6% BD-I, 0.4% BD-II and 1.4% subthreshold BD), whereas European and international studies show higher aggregate estimates (1.5% for BD-I and 0.8% for BD-II) (Merikangas et al., 2011). The age at onset for BD-I is approximately 18 years, and mid-twenties for BD-II. Mean age at onset for BD (BD-I and BD-II taken together) at European sites is 25.2 years, for US sites approximately 20.5 years and in the most recent Norwegian study 22.8 years (Larsson et al., 2010).

The course of BD is characterized by recurrent mood episodes with inter-episode euthymic periods. Patients spend approximately 40% of their time in an affective episode (Forte et al., 2015). Depression is the most prominent affective episode in BD, and patients with BD-II have longer periods with depression than patients with BD-I (Grande et al., 2016). BD is also associated with increased suicide rates, reduced quality of life and high somatic co-morbidity. Suicide attempts range between 33%-50%, where 15-20% of patients commit suicide (Grande et al., 2016). Suicide rates are particularly high in patients that are untreated, and quality of life is reduced (Vieta et al., 2018). Patients with BD have significant somatic co-morbidity which occurs at a younger age compared to the general population. The most prevalent somatic co-morbidities include cardiovascular diseases, diabetes and obesity which substantially contribute to the increased mortality rate (Grande et al., 2016).

Associated features

As with SCZ, BD is also associated with additional neuroimaging and cognitive features that are not a part of the diagnostic criteria. There are consistent findings on a group level with significant variation between individuals.

Brain imaging

A recent meta-analysis investigating cortical thickness in BD revealed that the anterior cingulate cortex areas lying within the prefrontal cortex show reduced gray matter volume. Further, the authors demonstrated decreased cortical thickness in the superior temporal, bilateral superior frontal regions, several prefrontal regions bilaterally as well as slightly reduced hippocampal volumes (Hanford et al., 2016; Hibar et al., 2018; Hibar et al., 2016).

Cognition

Patients with first episode BD show global cognitive impairment which suggests neurodevelopmental processes in BD (Bora and Pantelis, 2015; Demmo et al., 2016). Decreased performance on the verbal learning test is a consistent finding also in euthymic periods (Bourne et al., 2013). Patients that are at risk for developing BD did not show deficits in cognitive function. Longitudinal studies suggest that the cognitive impairments in first episode BD remain stable during the course of the illness (Demmo et al., 2017; Pfennig et al., 2017), and cognitive impairments may be associated with psychotic episodes in BD rather than BD-I/BD-II diagnosis (Simonsen et al., 2011).

Bipolar disorder not otherwise specified

This diagnosis of BD NOS is made when patients present with symptoms characteristic of a BD, however they do not meet the full criteria for any BD. This is often the case where there is insufficient information to make a more specific diagnosis.

5.2.2 Etiology and pathophysiology

The precise pathophysiological processes that underlie BD remain largely unknown. Nevertheless, decades of research has led to the development of several hypotheses.

5.2.2.1 Diathesis-stress hypothesis

As with SCZ, the etiology of BD is attributed to the interplay between genetic and environmental factors. This is supported by the fact that despite high heritability, genes alone do not explain all the variability in the development of BD (Misiak et al., 2017c).

Genetic risk factors

Recent twin studies and epidemiological studies show that BD is also highly heritable with heritability estimates up to 90% (Craddock and Sklar, 2013). Similarly to SCZ the genetic architecture of BD consists of common and rare genomic variants with polygenic make-up, as well as epigenetic factors. There is a considerable overlap between SCZ and BD in common genetic variants (Cardno and Owen, 2014; Tesli et al., 2014), implying that certain susceptibility genes increase the risk of major psychiatric illnesses in general and may not be specific to either disorder (Gandal et al., 2018). The PGC Bipolar Disorder Working Group (PGC-BD) confirmed the two most consistent GWAS findings in BD: *CACNA1C* and *ODZ4* (PGC-BD, 2011). Common genomic variants

that overlap with SCZ include a separate single nucleotide polymorphism for *CACNA1C*, and common variants near the genes *ZNF804A* and *ANK3* (Andreassen et al., 2013; O'Donovan et al., 2008; PG(GWAS)C-SCZ, 2011). At present, over 40 common risk variants (susceptibility genetic loci) for BD have been identified (Ikeda et al., 2018; Stahl et al., 2018). The role of copy number variations is less certain in BD and overall less prominent than for SCZ (Craddock and Sklar, 2013). Findings indicate that paternal age may also contribute to the risk of developing BD for fathers in the oldest age groups (de Kluiver et al., 2017). This has been associated with increased *de novo* mutations in the sperm of older men (Kong et al., 2012), thus supporting the role of *de novo* mutations in BD. Epigenetic findings include DNA methylation discoveries for several genes, and histone modifications (Ludwig and Dwivedi, 2016).

Environmental risk factors

Similar to SCZ, in addition to genetic risk factors, several environmental factors have also been associated with the development of BD. Some of these risk factors overlap with SCZ, whereas others seem more specific to BD. One consistent finding is parental loss particularly prior to 5 years of age (Faa et al., 2016). Epidemiological studies are inconclusive whether most infections increase the risk of BD, however, Toxoplasma gondii and influenza have been associated with BD (Rosenblat and McIntyre, 2017). Maternal stress and illicit drug use also increases the risk of developing BD (Marangoni et al., 2016). Further, the social zeitgeber theory proposes that "life stress" (*e.g.* loss of a spouse, working night shifts and jet lag; life events that disrupt social rhythms) can trigger affective episodes (Grandin et al., 2006). Trying to prevent such stress factors are therefore a part of the psychoeducation programs in BD.

5.2.2.2 Cell membrane dysfunction hypothesis

Altered signal transduction pathways in BD on the cell-membrane level, or downstream of the cell membrane but in close proximity of the membrane include G protein, adenyl cyclase, protein kinase C and the phosphatidylinositol pathway. The cell membrane dysfunction hypothesis proposes that these four key membrane-based signal transduction pathways could be overactive or oversensitive to receptor stimulation in BD (Kidd, 2004).

In addition, GWASs have identified genetic variants of the *ANK3* consistently associated with BD (Hughes et al., 2016). *ANK3* codes for Ankyrin-G protein, which is a scaffolding adapter that organizes membrane proteins, and link them to the cytoskeleton.

5.2.2.3 Dopamine hypothesis

The general properties of dopamine are described in section 5.1.2. Hypotheses from the 1970's postulated that if hyperdopaminergia underlies mania then hypodopaminergia may underlie depression (Ashok et al., 2017). The notion that hyperdopaminergia underlies mania is supported by observations of animal behavior after the administration of psychostimulants (*e.g.* cocaine and amphetamine) that increase dopamine in the neuronal synapse (Machado-Vieira et al., 2004). The dopamine hypothesis was later refined and described as a cyclical dysregulation in quantitative dopaminergic transmission (Berk et al., 2007). Recent studies involving neuroimaging support the dopamine hypothesis for mania, but are less conclusive for depression (Ashok et al., 2017).

5.2.2.4 Mitochondrial hypothesis

Several studies support a role for an underlying mitochondrial dysfunction in BD pathophysiology where phasic dysregulation of mitochondrial bioenergetics are associated with manic and depressive episodes (Morris et al., 2017).

5.2.2.5 Circadian dysregulation hypothesis

The circadian dysregulation hypothesis for BD proposes that a disturbance in biological rhythms can induce affective episodes in susceptible people (Muneer, 2017). Such biological rhythms include alterations in melatonin, cortisol rhythms, disruption of sleep/wake cycle, chronotype (an individual's propensity to sleep and carry out activities at a particular time during the day) and variations of clock genes (Abreu and Braganca, 2015).

5.2.2.5 Kindling hypothesis – epilepsy and BD

Kindling is the process when repetitively experienced stressors lead to the development of clinical symptoms (such as seizures and depressive symptoms). It was first described in 1969 for epilepsy in animal models and later extended to BD. Several studies suggest a relation between epilepsy and BD due to their episodic nature, similar course and the efficacy of antiepileptic drugs in both disorders. The underlying pathological mechanisms are not established, nevertheless, the kindling hypothesis applies for both epilepsy and BD (Post et al., 2001). Further, common pathologies may include GABAergic and glutamatergic mechanisms, as well as ion channel disturbances (especially calcium and potassium) (Mazza et al., 2007).

5.2.2.6 Amygdala and mood dysregulation

The amygdala plays a central role in emotion-related processes in the brain such as fear conditioning and emotional processing (*i.e.* emotional learning, perception and experience). MRI and fMRI studies show altered amygdala volumes and increased activity of the amygdala in BD, although results are somewhat inconsistent for amygdala volumes. Based on the role of amygdala in emotional processing together with imaging findings, a dysregulation of the amygdala could contribute to the pathophysiology of BD (Garrett and Chang, 2008).

5.2.3 Treatment

At present, there are several treatment options for BD. These include medication (lithium, antiepileptic drugs, antipsychotics and antidepressants), psychosocial interventions and electroconvulsive therapy. The treatment may vary for acute episodes (depression or mania), and for the prevention of new episodes.

Medication

To date, lithium remains the first-line treatment for BD. Lithium is a trace element and can be found as a natural ingredient in the drinking water and in the soil. The main source of lithium is tap water, vegetables and animal-derived foods (Schrauzer, 2002). Lithium has a small therapeutic window, where plasma levels of 0.5-1.2 mmol/l are therapeutic, plasma levels above 1.2 mmol/l are toxic (causing nausea, vomiting, diarrhea, gross tremor, confusion and lethargy) and plasma levels above 2.0 are lethal (Jakobsson et al., 2017). The precise actions of lithium that lead to its anti-manic and antidepressant effect are still largely unknown, although inhibition of the phosphatidylinositol pathway (Kidd, 2004) and glycogen synthase kinase 3 beta has been proposed as the most likely mechanism (Jakobsson et al., 2017).

Antiepileptic drugs are also effective in BD. Valproate is used to treat mania (most likely through its ability to inhibit γ-aminobutyric acid – GABA – catabolism, preventing GABA reabsorption and suppressing voltage-sensitive sodium channels) (Zhu et al., 2017), while lamotrigine is effective in the treatment of depressive symptoms especially as a prophylactic (Parker and McCraw, 2015) (possibly by affecting the limbic system through modulation of monoamines serotonin and dopamine (Johannessen Landmark, 2008)).

Antipsychotic drugs are indicated in acute mania and may be somewhat superior to lithium and valproate (Geddes and Miklowitz, 2013). Antipsychotics also have a role in the treatment of acute

depressive episodes in BD patients (Geddes and Miklowitz, 2013). A small group of antipsychotics have been shown to prevent mania (olanzapine, quetiapine and risperidone) and bipolar depression (quetiapine and lurasidone) (Goodwin et al., 2016).

The use of antidepressants in the treatment of bipolar depression remains controversial. The International Society for Bipolar Disorders Task Force investigated existing evidence on the efficacy and safety of antidepressants in monotherapy and in combination with mood stabilizers in the treatment of bipolar depression. They concluded that the existing data in this field is remarkably limited and methodologically poor, and it is difficult to make firm clinical recommendations on its basis (Pacchiarotti I, 2013). However, most guidelines do not recommend antidepressants as monotherapy (Goodwin et al., 2016).

Psychosocial interventions

Family focused therapy extends psychoeducation and psychological intervention to include closest relatives/spouses of patients with BD, and has been found superior, in combination with medication, to treatment exclusively with psychotropic drugs (Miklowitz and Chung, 2016). Similarly, lifestyle and dietary modifications (to reestablish circadian rhythm and prevent future episodes) (Kidd, 2004) and cognitive behavior therapy (Driessen and Hollon, 2010) are also effective in the treatment of BD in addition to medication. Novel psychosocial treatment approaches include digital platforms, where mobile applications enable continuous monitoring of symptoms as well social and physical activity facilitating earlier detection of mood episodes and aid in preventing relapse (Rajagopalan et al., 2017).

Electroconvulsive therapy

Electroconvulsive therapy (ECT) was introduced over 75 years ago after observations of clinical improvement from case reports of epileptic seizures during mood episodes. It is considered a highly effective treatment for acute affective episodes (mania, depression and mixed episodes) (Medda et al., 2014). ECT is an effective treatment alternative in post-partum psychosis, and is regarded as relatively safe during the first trimester of pregnancy (Bergink et al., 2016; Calaway et al., 2016). Case reports also support the role for ECT in patients with malignant catatonia when treatment with benzodiazepines is ineffective (Dessens et al., 2016). Further, ECT was found more effective in the acute phase of treatment-resistant bipolar depression than pharmacology treatment (Schoeyen et al., 2015).

A novel treatment under investigation is transcranial magnetic stimulation in both bipolar mania and bipolar depression. There are some studies showing limited efficacy, nevertheless, clinical studies are lacking, and firm conclusions cannot be drawn at present in BD (Brady and Keshavan, 2015).

5.3 The immune system

In this section I will give an overview of the immune system, its activity in the brain, and present important immune -related pathways. The role of the immune system in severe mental disorders will be introduced in the next section (5.4).

Immunology is the study of the body's defense against infection. Pathogens trigger an immune response, and we distinguish between a non-specific or innate immune response and a specific or adaptive immune response that is developed during life against a particular pathogen (Murphy, 2012). However, the immune system is not limited to combating bacteria and viruses, but also plays a major role in embryonic development and adult brain homeostasis, and controls vital processes such as neurogenesis, apoptosis and synaptic plasticity (Li and Barres, 2017). It is important to note that an immune response may also be trigged by a "sterile" insult such as physical, chemical or metabolic stress.

The main cells of the peripheral immune system include monocytes and macrophages, granulocytes (neutrophil, eosinophil and basophil), lymphocytes (T-cells, B-cells and Natural Killer cells), mast cells and dendritic cells. These immune cells are produced in the bone marrow and are released into the circulatory system.

5.3.1 Inflammation

The five signs of inflammation are heat (*calor*), pain (*dolor*), redness (*rubor*), swelling (*tumor*) and loss of function (*functio laesa*). The first four signs were identified by the Roman scholar Celsus two millennia ago, and the fifth sign was added by the Greek physician Galen 150 years later. During an immune response, cells of the immune system such as monocytes/macrophages and dendritic cells produce and secrete cytokines and chemokines which together with other inflammatory mediators cause the signs of inflammation.

Sterile inflammation occurs in the absence of external pathogens, and is a common event. It is a powerful homeostatic mechanism aimed at maintaining/restoring tissue integrity. When the stress response persists and the inflammation is not resolved, a vicious circle develops that has a key role in the pathophysiology of many human disorders.

Cytokines

Cytokines are small proteins that exert their effect by binding to specific receptors in a paracrine or endocrine manner. A special group of cytokines that lead to the recruitment of lymphocytes and monocytes are called chemokines (due to their chemoattractant properties). Important cytokines secreted by monocytes/macrophages include interleukin-1β (**IL-1β**), tumor necrosis factor alpha (previously TNF-α, today simply **TNF**) and interleukin-6 (**IL-6**). These are termed upstream cytokines since they are the upstream factors of the inflammatory cascade and therefore represent attractive therapeutic targets. IL-1β activates vascular endothelium and lymphocytes and induces fever and production of IL-6. TNF also activates vascular endothelium and increases vascular permeability, and can induce fever, mobilization of metabolites and shock. IL-6 activates lymphocytes and induces fever and acute-phase protein production in hepatocytes such as C-reactive protein (**CRP**) (Murphy, 2012). CRP was identified approximately in 1930 and is a protein that is highly sensitive to inflammation. CRP levels increase with viral and bacterial infections as well as trauma, post-surgical period and other non-pathogenic inflammatory diseases (Hausfater, 2014). Further, CRP reflects general inflammation in addition to endothelial and macrophage activation, and is a well-known biomarker for infection (Zheng and Xie, 2017).

These upstream regulators orchestrate numerous downstream effectors that regulate the inflammatory response including anti-inflammatory mediators that may contribute to terminate the response when inflammation is resolved. Thus, an imbalance between up- and down-stream regulators and pro- and anti-inflammatory mediators may result in a persistent low-grade inflammatory environment that may "prime", contribute to and aggravate the progression of disease.

Macrophages can also be activated by CD40 ligands (**CD40-L**) that are secreted by immune cells including monocytes, T helper cells and platelets. CD40-L are found on the surface of these cells, but is also secreted resulting in soluble CD40-L. CD-40L binds to the CD40 receptors on the surface of macrophages (Murphy, 2012).

Heat, redness and swelling result from vascular activation, where vessels dilate to increase local blood flow, and they become permeable to encourage the extravasation of immune cells from the circulatory system into the tissues. The von Willebrand Factor (**vWF**) was first discovered a century ago. It plays a pivotal role in vascular inflammation and the formation of thrombosis (Gragnano et al., 2017). vWF is produced by endothelial cells and megakariocytes, and also mediates leukocyte extravasation (Gragnano et al., 2017). Another example of an immune marker associated with

vascular inflammation is osteoprotegerin (**OPG**). OPG belongs to the TNF superfamily and its classical function is bone remodeling. However, in recent years OPG has also been associated with vascular calcification, which is a hallmark of atherosclerosis (Rochette et al., 2017).

Complement system

The complement system is part of the innate immune system and comprises of more than 30 plasma proteins that are produced mainly by the liver. There are three main complement pathways: the classical pathway (antibody-triggered pathway), the alternative pathway (pathogen trigged pathway) and the lectin pathway (activated by lectin-type proteins). The aim of the complement system is either to kill pathogens directly or to facilitate phagocytosis (Murphy, 2012).

5.3.2 Immune system in the brain

Initially the brain was regarded as an immune privileged organ due to its lack of lymphatic drainage and inefficient immune response against allogeneic graft (Louveau et al., 2015a). Today, we still regard the brain as immune privileged, however, this concept has been refined due to the extensive communication between the brain and the peripheral immune system, as well as the discovery of a lymphatic system in the brain (Louveau et al., 2015b).

Neuroglia

There are three types of neuroglia: microglia, astrocytes and oligodendrocytes.

As of yet, microglial cells remain the only parenchymal immune cells of the brain. They are the macrophages of the brain, and it is now clear that the role of microglia extends well beyond inflammatory responses (Cunningham et al., 2013). They are exclusively derived from embryonic haematopoietic progenitor cells found in the yolk sac and fetal liver (Prinz et al., 2017). Microglia migrate into the central nervous system (CNS) during embryonic development (approximately week 8 in humans). They maintain their population in the brain by self-renewal, and participate in numerous developmental events like neurogenesis, apoptosis, synapse elimination and the establishment and remodeling of neural circuits (Howes and McCutcheon, 2017; Li and Barres, 2017). In addition to microglia, there are three other non-parenchymal macrophages in the CNS: perivascular macrophages, meningeal macrophages and choroid-plexus macrophages. With the exception of microglia, there are no immune cells residing in the CNS in the healthy adult brain (Prinz et al., 2017). However, lymphocytes and neutrophil granulocytes can enter the CNS parenchyma during severe conditions (Ransohoff and Brown, 2012). Elimination of these cells and

the fluids and macromolecules that accumulate during inflammatory processes occurs via the meningeal lymphatic system which drains into the cervical lymphatic nodes (Louveau et al., 2015a).

In addition to microglia, the two other types of neuroglia (*i.e.* astrocytes and oligodendrocytes) also partake in inflammatory processes in the brain. Astrocytes are involved in the exchange of chemicals between the circulatory system and the nervous tissue, while oligodendrocytes produce the myelin sheath that surrounds the axons of neurons (Crossman, 2015). While microglia are derived from the hematopoietic lineage, astrocytes and oligodendrocytes share common neuroepithelial origins with neurons (Sloan and Barres, 2014). CNS inflammation triggers an astrocyte response which includes synapse phagocytosis, changes in neurotrophin secretion, clearance of debris and dead cells, reparation of the blood-brain-barrier (BBB) and scar formation (Liddelow and Barres, 2017). Until recently, oligodendrocytes have been viewed as "victims" of inflammatory insults due to their general vulnerability. It is now accepted that oligodendrocytes are able to produce cytokines (such as IL-1β and IL-6), chemokines and other immunomodulatory molecules making them active participants in immune responses (Zeis et al., 2016).

Cytokines and chemokines in the brain

A role for cytokines and chemokines in the brain developed over 40 years ago when researchers discovered that microglia and astrocytes produce cytokines. During the following years a novel role emerged for the immune system where cytokines were proposed to partake in activating neurons and glial cells, and controlling their proliferation, differentiation and survival (Munoz-Fernandez and Fresno, 1998). Cytokines and chemokines are now shown to influence cell migration, proliferation and differentiation in the CNS, and significant chemokines in the CNS include: CXCL8, CCL2, CCL3 and CCL5 (Stuart and Baune, 2014). Pro-inflammatory cytokines (*e.g.* IL-1β, TNF and IL-6) have been studied more extensively. Animal studies suggest a direct role for these cytokines in cognitive functions, such as verbal learning and memory. Several animal studies have demonstrated through direct cytokine injection into the brains of rats, and *via* cytokine receptor knock-out mice that cytokines are indeed necessary for learning, and that their excessive levels lead to memory impairments (Donzis and Tronson, 2014; Marin and Kipnis, 2013). In addition to their role in cognitive function, cytokines may also influence monoamine neurotransmitter systems such as serotonin and dopamine (Baganz and Blakely, 2013; Felger and Miller, 2012). Interestingly, dopamine also influences microglia and the innate and adaptive immune systems (Pinoli et al., 2017).

Blood Brain Barrier

The BBB is a physical yet dynamic barrier that tightly controls the passage of molecules from the circulatory system (brain vasculature) into the brain parenchyma. On the luminal side, cerebral endothelial cells form the lumen and the ends of the endothelial cells form the tight junctions. The endothelial cells are capsulated by the basal lamina on the abluminal surface, and the basal lamina is in turn capsulated by pericytes and the end feed of astrocytes (Phan et al., 2017). There is considerable cross-talk between the peripheral immune system and the brain through the blood brain barrier. The most significant mechanisms include (i) the neural pathway where peripherally produced cytokines activate the vagal nerve and signal to the brain (Erickson et al., 2012), (ii) the humoral pathway where cytokines can access the brain through circumventricular systems that lack intact blood brain barrier (Khandaker and Dantzer, 2016), (iii) cytokines can also activate the epithelial cells of the brain, which in turn produce and secrete cytokines into the brain parenchyma (Verma et al., 2006), (iv) and finally cytokines can enter the CNS via active transport through the BBB (Erickson et al., 2012). Further, activated microglia can disrupt the endothelial function of the BBB making the brain more vulnerable to immune mediators from the periphery (Pollak et al., 2018).

5.3.3 Notch signaling pathway

Notch signaling is a molecular pathway widely used in organ development to specify tissue differentiation through influencing cell-fate (Fazio and Ricciardiello, 2016). It also regulates tissue homeostasis in the adult brain and synaptic plasticity (Alberi et al., 2013). In the human immune system it is necessary for T-cell development (Murphy, 2012). Further, Notch signaling may also influence the development of macrophage functional phenotypes (classically activated/pro-inflammatory M1 phenotype and alternatively activated/anti-inflammatory M2 phenotypes) (Fazio and Ricciardiello, 2016), where activation of Notch signaling promotes macrophage polarization to M1(pro-inflammatory) phenotype (Cai et al., 2016). However, in the CNS Notch activation could reduce the pro-inflammatory activity of microglial cells (Grandbarbe et al., 2007).

In addition to its role in mediating inflammation, Notch signaling is known as a master regulator of neural stem cells and neural development, and orchestrates nervous system development and patterning by regulating neurogenesis, axonal growth, synaptogenesis and predisposing neurons to apoptosis (Ables et al., 2011).

5.4 The immune system in severe mental disorders

In this section I will summarize previous research linking the immune system to severe mental disorders. Figure 1 illustrates a summary of findings that implicate the immune system in severe mental disorders

A role for the immune system in the pathogenesis of SCZ was proposed already in 1929 when Tramer reported an increased risk of developing SCZ in offspring born during the winter season (Franzek and Beckmann, 1996; Tramer, 1929). This observation led to the hypothesis that microbial infections in the pregnant mother and perinatal infections could contribute to the development of SCZ. The similarities between sickness behavior and depression, and the observation that depression commonly occurs in illnesses associated with inflammation prompted the idea that the immune system may also be involved in the pathophysiology of mood disorders (Goldstein et al., 2009).

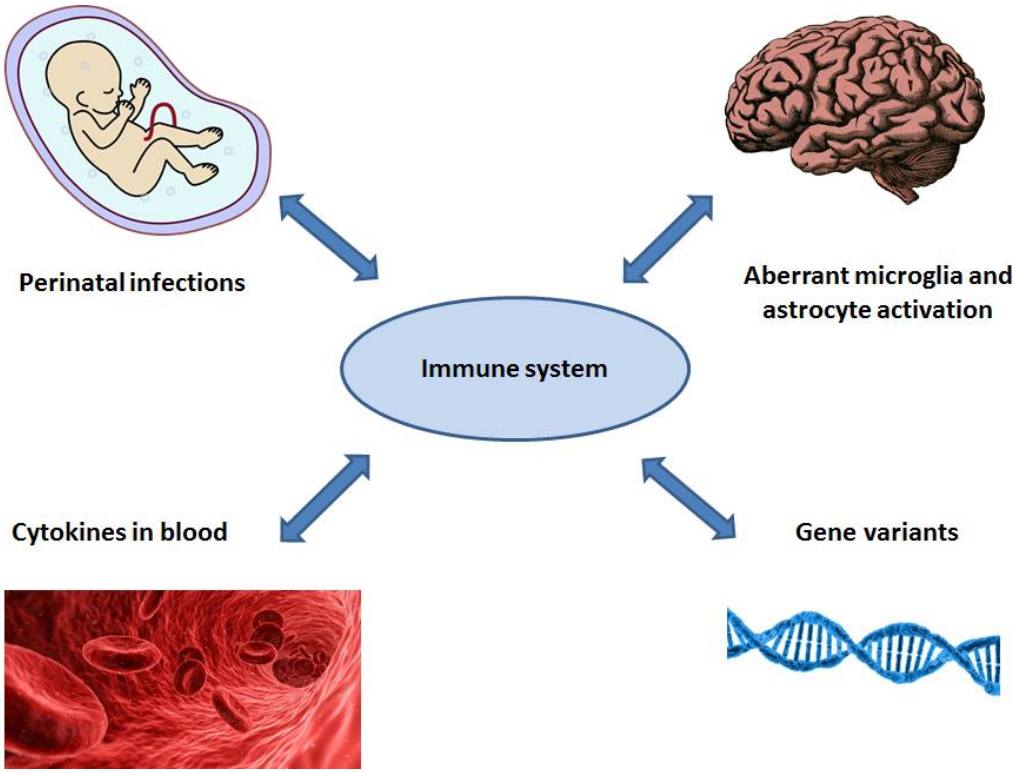

Figure 1. Summary of findings that implicate the immune system in severe mental disorders. Perinatal infections increase the risk for developing severe mental disorders. Post mortem studies indicate possible microglia and astrocyte activation, and the "two-hit hypothesis" from rodent studies further implicate microglial activation. Genome wide association studies have identified gene variants associated with SCZ and BD. Aberrant cytokine levels are frequently observed on a group level in severe mental disorders with a pro-inflammatory shift.

5.4.1 Macrophage-T-lymphocyte hypothesis

In 1992 Ronald S. Smith proposed a comprehensive macrophage-T-lymphocyte theory for SCZ (Smith, 1992), which he later extended to depression (Maes et al., 1995). The basis for this theory was the observation that following IL-2 administration to cancer patients, a significant proportion of patients with no previous history of psychiatric illness developed psychotic symptoms such as delusions and hallucinations. He suggested that the prodromal phase of SCZ is characterized by macrophage activation where macrophage derived cytokines mimic the symptoms of depressive episodes. This macrophage activation would then activate lymphocytes, however, at a certain point, macrophages would fail to suppress and control lymphocytes, and this would result in the psychotic symptoms observed in the active phase of SCZ (Smith, 1992). During the past two decades extensive research has been invested in measuring peripheral blood levels of leukocytes, and plasma and serum levels of cytokines and chemokines produced by immune cells. Studies performed in BD and in first episode patients with SCZ imply aberrant lymphocyte numbers (Miller et al., 2013). Overall, there is evidence supporting a pro-inflammatory cytokine imbalance in both BD and SCZ. The most consistent cytokine findings in SCZ include the monocyte/macrophage derived TNF, IL-1β and IL-6, and the T-lymphocyte derived IFN-γ and IL-12 (Miller et al., 2011). There is a similar pattern in BD with TNF and IL-6 being replicated findings, however IL-2 and IL-4 also seem to be elevated in BD (Munkholm et al., 2013).

5.4.2 Two hit model

The two hit model proposes that perinatal activation of microglia (such as infections) leads to a primed state of microglia where microglia would be encouraged to react with a pro-inflammatory M1 phenotype shift following insults and stress later in life (such as new infections). This in turn leads to the development of symptoms and tissue damage evidenced by cortical loss (Howes and McCutcheon, 2017). This hypothesis is strongly built on observations from animal studies (Grayson et al., 2016).

5.4.3 Immune genes and intrinsic vulnerability

GWAS have identified several genomic variants associated with SCZ in the major histocompatibility complex (MHC) and the extended MHC region on chromosome 6 (PGC-SCZ, 2014; Stefansson et al., 2009). The MHC region of the genome is a cluster of genes that encode numerous immune system related proteins. Among them are the human leukocyte antigen genes that encode proteins necessary for MHC molecules. The *NOTCH4* gene is also located within the

MCH region, and is among the GWAS findings associated with SCZ. Our group has found that *NOTCH4* is also implicated in BD, as BD patients had elevated expression levels of *NOTCH4* as measured by mRNA compared to HC and SCZ in whole blood (Dieset et al., 2012). In addition to the MHC region, further immune related common gene variants were identified in the largest GWAS up to 2014. These included CD19 and CD20 lines that are important in the B-lymphocyte lineage and are involved in acquired immunity (PGC-SCZ, 2014). A recent genetic study identified a potential role for the classical complement cascade in SCZ through investigating the expression of the *C4* gene and examining C4 protein localization and secretion (Sekar et al., 2016).

5.4.4 Cytokines in severe mental disorders

Numerous cytokines and chemokines have been investigated in severe mental disorders, and it is beyond the scope of this thesis to give a detailed overview. Therefore, I will focus on the cytokines that have been explored by our research group the past decade, and that will be further analyzed in the studies of this thesis.

Among the cytokines associated with severe mental disorders the most consistent findings support an imbalance in monocyte/macrophage derived cytokines (TNF, IL-1β and IL-6), and the T-lymphocyte derived IFN-γ and IL-12 (Miller et al., 2011) in SCZ; and TNF, IL-6, IL-2 and IL-4 in BD (Munkholm et al., 2013). NORMENT researchers have previously demonstrated that OPG and vWF levels were elevated in psychotic disorders (Hope et al., 2010; Hope et al., 2009). However, in larger samples these results were not replicated (Morch et al., 2016). Others have found that CD40L was associated with depression (Hufner et al., 2014), and elevated CRP levels were identified in SCZ and BD (Fernandes et al., 2016). Thus, cytokines representing different aspects of the immune system are associated with severe mental disorders, and are in need of further exploration to identify new therapeutic targets.

5.5 Hippocampus and memory

In this section I will give a brief overview of the anatomy of the hippocampus and memory formation, and I will also present the connection between the hippocampus and the immune system.

5.5.1 Anatomy of the hippocampus

The hippocampal formation is located medially in the temporal lobe lying beneath the lateral ventricle and the choroid plexus. Despite over a century of neuroanatomical study there is no consensus on the delineation of the hippocampal formation, and a broader definition includes the dentate gyrus, cornu ammonis (CA, or hippocampus proper) and the parahippocampal gyrus which consists of the subicular complex and the entorhinal cortex (Schultz and Engelhardt, 2014). Figure 2 shows the anatomy of the hippocampal formation. The dentate gyrus is a C-shaped structure that lies between the hippocampus proper and the parahippocampal gyrus. The hippocampus was previously divided into 4 fields: CA1-4, however, today the CA4 field is considered to be a part of the dentate gyrus and is not part of the hippocampus (Schultz and Engelhardt, 2014). In the following sections the hippocampus will thus be defined as the CA1-3 fields, while the dentate gyrus includes the CA4 field.

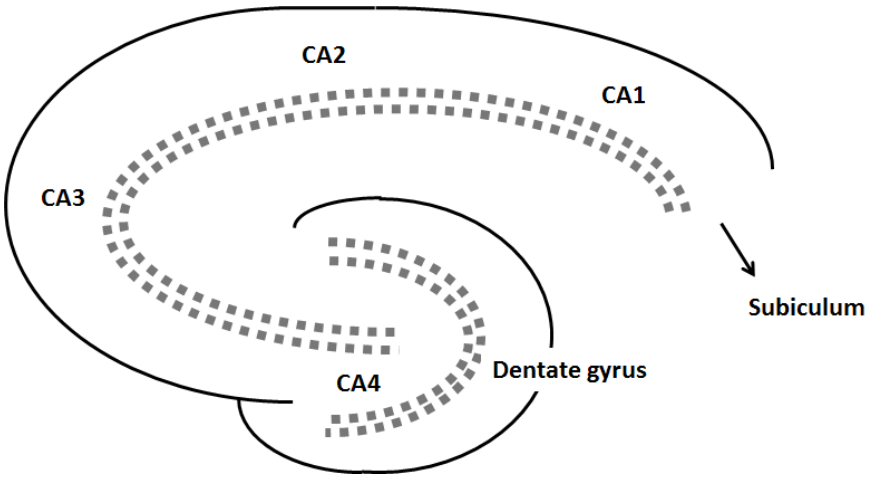

Figure 2 Cross sectional drawing of the hippocampal formation.

The cellular structure of the hippocampal formation is diverse. The dentate gyrus has three cortical layers where the principle layer consists of granular cells. The innermost layer of the dentate gyrus is called the hilus and is sometimes referred to as CA4. The pyramidal neurons in this layer are often considered as constituents of CA3 (Schultz and Engelhardt, 2014). The hippocampus has only one cortical layer, the pyramidal cell layer. Mossy fibers from the dentate gyrus contact the dendrites of CA3 neurons. CA2 is poorly delineated from both CA3 and CA1. CA1 overlaps with

the subiculum at its border, and together they form a transitional zone. The subiculum has three cortical layers while the presubiculum only contains a single layer. Finally, the entorhinal cortex has all six cortical layers (Schultz and Engelhardt, 2014).

5.5.2 Memory formation

Episodic memory is formed through the cortico-hippocampal circuit. The key elements of memory formation are illustrated in Figure 2. The entorhinal cortex receives information from the association cortices and this information is then processed through disynaptic and trisynaptic pathways within the hippocampus predominantly involving the dentate gyrus, CA3 and CA1. CA1, which provides the major output of the hippocampus, sends output information back to the entorhinal cortex *via* the subiculum (Basu and Siegelbaum, 2015). Through this loop the input is integrated and compared to stored memory-related information.

Lesions of different hippocampal areas have identified that the entorhinal cortex – CA1 region is the most important element in memory formation in the cortico-hippocampal circuit (Basu and Siegelbaum, 2015).

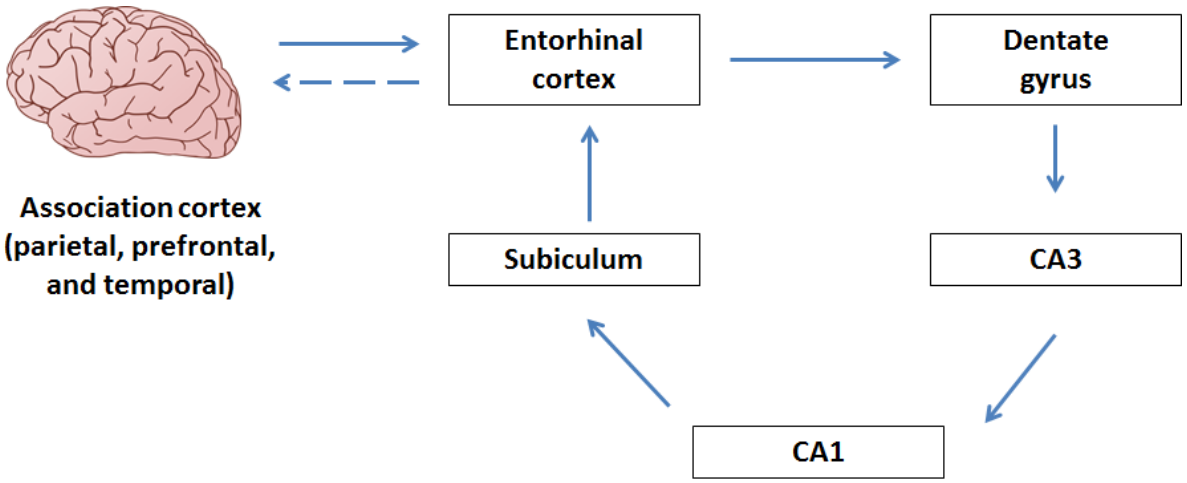

Figure 2 Anatomy of hippocampal memory formation. Input enters the loop via the entorhinal cortex, which sends projections onto the dentate gyrus. CA3 receives axons from the dentate gyrus and sends processed information to CA1 for further processing. CA1 is responsible for the majority of output information, which is sent back to the entorhinal cortex *via* the subiculum.

Long-term potentiation and depression

Donald O. Hebb, a Canadian psychologist, laid the foundations for synaptic plasticity in his model for learning and memory in 1949. He proposed that memory occurs as a result of a coincidence between pre- and postsynaptic activity (Nicoll, 2017). Twenty years later scientist described the phenomena of long-term potentiation (LTP) and depression (LTD) which form the basis of memory consolidation. Figure 4 illustrates the central molecular processes that occur between two nerve-endings during LTP and LTD. LTP: Glutamate is entered into the synapse from the presynaptic cell (*e.g.* axon from CA3), and when this finds the postsynaptic cell (*e.g.* dendrite on CA1) in a depolarized state, it binds to NMDA (as depolarization repels the magnesium blocking NMDA receptors) and AMPA receptors which induce calcium influx. Calcium entry through NMDA receptors activate intracellular kinases resulting in LTP and structural changes in the postsynaptic dendrite including delivery of AMPA receptors onto the surface of postsynaptic endings (Nicoll, 2017; Rozov et al., 2017). In the case of LTD, glutamate finds the postsynaptic ending in a less depolarized state, which is insufficient to repel the magnesium blocking the NMDA receptor. Hence, there is considerably less calcium influx, which leads to the activation of phosphatases, resulting in AMPA endocytosis (Rozov et al., 2017).

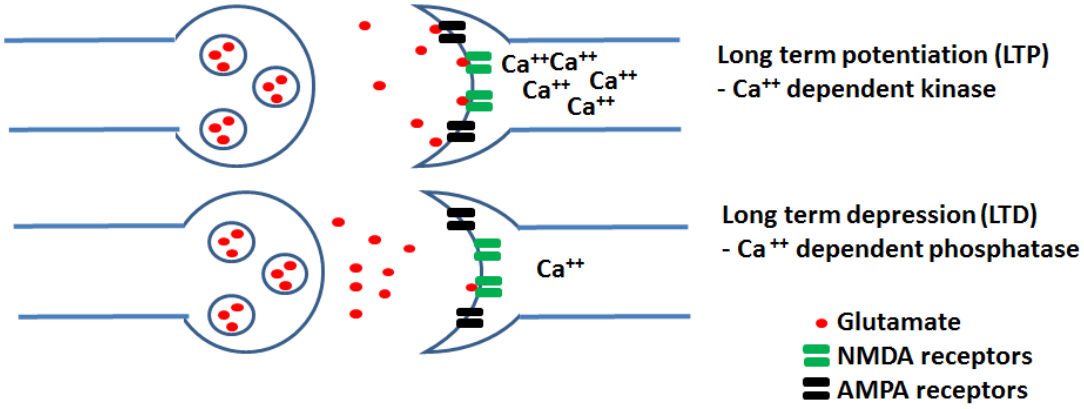

Figure 4 Memory formation on the molecular level. Presynaptic glutamate is released into the synapse. If the postsynaptic nerve ending is depolarized, then glutamate can bind to the NMDA receptors and calcium influx follows. If the postsynaptic nerve ending is not sufficiently depolarized, then NMDA channels are blocked by magnesium molecules, and calcium influx is limited. The excessive calcium influx activates calcium dependent kinases, which cause increased AMPA receptor transport to the nerve ending which further increases intracellular calcium influx. Modest calcium influx activates calcium dependent phosphatases, which leads to the reduction of AMPA on cell surface due to endocytosis.

Cytokines in memory formation

As previously mentioned, the role of the immune system is not limited to inflammation and combating bacteria, but is also involved in physiological processes of the brain. Animal studies demonstrate that memory formation in the hippocampus is influenced by cytokines and microglia (Arisi, 2014), and immune genes have been found to be upregulated in the dentate gyrus during high-frequency stimulation evoked LTP (Havik et al., 2007). IL-1β impairs hippocampal memory by interfering with NMDA receptor-mediated field potentials and impairing calcium influx into postsynaptic nerve endings in the dentate gyrus, thus inhibiting LTP (Butler et al., 2004). TNF was found to be necessary for memory formation under physiological conditions, and has been shown to inhibit LTP through complex mechanisms (Aloe et al., 1999; Butler et al., 2004). IL-6 has also been linked to hippocampal memory through increased *IL-6* gene expression following LTP, although the precise mechanisms are unclear (Balschun et al., 2004).

5.6 Knowledge gaps

In this section I will identify knowledge gaps in our understanding of how the immune system is involved in the pathophysiology of severe mental disorders, thus compiling the rationale for the current PhD thesis.

As previously introduced, severe mental disorders are associated with decreased hippocampal volumes, impaired verbal memory and indications of peripheral low grade inflammation. Cognitive deficits have clinical implications, and interventions targeting the cognitive deficits may aid in preventing disability in psychotic disorders (Kar and Jain, 2016). Animal studies show that the hippocampus and its functions are influenced by cells and molecules of the immune system (Arisi, 2014). Nevertheless, human studies investigating cognition, brain imaging and cytokines in severe mental disorders were scarce at the time of initiating our first study. In addition, plasma levels of immune markers show considerable individual variation also within the healthy population (Belzeaux et al., 2017), thus substantial sample sizes are needed to yield reliable results. Therefore, studies investigating cytokines, cognitive testing and brain imaging in well powered samples are needed to provide robust findings. This can be used to identify new therapeutic targets to treat severe mental disorders. Further, there is evidence supporting an association between hippocampal volume and plasma cytokines (Schmidt et al., 2016). However, hippocampal subfield volumes in relation to circulating cytokines have not yet been investigated in severe mental disorders in well-powered samples prior to the current study.

Several studies indicate that severe mental disorders are associated with low grade peripheral inflammation. However, there are no comprehensive studies that investigate TNF pathway gene expression in blood and brain in addition to cytokine levels in plasma. Further, TNF pathway activity has not been examined by measuring the TNF and its receptors ratio previously, nor has the relationship between TNF pathway activity and clinical symptoms and psychotropic medication been explored. These are most relevant areas to examine in order to better understand potential outcomes of anti-inflammatory treatment.

A final area that deserves attention is the role of developmental pathways, which are also linked to immune functions, in the pathophysiology of severe mental disorders. The Notch signaling pathway has been associated with SCZ and BD through genetic studies and gene expression levels (Dieset et al., 2012; Stefansson et al., 2009). Despite the fact that Notch was first associated with SCZ almost two decades ago (Wei and Hemmings, 2000), little is known about Notch signaling in severe mental disorders. As this pathway has great relevance both for neurodevelopment and fine-tuning the

immune system, understanding potential abnormalities in this pathway in severe mental illness may yield novel drug targets for the treatment of SCZ and BD.

6 AIMS

The overall aim of this thesis was to increase our knowledge about the pathophysiological mechanisms underlying severe mental disorders with a focus on immune related pathways.

In the first study we investigated the hypothesis that plasma levels of inflammatory markers are negatively associated with performance on verbal memory tests. In addition, we investigated whether such associations would be specific to patients. Further, we aimed to identify an association between immune markers and hippocampal subfield volumes. Our last aim was to determine whether performance on verbal memory testes correlated with hippocampal subfield volumes.

In the second study we investigated differences in TNF pathway activity between patients and controls by quantifying proteins, mRNA levels and the ratio between TNF and its receptors. Further, we examined whether TNF pathway activity would be associated with performance on working memory tasks. Finally, we explored possible relationships between psychotropic drugs, clinical symptoms and TNF pathway protein and mRNA levels.

In the last study we characterized the Notch signaling pathway in patients with severe mental disorders and in HC by performing a pathway analysis on the mRNA level. Our secondary aim was to gain knowledge regarding the relationship between Notch signaling components and the use of psychotropic drugs.

7 METHODS

7.1 Study design and ethics

The studies included in this thesis are parts of the TOP-study which was carried out at the Norwegian Centre for Mental Disorders Research, Oslo University Hospital, and collaborating Norwegian hospitals. The TOP-study is approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate (REK # 2009/2485). The blood samples collected during inclusion are kept in a biobank approved by the Norwegian Directorate of Health. All participants have given written informed consent to inclusion into the study.

For the TNF-pathway study the NORMENT group collaborated with the Lieber Institute, Baltimore, Maryland, United States, to obtain data on post-mortem gene expression. Human brain samples were collected at the National Institute of Mental Health through the Offices of the Chief Medical Examiner of the District of Columbia and of Northern Virginia and through tissue donations via funeral homes. Informed consent to study brain tissue was obtained from the surviving next-of-kin for all cases, according to Protocol #90-M-0142 approved by the National Institute of Mental Health /National Institutes of Health Institutional Review Board.

7.2 Participants of the TOP-study

7.2.1 Patients

Inclusion criteria

Patients were included into the TOP-study if they were aged between 18 and 65 years, had IQ above 70 and had a SCZ spectrum or BD spectrum disorder diagnosis.
Additional inclusion criteria into the specific studies:
Immune inclusion criteria: Patients were not included in the 3 studies if they had CRP levels of 20 mg/L and above, if they had coexisting autoimmune or inflammatory diseases, cancers or ongoing infections, and if they were using anti-inflammatory drugs.
Neurocognitive inclusion criteria: We included patients in the neurocognitive analyses if they had Norwegian (or another Scandinavian language) as their first language, if they attended all their compulsory schooling in Norway, and had no history of severe head injury. To ensure adequate individual effort patients were only included in the neurocognitive analyses if they had scored

above 14 on the forced recognition trial of the California Verbal Learning Test (CVLT-II) (Delis et al., 2004).

Diagnostic interview and symptom assessment

The clinical assessment and diagnostic interviews were carried out by a team of psychologists and physicians who were all trained until satisfactory inter-rater reliability was obtained (Ventura et al., 1998). Patients were interviewed using the Structural Clinical Interview for DSM-IV (First, 2002), and a diagnosis was made using all available information. Inter-rater reliability was satisfactory, with an overall agreement for DSM-IV diagnostic categories of 82% with $\kappa = 0.77$ (95% confidence interval = 0.60–0.94).

We evaluated affective and psychotic symptoms using the following interviews: Young Mania Rating Scale (YMRS) (Young et al., 1978) to assess hypomanic/manic symptoms; Inventory of Depressive Symptoms (IDS) (Rush et al., 1996) and Calgary Depression Scale for Schizophrenia (CDSS) (Addington et al., 1990) to measure depressive symptoms; and the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) to assess psychotic symptoms [positive, negative, excited, cognitive using PANSS 5-factorial model (Wallwork et al., 2012)]. We also evaluated general functioning and symptoms with the Global Assessment of Functioning split version function (GAF-F) and symptom scale (GAF-S) (Pedersen et al., 2007). Interrater reliability was acceptable for PANSS subscales with intra-class correlation coefficients (ICCs [1.1] (Shrout and Fleiss, 1979)) ranging from .71 to .73.

Cognitive assessment

The neurocognitive assessments were carried out by psychologists trained in standardized neuropsychological testing. We used the Wechsler Memory Scale-Third Edition (WMS-III) Logical Memory immediate (LM-learning) and delayed recall (LM-recall) (Wechsler et al., 2008), and the California Verbal Learning Test summed recall over learning list (CVLT-learning) and delayed free recall (CVLT-recall) (Delis et al., 2004) to assess verbal memory in patients and HC. We evaluated working memory using the Digit Span Test—backward (Wechsler Adult Intelligence Scale [WAIS] -III), letter number sequencing (WAIS-III) and the Working Memory—Mental Arithmetic (WM-MA) Test—commissions (Simonsen et al., 2011).

7.2.2 Controls

We included randomly selected healthy volunteers obtained from the National Population Registry from the same catchment area as the patients (Statistics Norway, www.ssb.no), and the volunteers were contacted by a letter of invitation. All control subjects were screened with interview for current and previous symptoms of severe mental illness, and for illnesses using the Primary Care Evaluation of Mental Disorders. They were also interviewed about familial history of severe mental illness, drug abuse, and somatic disease. HC were included if they had no history of severe psychiatric disorders (major depression, BD spectrum, SCZ spectrum) neither in any of their first-degree relatives, and no substance or alcohol abuse. In addition, they had the same inclusion criteria into the 3 studies as the patients (immune and neurocognitive inclusion criteria).

7.3 Postmortem cohort

Patients

Next-of-kin were contacted by telephone to gather basic demographic information and medical, substance use, and psychiatric history within 1 week of donation. Diagnosis was obtained through family interviews with the next-of-kin consisting of the Structured Clinical Interview for DSM-IV–clinician version (M.B. First, 1997), the National Institute of Mental Health psychological autopsy interview adapted in part from Columbia University Psychological Autopsy Interview (Kelly and Mann, 1996), the University of Pittsburgh postmortem interview (Lewis, 2002) as well as by psychiatric record reviews with the Diagnostic Evaluation After Death (S. Zalcman, 1983). Cases with cerebrovascular disease (infarcts or hemorrhages), subdural hematoma, neuritic pathology, or other significant pathological features were excluded from further study.

Controls

Control subjects were identified through telephone screening of next-of-kin as well as medical examiner documentation. The criteria for control subjects was no history of significant psychological problems or psychological care, psychiatric admissions, or drug detoxification, no known history of psychiatric symptoms or substance abuse as well as negative toxicology results. Cases were not considered as part of the normal control group if the manner of death was suicide or if death was due to drug overdose or poisoning.

7.4 Plasma protein assessment

Blood sampling procedure

Blood sampling was carried out in the morning for the majority of patients (median time 10 am) and mainly in the afternoon for the healthy control group (median time 3 pm) with large degree of overlap. Blood samples were drawn into EDTA tubes, stored at room-temperature for 45 minutes and placed in refrigerator at 4^{o}C. They were then transported to the Biobank the following workday, where 2 x 9 ml EDTA tubes were centrifuged at 1800 *g* for 15 minutes. Plasma was collected and stored at -80^{o}C in multiple aliquots. The same procedure was used for both patients and controls.

Measurement of immune markers

We measured plasma levels of immune markers using enzyme-linked immunosorbent assays. We quantified TNFR1, TNFR2, ADAM17, IL-1Ra, IL-6 and OPG using R&D systems (Minneapolis, MN, USA), DLL1 and DLK1 R&D systems (Abingdon, UK), soluble CD40L using Bender Medsystem (Vienna, Austria), CRP and vWF using Dako-Cytomation (Oslo, Norway) while TNF levels were obtained using Cloud Corp (Houston, TX, USA). Intra- and inter-assay coefficients of variance for proteins were less than 10%.

7.5 MRI

We obtained structural brain imaging data of patients and controls that consented to magnetic resonance imaging (MRI) between 2003 and 2007. They were scanned consecutively using a 1.5 Tesla scanner equipped with an 8-channel head coil (Siemens Magnetom Sonata scanner, Siemens Medical Solutions). All scans were visually inspected following standard procedures, and when brain pathology was detected (*e.g.* tumors, bleeding, infarcts or obstructive hydrocephalus) we excluded patients and controls, and they were referred to their general practitioner for follow-up. We used a sagittal T1-weighted magnetization prepared rapid gradient echo (MPRAGE) pulse sequence with the following parameters to assess brain volumes: time of repetition (TR)/echo time (TE)/inversion time (TI) = 2730 ms/3.93 ms/1000 ms, flip angle (FA) = 7°, field of view (FOV) = 240 mm, acquisition matrix = 256 9 192, voxel size = 1.33 9 0.94x1 mm3, and 160 slices. We repeated the sequence, and the two runs were combined during post-processing to increase signal-to-noise ratio.

The MRI processing procedures were fully automated without manual editing. MRI processing using FreeSurfer (Fischl, 2012) included motion correction and averaging of the two T1 weighted

volumes (Reuter et al., 2010), removal of non-brain tissue using a hybrid watershed/surface deformation procedure (Segonne et al., 2004) and automated Talairach transformation. Further, we carried out segmentation of the subcortical white matter and deep gray matter volumetric structures by combining information on image intensity, probabilistic atlas location, and the local spatial relationships between structures to automatically assign a neuroanatomical label to each voxel in the MRI volume (Fischl et al., 2002; Van Leemput et al., 2009). We used an automated segmentation method in FreeSurfer (Fischl, 2012) (http://surfer.nmr.mgh.harvard.edu) for hippocampal subfield segmentation.

The hippocampal subfield segmentation is based on a Bayesian modeling approach and manual delineations of each hippocampal subfield (Van Leemput et al., 2009). The obtained subfield volumes have been compared to manual hippocampal subfield tracings and were shown to be most reliable for the larger subfields including CA2/3, CA4/DG, and subiculum, with acceptable reliability for the CA1, presubiculum, and fimbria (Van Leemput et al., 2009).

7.6 mRNA expression – whole blood

RNA isolation: We collected blood samples in Tempus Blood RNA Tubes. We quantified *TNF*, *TNFR1*, *TNFR2* and *ADAM17* mRNA using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The sequencing library construction was performed using the TruSeq RNA Sample Preparation v2 kit by Illumina.
We extracted total RNA for Notch signaling pathway genes with ABI PRISM 6100 Nucleic Acid PrepStation and TEMPUS 12-port RNA Isolation Kit according to manufacturer's protocol. High-Capacity cDNA Reverse Transcription Kit was used for reverse transcription of 1 µg RNA.

Quantitative polymerase chain reaction (qPCR): We performed real-time reverse transcription polymerase chain reaction (real time RT-PCR) to quantify *TNF*, *TNFR1*, *TNFR2* and *ADAM17* mRNA with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Approximately 0.5 µg total RNA was used in 96-well PCR plates (Applied Biosystems). We placed 80 samples on each plate and they were randomly distributed to atone for the differences in reverse transcription or subsequent real-time PCR efficiency. Quantification of mRNA was performed with the quantitative polymerase chain reaction Master Mix for SYBR Green I (Applied Biosystems) in 10 µL duplicate reactions in 384 well plates on an ABI Prism 7900 (Applied Biosystems) using the 2(-Delta Delta C[T]) method with the average of four pools of cDNA. These were included on each plate in the RT-PCR and followed the samples in the real time PCR, as reference. The CV was

under 10 % for the average of these calibrators between all the plates analyzed. We designed sequence specific mRNA primers for the full-length *TNF, TNFR1, TNFR2* and *ADAM17* mRNA using Primer Express software version 3.0 (Applied Biosystems). We evaluated melt curves for all primers and normalized data to ß-actin.

Global Transcriptomics Analyses: We used the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/pathway.html) to select Notch pathway related genes. We used 200 ng of total RNA for each sample and we labelled and amplified the samples using the Illumina TotalPrep-96 RNA Amplification Kit (Thermo Fisher, Waltham, MA, USA). We performed global analysis of gene expression with Illumina HumanHT-12 v4 Bead Chip (Illumina, San Diego, CA, USA) consisting of more than 47 000 probes. We exported raw microarray scan files using the Illumina GenomeStudio software and loaded into R for downstream analysis using specific packages provided by BioConductor (Ritchie et al., 2011). We used Lumi to detect outliers (Du et al., 2008). The R package (version 3.24.4.) was applied to correct for technical batch effects, such as RNA extraction batch, RNA extraction method, DNase treatment batch, cRNA labelling batch and chip hybridization. Further quality control, quantile-normalization and log2-transformation were performed using Limma (Ritchie et al., 2015).

7.7 mRNA expression – post mortem

RNA isolation: total RNA was extracted from approximately 100 mg of post-mortem tissue homogenates of dorsolateral prefrontal cortex gray matter approximating BA9/46 in postnatal samples using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The sequencing library construction was performed using the TruSeq RNA Sample Preparation v2 kit by Illumina.

Global Transcriptomics Analyses: Illumina Real Time Analysis module was used to perform image analysis and base calling. FASTQ files containing the sequencing reads were generated using the BCL converter (CASAVA v1.8.2). These reads were aligned to the human genome (UCSC hg19 build) using the spliced-read mapper TopHat (v2.0.4) with the reference transcriptome to initially guide alignment on the basis of known transcripts of Ensemble Build GRCh37.67 (the "-G" argument in the software). A normalized reads per kilobase million (RPKM) metric were calculated for each gene by dividing the number of reads mapping to the gene divided by the length of the gene (in kilobases).

7.8 Statistical analyses

7.8.1 Software

We used SPSS software package for Windows version 22.0 (papers I and II) and version 24.0 (paper III) to assess data normality, investigate differences between groups and correlations, as well as to control for confounding factors. In addition, we performed the statistical analyses for global transcriptomics by using Limma (Ritchie et al., 2015) and R package (version 3.24.4.).

7.8.2 Data normality

We assessed data normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Where data showed significantly skewed distributions we attempted transformation of the variables (*e.g.* natural logarithmic). If the variable showed normal distribution after transformation, we used the transformed variables and performed parametric analyses. When data remained skewed despite attempts at data transformation, we used non-parametric test.

7.8.3 Correlations

We used Spearman's Rank Correlation for skewed data and Pearson's Correlation for normally distributed data to investigate associations between variables.

7.8.4 Differences between groups

We investigated significant differences in mean values between groups using T-tests and one-way analysis of variance for normally distributed variables. For variables with skewed distributions we used Mann-Whitney U test and Kruskal-Wallis test to investigate significant differences between groups.

7.8.5 Confounding factors

There are several conditions that alter monocyte/macrophage activation and the production of cytokines. Smoking has a well-established role in modulating both the innate and the adaptive immune system leading to an increase in TNF and TNF receptor production, however, nicotine *per se* may have anti-inflammatory properties (Arnson et al., 2010; Nizri et al., 2009). Adiposity is another important factor where activated macrophages in adipose tissue represent a rich source of cytokines (Howe et al., 2013). Age and sex are also associated with altered cytokine levels

(Rainville et al., 2017; Ventura et al., 2017), while ethnicity affects leukocyte and neutrophil counts (Padiyar and Hricik, 2011).

We investigated the effects of possible confounding factors. Known confounding factors were selected based on existing literature. The immune system and the Notch signaling pathway both show diurnal variation, we therefore also investigated differences in time of blood sampling and the expression of the clock gene *BMAL1* (Cermakian et al., 2013; Jensen et al., 2012; Ko and Takahashi, 2006). We then observed whether there was a significant difference in demographic variables between groups (*e.g.* age, sex, smoking, body mass index, time of blood sampling and ethnicity). Possible confounding factors were then explored further by investigating associations between variables (*e.g.* age/TNFR1, body mass index/TNFR1) and differences between groups (*e.g.* smoking/non-smoking, Caucasian/non-Caucasian). Alpha was set at 0.2 for these analyses, and the thus identified confounding factors were controlled for in Analysis of covariance (ANCOVA) models. We repeated the regression analyses after removing studentized deleted residuals below -3 and above 3 to ensure that our results were not driven by outliers.

7.8.6 Multiple testing

We controlled for multiple testing by lowering the alpha threshold based on the Bonferroni method for most of our analyses. The Bonferroni adjustment is a conservative statistical method to reduce the probability of type I errors by deflating the alpha threshold based on the number of independent null hypotheses (Ranstam, 2016). A general concern of the Bonferroni method is that it might increase the probability of type II errors, which are regarded as equally important and relevant in clinical practice (Perneger, 1998). We therefore distinguished between primary analyses and secondary analyses, and calculated different alpha thresholds to reduce the risk of type II errors.

We employed false discovery rate (Chadeau-Hyam et al., 2013) to control for multiple testing for the TNF pathway global transcriptomics analyses. This was done in collaboration with the National Institute of Mental Health group.

8 SUMMARY OF RESULTS

8.1 First Study

Association between cytokine levels, verbal memory and hippocampus volume in psychotic disorders and healthy controls

We first investigated associations between cytokine levels and verbal memory as measured using CVLT and WMS-II Logical Memory in 117 BD, 109 SCZ patients and 236 healthy controls. We controlled for age, sex and diagnosis. We found a significant association between plasma levels of sTNFR1 and verbal memory (β = -0.16, $p = 4 \times 10^{-4}$), indicating higher memory performance with lower sTNFR1 levels. These results remained significant after correcting for multiple testing. We found no significant associations between plasma levels of IL-1Ra, IL-6, OPG, vWF, CRP and soluble CD40L and verbal memory after correction for multiple testing, however, vWF showed nominally significant negative correlation with verbal memory tests (β = -15, p = 0.005).

We observed no significant interaction effect between diagnosis and sTNFR1 on memory scores, which supports that our finding is not strongly related to severe mental disorders, but is also present in individuals without a psychotic disorder.

We also examined whether inflammatory markers are associated with hippocampal subfield volumes in a subsample (BD=67, SCZ=46 and HC=111) that did not differ significantly from the main sample in demographics. We found no significant associations between hippocampal subfield volumes and any of the inflammatory markers.

Finally, we found a nominally significant positive association between hippocampal subfield volumes (CA2/3, CA4/DG and the subiculum) and performance on the California Verbal Learning Test – learning after controlling for confounders and correction for multiple testing (β = 0.22 and p = 0.003).

8.2 Second Study

A study of TNF-pathway activation in schizophrenia and bipolar disorder in plasma and brain tissue

In this study we measured plasma levels of TNF-pathway proteins and the expression of TNF-pathway genes in whole blood as well as in the brain.

We had a large sample (N=1003) of 247 BD, 569 SCZ and 624 HC participants with plasma levels of TNF-pathway markers. Our results show slightly elevated levels of plasma soluble TNF, TNFR1 and TNFR2 in patients *vs.* HC after controlling for confounding factors and adjusting for multiple testing (t=2.56 – 4.47 and p <0.001). Further, the SCZ group had significantly lower levels of soluble ADAM17 proteins compared to HC (t=-2.93 p <0.01). We also demonstrated that the ratio between soluble TNF and its receptors is increased in patients compared with HC (t=4.07 p<0.001).

We investigated TNF-pathway gene expression in whole blood in a subsample of patients and controls (BD=143, SCZ=224 and HC=184). Our analyses revealed significantly decreased *TNF* mRNA in patients compared to HC after controlling for confounders and correcting for multiple testing (t=-3.91 p<0.001). In addition, we found a highly significant difference in *ADAM17* mRNA levels in BD *vs.* HC (t=5.92 p<0.001) and BD *vs.* SCZ (t=8.03 p<0.001) with no differences between SCZ and HC (in other words BD > HC, SCZ).

The results from the post-mortem cohort (BD=44, SCZ=79 and HC=86) show no significant alterations in TNF-pathway gene expression in the dorsolateral prefrontal cortex after controlling for confounders and correction for multiple testing. Interestingly, *ADAM17* mRNA levels were significantly increased in the BD group compared to HC (t=1.62, p=0.05), however, this result did not remain significant after correcting for multiple testing.

We investigated whether working memory performance, which has frequently been associated with the dorsolateral prefrontal cortex, was associated with TNF-pathway markers. Indeed, we demonstrated that higher levels of both TNF (F (5,122)=3.35, R^2=0.09, β=-0.26, p=0.003) and the TNF/TNFRs ratio (F (5,120)=2.90, adjusted R^2=0.07, β=-0.23, p=0.008) were significantly associated with lower scores on working memory after controlling for age, sex, PANSS score and antipsychotics and correcting for multiple testing.

Our secondary analyses examined the relationship between the use of psychotropic drugs and clinical symptoms and TNF-pathway proteins. We discovered that higher lithium levels were significantly associated with higher TNF levels (n=19, β=0.42, p<0.01) and TNF/TNFRs ratio (β=0.39, p<0.05) after controlling for confounders, but was rendered non-significant after correction for multiple testing. Further, Patients using lithium (n=19) had higher levels of *ADAM17* mRNA (t=2.24, p<0.05) and sTNFR1 levels (t=2.34, p<0.05) compared to non-medicated (n=85) patients. We found no other associations with medication and TNF-pathway proteins. We identified nominally significant correlations between clinical symptoms (*i.e.*, PANSS, GAF and CDSS) and TNF-pathway proteins and TNF pathway related gene expression after controlling for confounders

and correcting for multiple testing. These findings, however, are inconsistent. In SCZ, higher TNF and TNFR1 levels were associated with increased symptom load (*i.e.* lower scores on GAF (β=-0.14, p<0.01) but we observed no such pattern in the BD group (β=0.01). Similarly, TNFR2 levels showed a negative correlation with depressive symptoms in BD (β=-0.22, p<0.01), but not in SCZ (β=-0.02). Further, we found a negative association between *TNF* mRNA levels and duration of illness in the SCZ group (β=-0.14, p<0.05) however we saw the opposite in the BD group (β=0.13, p>0.05). Therefore we cannot establish any consistent associations between clinical symptoms and TNF-pathway proteins.

8.3 Third Study

Exploring the Notch signaling pathway in schizophrenia and bipolar disorder

We carried out a pathway analysis in the third study by measuring the expression of Notch signaling pathway related genes and by quantifying the plasma levels of Notch related soluble proteins. We selected 49 genes and measured their mRNA levels by microarray in 241 BD, 338 SCZ patients and in 263 HC, and investigated the plasma levels of two Notch pathway related proteins in a larger sample (BD=246, SCZ=551 and HC=639).

Our plasma analyses revealed elevated plasma levels of the canonical Notch ligand DLL1 in both SCZ and BD compared to HC after controlling for confounders and correcting for multiple testing (t=8.80 p<0.001 and t=3.26 p<0.01 respectively). Further, SCZ patients showed significantly higher levels of DLL1 compared to BD (t=3.00 p<0.01). We found significant increases in the non-canonical Notch ligand DLK1 levels in patients (both SCZ and BD) compared to HC, which did not remain significant after correction for multiple testing (t=2.23 p<0.05 and t=2.00 p<0.05 respectively).

Significant mRNA findings are summarized in paper 3: Table 3, figures 1 and 2, as well as in supplementary figure 1. In SCZ, we found increased expression of *RFNG*, *DTX3L* and *KAT2B*, and decreased expression of *PSEN1*, *CREBBP*, *CTBP1*, *CTBP2*, *HDAC1*, *HDAC2* and *RBPJ* after controlling for confounders and correcting for multiple testing (p<0.001). In BD, we observed fewer alterations in gene expression with two significantly up-regulated genes (*RFNG* and *KAT2B*) and two significantly downregulated genes (*PSEN1* and *CREBBP*) (p<0.001). Significant differences between SCZ and BD patients were limited to decreased *LFNG* expression in SCZ, and increased *NOTCH2* expression in SCZ (p<0.001). Further, 22 gene mRNA levels were nominally

altered between patient and HC groups. These are summarized in supplementary table 1 in paper III.

Our secondary analyses examined the relationship between psychotropic drugs and select gene mRNA levels (*PSEN1*, *RBPJ* and *RFNG*). We showed that patients using lithium (n=35) had significantly higher *RBPJ* expression compared to patients not taking lithium (n=366, p<0.001). We repeated our analyses in the BD group and controlled for confounders and corrected for multiple testing. Our result remained significant (t=3.51 p=0.001 F=3.42 n=148). We did not find a dose dependent relationship between lithium and *RBPJ* expression. Nominally significant findings included a dose dependent positive association between antidepressants and DLL1 (n=30, β=0.15, p<0.05), and a group effect of antipsychotics and mood stabilizers on DLK1 levels (t=2.08 p<0.05 and t=2.38 p<0.05 respectively).

9 DISCUSSION

9.1 Main findings

9.1.1 TNF, memory and hippocampus morphology

In the first paper we reported a modest association between plasma levels of TNFR1 and verbal memory in a large sample of patients with severe mental disorder and controls. Further, this association was similar in SCZ, BD and in HC, and we observed no interaction effect of diagnosis. Finally, we found a significant association between verbal learning and hippocampal volume, which did not remain significant after correcting for multiple testing.

Our TNF findings are in line with other studies investigating associations between cognition and inflammatory markers, however there are generally few studies in this field, and several studies could not establish a significant correlation between TNF markers and verbal memory (Cheung et al., 2013; Misiak et al., 2017b; Rosenblat et al., 2015).

We did not find significant associations between the 6 other immune markers (IL-1Ra, IL-6, CRP, CD40L, vWF and OPG) and hippocampal memory. These observations do not preclude the involvement of these immune markers in hippocampus related memory rather they suggest a lack of connection between peripheral immune marker levels and memory performance. Indeed, IL-1β and IL-6 have been proposed to directly affect hippocampal learning and memory. This is supported by animal studies that show that IL-1β is involved in the formation of long term potentiation in the hippocampus (Tsai, 2017). Other studies show that IL-6 impairs long-term potentiation thus potentially interfering with memory formation (Arisi, 2014). A recent meta-analysis supported a negative association between CRP levels and verbal learning/memory, however, the authors concluded that findings regarding cytokines are discordant and the number of studies are scarce (Misiak et al., 2017b). CD40L may reflect macrophage activity, and the CD40-CD40L pathway is involved in neuro-inflammation. An animal study demonstrated that downregulation of CD40 signaling improved long-term cognitive functions in rats that survived sepsis, however, there are no other studies investigating the association between CD40 and verbal memory in humans (Michels et al., 2015). We have previously demonstrated elevated levels of vWF and OPG in severe mental disorders vs. HC in our TOP-sample, which may reflect endothelial activation (Hope et al., 2010; Hope et al., 2009). Endothelial activation could lead to increased permeability of the BBB, thus

aiding the penetration of cytokines into the brain parenchyma. However, vWF and OPG levels were not associated significantly with verbal memory in our sample after correcting for multiple testing.

We found no significant associations between hippocampal subfield volumes and immune markers, although we did observe a trend towards a negative association. This may be due to study design and type II errors, rather than lack of association between circulating cytokines and hippocampal volumes. These methodological challenges will be discussed in section 9.3. We observed a trend level association between increased OPG levels and hippocampal volume reduction, which may indicate a role for endothelial activation in hippocampal size reduction. The existing knowledge and our trend level finding suggest that further study of inflammatory markers and hippocampal volumes is warranted.

Finally, we found a nominally significant moderate association between larger hippocampal subfield volumes and performance on verbal memory/learning tests. This is in line with previous studies investigating the relationship between scores on the California Verbal learning test and hippocampal size (Chepenik et al., 2012; Tischler et al., 2006). Although we cannot draw direct causality from this cross-sectional observation, it does seem to support the bigger is better hypothesis for hippocampal volumes (Roth et al., 2010).

9.1.2 TNF pathway in peripheral blood and prefrontal cortex

In the second paper, we found that both SCZ and BD patients independently had slightly elevated plasma levels of TNF-pathway related proteins. We also determined that the relationship between pro-inflammatory and anti-inflammatory TNF pathway proteins was skewed to an increased pro-inflammatory profile. Further, despite the elevated TNF plasma levels, *TNF* mRNA expression in circulating immune cells was reduced in patients. We found no significant differences in TNF-pathway related mRNA expression in the dorsolateral prefrontal cortex (DLPFC), although, we did observe that a shift toward a pro-inflammatory imbalance in the TNF pathway was weakly but significantly associated with lower working memory scores. Figure 5 summarizes the TNF-pathway related proteins that we investigated in our second study. Briefly, TNF, TNFR1 and TNFR2 are transmembrane proteins with an extracellular domain that can be proteolytically cleaved from the membrane surface by the metalloprotease ADAM17. The soluble TNF receptors can bind the soluble TNF, thus reducing its availability (Moelants et al., 2013).

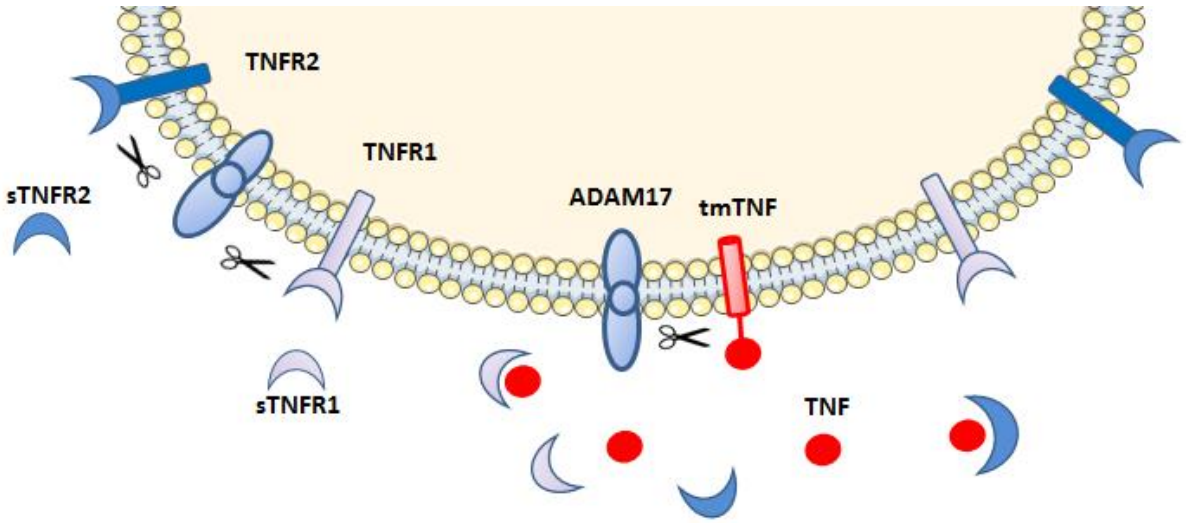

Figure 5 Summary of TNF-pathway related proteins. Transmembrane TNF (tmTNF) and its receptors TNFR1 and TNFR2 are transmembrane proteins with an extracellular domain that can be proteolytically cleaved from the membrane surface by the metalloprotease ADAM17. The cleaved proteins are soluble TNF, soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2). sTNFR1 and sTNFR2 are decoy receptors and compete with membrane bound receptors to bind sTNF thus reducing its availability (Moelants et al., 2013).

Our TNF, sTNFR1 and sTNFR2 findings are in line with previous studies that investigated cytokine levels from peripheral blood in patients with severe mental disorders (Miller et al., 2011; Munkholm et al., 2013; Upthegrove et al., 2014), however, no studies have investigated the relationship between TNF and its receptors. The TNF/TNFRs ratio reflects TNF-pathway activity and correlates with TNF bioactivity (Aukrust et al., 1999). Our results show that not only are the individual TNF and TNFRs proteins increased in patients, but also TNF relative to its receptors is increased in patients *vs*. controls. This further supports a pro-inflammatory imbalance in severe mental disorders.

We observed that while TNF protein levels were increased in patients, they had significantly decreased *TNF* expression in circulating immune cells. This finding is in contrast with other studies that determined increased *TNF* expression in monocytes and lymphocytes (Drexhage et al., 2010; Liu et al., 2010; Pandey et al., 2015). There could be a few reasons for this. Firstly, we examined gene expression in whole blood, where the majority of cells (approximately 70%) are neutrophil granulocytes (Dieset et al., 2012), thus we investigated the overall production of TNF in circulating cells, and our analyses were not restricted to monocytes or lymphocytes. Secondly, we have a considerably larger sample size (approximately 10-fold compared with the other studies). We found no differences in *TNFR1* and *TNFR2* expression levels, which is not in conflict with other findings, as these have not been investigated previously.

Another important finding from this study was the highly significantly increased expression of *ADAM17* in BD patients. This is a novel finding as previous studies have not investigated *ADAM17* expression in peripheral blood in severe mental disorders. Although ADAM17 plays an important part in the TNF-pathway, it is not specific to that pathway and partakes in other relevant pathways such as Notch and neuregulin (Mei and Xiong, 2008; Murphy et al., 2008).

We also investigated gene expression in the DLPFC in a separate sample using postmortem tissue. We found no significant differences between groups after correcting for multiple testing. However, we observed a trend towards increased *ADAM17* also in the BD group *vs.* HC. There is a small study that investigated *ADAM17* expression in Brodmann Area 9 and found increased expression in SCZ patients, however, the sample size was particularly small (6 patients and 6 controls) (Marballi et al., 2012). Other postmortem studies investigating the TNF-pathway in the prefrontal cortex in severe mental disorders found increased *TNFR1* expression in SCZ (n=19), and decreased *TNFR2* in BD (n=10) in the DLPFC (Dean et al., 2013). There are no previous studies comparable in size that have investigated the TNF-pathway in severe mental disorders.

Interestingly, we found nominally significant associations between TNF levels (as well as TNF-ratio) and serum concentrations of lithium. This observation supports previous studies that also found that lithium increases the production of pro-inflammatory cytokines (Maes et al., 1999).

The last finding of this study was reduced performance on working memory tests with increasing TNF ratio levels, independent of diagnosis. There are no previous studies investigating the association between working memory and the TNF-pathway.

9.1.3 Aberrant Notch signaling

In the third paper we determined significantly elevated plasma levels of DLL1 as well as altered gene expression of key Notch signaling proteins. Further, we showed for the first time that patients taking lithium had significantly higher *RBPJ* expression. Figure 6 illustrates the Notch signaling pathway.

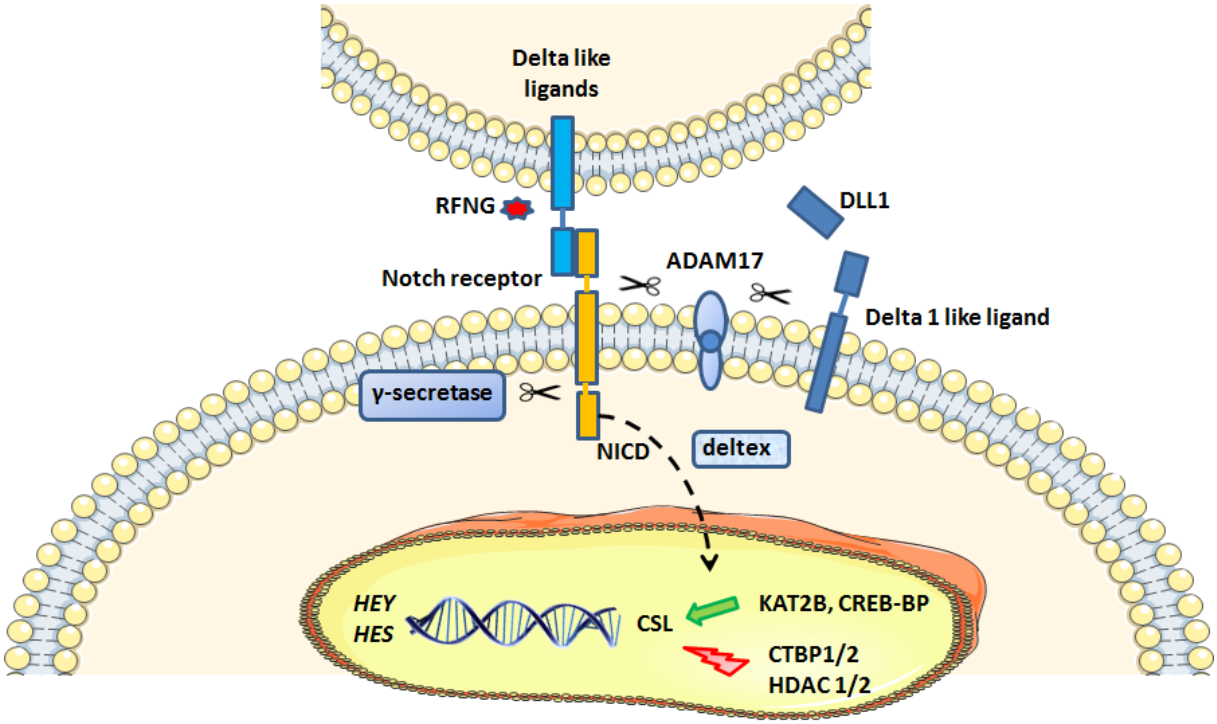

Figure 6 Notch signaling. Abbreviations with gene names: RFNG (radical fringe) /*RFNG*, DLL1 (delta like ligand 1) /*DLL1*, CSL (recombining binding protein suppressor of hairless)/*RBPJ*, CREB-BP (CREB binding protein)/*CREBBP*, CTBP1 (C-terminal-binding protein 1)/ *CTBP1*, CTBP2 (C-terminal-binding protein 2)/ *CTBP2*, HDAC1 (histone deacetylase 1)/ *HDAC1*, HDAC2 (histone deacetylase 2)/ *HDAC2*.
Notch activation is initiated by the Notch receptor binding a Notch ligand (delta-like-ligand). This leads to proteolytic cleavage of the receptor at two sites (i) by ADAM17 and (ii) γ-secretase. Following proteolytic processing the Notch intracellular domain (NCID) translocates into the nucleus where it binds to CSL and together with co-activators (KAT2B and CREB-BP) or co-repressors (CTBP1/2 and HDAC 1/2) they influence the expression of target genes (*HEY* and *HES*).

DLL1 is a transmembrane protein and is one of 3 delta ligands (DLL1, DLL3 and DLL4). It has an extracellular domain that can be proteolytically cleaved by ADAMs (Figure 6). We found significantly increased DLL1 protein levels in both SCZ and BD independently. There are no previous studies investigating DLL1 plasma levels in severe mental disorders. DLL1 likely inhibits Notch signaling (Mishra-Gorur et al., 2002) and the increased DLL1 levels may thus lead to attenuated Notch signaling. We did not observe increased *DLL1* expression in immune cells, indicating that the higher circulating DLL1 levels might be the result of increased shedding.

Our gene expression findings are also novel as this is the first study to conduct an exploratory pathway analysis on the Notch signaling pathway in severe mental disorders. In SCZ, we found increased expression of *RFNG*, *DTX3L* and *KAT2B*, and decreased expression of *PSEN1*, *CREBBP*, *CTBP1*, *CTBP2*, *HDAC1*, *HDAC2* and *RBPJ*. In BD, we observed fewer alterations in gene expression with two significantly up-regulated genes (*RFNG* and *KAT2B*) and two significantly downregulated genes (*PSEN1* and *CREBBP*). Significant differences between SCZ and BD patients were limited to decreased *LFNG* expression in SCZ, and increased *NOTCH2* expression in SCZ.

RFNG or radical fringe is a secreted protein that is specific to the Notch signaling pathway. Animal studies suggest that RFNG inhibits Notch signaling by decreasing the expression of the target gene *HES* (Mikami et al., 2001). Therefore, the increased expression of *RFNG* in both SCZ and BD support attenuated Notch signaling in severe mental disorders.

DTX3L is a gene that codes for deltex proteins. In general, deltex facilitates intracellular transportation of the notch intracellular domain to the nucleus (Figure 6) however it may also hamper Notch signaling by destabilizing Notch receptor when it interacts with other proteins (Hori et al., 2012). Therefore the significance of increased *DTX3L* expression is somewhat unclear.

PSEN1 codes for the presenilin-1 protein, which is the catalytic core of the γ-secretase complex (see Figure 6). γ-secretase is not specific to Notch signaling nevertheless the amyloid precursor protein and Notch receptors are its predominant substrates (Stanga et al., 2017).

Within the nucleus, the proteins coded by *CREBBP, KAT2B*, *CTBP1*, *CTBP2*, *HDAC1* and *HDAC2* act as co-activators (CREB-BP, KAT2B) or co-repressors (CTBP1/2 and HDAC1/2) facilitating or hampering transcription respectively. *RBPJ* codes for the Suppressor of Hairless/CBP-1/Lag-1 protein also known as CSL. Depending on whether it binds with co-activator or co-repressor proteins, CSL switches on or switches off the transcription of target genes. The significantly altered gene expression of co-activators/repressors as well as CSL further distinguishes the Notch pathway as a pathway of interest in severe mental disorders.

During the last decades researchers have identified several susceptibility genes for SCZ in connection to the Notch signaling pathway (Sundararajan et al., 2018). Among them, *NOTCH2*, *NOTCH3* and *NOTCH4* are genes coding transmembrane notch receptors. *NOTCH1* and *NOTCH2* expression was decreased in BD (nominally significant findings) and unaltered in SCZ. Further, we have previously demonstrated increased expression of *NOTCH4* and *ADAM17* however expression of both genes did not differ in patients *vs.* controls in this study. This discrepancy might be due to a

difference in sensitivity for microarray analyses compared with single gene expression analyses. This will be further discussed in section 9.4.

9.2 Potential immune related mechanisms in severe mental disorders

In this section, I will discuss our findings in a broader perspective with implications for disease mechanisms.

9.2.1 Memory

Our results may imply that the TNF pathway could be involved in memory processes independent of disease. The first studies to investigate the effect of TNF in learning and memory were conducted two decades ago. In one of the studies researchers chronically injected TNF into the ventricles of rats and found that this led to cognitive impairment (Bjugstad et al., 1998). Since then, several studies have investigated TNF levels in peripheral blood and performance on verbal learning/memory in humans in an effort to establish a connection. The basic mechanisms underlying memory formation were presented in the introduction section 5.5.2. The proposed mechanism underlying the role of TNF in memory formation is through its ability to influence intracellular calcium concentration and transcription factors such as NF-κB. Further, glial TNF increases the expression of AMPA receptors on the surface of neurons (Pickering et al., 2005). These processes interfere with the development of long term potentiation, thus impeding memory formation.

We found that peripheral levels of sTNFR1, which indicate activity in the peripheral TNF pathway, were negatively associated with performance on memory tests. The cross sectional design of our study limits causal inferences, however, it is plausible that elevated peripheral inflammatory markers may influence memory *via* the cross-talk between the peripheral immune system and the brain through the blood brain barrier as described in section 5.3.2. This may then have clinical implications and will be discussed in section 9.3.

9.2.2 Peripheral immune system

The TNF and Notch signaling pathways partake in multiple processes in the periphery that may not be related to the brain. Indeed our investigations were carried out using blood samples from the circulatory system, and our results would therefore be influenced by peripheral immune changes.

TNF pathway

In addition to its growing significance in neurological and neuropsychiatric disorders, TNF also plays a central role in other pathophysiological conditions such as cancer, cardiovascular diseases, autoimmune diseases, chronic inflammatory diseases and metabolic diseases. Thus the increased TNF activity that we found in severe mental disorders may in part be attributed to co-morbidity. We aimed to reduce the probability that our results would be associated to co-morbidity, and this is further described in the general methodological issues section, 9.4.

TNF partakes in the development of cancer through aiding the survival and proliferation of malignant cells (Balkwill, 2009). This was initially surprising, as TNF, as its name suggests (tumor necrosis factor), was first introduced as an agent that destroys, necrotizes cancer through its cytotoxic properties (Wanebo, 1989). Today we recognize that the role of the TNF pathway is complex and it has an important role in cell fate determination inducing cell proliferation as well as apoptosis (Aggarwal et al., 2012). It is interesting that the incidence of cancer in severe mental illnesses is slightly reduced compared to the general population on a group level, however lung and breast cancer have higher prevalence most likely due to the increased smoking habits of patients (Howard et al., 2010; Li et al., 2017).

The TNF pathway has a prominent role in cardiovascular diseases. Myocardial tissue produces TNF under pathological circumstances, and TNF levels have been associated with heart failure and cardiomyopathy (Feldman et al., 2000). Further, TNFR1 mediated effects of TNF are also associated to the formation of atherosclerosis (Kusters and Lutgens, 2015). TNF is also produced by adipocytes and TNF levels are elevated in obesity and lead to insulin resistance (Tzanavari et al., 2010). Cardiovascular and cardiometabolic diseases have high incidence rates and are the most common causes of death in severe mental disorders (Brown et al., 2010; Correll et al., 2017; Ringen et al., 2014). The elevated TNF levels and TNF-ratio are therefore important findings that further underscore an imbalance in the immune system towards a pro-inflammatory state and implicate the TNF pathway as a potential therapeutic target in severe mental illnesses.

TNF plays a central role in autoimmune diseases such as rheumatoid arthritis. This is further evidenced by the findings that TNF inhibitors are effective in treating the symptoms of rheumatoid arthritis (Monaco et al., 2015). The inverse association between rheumatoid arthritis and SCZ was already recognized in the 1930's before the development of neuroleptics (Vinogradov et al., 1991). Interestingly, other autoimmune diseases such as celiac disease and psoriasis are positively associated with SCZ (Chen et al., 2012). The connection between autoimmune diseases and SCZ

does not seem to extend to BD. With the exception of pernicious anemia there are no consistent associations between BD and autoimmune diseases (Eaton et al., 2010). We observed that both SCZ and BD patients independently had increased TNF levels and TNF/TNFRs ratio. Thus, our findings suggest that autoimmune diseases are unlikely to explain the differences in TNF pathway activity between patients and controls.

Notch pathway

The Notch signaling pathway has a distinct role in both adaptive and innate immunity as well as in hematopoiesis.

During hematopoiesis Notch signaling controls the differentiation of several cell lineages. The best studied is the differentiation of bone marrow progenitor cells. These cells reach the thymus via the bloodstream and their differentiation is controlled by Notch signaling in the thymus. Notch signaling ensures T-cell lineage commitment (Radtke et al., 2010). Notch signaling is also essential for the differentiation of splenic B-cells, and influences other hematopoietic lineages such as megakariocytes and dendritic cells (Radtke et al., 2010).

Notch signaling has an important and well recognized role in T-cell mediated immunity where it regulates T helper differentiation as well as regulatory T-cell function and differentiation (Yuan et al., 2010). However, the mechanisms underlying these processes require further elucidation.

In addition to its role in T-cell mediated immunity and hematopoiesis, the Notch signaling pathway has an emerging role in fine tuning innate immunity and inflammatory processes. This is accomplished through the canonical Notch signaling which can activate macrophages and influence the production of macrophage derived cytokines such as TNF. Indeed, macrophages constitutively express Notch pathway components (Shang et al., 2016).

We demonstrated that Notch signaling may be attenuated in SCZ and to a lesser extent in BD. This could influence adaptive immunity by altering T-cell lineages (skewing T helper 1 and 2 ratios as well as causing subtle changes in regulatory T-cell function). Within innate immunity, the weakened Notch signaling might reflect a compensatory mechanism in the periphery – a response to the increased TNF pathway activity – as impaired Notch signaling has been associated with a decrease in macrophage activity (Outtz et al., 2010). Indeed, monocyte/macrophage activation is associated with increased Notch ligand production, and we found elevated levels of DLL1. However, although there is an established connection between Notch signaling and the immune

system and inflammation, it is unclear how interfering with Notch signaling would affect inflammation and autoimmune diseases (Radtke et al., 2010).

9.2.3 Immune activity in the brain?

As previously described in section 5.4.4, numerous studies have shown increased peripheral TNF pathway cytokine levels in severe mental disorders. But where do the TNF pathway cytokines come from? Could they be produced in the brain with increased cytokine levels in the periphery as a result? The NORMENT group collaborated with the Lieber Institute to measure TNF-pathway gene expression in the periphery and in the central nervous system (*i.e.* the dorsolateral prefrontal cortex). We found that *TNF* expression (as measured by mRNA) is significantly lower in peripheral blood in severe mental disorders compared to HC. This may suggest a compensatory downregulation of *TNF* in these cells. Nevertheless, soluble TNF levels did not correlate with *TNF* mRNA levels in our sample, which weakens this conclusion (Paper II, supplementary table 1). We found no significantly increased expression of TNF pathway related genes in the DLPFC after correcting for multiple testing. In spite of these findings, we did observe a trend towards increased expression of *ADAM17* in the BD group also in the brain. This is consistent with our peripheral cytokine findings. The function of ADAM17 is described in sections 9.1.2 and 9.1.3. It partakes in the activation of several pathways including the TNF and NOTCH pathways. Increased mRNA levels may suggest increased shedding of TNF pathway proteins in the brain. These results taken together may imply that the subtle inflammation in severe mental disorders is unlikely to originate from circulating whole blood cells. Nevertheless, if increased *ADAM17* mRNA levels reflect increased enzyme activity, then this may lead to increased shedding of TNF in BD, possibly inducing downregulation of mRNA in the blood. It remains unclear whether the subtle pro-inflammatory state in severe mental disorders is a primary disease mechanism in SCZ and BD, or whether it is secondary due to inflammatory processes following tissue damage in the brain or somatic co-morbidity in the periphery.

9.3 Clinical implications

TNF pathway

We showed that there is a subtle dysregulation of the TNF-pathway, and that increased TNF activity is associated with slight disturbances in hippocampal memory and working memory in the DLPFC. This may indicate a role for medication that impedes the TNF-pathway in the treatment of severe mental disorders. TNF inhibitors are a recognized and approved treatment of peripheral inflammatory diseases such as rheumatoid arthritis (Arentz-Hansen et al., 2007), psoriasis (Conrad and Gilliet, 2018) and inflammatory bowel disease (Cohen and Sachar, 2017). The low grade inflammation with increased TNF activity in severe mental disorders may then warrant investigation of treatment with TNF inhibitors. There are five TNF inhibitors approved today where etanercept is receptor based, while infliximab, adalimumab, golimumab and certolizumab are monoclonal antibody based TNF inhibitors (Kemanetzoglou and Andreadou, 2017). They all inhibit the TNF pathway by blocking transmembrane TNF interaction with its receptors. TNF inhibitors have been tested in neurological and neuropsychiatric disorders such amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Alzheimer's disease but failed in MS and ALS treatment. This was partially attributed to the dual role of TNF in the CNS. Indeed, TNF has both neurotoxic and neuroprotective properties (Olmos and Llado, 2014). Recent evidence highlights the different properties of soluble and transmembrane TNF and their interaction with TNF receptors. Soluble TNF and TNFR1 signaling has been associated with pro-inflammatory processes that promote autoimmune and neurodegenerative diseases, whereas transmembrane TNF, TNFR2 and intracellular TNFR1 signaling play an important role in host defense, neuroprotection and brain repair mechanisms (Probert, 2015). Therefore, selective TNF inhibitors targeting soluble TNF but not transmembrane TNF could have a role in the treatment of severe mental disorders.

Another possible therapeutic target could be the ADAM17 regulator iRhom2 that facilitates TNF shedding from the membrane surface. Blocking this protein would prevent the release of pathogenic soluble TNF (Kalliolias and Ivashkiv, 2016).

Notch signaling pathway

As previously discussed, Notch signaling is relevant for neurodevelopment, peripheral immune processes and inflammation. We have demonstrated altered Notch signaling in severe mental disorders, which may have implications for underlying disease mechanisms.

There is strong evidence supporting a role for Notch signaling in inflammation and immunity, where Notch signaling controls and fine tunes both T-cell function and differentiation as well as innate immunity and monocyte/macrophage activation (Shang et al., 2016). The macrophage T-lymphocyte hypothesis for SCZ and BD suggest chronic activation of T-cells and macrophages (Beumer et al., 2012; Maes et al., 1995; Smith, 1992) and our findings of elevated TNF levels and TNF/TNFRs ratio support this hypothesis. We also found altered expression of key Notch signaling related genes suggesting aberrant Notch pathway activity in SCZ and, to a lesser extent, in BD. Our results together with the established interplay between the immune system and the Notch signaling pathway further support that drugs targeting the Notch pathway may have a role in the treatment of severe mental disorders in the future. At present, the molecular processes underlying the interplay between the immune system and Notch signaling need further elucidation. Nevertheless, Notch pathway's role in fine tuning the immune system makes it a promising therapeutic target.

The Notch signaling pathway has been extensively studied in hematological malignancies such as acute lymphoblastic leukemia (ALL), B-cell malignancies like chronic lymphocytic leukemia and multiple myeloma. Further, aberrant Notch signaling has also been demonstrated in solid tumors including breast cancer, cervical cancer and colon cancer (Olsauskas-Kuprys et al., 2013). To date over 100 gamma secretase inhibitors have been developed to treat cancer. Significant gastrointestinal side-effects limit its use, and require close monitoring and adjustment of doses (Olsauskas-Kuprys et al., 2013). Therefore, several Notch ligand and Notch receptor targeting antibodies are being developed to improve the specificity of Notch-directed therapeutics (Gordon and Aster, 2014). Interestingly, valproic acid, an antiepileptic agent that is used in the treatment of epilepsy and manic episodes in BD has been shown to have Notch signaling pathway activating properties, and has also been proposed a role in the treatment of osteoporosis (Ji et al., 2017). In zebrafish, valproic acid was shown to inhibit histone deacetylase (a co-repressor of RBP-J), and thus leads to upregulation of Notch signaling (Dozawa et al., 2014). Further, valproic acid has been shown to activate Notch-1 signaling, and has been proposed as a candidate drug in cancer treatment (Greenblatt et al., 2007). However, other studies suggest that lithium and valproic acid have Notch inhibiting properties in mice (Wang et al., 2015). These findings further support Notch pathway as a

novel target for new drug development in treating severe mental disorders, nevertheless, further research is needed to elucidate this pathway.

9.4 Methodological issues

The three studies in this thesis have some limitations that will be addressed in this section. Methodological challenges affecting all the studies are described under General methodological issues, while problems limited to individual studies are discussed in the Specific methodological issues section.

9.4.1 General methodological issues

All three studies have a cross-sectional design which limits claims about causality.

The immune system is affected by many pathological conditions. Patients with severe mental disorders have substantial premature mortality due to cardiovascular diseases and cancers (Crump et al., 2013a; Crump et al., 2013b). Therefore, the studies investigating the immune system were designed to limit confounders such as established inflammatory diseases (*i.e.* the inclusion criteria included CRP levels below 20, no known autoimmune diseases, no malignancies and no ongoing inflammatory diseases). Further, we controlled for body mass index to control for obesity. There may be an increased risk of developing specific cancer types in severe mental disorders, such as lung cancer. This is thought to be associated with smoking habits (Howard et al., 2010), we therefore controlled for smoking in our TNF analyses. In conclusion, we attempted to reduce the effect of confounding factors on our results by tailoring the inclusion criteria for immune studies and also by controlling for established confounders in statistical analyses. Nevertheless, we cannot rule out, that our results may be confounded by co-morbid diseases.

Due to the naturalistic design of the main study most patients were using psychotropic medication. We controlled for co-medication in our analyses and also investigated medication in monotherapy wherever possible.

The immune system and the Notch signaling pathway show diurnal variation (Cermakian et al., 2013; Jensen et al., 2012). It is therefore important to address the significant difference in the time of blood sampling between patients and controls. Both patient and control groups were included at all times of day with the majority of patients during the morning, and the majority of controls during the afternoon. There was a large degree of overlap, while the average time difference was approximately 5 hours. This was mainly due to differences in employment status and ability to take off from work. We conducted additional analyses to investigate the effect of time of blood sampling on cytokine levels. TNFR1 was not correlated with time of blood sampling in our control sample, however, we cannot exclude that our results were confounded by the differences in time of blood

sampling as we also had missing data, and diurnal variation may in part affect the immune system. In our Notch analyses we adjusted for the expression of the biological clock gene (*BMAL1*) to ensure that the differences in Notch signaling pathway related gene expression were not due to differences in circadian rhythms between patients and controls.

Cytokines and plasma proteins are sensitive to handling. To avoid systematic errors we used the same procedure for both patient and control groups in blood sampling (except for the time of day), storage, freezing and thawing.

We observed several significant findings ($p<0.05$) but due to correction for multiple testing the alpha threshold was lowered, and these results did not remain significant. We decided to use a conservative approach to avoid type I errors, but this makes studies susceptible to type II errors (Armstrong, 2014).

Finally, it is important to recognize that measuring plasma markers and gene expression in whole blood may not reflect protein levels and gene expression in the brain. However, there is an established communication between the periphery and the CNS through the BBB, which makes measuring plasma markers relevant (D'Mello and Swain, 2017). There are also several studies that suggest that the mRNA expression levels may overlap between brain and blood to a certain degree (Tylee et al., 2013).

9.4.2 Specific methodological issues

The first study investigating associations between cytokine levels, memory and hippocampal volumes have two main limitations in addition to those already described in the general methodological issues section. Firstly, participants of this study were scanned using a 1.5 tesla scanner, and hippocampal subfield volumes may not be reliably measured on a 1.5 T scanner due to insufficient resolution (Giuliano et al., 2017). The reduced sensitivity may have contributed to our lack of findings, and future studies using data with higher resolution may yield different results. Further, the latest version of FreeSurfer may give more precise automated hippocampus segmentation results compared to the older versions (Schmidt et al., 2018). Secondly, there were substantial differences in the number of days/months/years between blood sampling and brain imaging (up to 5 years). This, however, is not a major limitation as recent reviews show that the size of the hippocampus stays relatively stable after the onset of SCZ (Bartholomeusz et al., 2017; Chiapponi et al., 2013).

In the second study we measured mRNA levels from dorsolateral prefrontal cortex in addition to plasma proteins and leukocyte mRNA. We found no significant differences between patients and controls in TNF pathway mRNA levels in the brain. However, it is important to denote that plasma levels of mRNA were measured using quantitative PCR, while mRNA levels in the brain were measured using global transcriptomic analyses which are less sensitive than quantitative PCR. Further, the brain analyses were performed on whole cortical samples therefore we cannot draw conclusions regarding microglial gene expression, which would be most relevant to investigate in severe mental disorders. Analyzing postmortem tissue has its own limitations. Several factors have been identified (*e.g.* pH, postmortem interval, age, agonal state, and smoking) that influence postmortem tissue and RNA quality. These factors were carefully considered, thus increasing the reliability of the RNA assessments (Lipska et al., 2006).

In the third article we conducted a pathway analysis of the Notch signaling pathway. We used global transcriptomics analyses, and have not confirmed our results using qPCR. Our results should therefore be interpreted with caution as microarray analyses are less sensitive than qPCR. Further, we did not measure intracellular protein levels or phosphorylation status, which are necessary to understand pathway activity. Lastly, using blood as proxy for the brain has major limitations, and should be considered when drawing conclusions (Sullivan et al., 2006).

9.5 Implications for further research

The three studies that form the foundation of this thesis have novel results that prompt future research.

The immune system's role in cognitive processes has gained increasing attention over the past decades and is also recognized in fields outside of psychiatry such as in infectious diseases (*e.g.* HIV, HIV-associated neurocognitive disorders) and surgery (*e.g.* postoperative cognitive delirium) (Cortese and Burger, 2017; Hong and Banks, 2015). Our study supports that a slight shift towards a pro-inflammatory state is modestly associated with impaired verbal memory. It is then plausible that drugs with additional anti-inflammatory properties may be beneficial in the treatment of patients who also show signs of cognitive difficulties, and indeed, several studies have already been carried out to investigate such drugs (Ayorech et al., 2015; Khandaker and Dantzer, 2016).

The second study shows increased TNF-pathway activity in patients with severe mental disorders compared to HC and further highlights the TNF-pathway as a potential therapeutic target. The cause of this pro-inflammatory imbalance remains elusive. The pathophysiology of SCZ and BD may in part be attributed to a primary immune dysfunction in a subgroup of these patients. There is growing interest in identifying these phenotypes both within SCZ and BD as these subgroups may benefit from anti-inflammatory treatment that in consequence target the primary disease mechanisms (Khandaker and Dantzer, 2016; Rosenblat and McIntyre, 2017). In addition to a potential primary immune dysfunction, the pro-inflammatory state in severe mental disorders may also be due to secondary pathologies such as comorbid cardiovascular disease, autoimmune diseases, cancers and obesity (Feldman et al., 2000; Kusters and Lutgens, 2015; Monaco et al., 2015; Tzanavari et al., 2010). Importantly, these patients have a shortened lifespan by 10-20 years which is mainly due to somatic co-morbidity (Crump et al., 2013a; Laursen et al., 2012). Anti-inflammatory drugs targeting the TNF-pathway may therefore be relevant both in treating the primary immune dysfunction or the secondary pro-inflammatory state.

In the third study we show aberrant Notch signaling in SCZ and to a lesser extent in BD. The Notch signaling pathway has an established role in regulating different aspects of immunity, and it also partakes in adult brain homeostasis (Ables et al., 2011). Future research could further investigate the relationship between the TNF pathway and the Notch signaling pathway in severe mental disorders to explore whether the imbalance in these pathways is related. Such associations could further support the Notch signaling pathway as a therapeutic target in severe mental disorders. In addition, a similar pathway analysis in brain tissue (post-mortem) could clarify whether the changes

observed in circulating leukocytes reflect general Notch pathway activity or are restricted to blood cells.

Finally, our secondary analyses investigating associations between the TNF and Notch signaling pathways and psychotropic drugs revealed a potential connection between lithium and these pathways. Lithium plays a central role in the treatment of BD although it has significant side effects. Our findings support that the use of lithium may increase TNF pathway activity in the periphery. Future studies could be directed at investigating whether the increased TNF in lithium treatment is associated with significant clinical side-effects. In addition, we have identified two potential targets for lithium (*RBPJ* and *ADAM17*) which need further elucidation.

Overall, an imbalance in the immune system in severe mental disorders is increasingly recognized, and supported by the findings of the present thesis. Nevertheless, there are still unanswered questions. What is the primary mechanism for this imbalance? Is it due to the illness itself, or is it secondary to comorbid somatic illnesses or secondary to neuro-inflammatory processes? It is challenging to investigate processes of the brain in humans. Imaging techniques utilizing microglia activation tracers are well underway (Tronel et al., 2017), but are still lacking for astrocytes (Poutiainen et al., 2016). Future research is thus dependent on developing novel tools to investigate protein levels, phosphorylation status of enzymes and microglia and astrocyte activation in the brain.

10 Conclusions

In this section I will highlight the main conclusions of the thesis. The involvement of the immune system in the pathophysiology of severe mental disorders is receiving increasing attention. This thesis was aimed at gaining further knowledge about immune pathways previously implicated in SCZ and BD.

- Increased TNF pathway activity, reflected by elevated plasma levels of soluble TNFR1 and TNF, is associated with lower verbal memory and working memory performance. These findings support a connection between a systemic pro-inflammatory imbalance and aberrant cognitive functioning. We found no significant associations between cytokines and hippocampal subfield volumes, neither between performance on the CVLT and hippocampal subfield volumes after correcting for multiple testing.

- Patients with severe mental disorders have increased TNF pathway activity compared to HC. Circulating leukocytes and cells of the dorsolateral prefrontal cortex are an unlikely source. The cause of the pro-inflammatory imbalance in SCZ and BD remains elusive and future studies are needed to identify sub-groups with dysregulated immune system.

- Lithium may be associated with elements of both the TNF and Notch signaling pathways. This could be relevant in the treatment of lithium side effects as well as uncovering additional therapeutic targets in severe mental disorders.

- We found no significant associations between TNF pathway activity and clinical symptoms of SCZ and BD.

- SCZ patients may have attenuated Notch signaling in circulating leukocytes with similar, albeit less prominent, evidence in BD. Notch signaling is complex and although we cannot draw definitive conclusions from our findings, we do observe distinct differences between patients and controls. Notch signaling fine tunes the immune system thus making it a significant therapeutic target.

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I

Association between cytokine levels, verbal memory and hippocampus volume in patients with psychotic disorders and healthy controls

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Abstract

Background: There is growing evidence that the immune system is implicated in the pathophysiology of schizophrenia and bipolar disorders, and immune markers have been shown to play a role in verbal memory processes. In this study we investigated whether elevated plasma levels of immune markers were associated with verbal memory and hippocampal subfield volumes in patients with severe mental illnesses and in healthy controls.

Methods: 230 patients with a broad DSM-IV schizophrenia spectrum illness or bipolar disorder and 236 healthy controls from the same catchment area were consecutively recruited. Memory was assessed using the Wechsler Memory Scale-Third Edition (WMS-III) Logical Memory immediate and delayed recall, and the California Verbal Learning Test summed recall over learning list (CVLT learning) and delayed free recall. We measured plasma levels of soluble tumor necrosis factor receptor 1 (sTNF-R1), interleukin-1 receptor antagonist, interleukin-6, von Willebrand factor, osteoprotegerin, high-sensitivity C-reactive protein and sCD40 Ligand. Hippocampal subfield estimates were obtained using FreeSurfer.

Results: We found a moderate negative association between sTNF-R1 and performance on verbal memory learning and recall tests as measured by the WMS-III Logical Memory after correcting for age, sex and diagnosis. We also found a moderate positive association between CVLT learning and hippocampal volumes.

Limitations: The time difference between blood sampling and MRI, skewed immune variables, and the cross-sectional nature of the study comprise the main limitations.

Conclusions: The findings suggest a role for immune involvement in memory independent of severe mental disorders, and support the "bigger is better" hypothesis of hippocampal subfield volumes.

Introduction

Schizophrenia (SCZ) and bipolar disorder (BD) are severe mental disorders with an estimated heritability of approximately 70 – 80 % (Craddock and Sklar, 2013; van Dongen and Boomsma, 2013). They rank among the most costly disorders causing long-term disability and are relatively common with a combined life time prevalence of approximately 3 %. Despite extensive research, the pathogenesis of these disorders remains largely unknown. A considerable genetic overlap exists between the two disorders which suggests common disease mechanisms (Cardno and Owen, 2014). Among other factors, the immune system has been implicated in the pathophysiology of SCZ and BD through findings from genome wide association studies for SCZ (Shi et al., 2009), as well as experimental studies and clinical studies of circulating levels of inflammatory markers in both disorders (Altamura et al., 2013; Consortium, 2011; Hope et al., 2011; Hope et al., 2010; Hope et al., 2013; Munkholm et al., 2013). However, the precise role of the immune system in the development of these disorders is yet to be unravelled.

Cognitive dysfunction is an important characteristic of severe mental disorders (Simonsen et al., 2011). Impaired verbal memory is a consistent finding in SCZ, and is also seen in patients with BD (Hellvin et al., 2012; Simonsen et al., 2011). Several lines of evidence obtained mainly through animal studies have implicated the immune system in memory processes both under normal conditions and in impaired memory (Yirmiya and Goshen, 2011). One way to study enhanced inflammatory activity in humans is by measuring the plasma levels of immune markers that are not only expressed by immune cells, but also by cells of the central nervous system including neurons, astrocytes and microglia (Arisi, 2014). We have previously shown increased levels of pro-inflammatory markers in patients with severe mental disorders such as tumor necrosis factor (i.e., soluble TNF receptor 1 [sTNF-R1]) as well as other markers of immune activation including von Willebrand factor (vWF) (Hope et al., 2009), which reflects endothelial cell activation, and osteoprotegerin (OPG) (Hope et al., 2010), which indicates vascular inflammation. We have also

investigated other immune markers in relation to psychotic and affective symptoms such as the pro-inflammatory immune markers interleukin-1 (i.e., IL-1 receptor antagonist [IL-1Ra]) and interleukin-6 (IL-6), sCD40 ligand (sCD40L), which reflects platelet-mediated inflammation, and high sensitivity C-reactive protein (hsCRP), which is a reliable down-stream marker of inflammation (Hope et al., 2011; Hope et al., 2013). Several studies have investigated associations between verbal memory and the immune system (Hudetz et al., 2011; Kesler et al., 2013), however, such associations have not been previously explored in patients with severe mental disorders.

The hippocampal formation is a unique anatomical complex comprising several subfields, and plays an important role in verbal learning and memory (Tamminga et al., 2010). Reductions in hippocampal formation volumes are a consistent finding in SCZ, but can also be seen in patients with BD (Rimol et al., 2010). Since immune processes seem to affect hippocampal function and neurogenesis (McAfoose and Baune, 2009), they may also be associated with changes in hippocampal volume. However, little is known about the association between immune-related markers and hippocampal structural properties in general, and associations between disease specific reductions in hippocampal volumes and plasma levels of immune markers have seldom been studied. Due to the heterogeneous functional and structural anatomy of the hippocampus, it has been hypothesized that subfield-specific measures of the hippocampal formation will prove more sensitive to disease effects than whole hippocampus volume measures (Mueller and Weiner, 2009). Indeed, distinct hippocampal subfield volume reductions with associations to cognitive performance have recently been reported in patients across the psychosis spectrum (Haukvik et al., In press; Mathew et al., 2014), but if such associations are related to inflammatory markers is not known.

Based on current knowledge from previous comparative and human studies and theoretical considerations, we hypothesized that plasma levels of inflammatory markers would be negatively associated with performance on verbal memory tests in patients with severe mental disorders and in young healthy controls. Secondly, we hypothesized that these inflammatory markers would show a

negative correlation with hippocampal volumes, indicating that high levels of inflammatory markers would be associated with reduced hippocampal subfield volumes. Lastly, in order to explore the potential neurocognitive relevance of any volumetric associations with cytokine levels, we also tested for associations between performance on verbal memory tests and hippocampal volumes probing the "bigger is better" hypothesis in hippocampal subfields. According to this hypothesis larger volume should result in stronger function regardless of the causal factors underlying the size of the structure (Mathew et al., 2014; Van Petten, 2004).

Methods

Study Design and Ethics

The Thematically Organized Psychosis (TOP) Study at the Oslo University Hospital and collaborating Hospitals across Norway, is a large ongoing study. The study sample for the present article consisted of patients and healthy control subjects included between May 2003 and September 2007. The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate, and the biobank is approved by the Norwegian Directorate of Health.

Participants

The recruitment protocol in the TOP study has previously been described in detail (Dieset et al., 2012), and here we only present the sample used in the current study**.**

Patients: The main inclusion criteria were DSM-IV diagnoses of schizophrenia spectrum disorders or bipolar spectrum disorders, IQ > 70, having either Norwegian as a mother tongue or receiving all compulsory education in Norway, age between 18 and 65 years. Patients were excluded if they had self-reported head-injury with EEG, CT or MRI scan findings following the injury, coexisting neurological illness or autism spectrum disorder (ASD), and if they scored below 15 on the Californian Verbal Learning Test (CVLT) forced recognition (Delis et al., 2004), indicating suboptimal motivation. In total 68 patients were excluded from the present analysis, including 5 due

to previous head injuries or neuroradiological findings, concurrent neurological illness or ASD, 49 did not receive their compulsory education in Norway, or did not have Norwegian as their first language, and 4 due to cut-off on CVLT forced recognition.

The schizophrenia spectrum group (N = 112) comprised patients with schizophrenia (N = 89), schizophreniform disorder (N = 8) and schizoaffective disorder (N = 15). The bipolar spectrum disorder group (N = 118) included patients with bipolar I disorder (BD I, N = 69), bipolar II disorder (BD II, N = 42) and bipolar disorder not otherwise specified (BD NOS, N = 7).

Controls: A representative age- and sex-matched group of healthy volunteers (N = 236) from the same catchment area was randomly selected from the National Population Registry and contacted by a letter of invitation. All control subjects were screened for illness using the Primary Care Evaluation of Mental Disorders and interviewed about severe psychiatric disorders, drug abuse, and somatic disease. Exclusion criteria were any history of severe psychiatric disorders (major depression, bipolar disorders, and schizophrenia) in the control subjects or in any of their first-degree relatives, substance or alcohol abuse or dependency, and the previously described exclusion criteria for patients.

None of the patients or the controls had any acute infections at the time of blood sampling, as indicated by plasma levels of CRP below 20, and no signs of infection during the physical examination.

Of the 466 participants included in the present study (Main group) 225 subjects also underwent MRI (MRI subgroup). The MRI subgroup included 111 healthy controls, in addition to patients diagnosed with schizophreniform disorder (N = 6), schizoaffective disorder (N = 4) and schizophrenia (N = 37), BD I (N = 36), BD II (N = 28), and BD NOS (N = 3) diagnoses. All patients were examined by a physician to assess current health and potential neurological signs.

Clinical Assessments

Diagnosis was obtained with the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Clinical symptoms were evaluated using the Young Mania Rating Scale, Inventory of Depressive Symptoms and the Positive and Negative Syndrome Scale, while functioning was measured using the Global Assessment of Functioning split version function and symptom scale. The clinical assessment team consisted of clinical psychologists or psychiatrists, who all were trained until satisfactory inter-rater reliability was obtained. For details, see (Lagerberg et al., 2014; Ventura et al., 1998).

Memory Assessments

Verbal memory was assessed by trained clinical psychologists using the Wechsler Memory Scale-Third Edition (WMS-III) Logical Memory immediate (LM-learning) and delayed recall (LM-recall) (Wechsler et al., 2008), and the California Verbal Learning Test summed recall over learning list (CVLT-learning) and delayed free recall (CVLT-recall) (Delis et al., 2004). Neurocognitive testing was routinely performed the same day following blood sampling, except for a subgroup (7.9%) where there was a mean interval between assessments of 8 days (median, IQR = 4 – 15). Forty two subjects have missing data regarding time of assessment.

Immune Markers

Assessment of immune markers: We have previously described in detail the blood sampling procedures and analysis of immune markers in a partly overlapping sample (Hope et al., 2009).

MRI acquisition

Imaging data was obtained between august 2003 and December 2007 on a 1.5 Tesla Siemens Sonata scanner using an 8-channel head coil. The pulse sequence used for volumetric assessments was a sagittal T1-weighted Magnetization Prepared Rapid Gradient Echo (MPRAGE) with the following parameters: time of repetition (TR)/echo time (TE)/inversion time (TI) =

2730ms/3.93ms/1000ms, flip angle (FA) = 7°, field of view (FOV) = 240mm, acquisition matrix = 256x192, voxel size = 1.33x0.94x1 mm3, and 160 slices. The sequence was repeated and the two runs were combined during postprocessing in order to increase signal-to-noise ratio. Patients and healthy controls were scanned consecutively.

MRI processing and analysis

FreeSurfer (http://surfer.nmr.mgh.harvard.edu) was used to obtain estimates of the hippocampal subfield volumes, total hippocampal formation volume, and intracranial volume (ICV) (Fischl, 2012). Details regarding the procedures involved have previously been described (Van Leemput et al., 2009). Briefly, the processing includes motion correction and averaging (Reuter et al., 2010) of the two T1 weighted volumes, removal of non-brain tissue using a hybrid watershed/surface deformation procedure (Segonne et al., 2004), automated Talairach transformation, and segmentation of the subcortical white matter and deep gray matter volumetric structures by combining information on image intensity, probabilistic atlas location, and the local spatial relationships between structures to automatically assign a neuroanatomical label to each voxel in the MRI volume (Fischl et al., 2002; Fischl et al., 2004). The MRI processing procedures were fully automated without manual editing. All segmented scans were visually inspected following standard procedures.

The novel approach for hippocampal subfield segmentation is based on a Bayesian modelling approach and manual delineations of each hippocampal subfield (Van Leemput et al., 2009). The subfield volumes obtained have been compared to manual hippocampal subfield tracings, and shown to be most reliable for the larger subfields including CA2/3, CA4/DG, and subiculum, with acceptable reliability for the CA1, presubiculum, and fimbria (Van Leemput et al., 2009).

Statistical analysis

We performed logarithmic transformation for all immune markers except for sCD40-L where we used square root transformation due to skewed distributions, and we used the transformed variables in the general linear model analyses.

We used raw data in all our analyses regarding verbal memory. The CVLT-learning and recall variables also showed skewed distributions, which remained skewed after logarithmic transformation. However, the distribution of the standardized residuals in the general linear model showed normal distributions for analyses including the CVLT-learning, but not for the CVLT-recall.

We corrected for multiple testing. Three of the investigated immune markers (sTNF-R1, IL1-Ra and IL-6) had previously been found to play a role in memory, and several of the verbal memory tests and MRI volumes were highly correlated ($r = 0.92$ for LM learning and recall; $r = 0.85$ for CVLT learning and recall; and $r = 0.77 – 0.86$ for hippocampus subfields, except for the presubiculum, which had a slightly lower correlation to the rest of the subfields $r = 0.54 – 0.77$). Due to the strong priori hypotheses and the strong correlations between measures, yielding the statistical tests non-independent, we allowed a higher significance threshold for these immune markers and the tests investigating associations between verbal memory and hippocampal subfields ($p < 0.005$) whereas we used Bonferroni correction for the remaining immune markers and tests ($p < 0.002$).

Due to differences in the time elapsed between blood sampling and neuropsychological testing, and missing data we repeated our analyses for the subgroup of subjects where both procedures were performed on the same day, and compared results with the main findings. We also controlled for the differences in elapsed time to MRI scanning for the MRI subgroup by covarying for time interval in the statistical models.

Results

The immune marker, memory test and hippocampal volume results from partly overlapping samples have been reported previously in separate analyses (Hope et al., 2009; Rimol et al., 2010; Simonsen et al., 2011).

Demographics and clinical characteristics

The socio-demographic and clinical characteristics of the participants are presented in Table 1 for the Main group and in Supplementary Table 1 for the MRI subgroup.
There was no significant difference in demographic and clinical characteristics of the MRI subgroup (N = 225) and the Main group (N = 466), however, the two groups slightly differed in terms of intra group *post hoc* analyses of ethnicity, self-reported head injury, age, alcohol use, years of education and YMRS scores (see Table 1 and Supplementary Table 1).

Verbal memory and immune markers

Levels of immune markers, scores on verbal memory and hippocampal volumes for the current sample are summarized in Supplementary tables 2 and 3. We have previously reported increased sTNF-R1 and vWF in the Main group and the MRI subgroup mirrored these results showing significantly higher plasma levels of sTNF-R1 and vWF compared to the healthy controls. There were no significant differences between the Main group and the MRI subgroup in plasma levels of immune markers and performances on verbal memory tests.

We found moderate but significant negative associations between sTNF-R1 and LM-learning ($r = -0.17$, $p = 2 \times 10^{-4}$) and LM-recall ($r = -0.16$, $p = 0.001$) after adjusting for age, sex and diagnosis (Fig. 1, Table 2). Both findings remained significant after correcting for multiple testing. We observed no interaction effect of diagnosis and sTNF-R1 on memory scores, indicating that the associations between sTNF-R1 and verbal memory were not significantly different between groups.

We also found significant negative associations between vWF and CVLT learning and recall (r = -15, p = 0.005), however, these findings did not remain significant after adjusting for age, sex and diagnosis, or when only adjusting for age and sex, and correcting for multiple testing.

There were no significant associations between the other cytokines and verbal memory after correcting for age, sex and diagnosis (Supplementary Table 4).

Verbal memory and hippocampal volumes

The associations between CVLT recall and subiculum have been recently investigated by Haukvik et al. in a partially overlapping sample (Haukvik et al., In press).

Both CVLT learning and CVLT recall were moderately positively associated with the total hippocampal formation volume and hippocampal subfield volumes (β = 0.22 - 0.21 and p = 0.003 – 0.005, respectively), except for CA1 and CVLT learning, after correcting for age, sex, estimated intracranial volume and diagnosis (Fig. 2, Table 2 and Supplementary Table 5). However, only association with CVLT learning remained significant after correction for multiple testing. There were no significant interaction effects between hippocampal volumes and diagnosis on verbal memory scores, indicating similar associations between hippocampal volumes and verbal memory in patients and controls, respectively.

LM-learning and LM-recall were not associated with hippocampal volumes in our analyses.

Hippocampal volumes and cytokines

There were no significant associations between cytokines and hippocampal subfields after correcting for age, sex, diagnosis and estimated intracranial volume (Supplementary Table 6). However, we found a trend (p = 0.07) towards a negative association (β = - 0.10) between OPG and

the volume of the total hippocampal formation after adjusting for age, sex, estimated intracranial volume and diagnosis (Table 2).

Discussion

The main finding of the present study was a moderate negative association between sTNF-R1, a marker of TNF activity, and performance on verbal memory learning and recall tests as measured by the WMS-III logical memory in both patients with severe mental disorders and in young healthy controls. We also found a moderate positive association between estimates of hippocampal morphology and verbal memory performance assessed using the CVLT test. These findings may indicate a role for TNF related pathway involvement in memory processes that is not limited to patients with severe mental illness but is also present in young healthy controls.

There are several lines of evidence suggesting that inflammatory and immune-mediated mechanisms could interfere with memory performance. Although normal levels of TNFα do not seem to be involved in long term potentiation (LTP) induction or maintenance (Yirmiya and Goshen, 2011), TNFα at pathophysiological concentrations inhibit LTP in the dentate gyrus and CA1 regions of the hippocampus (Butler et al., 2004), thus negatively influencing memory processes. Our findings of negative associations between sTNF-R1 and performances on the WMS-III logical memory tests were independent of diagnosis, and we found no significant interaction effects of diagnosis and sTNF-R1 on verbal memory scores, suggesting that the potential cytokine-mediated regulation of verbal memory is not limited to patients with SCZ and BD where a cytokine imbalance is likely, but may be a "naturally" occurring mechanism, that doesn't depend on pathological plasma levels of TNFα in humans. Another putative mechanism by which TNFα may influence memory is through its ability to regulate levels of major histocompatibility complex (MHC) class I proteins in the brain (Drew et al., 1993). There is now growing evidence suggesting that MHC class I proteins play a crucial role in synaptic plasticity and hippocampal function (Shatz,

2009). They are critical for Hebbian synaptic plasticity (the theory suggesting that long-term potentiation and long-term depression are the underlying mechanisms for memory consolidation (McAfoose and Baune, 2009)) and have been proposed to limit synaptic strength and alter the trafficking and function of NMDA and AMPA receptors (Elmer and McAllister, 2012; Nelson et al., 2013).

One could argue that plasma levels of cytokines are less relevant for the brain. However, circulating cytokines bypass the blood-brain-barrier (BBB) by activation of vagal afferents (Erickson et al., 2012) and endothelial cells in the BBB can be activated by circulating cytokines, which in turn produce and secrete cytokines into the brain parenchyma (Verma et al., 2006). In addition cytokines can directly reach the brain by leakage through the BBB or by active transport through the BBB (Erickson et al., 2012). It is therefore possible that the cytokines influencing cognitive functions in the brain are the result of the complex interplay between central and peripheral cytokines (Jones and Thomsen, 2013).

We did not find significant negative associations between plasma levels of cytokines and hippocampal volumes after correcting for age, sex, diagnosis and estimated intracranial volume. This may be due to the reduced statistical power as our MRI sample size was half the size of the Main sample. We did, however, observe a trend level negative association between OPG and hippocampal volumes. The potential role of OPG in hippocampal volume reduction has not yet been investigated, and its association to severe mental disorders has not been firmly established. Further studies with larger sample sizes are necessary to confirm these results.

We also found a moderate positive association between hippocampal subfield volumes and performance on the CVLT learning after correcting for age, sex, diagnosis and estimated intracranial volume, which remained significant after correction for multiple testing. Several studies have also found positive associations between performances on CVLT learning and hippocampal volumes (Chepenik et al., 2012; Tischler et al., 2006), as well as on distinct hippocampal subfield

volumes (Mathew et al., 2014), supporting the validity of our findings. We did not find any interaction effect between diagnosis and hippocampal subfield volumes on memory performances suggesting that bigger hippocampal subfield volumes may modestly reflect higher performance on CVLT learning both in patients with SCZ and BD, as well as in young healthy adults.

Limitations

There are some limitations to our study. Firstly, there is a considerable time difference between blood sampling and brain scanning. Although some studies indicate changes in hippocampal volumes following physical training (Erickson et al., 2011) or treatment with Lithium (Yucel et al., 2007), other studies investigating brain morphology find only subtle or no significant changes over time in patients with schizophrenia (Nesvag et al., 2012; Whitworth et al., 2005). Secondly, we used MRI scans obtained on a 1.5 T scanner, which may have decreased sensitivity to disease-related biological variability compared to data obtained from higher-field magnets which may allow for signal to noise ratio. The third limitation of the present study arises from the statistical challenges presented when working with skewed data. In order to avoid violating statistical test assumptions we used transformed variables in our analyses thus reducing their variability. Finally, the cross-sectional nature of the study prevents us from determining causality.

Conclusion

To conclude, our findings support the growing consensus that the immune system is involved in memory processes, where elevated plasma levels of sTNF-R1 modestly reflect impaired verbal memory and recall in both patients with severe mental disorder as well as in healthy controls, suggesting a role for immune involvement in memory processes independent of severe mental disorders. Increased hippocampal volumes also seem to be associated with higher scores on

memory tests supporting the “Bigger is better” hypothesis not only in patients with SCZ and BD, but also in young healthy controls.

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Table 1. Demographic and clinical characteristics of participants in the Main group (N = 466).

Parameters	Bipolar Disorder (N = 118)	Schizophrenia (N = 112)	Healthy Controls (N = 236)	Post Hoc Analysis
Male sex, N (%)	46 (39.0)	61 (54.5)	104 (44.1)	NS
Ethnicity (European)[a]	107 (90.7)	100 (89.3)	234 (99.6)	CTR >SCZ, BD
Tobacco (users)[b]	63 (53.8)	60 (53.6)	32 (20.4)	SCZ, BD > CTR
Cannabis (users)[c]	11 (9.3)	16 (14.4)	1 (0.4)	SCZ, BD > CTR
Head injury[d]	8 (6.8)	5 (4.5)	2 (0.9)	SCZ, BD > CTR
Medication:				
Antipsychotics	54 (45.8)	98 (87.5)	-	SCZ > BD
Lithium	15 (12.7)	1 (0.9)	-	BD > SCZ
Antidepressants	47 (39.8)	33 (29.5)	-	NS
Mood stabilizers	66 (55.9)	18 (16.1)	-	BD > SCZ
Hypnotics	14 (11.9)	10 (8.9)	-	NS
Age (years), median (IQR)	32 (25 – 46)	30 (25 - 38.5)	35 (27 – 44)	CTR, BD > SCZ
Alcohol (IU)[e]	4 (2 – 15)	0 (0 – 7.8)	6 (2 – 15)	CTR, BD > SCZ
Education (years)	14 (12 – 15)	12 (11 – 14.5)	15 (12 – 16)	CTR > BD >SCZ
IQ [f]	109 (100 – 116)	103 (89 – 114)	114 (108 – 119)	CTR > BD >SCZ
Sampling – Testing (days)[g]	0 (0 – 0)	0 (0 – 6)	0 (0 – 0)	SCZ > BD, CTR
Body Mass Index	25.2 (22.2 – 28.1)	25.5 (23.1 – 29.3)	-	NS
PANSS total score [h]	45 (39 – 45)	58 (49 – 69)	-	SCZ > BD
YMRS total score [i]	2 (0 – 5)	5 (1 – 10)	-	SCZ > BD
IDS total score [j]	13 (6 – 24)	11 (7 – 21)	-	NS
GAF-F [k]	57 (48 – 67)	42 (36 – 50.5)	-	BD > SCZ
GAF-S [l]	60 (53 – 66)	40 (35 – 51)	-	BD > SCZ

Missing: [a] N = 1, [b] N = 80, [c] N = 1, [d] N = 2, [e] N = 29, [f] N = 3, [g] N = 42, [h] N = 2, [i] N = 2, [j] N = 35, [k] N = 1, [l] N = 1,

Abbreviations: CTR = Controls; BD = Bipolar Disorder Spectrum; SCZ = Schizophrenia Spectrum; NS = Non-Significant; IU = International Units two weeks prior to inclusion in the study; IQ = Wechsler Abbreviated Scale of Intelligence; PANSS=Positive and Negative Syndrome Scale; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; GAF-F = Global Assessment of Functioning - Function Scale; GAF-S = Global Assessment of Functioning - Symptom Scale.

Categorical data are given as percent, while continuous data are given as median with interquartile range. Post Hoc Analysis is performed using Pearson Chi-square for categorical data, and Kruskal-Wallis and Mann-Whitney tests for continuous data.

Table 2. Significant associations between cytokines, verbal memory and hippocampal subfield volumes.

Parameters	Parameters	Total population (N = 466)		Patients (N = 230)		Controls (N = 236)	
Memory test:	*Cytokines:*	Uni.	Adj.[1]	Uni.	Adj.[2]	Uni.	Adj.[2]
LM learning	sTNF-R1	**-.21*** **	**-.17*** **	**-.17***	**-.18****	**-.18****	**-.18****
LM recall	sTNF-R1	**-.20*** **	**-.15****	**-.15***	**-.16***	**-.17***	**-.17****
CVLT learning	sTNF-R1	**-.10***	-.06	-.07	-.07	-.05	-.06
	vWF	**-.15****	-.08	**-.15***	-.12	-.08	-.04
CVLT recall	vWF	**-.14****	-.07	-.12	-.08	-.08	-.05
		(N = 225)		(N = 114)		(N = 111)	
Hippocampus:	*Cytokines:*	Uni.	Adj.[3]	Uni.	Adj.[4]	Uni.	Adj.[4]
CA4/DG	OPG	**-.13***	-.08	-.18	-.15	-.06	.04
Memory test:	*Hippocampus:*	Uni.	Adj.[3]	Uni.	Adj.[4]	Uni.	Adj.[4]
CVLT learning	Subiculum	**.14***	**.22****	**.20***	**.28****	-.05	.11
CVLT recall	Subiculum	.11	**.21****	.13	**.24***	-.02	.16

* p < 0.05 level (2-tailed) ** p < 0.01 level (2-tailed) *** p < 0.001 level (2-tailed)
Univariate association (= Uni.) and Adjusted association (=Adj.) are given as standardized beta.
[1] Adjusted for age, sex and diagnosis; [2] Adjusted for age and sex; [3] Adjusted for age, sex, estimated intracranial volume and diagnosis; [4] Adjusted for age, sex and estimated intracranial volume.
Abbr.: LM = Logical Memory subtest of the Wechsler Memory Scale III; CVLT = California Verbal Learning Test; sTNF-R1 = soluble Tumor Necrosis Factor Receptor 1; vWF = von Willebrand Factor; DG = Dentate Gyrus; OPG = Osteoprotegerin.

Supplementary Table 1. Demographic and clinical characteristics of participants in the MRI subgroup (N = 225).

Parameters	Bipolar Disorder (N = 67)	Schizophrenia (N = 47)	Healthy Controls (N = 111)	Post Hoc Analysis
Male sex, N (%)	29 (43.3)	26 (55.3)	49 (44.1)	NS
Ethnicity (European)[a]	64 (95.5)	40 (85.1)	109 (99.1)	CTR > SCZ
Tobacco (users)[b]	39 (58.2)	23 (48.9)	16 (20.3)	SCZ, BD > CTR
Cannabis (users)[c]	9 (13.4)	6 (13.0)	1 (0.9)	SCZ, BD > CTR
Head injury[d]	5 (7.5)	2 (4.3)	1 (0.9)	NS
Medication:				
Antipsychotics	31 (46.3)	40 (85.1)	-	SCZ > BD
Lithium	7 (10.4)	0 (0)	-	BD > SCZ
Antidepressants	26 (38.8)	16 (34.0)	-	NS
Mood stabilizers	37 (55.2)	6 (12.8)	-	BD > SCZ
Hypnotics	8 (11.9)	4 (8.5)	-	NS
Age (years)	31 (25 – 38.5)	30 (26 – 34.5)	36 (28 – 44.5)	CTR > SCZ
Alcohol (IU)[e]	4 (0 – 17.3)	1 (0 – 8)	6 (2 – 15)	CTR > SCZ
Education (years)	14 (12 – 15)	12 (10 – 14)	15 (12 – 16)	CTR, BD >SCZ
IQ	111 (105 – 117)	106 (93.5 – 116.5)	116 (109 – 120)	CTR > BD >SCZ
Sampling – Testing (days)[f]	0 (0 – 0)	4 (0 – 7)	0 (0 – 0)	SCZ > BD, CTR
Sampling – Scanning (days)[g]	263.5 (60 –612.5)	75 (35 – 393)	406 (115 – 1007)	CTR > BD > SCZ
Body Mass Index	24.7 (22.3 – 27)	25.1 (22.9 – 28.2)	-	NS
PANSS total score[h]	45 (39 – 49.5)	55 (44.5 – 70.5)	-	SCZ > BD
YMRS total score	1 (0 – 4)	3 (0 – 8)	-	NS
IDS total score[i]	14.5 (7 – 25)	9 (6 – 19)	-	NS
GAF-F	59 (48.5 – 67)	42 (38 – 50)	-	BD > SCZ
GAF-S	61 (54 – 65)	41 (36 – 51)	-	BD > SCZ

Missing: [a] N = 1; [b] N = 32; [c] N = 1; [d] N = 1; [e] N = 13, [f] N = 20, [g] N = 15, [h] N = 3, [i] N = 24

Abbreviations: IQR = Interquartile Range; CTR = Controls; BD = Bipolar Disorder Spectrum; SCZ = Schizophrenia Spectrum; NS = Non-Significant; IU = International Units two weeks prior to inclusion in the study; IQ = Wechsler Abbreviated Scale of Intelligence; PANSS=Positive and Negative Syndrome Scale; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; GAF-F = Global Assessment of Functioning - Function Scale; GAF-S = Global Assessment of Functioning - Symptom Scale.

Categorical data are given as percent, while continuous data are given as median with interquartile range. Post Hoc Analysis is performed using Pearson Chi-square for categorical data, and Kruskal-Wallis and Mann-Whitney tests for continuous data.

Supplementary Table 2. Immune and verbal memory characteristics for the Main group (N = 466).

Parameters	Bipolar Disorder (N = 118)	Schizophrenia (N = 112)	Healthy Controls (N = 236)	Post Hoc Analysis
Cytokines				
sTNF-R1	1.01 (0.90 – 1.17)	1.05 (0.82 – 1.26)	0.91 (0.78 – 1.10)	SCZ, BD > CTR
IL1-RA	0.29 (0.14 – 0.73)	0.41 (0.17 – 0.95)	0.34 (0.14 – 0.75)	NS
hsCRP	0.35 (0.14 – 0.73)	0.33 (0.10 – 0.95)	0.30 (0.10 – 1.06)	NS
vWF	97(60 – 129)	93 (70.5 – 129.5)	77 (52 – 109)	SCZ, BD > CTR
OPG	2.67 (2.04 – 3.25)	2.51 (2.01 – 2.87)	2.42 (1.92 – 2.98)	NS
IL-6	0.19 (0.10 – 0.40)	0.18 (0.10 – 0.49)	0.17 (0.10 – 0.35)	NS
CD40-L	1.42 (0.79 – 2.69)	1.69 (0.78 – 2.76)	1.65 (0.88 – 2.63)	NS
Verbal Memory Tests				
CVLT learning	57 (48 – 64)	49 (42 – 55)	59 (52 – 65)	CTR, BD > SCZ
CVLT recall	14 (12 – 15)	11 (9 – 14)	14 (12 – 15)	CTR, BD > SCZ
LM learning	25.08 (6.68)	21.57 (6.86)	26.95 (6.18)	CTR > BD > SCZ
LM recall	21.95 (7.34)	17.84 (7.23)	24.16 (6.87)	CTR > BD > SCZ

Abbreviations: IQR = Interquartile Range; CTR = Controls; BD = Bipolar Disorder Spectrum; SCZ = Schizophrenia Spectrum; NS = Non-Significant; sTNF-R1 = Soluble Tumor Necrosis Factor Receptor 1; IL1-RA = Interleukin 1 Receptor antagonist; hsCRP = High Sensitivity C-Reactive Protein; vWF = von Willebrand Factor; OPG = Osteoprotegerin; IL-6 = Interleukin 6; CD40-L = CD40 Ligand; CVLT = California Verbal Learning Test; SD = Standard Deviation; LM = Logical Memory subtest of the Wechsler Memory Scale III.

Cytokines and CVLT tests are given as median with IQR. LM test are given as mean and standard deviation. Post Hoc analysis is performed using Kruskal-Wallis and Mann-Whitney tests for cytokines and CVLT tests, and ANOVA, Tukey for LM tests.

Supplementary Table 3. Immune, verbal memory and hippocampal volume characteristics for the MRI subgroup (N = 225).

Parameters	Bipolar Disorder (N = 67)	Schizophrenia (N = 47)	Healthy Controls (N = 111)	Post Hoc Analysis
Cytokines				
sTNF-R1	1.00 (0.89 – 1.14)	1.05 (0.83 – 1.26)	0.87 (0.77 – 1.05)	SCZ, BD > CTR
IL1-RA	0.27 (0.13 – 0.65)	0.42 (0.20 – 0.99)	0.37 (0.18 – 0.88)	NS
hsCRP	0.35 (0.18 – 0.89)	0.27 (0.12 – 0.85)	0.27 (0.10 – 0.85)	NS
vWF	90 (58 – 124)	102 (75.5 – 131.5)	79 (52 – 101)	SCZ, BD > CTR
OPG	2.38 (1.91 – 2.87)	2.43 (2.00 – 2.87)	2.37 (1.84 – 2.75)	NS
IL-6	0.15 (0.10 – 0.30)	0.16 (0.10 – 0.31)	0.19 (0.10 – 0.37)	NS
CD40-L	1.37 (0.77 – 2.45)	1.95 (0.75 – 2.70)	1.68 (1.10 – 2.88)	NS
Verbal Memory Tests				
CVLT learning	58 (49 – 66)	49 (43 – 54)	59 (53 – 65)	CTR, BD > SCZ
CVLT recall	14 (12 – 16)	11 (9 – 14)	14 (12 – 16)	CTR, BD > SCZ
LM learning	25.84 (7.02)	22.38 (6.70)	28.30 (5.99)	CTR > BD > SCZ
LM recall	22.70 (7.82)	18.64 (6.67)	25.36 (6.45)	CTR > BD > SCZ
MRI Volumes (cm^3)				
Hippocampal formation	8.15 (0.88)	7.93 (0.88)	8.36 (0.74)	CTR > SCZ
CA1	0.66 (0.10)	0.65 (0.09)	0.67 (0.07)	NS
CA2/3	2.00 (0.27)	1.94 (0.28)	2.07 (0.23)	CTR > SCZ
CA4/DG	1.12 (0.15)	1.08 (0.15)	1.16 (0.12)	CTR > SCZ
Presubiculum	0.91 (0.12)	0.86 (0.10)	0.91 (0.10)	CTR, BD > SCZ
Subiculum	1.28 (0.17)	1.23 (0.14)	1.31 (0.13)	CTR > SCZ
ETIV	1601.67 (164.56)	1602.28 (159.14)	1590.30 (162.91)	NS

Abbreviations: IQR = Interquartile Range; CTR = Controls; BD = Bipolar Disorder Spectrum; SCZ = Schizophrenia Spectrum; NS = Non-Significant; sTNF-R1 = Soluble Tumor Necrosis Factor Receptor 1; IL1-RA = Interleukin 1 Receptor antagonist; hsCRP = High Sensitivity C-Reactive Protein; vWF = von Willebrand Factor; OPG = Osteoprotegerin; IL-6 = Interleukin 6; CD40-L = CD40 Ligand; CVLT = California Verbal Learning Test; SD = Standard Deviation; LM = Logical Memory subtest of the Wechsler Memory Scale III; ETIV = Estimated Total Intracranial Volume.
Cytokines and CVLT tests are given as median with IQR. LM tests and hippocampal volumes are given as mean and standard deviation. Post Hoc analysis is performed using Kruskal-Wallis and Mann-Whitney tests for cytokines and CVLT tests, and ANOVA, Tukey for LM tests and hippocampal volumes.

Supplementary Table 4. Analysis of associations between verbal memory and cytokines in whole sample (N = 466) after correcting for age, sex and diagnosis.

Memory Test	Cytokine	Age	Sex (M)	Group (P)	Cytokine	Full Model	
		t	t	t	t	F	R^2
LM learning	**sTNF-R1**	**-.18**	**-1.75**	**-5.18*** **	**-3.78*** **	**13.17**	**.10**
	IL1-RA	-.44	-1.65	-5.77***	-1.34	9.79	.08
	CRP	-.57	-1.59	-5.89***	1.13	9.64	.08
	vWF	-.51	-1.70	-5.86***	.68	9.43	.08
	OPG	-.43	-1.64	-5.82***	.07	9.30	.08
	IL-6	-.40	-1.65	-5.79***	-.68	9.43	.08
	CD40-L	-.44	-1.66	-5.83***	-.28	9.32	.08
LM recall	**sTNF-R1**	**-.96**	**-2.45***	**-5.73*** **	**-3.42** **	**14.93**	**.12**
	IL1-RA	-1.20	-2.35*	-6.28***	-1.40	12.25	.10
	CRP	-1.27	-2.31*	-6.37***	.73	11.85	.09
	vWF	-1.18	-2.36*	-6.25***	.11	11.71	.09
	OPG	-1.17	-2.33*	-6.33***	.08	11.71	.09
	IL-6	-1.15	-2.35*	-6.30***	-.73	11.85	.09
	CD40-L	-1.22	-2.35*	-6.34***	-.66	11.83	.09
CVLT learning	sTNF-R1	-3.07**	-5.68***	-6.02***	-1.27	20.45	.15
	IL1-RA	-3.18**	-5.65***	-6.26***	-1.38	20.54	.15
	CRP	-3.18**	-5.61***	-6.33***	.37	20.02	.15
	vWF	-2.91**	-5.53***	-5.90***	-1.76	20.89	.15
	OPG	-3.21**	-5.54***	-6.34***	.56	20.07	.15
	IL-6	-3.19**	-5.66***	-6.36***	.88	20.21	.15
	CD40-L	-3.17**	-5.64***	-6.32***	-.28	20.00	.15
CVLT recall	sTNF-R1	-3.65***	-5.67***	-5.17***	-.84	18.34	.14
	IL1-RA	-3.73***	-5.65***	-5.35***	-.87	18.35	.14
	CRP	-3.70***	-5.63***	-5.39***	.19	18.14	.14
	vWF	-3.50**	-5.55***	-5.04***	-1.47	18.76	.14
	OPG	-3.65***	-5.62***	-5.37***	-,06	18.14	.14
	IL-6	-3.75***	-5.67***	-5.44***	.93	18.39	.14
	CD40-L	-3.75***	-5.65***	-5.39***	-.64	18.25	.14

* $p < 0.05$ level (2-tailed) ** $p < 0.01$ level (2-tailed) *** $p < 0.001$ level (2-tailed)
Abbreviations: M = male, P = patients, LM = Logical Memory subtest of the Wechsler Memory Scale III, CVLT = California Verbal Learning Test, sTNF-R1 = Soluble Tumor Necrosis Factor Receptor 1, IL1-RA = Interleukin 1 Receptor antagonist, hsCRP = High Sensitivity C-Reactive Protein, vWF = von Willebrand Factor, OPG = Osteoprotegerin, IL-6 = Interleukin 6, CD40-L = CD40 Ligand.

Supplementary Table 5. Analysis of associations between verbal memory and hippocampal volumes in subsample (N = 225) after correcting for age, sex, estimated total intracranial volume and diagnosis (patient or control).

Verbal memory Test	MRI volume	Age	Sex (M)	ETIV	Group (P)	MRI volume	Full Model	
		t	t	t	T	t	F	R^2
LM learning	Hippocampus	-1.5	-.03	.30	-4.37***	.49	4.64	.10
	CA1	-1.64	-.07	.42	-4.56***	.44	4.63	.10
	CA2/3	-1.56	-.08	.21	-4.31***	.78	4.73	.10
	CA4/DG	-1.60	-.08	.19	-4.28***	.93	4.78	.10
	Presubiculum	-1.62	-.05	.51	-4.59***	.06	4.59	.10
	Subiculum	-1.58	-.05	.24	-4.40***	.75	4.71	.10
LM recall	Hippocampus	-1.09	-.21	-.40	-4.22***	1.50	5.11	.11
	CA1	-1.39	-.28	.13	-4.63***	.58	4.69	.10
	CA2/3	-1.30	-.30	-.07	-4.37***	.91	4.80	.10
	CA4/DG	-1.34	-.30	-.13	-4.32***	1.14	4.90	.10
	Presubiculum	-1.39	-.30	-.03	-4.58***	.78	4.75	.10
	Subiculum	-1.32	-.26	-.09	-4.44***	.98	4.83	.10
CVLT learning	**Hippocampus**	-.56	-1.87	-1.77	-2.85**	**2.74****	6.10	.12
	CA1	-1.12	-2.02*	-1.07	-3.39**	1.71	5.09	.10
	CA2/3	-.86	-2.10*	-1.68	-2.85**	**2.82****	6.55	.13
	CA4/DG	-.99	-2.05	-1.53	-2.89**	**2.67****	6.03	.12
	Presubiculum	-1.12	-2.06*	-1.37	-3.30**	**2.04***	5.37	.11
	Subiculum	-.92	-1.97	-1.72	-2.97**	**2.97****	6.19	.13
CVLT recall	**Hippocampus**	-1.55	-1.84	-2.36*	-2.73**	**2.67****	7.04	.14
	CA1	-2.14*	-2.02*	-1.88	-3.20**	**2.16***	6.49	.13
	CA2/3	-1.86	-2.07*	-2.34*	-2.71**	**2.85****	7.26	.14
	CA4/DG	-1.99*	-2.02*	-2.18*	-2.76**	**2.67****	7.05	.14
	Presubiculum	-2.12*	-2.04*	-2.07*	-3.15**	**2.18***	6.51	.13
	Subiculum	-1.93	-1.93	-2.31*	-2.86**	**2.85****	7.27	.14

* p < 0.05 level (2-tailed) ** p < 0.01 level (2-tailed) *** p < 0.001 level (2-tailed)

Abbreviations: M = male, ETIV = estimated total intracranial volume, P = patients, LM = Logical Memory subtest of the Wechsler Memory Scale III, CVLT = California Verbal Learning Test

Supplementary Table 6. Analysis of associations between hippocampal subvolumes and cytokines in MRI subgroup (N = 235) after correcting for age, sex, estimated total intracranial volume and diagnosis (patient or control).

MRI volumes	Cytokine	Age	Sex (M)	ETIV	Group (P)	Cytokine	Full Model	
		t	t	t	t	t	F	R^2
Hippocampus	sTNF-R1	-2.58*	-.56	7.91***	-3.81***	-.48	22.36	.34
	IL1-RA	-2.60*	-.46	7.67***	-3.98***	.37	22.33	.34
	IL-6	-2.60*	-.50	7.88***	-3.98***	.71	22.44	.34
	vWF	-2.49*	-.46	7.89***	-3.72***	-.94	22.56	.34
	OPG	-2.55*	-.55	7.80***	-3.98***	-1.79	23.26	.35
	CRP	-2.63**	-.60	7.95***	-3.96***	-.80	22.49	.34
	CD40-L	-2.77**	-.74	8.08***	-4.11***	-1.52	22.99	.34
CA1	sTNF-R1	.79	.61	5.20***	-1.87	-.66	10.63	.20
	IL1-RA	.83	.84	4.79***	-2.04*	1.47	11.06	.20
	IL-6	.76	.68	5.13***	-2.04*	.71	10.65	.20
	vWF	.73	.68	5.16***	-2.02*	.06	10.52	.19
	OPG	.81	.66	5.07***	-2.03*	-1.24	10.91	.20
	CRP	.75	.65	5.16***	-2.04*	-.23	10.54	.19
	CD40-L	.63	.51	5.28***	-2.13*	-1.08	10.81	.20
CA2/3	sTNF-R1	-.88	.52	7.05***	-3.54***	-1.49	20.24	.32
	IL1-RA	-.99	.69	6.75***	-3.90***	-.00	19.60	.31
	IL-6	-.98	.69	6.87***	-3.89***	-.51	19.67	.31
	vWF	-.88	.73	6.88***	-3.67***	-.78	19.77	.31
	OPG	-.93	.67	6.80***	-3.89***	-1.20	20.01	.31
	CRP	-.98	.74	6.85***	-3.92***	.42	19.65	.31
	CD40-L	-1.15	.44	7.10***	-4.09***	-1.58	20.32	.32
CA4/DG	sTNF-R1	-.22	.34	6.34***	-3.52***	-1.38	16.41	.27
	IL1-RA	-.32	.48	6.09***	-3.86***	-.07	15.89	.27
	IL-6	-.31	.49	6.18***	-3.87***	-.48	15.95	.27
	vWF	-.19	.54	6.20***	-3.59***	-1.00	16.16	.27
	OPG	-.25	.46	6.11***	-3.85***	-1.38	16.40	.27
	CRP	-.32	.48	6.20***	-3.86***	-.09	15.89	.27
	CD40-L	-.47	.26	6.39***	-3.98***	-1.45	16.46	.27
Presubiculum	sTNF-R1	.58	.72	7.14***	-2.14*	-.58	19.04	.30
	IL1-RA	.48	.69	7.17***	-2.32*	-.92	19.19	.31
	IL-6	.53	.79	7.11***	-2.31*	-.05	18.94	.30
	vWF	.62	.82	7.10***	-2.12*	-.70	19.08	.30
	OPG	.56	.78	7.06***	-2.30*	-.47	19.01	.30
	CRP	.52	.66	7.18***	-2.27*	-.99	19.22	.31
	CD40-L	.44	.64	7.18***	-2.37*	-.90	19.18	.31
Subiculum	sTNF-R1	-.57	-.12	6.77***	-2.98**	-1.04	16.15	.27
	IL1-RA	-.63	.04	6.47***	-3.24**	.43	15.90	.27
	IL-6	-.63	.01	6.65***	-3.24**	.91	16.08	.27
	vWF	-.53	.04	6.67***	-3.00**	-.96	16.10	.27

OPG	-.59	-.04	6.59***	-3.24**	-1.38	16.37	.27
CRP	-.66	-.11	6.73***	-3.22**	-0.85	16.05	.27
CD40-L	-.83	-.28	6.92***	-3.39**	-1.73	16.67	.28

* $p < 0.05$ level (2-tailed) ** $p < 0.01$ level (2-tailed) *** $p < 0.001$ level (2-tailed)
Abbreviations: M = male, ETIV = estimated total intracranial volume, P = patients, LM = Logical Memory subtest of the Wechsler Memory Scale III, CVLT = California Verbal Learning Test, sTNF-R1 = Soluble Tumor Necrosis Factor Receptor 1, IL1-RA = Interleukin 1 Receptor antagonist, hsCRP = High Sensitivity C-Reactive Protein, vWF = von Willebrand Factor, OPG = Osteoprotegerin, IL-6 = Interleukin 6, CD40-L = CD40 Ligand.

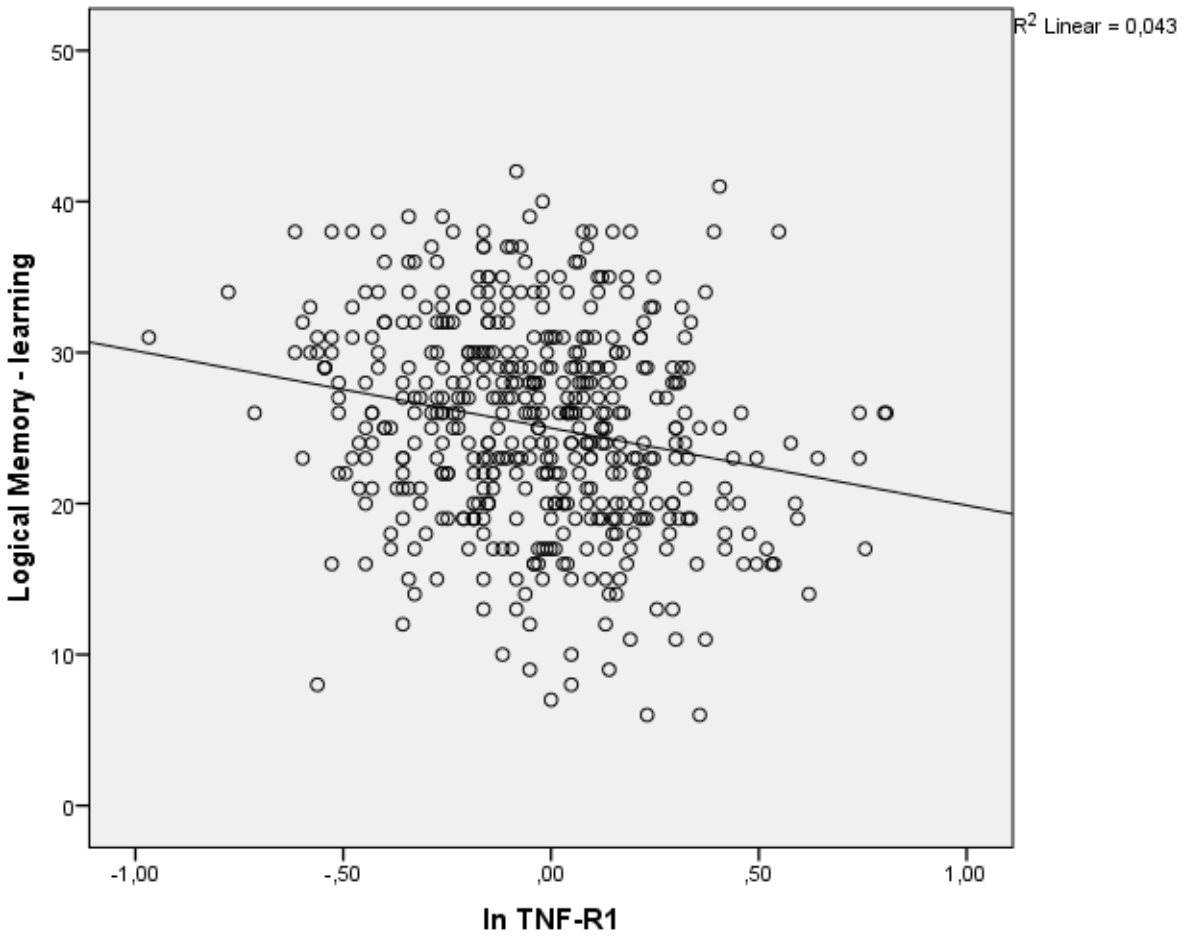

Figure 1. Scatterplot for WMS-III logical memory learning and plasma levels of sTNF-R1 in the Main sample (N = 466).

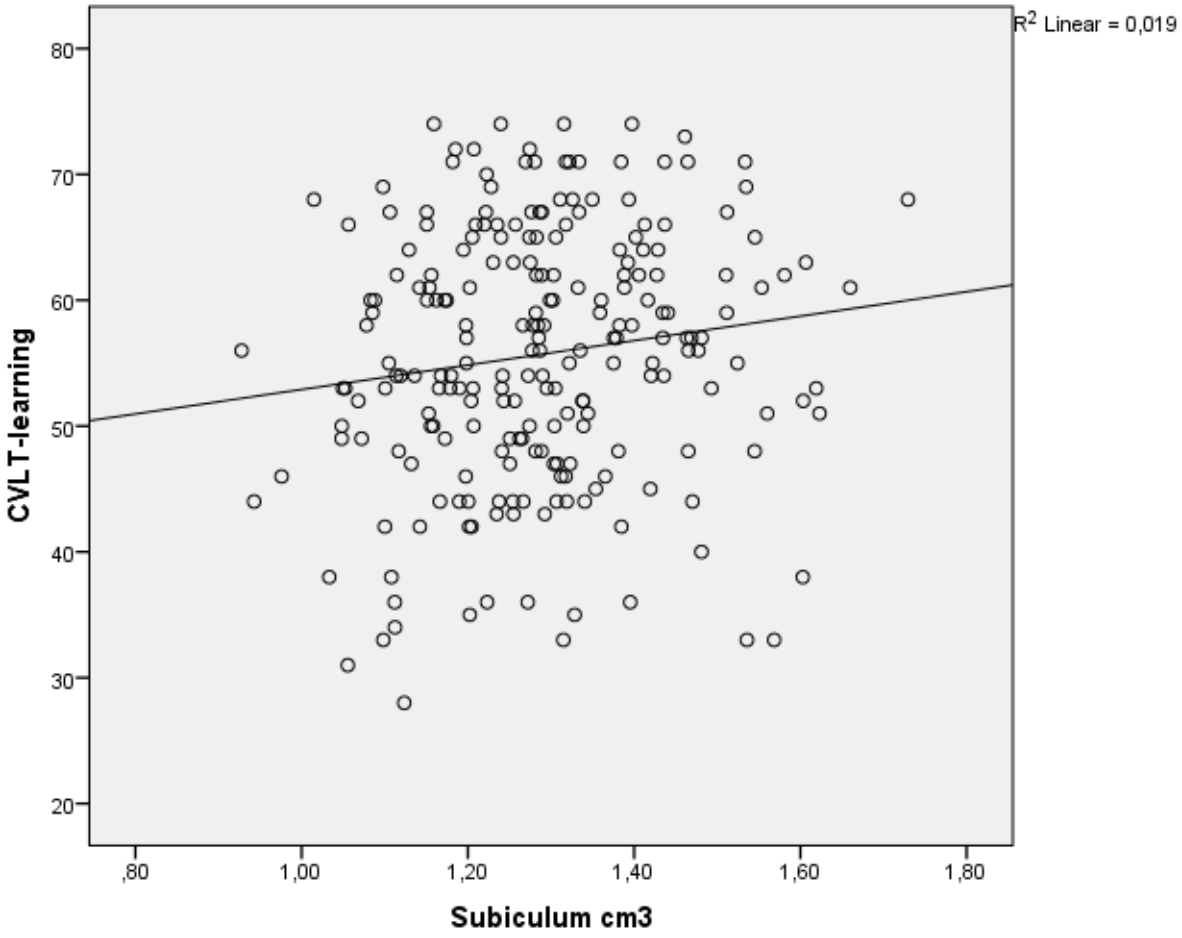

Figure 2. Scatterplot for CVLT learning and Subiculum volume (cm^3) in the MRI group (N = 225).

II

A study of TNF-pathway activation in schizophrenia and bipolar disorder in plasma and brain tissue

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Abstract

Objective: A pro-inflammatory imbalance in the tumor necrosis factor (TNF) system may contribute to the pathogenesis of schizophrenia (SCZ) and bipolar disorders (BD) and related comorbidities. We investigated the relative distribution of TNF related molecules in blood and dorsolateral prefrontal cortex (DLPFC) in these disorders.

Method: We measured plasma levels of TNF, soluble TNF receptor 1 (sTNFR1), soluble TNF receptor 2 (sTNFR2) and A Disintegrin And Metalloprotease-17 (ADAM17) using enzyme immunoassays, and calculated the TNF/sTNFRs ratio (TNF/sTNFR1+sTNFR2) in a sample of 816 SCZ and BD spectrum patients and 624 healthy controls (HC). *TNF*, *TNFRSF1A (TNFR1)*, *TNFRSF1B (TNFR2)* and *ADAM17* mRNA levels were determined in whole blood, and post-mortem DLPFC obtained from an independent cohort (n=80 SCZ, n=44 BD, and n=86 HC).

Results: In peripheral blood, we show increased TNF-related measures in patients compared to HC, with an increased TNF/sTNFRs ratio ($p = 6.00x10^{-5}$), but decreased *TNF* mRNA expression ($p=1x10^{-4}$), with no differences between SCZ and BD. Whole blood *ADAM17* mRNA expression was markedly higher in BD *vs*. SCZ patients ($p=1.40x10^{-14}$) and *vs*. HC ($p=1.22x10^{-8}$). In post-mortem DLPFC, we found no significant differences in mRNA expression of TNF pathway genes between any groups.

Conclusions: SCZ and BD patients have increased plasma TNF pathway markers without corresponding increase in blood cell gene expression. *ADAM17* expression in leukocytes is markedly different between the two disorders, while alterations in TNF related gene expression in DLPFC is uncertain. Further studies are necessary to elucidate the aberrant regulation of the TNF pathway in severe mental disorders.

Key words: DLPFC; cytokines; working memory; mRNA; post-mortem.

Introduction

A dysregulation of the immune system due to chronically activated macrophages and T-cells has been proposed to contribute to the pathogenesis of schizophrenia (SCZ)(Smith, 1992) and bipolar disorder (BD)(Munkholm et al., 2013). Several lines of evidence support a pro-inflammatory profile in both diseases, where alterations in the tumor necrosis factor (TNF)-pathway, consisting of soluble and membrane bound TNF (formerly TNF-alpha) and its two receptors, have been reported in peripheral blood(Munkholm et al., 2013; Upthegrove et al., 2014). However, the regulation and the site of production of TNF related molecules in these disorders are far from clear.

TNF is a pro-inflammatory cytokine expressed by macrophages, other leukocyte subsets and endothelial cells(Aggarwal, 2003; Imaizumi et al., 2000; Tecchio et al., 2014), as well as by neurons, astrocytes and microglia that have a macrophage phenotype. It signals through two distinct membrane-bound receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) of which TNFR1 is widely distributed in a range of cells and tissues whereas TNFR2 is more selectively expressed by immune cells, endothelial cells, specific neuronal subtypes and glia cells(Naude et al., 2011). Under physiological conditions TNF has been known to regulate synaptic transmission, neurotransmission, homeostatic synaptic scaling, neurogenesis and long term potentiation(Bernardino et al., 2008; Clark and Vissel, 2015; McCoy and Tansey, 2008). TNF and its receptors are produced as transmembrane proteins with an extracellular domain that can be proteolytically cleaved from the cell surface by metalloproteases like A Disintegrin And Metalloprotease-17 (ADAM17) resulting in soluble TNF, soluble (s)TNFR1 and sTNFR2. Increased circulating TNF, sTNFR1 and sTNFR2 levels have been reported by several studies in SCZ(Upthegrove et al., 2014) and BD(Doganavsargil-Baysal et al., 2013; Munkholm et al., 2013), but to this end, no large studies have evaluated if this leads to a pro-inflammatory imbalance between TNF and its soluble receptors, i.e., the TNF/sTNFRs ratio that reflects activity in the TNF system and correlates with TNF bioactivity(Aukrust et al., 1999).

Previously, modestly sized studies have demonstrated increased *TNF* mRNA expression in monocytes and lymphocytes in SCZ and BD(Drexhage et al., 2010; Liu et al., 2010; Pandey et al., 2015). To our knowledge, however, *TNF*, *TNF receptor superfamily [TNFRSF] 1A and TNFRSF1B* (denoted by *TNFR1* and *TNFR2*, respectively, in the present manuscript*)*, and *ADAM17* mRNA expression in whole blood cells have not been investigated in a well-powered sample of patients with severe mental disorders.

The dorsolateral prefrontal cortex (DLPFC) is associated with a range of complex behaviors frequently referred to as executive functions, including working memory(Arnsten and Jin, 2014), cognitive and behavioral flexibility, and abstract reasoning, all of which have been implicated in SCZ and to a lesser extent in BD(Brandt et al., 2014; Forbes et al., 2009; Porter et al., 2015). We have previously found that general cognitive abilities were negatively associated with plasma TNFR1 levels in adults with SCZ and BD(Hope et al., 2015). Moreover, a post-mortem study found elevated levels of *TNFR1* mRNA in the frontal cortex in SCZ and increased transmembrane TNF in BD suggesting alterations in the TNF-pathway in the central nervous system in both illnesses(Dean et al., 2013). However, large postmortem studies of TNF-pathway gene expression in the DLPFC are lacking.

The aim of the present study was to further determine the role of TNF-pathway related molecules at the protein and mRNA levels in BD and SCZ patients. First we investigated these markers, including the balance between TNF and its receptors, in peripheral blood in a large cohort (n=1440). We then investigated whether TNF proteins and their proportion is associated with working memory, a task affiliated with the DLPFC. Lastly, mRNA levels of these molecules were examined in the DLPFC in an independent cohort (n=210). We hypothesized that patients with SCZ and BD would present with a distinct systemic and cortical pattern of TNF-pathway markers reflecting the relative contribution of these different compartments to the sustained systemic TNF activation that is proposed to be operating in these patients.

Methods

Plasma and leukocyte cohort

Study Design and Ethics: The TOP Study at the NORMENT Centre, Oslo University Hospital, and collaborating Norwegian hospitals(Dieset et al., 2012) was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate. The biobank was approved by the Norwegian Directorate of Health. All participants provided written informed consent after receiving a description of the study.

Participants: The main inclusion criteria were DSM-IV diagnoses of schizophrenia spectrum disorders or bipolar spectrum disorders, IQ > 70 and age between 18 and 65 years (for details see(Dieset et al., 2012)). Healthy volunteers without any history of severe psychiatric disorders (or in any of their first-degree relatives), or substance/alcohol abuse/dependency from the same catchment area were randomly selected from the National Population Registry (www.ssb.no). (For details see(Dieset et al., 2012)) For the present analyses, patients and controls were not included if they had coexisting autoimmune or inflammatory disease, cancer, ongoing infections, used anti-inflammatory drugs or had C-reactive Protein (CRP) levels above 20 mg/L.

Clinical Assessments: Diagnosis was obtained using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Clinical symptoms were evaluated using the Young Mania Rating Scale (YMRS), Inventory of Depressive Symptoms (IDS), Calgary Depression Scale for Schizophrenia (CDSS), Positive and Negative Syndrome Scale (PANSS), while functioning was measured using the Global Assessment of Functioning split version function (GAF-F) and symptom scale (GAF-S). The clinical assessment team consisted of clinical psychologists and psychiatrists, who were all trained until satisfactory inter-rater reliability was obtained(Lagerberg et al., 2014; Ventura et al., 1998). Psychotic symptoms were examined in the SCZ group using the PANSS 5-factorial

model(Wallwork et al., 2012), while depressive and hypomanic/manic symptoms were investigated in the BD group using YMRS, IDS and CDSS.

Neurocognitive Assessment: Psychologists trained in standardized neuropsychological testing performed neurocognitive assessment. Working memory was assessed with the Digit Span Test—backward (Wechsler Adult Intelligence Scale [WAIS] -III), letter number sequencing (WAIS-III) and the Working Memory—Mental Arithmetic (WM-MA) Test—commissions(Simonsen et al., 2011). For details see (Demmo et al., 2016).

Cytokine Assessment: We used enzyme-linked immunosorbent assay to quantify protein levels due to its high specificity and sensitivity(Keustermans et al., 2013), and measured plasma levels of TNF using a high sensitivity enzyme immunoassay (EIA) from Cloud Corp (Housten, TX) while sTNFR1, sTNFR2 and sADAM17 was analyzed using EIAs from R&D systems (Minneapolis, MN). Intra- and inter-assay coefficients of variance for proteins were less than 10%. The ratio between TNF and sTNFRs may provide an estimate of the molar balance in serum between TNF molecules and sTNFRs. In molecular terms, this ratio was defined as TNF (pmol/L)/(sTNFR1 + sTNFR2)(pmol/L) X 100, assuming a molecular mass of (17 x 3) kD and 30 kD for TNF (trimer) and both types of sTNFRs, respectively.

RNA isolation and RT-PCR: Total RNA was isolated from whole blood using the Tempus 12-Port Isolation kit (Applied Biosystems; Ambion, Austin, TX, USA) and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The tubes were stored at (-)80^{o}C. Reverse transcription was performed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with ~0.5 μg total RNA in 96-well PCR plates (Applied Biosystems). Each plate contained 80 samples that were randomly distributed to atone for the differences in reverse transcription or subsequent real-time PCR efficiency.

Quantification of mRNA is described in detail in the Supplementary Text. We used Primer Express software version 3.0 (Applied Biosystems) to design sequence specific mRNA (primer spanning

exon-exon junction) oligonucleotide primers for the full-length *TNF, TNFR1, TNFR2* and *ADAM17* mRNA. Melt curves were evaluated for all primers. Data were normalized to ß-actin.

Brain cohort

Post-Mortem Brain sample collection:

Details of tissue acquisition, handling, processing, dissection, clinical characterization, diagnoses, neuropathological examinations, RNA extraction and quality control measures were described previously(Lipska et al., 2006). Toxicological analysis was performed on every case. For control cases, subjects with evidence of macro- or microscopic neuropathology, drug use, alcohol abuse or psychiatric illness were excluded.

Post-Mortem Brain RNA extraction and sequencing

Details of post-mortem DLPFC RNA extraction and sequencing, and RNA Seq data processing were previously described(Jaffe et al., 2015). Total RNA was extracted from ~100 mg of post-mortem tissue homogenates of DLPFC gray matter approximating BA9/46 in postnatal samples using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The poly(A)-containing RNA molecules were purified from 1 μg DNase-treated total RNA and, following purification, fragmented into small pieces using divalent cations under elevated temperature. Reverse transcriptase and random primers were used to copy the cleaved RNA fragments into first-strand cDNA, and the second-strand cDNA was synthesized using DNA polymerase I and RNase H. We performed the sequencing library construction using the TruSeq RNA Sample Preparation v2 kit by Illumina (See Supplementary Text for details).

Post-Mortem Analyses: RNA sequencing data processing

The Illumina Real Time Analysis (RTA) module performed image analysis, base calling and ran the BCL converter (CASAVA v1.8.2), generating FASTQ files containing the sequencing reads. These

reads were aligned to the human genome (UCSC hg19 build) using the spliced-read mapper TopHat (v2.0.4) using the reference transcriptome to initially guide alignment, on the basis of known transcripts of Ensemble Build GRCh37.67 (the "-G" argument in the software). A normalized reads per kilobase million (RPKM) metric were calculated for each gene by dividing the number of reads mapping to the gene divided by the length of the gene (in kilobases).

Statistical analysis

Plasma and leukocyte cohort analyses: Statistical analyses were performed using the SPSS software package for Windows, version 22.0. Data normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Differences in demographic data between groups were investigated using the chi-square test for categorical variables, the Kruskal-Wallis test for continuous variables, and Tukey and the Mann-Whitney U test for post hoc analyses. We used T-tests for normally distributed variables, and non-parametric tests (Mann Whitney U test) for skewed distributions to investigate differences between groups. Correlations were examined by using Spearman's Rank correlation.

Potential confounders (age, sex, smoking status, body mass index [BMI], time for blood sampling and race) were investigated using non-parametric tests (Spearman's rank correlation and Mann-Whitney U test), and confounders with a p-value lower than 0.2 were controlled for in linear regression models. Results are given as standardized beta or T-test from the regression analyses.

We calculated an aggregate working memory score from the three working memory tasks and controlled for age, gender, diagnosis and investigated interaction effects of diagnosis in general linear models.

We corrected for multiple testing according to Bonferroni, and alpha was set at $p < 0.007$ for the main analyses in the circulation (correcting for 7 tests: TNF, sTNFR2, sADAM17, *TNF* mRNA,

TNFR1 mRNA, *TNFR2* mRNA and *ADAM17* mRNA), and $p < 0.01$ for the aggregate working memory score.

Post-mortem analyses: Differential expression analysis was performed using LIMMA R package(Ritchie et al., 2015). For SCZ versus HC samples, gene RPKM was regressed against a binary diagnosis variable, covarying for 8 principal components and covariates of age, sex, and mitochondrial mapping rate, with the number of components determined for the expression matrix of a set of 562 immune genes (Birnbaum et al, under review) using the sva R package (surrogate variable analysis)(Leek et al., 2012). The optimum number of components was determined based on an iterative algorithm to remove the impact of mRNA quality on differences in expression between patients and controls that is not accounted for by controlling only for RIN and demographic variables. For BD versus HC samples, gene RPKM was regressed against a binary diagnosis variable, covarying in this case for 10 principal components and age, sex, and mitochondrial mapping rate. A multiple testing correction, FDR, was employed to correct for all immune genes in the matrix of immune genes from which the principal components were derived.

Results

Demographics and clinical characteristics

The socio-demographic and clinical characteristics of the participants are presented in Tables 1 and 2.

Plasma levels of TNF related molecules

The plasma levels of TNF related molecules in absolute values are summarized in Table 3. The patient group as a whole as well as the SCZ and the BD groups separately had significantly higher levels of TNF, sTNFR1and sTNFR2 as well as the TNF/sTNFRs ratio as an estimate of TNF activity compared to HC, also in adjusted analysis (i.e. confounders). In contrast, patients had lower levels of sADAM17 compared to HC in adjusted analysis. This pattern, however, was restricted to the SCZ group, with no significant difference between the BD and HC group (Table 4).

TNF, TNFR1, TNFR2 and ADAM17 mRNA expression in whole blood

The mRNA expression levels of TNF related molecules in whole blood are summarized in Table 3. The patient group as a whole as well as the SCZ and BD groups independently had lower levels of *TNF* mRNA compared to HC, with no difference between SCZ and BD (Table 4). In contrast, there were no differences in ***TNFR1*** **mRNA** and ***TNFR2*** **mRNA** between patients and controls (Table 4). However, patients had significantly higher levels of ***ADAM17*** **mRNA** compared to HC after controlling for confounding factors. This effect was clearly driven by the BD group (Table 4).

TNF proteins and working memory

Performance on working memory tasks is presented in Supplementary Table 5. We found significant associations between working memory and TNF-ratio and TNF that were significantly stronger in the SCZ group compared to CTR and BD (data not shown), and remained significant after correcting for multiple testing and controlling for age, sex, PANSS score and antipsychotics in

the SCZ group (TNF-ratio: F (5,120)=2.90, adjusted R^2=0.07, β=-0.23, p=0.008) (TNF: F (5,122)=3.35, R^2=0.09, β=-0.26, p=0.003) (Supplementary Table 6).

TNF, TNFR1, TNFR2 and ADAM17 mRNA expression in cortex

As shown in Supplementary Figure 1 and Table 4, there were no differences in mRNA levels of *TNF* (FDR= 0.99), *TNFR1* (FDR=0.84), *TNFR2* (FDR=0.92) or *ADAM17* (FDR=0.95) between SCZ and HC. Similarly, no differences in mRNA levels of *TNF* (FDR=0.76), *TNFR1* (FDR=0.77), *TNFR2* (FDR=0.99) or *ADAM17* FDR=0.39) were detected between BD and HC.

Correlation between TNF-related molecules in plasma (protein) and whole blood (mRNA)

The associations between protein and mRNA of the TNF related molecules in plasma and whole blood, respectively, are presented in Supplementary Table 1. We found moderate but highly significant associations between *TNFR1*, *TNFR2* and *ADAM17* mRNA, and significant weak associations between sTNFR1 and sTNFR2 in both the HC group and the patient group after controlling for confounders. Importantly, however, except for a weak correlation between mRNA and protein levels of TNFR1 in the patient group as a whole and those with SCZ, there was no significant correlation between protein and mRNA levels of the different TNF related molecules (Supplementary Table 1).

Role of medication

Patients using lithium (n=19) had higher levels of *ADAM17* mRNA and sTNFR1 levels compared to non-medicated (n=85) patients in the BD group. Serum levels of lithium were associated with higher TNF levels and increased TNF/sTNFRs ratio (Supplementary Table 2), however, these results do not remain significant after correction for multiple testing. We found no other significant associations between medication groups (antipsychotics, mood stabilizers and antidepressants), medication dosage and cytokines/mRNA.

Clinical characteristics and TNF pathway expression and cytokines

We found weak correlations with small effect sizes between clinical symptoms (i.e., PANSS, GAF and CDSS) and TNF pathway related gene expression and corresponding proteins. These results, however, do not survive correction for multiple testing (Supplementary Table 3-4).

PANSS, GAF and duration of illness in the SCZ group: We found weak negative associations after controlling for confounders between *TNF* mRNA and PANSS excited symptoms, *TNFR1* mRNA and PANSS negative symptoms, TNF and GAF-symptom scale, sTNFR1 and GAF-symptoms scale, and *TNF* mRNA and duration of illness. Thus, it seems that decreased *TNF* and *TNFR1* mRNA expression was associated with increased disease symptom severity, while increased circulating TNF and sTNFR1 was associated with increased symptom severity.

CDSS, IDS, YMRS, GAF and duration of illness in the BD group: We found a weak positive association after controlling for confounders between *TNF* mRNA and duration of illness in the BD group (the opposite of what we found in the SCZ group). Of the clinical symptoms only CDSS showed associations with cytokines and mRNA; increasing sTNFR2 levels and *TNFR2* mRNA expression were associated with decreased depressive symptoms, while increased *TNF* mRNA levels were associated with increased depressive symptoms.

Discussion

We found that patients with SCZ and BD had slightly increased plasma levels of TNF-related molecules with significantly increased TNF/sTNFRs ratio, which has been shown to be a surrogate marker of TNF bioactivity(Aukrust et al., 1999), compared to HC suggesting a subtle but potentially biologically relevant pro-inflammatory imbalance in the TNF system in these disorders. However, while TNF levels were elevated in plasma, *TNF* mRNA was decreased in whole blood suggesting other cellular sources of TNF than circulating leukocytes (Figure 1). The increased *ADAM17* mRNA expression detected in BD compared to SCZ in peripheral blood could potentially contribute to increase shedding of membrane-bound TNF in BD, suggesting a differential role for *ADAM17* in BD compared to SCZ. We have previously reported that elevated plasma levels of sTNFR1 were associated with lower scores on several cognitive tests(Hope et al., 2015; Hoseth et al., 2016). Here, we found that a shift toward a pro-inflammatory imbalance in the TNF pathway was weakly but significantly associated with lower working memory scores potentially suggesting a link between the DLPFC and the TNF pathway, however, we observed no significant alterations in TNF-system mRNA expression in the DLPFC of patients compared to HC.

The increased levels of TNF, sTNFR1 and sTNFR2 in SCZ and BD patients *per se* may not necessarily reflect a pro-inflammatory shift in the TNF-pathway considering that soluble TNF receptors partake in regulating TNF activity by acting as decoy receptors and competing with membrane-bound receptors for TNF(Moelants et al., 2013). However, our results suggest that while both plasma levels of TNF and its soluble receptors are elevated in SCZ and BD, there is also a pro-inflammatory imbalance as shown by increased TNF/sTNFRs ratio(Aukrust et al., 1999). In spite of this pro-inflammatory imbalance in plasma, and in contrast to previous more small-scaled studies(Drexhage et al., 2010; Liu et al., 2010), *TNF* mRNA was down-regulated in circulating leukocytes from both BD and SCZ patients implying other cellular sources for the elevated plasma levels in these disorders, such as endothelial cells and tissue macrophages (Figure 1). Based on the distinct upregulation of *ADAM17* mRNA in leukocytes in BD and the role of ADAM17 in the

release of TNF and its receptor from their membrane to their soluble form, it is, however, possible that increased shedding of these molecules could contribute to their increased plasma levels in BD patients, potentially representing a distinct pattern of this disorder.

We predicted that these results in peripheral blood would be recapitulated in the DLPFC. Using a conservative method to control for mRNA quality in brain tissue, which is a major confounder of prior studies that control only for RIN and demographics, we find no evidence of the changes in peripheral blood in prefrontal cortical samples from either patients with SCZ or BD. It is important to recognize that our mRNA quality adjustment is rigorous and conservative and though potentially sensitive to type II error, is robust in controlling for Type I error. The lack of changes in the expression of TNF related signaling molecules in the DLPFC *per se* cannot rule out altered TNF-pathway activity in other regions of the brain or the possibility that inflammatory molecules in peripheral blood have CNS effects without changing intrinsic gene expression. Indeed, cytokines in peripheral blood are able to cross the blood brain barrier(Erickson et al., 2012) potentially influencing learning and memory(Hoseth et al., 2016), cognition(Hope et al., 2015) and neural activity and viability(Vezzani and Viviani, 2015). Our findings raise questions, however, about whether TNF signaling in peripheral blood is a marker of a primary pathophysiological process or secondary to other disease mechanisms. Notably, enhanced TNF activity is associated with several co-morbid metabolic conditions such as type 2 diabetes and cardiovascular disease(Carlsson et al., 2016; Moon et al., 2004). However, it is important to underscore that the cortex analyses were performed on whole cortex samples and not on isolated cells, and an up- or down-regulation for example in microglia may be masked by expression in other cells. Correlations between clinical features, treatment and TNF measures in peripheral blood were largely uninformative showing a rather complex pattern. However, increasing depressive symptoms were associated with a moderate increase in *TNF* mRNA, and lower levels of sTNFR2, *TNFR1* and *TNFR2* mRNA suggesting a pro-inflammatory imbalance in circulating immune cells of these patients, further supporting a potential link between TNF activity and depression as described by

others(Soczynska et al., 2009). We found no significant difference in plasma/whole blood cytokine/mRNA levels between patients using antipsychotics in monotherapy and non-medicated patients, and no significant correlation between defined daily dosage of antipsychotics and cytokine/mRNA levels. This is in contrast to previous smaller studies that have found that antipsychotics may influence the expression of *TNF*(Paterson et al., 2006). However, plasma levels of TNF and TNF/sTNFRs ratio increased with higher lithium serum concentrations, and patients using lithium had higher sTNFR1 and *ADAM17* mRNA levels compared to non-medicated bipolar patients, suggesting that lithium may promote activation of the TNF system.

There are some limitations to our study. Firstly, there is a difference in the time of blood sampling for our HC group compared to the patient group, but this was controlled for in the analysis of cytokine and mRNA levels. Secondly, current RNA sequencing quantification and mapping methods may lead to potential underestimation of mRNA levels. Further, although previous studies have measured TNF related proteins in postmortem brain(Marballi et al., 2012), we were unable to obtain reliable data on postmortem TNF pathway proteins. Finally, our data on the association of TNF molecules and clinical symptoms should be interpreted with caution as no consistent patterns were observed.

This is by far the largest study that investigates the TNF system in patients with severe mental disorders, including both plasma levels as well as mRNA expression in whole blood and in brain. Our data suggest a complex regulation of TNF related molecules in peripheral blood of BD and SCZ patients with increased TNF/sTNFRs ratio in plasma, reflecting enhanced TNF activity as a major finding. The highly significant difference in *ADAM17* mRNA expression between SCZ and BD may also implicate other ADAM17 substrates and respective systems in the pathology of BD, and requires further elucidation. Our findings also suggest that circulating leukocytes are not a major source of soluble TNF levels in plasma. The lack of findings in the DLPFC leaves unresolved the issue of how peripheral blood alterations relate to the TNF system in the brain. Further studies

are needed to investigate other cortical regions of the brain and the impact of our findings on the pathogenesis of severe mental disorders.

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Table 1. Demographic and clinical characteristics of participants.

Parameters	Plasma (cytokine/protein) cohort				Leukocyte (mRNA) cohort			
	SCZ (N = 569)	BD (N = 247)	HC (N = 624)	Post Hoc Analysis	SCZ (N = 224)	BD (N = 143)	HC (N = 184)	Post Hoc Analysis
Male sex, N (%)	340 (59.8)	103 (41.7)	330 (52.9)	SCZ > HC > BD	127 (56.7)	51 (35.7)	108 (58.7)	HC, SCZ > BD
Ethnicity (Caucasian)	453 (79.6)	222 (89.9)	611 (97.9)	HC > BD > SCZ	178 (79.5)	132 (92.3)	184 (100)	HC > BD > SCZ
Tobacco (users)[a]	300 (54.8)	131 (54.6)	55 (19.9)	SCZ, BD > HC	118 (57.3)	66 (56.4)	24 (20.5)	SCZ, BD > HC
Medication:								
Antipsychotics	488 (85.8)	128 (51.8)	-	SCZ > BD	189 (84.4)	76 (53.1)	-	SCZ > BD
Lithium	9 (1.6)	50 (20.3)	-	BD > SCZ	4 (1.8)	20 (14.0)	-	BD > SCZ
Antidepressants	179 (31.5)	95 (38.8)	-	BD > SCZ	36 (16.1)	65 (46.1)	-	BD > SCZ
Mood stabilizers	76 (13.4)	98 (40.2)	-	BD > SCZ	61 (27.2)	54 (38.0)	-	BD > SCZ
Age (years)	27 (15)	32 (18)	32 (13)	BD, HC > SCZ	29 (15)	31 (16)	31.5 (13)	NS
Body Mass Index[b]	25.7 (7.1)	25.4 (5.6)	24.4 (4.4)	BD, SCZ > HC	25.9 (7.4)	25.4 (5)	24.5 (4.29)	SCZ > HC
Duration of Illness (years)	4 (8)	9 (13)	-	BD > SCZ	4.5 (7.8)	8 (10.5)	-	BD > SCZ
Time of blood sampling[c]	09:45 (1:10)	09:27 (0:55)	12:42 (6:50)	HC > SCZ > BD	09:50 (1:00)	09:30 (1:00)	11:10 (6:50)	HC > SCZ > BD
PANSS total score	62 (22)	45 (13)	-	SCZ > BD	63 (22)	44 (13)	-	SCZ > BD
YMRS total score	4.0 (9)	2 (6)	-	SCZ > BD	5 (9)	1 (4)	-	SCZ > BD
IDS total score	17 (20)	15 (18)	-	NS	17 (21)	14 (19)	-	NS
CDSS total score	5 (7)	3 (6)	-	SCZ > BD	5 (8)	2.5 (7)	-	SCZ > BD
GAF-S	40 (13)	55 (16)	-	BD > SCZ	39 (13)	57 (15)	-	BD > SCZ
GAF-F	41 (15)	50 (20)	-	BD > SCZ	40 (13)	51 (17)	-	BD > SCZ

Missing: [a] N = 369, [b] N = 319, [c] N = 422 in the plasma cohort

Abbreviations: HC = Healthy Controls; SCZ = Schizophrenia Spectrum; BD = Bipolar Disorder; NS = Non-Significant; PANSS=Positive and Negative Syndrome Scale; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; CDSS= Calgary Depression Scale for Schizophrenia; GAF-F = Global Assessment of Functioning - Function Scale; GAF-S = Global Assessment of Functioning - Symptom Scale. Categorical data are given as percent in brackets, while continuous data are given as median with interquartile range. Post hoc analysis is performed using Pearson Chi-square for categorical data, and Kruskal-Wallis and Mann-Whitney tests for continuous data.

Table 2. Post-mortem brain cohort samples descriptives.

Parameters	Brain cohort		
	SCZ (N = 80)	BD (N = 44)	HC (N = 88)
Male sex, N (%)	54 (73.8)	23 (52.3)	69 (78.4)
Ethnicity (Caucasian)	80 (100)	44 (100)	88 (100)
Mean age (SE)	46.5 (1.8)	46.8 (2.1)	44.7 (1.7)
Mean RIN (SE)	8.2 (0.06)	8.3 (0.08)	8.4 (0.05)

Abbreviations: SCZ = schizophrenia; BD = Bipolar Disorder; HC= Healthy Controls, SE = Standard Error, RIN = RNA integrity number

Table 3. Unadjusted data: Protein levels in plasma, relative mRNA expression in peripheral blood, and mRNA expression in the dorsolateral prefrontal cortex.

Parameters	Schizophrenia		Bipolar Disorder		Healthy Controls	
	N	M (IQR)	N	M (IQR)	N	M (IQR)
Plasma cytokine/protein						
TNF (pg/mL)	352	1.20 (1.28)	168	1.16 (1.04)	358	1.09 (1.03)
sTNFR1 (ng/mL)	569	1.76 (0.74)	247	1.67 (0.52)	624	1.60 (0.77)
sTNFR2 (ng/mL)	553	4.76 (1.43)	239	4.60 (1.47)	591	4.51 (1.44)
ADAM17 (pg/mL)	548	189 (282)	240	227 (358)	594	214 (341)
Leukocyte mRNA						
TNF mRNA	224	0.89 (0.44)	142	0.92 (0.41)	184	1.00 (0.58)
TNFR1 mRNA	224	1.02 (0.32)	143	1.08 (0.46)	184	1.00 (0.31)
TNFR2 mRNA	224	0.98 (0.36)	143	1.00 (0.46)	184	1.00 (0.43)
ADAM17 mRNA	224	1.02 (0.29)	142	1.24 (0.46)	184	1.00 (0.38)
DLPFC mRNA						
TNF mRNA	80	0.03 (0.05)	44	0.03 (0.06)	86	0.02 (0.05)
TNFR1 mRNA	80	3.87 (3.10)	44	3.19 (2.43)	86	2.35 (0.77)
TNFR2 mRNA	80	1.74 (1.20)	44	1.37 (0.63)	86	1.44 (0.64)
ADAM17 mRNA	80	3.25 (0.95)	44	2.83 (0.91)	86	3.40 (0.94)

Abbreviations: TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; DLPFC= dorsolateral prefrontal cortex;IQR=interquartile range; M = median, ng = nanogram; pg = pictogram.
Data are given as median with interquartile range due to skewed distributions.

Table 4. Differences in cytokine and mRNA levels between patients and controls after controlling for confounders.

	Plasma cohort (soluble cytokine)			Leukocyte cohort (mRNA)			Brain cohort (mRNA)		
	n	*β*	*t*	*n*	*β*	*t*	*t*	FC	FDR
Patients vs. HC									
TNFR1	1003	.14	**4.47*** **	472	.05	1.11			
TNFR2	1000	.10	**2.94** **	315	.11	1.56			
TNF	459	.17	**3.48** **	564	-.16	**-3.91*** **			
ADAM17	1415	-	**-2.08***	428	.16	**3.15** **			
TNF/sTNFRs ratio	455	.20	**4.07*** **						
SCZ vs. HC									
TNFR1	748	.15	**3.82*** **	309	.01	.15	.79	1.02	.84
TNFR2	828	.09	**2.56***	239	.14	1.71	-.40	.99	.92
TNF	319	.16	**2.38***	323	-.18	**-3.02** **	-.04	1.00	.99
ADAM17	744	-	**-2.93** **	323	.02	.28	-.33	.99	.95
TNF/sTNFRs ratio	317	.16	**2.48***						
BD vs. HC									
TNFR1	479	.17	**3.50** **	264	.05	.81	-.61	.99	.77
TNFR2	516	.15	**3.42** **	234	.04	.57	-.01	1.0	.99
TNF	189	.19	**2.61** **	326	-	**-2.56***	.64	1.01	.76
ADAM17	315	-.03	-.48	222	.40	**5.92*** **	1.62	1.05	.39
TNF/sTNFRs ratio	195	.22	**2.72** **						
BD vs. SCZ									
TNFR1	478	-.07	-1.46	367	.06	1.12			
TNFR2	696	-.06	-1.54	367	-.07	-1.25			
TNF	347	-.07	-1.28	366	.01	.11			
ADAM17	510	.06	1.42	366	.39	**8.03*** **			
TNF/sTNFRs ratio	344	-.02	-.39						

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

Abbreviations: SCZ=schizophrenia; BD=bipolar disorder; HC=healthy controls; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2)

The brain cohort: *n*(HC) = 86, *n*(SCZ)=79, *n*(BD) = 44.

Results are given as T-values from linear regression analyses after controlling for confounding factors.

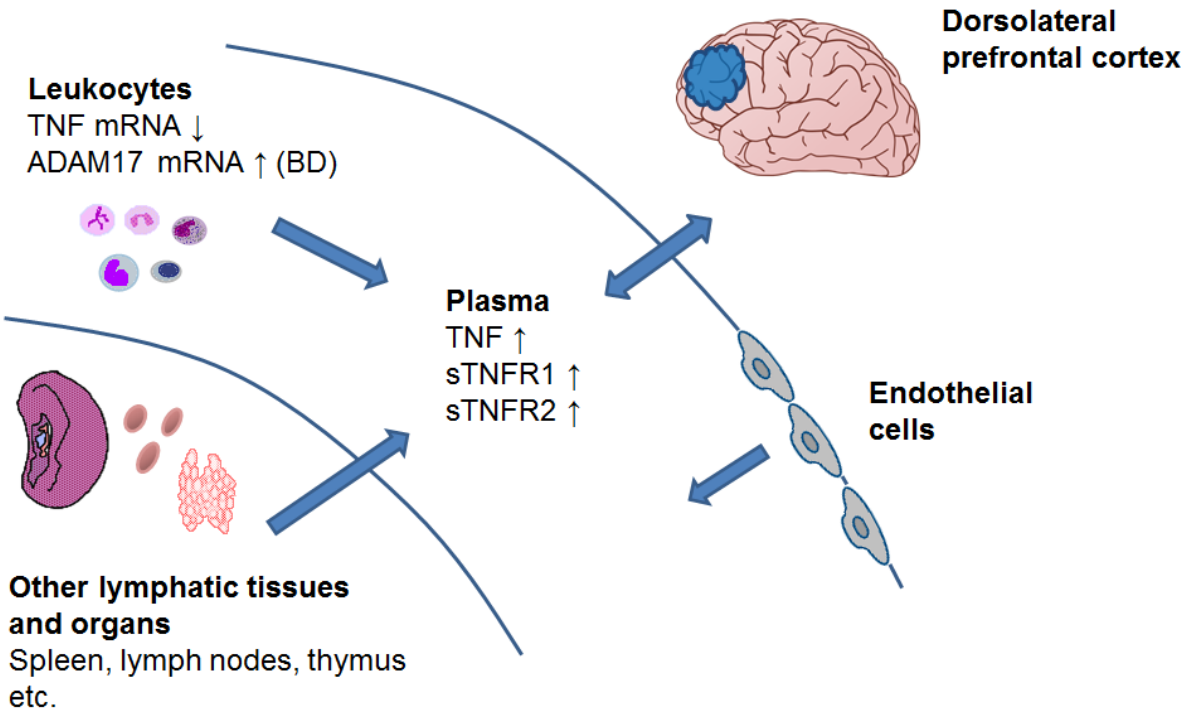


Figure 1. Elevated soluble plasma cytokine levels in SCZ and BD may be a result of increased shedding from their membrane bound form in BD, but blood leukocytes seem an unlikely source as *TNF* mRNA is downregulated in both disorders. There are no strong indications of alterations in TNF related signaling molecules in prefrontal cortex, however, soluble plasma cytokines can pass through the blood brain barrier, and differential expression of these molecules may occur in other regions of the brain. The increase in plasma cytokines may also be a result of heightened immune activity in other lymphatic tissues and organs as well as endothelial cells.

Supplementary Text

Quantification of whole blood mRNA

Quantification of mRNA was performed with the q polymerase chain reaction Master Mix for SYBR Green I (Applied Biosystems) in 10 μL duplicate reactions in 384 well plates on an ABI Prism 7900 (Applied Biosystems) using the 2(-Delta Delta C[T]) method with the average of four pools of cDNA. These were included on each plate in the RT-PCR and followed the samples in the real time PCR, as reference. The CV was under 10 % for the average of these calibrators between all the plates analyzed.

Sequencing library construction

The sequencing library construction was performed using the TruSeq RNA Sample Preparation v2 kit by Illumina. Briefly, cDNA fragments undergo an end repair process using T4 DNA polymerase, T4 polynucleotide kinase and Klenow DNA polymerase with the addition of a single adenosine using a Klenow polymerase lacking 3′ to 5′ exonuclease activity, and then ligated to the Illumina paired-end (PE) adapters using T4 DNA ligase. An index/barcode was inserted into Illumina adapters, allowing samples to be multiplexed in one lane of a flow cell. These products were then purified and enriched with PCR to create the final cDNA library for high throughput DNA sequencing using an Illumina HiSeq 2000.

Supplementary Table 1. Associations between protein and mRNA in the blood after controlling for confounders.

	sTNFR1	sTNFR2	sADAM17	TNF mRNA	TNFR1 mRNA	TNFR2 mRNA	ADAM17 mRNA
in HC							
TNF	.10	-.02	.02	-.01	-.03	-.04	-.12
sTNFR1		**.20****	.08	-.21	.03	-.02	-.06
sTNFR2			-.01	.00	-.03	-.12	-.06
sADAM17				-.02	.11	.11	-.01
TNF mRNA					-.13	-.03	-.06
TNFR1 mRNA						**.48*****	**.68*****
TNFR2 mRNA							**.44*****
in SCZ+BD							
TNF	.08	**.15***	**.13***	-.05	-.01	.06	.02
sTNFR1		**.33*****	.01	-.07	**.15***	-.06	-.01
sTNFR2			-.02	**-.18****	.08	-.02	-.05
sADAM17				-.08	.00	-.04	.04
TNF mRNA					-.08	**-.12***	**.16****
TNFR1 mRNA						**.48*****	**.44*****
TNFR2 mRNA							**.39*****
in SCZ							
TNF	-.01	**16***	.12	-.06	-.05	-.05	-.01
sTNFR1		**.41*****	.01	-.04	**.20****	.10	-.02
sTNFR2			.04	-.04	.09	-.02	.05
sADAM17				-.05	.00	-.03	-.03
TNF mRNA					.00	**-.16***	.12
TNFR1 mRNA						**.44*****	**.51*****
TNFR2 mRNA							**.45*****
in BD							
TNF	**.16***	.09	**.19***	-.01	-.04	.11	.02
sTNFR1		**.19***	.07	-.22	.15	.15	.10
sTNFR2			-.06	**-.30****	.15	.02	-.07
sADAM17				-.17	-.04	-.06	-.08
TNF mRNA					**-.27****	-.08	**.26****
TNFR1 mRNA						**.50*****	**.34****
TNFR2 mRNA							**.48*****

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

Abbreviations: SCZ = Schizophrenia; BD = Bipolar Disroder; HC = Healthy Controls; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2); ADAM17=A Disintegrin And Metalloprotease-17 protein. Results are given as standardized beta from linear regression analyses.

Supplementary Table 2. Associations between daily defined dose of medication and protein/mRNA levels, and group effect of medication vs. non-medicated patients after controlling for confounders and diagnosis.

	Serum cohort					mRNA cohort			
	TNF	sTNFR1	sTNFR2	TNF/sTNFRs ratio	sADAM17	TNF mRNA	TNFR1 mRNA	TNFR2 mRNA	ADAM17 mRNA
DDD associations:									
Antipsychotics	.07	-.07	-.02	-.04	.04	.09	.21	-.04	.11
Lithium	**.42****	-.14	-.01	**.39***	.01	-.40	.15	.15	.42
Mood stabilizers	.02	.08	.11	-.03	.06	-.01	-.08	.04	-.03
Antidepressants	.07	-.01	.14	.03	-.07	-.04	-.02	.08	-.08
Group effects:									
Antipsychotics	-1.11	1.79	.29	-.08	-.15	-1.32	-.82	.02	-1.19
Lithium	.24	**2.34***	.52	.41	-.33	-.37	-.24	-.29	**2.24***
Mood stabilizers	-.10	1.50	.75	-.23	-1.17	-1.24	-.48	-.75	-1.09
Antidepressants	.08	1.69	.45	.28	-1.00	-1.28	-.25	-1.24	-1.52

*$p < 0.05$ **$p < 0.01$

Abbreviations: DDD = daily defined dose; TNF=tumor necrosis factor; TNF=soluble TNF; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2); ADAM17=A Disintegrin And Metalloprotease-17 protein.

Results are given as standardized beta from linear regression analyses for associations and as T-values from linear regression analyses for medication effects.

Only antipsychotics were investigated as monotherapy due to small sample sizes in the other medication groups. We used serum concentration of lithium instead of DDD, and differences in *ADAM17* mRNA levels were examined in the bipolar disorder group.

Supplementary Table 3. Associations between protein/ mRNA and clinical characteristics in **schizophrenia**.

	DOI	Pos.	Neg.	Disorg.	Excited	Depr.	GAF-S	GAF-F
TNF	.05	.03	.04	.10	.05	.09	**-.14***	-.12
sTNFR1	.05	.05	.08	.04	.03	-.03	**-.14****	-.08
sTNFR2	.01	.06	.08	.02	.02	-.02	-.08	-.08
ADAM17	.03	.01	.02	.00	-.06	-.04	-.01	.01
TNF/sTNFRs ratio	.10	.02	-.03	.07	.05	.11	-.11	-.10
TNF mRNA	-.14*	-.07	.02	-.10	**-.17***	.02	.13	.13
TNFR1 mRNA	-.06	-.05	**-.14***	.06	-.07	-.07	.00	-.08
TNFR2 mRNA	-.07	-.08	-.06	.06	.07	-.09	.08	.00
ADAM17 mRNA	-.08	-.09	-.12	-.04	-.12	-.06	.07	.00

*p< 0.1; **p<0.01

Abbreviations: DOI=Duration of illness; PANSS=Positive and Negative Syndrome Scale, 5-factorial model(Wallwork et al., 2012); Pos.=PANSS Positive symptoms; Neg.=PANSS Negative symptoms; Disorg.=PANSS disorganized symptoms; Depr.=PANSS depressive symptoms; GAF-F=Global Assessment of Functioning - Function Scale; GAF-S=Global Assessment of Functioning - Symptom Scale; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2).

Associations are given as standardized beta after controlling for confounders in linear regression analyses. ADAM17 mRNA was not associated with the selected confounders and is therefore investigated using Spearman's Rank correlation.

Supplementary Table 4. Associations between protein/mRNA and clinical characteristics in **bipolar disorder**.

	DOI	CDSS	IDS	YMRS	GAF-S	GAF-F
TNF	.03	-.08	-.04	.07	.01	.07
sTNFR1	-.02	.08	.09	.07	.02	.06
sTNFR2	-.09	**-.22****	-.06	-.08	.09	.05
ADAM17	-.03	-.04	.01	.10	-.10	-.12
TNF/sTNFRs ratio	.05	.04	.01	.12	-.08	-.06
TNF mRNA	.13	**.35****	.14	.01	-.06	.01
TNFR1 mRNA	-.04	-.18	.00	.03	.03	.02
TNFR2 mRNA	-.12	**-.27***	-.07	.04	.06	.01
ADAM17 mRNA	.05	-.17	-.06	.03	.03	.07

*p< 0.1; **p<0.01

Abbreviations: DOI=Duration of illness; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; CDSS=Calgary Depression Scale for Schizophrenia; GAF-F=Global Assessment of Functioning - Function Scale; GAF-S=Global Assessment of Functioning - Symptom Scale; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2).

Associations are given as standardized beta after controlling for confounders in linear regression analyses.

Supplementary Table 5. Performance on working memory tasks.

Parameters	Schizophrenia		Bipolar Disorder		Healthy Controls		ANOVA/Kruskal-Wallis and Post Hoc analyses
	N	M (SD)	N	M (SD)	N	M (SD)	
Digit Span Test—backward (WAIS-III)	128	4.26 (1.06)	88	4.69 (1.30)	28	4.96 (0.92)	CTR,BD > SCZ
Letter Number Sequencing (WAIS-III)	128	9.54 (2.80)	88	10.07 (2.16)	28	11.54 (2.55)	CTR > BD,SCZ
WM-MA	128	2.68 (1.09)	88	3.00 (0.96)	28	3.46 (0.72)	CTR > BD,SCZ
Working Memory Aggregate Score	128	0.74 (0.77)	88	1.02 (0.71)	28	1.43 (0.63)	CTR > BD > SCZ

Abbreviations: WAIS-III= Wechsler Adult Intelligence Scale-III; WM-MA= Working Memory—Mental Arithmetic. M=mean, SD=standard deviation. Digit span test-backward and letter number sequencing test are given as raw score. WM-MA is given as d-prime. Working memory aggregate score is calculated from z-scores of the 3 tasks. Differences between groups were investigated using ANOVA for working memory aggregate score, and Kruskal-Wallis test for the remaining 3 tests. Post Hoc analyses were performed using Tukey and Mann-Whitney test respectively.

Supplementary table 6. Stepwise linear regression analyses investigating working memory as dependent variable in the schizophrenia group.

	TNF-ratio (*β*)	age (*β*)	sex (*β*)	PANSS total (*β*)	Antipsychotics (*β*)	*adj. R^2*	*F (df)*
model 1	-.25**					.05	*F*(1,124) = 8.05***
model 2	-.26**	-.07	-.10			.05	*F* (3,122) = 3.40*
model 3	-.24**	-.10	-.10	-.16		.07	*F* (4,121) = 3.48*
model 4	-.23**	-.10	-.10	-.17	-.07	.07	*F* (5,120) = 2.90*

	TNF (*β*)	age (*β*)	sex (*β*)	PANSS total (*β*)	Antipsychotics (*β*)	*adj. R^2*	*F (df)*
model 1	-.28**					.07	*F* (1,126) = 10.61**
model 2	-.28**	-.06	-.10			.07	*F* (3,124) = 4.27**
model 3	-.27**	-.09	-.11	-.15		.09	*F* (4,123) = 4.02**
model 4	-.26**	-.09	-.10	-.16	-.07	.09	*F* (5,122) = 3.35**

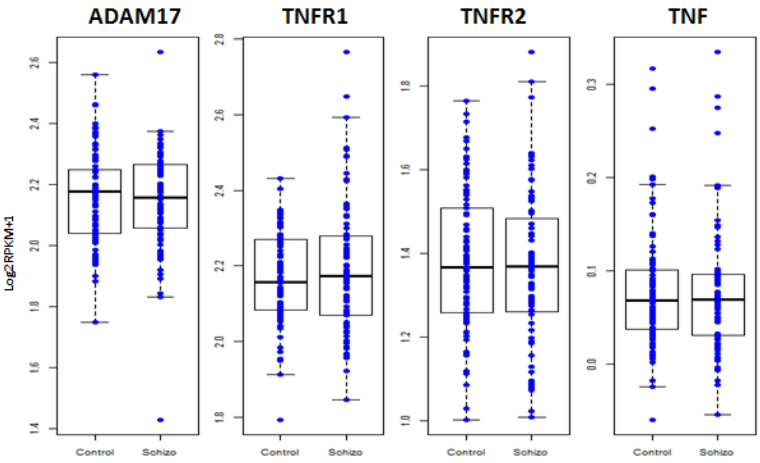
*p<0.05 **p<0.01 ***p<0.001

Abbreviations: TNF=Tumor Necrosis Factor; PANSS=Positive and Negative Syndrome Scale.

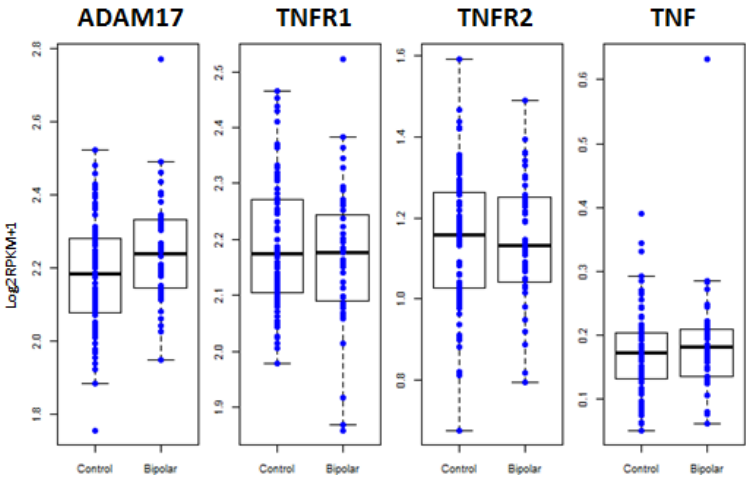
Results are given as standardized β from linear regression analysis.

Supplementary Figure 1. Results from post-mortem prefrontal cortex RNA sequencing.

SCZ vs HC



BD vs HC



No significant difference in *TNFR1*, *TNFR2* and *ADAM17* and *TNF* mRNA expression between patients and controls in the post-mortem sample. SCZ (n=80), BD (n=44) and HC (n=86). Y-axis is Log2RPKM+1 adjusted for principal components, age sex, and mitochondrial mapping rate. FDR is multiple testing correction for expression matrix of n=562 immune genes (elsewhere reported).

Reference to Supplementary Table 3

1. Wallwork RS, Fortgang R, Hashimoto R, Weinberger DR, Dickinson D. Searching for a consensus five-factor model of the Positive and Negative Syndrome Scale for schizophrenia. *Schizophr Res.* 2012;137(1-3):246-250.

III

Title page

Attenuated Notch signaling in schizophrenia and bipolar disorder

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Key words: schizophrenia; bipolar disorder; Notch; neurodevelopment; psychosis; lithium

Abstract

The Notch signaling pathway plays a crucial role in neurodevelopment and in adult brain homeostasis. We aimed to further investigate Notch pathway activity in bipolar disorder (BD) and schizophrenia (SCZ) by conducting a pathway analysis. We measured plasma levels of Notch ligands (DLL1 and DLK1) using enzyme immunoassays in a large sample of patients (SCZ *n*=551, BD *n*=246) and healthy controls (HC *n*=639). We also determined Notch pathway related gene expression levels by microarray analyses from whole blood in a subsample (SCZ *n*=338, BD *n*=241 and HC *n*=263). We found significantly elevated Notch ligand levels in plasma in both SCZ and BD compared to HC. Significant gene expression findings included increased levels of *RFNG* and *KAT2B* ($p<0.001$), and decreased levels of *PSEN1* and *CREBBP* in both patient groups ($p<0.001$). *RBPJ* was significantly lower in SCZ *vs* HC ($p<0.001$), and patients using lithium had higher levels of *RBPJ* ($p<0.001$). We provide evidence of altered Notch signaling in both SCZ and BD compared to HC, and suggest that Notch signaling pathway may be disturbed in these disorders. Lithium may ameliorate aberrant Notch signaling. We propose that drugs targeting Notch pathway could be relevant in the treatment of psychotic disorders.

Introduction

Schizophrenia (SCZ) and bipolar disorder (BD) are severe mental disorders that have prompted extensive research as they are among the leading causes of worldwide disability(Chong et al., 2016; Ferrari et al., 2016). The neurodevelopmental hypothesis for SCZ suggests that the neuroanatomical defects associated with SCZ are caused by dysregulation of brain development(Weinberger, 1987). In BD, brain morphological alterations are more subtle, but these together with behavioral changes prior to onset of illness support neurodevelopmental abnormalities also for BD(O'Shea and McInnis, 2016). In addition to developmental abnormalities, accelerated grey matter decline, aberrant brain connectivity and biochemical changes in the adult brain may indicate neurodegenerative processes in SCZ(Bartholomeusz et al., 2017; Pino et al., 2014); this may also be present in BD, albeit substantially less prominent(Savitz et al., 2014).

Notch signaling is well known as a master regulator of neural stem cells and neural development, and orchestrates nervous system development and patterning by regulating neurogenesis, axonal growth, synaptogenesis and predisposing neurons to apoptosis also in the adult brain(Ables et al., 2011). These facts make it a pertinent candidate for exploration in psychotic disorders. The Notch signaling pathway was first associated with SCZ through genetic findings linking the *NOTCH4* gene to SCZ in British parent-offspring trios(Wei and Hemmings, 2000), and later confirmed by larger genome wide association studies(Stefansson et al., 2009). Initial studies investigating Notch in BD were inconclusive(Ahearn et al., 2002; Prathikanti et al., 2004), however, in 2012 we found increased gene expression of *NOTCH4* in BD(Dieset et al., 2012).

In addition to being important in regulating neural cell proliferation, differentiation, and neural cellular growth(Ables et al., 2011), Notch is a crucial contributor in adaptive and

innate immune responses(Radtke et al., 2010). Notch and its ligands (*e.g.* delta-like 1, DLL1) have also been implicated in endothelial cell dysregulation and vascular inflammation(Butko et al., 2016; Quillard and Charreau, 2013) as well as macrophage activation(Shang et al., 2016), and is involved in the interaction between immune cells and the brain during ischemic stroke. This may be relevant also in relation to severe psychotic disorders as immune-pathogenic mechanisms have been implicated in SCZ and BD, partly based on the demonstration of low-grade systemic inflammation including T cell activation in these patients(Bergink et al., 2014).

Based on the emerging significance of neuro-inflammation and immunogenetics in SCZ and BD, we hypothesized that Notch signaling components in inflammatory cells, both as a proxy for CNS tissues, but also representing important cells that could modulate different neural processes, would be dysregulated. Thus, we aimed to characterize the Notch signaling pathway in patients with SCZ, BD and healthy controls (HC) by conducting a pathway analysis on the mRNA level in whole blood, as well as investigating plasma levels of secreted Notch ligands. Our secondary aim was to investigate whether potential alterations in Notch pathway mRNA expression or secreted ligands are associated with the use of psychotropic medication.

Results

Demographics and clinical characteristics

Socio-demographic and clinical characteristics of the participants are shown in Table 1. The plasma protein cohort and the whole blood mRNA cohort showed the same differences between SCZ, BD and HC groups with the exception of one clinical characteristic: SCZ patients had higher CDSS scores in whole blood cohort, but not in the plasma protein cohort. The main differences between patients and HC were age, ethnicity and sex.

Plasma levels of Notch ligands

The plasma levels of the secreted Notch ligands and group comparisons are summarized in Table 2. Patients had significantly higher plasma levels of DLL1 compared to HCs, with SCZ having significantly higher levels than BD after controlling for age and gender. DLK1 levels showed nominally significant increase in both patient groups compared to HC with no differences between BD and SCZ.

Gene expression in whole blood

The mRNA expression of Notch pathway genes are summarized in Figures 1 and 2 and Table 3, and nominally significant findings ($0.001 < p < 0.05$) in Supplementary Table 1. Effect size estimates were small in general with the highest value on 0.16 for *KAT2B*. Compared to HC, both SCZ and BD had increased levels of *RFNG* and *KAT2B*, and decreased levels of *PSEN1* and *CREBBP* mRNA expression. In addition, the SCZ group had increased *DTX3L* and decreased *RBPJ, CTBP* and *HDAC* mRNA expression compared to HC (Figure 1). The main

significant differences between SCZ and BD were lower *LFNG* and increased *NOTCH2* in SCZ *vs* BD (Table 3 and Supplementary figure 1).

Role of medication

We found that patients using lithium (n=35) had significantly higher *RBPJ* expression compared to patients not taking lithium (n=366, p<0.001) (Supplementary table 2). As the BD group had higher levels of *RBPJ* mRNA we also controlled for diagnosis. Our result remained significant after controlling for diagnosis (*t*=3.35 *p*=0.001 *F*=5.63 *n*=400) and when investigating lithium in the BD group alone (*t*=3.51 *p*=0.001 *F*=3.42 *n*=148). This association does not seem to be dosage dependent, as we observed no correlation between serum levels of lithium and *RBPJ* expression. We found nominally significant effects of antipsychotics and mood stabilizers on DLK1 levels, and a weak dose dependent association between antidepressants and DLL1 levels (Supplementary table 2).

Discussion

In the present study we evaluated circulating levels of secreted Notch ligands from plasma and mRNA expression of Notch family members and transcripts that may influence Notch signaling in whole blood in a large population of patients with SCZ and BD as well as in heathy controls. We found significant differences in the concerted regulation of Notch mRNA species between patients and controls, in addition to an increase in circulating levels of secreted Notch ligands. Our main findings include (i) raised levels of DLL1, which is a soluble Notch ligand with potential inhibiting effect on Notch signaling, (ii) down-regulation of *PSEN1* and *RBPJ* (SCZ), potentially impairing intracellular Notch signaling, and (iii) up-regulation of *RFNG* mRNA, which may inhibit ligand-receptor interactions, further impairing Notch signaling.

DLL1 and DLK1 are soluble Notch ligands that are cleaved from their respective transmembrane forms by ADAM proteins, and likely inhibit Notch signaling(Falix et al., 2012; Mishra-Gorur et al., 2002). Thus, the increased plasma levels of DLL1 and potentially DLK1 in SCZ and BD could reflect attenuated Notch activity in these disorders. We have previously identified elevated ADAM17 mRNA levels in BD, suggesting that ADAM17 may be involved in the heightened shedding of Notch ligands in our patient group(Hoseth et al., 2017). Fringe proteins modulate Notch signaling by sensitizing notch receptors to delta ligands and attenuating notch-serrate interactions(Takeuchi and Haltiwanger, 2014). Radical fringe (RFNG) is abundantly expressed in the rat brain, and has been found to negatively modulate Notch signaling(Mikami et al., 2001). Both SCZ and BD patients had significantly elevated *RFNG* expression compared to HC. This further supports a possible impediment in Notch signaling in patients *vs*. controls. PSEN1- controlled γ-secretase activity is essential for Notch signaling as it releases the Notch intracellular domain (NICD) that subsequently enters

the nucleus to regulate gene activity. Thus, decreased *PSEN1* expression in patients also favors attenuated Notch signaling, but may also have multiple functions outside of the γ-secretase complex(Duggan and McCarthy, 2016). The implications of enhanced expression of *DTX3L* are less clear. DTX3L belongs to the deltex family, which can facilitate intracellular trafficking of the NICD from the membrane to the nucleus. However, several proteins interact with deltex, and together they destabilize Notch receptors, acting as Notch signaling inhibitors(Hori et al., 2012).

In the nucleus, the RBP-J protein enables NICD to bind to DNA and regulate the expression of notch target genes (*HES* and *HEY*). SCZ patients had decreased *RBPJ* mRNA expression compared to HC. *RBPJ* deletion in mouse embryonic brain causes neural stem/progenitor cells to prematurely differentiate into neurons thus depleting the neuronal stem cell population(Imayoshi et al., 2010). There is also evidence indicating that RBP-J is necessary for hippocampal-dependent learning and memory in mice(Liu et al., 2015). Thus, a shift towards downregulation could hamper proliferation in the brain and interfere with hippocampal functions. The downregulated co-repressors (*i.e. CTBP*s and *HDAC*s) and upregulated co-activator *KAT2B* could imply a shift in favor of increased target gene transcription especially in SCZ *vs* controls. However, as the mRNA expression of *RBPJ* is downregulated, the significance of these alterations is unclear, possibly reflecting compensatory mechanisms. Furthermore, these transcriptional regulators are not restricted to Notch signaling and could be related to other signaling pathways(Stankiewicz et al., 2014). In contrast to our finding of decreased *HDAC1/2* in SCZ, a recent smaller study demonstrated increased *HDAC1* mRNA in blood leukocytes and in the hippocampus and prefrontal cortex of patients with SCZ who were subjected to early life stress(Bahari-Javan et al., 2017).

Activation of the Notch signaling pathway induces transcription of target genes (*HES* and *HEY*). Hes and hey proteins predominantly act as transcriptional repressors(Fischer and Gessler, 2007) and, depending on cellular context(Aster, 2014), impede cell differentiation, and prompt cell proliferation also in the adult brain(Imayoshi et al., 2010). We did not observe altered expression of target genes in this study, thus our finding of an attenuated pattern of Notch signaling pathway should be interpreted with some caution.

We show for the first time in a naturalistic study that patients taking lithium had higher levels of *RBPJ* expression compared to patients not medicated with lithium but using other psychotropic medication. This finding is in line with a previous study that proposed that lithium may activate the Notch pathway through the inhibition of glycogen synthase kinase-3β(Espinosa et al., 2003).

Our finding of a possibly hampered Notch signaling may seem at odds with the enhanced T cell response and chronic low grade systemic inflammation in SCZ and BD. However, the role of the adaptive immune system and T cell-mediated immune responses in these patients are still unclear, with some studies favoring a TH2 shift at least in SCZ(Brambilla et al., 2014; Debnath, 2015). Notch has emerged as an important regulator of TH-cell differentiation(Amsen et al., 2009) and stimulation with Notch ligands, including DLL1, promotes a TH1 phenotype which may be inhibited by soluble DLL1(Maekawa et al., 2003). Thus, our finding of increased plasma DLL1 and hampered Notch signaling as reflected by decreased *RBPJ*, is compatible with an altered T cell function potentially favoring development of Th2 cells. A recent meta-analysis suggested a shift towards a TH2 phenotype in SCZ based on circulating levels of typical TH1 and TH2 cytokines while in vitro studies favored a TH1 response in the patients. Thus, the role of Notch on T-cell mediated immune responses in the psychiatric disorders needs further study.

There are some limitations to the current study. Firstly, using whole blood as proxy for the brain has limitations, especially when we take into consideration that the Notch signaling pathway is almost exclusively dependent on cell-cell interaction. However, current technology does not yet enable us to investigate gene expression *in vivo* in the brain. We are thus dependent on either post-mortem studies which are limited by artificial changes in gene expression, or alternatively, evaluation of easily accessible tissues that may reflect processes in the brain to a certain degree(Sullivan et al., 2006). Secondly, the use of whole blood does not necessarily reflect the levels in various leukocyte subsets such as T cells and monocytes. Third, due to the naturalistic nature of our study most patients were using a combination of psychotropic medication which prevented exploration of medication in monotherapy. Fourth, the patients were not completely matched with controls in relation to age, ethnicity and sex, but these factors were adjusted for in the statistical analyses.

In conclusion, we demonstrate altered Notch signaling in whole blood in SCZ compared to HC. Although less clear, our results also indicate altered Notch signaling in BD compared to HC. Further, we show an association between the use of lithium and Notch pathway activation. The demonstrated pattern of Notch molecules suggest that the Notch signaling pathway may be compromised in patients compared to controls. There is emerging evidence from rodent studies that Notch signaling is important in adult brain, and partakes in the regulation of adult neural stem cell migration, morphology, synaptic plasticity and survival of neurons(Ables et al., 2011). Future studies should be aimed at further exploring the Notch signaling pathway in severe mental disorders, and investigate if therapy targeting this system could be relevant in psychotic disorders.

Methods

The present study is part of the Norwegian Centre for Mental Disorders Research, University of Oslo and Oslo University Hospital, and collaborating Norwegian hospitals(Dieset et al., 2012). The study is approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate and all research was performed in accordance with these guidelines and regulations. We obtained written informed consent from all participants. The biobank is approved by the Norwegian Directorate of Health.

Participants

We included patients in the study if they had DSM-IV diagnoses of schizophrenia spectrum disorders or bipolar spectrum disorders, IQ > 70, and age between 18 and 65 years (for details see(Dieset et al., 2012)). We randomly selected healthy controls without a prior history of severe psychiatric disorders (or in any of their first-degree relatives), or substance/alcohol abuse/dependency from the same catchment area from the National Population Registry (www.ssb.no) (for details see(Dieset et al., 2012)). For the present analyses, we included patients and controls if they had no coexisting autoimmune or inflammatory disease, cancer or ongoing infections, were not using anti-inflammatory drugs, and had C-reactive Protein (CRP) levels below 20 mg/L.

Clinical Assessments: The clinical assessment and diagnostic interviews were carried out by a team of psychologists and physicians who were trained until satisfactory inter-rater reliability was obtained(Lagerberg et al., 2014; Ventura et al., 1998). We used the Structured Clinical Interview for DSM-IV Axis I Disorders(First et al., 1995) to obtain diagnoses. We evaluated clinical symptoms using the Young Mania Rating Scale (YMRS)(Young et al., 1978), Inventory of Depressive Symptoms (IDS)(Rush et al., 1996), Calgary Depression Scale for Schizophrenia (CDSS)(Addington et al., 1990) and Positive and Negative Syndrome Scale (PANSS)(Kay et al., 1987). Global functioning and symptom load was measured with the Global Assessment of Functioning (GAF), split

version. The scale has two measures reflecting functioning (GAF-F) and symptom load (GAF-S)(Pedersen et al., 2007).

Plasma protein assessment

DLL1 and DLK1 were measured in duplicate using commercially available antibodies (R&D Systems, Abingdon, UK) in a 384 format using a combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT, USA) dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an ELISA plate reader (Bio-Rad, Hercules, CA, USA). Intra- and inter-assay coefficients of variation were 3.9% and 3.7% for DLL1 and 5.0% and 8.4% for DLK1, respectively. We observed no diurnal variation for DLL1 and DLK1: in a smaller group of HC (n=13) blood was collected at 4 time-points within 24 hours (mean intra-individual CV±SD was 5.8±5.2%, p=0.27 for DLL1; and CV±SD=5.8±6.4%, p=0.86 for DLK1). Further, we found no postprandial variation for DLL1 and DLK1 (n=13, fasting vs. non-fasting; mean intra-individual CV±SD was 11.1±7.6%, p=0.15 for DLL1 and CV±SD = 9.8±11.0%, p=0.84 for DLK1). Detection limits were 10 pg/mL and 25 pg/mL for DLL1 and DLK1, respectively, as defined as 3xSD of assay buffer (n=10).

RNA isolation and microarray analysis

RNA isolation: Blood samples were collected using Tempus Blood RNA Tubes. Total RNA was extracted with ABI PRISM 6100 Nucleic Acid PrepStation and TEMPUS 12-port RNA Isolation Kit according to manufacturer's protocol. High-Capacity cDNA Reverse Transcription Kit was used for reverse transcription of 1 µg RNA.

Global Transcriptomics Analyses: We selected 49 Notch pathway related genes using the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/pathway.html, hsa04330, version date 5/9/17). In addition, we included *HEY1* as it is a well-recognized Notch signaling

pathway target gene (Fischer and Gessler, 2007). For each sample 200 ng of total RNA was biotin labelled and amplified using the Illumina TotalPrep-96 RNA Amplification Kit (Thermo Fisher, Waltham, MA, USA). Global analysis of gene expression was performed with Illumina HumanHT-12 v4 Bead Chip (Illumina, San Diego, CA, USA) consisting of more than 47 000 probes (ie. transcripts). For this purpose, 842 samples (263 HC, 338 SCZ and 241 BD) passed labeling and scanning. Raw microarray scan files were exported using the Illumina GenomeStudio software and loaded into R for downstream analysis using specific packages provided by BioConductor (Ritchie et al., 2011). Lumi was used to detect outliers(Du et al., 2008). ComBat from the SVA R package was used to correct for technical batch effects, like RNA extraction batch, RNA extraction method, DNase treatment batch, cRNA labelling batch and chip hybridization (Leek et al., 2012). Further quality control, quantile-normalization and log2-transformation were done using Limma(Ritchie et al., 2015).

Association between medication and protein/mRNA

We used the defined daily dose (DDD) of psychotropic medications, which is *the assumed average maintenance dose per day for a drug used for its main indication in adults, to investigate associations between medication and proteins/mRNA.* We calculated the dose relative to DDD for antipsychotics, mood stabilizers and antidepressants according to the guidelines from the World Health Organization Collaborating Center for Drug Statistics Methodology (https://www.whocc.no/atcdd), and *used serum concentration levels for lithium. We selected* key Notch signaling pathway related genes whose expression was significantly altered in our analyses (*PSEN1*, *RBPJ* and *RFNG*) in addition to plasma proteins.

Statistical analysis

Plasma proteins: We used the SPSS software package for Windows, version 24.0 for the statistical analyses of demographic data and plasma proteins. We assessed data normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. As distributions were skewed, we used the Kruskal-Wallis test, the Mann-Whitney U test and the chi-square test to investigate differences in demographic

data and plasma proteins between groups. Associations between medication and proteins/mRNA were investigated by Spearman's Rank correlation test. We controlled for age and sex in linear regression models. We inspected the histogram and the normal P-P plot of the regression standard residuals. In case of skewed distribution of the residuals we repeated our analyses using ln transformation of the dependent variable, and then by removing outliers defined as above -3 and below 3 studentized deleted residuals saved from the regression analyses.

Global Transcriptomics: Analysis was performed on batch-adjusted log2-transformed values. We used the R software environment to investigate group differences in mRNA expression. We fitted a linear model using age, sex and *Bmal1* expression as covariates. Bmal1 is a critical component of the molecular clock(Ko and Takahashi, 2006), and we used its expression level to adjust for differences in time of blood sampling and circadian rhythm between patients and HC.

Medication: We used *ANCOVA to explore the effect of medication while controlling for age, sex and other medication groups.*

Correction for multiple testing: We corrected for multiple testing according to the Bonferroni method. Alpha was set at $p < 0.001$ for our main microarray analyses (investigating 49 genes), and at $p < 0.03$ for the two plasma proteins. Our secondary analyses investigating the effects of medication were explorative in nature, and we also applied Bonferroni to correct for these analyses separately from the main analyses. Alpha for the secondary analyses was thus set at $p < 0.003$ (correcting for 20 tests).

Data Availability

The datasets generated and analyzed during the current study are not publicly available due to Institutional Review Board restrictions but are available from the corresponding author on reasonable request.

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Author contributions

EZH, OAA, TU designed the study and wrote first version of manuscript. EZH, ID, RHM, SH, ESG, IM, VMS, PA and NES provided data. HRB, FK and EZH did data analyses. EZH, IM, OAA, SD, VMS, PA and TU interpreted the results. All authors critically revised this manuscript.

Competing Financial Interest Statement

The authors declare no conflicts of interest or biomedical financial interests.

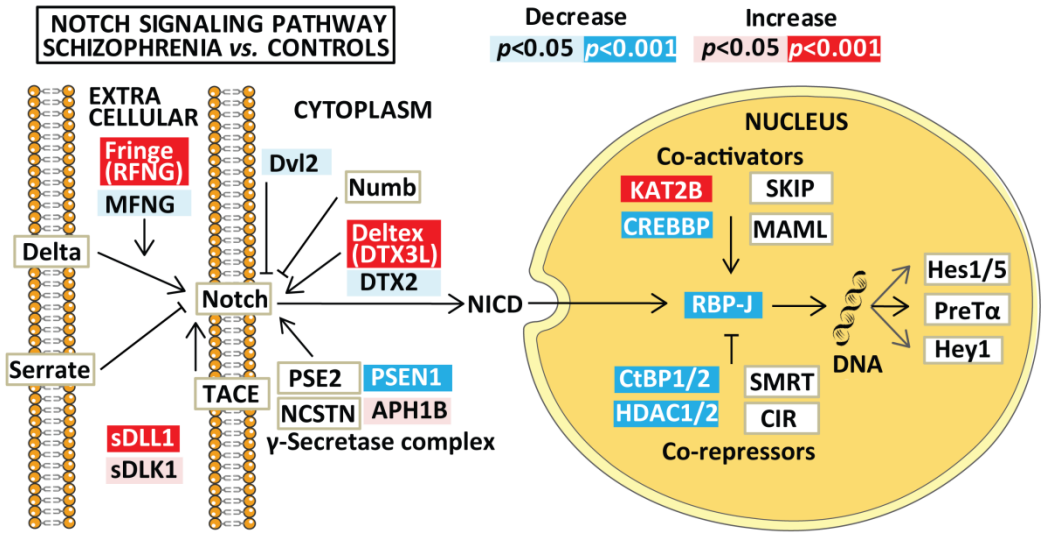

Figure 1

Summary of significant and nominally significant findings in Notch pathway mRNA expression between the schizophrenia and the healthy control group after controlling for age, gender and *Bmal1*.

Results are given as *p*-values, adjusted for multiple testing, where significant results are indicated in red/dark blue (for increased/decreased mRNA expression) and nominally significant results ($0.001 < p < 0.05$) are shown as pink/light blue (for increased/decreased mRNA expression). Non-significant results are depicted as boxes with white background. The figure is based on the Notch signaling pathway in the KEGG database (hsa04330, version date 5/9/17). Hey1 is included by the authors due to its known role as a target gene for Notch signaling (Fischer and Gessler, 2007).

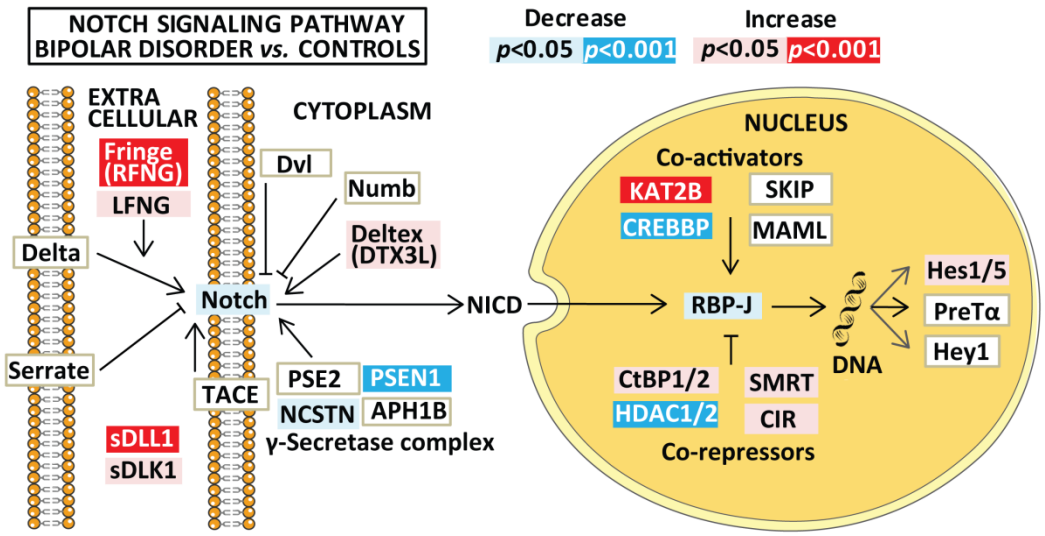

Figure 2

Summary of significant and nominally significant findings in Notch pathway mRNA expression between the bipolar disorder and the healthy control group after controlling for age, gender and *Bmal1*.

Results are given as *p*-values, adjusted for multiple testing, where significant results are indicated in red/dark blue (for increased/decreased mRNA expression) and nominally significant results ($0.001 < p < 0.05$) are shown as pink/light blue (for increased/decreased mRNA expression). Non-significant results are depicted as boxes with white background. The figure is based on the Notch signaling pathway in the KEGG database (hsa04330, version date 5/9/17). Hey1 is included by the authors due to its known role as a target gene for Notch signaling (Fischer and Gessler, 2007).

Table 1. Demographic and clinical characteristics of participants.

Parameters	Plasma (Notch ligand) cohort				Whole blood (mRNA) cohort			
	SCZ (N = 551)	BD (N = 246)	HC (N = 639)	Post Hoc Analysis	SCZ (N = 338)	BD (N = 241)	HC (N = 263)	Post Hoc Analysis
Male sex, N (%)	334 (60.6)	97 (39.4)	364 (57.0)	SCZ > HC > BD	275 (61.5)	90 (39.5)	144 (54.8)	SCZ > HC > BD
Ethnicity (Cauc. %)	444 (80.6)	213 (86.6)	629 (98.4)	HC > BD > SCZ	228 (90.1)	140 (94.6)	208 (100)	HC > BD > SCZ
Medication N(%):								
Antipsychotics	510 (84.6)	167 (66.0)	-	SCZ > BD	234 (92.5)	114 (77.0)	-	SCZ > BD
Lithium	12 (2.0)	51 (20.2)	-	BD > SCZ	3 (1.2)	32 (21.6)	-	BD > SCZ
Antidepressants	179 (31.5)	95 (38.8)	-	BD > SCZ	35 (13.8)	66 (44.6)	-	BD > SCZ
Mood stabilizers	56 (9.3)	87 (34.4)	-	BD > SCZ	78 (30.8)	57 (38.5)	-	BD > SCZ
Age (years)	27 (13)	29 (18)	31 (13)	BD, HC > SCZ	29 (14)	35 (20)	31.0 (13)	BD, HC >SCZ
DOI (years)	4 (8)	4 (10)	-	BD > SCZ	4 (9)	5 (13)	-	BD > SCZ
PANSS total score	62 (22)	44 (13)	-	SCZ > BD	64 (24)	45 (14)	-	SCZ > BD
YMRS total score	3 (9)	2 (5)	-	SCZ > BD	3 (9)	2 (6)	-	SCZ > BD
IDS total score	17 (19)	17 (16)	-	NS	18 (18)	15 (16)	-	NS
CDSS total score	5 (8)	4 (6)	-	NS	5 (7)	3 (7)	-	SCZ > BD
GAF-S	40 (15)	57 (16)	-	BD > SCZ	40 (14)	54 (17)	-	BD > SCZ
GAF-F	42 (14)	51 (19)	-	BD > SCZ	42 (15)	50 (17)	-	BD > SCZ

Abbreviations: SCZ = Schizophrenia; BD = Bipolar Disorder; HC = Healthy Controls; Cauc.= Caucasians; NS = Non-Significant; DOI=Duration of illness; PANSS=Positive and Negative Syndrome Scale; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; CDSS= Calgary Depression Scale for Schizophrenia; GAF-S = Global Assessment of Functioning - Symptom Scale; GAF-F = Global Assessment of Functioning - Function Scale.

Categorical data are given as percent in brackets, while continuous data are given as median with interquartile range. Post hoc analysis is performed using Pearson Chi-square for categorical data, and Mann-Whitney U tests for continuous data. Differences between groups are significant when $p<0.05$.

Table 2. Differences between groups for plasma markers of Notch pathway after controlling for age and gender.

plasma ligands	*M(IQR)*			SCZ vs. HC			BD vs. HC			SCZ vs. BD		
	SCZ	BD	HC	*df*	*t*	*F*	*df*	*t*	*F*	*df*	*t*	*F*
DLL1	5 (1.8)	4.7 (1.6)	4.5 (1.3)	1232	8.8***	32.01***	889	3.26**	10.81***	848	3.00**	9.13***
DLK1	186 (170)	188 (164)	180 (149)	1243	2.23*	10.62***	893	2.00*	11.38***	854	0.08	9.06***

*$p < 0.05$ **$p < 0.03$ ***$p < 0.001$

Abbreviations: M=median; IQR=interquartile range; SCZ=schizophrenia; BD=bipolar disorder; HC=healthy controls; DLL1= Delta-like protein 1; DLK1= Delta Like Non-Canonical Notch Ligand 1.

ANCOVA using linear regression models. Results are significant if $p<0.03$, and nominally significant if $0.03<p<0.05$ (Bonferroni correction).

Table 3. Significant differences between patients and controls in Notch signaling pathway gene mRNA expression after controlling for age and gender.

	Genes	specificity	SCZ vs. HC	BD vs. HC	SCZ vs. BD
			B	*B*	*B*
Receptor and Cytoplasm	*LFNG*	+++	-.01	.07**	-.09***
	RFNG	+++	.11***	.07***	.03
	NOTCH2	+++	.01	-.04**	.06***
	DTX3L	+++	.05***	.00	.05**
	PSEN1	++	-.03***	-.02***	-.01
Nucleus	*KAT2B*	++	.16***	.13***	.03
	CREBBP	+	-.10***	-.09***	-.01
	CTBP1	++	-.03***	-.01	-.02*
	CTBP2	++	-.03***	-.01	-.02**
	HDAC1	++	-.08***	-.04*	-.04*
	HDAC2	++	-.06***	-.05*	-.01
	RBPJ	+	-.07***	-.01	-.06**

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

Specificity: + (unspecific, involved in many pathways), ++ (involved in up to 3 additional pathways *e.g.* Wnt, NF-kappa), +++ (exclusively Notch pathway related gene).

Abbreviations: SCZ=Schizophrenia; BD=Bipolar disorder; HC=Healthy controls; B=Unstandardized regression coefficient.

Gene names are listed according to the HUGO Gene Nomenclature Committee.

Results are given as effect size estimates from the linear regression analysis after correction for age, sex and *BMAL1* expression. Results are significant if $p<0.001$, and nominally significant if $0.001<p<0.05$ (Bonferroni correction).

Title page

Attenuated Notch signaling in schizophrenia and bipolar disorder

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Supplementary Table 1. Nominally significant differences ($0.001 < p < 0.05$) between patients and controls in Notch signaling pathway gene mRNA expression after controlling for age and gender.

	Genes	specificity	SCZ vs. HC	BD vs. HC	SCZ vs. BD
			B	*B*	*B*
Receptor and Cytoplasm	*MFNG*	+++	-.05**	-.03*	-.01
	LFNG	+++	.01	.02*	-.02
	RFNG	+++	.02**	.02**	.00
	DTX1	+++	.02*	.01	.02
	NOTCH1	+++	-.04	-.08**	.04
	DTX2	+++	.01	.02*	-.02
	DTX2	+++	-.04*	-.03	-.01
	PSEN1	++	-.03**	-.03**	.00
	PSEN2	++	-.01	-.02*	.01
	APH1B	+++	.04**	.03	.01
	DVL1	++	.01	-.01	.02*
	DVL1	++	-.01	.00	-.01*
	DVL2	++	-.02*	-.02	.00
	NCSTN	+++	.00	-.05**	.05**
Nucleus	*CIR*	+++	-.01	.05*	.05*
	CTBP1	++	.01	.03**	-.02
	KAT2A	++	-.04*	.02	-.05**
	HES1	++	.01	.02*	-.01
	NCOR2	+++	.01	.01*	.00
	NCOR2	+++	-.03	.02	-.05**
	RBPJ	+++	-.01	.00	-.01*
	RBPJ	+++	-.01*	-.02**	.01

*$p < 0.05$ **$p < 0.01$

Specificity: + (unspecific, involved in many pathways), ++ (involved in up to 3 additional pathways *e.g.* Wnt, NF-kappa), +++ (exclusively Notch pathway related gene).

Abbreviations: SCZ=Schizophrenia; BD=Bipolar disorder; HC=Healthy controls; *B*=Unstandardized regression coefficient.

Gene names are listed according to the HUGO Gene Nomenclature Committee. The genes represent probes, and are different from the probes used in Table 1.

Results are given as effect size estimates from the linear regression analysis after correction for age, sex and *BMAL1* expression.

Supplementary Table 2. Associations between daily defined dose of medication, Notch ligand levels in plasma and mRNA expression. Group effects of medicated vs. non-medicated patients.

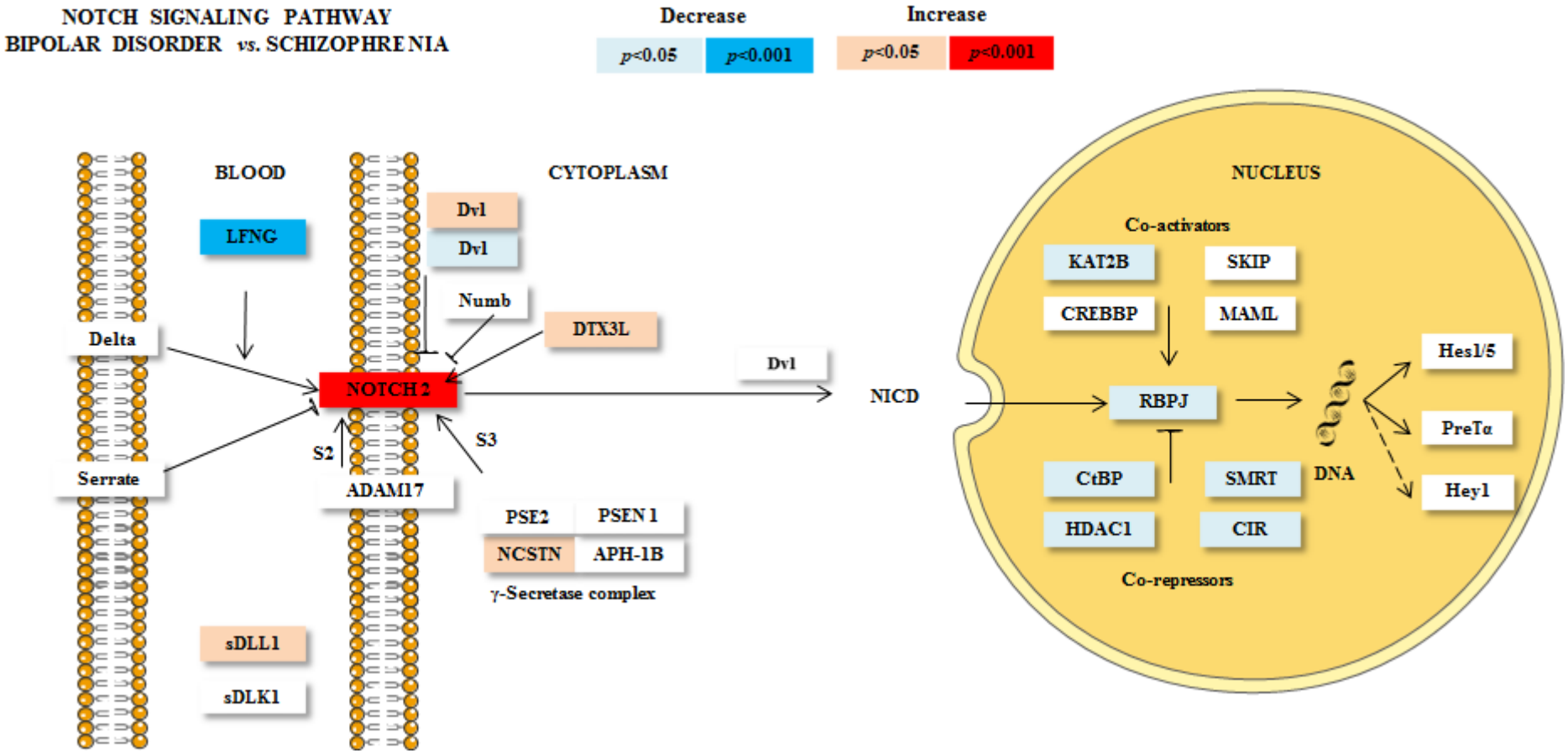
	n	Notch ligands		*n*	mRNA		
		DLL1	DLK1		PSEN1	RBPJ	RFNG
DDD associations:							
Antipsychotics	603	.06	.06	299	.08	.00	.05
Lithium	54	.16	.10	31	.11	-.12	.06
Mood stabilizers	142	.08	.03	101	.05	-.20	-.14
Antidepressants	30	**.15***	-.04	120	.18	.03	-.10
Group effects:	yes (*n*) / no (*n*)			yes (*n*) / no (*n*)			
Antipsychotics	690 / 124	1.87	**2.08***	348 / 53	.75	-1.18	.10
Lithium	63 / 751	-.91	-.36	35 / 366	-.52	4.46***	-1.57
Mood stabilizers	145 / 669	-.40	**2.38***	101 / 300	-.32	1.30	-.33
Antidepressants	248 / 566	.35	.11	135 / 266	.51	.94	-.16

*$p < 0.05$ ***$p < 0.001$

Abbreviations: DDD = daily defined dose; DLL1= delta like canonical Notch ligand 1; DLK1= Delta Like Non-Canonical Notch Ligand 1; yes=using the specified medication; no=not using the specified medication. Gene names are listed according to the HUGO Gene Nomenclature Committee.

We used serum concentration of lithium instead of DDD.

Associations and group effects are given as *t* from analyses of covariance with age, gender and other medication groups as covariates. Results are significant if $p<0.003$ after correction for multiple testing.



Supplementary Figure 1 Summary of significant and nominally significant findings in Notch pathway mRNA expression between the schizophrenia and the bipolar disorder group after controlling for age, gender and *Bmal1*.
Results are given as *p*-values, adjusted for multiple testing, where significant results are indicated in red/dark blue (for increased/decreased mRNA expression) and nominally significant results ($0.001 < p < 0.05$) are shown as pink/light blue (for increased/decreased mRNA expression)