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DYSREGULATION OF THE KYNURENINE PATHWAY IN PSYCHOTIC DISORDERS – IMMUNOLOGICAL ASPECTS

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DYSREGULATION OF THE KYNURENINE PATHWAY IN PSYCHOTIC DISORDERS – IMMUNOLOGICAL ASPECTS

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ABSTRACT

Kynurenic acid (KYNA), a metabolite of the kynurenine pathway, is emerging as a key factor in the development of psychotic disorders. Increased levels of KYNA are found in the brain and cerebrospinal fluid (CSF) of patients with schizophrenia and bipolar disorder, and are associated to psychotic symptoms. The unique pharmacological profile of KYNA allows it to exert actions on all the main neurotransmitter systems of the brain. The kynurenine pathway is under immunological control and can be induced by inflammatory signaling, hence linking findings of an immune involvement in psychotic disorders with established hypotheses of dopamine and glutamate in schizophrenia. KYNA has also been linked to impaired cognitive functioning, often accompanying psychotic disorders. Along this background, we set out to further investigate the kynurenine pathway and its induction by immune signaling, in the main psychotic disorders schizophrenia and bipolar disorder.

Starting with a GWAS against CSF levels of KYNA, and with the aid of clinical association studies, post mortem analyses and cell culture experiments, we identified a gene variant associated to a decreased function of SNX7 in patients with bipolar disorder. SNX7 was shown to influence the activity of caspase-8, an activator of interleukin (IL)-1β. Using primary human astrocyte cultures, we show that IL-1β selectively induces TDO and hence increases the production of KYNA. In this study, KYNA was also associated to dopamine signaling, psychotic symptoms and impaired executive functions in patients with bipolar disorder.

In patients with schizophrenia, we show that levels of CSF quinolinic acid (QUIN), produced in the other branch of the kynurenine pathway, are not elevated, but the QUIN/KYNA ratio is lowered. These findings suggest an imbalance in the upregulated kynurenine pathway in schizophrenia.

Further tryptophan, along with the kynurenine metabolites KYNA and QUIN, the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), the dopamine metabolite homovanillic acid (HVA), and the cytokines IL-6, IL-8 and tumor necrosis factor (TNF)-α were investigated in the CSF of a cohort of twins with various psychiatric morbidity. Data presented here support a considerable environmental contribution to the levels of these factors. Associations between CSF levels of KYNA and levels of HVA as well as 5-HIAA were observed, as were associations between these CSF metabolites and psychopathology measures, extended to enlace schizotypal traits also present in relatives of patients with psychotic disorders.

Finally, we investigated if dermal *ex vivo* fibroblasts could serve as a model for studying the kynurenine pathway. All pathway enzymes were indeed expressed by the fibroblasts. KYNA was produced following stimulation with typical pathway inducing cytokines, i.e. interferon-γ and TNF-α, hence indicating the possibility to develop a model of easily accessible cells for studies of molecular and genetic aspects of psychotic disorders.

Taken together, the data presented in this thesis strengthens the position of KYNA as a highly interesting target for further studies of psychotic disorders as well as for future therapeutic interventions. These data do not only confirm prior studies implicating a role for KYNA in psychotic disorders, but contributes with in-depth knowledge of potential molecular mechanisms linking a genetic makeup to aberrant immune signaling, CSF KYNA levels, psychotic symptoms and impaired cognitive function in patients with psychotic disorders.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

ADE	Affective Disorder Evaluation
AhR	aryl hydrocarbon receptor
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
APC	antigen presenting cell
AUDIT	Alcohol Use Disorders Identification Test
α7nACh	α7 nicotinic acetylcholine
BA	brodmann area
BBB	blood-brain barrier
BD	bipolar disorder
BMI	body mass index
BPRS	Brief Psychiatric Rating Scale
BSA	bovine serum albumin
CASP8	caspase 8 human gene
CD	cluster of differentiation
c-FLIP	cellular FLICE-like inhibitory protein
CI	confidence interval
CNS	central nervous system
CNV	copy number variation
COX	cyclooxygenase
CSF	cerebrospinal fluid
Ct	cycle threshold
CVO	circumventricular organ
DALY	disability adjusted life year
DC	dendritic cells
D-KEFS	Delis-Kaplan Executive Functioning System
DLPFC	dorsolateral prefrontal cortex
DMEM	Dulbecco's modified eagle medium
dNTP	deoxyribonucleotide triphosphate
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	DL-Dithiothreitol

DUDIT	Drug Use Disorders Identifications Test
DZ	dizygotic
EC50	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (exempli gratia lat.)
FBS	fetal bovine serum
FGF	fibroblast growth factor
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GAF	Global Assessment of Functioning
GC-MS	gas chromatography–mass spectrometry
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPR35	G protein-coupled receptor 35
GWAS	genome-wide association studies
HAAO	3-hydroxyanthranilate 3,4-dioxygenase
HAMD	Hamilton Rating Scale for Depression
HDRS	Hamilton Depression Rating Scale
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
hsCRP	high-sensitivity C-reactive protein
HVA	homovanillic acid
IC50	half maximal inhibitory concentration
IDO	indoleamine 2,3-dioxygenase
i.e.	that is (id est lat.)
IFN	interferon
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist

IR	infrared
i.c.v.	intracerebroventricular
IQR	interquartile range
KAT	kynurenine aminotransferase
kDa	kilodalton
Km	Michaelis-Menten constant
KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
KYNA	kynurenic acid
KYNU	kynureninase
LD	linkage disequilibrium
LLOD	lowest level of detection
lncRNA	long non-coding RNA
LOD	limit of detection
LPS	lipopolysaccharide
LTP	long term potentiation
MAF	minor allele frequency
MDS	multidimensional scaling
MEM	minimum essential medium
MHC	major histocompatibility complex
MIA	maternal immune activation
M.I.N.I.	Mini International Neuropsychiatric Interview
mitAAT	mitochondrial aspartate aminotransferate
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MZ	monozygotic
NaCl	sodium chloride
NAD+	nicotinamide adenine dinucleotide
NF-kB	nuclear factor-kB
NK	natural killer
NMDA	N-methyl-D-aspartate
NR	glutamate [NMDA] receptor subunit
OR	odds ratio

PAMP	pathogen-associated molecular pattern
PANSS	Positive and Negative Syndrome Scale
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
PPI	prepulse inhibition
PtdIns3P	phosphatidylinositol 3-phosphate
QPRT	quinolinate phosphoribosyltransferase
QUIN	quinolinic acid
SANS	Scale for Assessment of Negative Symptoms
SAPS	Scale for Assessment of Positive Symptoms
SCAN	Schedules for Clinical Assessment in Neuropsychiatry
SCID	Structured Clinical Interview for DSM-IV
SD	standard deviation
SEM	standard error of the mean
SMRI	Stanley Medical Research Institute
SNP	single nucleotide polymorphism
SNX	sorting nexin
SPQ-B	Schizotypal Personality Questionaire
STEP-BD	systematic treatment enhancement program for bipolar disorder
TBS	Tris buffered saline
TDO	tryptophan 2,3-dioxygenase
TLR	Toll-like receptor
TMT	Trail Making Test
TNF	tumor necrosis factor
TR	text revision
TRP	tryptophan
wt	wild-type
YMRS	Young Mania Rating Scale
3-HK	3-hydroxykynurenine
3-HAA	3-hydroxyanthranilic acid
5-HIAA	5-hydroxyindoleacetic acid

1 INTRODUCTION

Mental sickness has been, and still is, one of the most frightful and stigmatizing experiences a person can go through. Even in today's modern illuminated western society, people with mental illness are met by prejudice and fear, reactions that are normal and very humane when standing face to face with the different and unknown. The prejudice and fear are however not limited to the diseases per se. Few topics engage so many, and it is difficult to find a person who does not culture an opinion on the treatment of psychiatric diseases. Opinions are frequently only in the form of a cautious apprehension, but all too often there is a fierce opposition to the methods used in modern psychiatry. This opposition needs to be faced by knowledge; a knowledge that can only spring out of research and evidence-based medical approaches, made available to the society.

1.1 SCHIZOPHRENIA

Even though descriptions of madness are as old as the written language, the German psychiatrist Emil Kraepelin was the first to describe the congregation of symptoms that later was to become known as schizophrenia (Kraepelin, 1919), portraying a disorder characterized by loss of reality perception. Since then, thousands of research reports regarding the symptomatology, etiology and molecular neurobiology have been added to this first report of the syndrome, but the disorder remains enigmatic. In the absence of biological markers, schizophrenia is defined by an observable clinical syndrome, comprising psychotic symptoms, social and emotional dissociation and diminished cognitive abilities. The symptoms can be classified into three rather distinct clusters (Andreasen and Olsen, 1982); Positive, i.e. psychotic symptoms, negative symptoms involving deficits in social and emotional functioning, and cognitive impairment. Positive and negative symptoms often follow different courses over time (Eaton et al., 1995), respond differently to pharmacological treatment and may have different etiologies. Positive symptoms often respond well to treatment with antipsychotic medication, are associated with good premorbid adjustment, higher educational level and later age at onset. Negative symptoms are more difficult to treat and often persist despite treatment, are associated with poor premorbid adjustment, lower educational level, and more pronounced cognitive impairment (Andreasen et al., 1990). It is often

the negative and cognitive aspects of the disorder presenting a challenge for patients to manage everyday tasks (Green, 1996).

The global lifetime prevalence of schizophrenia has been reported to be approximately 0.4-0.9%, with newer revisions tending to show lower estimates (McGrath et al., 2008; Perälä et al., 2007; Saha et al., 2005). Heredity estimates reach 80-85% (Cardno and Gottesman, 2000). No difference in prevalence between developed and developing countries (World Health Organization, 1979), nor between men and women (Murray and Os, 1998) exists.

Patients with schizophrenia suffer from excess morbidity and die 12-15 years earlier than the general population (Crump et al., 2013a). A recent review assessed suicide rates to 5%, although estimates as high as 20% has also been reported (Hor and Taylor, 2010; Osborn et al., 2008). The main reason for the increased mortality is however the presence of concomitant somatic illness, mainly cardiovascular (Crump et al., 2013b; Schoepf et al., 2014). A vast need for healthcare recourses, in combination with the fact that a large proportion of patients will face social exclusion and unemployment throughout their lives, produces substantial societal costs, both in Sweden and worldwide, seldom fully appreciated by decision makers (Ekman et al., 2013; Knapp et al., 2004). These devastating consequences make schizophrenia rank among the top ten causes of disability-adjusted life years (DALY's) in the world (Lopez and Murray, 1998; Rössler and Salize, 2005).

The onset of schizophrenia generally occurs during late adolescence or early adult life and patients typically come to the attention of mental health care during their first psychotic episode. The psychosis is however often preceded by a period of prodromal symptoms that can be present for years before the psychotic outbreak (Yung and McGorry, 1996). The presence of subtle symptoms already during the first years of life in some patients who later develop schizophrenia is indicative of schizophrenia being a neurodevelopmental disorder (Marenco and Weinberger, 2000; Weinberger, 1988).

Several studies claim that there is a continuum of schizophrenia-like symptoms in the population, ranging from sub-clinical personality traits, through the schizotypal personality disorders and with manifest schizophrenia and psychotic bipolar disorder at the far end of the spectrum (See Siever and Davis, 2004 for review). Milder phenotypes

of the continuum can be present as sub-clinical traits in unaffected relatives of patients with schizophrenia and offers a valuable opportunity to study the pathophysiology of these particular traits, unbiased by the many factors, including medication, a chronic schizophrenia diagnosis presents.

Two main theories of the pathophysiology of schizophrenia are widely acknowledged. The dopamine hypothesis, first formulated in 1966 and based on the pioneering work of Arvid Carlsson (Carlsson and Lindqvist, 1963; Van Rossum, 1966), claims that dopaminergic hyperactivity is responsible for the symptoms observed in schizophrenia. The initial dopamine hypothesis was focusing on increased dopaminergic transmission in subcortical regions but could not explain the presence of negative symptoms and cognitive dysfunctions in schizophrenia. During the last decades of the 20th century it was hypothesized that altered functions of the dopamine system in the prefrontal cortex (PFC) could account for the negative and cognitive domains in schizophrenia (Knable and Weinberger, 1997). It became increasingly apparent that dopamine transmission through dopamine 1 (D1) receptors was crucial for normal prefrontal functions (Goldman-Rakic et al., 2000), and the dopamine hypothesis was expanded to include a prefrontal dopaminergic hypofunction, in addition to the increased subcortical transmission (Davis et al., 1991). The glutamate deficiency theory was born with the report by (Kim et al., 1980) of low cerebrospinal fluid (CSF) levels of glutamate in patients with schizophrenia and in similarity to the dopamine hypothesis it was backed up by a range of pharmacological evidence involving the glutamatergic N-methyl D-aspartate (NMDA) receptor. For example, synthetic NMDA receptor antagonists like ketamine produce psychotic symptoms often indistinguishable from schizophrenia in healthy individuals (Allen et al., 1978), and worsen symptoms in patients with schizophrenia (Malhotra et al., 1997). Likely, these theories mirror some of the extremely complex pathological processes leading to the development of schizophrenia.

In addition, and congruent with these well-established hypothesis, a great number of risk factors of both environmental and genetic origin have been associated with the development of schizophrenia, and likely the disorder is a result of the combination of the two.

1.2 BIPOLAR DISORDER

Previously known as manic-depressive disorder, bipolar disorder, as the name implies, is a disorder of affective fluctuations and is increasingly being viewed as a continuum of mood symptoms. Symptoms can range from mild depression and brief hypomania, as present in cyclothymia, through bipolar disorder II, characterized by depressive and hypomanic episodes, to the most severe and incapacitating bipolar disorder I. Bipolar disorder I is characterized by severe manic and depressive episodes, and may in extreme cases present with predominantly mania and psychosis (Müller-Oerlinghausen et al., 2002). About 50% of patients experience psychotic symptoms, frequently being grandiose delusions (Dunayevich and Keck, 2000). The psychotic states may however involve all types of psychosis, including mood-incongruent, bizarre and first rank symptoms as well as catatonia and formal thought disorder, symptoms previously believed to be specific to schizophrenia (Pope and Lipinski, 1978). The psychotic symptoms, also common in bipolar disorder II (Mazzarini et al., 2010), may appear during manic as well as during depressive episodes. Patients can also experience mixed episodes where symptoms of mania and depression are present simultaneously. Patients typically spend a larger proportion of their life in depressive, rather than hypomanic or manic states. (Phillips and Kupfer, 2013). Between the manic and depressive episodes, most patients experience a neutral mood, referred to as the euthymic state. People with bipolar disorder also suffer from cognitive impairment, in particular deficits in executive function, sustained attention, and verbal memory and learning are observed. Few studies have investigated premorbid cognitive function in patients who later develop bipolar disorder. The cognitive impairment is evident from the onset of the disorder, present also during euthymic states and likely cognition deteriorates over time (Ferrier et al., 1999; Martínez-Arán et al., 2004; Quraishi and Frangou, 2002; Robinson et al., 2006). Impairment in investigated cognitive domains often worsen during mood episodes, in particular manic, and has been shown to be associated with duration of illness, number of episodes, and number of hospital admissions (Cavanagh, 2002; Clark, 2002; Denicoff et al., 1999; Fossati et al., 2004).

The lifetime prevalence has been estimated to 0.6% for bipolar disorder I and 0.4% for bipolar disorder II. The age of onset is usually in the late adolescent, early adult period with a mean age of onset at 18 and 20 years for bipolar disorder I and II,

respectively (Merikangas et al., 2011). Gender, race and ethnicity does not impact the prevalence rates (Merikangas et al., 2011; Weissman et al., 2014). As opposed to the more severe bipolar disorder I with severe manic and depressive states critically impairing normal function, the other diagnostic subtypes have tended to be under-, or misdiagnosed. It has been shown that approximately 30% of patients receiving mood-stabilizing treatment do not have a bipolar diagnosis (Angst et al., 2010; Phillips and Kupfer, 2013).

Bipolar disorder heritability rates are assessed to 60-85% (Smoller and Finn, 2003).
Suicide is a common cause of death, with rates for completed suicide being 15 for males and 22 for females and the general mortality rates in patients with bipolar disorder are 2.5 times higher than in the general population (Osby et al., 2001). Despite the hardships of a serious mental illness, patients with bipolar disorder are often overrepresented in creative professions (Kyaga et al., 2011; Redfield Jamison and Freeman, 1993).

In contrast to schizophrenia, only a few environmental risk factors for bipolar disorder have been identified. In a study investigating a number of known schizophrenia risk factors in a bipolar cohort, the only factor shown to increase the risk of attaining the disorder was early parental loss, in particular maternal (Mortensen et al., 2003). Moreover, low social support is also associated with several features of the disorder, including greater risk for relapse and a worse course of the disorder, and stressful life events are known triggers of both the onset of bipolar disorder and subsequent affective episodes (Alloy et al., 2005).

There is evidence for a dopaminergic component also in mania, presented by the fact that dopamine-releasing drugs can induce also symptoms of mania and that dopamine-blocking antipsychotics are effective in treating mania. In similarity with the dopamine hypothesis of schizophrenia, mesolimbic dopamine hyperactivity is likely involved in the psychotic states in bipolar disorder (Cousins et al., 2009). Also glutamate dysregulation is likely contributing to symptoms of bipolar disorder, and both lithium and a number of antidepressants have been shown to modify glutamatergic signaling through NMDA receptors (Rapoport et al., 2009).

1.3 SIMILARITIES AND DIFFERENCES BETWEEN SCHIZOPHRENIA AND BIPOLAR DISORDER

Even if diagnostic systems offer easily distinguishable diagnostic criteria for schizophrenia and bipolar disorder, the distinction is not as obvious in the clinical reality, as schizophrenia and bipolar disorder share many features. Both strike at the sensitive period of adolescence or early adulthood, and present a lifelong and episodic disease course where both onset and subsequent episodes are often triggered by adverse life events (Alloy et al., 2005; Ventura et al., 1989). Psychotic symptoms in bipolar disorder might be hard to distinguish from schizophrenia and both mania and psychosis respond to dopamine blockade, indicating that dopaminergic hyperactivity is also underlying manic symptoms (Cousins et al., 2009). Conversely patients with schizophrenia often present with depression, specifically during the prodromal phase and first episode (Häfner et al., 1999) and it has been suggested that negative symptoms are a distinguishable entity also in depression (Winograd-Gurvich et al., 2006).

Parts of the genetic basis of schizophrenia are shared with bipolar disorder. In a large twin study, it was assessed that the monozygotic (MZ) co-twins of probands with schizophrenia had increased risks of schizophrenia (40.8%), as well as mania (8.2%) while the MZ co-twins of manic probands had increased risk of mania (36.4%) as well as schizophrenia (13.6%) (Cardno et al., 2002). Also, molecular genetic studies report a substantial overlap in susceptibility genes to both of the disorders (Lichtenstein et al., 2009; Purcell et al., 2009).

There is however a number of fundamental differences pointing to the fact that even though the disorders may share certain neurophysiological qualities they are not merely disorders on the same continuous scale differing only in severity. As already mentioned, bipolar disorder is not associated with the array of environmental risk factors that haunts schizophrenia (Mortensen et al., 2003). In contrast to patients with schizophrenia who often display a long period of reduced premorbid functioning before the psychotic break, patients with bipolar disorder are often high achievers prior to disease onset (Goldberg, 1999). The low premorbid functioning in schizophrenia is often reflected in structural and functional brain abnormalities observed in high-risk individuals who later develop schizophrenia, and becomes progressively worse during

the course of the disorder (Pantelis et al., 2003, 2005; Wright et al., 2000). The most commonly observed structural anomalies in schizophrenia are reduced cerebral volume, reduced gray matter in the medial temporal lobe, including amygdala and hippocampus, and enlarged ventricles (Honea et al., 2005; Wright et al., 2000). The research field is more ambiguous when it comes to structural abnormalities in bipolar disorder, and both positive and negative reports and meta-analyses have been published (McDonald et al., 2004; Selvaraj et al., 2012). This difference might also be reflected in the difference in cognitive impairment between the two disorders, where studies consistently report milder cognitive deficits in patients with bipolar disorder compared to schizophrenia. The only cognitive domains frequently reported to be impaired in bipolar disorder are deficits in verbal memory and executive function (Goldberg et al., 1993; Martínez-Arán et al., 2004). Having the shared genetic basis of psychosis and the differences in cognition and brain structures in mind, it has been suggested that schizophrenia arises due to a genetic vulnerability in combination with neurodevelopmental insults, while bipolar disorder does not (Murray et al., 2004).

1.4 THE KYNURENINE PATHWAY

Kynurenic acid, as the name implies, was discovered in canine urine in 1853 by Justus von Liebig (Liebig, 1853). However, it was not recognized as a metabolite of the essential amino acid tryptophan until 50 years later (Ellinger, 1904). The other metabolites in what was later termed the kynurenine pathway were subsequently identified, and it was found that the kynurenine pathway accounts for ~95% of the human tryptophan metabolism (Wolf, 1974).

The entry of tryptophan into the kynurenine pathway is guarded by the two rate-limiting enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO; Figure 1).

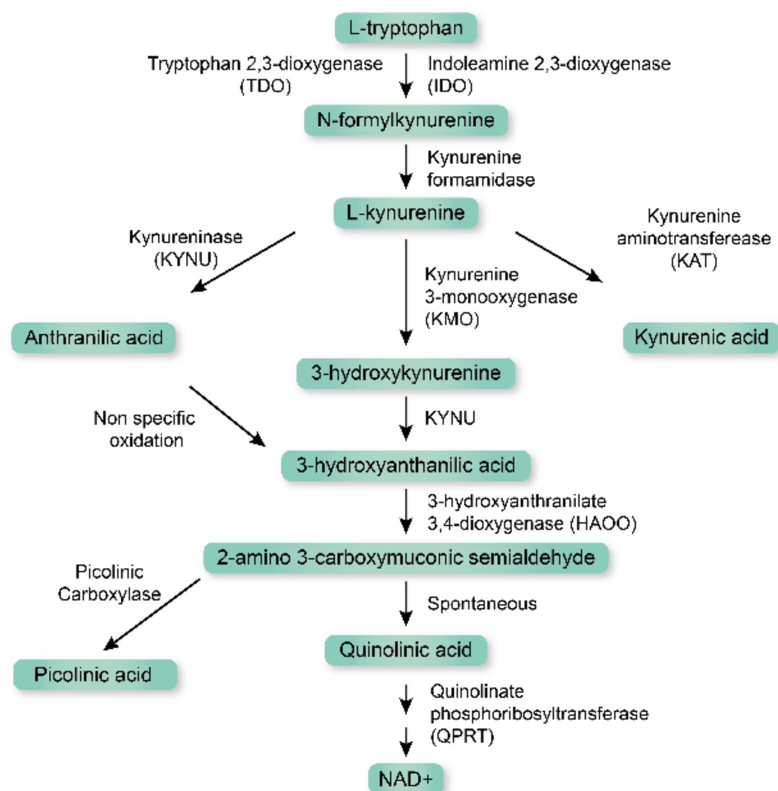

Figure 1. The kynurenine pathway

Following hydroxylation of tryptophan by IDO or TDO to form N-formylkynurenine, it is further metabolized to kynurenine by the action of kynurenine formamidase (Mehler and Knox, 1950). Kynurenine, the pivotal compound of the pathway, can then follow three separate paths; i) It can be metabolized to form KYNA through the actions of one of the kynurenine aminotransferase (KAT) enzymes (KAT 1, KAT 2, KAT 3 or mitAAT). ii) Form anthranilic acid through the action of kynureninase (KYNU). iii) Form 3-hydroxykynurenine (3-HK), through the action of kynurenine 3-monooxygenase (KMO; Moroni, 1999).

KYNA is an end-metabolite, while anthranilic acid and 3-HK are further metabolized to 3-hydroxyanthranilic acid (3-HAA) by a non-specific oxidation or KYNU respectively. 3-HAA is then converted to 2-amino-3-carboxymuconic acid semialdehyde by 3-hydroxyanthranilate 3,4-dioxygenase and then further converted to quinolinic acid (QUIN) by a nonenzymatic process. Finally, QUIN is metabolized by quinolinate phosphoribosyltransferase (QPRT) and incorporated into nicotinamide adenine dinucleotide (NAD+; Guidetti et al., 1995; Guillemin et al., 2001a, 2005a, 2007; Moroni, 1999; Stone, 1993).

Synthesis of the two principal metabolites of the kynurenine pathway, KYNA and QUIN, are spatially separated in the brain. KAT enzymes are preferentially expressed in astrocytes, which do not express KMO, thereby making astrocytes the main KYNA producing cell type in the brain (Guidetti et al., 2007; Guillemin et al., 2001a). KMO and KYNU, on the other hand, are primarily expressed by microglia and macrophages, which express only minute amounts of KAT, and QUIN is, therefore, mainly produced in these cells (Lehrmann et al., 2001).

KYNA does not easily pass the blood brain barrier (BBB; Fukui et al., 1991), but is removed from the central nervous system (CNS) by a probenecid sensitive transport mechanism (Moroni et al., 1988) and further eliminated by urinary excretion (Turski and Schwarcz, 1988). It is also suggested that astrocytes can take up and eliminate QUIN by means of the enzyme QPRT that is highly expressed in these cells (Guillemin et al., 2001a). In addition to the main metabolites KYNA and QUIN, the metabolites 3-HAA and 3-HK also have neuroactive properties.

1.4.1 Neurochemical properties of kynurenines

KYNA is the only known endogenous metabolite to antagonize *N*-methyl D-aspartate (NMDA) receptors. At low micromolar concentrations (IC50 ≈ 8-15 μM) it competitively blocks the strychnine-insensitive glycine site of the NMDA receptor (Birch et al., 1988; Kessler et al., 1989; Parsons et al., 1997). At higher concentrations (IC50 ≈ 0.2-0.5 mM) it antagonizes the glutamate recognition site of the NMDA receptor. In even higher concentrations in the millimolar range, it also competitively antagonizes glutamatergic receptors of the AMPA and kainate types (Kessler et al., 1989), however the physiological relevance of this is not clear. At low concentrations (IC50 ≈ 7 μM), KYNA non-competitively antagonizes the α7 nicotinic acetylcholine (α7nACh) receptor (Hilmas et al., 2001). Recently KYNA was also proposed as an endogenous agonist of the GPR35 receptor (Wang et al., 2006), and the finding that KYNA is a ligand of the aryl hydrocarbon receptor (AhR; DiNatale et al., 2010) has spurred research of the actions of KYNA through the AhR in the field of inflammatory research. In addition to its receptor-mediated actions, KYNA might be a free radical scavenger and an endogenous antioxidant (Lugo-Huitrón et al., 2011).

As opposed to KYNA, QUIN is an excitatory neurotoxin, mediating its effects through its agonistic actions on the NMDA receptor (IC50 ≈180μM; Stone, 1993), and has also been described as a free radical generator (Rios and Santamaria, 1991), a feature it shares with 3-HK (Eastman and Guilarte, 1989). Both 3-HK and 3-HAA also generate superoxide and hydrogen peroxide (Goldstein et al., 2000). Moreover, QUIN was recently shown to stimulate the release of glutamate from neurons, inhibit glutamate uptake into astrocytes and inhibit astrocytic glutamine synthetase (Tavares et al., 2000, 2002; Ting et al., 2009), and its actions are further investigated in a number of different contexts (see Guillemin, 2012 for review).

1.4.2 Regulation of the kynurenine pathway

The first point for regulation of the kynurenine pathway is the initial and rate-limiting step involving IDO and TDO. These two enzymes have different cellular distribution and respond to different stimuli (Batabyal and Yeh, 2007). IDO is mainly present in certain immune cells, such as dendritic cells and macrophages (Moffett and Namboodiri, 2003), intestine, lung, placenta and brain (Stone, 1993), and it is generally accepted that it is activated by immune stimuli such as cytokines (King and Thomas, 2007). TDO has long been recognized as a predominantly hepatic enzyme; however, it is now known to be also expressed in the CNS (Guillemin et al., 2007; Kanai et al., 2009, 2010; Ohira et al., 2010). Hepatic TDO is chiefly regulated by levels of tryptophan and glucocorticoids (Stone, 1993).

Since the main KYNA producing enzymes, KAT 1 and KAT 2 have Michaelis Menten constants (*Km*) in the millimolar range (Guidetti et al., 1997; Okuno et al., 1991), the production of KYNA is mainly governed by the availability of the immediate precursor, kynurenine (Schwarcz and Pellicciari, 2002). The production of KYNA is hereby critically dependent on the activity of IDO and TDO. This notion is supported by experiments where kynurenine is administered systemically to both rats and primates, giving rise to elevated levels of KYNA (Jauch et al., 1993; Swartz et al., 1990; Wu et al., 1992). Glutamine and phenylalanine are also competitive substrates for KAT 1 and KAT 2, and therefore the intracellular concentrations of these amino acids, may indirectly affect the rate of KYNA formation (Chang et al., 1997). KMO, converting kynurenine to 3-HK has a *Km*≈20 μM (Bender and McCreanor, 1985) and

can be considered a critical enzyme also for KYNA formation, in the sense that a lower activity of this enzyme will accumulate kynurenine that in turn will be processed by the KAT enzymes. This is likely the scenario under physiological conditions, as well as in psychiatric disorders such as schizophrenia, where KYNA, but not 3-HK and QUIN are found elevated (Schwarcz et al., 1988). Increased activity of KMO has been observed in a mouse model of Huntington's disease, likely explaining the elevated levels of QUIN and 3-HK in the early stages of this disorder.

A number of approaches are frequently employed to increase the brain concentration of KYNA in an experimental setting. Drugs inhibiting KMO will indirectly increase the production of KYNA through increased kynurenine availability (Russi et al., 1992; Speciale et al., 1996). As previously mentioned, it is also possible to administer the immediate precursor kynurenine, which readily passes the BBB, and can, therefore, be administered systemically. Administering probenecid impairs the transport of KYNA from the brain and thereby also increases the central concentration (Moroni et al., 1988).

1.4.3 Regulation of the kynurenine pathway by immunological stimuli

The research of a link between the immune system and the kynurenine pathway has been around since the finding that IDO is induced by lipopolysaccharide (LPS; Yoshida and Hayaishi, 1978) and evidence of this connection are presently mounting. It was soon recognized that the relation between infection and activation of the kynurenine pathway is mediated by cytokines, molecules coordinating the immune response to infection and other inflammatory stimuli (Yoshida et al., 1981).

The pro-inflammatory cytokine interferon-γ (IFN-γ) is considered the principal activator of IDO and thus the kynurenine pathway (See Taylor and Feng, 1991, for review). Other cytokines, such as tumor necrosis factor (TNF) and interleukin (IL)-1 has however been shown to act synergistically with IFN-γ to induce IDO enzyme activity *in vitro* (Babcock and Carlin, 2000; Robinson et al., 2003), or to activate IDO independent of IFN-γ (Fujigaki et al., 2006; Guillemin et al., 2001b). IFN-γ has the capacity to induce the mRNA expression of KAT-I and KAT-II in human astrocytes *in vitro* (Guillemin et al., 2001a), but also give rise to elevated levels of QUIN in the

mouse brain (Saito et al., 1991, 1992). A neurotropic influenza virus administered to neonatal mice, activates several enzymes of the kynurenine pathway, including both IDO and the QUIN branch enzymes KMO, KYNU, and HAOO, and give rise to a transient increase in KYNA levels (Asp et al., 2010; Holtze et al., 2008). Studies on macaques show elevated levels of both KYNA and QUIN in macaques infected with the simian immunodeficiency virus (Heyes et al., 1992a, 1998) and elevated levels of QUIN following infection with polio (Heyes et al., 1992b). Several studies have investigated the effect of the parasite Toxoplasma gondii on the kynurenine pathway. For example, mice infected with this parasite show induction of IDO, and elevated levels of kynurenine and QUIN, an effect likely mediated through IFN-γ (Fujigaki et al., 2002; Notarangelo et al., 2014; Silva et al., 2002).

As opposed to IDO, TDO was initially not considered to be regulated by immunological stimuli (Heyes et al., 1998; Saito et al., 1992, 1993). Later, however, increased expression of TDO mRNA was observed in human placenta following bacterial intra-uterine infection, as well as in placental cells following LPS stimulation (Manuelpillai et al., 2003, 2005). Another study reported on antimicrobial and immunoregulatory effects of TDO in an inducible TDO expressing cell system (Schmidt et al., 2009).

In clinical settings, it has also been observed that the kynurenine pathway is frequently activated during infections, as well as during inflammatory conditions of the CNS (Heyes et al., 1992c). Elevated levels of KYNA have been found in patients with tick borne encephalitis (Holtze et al., 2012a) herpes simplex virus type 1 encephalitis (Atlas et al., 2013), cerebral malaria, (Dobbie et al., 2000; Medana et al., 2003) and HIV-1, where also kynurenine and QUIN are elevated (Achim et al., 1993; Atlas et al., 2007; Baran et al., 2000; Heyes et al., 1991, 1998, 2001). Elevated levels of QUIN have also been observed in Lyme disease (Halperin and Heyes, 1992). In addition, immunotherapy with IFN-α has been found to activate the kynurenine pathway (Raison et al., 2010).

1.4.4 Physiological significance of the kynurenine pathway

Since the discovery that intracerebroventricular (i.c.v) injection of QUIN causes seizures in mice, (Lapin, 1978), and subsequently the discovery that KYNA is synthesized in the brain (Moroni et al., 1988; Turski et al., 1988), the physiological relevance of central kynurenines have gained increased attention. Since the concentration of both QUIN and KYNA are found in the rodent brain in concentrations far below the levels needed to exert an action on the different receptors *in vitro* (see 1.2.2), it has been an issue of controversy whether metabolites of the kynurenine pathway exert any physiological actions in the CNS. There is however a number of rationalizations to the apparent mechanistic gap between observed effects on one hand and brain concentrations and receptor efficacy on the other hand.

During the late 1990's, it became increasingly apparent that astrocytes actively contribute to synaptic transmission. Astrocytes tightly engulf the synapse in an interaction termed "the tripartite synapse" (Araque et al., 1999), where they can respond to released neurotransmitters by releasing factors of their own, thereby modulating the synaptic transmission (See Araque et al., 2014 for review). Since astrocytes are the prime source of KYNA the concentration at the synaptic cleft in all probability reaches the required levels to block NMDA and α7nACh receptors.

The often-dramatic effects of experimentally administered QUIN in the CNS are, on the other hand, attributed to a combination of factors. Firstly, QUIN seem to lack efficient removal mechanisms in the CNS (Foster et al., 1984). Secondly, the excitotoxic actions through NMDA receptors are likely potentiated by the simultaneous generation of free radicals (Behan and Stone, 2002). QUIN selectively activates NR2A and NR2B subtypes of the NMDA receptor, predominantly present in the forebrain (Prado De Carvalho et al., 1996).

Although the complexity of the interactions between kynurenines and neuronal circuits is just beginning to dawn on us, it is beyond doubt that they play a role in human physiology and pathology. During the last decades, the interest in the role of the kynurenine pathway in a range of different medical conditions has almost exploded, hence opening up the arena for new insights.

The impact of kynurenines in neurodegenerative disorders has been studied for decades. KYNA has been reported elevated in Alzheimer's disease (Baran et al., 1999), and in amyotrophic lateral sclerosis (ALS; Iłzecka et al., 2003). However, the kynurenine pathway metabolite most often discussed in relation to neurological and neurodegenerative disorders is the NMDA receptor agonist QUIN, shown to act as a neurotoxin in the CNS (Schwarcz et al., 1983; Stone and Perkins, 1981). QUIN has been most thoroughly investigated in Huntington's disease, where QUIN levels are elevated in the initial stages of the disease, especially within the most affected brain regions (Guidetti et al., 2004). This increase in QUIN is matched by a similar increase in 3-HK levels (Guidetti et al., 2004; Pearson and Reynolds, 1992), and it is likely that the simultaneous actions of these two compounds work in a synergistic fashion (Guidetti and Schwarcz, 1999). Also in Alzheimer both 3-HK and QUIN are found in higher concentrations, and QUIN immunoreactivity is found mainly in association with amyloid plaques (Bonda et al., 2010; Guillemin et al., 2005b). Pathological changes in Parkinson and ALS are likely also under the influence of kynurenine metabolism (Chen et al., 2010; Zinger et al., 2011)

It is conceivable that the elevated levels of KYNA sometimes observed in patients with neurodegenerative disorders might be a consequence of the elevated levels of QUIN, where the synthesis of KYNA is increased in order to counteract the neurotoxic effects of centrally elevated QUIN. This assumption is supported by several studies showing that KYNA has neuroprotective properties, likely mediated through the antagonizing effect on NMDA receptors, and possibly also α7ACh receptors (Foster et al., 1984; Miranda et al., 1997; Sapko et al., 2006; Stone, 1993; Zwilling et al., 2011; Atlas et al., 2013). In patients with HIV-1, serving as the most severe prototype of a viral disease, CNS QUIN and 3-HK are also elevated, too a much higher degree than the previously mentioned increase in KYNA in these patients (Heyes et al., 1990, 1991, 1992c).

Not only is the kynurenine pathway activated by immunological stimuli (see 1.4.3), but it has also become increasingly apparent that it takes part in a number of immunological processes. Already during the early 1980's it was noted that tryptophan degradation accompany immune activation (Pfefferkorn, 1984), and it was generally believed that the observed antimicrobial effect was a result of the removal of the essential nutrient tryptophan from the pathogen, thus inhibiting its growth. Several studies since have noted that IDO activation contributes to the development of

tolerance by inhibiting T-cell responses (Mándi and Vécsei, 2012; Mellor and Munn, 2004). IDO has been shown to mediate tolerance to tumors (Munn and Mellor, 2007), preventing fetal rejection (Munn et al., 1998), and has been implicated in mediating protection against autoimmunity (Fallarino et al., 2009; Grohmann et al., 2003; Saxena et al., 2007; Yan et al., 2010). At least some of these processes are plausibly mediated by the metabolites downstream of IDO. Kynurenine has been described to inhibit natural killer (NK)-cell responses (Della Chiesa et al., 2006), and kynurenine, 3-HK and 3-hydroxyanthranilic acid has been shown to suppress T-cell responses (Terness et al., 2002).

KYNA has also been shown to display immunogenic properties. For example, it attenuates the production of TNF-α in mononuclear cells, an effect believed to be mediated through its binding to the GPR-35 receptor (Wang et al., 2006), and it also inhibits the production of CD14+ peripheral blood monocytes (Tiszlavicz et al., 2011). Inhibition of TNF-α production by KYNA may be contributing to its neuroprotective effects (Mándi and Vécsei, 2012).

As a consequence of the realization of the role the kynurenine pathway plays in immunological and tolerogenic processes, kynurenine pathway metabolites are now also being investigated in a multitude of conditions, such as inflammatory pain certain autoimmune disorders, and cancer.

1.4.5 The kynurenine pathway in psychiatric disorders

It was early recognized that tryptophan metabolism might impact psychiatric disorders. As early as in the 1970's, the connection between tryptophan metabolism and depression was documented (Curzon and Bridges, 1970; Lapin, 1973). The focus of depression research however soon shifted from kynurenines to serotonin, as the use of serotonin reuptake inhibitors became a full-blown success story. Nonetheless, as the serotonin hypothesis of depression have failed to explain many of the observed features of the disorder as well as the slow onset of the treatment effects of serotonin reuptake inhibitors, interest has shifted back to kynurenines in recent years. Increased breakdown of tryptophan has been observed in depressed patients (Maes et al., 2000), as well as in patients receiving IFN-α treatment who subsequently develop depression

(Capuron et al., 2003). IDO activation by the immune system is a central finding in experimental models connected to the development of depressive-like behavior that temporally occurs when the sickness behavior per se is waning (Dantzer et al., 2008). It is however less clear how the increased activity of the kynurenine pathway can trigger depressive behavior, and which metabolites play a role in the pathological mechanisms. A plausible scenario is that a dysregulation of the pathway, favoring production of neurotoxic metabolites such as 3-HK and QUIN, is responsible for the symptoms and observed loss in hippocampal volume in depression (Müller and Schwarz, 2007; Sheline et al., 1996). Increased QUIN immunoreactivity and decreased astrocyte density is found in post mortem brains of patients with depression (Si et al., 2004; Steiner et al., 2011). In two recently published studies on suicide, of which approximately half the patients suffered from depression CSF QUIN was elevated while CSF KYNA was not. In one study QUIN correlated to ratings on the suicide intent scale (Erhardt et al., 2013) and the other study reported an association between low levels of KYNA and severity of depression, as well as ratings on the suicide assessment scale (Bay-Richter et al., 2014). These studies are indicative of an NMDA mediated mechanism of depressive symptoms, where lower levels of KYNA could augment the vulnerability of, in particular hippocampal, neurons to the neurotoxic effects of NMDA receptor over-activation by QUIN. This is also in line with the findings that NMDA receptor antagonists such as ketamine produce a rapid and long lasting antidepressant effect in depressed patients (Berman et al., 2000). The complexity of the situation is however illustrated by the finding that in patients receiving IFN-α treatment for chronic hepatitis or cancer, both QUIN and KYNA are equally elevated in the CSF (Raison et al., 2010). Depression following IFN-α treatment is however a specific subtype of induced depression, and this finding might not be representative for the entire group of patients suffering from endogenous depression.

Findings of an abnormal tryptophan metabolism in schizophrenia date back more than 50 years. Following a tryptophan load, patients with schizophrenia were observed to excrete 2.4 times the amount of tryptophan metabolites as normal controls (Benassi et al., 1961). The discovery of KYNA in the human brain (Moroni et al., 1988; Turski et al., 1988) spurred further research on the link between tryptophan metabolism and schizophrenia, and KYNA, was soon found in elevated concentrations in the brain and CSF of patients with schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001). The

metabolite in focus since the initial finding has been KYNA, and numerous studies confirm these findings of elevated levels of both KYNA, and more recently, its precursor kynurenine in the CNS of patients with schizophrenia (Erhardt et al., 2001a; Linderholm et al., 2012; Miller et al., 2006; Nilsson et al., 2005; Sathyasaikumar et al., 2011; Schwarcz et al., 2001). Importantly, increased expression of TDO, but not IDO has also been observed in the brains of patients with schizophrenia (Miller et al., 2004, 2006). It was confirmed that the elevated levels of KYNA in patients with schizophrenia was not a medication effect in a study where both medicated and drug-naïve patients had higher levels than healthy controls (Nilsson et al., 2005). Elevated CSF levels of KYNA, and brain levels of kynurenine, has also been observed in bipolar disorder, where the highest levels were observed in the group with psychotic features (Miller et al., 2006; Olsson, 2010; Olsson et al., 2012a). Further supporting a role for KYNA specifically in psychosis, are the findings that KYNA is elevated in patients with HIV with psychotic symptoms (Atlas et al., 2007). Also, patients who are suffering anti-NMDA receptor encephalitis, a condition in which autoantibodies target brain NMDA receptors, frequently present with psychosis (Dalmau et al., 2011).

1.4.6 The kynurenic acid hypothesis of schizophrenia

Since KYNA has known neuroactive properties, being an antagonist at the glycine site of the NMDA receptor and of the α7nACh receptor, it was a natural step to start to investigate the relevance of elevated levels of this compound in experimental models.
Synthetic NMDA receptor antagonists can produce psychotic symptoms, often indistinguishable from schizophrenia, in healthy people, and aggravate symptoms in patients with schizophrenia (Allen et al., 1978; Malhotra et al., 1997). Synthetic NMDA antagonist has also been shown to impact the activity of midbrain dopamine neurons in rats (French, 1994; French et al., 1993).

In line with this, endogenous KYNA has been shown to tonically modulate firing of rat midbrain dopaminergic neurons, with elevated levels of KYNA leading to increased firing (Erhardt and Engberg, 2002; Erhardt et al., 2001b; Linderholm et al., 2007; Nilsson et al., 2006), and lower levels of KYNA decreasing the firing rate of dopamine neurons (Schwieler et al., 2006, 2008). Elevated levels of KYNA in the rat also changes the dopamine response to amphetamine (Olsson et al., 2009), in line with the fact that

patients with schizophrenia show an abnormal dopaminergic response to amphetamine compared to healthy controls (Abi-Dargham et al., 1998; Laruelle, 1998; Laruelle and Abi-Dargham, 1999). These data are indicative of an involvement of KYNA in the positive symptoms of schizophrenia.

KYNA also bi-directionally modulates the release of glutamate both in cortical areas and the hippocampus (Konradsson-Geuken et al., 2010; Pocivavsek et al., 2011), as well as the release of cortical acetylcholine (Zmarowski et al., 2009), and cortical GABA (Beggiato et al., 2014). Glutamatergic and cholinergic neurotransmission is heavily implicated in cognitive functioning. It is well-known that patients with schizophrenia frequently show impairments in working memory as well as in verbal learning and memory, domains that are considered core features of the disease (Nuechterlein et al., 2004). Behavioral experimental studies confirm the involvement of KYNA in cognitive domains. Elevated levels of KYNA are shown to have an impairing effect on spatial memory and learning as well as on social interaction (Alexander et al., 2012; Chess and Bucci, 2006; Chess et al., 2007; Pocivavsek et al., 2012; Trecartin and Bucci, 2011). Also, lowering the endogenous levels of KYNA enhances glutamate release, hippocampal plasticity and cognitive function (Potter et al., 2010). Elevated levels of KYNA also disrupts prepulse inhibition (PPI) in experimental animals (Erhardt et al., 2004). A defect in PPI, believed to reflect a deficit in sensorimotor gating, is also observed in patients with schizophrenia (Braff and Geyer, 1990).

Altogether the kynurenic acid hypothesis of schizophrenia suggests that elevated levels of KYNA cause alterations in dopaminergic, glutamatergic, GABAergic and cholinergic neurotransmission, leading to the observed symptoms in schizophrenia. By incorporating actions on these neurotransmitter systems, the kynurenic acid hypothesis is not only in line with the dopamine hypothesis and the glutamate deficiency theory of schizophrenia, but rationally bring these hypotheses together under a common umbrella.

1.5 IMMUNE PROCESSES AND NEUROINFLAMMATION

The classical inflammatory response as we know it is based on observations of the peripheral immune system and is initiated by cells of the innate immune system, reacting to pathogens or tissue damage. Activation is achieved through signaling via toll-like receptors (TLRs), pattern recognition receptors capable of recognizing pathogen-associated molecular patterns (PAMPs), leading to phagocytosis of pathogens or damaged tissue components by professional antigen presenting cells (APCs). Dendritic cells (DCs), macrophages or other APCs then present antigen in conjunction with MHC-II molecules expressed on their surface. Activation of TLRs is also coupled to an induction of a proinflammatory cascade leading to a swift release of cytokines, chemokines and other signaling molecules recruiting other types of inflammatory cells. APCs migrate to lymphatic nodes where T-cells are capable of responding to the antigen presentation by in turn releasing cytokines. Cytokines further drive the propagation of both the innate and adaptive immune responses through the proliferation and differentiation of several different T-cell subsets and antibody producing B-cells.

The brain was for the most part of the previous century considered to be an immune privileged site. Although the definition of immune privilege has been floating and imprecise, a number of factors are generally attributed to the concept. Firstly, the brain parenchyma lack functional DCs and no other cell type has been observed performing the crucial tasks of antigen uptake, migration from CNS to lymphatic nodes and presentation of antigen to T-cells. This fact constitutes the cellular basis of the immune privilege of the CNS (Ransohoff and Brown, 2012). The second factor invoked to prove CNS immune privilege is a lack of adaptive immune responses following injection of immunogens into the brain parenchyma, whilst the same immunogen is capable of inducing a rapid response in the periphery.

It is now acknowledged that the immune privilege of the CNS is not absolute. Immunological processes do occur within the CNS, in an intricate way controlling the environment of the brain in close contact with peripheral processes. The concept of neuroinflammation, born during the early 1990's, however acknowledged that CNS immune reactions are under rigid control since an inflammatory reaction, as we know it from the periphery, would have deleterious effects on neurons, and other CNS cell types with poor regenerating capacity. In addition to the BBB, often quoted as the sole

reason for the CNS immune privilege, the brain parenchyma is held in an immunosuppressed state by the release of only partially identified factors from both neurons and astrocytes (see Galea et al., 2007 for overview).

In spite of the BBB, there are a number of routes for communication between the periphery and the central immune system. Immune activation by peripheral TLRs leads to central activation of immunological mechanisms, usually producing fever and sickness behavior (Dantzer et al., 2008). Primary afferents, such as the vagal or the trigeminal nerves, constitute main sensory routes from the periphery to the brain and are known conveyors of immune signals to the CNS (Bluthé et al., 1994; Goehler et al., 2000). In addition, a humoral pathway, involving BBB endothelial cells as well as macrophage-like cells lining the circumventricular organs (CVOs), may contribute to the relay of immune signals from the periphery to brain. This route involves the activation of the NF-κB and cyclooxygenase (COX)-2 in BBB endothelial cells by peripheral IL-1β (Laflamme et al., 1999). It is also believed that phagocytic cells at the CVOs take up IL-1β from the periphery, in order to release it inside the brain where it can act on adjacent microglia (Konsman et al., 1999). Passage of cytokines into the brain at the leaky regions of the CVOs by volume diffusion has also been described (Vitkovic et al., 2000a). The cytokine signal is then believed to propagate by means of volume transmission within the brain (Agnati et al., 1995). The relative importance of a humoral route of immune signaling from periphery to brain has however been questioned (Dantzer et al., 2000), as has the presence of a saturable active transport system for cytokines such as IL-1. Such a system has been described (Banks et al., 1995, 1991, 2001), but the molecular identity of these transporters remain evasive, and it is not known if they do contribute to physiological or pathological processes.

Immunological reactions in the brain parenchyma are orchestrated by cells specific to the CNS, with mainly innate immune processes taking place. Microglia, a cell type of myeloid origin, often referred to as the resident macrophages of the brain, are considered to be the primary cell of the innate immune system in the CNS. They are normally present in what is referred to as a resting, or quiescent state, in which they nevertheless are highly active, constantly scanning their microenvironment for signs of infection or tissue damage (Nimmerjahn et al., 2005). Upon activation, microglia attain a more macrophage-like morphology, go into a proliferatory state, become motile, and capable of phagocytosis (Glezer et al., 2007). Activation of microglial immune

processes is also achieved through activation of TLRs. In the CNS, TLRs are also expressed by astrocytes and to some extent also by neurons (Dong and Benveniste, 2001; Falsig et al., 2008) and the interplay between these three cell-types is crucial for an efficient regulation of immune processes within the brain parenchyma. The complexity of the interactions between the several players in the multipartite synapse is still under intense investigation, but glial cells are increasingly being viewed as essential elements in neuronal, as well as immunological signaling (Halassa et al., 2007; Tremblay et al., 2011; Xanthos and Sandkühler, 2014)

Today the term 'neuroinflammation' is as loosely defined as its predecessor 'immune privilege' once was. The term is often applied to the release of cytokines within the brain, and in connection with diseases known to hold an inflammatory component, however it is increasingly apparent that what we call neuroinflammation is not necessarily a pathological process. Several individual cytokines are believed to be constitutively expressed in particular brain regions. IL-1β, a pleiotropic cytokine with mainly pro-inflammatory actions, has been found to contribute to long-term potentiation (LTP) in rats (Schneider et al., 1998), both *in vivo* and *in vitro*, and disrupted IL-1β signaling leads to memory deficits in rodents. Interestingly mice with no functional IL-1 receptor or infusion of IL-1 receptor antagonist (IL-1Ra; Avital et al., 2003; Yirmiya et al., 2002) show the same type of hippocampal-dependent memory deficits as transgenic animals overexpressing IL-1β (Hein et al., 2011), possibly indicating a U-shaped optimum for physiological IL-1β levels in the CNS. IL-1β, production is under tight regulation, and the final step for acquiring a functional IL-1β molecule is the proteolytic cleavage of pro-IL-1β into mature IL-1β. This process has been solely attributed to caspase-1, a member of a family of cysteine proteases mainly involved in the regulation of apoptosis (Thornberry, 1998). Recently however a role for caspase-8 in the regulation and cleavage of IL-1β has been revealed (Gringhuis et al., 2012; Maelfait et al., 2008). Caspase-8, primarily known for its actions as an initiator of the death receptor-induced extrinsic pathway to apoptosis (Dupaul-Chicoine and Saleh, 2012), has now been ascribed a number of new roles, several of which are in an immune-related context (Burguillos et al., 2011; Oberst and Green, 2011).

Also, TNF-α has been shown to be crucial for synaptic scaling in the normal brain (Stellwagen and Malenka, 2006), but can contribute to neuronal cell death if dysregulated (Allan et al., 2005; Hermann et al., 2001). These findings suggest that the

term 'immune signaling' might be a more suitable expression than 'neuroinflammation,' for observations of individual cytokines in the CNS, both in health and disease. Along this background, it was recently proposed that inflammatory reactions occur in the CNS in response to altered neuronal activity (Xanthos and Sandkühler, 2014), an idea that is in line with the many neuromodulatory actions of cytokines in the CNS (see Vitkovic et al., 2000b for review).

1.5.1 Immune system involvement in psychotic disorders

The idea that psychotic disorders are affected by immunological factors is not new. Over a century ago researchers made the connection between fever and 'madness', and in 1927 Julius Wagner-Jauregg was awarded the Nobel Prize for his pioneering work on pyrotherapy (artificial fever) in mental patients. Lately, the interest in an immunological contribution to psychotic disorders has reemerged, to a large extent due to a paradigm shift in how we view the immune system of the brain (see Müller, 2014; Watkins et al., 2014 for reviews).

Epidemiological evidence has traditionally been the strongest link to an immunological component in schizophrenia. Several of the confirmed risk factors for schizophrenia, including winter birth (Davies et al., 2003), urban living (McGrath et al., 2004), maternal infection during pregnancy (Brown and Derkits, 2010), and infection during early childhood (Dalman et al., 2008; Khandaker et al., 2012) involve an immune component, directly or indirectly. Early life exposure to immune activation is known to alter brain function later in life. In models of maternal immune activation (MIA), cytokine profiles in the brain differ in an age and region-specific manner (Garay et al., 2013). Basal neurotransmission is also altered following MIA (Winter et al., 2009) and administration of IL-1β to mice at birth changes the dopamine content of hypothalamus later in life (Kabiersch et al., 1998). Early immune activation is also capable of changing a number of behavioral parameters associated with psychotic morbidity, including deficits in PPI (Asp et al., 2010; Bitanihirwe et al., 2010; Boksa, 2010).

Epidemiological and experimental data are also supported by clinical findings of elevated levels of cytokines in patients with psychotic disorders. Numerous studies have over the years reported variable and often contradicting findings. In contrast to the

abundance of studies measuring peripheral levels of cytokines in psychotic disorders, reports on brain or CSF levels of cytokines are sparse and sample sizes generally small. Although peripheral measurements of cytokines might indicate that dysregulation of immune signaling in psychotic disorders is not confined to the CNS, studies of peripheral immune signaling are not very informative about the potential pathological processes in the brain. Brain immune events are, as previously described, radically different and relatively isolated from their peripheral counterpart, and several authors point out the fact that there is a noteworthy lack of correlation between levels of peripheral and central cytokines (de Jager et al., 2009; Lindqvist et al., 2009; Schwieler et al., 2014). Early studies of central levels of cytokines suffered from a number of serious methodological limitations, specifically in regard to inappropriate control groups and insensitive assays. The results from early studies are, therefore, contradicting and provide little value to the research of brain cytokines today. More recently, elevated levels of CSF IL-1β in first episode patients with schizophrenia has however been observed in studies from our laboratory (Söderlund et al., 2009) and chronic patients with schizophrenia were reported to have elevated CSF levels of IL-6 (Schwieler et al., 2014). Two more recent studies however also reported elevated CSF IL-6 levels in patients with schizophrenia compared to healthy controls (Garver et al., 2003; Sasayama et al., 2013). In a recent meta-analysis of cytokine alterations in schizophrenia including seven studies of CSF cytokines the only difference in CSF cytokine levels observed was decreased levels of IL-1β in patients compared to controls (Miller et al., 2011), however the author points out that the result should be cautiously interpreted in the light of the small number of patients included. A factor to consider in interpretation of these studies is the disease status of included patients. It has been suggested that cytokine profiles in CSF of first episode patients or in acute exacerbations of psychotic symptoms will differ from stable chronic states. The acute psychotic states would hence be associated with a more pro-inflammatory profile while anti-inflammatory mediators take the upper hand during resolution of psychosis (Miller et al., 2011). Post-mortem investigations of brain mRNA and protein expression of cytokines in schizophrenia constitutes a useful complement to the CSF studies, corroborating the findings of inflammatory signaling in the brain of patients with schizophrenia. Hence, IL-1Ra mRNA was found to be decreased in PFC of patients with schizophrenia (Toyooka et al., 2003) while TNF-α and TNF receptor 1 mRNA was increased (Dean et al., 2013; Paterson et al., 2006). In line with the CSF studies, IL-6 and IL-8 mRNA was also observed to be increased in the PFC of patients with

schizophrenia (Fillman et al., 2013). In this study, the elevation of IL-1β did not reach significance but mRNA expression of IL-1β was linked to density of MHC-II expressing cells in schizophrenia but not in controls (Fillman et al., 2013). In another recent report both protein and mRNA levels of IL-1β and TNF-α were elevated in the PFC of patients with schizophrenia (Rao et al., 2013).

The idea that also the pathology of bipolar disorder might be under the influence of immunological factors is of a more recent date, and thus, the reports of central levels of cytokines are limited to one CSF study and one post-mortem study. Elevated levels of IL-1β was hence observed in the CSF of patients with bipolar disorder, with the highest levels observed in patients with a lifetime history of psychosis (Söderlund et al., 2011). Increased trans-membrane TNF-α mRNA was observed in the PFC of patients with bipolar disorder, however no information of the presence of psychosis in this cohort was provided (Dean et al., 2013).

In summary, in spite of the frequently contradictory clinical findings, evidence of an immunological component in psychotic disorders is converging.

1.6 GENETIC ASPECTS OF PSYCHOTIC DISORDERS

In spite of the high heredity estimates for schizophrenia and bipolar disorder and decades of search for genes connected to the pathology of these disorders, no convincing gene candidates have emerged. The number of suggested risk genes is however constantly growing, even more so in the era of genome wide association studies (GWAS). Most of the identified candidates over the years have failed to stand replications and lack support by functional experimental studies. A number of critical factors hamper the search for genes in psychotic disorders. The frequently invoked polygenic background of psychotic disorders cannot alone explain the lack of success in the search of culprit genes. Adding evidence of strong gene-interaction effects on the development of these disorders and hypotheses that different subtypes of schizophrenia and bipolar disorder are under the influence of specific genes, presents a multi-layered complexity providing a worst-possible scenario for the search of genes related to the pathophysiology of psychotic disorders. Even though genes identified in GWAS screenings provide entirely new areas in which to focus our interest, such as the many

hits in the MHC region (Jia et al., 2012; de Jong et al., 2012), a more fruitful approach to understanding the genetics behind these complex disorders might be to dissect the array of symptoms clustered together as "schizophrenia" or "bipolar disorder" into more manageable specific phenotypes. While these clusters based on DSM diagnoses are undoubtedly clinically and administratively useful they have proved to hold little value in the search for heritable factors crucial for the development of psychotic disorders (Weinberger, 1999). Hence the endophenotype concept in psychiatry states that there are measurable components connecting the disease and the distal genotype and that these components can aid the studies of complex disorders (Gottesman and Gould, 2003). Employing this approach, biological markers that can objectively be measured, presents ideal candidates. A number of phenotypes are under investigation in this context, such as deficits in sensorimotor gating (Braff et al., 2001), eye tracking dysfunctions (Calkins et al., 2008) as well as neurocognitive deficits (Burdick et al., 2006; Cornblatt and Malhotra, 2001; Glahn et al., 2003). Also KYNA, a compound readily quantitated in the CSF and elevated in patients with schizophrenia and bipolar disorder can be considered an aspirant. This radically different way of investigating genetic foundations might, therefore, manage a task that traditional genetic approaches have failed to do.

Twin studies have provided an invaluable contribution in determining the heredity of specific psychotic disorders (Kendler, 1983), as well as in establish the extent of overlap in the genetics between closely related disorders such as schizophrenia, schizoaffective disorder and bipolar disorder (Cardno et al., 2002). Twin studies however continue to play an essential role in identifying new phenotypes and their covariates within diagnostic syndromes, defining their boundaries as well as extending the phenotype concept to include potential biological markers (Cardno and Gottesman, 2000).

2 AIMS

The overall aim of this thesis was to further explore the role of kynurenine pathway metabolites in the pathology of the main psychotic disorders schizophrenia and bipolar disorder, with a specific focus on the influence of cytokine signaling on the kynurenine pathway.

The specific aims are:

1. To investigate potential associations between CSF levels of KYNA, HVA and lifetime history of psychosis in patients with bipolar disorder.

2. To investigate the genetic variability underlying CSF levels of KYNA in patients with bipolar disorder

3. To map molecular signaling pathways mediating the genetic influence on CSF levels of KYNA

4. To investigate if IL-1β alone can induce the kynurenine pathway in human astrocytes

5. To explore potential associations between KYNA, IL-1β and set-shifting performance in patients with bipolar disorder.

6. To investigate the levels of QUIN in the CSF of patients with chronic schizophrenia, and relate these levels to the levels of CSF KYNA from the same patients.

7. To examine the possibilities of using *ex vivo* dermal fibroblasts as a model system for studies of the kynurenine pathway.

8. To investigate CSF levels of tryptophan, KYNA, QUIN, HVA, 5-HIAA, IL-6, IL-8 and TNF-α in twins with severe psychiatric disorders, and to relate the levels of these metabolites to psychopathology measures.

3 MATERIALS AND METHODS

3.1 ETHICS

All studies involving human subjects were performed in compliance with the Declaration of Helsinki for experiments involving human subjects. Patients received verbal as well as written information and provided an oral and written consent to participate in the study. All studies were approved by the ethical review boards of Karolinska Institutet (**paper I, II and IV**), or Linköping University and the Swedish Medical Products Agency (paper II).

3.2 CELL CULTURE STUDIES

3.2.1 Cytokines and antibodies

For **paper I**, human recombinant IL-1β (PHC0815) and IFN-γ (PHC4031) were purchased from Invitrogen (GIBCO®). Antibodies against IDO1 (ab55305), TDO2 (ab 84926), Iba1 (ab5076), Nestin (ab6142) and S100B (ab52642) were purchased from Abcam (Cambridge, UK), GFAP (Z0334) from DAKO (Copenhagen, Denmark), NeuN (MAB377), from Chemicon (Millipore/Chemicon Billerica, Massachusetts, USA), Vimentin (180052) from Zymed (Invitrogen) and β-actin (A5441) from Sigma-Aldrich. Secondary antibodies for Western Blots were IR Dye 800 CW goat anti-mouse IgG (926-322, 1:10000) and IR Dye 680 CW goat anti-rabbit IgG (92632221, 1:10000) for use with the Odyssey system (LI-COR Biosciences, Linkoln, Nebraska, USA) or Horse Radish Peroxidase coupled antibodies (7076 and 7074, 1:75000) from Cell Signaling Technology (Danvers, Massachusetts, USA). For immunofluorescent detection Alexa-Fluor conjugated secondary antibodies (1:300, A11029, A11032, A11034, A110379) from Invitrogen, were used. For **paper II**, TNF-α and IFN-γ 200 were purchased from PeproTech, London, U.K.

3.2.2 Fibroblast Cultures

To establish fibroblast cultures, a cutaneous biopsy was taken from the arm of seven consenting volunteers recruited at Karolinska University Hospital Huddinge. Biopsies were minced and placed in 35 mm dishes (Corning Incorporated, Corning NY, USA) under a sterile glass coverslip and cultured in DMEM Glutamax, 10 mM HEPES, 1X MEM amino acids, 1X sodium pyruvate supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 15% fetal calf serum (all from Invitrogen, Paisley, UK), in a humidified 37°C, 5% CO2 incubator. After 2 passages, cells were seeded into 6-well plates (Corning Inc.). At confluence, cytokine stimulation was performed during 48 hours using human recombinant TNF-α 100 U/ml and/or IFN-γ 200 U/ml (PeproTech, London, U.K.) in serum-free media, otherwise as above. Experiments were ended by removal and freezing of the supernatants and addition of lysis buffer to the cell monolayer for RNA extraction.

3.2.3 Human Astrocyte Culture

Human embryonic primary cortical astrocytes were purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA) and cultured according to manufacturers recommendations with some modifications. Briefly, cells were cultured in Poly-L-lysine (P4707;Sigma) coated cell culture flasks in astrocyte media, supplemented with 2% fetal bovine serum and a mix of growth factors comprising a final concentration of BSA (10 µg/ml), insulin 5µg/ml, FGF-2 (2ng/ml), IGF-I (2ng/ml), hydrocortisone (1µg/ml) and progesterone (20ng/ml) and containing penicillin/streptomycine (1%; ScienCell™). Cells were kept in 37°C in a humidified atmosphere in a 5% CO2 – 95% air mixture. All experiments were performed on cells in passage 4. Cells were serum starved (0.02% FBS and 0.01 % growth supplement mix) for 24 hours prior to experiments and were then stimulated with IL-1β (10 ng/ml) for 1, 3 and 24 hours for gene expression analysis and 24 and 48 hours for protein expression analysis. It is well established that IFN-γ drives IDO expression and thus IFN-γ (200ng/ml) was included as a positive control. All experiments were performed in triplicates and repeated twice.

3.2.4 RNA extraction and reverse transcription

Following stimulation of cells in **paper I**, total RNA was extracted using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. The amount and purity of total RNA was measured using a NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Complementary DNA was synthesized using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) in a thermal cycler (Icycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following program: 10 min at 25°C, 30 min at 48°C, 5 min at 95°C. Negative transcription controls were run, with no added reverse transcriptase to control for the presence of genomic DNA in subsequent real time PCR analyses. In **paper III** total RNA was extracted from the cells using the RNeasy Mini kit (Qiagen, GmbH, Hilden, Germany). The amount and purity of the RNA was assessed by spectrophotometry using a Nanodrop ND-1000. Total RNA (250 ng) was subsequently treated with 1 unit of amplification grade DNase I (Invitrogen) for 15 min at room temperature and inactivated by the addition of 2.5 mM EDTA followed by incubation at 65°C for 10 min according to the manufacturer’s instructions. The DNase-treated RNA was subsequently reverse transcribed in 20 µl reactions containing the following reagents from Invitrogen; 250 ng of Oligo(dT) primer, 1 × First Strand Buffer, 10 mM DTT and 500 µM of each dNTP and 100 U Superscript II. cDNA synthesis was allowed to proceed for 1 h at 42°C before inactivation at 72°C for 10 min.

3.2.5 Real-time PCR and data analysis

In **paper I**, TaqMan PCR amplification reactions were performed using TaqMan Universal Master mix and 250ng of cDNA in MicroAmp Optical Plates with MicroAmp Optical Adhesive Film (Applied Biosystems). Quantitative PCR was performed using the Fast Real-Time PCR System (Applied Biosystems) with incubations at 50°C for 2 min. and at 95°C for 10 min. to activate the AmpliTaq polymerase, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Pre-developed specific primers (TaqMan® Gene Expression Assays, Applied Biosystems,) were used to detect *IDO1* (Assay ID Hs00158027_m1) and *TDO2* (Assay ID Hs00194611_m1) and *HPRT1* (Assay ID Hs01003270_g1).

Sample threshold cycle (Ct) values in standard curve samples containing *IDO1, TDO2* and *HPRT1* mRNA (**paper I**) were used to calculate the cDNA concentration equivalents in the test samples. Gene expression data of the gene of interest was then normalized to *HPRT1* reference gene expression to obtain relative concentration and is presented as relative units. In **paper III**, one µl cDNA templates were added to triplicate 25 µl reaction mixtures using Platinum SYBR® Green qPCR Supermix UDG (Invitrogen). An ABI Prism 7500 real-time thermocycler was used (Applied Biosystems, Palo Alto, CA, USA). Primers (Invitrogen) are provided in Table 1. Threshold cycle (Ct) values from the exponential phase of the PCR amplification plot for each target transcript was normalized to that encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). From these values, fold-differences in the levels of transcripts between individual untreated and treated cell cultures were calculated according to the formula 2-ΔΔCt (Livak and Schmittgen, 2001).

Target transcript	Gene	Polarity	Sequence (5′→3′)
IDO1	*INDO*	Sense	GCATTTTTCAGTGTTCTTCGCATA
		Anti-sense	CATACACCAGACCGTCTGATAGCT
TDO	*TDO2*	Sense	GAACATCTTTTTATCATAACTCATCAAGCT
		Anti-sense	ACAACCTTAAGCATGTTCCTTTCAT
KMO	*KMO*	Sense	TGTAATCCTCCAAGCTTCAATCTG
		Anti-sense	CTAGTAGATGCCCACTGAATATTTGTG
HAAO	*HAAO*	Sense	GGACGTTCTGTTTGAGAAGTGGTT
		Anti-sense	AGCTGAAGAACTCCTGGATGATG
KAT1	*CCBL1*	Sense	CCTGCTAAGGCTCAGGTATAACCT
		Anti-sense	GGACTCAAGCCTAAAGGCAACTC
KAT2	*AADAT*	Sense	CACATCTGGCAGCCAACAAG
		Anti-sense	CACTGGCAACATTAATAATGTTGCA
KAT3	*CCBL2*	Sense	ACTATCAGCCATCCCCGTTTC
		Anti-sense	AATGAAGCAAAAACGCACAAACT
KAT4	*GOT2*	Sense	TGTGGTGTGCAGCCTCTCAT
		Anti-sense	AAGCCTGAACCCAGCTAGCA
KYNU	*KYNU*	Sense	ACAGGATCTGCCTCCAGTTGA
		Anti-sense	TGGCCCACTTATCTAGTTCTTCTTC
QPRT	*QPRT*	Sense	ACACCGGCCATGGGTTAAC
		Anti-sense	GCCCCATTGGCCACTGA
GAPDH	*GAPDH*	Sense	CACATGGCCTCCAAGGAGTAA
		Anti-sense	TGAGGGTCTCTCTCTTCCTCTTGT

Table 1. Gene symbols and primer sequences for transcripts analyzed by real-time PCR in paper III.

3.2.6 Protein extraction and Western Blotting

In **paper I**, total protein was extracted using ice-cold lysis buffer (50 mm Tris buffer, pH 7.4, containing 0.5% Triton X-100, 150mm NaCl, 1 mM EDTA and protease inhibitors) and homogenized by sonication. After centrifugation the supernatants were mixed with loading buffer; 10X Orange Loading Dye for Odyssey (Licor; 929-10100) or 4X Loading Dye (NuPage; NP0007) for film, denatured at 95°C for 5 minutes, subjected to NuPAGE 10% or 4-12% Bis–Tris gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Invitrogen, LC2001). Membranes were blocked in 5% non-fat milk (Bio-Rad 170-6404) in Tris buffered saline with 1% tween (TBS-T) or Odyssey blocking buffer (927-40000) for 1 hour prior to antibody labeling. The membrane was incubated with primary antibody against TDO2 (1:2000) or IDO1 (1:1000) over night in 4°C, washed in TBS-T and then incubated with secondary antibody for 1 hour in room temperature. Following wash in TBS-T, membranes were developed using either the Licor Odyssey scanner or SuperSignal West Pico Chemiluminescence substrate (SuperSignal, Pierce Biotechnology, Inc., Rockford, IL, USA, 34080). Protein levels were normalized to β-actin (1:5000) and blots were analyzed using Quantity One 1-D Analysis Software (Bio-Rad).

3.2.7 Immunocytochemistry

In order to investigate cell purity of cultures in **paper I**, cells were cultured on cover slides coated with Poly-L-lysine (P4707) and laminin (L2020; both from Sigma). Cells were serum starved for 24 hours and then fixed in 4% paraformaldehyde in phosphate buffer for 15 minutes. After permeabilization in PBS containing 0.1% Triton-X100 (PBS-TX) for 5 minutes, the cells were blocked in PBS-TX containing 5% normal goat serum for 1 hour in room temperature. Cells were then incubated with primary antibodies against GFAP, Iba1, NeuN, S100B, nestin and vimentin (1:100) in PBS-TX, over night in 4°C. To further investigate the protein expression of IDO1 and TDO2 in these cells (paper I), they were also labeled with antibodies against these proteins (IDO1 and TDO2 1:100), following IL-1β (10ng/ml) stimulation for 48 hours. Cells were washed with PBS and then incubated with Alexa Fluor® conjugated secondary antibodies for 1 hour at room temperature. Following antibody incubation, cover slides

were dried and mounted on glass slides using cover slip mounting medium containing DAPI (Prolong Gold with DAPI, P36934, Invitrogen). Confocal images were captured using a confocal microscope system (Zeiss, LSM 710, Munich, Germany) operated by LSM software ZEN 2009.

3.3 HUMAN SUBJECTS

In **paper I**, subjects were collected from several sources. For *sample I*, patient data were collected from euthymic bipolar disorder patients that were enrolled in a long-term follow-up program (St. Göran bipolar project) at a bipolar outpatient unit at the Northern Stockholm psychiatric clinic. *Sample II* consisted of patients from St. Göran bipolar project who did not undergo lumbar puncture as well as patients with clinical diagnosis of bipolar disorder I from Karolinska Hospital in Huddinge or from ordinary psychiatric outpatient units in Stockholm. The diagnostic procedure has been outlined in detail previously. All included subjects had been diagnosed with either bipolar disorder I or bipolar disorder II.

The analyses included all subjects with existing data (not all patients volunteered to undertake all investigations). All patients, and their parents, in *sample I* were born in Sweden.

The 46 general population controls were randomly selected by Statistics Sweden. All were born in Sweden and by parents also born in Sweden.

Sample II consisted of 420 patients with bipolar disorder I, 108 patients with bipolar disorder II, 32 patients with bipolar disorder none otherwise specified, and 5 patients with schizoaffective disorder of bipolar type. The mean age in this sample was 50±16 years and 70% were females. 59% of the patients in *sample II* had a history of psychosis compared to 47% in *sample I*. This difference may be related to a higher proportion of bipolar disorder I and to a broader definition of psychosis in *sample II*.

In *sample III*, patients contributing with data in the analyses of CSF IL-1β were also from the St. Göran bipolar project. Controls were collected at Linköping University Hospital (Sweden). Cytokine levels of healthy volunteers were analyzed and published

in a previous study (Söderlund et al., 2011). Three of a total of 30 patients were here excluded. One of these three patients had an alcohol abuse and a cardiovascular disease (CSF IL-1β concentration 10.31 pg/ml, the patient classified as psychotic), one had persistent auditory hallucinations, suspected cannabis and alcohol abuse (CSF IL-1β concentration 9.97 pg/ml, the patient classified as psychotic), and finally one at the age of 73 had a cerebrovascular disease (CSF IL-1β concentration 0.73 pg/ml, the patient classified as psychotic).

In **paper II**, Twenty-two Swedish Caucasian outpatients, diagnosed with schizophrenia (n = 18; 11 males, 7 females) or schizoaffective disorder (n = 4; 2 males, 2 females), according to the Diagnostic and Statistical Manual (DSM)-IV criteria were included in the study. All patients were recruited from an outpatient clinic located at Linköping University hospital (Sweden). All of the patients were prescribed olanzapine as the only antipsychotic drug. The patients had been on medication with olanzapine for between 0.1 and 11 years (median 2 years) using the same dose (5–25 mg/day) for at least 14 days prior to CSF sampling. Mean (± SD) age of patients was 37.1 ± 7.6 years (range 23–50 years). Full details of the study design and patient characteristics, including serum and CSF concentrations of olanzapine, have been published elsewhere (Skogh et al., 2011). As controls, 26 healthy Caucasian volunteers (18 males, and 8 females in the follicular phase of the menstrual cycle) were recruited among medical students, hospital staff, and their relatives. Controls were not allowed to use any medication for at least one month prior to sampling; however, coffee and smoking were allowed. The mean (± SD) age of the controls was 24.9 ± 5.8 years (range 18–49 years).

In **paper III** seven consenting volunteers recruited at Karolinska University Hospital Huddinge were used to establish fibroblast cultures from a cutaneous biopsy from the arm.

In **paper IV** thirteen twin pairs were recruited from a nationwide cohort of Swedish-born, same-sex twins with schizophrenia, bipolar disorder and healthy control-pairs ascertained through the Swedish Twin Registry. In four of the included twin-pairs neither proband nor co-twin was affected by bipolar disorder, schizophrenia or schizoaffective disorder. The sibling of one of the 26 recruited twins did not agree to participate in the lumbar puncture. None of the participants was hospitalized at the time

of examination. Prior to analysis two CSF samples were lost due to experimental error, leaving in total ten pairs and three single twins for analysis.

3.3.1 Assessment of subjects

In **paper I** medical chart reviews, and in some cases interviews with the treating physician were performed in addition to the collected research data, in order to assure a high diagnostic validity. In *sample I* the clinical diagnosis of bipolar disorder was established according to the Affective Disorder Evaluation (ADE), which was previously used in the STEP-BD project (Sachs et al., 2003). The ADE was translated and modified to suit Swedish conditions. To minimize inter-rater variability, the collected information was presented at a diagnostic case-conference, and a consensus panel of experienced board-certified psychiatrists who specialize in bipolar disorder made the final diagnostic decision at this conference. CSF samples where collected when the patients were symptom free and in a stable euthymic mood, as judged by a physician. For ethical reasons, the patients continued to take their prescribed medication.

Control subjects in *sample I and II* of **paper I** underwent a psychiatric interview by experienced clinicians using the M.I.N.I. to exclude psychiatric disorders (Sheehan et al., 1997). Moreover the controls completed the same investigations the patients had undertaken, including self-rating scales, somatic tests, blood tests, and lumbar puncture. Because the assessments of controls might reveal pathological findings, case conferences were held between examining clinicians, primary investigator, and the study coordinator to decide whether or not to include such persons in the study. It was thus decided to allow past minor depressive episodes, isolated episodes of panic disorder, eating disorders, or obsessive compulsive disorder that had remitted spontaneously or with brief psychotherapy counseling. Substance abuse was screened for at the telephone interview by the nurse, in the psychiatric interview, by AUDIT and DUDIT, as well as by determining serum levels of carbohydrate-deficient transferrin (Saunders et al., 1993).

Overconsumption of alcohol as revealed by carbohydratedeficient transferrin or responses indicating large consumption (48 standard drinks per time more than 2 times per week) and/or amnesia and/or loss of control more than once per month resulted in

the exclusion of these individuals from the study. Other exclusion criteria were neurological conditions other than mild migraines, untreated endocrinological disorders, pregnancy, dementia, recurrent depressive disorder, and suspected severe personality disorders (based on interview and SCID-II personality assessment) and a family history of schizophrenia or bipolar disorder in first-degree relatives. Individuals with a severe inflammatory disease (peripheral or central), a brain disease, and an ongoing drug treatment that could influence the immune system, or who fulfilled criteria for a DSM-IV-TR disorder were not allowed to participate as controls (Jakobsson et al., 2013).

In *sample II*, lifetime manic and depressive symptoms were assessed based on interviews and medical records focusing on the most severe manic episode using the modules for mania and depression in the Schedules for Clinical Assessment in Neuropsychiatry (SCAN; Wing et al., 1990). Phenotypes such as lifetime psychotic features during manic or depressive episodes were also assessed.

36 healthy male volunteers used in *sample III* of **paper I** and in **paper II**, were recruited from among medical students, hospital staff members and their relatives. They all underwent a medical check-up including laboratory tests (electrolytes, blood, thyroid, kidney and liver) and a physical examination. The volunteers were free of medication for at least 1 month and free from any form of substance abuse. Smoking and coffee was allowed. The volunteers underwent a semi-structured interview using the Structured Clinical Interview for DSM-IV Axis I disorders (SCID-I First et al., 1997a). The interview was directed toward affective disorders, anxiety disorders and drug abuse. The volunteers also completed the SCID-II questionnaire for personality disorders (First et al., 1997b). 30 healthy volunteers were considered to be eligible for inclusion in the study with respect to the clinical interview and SCID results. All were considered healthy by the psychiatrist performing the examinations and showed no signs of psychiatric or somatic illness or had any laboratory test results outside of the standardized reference ranges. None of the volunteers had a family history of major psychosis or suicide in first- or second-degree relatives.

For patients recruited from the St. Göran bipolar project, psychosis was strictly defined as hallucinations and/or delusions, not caused by a psychotropic substance as judged by the physician, and in an affective episode, thus fulfilling the criteria according to DSM-

IV-TR. For patients recruited from the outpatient unit at Karolinska Hospital in Huddinge or from ordinary psychiatric outpatient units in Stockholm the definition of psychosis was 'loss of reality and delusions, hallucinations or paranoia during manic or depressive episodes according to DSM-IV. Control subjects underwent the same clinical evaluation as patients and could choose in which other investigations to participate

In *sample I* of **paper I**, an experienced psychologist performed neuropsychological assessments in conjunction with the lumbar puncture. In the Trail Making Test (TMT) from the Delis-Kaplan Executive Functioning System (D-KEFS), Number Sequencing (connecting the numbers 1-16) and Letter Sequencing, (connecting the letters A-P) was first extracted. The "Switching cost" was the total time taken for combined letter/number switching minus the Combined Number Sequencing + Letter Sequencing. These variables are extracted as raw scores and then converted to a contrast score in which the scaled score 10 is average in the normal population, with 7-13 reflecting +/- 1 SD and lower scores are reflecting difficulties in set shifting thought to capture cognitive inflexibility. The derived scores are thought to enhance the test's sensitivity to executive functioning while minimizing the influence of non-cognitive factors.

In **paper II**, All patients were somatically healthy, as judged by routine laboratory analyses (electrolytes, hematology, kidney, liver, and thyroid function) and a physical examination. The Brief Psychiatric Rating Scale (BPRS; Overall and Gorham, 1962) and Global Assessment of Functioning (GAF; American Psychiatric Association, 1994) index were used to evaluate symptoms and the level of function, respectively.

In **paper IV** all twin individuals were interviewed with SCID-I for DSM-IV Axis I Disorders (First et al., 1997a) and SCID-II for DSM-IV Axis II Disorders (First et al., 1997b). Information about socioeconomic status, smoking habits, and current medication was collected. Psychiatric symptoms were rated using the following scales: Scale for Assessment of Negative Symptoms (SANS; Andreasen, 1983), Scale for Assessment of Positive Symptoms (SAPS; (Andreasen, 1984), Schizotypal Personality Questionaire (SPQ-B; Raine and Benishay, 1995), Hamilton Depression Rating Scale (HDRS; Williams, 1988), and Young Mania Rating Scale (YMRS; Young et al., 1978). The Global Assessment Function (GAF; American Psychiatric Association, 1994) was

used to assess DSM-IV Axis V. Because collection of the CSF in the twin participants was performed two months or more after the psychiatric assessment described above, a psychiatrist performed a complementary SPQ-B assessment adjacent to the CSF-collection to update information about psychiatric status, current medication and somatic status. Also information about heredity, age of onset, insight, period of active symptoms and lifetime somatic diagnosis was collected. Finally two clinically experienced researchers with access to information of the diagnostic assessments, medical records and a full history of lifetime psychiatric diagnostic codes of the Hospital Discharge Registry (1973–2009), but with no access to the CSF-results, decided on a final consensus diagnosis.

3.3.2 Zygosity determination

In **paper IV**, the zygosity of the twins, mono- or dizygotic, was validated using a robust panel of 47 highly multiplexed single nucleotide polymorphisms (SNPs) that provide reliable and high quality data on a range of different DNA templates (Hannelius et al., 2007). Of the 13 twin pairs recruited for the study, 7 were monozygotic and 6 dizygotic.

3.3.3 Database studies - Postmortem samples

In **paper I**, a post-mortem brain tissue collection deposited in the Braincloud database (http://braincloud.jhmi.edu) was used to study co-expression of *SNX7* and *CASP8*. Data on 272 postmortem tissue homogenates of dorsolateral prefrontal cortex (DLPFC; i.e., BA46/9) from healthy donors were obtained. The sample and the methods are previously described (Colantuoni et al., 2011). Levels of *CASP8* mRNA in relation to psychosis in bipolar disorder were studied using data obtained from the Stanley Medical Research Institute (SMRI) database (www.stanleygenomics.org). A prior meta-analysis (Elashoff et al., 2007) of 105 DLPFC (BA 46) samples using the SMRI microarray collection was queried comparing bipolar disorder cases with psychotic features to bipolar disorder cases without psychotic features. Diagnosis was here made according to DSM-IV-TR.

3.3.4 Rating Scales

There are several scales rating the severity of schizophrenia symptoms. Some frequently employed are the Positive and Negative Syndrome Scale (PANSS; Kay et al., 1987), The Scale for the Assessment of Positive Symptoms (SAPS; Andreasen, 1984), the Scale for the Assessment of Negative Symptoms (SANS; Andreasen, 1983) and the Brief Psychiatric Rating Scale (BPRS; Overall and Gorham, 1962). There are also scales rating symptoms in the schizotaxic range (Meehl, 1962), used to assess the presence of schizotypic, schizoid and paranoid personality disorders, such as the cluster A of the Structured Clinical Interview for the DSM-IV Axis II personality disorders (SCID-II), and the SPQ-B (Raine, 1991; Raine and Benishay, 1995).
In **paper IV** potential correlations between SANS, SAPS, SPQ-B, and SCID-II on one hand and CSF metabolites and cytokines on the other hand are investigated. Therefore these four scales will be shortly described.

3.3.4.1 SANS and SAPS

The SANS and the SAPS are composed of nine subscales, measuring the severity of delusions, hallucinations, positive formal thought disorder and bizarre behavior on the positive scale, and deficit symptoms such as avolition, anhedonia, flat affect, alogia and attentional disturbances on the negative sub-scale. Every sub-scale contains varying number of items, as well as a global rating for the specific sub-scale. Since the development of these scales, several studies claiming the presence of more than two dimensions of schizophrenia symptoms have been published (Arndt et al., 1991; Klimidis et al., 1993; Minas et al., 1992).

3.3.4.2 SPQ-B

The SPQ is a self-report questionnaire composed of nine sub-scales measuring different schizotypal traits. Originally the questionnaire was composed of 74 items, but has now been improved to contain only 22 items representing three sub-scales in the SPQ-Brief. The subscales measure cognitive-perceptual factors, interpersonal factors and disorganization factors (Raine and Benishay, 1995).

3.3.4.3 SCID-II

The SCID-II is a questionnaire for the evaluation of Axis-II personality disorders. It is composed of a questionnaire to be completed by the patient that is used as a screening

tool to shorten the subsequent interview. The questionnaire evaluates the presence of twelve different personality disorders. Cluster A contains the scales to measure the presence of schizoid, paranoid and schizotypal personality disorder and are used in this thesis.

3.4 CSF ANALYSES

3.4.1 Lumbar Puncture

In **paper I** and **II**, lumbar puncture was performed on all participants between 8 am and 11 am after a night of fasting and bed-rest. A disposable needle (BD Whitacre Needle, 0.7 × 90 mm) was inserted at the L4–L5 level. In **paper I**, a volume of 12 mL of CSF was collected, inverted to avoid gradient effects, divided into aliquots and stored at –70°C until analyzed. In **paper II**, CSF was allowed to drip into a plastic test tube. The CSF samples were protected from light, centrifuged at 1438 g for 10 minutes (Sigma 203 centrifuge) within 30 minutes after the puncture, and divided into 2-to 3-ml aliquots. Samples were stored at −70 °C pending analysis.

The same neurologist performed the 25 lumbar punctures in **paper IV**, with all subjects in a sitting position. Sixteen twin pairs were examined on the same day and the remaining two pairs within the same month. The skin in the lumbar region was washed with sterile cotton swabs and chlorhexidine 5 mg/mL (Fresenius Kabi, Homburg, Germany) before puncture. A disposable needle (Becton Dickinson (BD) 22 GA 3.00 IN, 0.70675 mm or BD Whitaker Needle 25 GA 3.50 IN, 0.50690 mm was inserted in vertebral interspace L3-4, or L4-5, and the very first 12 drops of CSF, approximately 0.6 mL, were collected in a sterile test tube for microscopic examination. The following 12 mL of CSF were allowed to drip spontaneously, or by suction six times using a 2 mL syringe due to slow flow, in a second test tube, which was gently inverted 10 times to secure homogeneous mixing of the components to avoid gradient effects.

3.4.2 Analysis of KYNA

Analysis of KYNA in CSF samples (**papers I, II, III, IV**) was performed using an isocratic reversed-phase high-performance liquid chromatography (HPLC) system, including a dual-piston, high-liquid delivery pump (Bischoff Chromatography), a ReproSil-Pur C18 column (4 × 150 mm, Dr. Maisch GmbH) and a fluorescence detector (Jasco Ltd.) with an excitation wavelength of 344 nm and an emission wavelength of 398 nm (18 nm bandwidth), essentially as previously described.26 A mobile phase of 50 mM sodium acetate (pH 6.2, adjusted with acetic acid) and 7.0% acetonitrile was pumped through the reversed-phase column at a flow rate of 0.5 mL/min. Samples of 50 µL were manually injected (Ecom). Zinc acetate (0.5 M not pH adjusted) was de- livered after the column by a peristaltic pump (P-500; Pharmacia) at a flow rate of 0.10 mL/min. Signals from the fluorescence detector were transferred to a computer for analysis with Datalys Azur (version 4.6.0.0; http://datalys.net). The retention time of KYNA was about 7–8 minutes. Initially, the sensitivity of the system was verified by analysis of a standard mixture of KYNA with concentrations from 0.5 to 30 nM, which resulted in a linear standard plot. To verify the reliability of this method, some samples were analyzed in duplicate, and the mean intra-individual variation was below 5%

In **paper I**, cell culture supernatants were collected and immediately frozen on dry ice and kept in -20°C until analysis. In order to precipitate residual protein, samples were centrifuged at 14000 rpm for 5 minutes and an equal volume of perchloric acid (0.4M) was added to the supernatants. The centrifugation procedure was repeated followed by addition of 70% perchloric acid and centrifugation 2 more times. Analysis of KYNA was performed as described for CSF samples with some modifications. Initially, the sensitivity of the system was verified by analysis of a standard mixture of KYNA with concentrations from 0.25 to 30 nM, which resulted in a linear standard plot. The lower detection limit of the system was set to 0.625 nM, and samples below this limit are reported as undetectable.

In **paper III**, fibroblast cell culture supernatants (1.0 ml) were collected and kept in -20°C until analysis. In order to precipitate residual protein, samples were centrifuged at 20800 g for 5 minutes and an equal volume of 0.4 M perchloric acid was added to the supernatants. After a second centrifugation 70% perchloric acid (300 µl) was added,

and thereafter the supernatants were centrifuged twice at 20800 g for 5 minutes. Analysis was performed as in **paper I**.

3.4.3 Analysis of kynurenine

To analyze kynurenine (**paper II**) samples were thawed in 4°C and 50µl were manually injected (Rheodyne, Cotati, California) into a HPLC system. Separation was achieved by reversed-phase liquid chromatography using a 20mM NaH2PO4 buffer (not pH adjusted) with 5.0% acetonitrile. The mobile phase was delivered by an HPLC pump (Bischoff Chromatography, Leonberg, Germany) through a ReproSil-Pur C18 column (4 3 150 mm, Dr Maisch GmbH, Ammerbuch, Germany) at a rate of 0.5 ml/min. Following separation, the analysate was first passed through a guard cell with an oxidizing potential of 50 mV. Samples were then quantified by sequential oxidation and reduction in a high-sensitivity analytical cell (ESA 5011; ESA Inc., Chelmsford, Massachusetts) controlled by a potentiostat (Coulochem III; ESA Inc.) with an applied potential of 600 mV for detection of kynurenine. The signals from the detector were transferred to a computer for analysis (Datalys Azur, Grenoble, France). The retention time of kynurenine was approximately 8–9 minutes. The sensitivity of the system was verified by analysis of standard mixtures of kynurenine, with concentrations from 5 to 100nM, resulting in a linear standard plot.

3.4.4 Analysis of tryptophan and quinolinic acid

In **paper II**, the analysis of tryptophan was performed as described for kynurenine. The retention time was approximately 15–16 minutes and the sensitivity of the system was verified by analysis of standard mixtures of tryptophan, with concentrations from 0.5 to 5µM, resulting in a linear standard plot.

In **paper IV**, analysis of tryptophan and quinolinic acid was performed using For QUIN, standard curves were prepared in the range of 0.005 to 0.5 µmol/L QUIN (Sigma-Aldrich), dissolved in Dulbecco´s Phosphate Buffer Saline (PBS; Gibco®, Life Technologies, Carlsbad, CA, USA), aliquoted and stored at -70°C until use. CSF and standard samples (50µL) were diluted 2x with internal standard solution in 5% formic

acid and filtered at 3000 g for 60 minutes at 10°C using 10 kDa Ultracel®-10 filter plates (Merck Millipore, Darmstadt, Germany). QUIN, TRP, D5-TRP was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the Internal Standard (13C315N1-QUIN) from Synfine research Inc., (Ontario, Canada) Internal standard was added to each standard and CSF sample to a final concentration of 0.5 µmol/L. Following centrifugation 7.5 µL of the filtrate was injected into a Waters Acquity HPLC system equipped with a SymmetryShieldTM RP18 2.1 × 100mm, 3.5µm particle column. The detection was performed using a Waters Xevo TQ-S triple quadrupole mass spectrometer operating in positive ionization MS/MS configuration. The mobile phase was run at a flow rate of 300 µL/minute and consisted of 2.1% formic acid (MS-grade, Sigma-Aldrich) in MilliQ water (A phase) and 95% acetonitrile (MS-grade, Sigma-Aldrich), 0.1% formic acid in MilliQ water (B phase), starting with 5% B for 2 minutes, following gradient elution up to 95% B, with a total run time of 10 minutes. The mass spectrometer was tuned for quinolinic acid and tryptophan and set at capillary voltage of 3.0V, cone voltage 6 and 18 V for quinolinic acid and tryptophan respectively, source temperature 150°C, desolvation temperature 500°C, desolvation gas flow of 1000 L/hr and collision energy of 16 eV. Mass spectral transition for quinolinic acid and tryptophan was m/z 168> 106; 205 > 118 and for the IS 172> 110; 210 > 123.

Calibration was performed using standards covering the range of the CSF concentration. Seven concentration points were used to establish a linear calibration curve and plotted using the ratio of analyte peak area over IS peak area after integration by Masslynx 4.1 software (Waters Corporation, Milford, MA, USA). Retention times for quinolinic acid and tryptophan was 1.2 and 3.6 minutes respectively.

3.4.5 Analysis of HVA and 5-HIAA

In **paper I** and **IV** the analysis of HVA and 5-HIAA was performed using selected ion monitoring with deuterium-labelled internal standards. To 0.2 ml of CSF, 400 pmol of [D3]-HVA and 200 pmol of [D2]-5-HIAA were added as internal standards. The samples were acidified with 4 M formic acid and saturated with sodium chloride before extraction twice with 0.5 ml of diethyl ether. The combined diethyl ether phases were evaporated under a stream of nitrogen and the derivatives were prepared by the addition of 20% pentafluoropropanol in pentafluoropropionic anhydride (50µl). The samples

were heated for 15 minutes at 75°C and were then evaporated in a desiccator under vacuum. Subsequently, pentafluoropropionic anhydride (50 µl) was added and allowed to react for another 5 minutes at 75°C. The reagent was again evaporated and the residue was dissolved in ethyl acetate (25 µl) (Swahn et al., 1976). After preparations the analysis was performed on a Finnigan 3200 GC-MS system (Blennow et al., 1993).

3.4.6 Analysis of cytokines

In **paper I** a sandwich immunoassay-based protein array multi plex system (Invitrogen AB) was used. It presented a guaranteed lowest detection limit of 1 pg/mL for each cytokine to quantify IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM)-CSF, IFN-γ and TNF-α. The samples were incubated with beads coated with the specific antibodies. Thereafter incubations were conducted with biotin-conjugated antibodies and streptavidin-phycoerythrin. Standard curves (Biosource International) ranging from 0.38 pg/mL to 1025 pg/mL of the respective cytokine were used for quantification. A Luminex reader (Luminex Corporation) was used to simultaneously quantify the concentrations of the cytokines.

In **paper IV**, CSF samples used for cytokine analysis had previously been thawed once. IL-1β, IL-6, IL-8 and TNF-α, was quantified in CSF using a customized Human Ultra-Sensitive 4-Plex Kit (MesoScale Discovery®, Gaithersburg, MD, USA) in 2012. The assays were analyzed as per the manufacturers protocol (http://www.mesoscale.com), with the modification of a longer primary incubation time (over night at 4°C) and a sample volume of 50 µl. Intra-assay coefficient of variation was below 20% for all analytes presented. The limit of detection (LOD) in our analysis were: IL-1β (0.19 pg/ml), IL-6 (0.05 pg/ml), IL-8 (0.04 pg/ml) and TNF-α (0.08 pg/ml).

3.5 GENOTYPING AND QUALITY CONTROL

Genotyping was done as part of enrollment of this cohort in a large multi-center GWAS effort. Subjects in *sample I* and in the control sample were genotyped using the Affymetrix 6.0 array (Santa Clara, CA, USA) at the Broad Institute in Boston, MA. Subjects in *sample II* were genotyped using the Affymetrix 6.0 chip or the Illumina

OmniExpress chip. Blood samples were obtained and DNA extracted from whole blood using standard methods at the Karolinska Institutet. SNPs were excluded for marked departure from Hardy-Weinberg equilibrium ($P<1x10^{-6}$), low minor allele frequencies (<1%), and non-random genotyping failure, inferred from the flanking haplotype background using the PLINK 'mishap' test ($P<1x10^{-10}$). Plate-based associations of $P<1x10^{-6}$ were taken as evidence of non-random plate failure, based on a comparison of allele frequency of each plate to all others and were removed on a plate-by-plate basis. (Bergen et al., 2012).

3.6 STATISTICS

The primary analysis in the GWAS in **paper I** was a linear regression of CSF KYNA residuals on single-SNP allele dosage. "Genome-wide significant" was set to $P<5\times10^{-8}$. Additional analyses with the first four multidimensional scaling (MDS) components as covariates to control for population substructure were also performed (Bergen et al., 2012) but suggest no effects related to genetic diversity. In *sample II,* the ethnicity of the participants was partly unknown and all reported results are adjusted for population stratification. In the correlation analyses "*r*" refers to Pearson´s correlation coefficient if not otherwise specified. The assumptions of each test were checked. All reported *P*-values are two sided. All analyses were done using the statistical software programs R (R Development Core Team, Vienna, Austria), IBM SPSS Statistics 20.0 (IBM SPSS Inc., Chicago, IL, USA), the script PROCESS for SPSS (Hayes, 2013), and Graph-Pad® prism 6.0 (http://www.graphpad.com).

In **paper I**, plotting CSF QUIN residuals revealed one patient as an outlier in regard to CSF QUIN (standardized residuals<3 SD). Data from this 29-year-old male patient (CSF QUIN = 85.1 nM) were removed from all further analyses. For one patient the QUIN levels were below the lowest level of detection (LLOD), and the QUIN value for that patient was substituted for the LLOD value (5 nM). Background characteristics between patients and controls were compared using t-tests or Chi-square tests. To study the effect of background characteristics on CSF QUIN concentration in the patient sample and in the controls sample we used correlation analyses or Mann–Whitney U-tests. All correlation analyses were performed using Spearman rank correlation tests. The comparisons of CSF kynurenine metabolites levels and the QUIN/KYNA ratio

between patients and healthy volunteers were performed using t-tests. Logistic regression analyses with age and sex as covariates were also performed. All reported *P*-values are two sided. All analyses were made using IBM SPSS Statistics 21.0 software (IBM SPSS Inc., Chicago, IL, USA).

In **paper III**, comparisons across treatments were done by repeated measures ANOVA with Bonferroni's Multiple Comparison Test using GraphPad® prism (GraphPad Software, Inc., San Diego, CA, USA).

In **paper IV**, background characteristics between proband and co-twins, and between di-, and monozygotic twins were compared using Mann-Whitney U-tests, Chi-square tests or Fisher exact tests. To study the effect of background characteristics on levels of CSF metabolites in the probands and co-twins we used correlation analyses or Mann-Whitney U-tests. All correlation analyses were performed using Spearman rank correlation tests.

Levels of KYNA, tryptophan, QUIN, HVA, 5-HIAA, IL-6, IL-8 and TNF were log-transformed to obtain a normal distribution of the data.
To account for the correlated structure of the data, a linear mixed model with a random intercept shared between twins in a pair, was fitted. For every analyzed parameter, potential confounders identified in the initial analyses with a *P*-value<0.1 were included as co-variates in the initial model. Co-factors were subsequently removed based on significance of co-factors and Akaike's Information Criterion aiming for a final best-fitted model with fewer parameters. Age and sex was included as co-variates in all analyses. All reported *P*-values are two sided. All analyses were made using IBM SPSS Statistics 21.0 software (IBM SPSS Inc., Chicago, IL, USA).

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Psychotic patients with bipolar disorder have elevated levels of CSF KYNA and CSF HVA

In *sample I*, CSF KYNA concentration was independent of bipolar disorder subtype (*P*=0.43). An effect of age on CSF KYNA concentration was observed (*P*=0.002). To account for potential age confounding, we used the normally distributed residuals from a linear regression of age vs. CSF KYNA when age was indicated as a potential confounder. CSF KYNA was measured in 76 bipolar disorder patients of whom thirty-six patients had a history of psychosis, while 40 patients had no history of psychosis. CSF KYNA was also measured in a control group of 46 healthy volunteers randomly selected from the general population. The psychotic, but not the non-psychotic bipolar disorder patients had a significantly higher mean CSF KYNA concentration than controls (*P*=0.015 and P=0.99 respectively). Moreover, the psychotic bipolar disorder group had significantly higher CSF KYNA concentration than non-psychotic bipolar disorder patients (*P*=0.026; Figure 2A).

CSF HVA concentration was successfully determined in 72 of the 76 patients and in 45 of the 46 healthy controls. A strong positive correlation to CSF KYNA was observed in patients (r=0.67, P=9.4x10^{-11}; Figure 2B), and in controls (r=0.65, P=1.0x10-6). Similar to the analysis of CSF KYNA, the psychotic bipolar disorder group had a significantly higher mean CSF HVA concentration than healthy controls (*P*=0.003), as well as non-psychotic bipolar disorder patients (*P*=0.024), while no significant difference in CSF HVA concentration was seen between controls and non-psychotic bipolar disorder patients (*P*=0.81; Figure 2C).

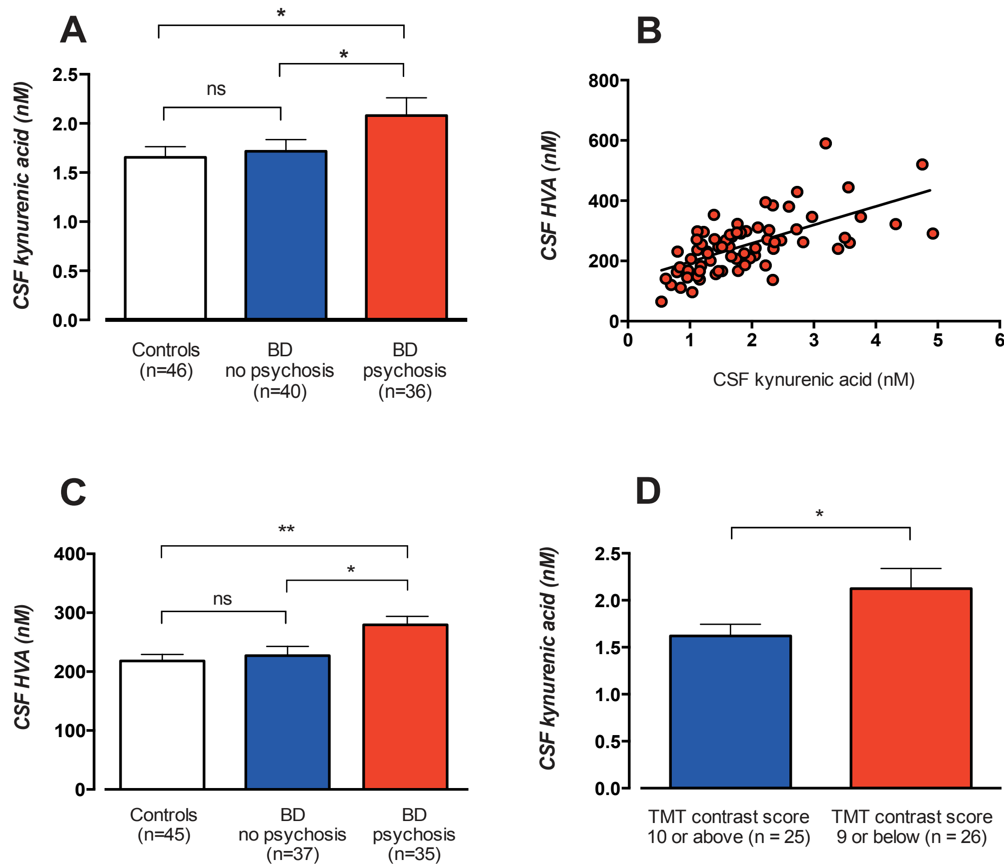

Figure 2. CSF concentrations of kynurenic acid (KYNA) in euthymic bipolar disorder patients and healthy controls. (A) Mean ± SEM of CSF KYNA levels in 46 healthy psychosis (1.72 ± 0.12 nM), and 36 bipolar disorder patients with a history of psychosis (2.08 ± 0.18 nM). One-way ANOVA with Tukey post hoc tests (using residuals from a linear regression of age vs. CSF KYNA); P=9.3x10^{-3}, post hoc tests; controls vs. non-psychotic bipolar disorder, P=0.99, controls vs. psychotic bipolar disorder, P=0.015controls (1.60 ± 0.10 nM), and non-psychotic bipolar disorder vs. psychotic bipolar disorder, P=0.026. (B) The correlation between CSF KYNA and CSF HVA (nM) in 72 of these 76 bipolar disorder patients. Pearson's r = 0.67; P=9.4x10^{-11}. (C) Mean± SEM CSF HVA concentration in 72 bipolar disorder patients with (280 ± 14.4 nM) or without (227 ± 15.6 nM) a history of psychosis in comparison to CSF HVA levels in 45 healthy controls (216 ± 10.6 nM). One-way ANOVA followed by Tukey post hoc tests revealed that the psychotic bipolar disorder group had a significantly higher mean CSF HVA concentration compared to controls (P=0.003), and non-psychotic bipolar disorder patients (P=0.024), while no significant different in CSF HVA concentration was seen between controls and non-psychotic bipolar disorder patients (P=0.81). (D) Mean±SEM CSF KYNA in the 51 bipolar disorder patients who underwent trail-making test (TMT). The mean CSF KYNA concentration was significantly higher in patients that scored below average on the contrast score (<10, n=26; 2.12 ± 0.12 nM) compared to patients scoring average or higher (≥10, n=25; 1.62 ± 0.21 nM, P=0.048). Two-sided P-values, statistical significance set to P<0.05. * P<0.05,** P<0.01,*** P<0.001.

4.1.2 CSF KYNA is associated to set shifting performance

In conjunction with the lumbar puncture, 51 of the 76 euthymic bipolar disorder patients agreed to carry out TMT (from the Delis-Kaplan Executive Functioning System; D-KEFS) to evaluate executive functions in terms of set-shifting ability. To separate the executive component of this test, we used the contrast score of condition 4 minus condition 1+2. A score of 10 reflects average performance in a normal population, while lower scores indicate diminished cognitive flexibility in terms of decreased set-shifting ability. The mean CSF KYNA concentration was significantly higher in patients that scored <10 compared to patients scoring ≥10 or higher (*P*=0.048, Figure 2D). The use of different pharmacological treatments was equally distributed across the two groups.

These findings are in line with previous studies suggesting that KYNA is intimately related to dopamine neurotransmission. KYNA has in a number of studies been shown to modulate the firing of midbrain dopaminergic neurons (see introduction) and CSF KYNA has also previously been shown to correlate with CSF levels of HVA (Nilsson-Todd et al., 2007; Nilsson et al., 2007). These results also support the notion of KYNA as a factor linked to psychosis, as the highest levels of both KYNA, and HVA were found in patients with a history of psychosis. These results also extend the findings that KYNA influences cognitive flexibility in the set shifting paradigm from experimental settings (Alexander et al., 2012, 2013), to the clinical reality. As cognitive dysfunction is often accompanying psychotic states, this constitutes another piece of evidence for the role of KYNA in psychotic disorders.

4.1.3 Genome-wide association study

Despite the modest sample size (n=76), one SNP (rs10158645) located within 1p.21.3, reached genome-wide statistical significance in relation to CSF KYNA ($P=2.6\times10^{-8}$; Figure 3A and 3B). To control for population substructure, we also analyzed the association using the first four multidimensional scaling (MDS) components as covariates. In line with a low grade of genetic diversity (all subjects and their parents were born in Sweden), this yielded a similar result as the unadjusted analysis (*P*=4.33x10-8).

The mean CSF KYNA concentration among patients with the T/T genotype (n=55) was 1.62 ± 0.095 nM, and in patients with the G/T genotype (n=21) 2.48 ± 0.23 nM. The CSF KYNA concentration in the one patient carrying the G/G genotype was 4.75 nM.

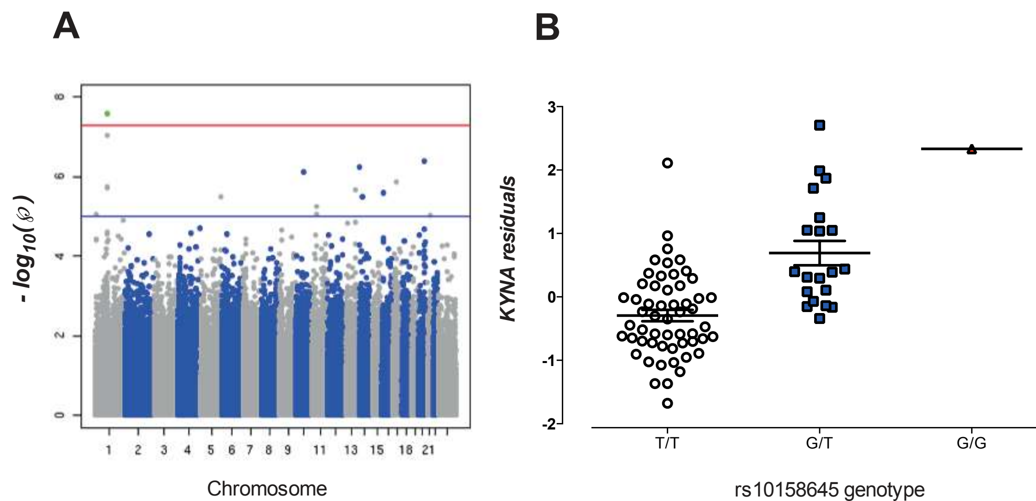

Figure 3. Genome-wide association study against kynurenic acid levels in cerebrospinal fluid (CSF) of 76 euthymic bipolar disorder patients. (A) Manhattan plot from the GWAS against age-adjusted CSF kynurenic acid (KYNA) levels in bipolar disorder. The single nucleotide polymorphism (SNP) rs10158645, located within 1p.21.3, reached genome-wide statistical significance in relation to CSF KYNA concentration among bipolar disorder patients (β=1.07, P=2.58x10^{-8}, MAF=0.15). (B) KYNA levels in carriers of the T/T genotype (n=55), the G/T genotype (n=21), and the T/T genotype (n=1) in rs10158645. The y-axis displays residuals from a regression of age vs. CSF KYNA concentration (nM).

Analogous to results in bipolar disorder patients, a significant effect of the minor allele in rs10158645 on CSF KYNA concentration (adjusted for age) was also seen in the control sample (n=46, P=0.036; Figure 4). Including an adjustment for population stratification gave a similar result (P=0.021).

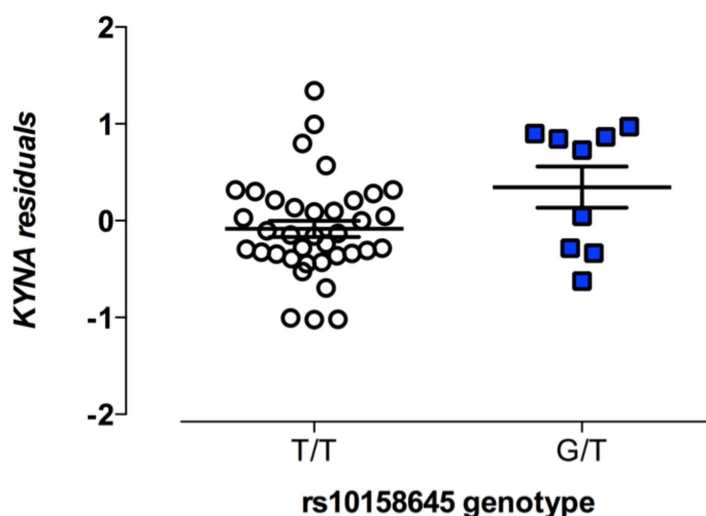

Figure 4. Levels of CSF kynurenic acid (KYNA) in the 46 healthy controls randomly selected from the general population and here stratified on rs10158645 genotype. 37 subjects carried the T/T genotype, 9 the G/T genotype while no carriers of the G/G genotype was found. Carriers of the G/T genotype had significantly higher CSF KYNA concentration (T/T: mean CSF KYNA=1.55±0.11 nM, and G/T: mean CSF KYNA 1.83±0.26 nM; *P*=0.036). The y-axis displays residuals from a regression of age vs. CSF KYNA concentration (nM).

4.1.4 Association of rs10158645 to psychosis

The minor allele of rs10158645 was associated with psychotic symptoms in *sample I* (n=76, OR=3.98, 95% CI: 1.37-11.5, *P*=0.011). The effect of rs1018645 genotype on psychosis was also studied using a replication sample of 565 bipolar disorder patients without CSF KYNA data (*sample II*). Similar to the result from *sample I*, we observed an increased risk of psychosis in *sample II* for patients carrying the minor G allele (OR=1.50, 95% CI: 1.06-2.03; *P*=0.020). Using a recessive model yielded an OR of 6.45 (95% CI: 1.48-28; *P*=0.013). Combining both samples (n=641) yielded an OR of 1.59 (95% CI: 1.17-2.16; *P*=0.002) under an additive model.

4.1.5 Association of rs10158645 with set-shifting performance

Of the 76 patients in *sample I*, 51 agreed to participate in TMT testing. In the replication sample, with no CSF KYNA data, 38 patients had carried out TMT. To achieve sufficient power, we tested the effect of rs10158645 on set-shifting ability

using a combined sample (n=89). Carriers of the minor allele scored close to average (n=60, 10.1 ± 0.30), while the non-carriers (n=29) had a significantly lower mean contrast score (8.93±0.47, *P*=0.035), indicating diminished cognitive flexibility.

4.1.6 Causal inference analyses proposes a mechanistic route from rs10158645 via KYNA and HVA to psychosis

The minor allele in rs10158645 was also associated with increased CSF HVA concentration (n=72, $P=7.3\times10^{-7}$). We endeavored to statistically evaluate a mediational model with rs10158645 allele frequency as the causal variable, CSF KYNA as the mediator, and CSF HVA as the outcome variable. Using a non-parametric bootstrap method (the PROCESS script for SPSS (Hayes, 2013), percentile bootstrap=20000), the indirect effect that estimates the amount of mediation was significant (point estimate=64.8, 95% bootstrap CI: 35.7-101), while the direct (direct effect=total effect–indirect effect) was smaller and non-significant (point estimate=42.6, 95% bootstrap CI:-0.53-85.7). To study possible reverse causal effects we interchanged the mediator and outcome variable. This suggested a better fit of the first model. These mediation analyses therefore propose that the causal minor allele in rs10158645 increases CSF HVA via KYNA, in agreement with previous experimental studies.

To study whether the effect of KYNA on psychosis in bipolar disorder was driven by its effect on dopaminergic transmission, we tested a mediation model in which CSF KYNA was the causal variable, CSF HVA the mediator, and psychosis the outcome variable. Again, we used PROCESS with percentile bootstrap (20000), age as a covariate, and the same 72 subjects. This model gave a significant indirect effect (point estimate=0.71, 95% bootstrap CI: 0.15-1.81) with a non-significant direct effect (point estimate=0.15, 95% bootstrap CI: -0.61-0.90), suggesting that the increased risk of psychosis caused by elevated levels of KYNA was mediated by dopamine neurotransmission. Numerous studies link KYNA to dopaminergic signaling. KYNA has also previously been shown to associate to psychosis in a clinical sample (Olsson et al., 2012a) and is repeatedly reported to be elevated in

patients with schizophrenia (Erhardt et al., 2001a; Linderholm et al., 2012; Nilsson et al., 2005; Schwarcz et al., 2001). This is however the first time that KYNA is implicated in a putative chain of events, leading from a gene variant to psychosis. The identification of the genome significant SNP, rs10158645, associated both to levels of KYNA and to psychosis, made it possible for us to utilize causal inference analyses, trying to dissect the path leading from genotype to psychosis. These analyses suggest a path starting with the gene variant, leading to elevated levels of KYNA, which in turn leads to an increase in dopamine neurotransmission, directly underlying the presence of positive psychotic symptoms. Furthermore rs10158645 was also associated to set-shifting performance, known to be impaired in patients with bipolar disorder (Bora et al., 2009; Yatham et al., 2010). Even though GWAS's aiming at identifying genetic variation underlying bipolar disorder and schizophrenia are proposing SNPs at a rapid pace, verifying the role of a gene variant associated to the heterogeneous cluster of symptoms present in psychotic disorders have proven exceptionally challenging. Since the goal of the GWAS in this study was identifying variation underlying the levels of KYNA, already proposed to constitute a component in the development of psychosis, further exploration of putative biological pathways might be less complex.

4.1.7 Functional consequences of gene expression of the rs10158645 genotype

In order to study the effect of the rs10158645 genotype on gene expression, fibroblast data from the Matched Co-Twin Analysis (Nica et al., 2011), was searched regarding an effect of rs10158645 genotype on gene expression (by default set to a search of +/- 1Mb, i.e., cis-effects). This revealed a significant association between the minor allele in rs10158645 and decreased expression of the nearby gene *SNX7* (*P*=2.0x10-4). A similar and significant association (*P*=0.0096) was also found in a replicate sample (unrelated subjects of the HapMap3 populations; n=198), based on data from lymphoblastoid cell lines.

rs10158645 is located in an intron of *RP5-896L10.1*, a long non-coding RNA (lncRNA) gene that is predominately expressed in the brain, and approximately 2 kB

from a predicted enhancer region (http://encodeproject.org/ENCODE/). Although sparsely studied, lncRNA have recently been shown to regulate the expression of nearby genes, i.e., cis-effects (Lai et al., 2013), suggesting that the observed cis-effect of rs10158645 on *SNX7* was mediated by increased *RP5-896L10* expression. Against this background, we deemed that the most likely functionality of the locus identified in the GWAS was in relation to *SNX7*.

4.1.8 Downregulation of SNX7 induces caspase-8

The SNX family proteins contain a phox (PX)-homology domain that binds phosphoinositides, and function in diverse intracellular trafficking and signaling processes (Teasdale and Collins, 2012). SNX7, together with SNX1,-2,-4,-5,-6 and -8, belongs to the PX-BAR subfamily that all have a C-terminal BAR domain in common, and the structurally predicted phosphoinositide specificity for SNX7 is phosphatidylinositol 3-phosphate (PtdIns3P) (Teasdale et al., 2012). Although SNX7 is rarely studied, a recent report showed that a down-regulation of SNX7 increases caspase-8 protein levels in hepatocytes, most likely by inducing the degradation of the short form of C-FLIP at the riboptosome platform (Xu et al., 2012). Supporting a functional link between SNX7 and caspase-8 in brain tissue, we found a strong inverse coexpression of SNX7 and CASP8 (r=-0.44; P=1.6x10^{-14}) in DLPFC by utilizing 272 postmortem human brain samples.

4.1.9 Caspase-8 and IL-1β are associated with psychosis in bipolar disorder

In microglia, caspase-8 is reported to cleave pro-IL-1β into its biologically active structure (Burguillos et al., 2011, Dupaul-Chicoine and Saleh, 2012). To test the relevance of caspase-8 induced activation of IL-1β for psychotic symptoms in bipolar disorder, we first studied brain DLPFC *CASP8* mRNA expression in a postmortem sample of 105 bipolar disorder patients. This revealed that *CASP8* expression was significantly increased in bipolar disorder with psychotic features compared to bipolar disorder without psychotic features with an approximate fold change of 1.15 (99%CI:

1.04-1.20). Secondly, we re-analyzed a sample of 27 male euthymic bipolar disorder patients and 30 male healthy controls with regard to CSF IL-1β data (Söderlund et al., 2011; *sample III*). Both the psychotic and the non-psychotic bipolar disorder group had significantly higher CSF IL-1β concentrations compared to controls ($P<1x10^{-6}$ and $P=5.8x10^{-5}$ respectively). However, the psychotic bipolar disorder group also had significantly higher CSF IL-1β concentration compared to non-psychotic bipolar disorder patients ($P=0.020$). Figure 5A). The control group was significantly younger than the patient group (40.9±2.34 years vs. 25.4±1.31 years, $P<1.0x10^{-6}$). CSF IL-1β concentration was, however, not associated with age, and adjusting for age gave similar results (data not shown). Sex, BMI, smoking status, and weekly alcohol consumption were not associated with CSF IL-1β concentration (data not shown). Comparison of pharmacological treatments between the patient groups suggested an equal distribution, and no significant effects of any pharmacological treatments on IL-1β concentration could be detected.

4.1.10 IL-1β is associated with set-shifting ability in bipolar disorder

Bipolar disorder patients with available CSF IL-1β data (*sample III*) were asked to carry out TMT. Of 16 participating patients, 9 scored ≥10 on the TMT contrast score, while 7 scored <10. The median CSF IL-1β concentration was significantly higher in patients with more difficulties in set-shifting, defined by a score <10, compared to patients scoring ≥10 ($P=0.012$; Figure 5B). A putative influx of peripheral IL-1β to the brain may influence central levels of IL-1β. Thus, to avoid uncontrolled confounding by peripheral inflammation we also measured serum high-sensitive CRP (hsCRP). hsCRP covaries with serum concentration of IL-1β (Hung et al., 2011) and is associated with cognitive impairment (Hoshi et al., 2010). Partial correlation analyses with hsCRP as covariate were performed to test for a possible confounding effect of peripheral inflammation, but there was still a significant negative correlation between CSF IL-1ß concentration and set-shifting ability (r_S[Spearman]=-0.59, $P=0.021$; Figure 5C).

IL-1β has previously been reported to be elevated in patients with both bipolar disorder and schizophrenia (Söderlund et al., 2009, 2011). The cause of the increased IL-1β levels in CSF of patients with psychotic disorders has however not been

thoroughly investigated on the molecular level, and is a constant topic for speculation. The proposed path linking the gene variant identified in the GWAS with the function of SNX7, and further to caspase-8 and IL-1β constitutes one of the first plausible examples of molecular events leading to the increase of IL-1β in psychotic disorders. IL-1β has also previously been implicated in memory function. Constitutively expressed low levels of IL-1β has been shown to play a role in the synaptic plasticity in the healthy brain, as it is necessary for development of LTP (Schneider et al., 1998). However, overexpression of IL-1β in the hippocampus of mice leads to deficits in spatial memory (Moore et al., 2009).

Altogether, and in conformity with the previously presented data for KYNA, these results further strengthen the role for the involvement of IL-1β in both psychosis and cognition.

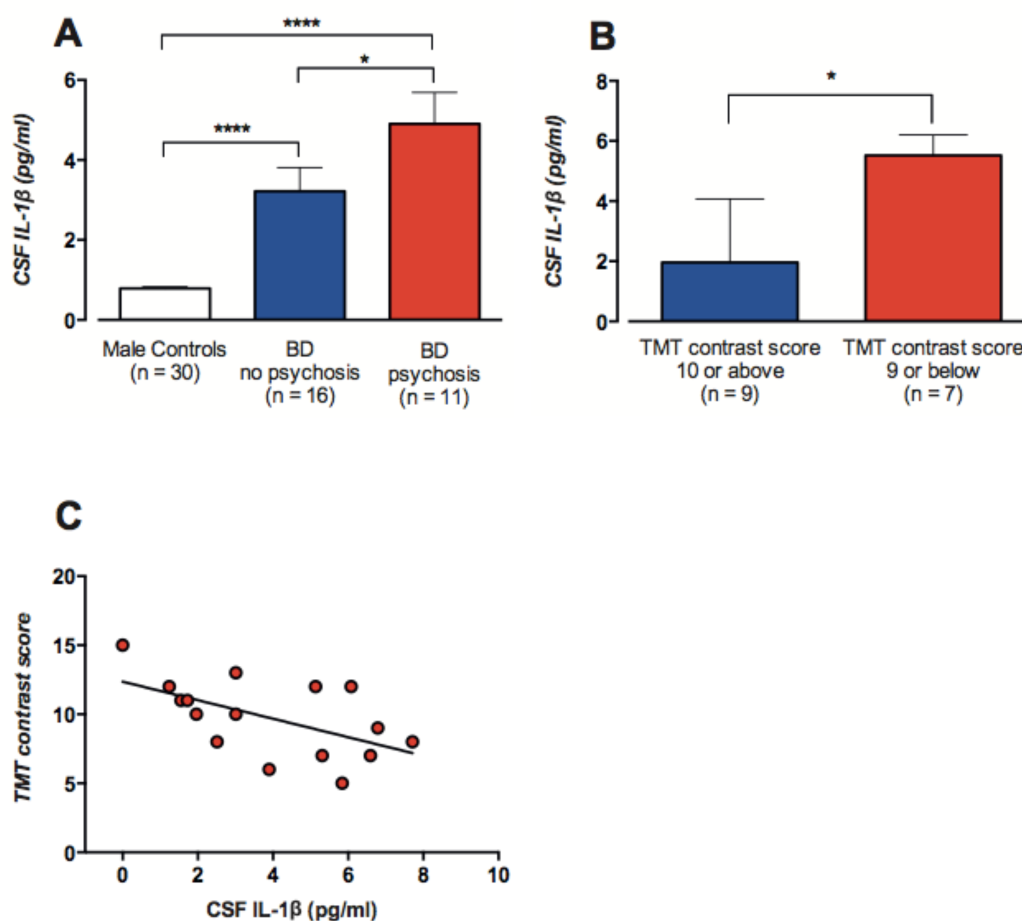

Figure 5. CSF concentrations of IL-1β euthymic bipolar disorder patients and healthy controls. (A) Mean ± SEM CSF IL-1β in 30 healthy male volunteers (0.79±0.044 pg/ml), 16 bipolar disorder (bipolar disorder) patients without a history of psychosis (3.06±0.58 pg/ml), and 11 bipolar disorder patients with a history of psychosis (5.35±0.71 pg/ml). One-way ANOVA with Tukey post hoc tests; controls vs. non-psychotic bipolar disorder, P=5.8x10^{-5}, controls vs. psychotic bipolar disorder, P<1x10^{-6}, and non-psychotic bipolar disorder vs. psychotic bipolar disorder, P=0.020. (B) 16 of these bipolar disorder patients carried out the trail making test (TMT). 9 patients scored 10 or above on the contrast score and 7 scored below 10. The median CSF IL-1ß concentration was higher in the group that scored <10 (5.84±2.89 [IQR] pg/ml) compared to the group that scored 10 or above (1.96±2.68 nM; Mann-Whitney U test, P=0.012). (C) A possible confounding effect of peripheral inflammation was assessed with correlation analyses. Increasing CSF IL-1ß concentration was associated with difficulties in set-shifting (r_s = -0.53, P=0.034), also when adjusting for hsCRP concentration (r_s = -0.59, P=0.021). Error bars represent mean ± SEM in 5A and in 5B the median and third quartile. Statistical significance set to P<0.05. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

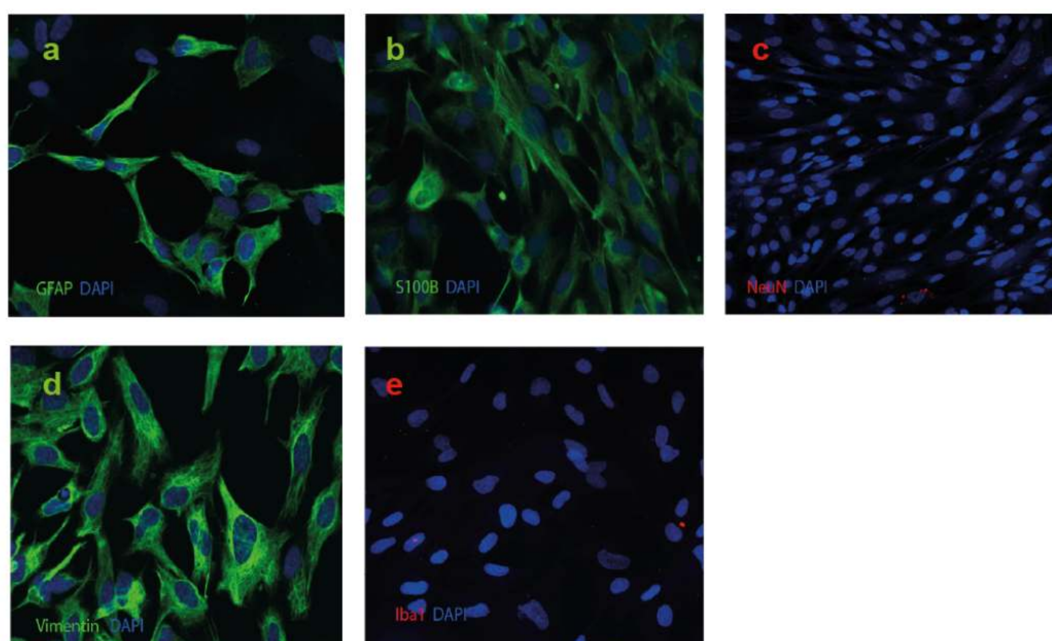

Figure 6. Immunofluorescent staining of the astrocyte markers GFAP at 40X magnification (a), S100B, at 40X magnification (b) and Vimentin at 20X magnification (d) Cells did not stain for the neuronal marker NeuN (c) or the microglial marker Iba1 (e), confirming the relative purity of the cultures. All immunofluorescent staining were combined with the nuclear stain DAPI.

4.1.11 IL-1β induces TDO in human cortical astrocytes

Although it is well established that the pro-inflammatory cytokine interferon (IFN)-γ is a potent inducer of the kynurenine pathway, less is known about the specific effects of IL-1β. We therefore investigated whether IL-1β also activates the kynurenine pathway by inducing the rate-limiting enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and/or tryptophan 2,3-dioxygenase-2 (TDO2), hereby accounting for the observed increase in CSF KYNA in bipolar disorder. Fetal human cortical astrocytes were cultured and stimulated with recombinant human IL-1β (10 ng/ml). IFN-γ (200 ng/ml) was used as positive control.

Prior to experiments the relative purity of cultures was established. Cells hence expressed the astrocyte markers GFAP (Figure 6a), S100B (Figure 6b), and Vimentin (Figure 6d). No expression of the neuronal marker NeuN (Figure 6c), or the microglial marker Iba1 (Figure 6e), was detected.

Stimulation with IL-1β (10 ng/ml) or IFN-γ (200 ng/ml) for 24 hours increased the expression of *IDO1* mRNA ($P<0.0001$) compared to vehicle treated cells (vehicle at 1 hour standardized to 100%; Figures 7A, 7B). Stimulation with IL-1β (10 ng/ml), increased the expression of *TDO2* mRNA ($P<0.0001$), while IFN-γ (200 ng/ml) did not ($P=0.40$), vehicle at 1 hour standardized to 100%; Figures 7C, 7D).

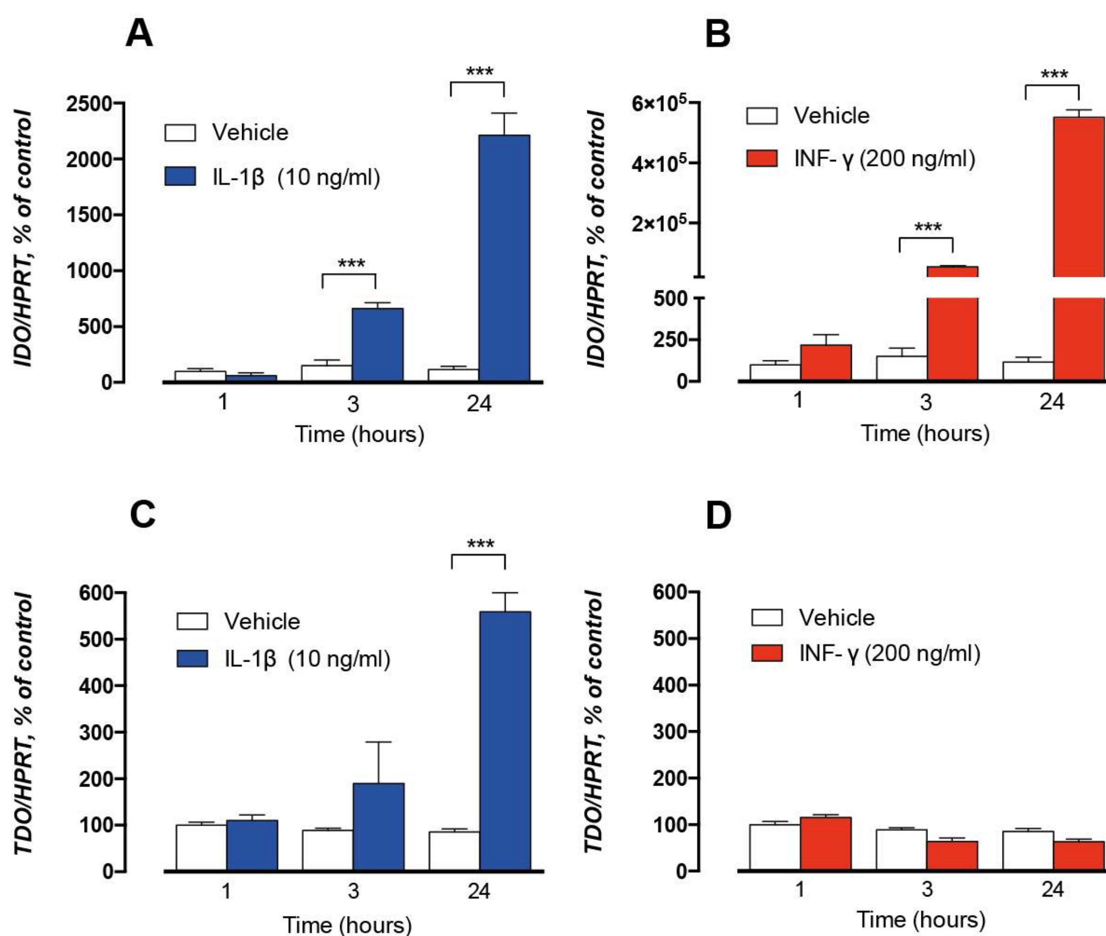

Figure 7. mRNA levels of *IDO1* and *TDO2* in fetal human cortical astrocytes stimulated with IL-1β and IFN-γ. *IDO1* and *TDO2* were normalized to *HPRT*. Bar graphs represent *IDO1* and *TDO2* mRNA levels expressed as % of vehicle treated control cultures, where vehicle at 1hr =100%. A. IL-1β stimulated the induction of transcription of *IDO1* (2213±198% vs. vehicle 116±29%, $P<0.0001$) B. IFN-γ was only able to stimulate *IDO1* transcription (551944±24844% vs. 116±65%, $P<0.0001$), C. IL-1β stimulated the induction of transcription of *TDO2* (559±41% vs. vehicle 86±6.48%, $P<0.0001$), IFN-γ did not induce the transcription of *TDO2* (63±5.71% vs. 86±6.48%, $P=0.40$). Data are reported as mean±SEM Statistical significance set to $P<0.05$. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Protein levels of IDO1 and TDO2 were analyzed by Western Blotting at baseline and after exposure to IL-1β (10 ng/ml) or IFN-γ (200 ng/ml) for 48 hours. Low levels of IDO1 protein expression were detected in unstimulated cells, but despite the marked increase of IDO1 mRNA levels following IL-1β stimulation, no changes in protein levels were observed after 48 h IL-1β exposure (*P*=0.41; Figure 8A). Stimulation with IFN-γ (200 ng/ml) was associated with a marked increase in IDO1 protein levels (*P*=0.001; Figures 8A). TDO2 protein expression was readily detectable at all time-points including baseline. Stimulation with IL-1β (10 ng/ml) for 48 hours increased protein levels of TDO2 compared to vehicle treated cells (*P*=0.006; Figure 8B), whereas stimulation with IFN-γ (200 ng/ml) did not affect protein levels of TDO2 (*P*=0.21; Figure 8B).

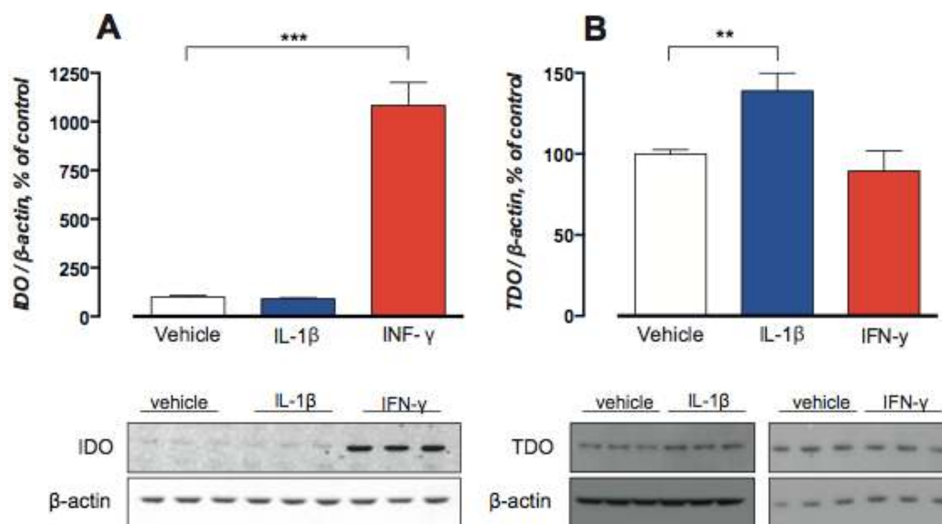

Figure 8. Protein levels of IDO1 and TDO2 in fetal human cortical astrocytes stimulated with IL-1β and IFN-γ. IDO1 and TDO2 immunopositive bands are normalized to β-actin. Bar graphs represent IDO1 and TDO2 protein levels expressed as % of vehicle treated control cultures. Data are reported as mean±SEM. Representative Western blots of IDO1 and TDO2 are shown below each bar graph. (A) Low levels of IDO1 protein expression were detected in unstimulated cells, but following 48 h IL-1β stimulation, no changes in protein levels were observed (90 ± 5.13% vs. 100 ± 7.85%; *P*=0.41). Stimulation with IFN-γ was associated with a marked increase in protein levels of IDO1 (1084 ± 117% vs. 100 ± 7.85%; *P*=0.001). (B) Stimulation with IL-1β for 48 hours increased protein levels of TDO2 compared to vehicle treated cells (139 ± 11.1% vs. 100 ± 2.70%, *P*=0.006), whereas stimulation with IFN-γ did not affect protein levels of TDO2 (80 ± 11.2% vs. 100 ± 8.70%; *P*=0.21). Statistical significance set to *P*<0.05. *P<0.05, **P<0.01, ***P<0.001,

Protein expression was also visualized using immunofluorescence techniques. In agreement with the Western Blot data, TDO2 following IL-1β exposure appeared to be localized in scattered cells expressing high levels of the protein (Figure. 9A), while IDO1 staining was weak (Figure 9B). Although IDO1 and TDO2 perform the same enzymatic function, they show little sequence and structure homology and are likely to perform the oxidative cleavage reaction using different mechanisms (Batabyal and Yeh, 2007). It is therefore not surprising to find that the enzymes are regulated by different stimuli.

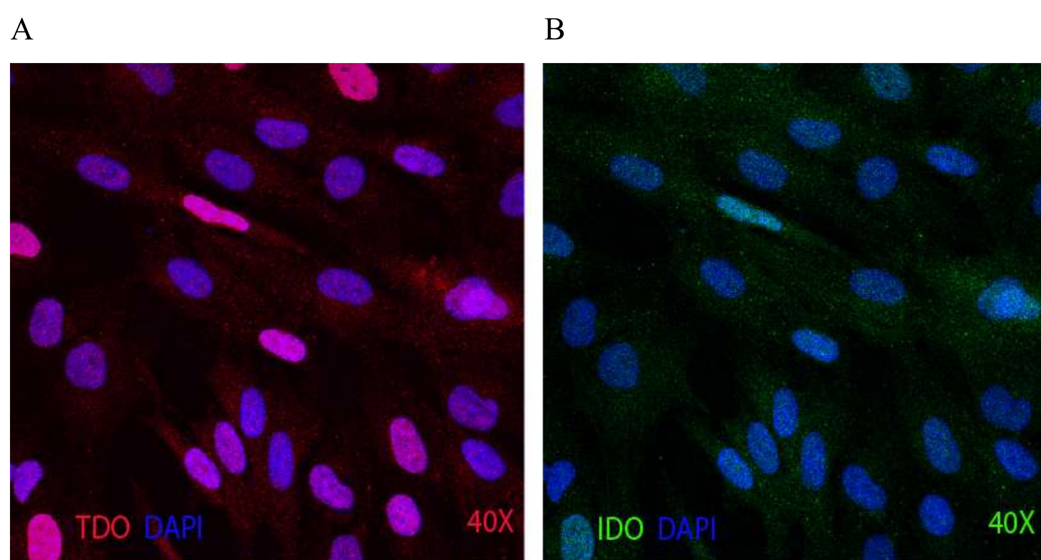

Figure 9. Protein levels of TDO and IDO visualized using immunofluorescence, captured at 40X magnification.

4.1.12 IL-1β induces the production of KYNA from human cortical astrocytes

KYNA was analyzed at 1, 3, and 24 hours. At 24 hours, cells stimulated with IL-1β showed 4 times higher KYNA concentrations compared to vehicle treated cells (*P*<0.0001; Figure 10A). Cells stimulated with IFN-γ had >85 times higher KYNA concentrations compared to vehicle treated cells (*P*<0.0001; Figure 10B). The increase in KYNA levels following IL-1β stimulation might seem high in relation to the relatively modest increase in TDO2 protein. However, the TDO2 enzyme is a homotetramer reliant on heme as a prosthetic group. TDO2 enzyme activity is therefore not solely dependent on the abundance of protein as measured by western

blot, since both the assembly of the tetramer as well as the activation of the heme moiety will influence enzyme activity (Li and James, 2007; Ren and Correia, 2000). Altogether, our *in vitro* data suggest that IL-1β induces TDO2, resulting in elevated levels of KYNA. This mechanism may critically contribute to the activation of the kynurenine pathway as seen in several disorders. In fact TDO, but not IDO, expression, is elevated in patients with schizophrenia and bipolar disorder, along with elevated levels of kynurenine (Miller et al., 2004, 2006), suggesting that these results mirror the clinical situation. Also in line with these results are a lack of findings of elevated levels of IFN-γ in the CSF of patients with schizophrenia (Miller et al., 2011)

In summary, results from this study suggest a molecular pathway, leading from rs10158645 affecting the function of SNX7, in turn activating capase-8 that cleaves pro-IL-1β into mature functional IL-1β. IL-1β in turn induces TDO, leading to increased production of KYNA, that in turn modulates dopamine neurotransmission, known to contribute to the development of psychosis and cognitive deficits accompanying psychotic states.

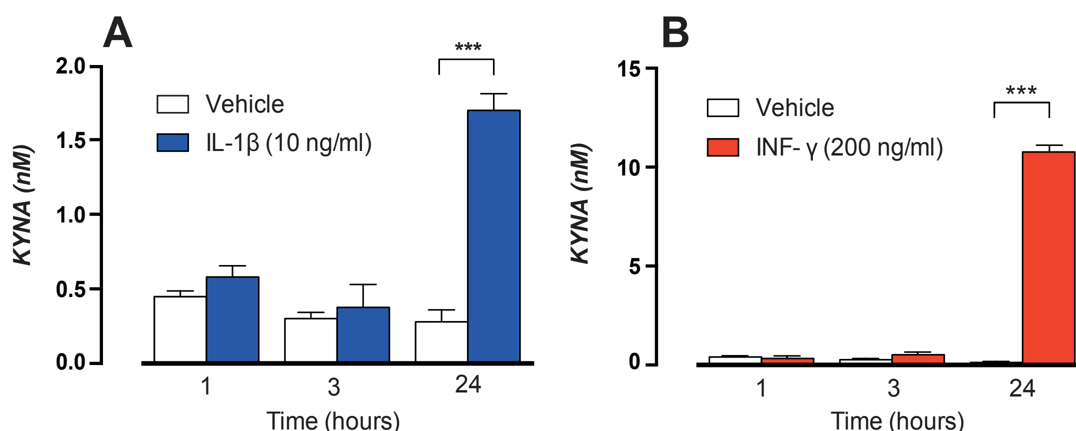

Figure 10. Kynurenic acid levels in fetal human cortical astrocytes stimulated with IL-1β and IFN-γ. Fetal cultured human cortical astrocytes stimulated with recombinant human IL-1β (10 ng/ml) and interferon (IFN)-γ (200 ng/ml). All cells were analyzed at time-points 1, 3, and 24 hours. Bar graphs represent % of vehicle (t=1h) at these time points. Experiments were performed in triplicate and repeated twice. Data are shown as mean±SEM. (A) At 24 hours, cells stimulated with IL-1β showed 4 times higher KYNA concentrations (1.70 ± 0.11 nM) than vehicle treated cells (0.42 ± 0.10 nM, $P<0.0001$). (B) At 24 hours, cells stimulated with IFN-γ had more than 85 times higher KYNA concentrations (10.8 ± 0.34 nM) than vehicle treated cells (0.14 ± 0.04 nM, $P<0.0001$). Statistical analyses: one-way ANOVA with Bonferroni post hoc tests. All *P*-values are two sided. Statistical significance set to $P<0.05$. *$P<0.05$, **$P<0.01$, ***$P<0.001$

4.2 PAPER II

Numerous studies show elevated levels of KYNA as well as kynurenine in the CSF and brain of patients with psychotic disorders (see introduction). In this study we aimed to investigate the CSF levels of QUIN, the main metabolite of the other branch of the kynurenine pathway, in patients with chronic schizophrenia.

Age was correlated to QUIN levels in controls (P=0.012) but not in patients, there was however a trend towards a correlation also in patients (P=0.113). Sex, height, weight, and BMI were not correlated to CSF QUIN in patients or in controls. In the patient group there was no correlation between CSF QUIN and psychiatric symptom ratings (BPRS and GAF) however, there was a tendency of a positive correlation between CSF QUIN and serum levels of olanzapine (ρ=0.414, P=0.062). There was no correlation between CSF QUIN levels and CSF olanzapine levels (ρ=0.333, P=0.140). The mean CSF QUIN concentration did not differ between smokers/non-smokers or male/females, in patients or in controls. (Table 2).

	Smoking		**Sex**		**Case**	
	Yes	No	Male	Female	Patient	Control
N	12	34	30	17	21	26
QUIN(mean±SEM)	16.5±1.8	20.5±1.0	19.2±1.3	19.4±1.0	20.6±1.5	18.2±1.1
P-value	0.074		0.913		0.198	

Table 2. QUIN levels in patients and controls and according to sex and smoking status.

4.2.1 No difference in QUIN, but a decreased QUIN/KYNA ratio in patients with schizophrenia compared to controls

The mean CSF QUIN concentration did not differ significantly between patients and controls (P=0.198; Figure 11A). Adjusting the comparison for potential confounding by age and sex gave a similar result (OR=0.93, 95% CI 0.80-1.10; P=0.355). The QUIN/KYNA ratio was however close to significantly decreased in patients compared to healthy controls (P=0.057; Figure 8B). Adjusting the analysis for differences in age and gender distribution in two groups strengthened the association between the patient group and a decreased QUIN/KYNA ratio

(OR=0.66, 95% CI 0.45-0.95; *P*=0.027). This result is likely to be independent of smoking status. The QUIN/KYNA ratio was higher in patients despite the fact that there was a nearly significant positive correlation between serum olanzapine and QUIN (ρ=0.414, *P*=0.062).

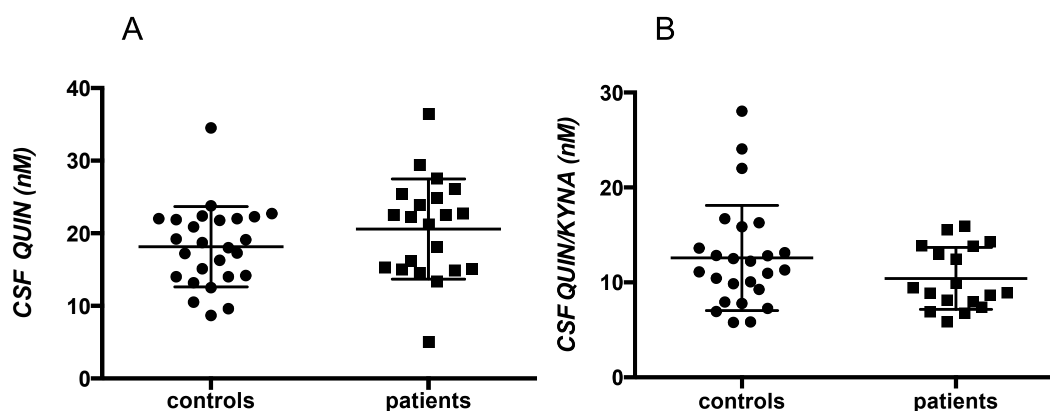

Figure 11. (A) Mean ± SEM CSF QUIN levels in patients with schizophrenia (20.6 ± 1.51 nM) and healthy controls (18.2 ± 1.08 nM; *P*=0.198). Following adjustment for age and sex OR=0.93, 95% CI 0.80-1.10; *P*=0.355 (B) the QUIN/KYNA ratio in patients with schizophrenia and healthy controls. Following adjustment for age and sex OR=0.66, 95% CI 0.45-0.95; *P*=0.027.

4.2.2 Relationship between tryptophan and its metabolites

A correlation between CSF QUIN and CSF kynurenine levels was detected in patients (ρ=0.53, *P*=0.014) but not in the controls (ρ=-0.32, *P*=0.117). A similar pattern was seen regarding CSF QUIN and CSF KYNA in patients (ρ=0.54, *P*=0.016) and in controls (ρ=0.12, *P*=0.565). No significant correlation between CSF tryptophan and CSF QUIN levels was detected in patients (ρ=0.32, *P*=0.164), or in controls (ρ=-0.18, *P*=0.373). See Table 3 for levels of kynurenine, KYNA and tryptophan. The fact that the correlation between QUIN on one hand and KYNA and KYN on the other hand was observed only in patients is indicating that the kynurenine pathway is upregulated in the patient group. Despite a correlation on the individual patient level, no difference in CSF QUIN levels between patients and controls was observed, suggesting that a putative enhancement of QUIN formation is substantially lower compared to the increase in kynurenine and KYNA in the patients. The lack of correlation between CSF QUIN and CSF

tryptophan might be attributed to the fact that no difference in the levels of neither tryptophan nor QUIN was observed.

	Patient	**Control**
N	21	26
tryptophan (μM)	1.7±0.03	1.8±0.07
N	21	26
kynurenine (nM)	57.2±3.5	37.3±4.3
N	19	26
KYNA (nM)	2.1±0.2	1.6±0.1

Table 3. CSF levels of tryptophan, kynurenine and KYNA in the present sample, presented as mean ± SEM. Part of these data have previously been published (Linderholm et al., 2012).

The higher QUIN/KYNA ratio in controls is in line with one previous study investigating levels of QUIN in post mortem brain of patients with schizophrenia (Schwarcz et al., 1988). Previous studies provide a mechanistic explanation for this. Thus, compared to the higher capacity of KAT enzymes, displaying *Km* values in the low millimolar range (Okuno et al., 1991), the KMO enzyme gets saturated at relatively low concentrations (*Km*≈20 μM; Bender and McCreanor, 1985), and can therefore act as a rate limiting step in the synthesis of QUIN. In a situation where L-kynurenine is elevated (e.g. by induction of TDO) such a limitation of KMO might guard against excessive production of QUIN. A suboptimal function of KMO could also present an explanatory mechanism of the observed increase in KYNA but not in QUIN (Holtze et al., 2012b; Lavebratt et al., 2014; Sathyasaikumar et al., 2011). Such a scenario would thus shunt the metabolism of L-kynurenine towards formation of KYNA, which indeed is observed in the present study. This study therefore renders support to the hypothesis that KYNA is elevated in patients with schizophrenia, likely due to a lower input of kynurenine into the QUIN branch of the pathway.

In summary, the present findings indicate an over-activated but also an imbalanced kynurenine pathway in patients with schizophrenia, favoring the production of KYNA over QUIN.

4.3 PAPER III

4.3.1 Detection of transcripts encoding kynurenine pathway enzymes

All the investigated kynurenine pathway transcripts (IDO1, TDO, KAT1, KAT2, KAT3, KAT4, KMO, KYNU, HAAO, QPRT) were detected in untreated fibroblast cell cultures. The levels of expression varied considerably across the different genes, with transcripts encoding IDO1 detected at the lowest level and those encoding KAT3 detected at the highest level. The variation across individual cultures (n=7), ranged from 2.5 (KAT3) to 145-fold (KYNU).

This is the first report that human skin fibroblast in culture express detectable levels of transcripts encoding the different enzymes of the kynurenine pathway. Substantial differences in the basal levels of expression across genes and individuals were observed which are likely to be explained by genetic and epigenetic variation between individual cultures.

4.3.2 Modulation of transcript-levels by IFN-γ and/or TNF-α

The levels of transcripts encoding IDO1 were significantly increased (>105-fold) in cultures stimulated with IFN-γ ($P<0.001$) as well as IFN-γ together with TNF-α ($P<0.001$) compared to unstimulated cultures, although no effect of TNF-α alone was observed (Figure 12A). Transcripts encoding tryptophan 2,3-dioxygenase (TDO), on the other hand, were significantly down-regulated in cultures stimulated with a combination of IFN-γ and TNF-α (20-fold; $P<0.001$), as compared to unstimulated cells or cells stimulated with the individuals cytokines (Figure 12B). Moreover, levels of transcripts encoding the kynurenine aminotransferases (KATs) were either unaffected (KAT2) or down-regulated (KAT1 and KAT3) following stimulation with a combination of IFN-γ and TNF-α (2.6- fold, $P<0.001$ and 1.7-fold, $P<0.01$ respectively, Figure 12C, D and E). Levels of transcripts encoding mitochondrial aspartate aminotransferase (mitAAT, i.e KAT4) were significantly down regulated (1.5-fold) in cultures treated with IFN-γ ($P<0.05$) and further decreased with the combination of IFN-γ and TNF-α (2.7-fold; $P<0.001$, Figure 12F). Levels of transcripts encoding kynurenine 3-monooxygenase (KMO) observed in the fibroblast cultures were not significantly affected by the cytokine treatment (Figure 12G).

Levels of transcripts encoding kynureninase (KYNU) were upregulated following IFN-γ (8-fold; $P<0.01$) or TNF-α stimulation (28-fold; $P<0.001$). A further increase in the levels of KYNU transcripts was observed with the combination of IFN-γ and TNF-α (650-fold; $P<0.001$, Figure 12H). Levels of transcripts encoding 3-hydroxyanthranilate 3,4-dioxygenase (HAAO) were upregulated only in cultures treated with the combination of IFN-γ and TNF-α (12-fold, $P<0.001$, Figure 12I). Levels of transcripts encoding quinolinate phosphoribosyltransferase (QPRT) were down-regulated by the combination of IFN-γ and TNF-α (5-fold, $P<0.001$), but unaffected by the individual cytokines (Figure 12J).

In line with previous studies (Byrne et al., 1986; Däubener and MacKenzie, 1999), human fibroblast cultures appear to be able to increase the rate of tryptophan degradation along the kynurenine pathway in response to IFN-γ stimulation, with a further increase when applying IFN-γ and TNF-α simultaneously. Our present findings support the notion that IDO1 is the major determinant of this response in human fibroblasts, as is also the case in many other cell types (King and Thomas, 2007).

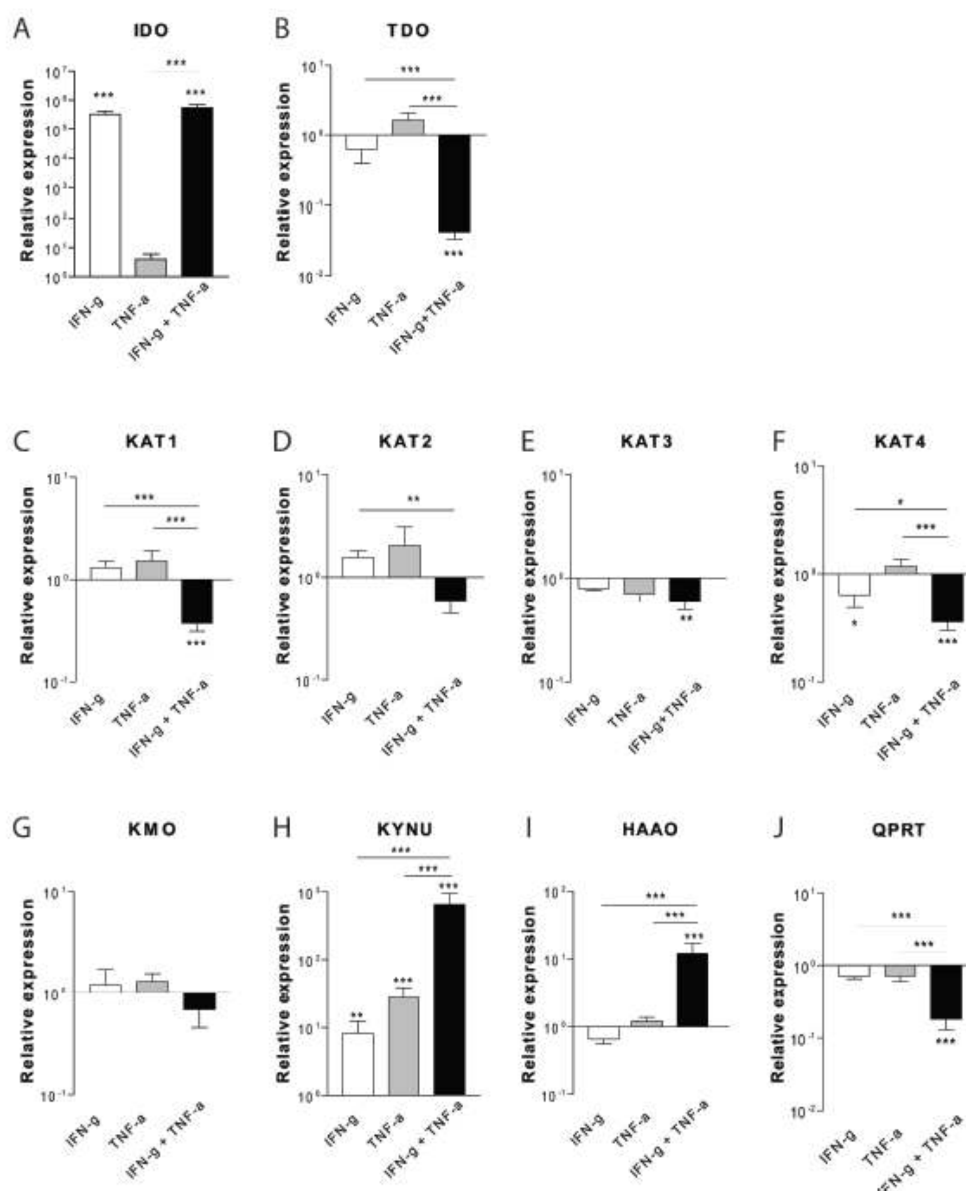

Figure 12. Relative levels of transcripts encoding enzymes in the kynurenine pathway (A-J) following treatment with IFN-γ (200 U/ml), TNF-α (100 U/ml) or the combination of these two cytokines (IFN-γ+TNF-α) during 48 hrs in serum-free cell culture medium (n = 7). Levels of all transcripts are normalized to levels observed in untreated control cells (baseline). **P*<0.05, ***P*<0.01, ****P*<0.001.

To address potential functionality of the kynurenine pathway in these human fibroblast cultures, we measured the accumulation of KYNA, one of the end metabolites of the pathway in the supernatants. Significantly higher levels were detected in supernatants of cells treated with IFN-γ or with IFN-γ and TNF-α compared to supernatants from unstimulated cells (*P*<0.0001; Figure 13). TNF-α alone did not cause a significant increase in the accumulation of KYNA.

In summary, the present results suggest that fibroblast cultures can be used to study disease-related abnormalities in the kynurenine pathway of tryptophan degradation. However, in order to extrapolate data from experiments in dermal fibroblasts to mechanisms in the CNS, further validation of this model is needed.

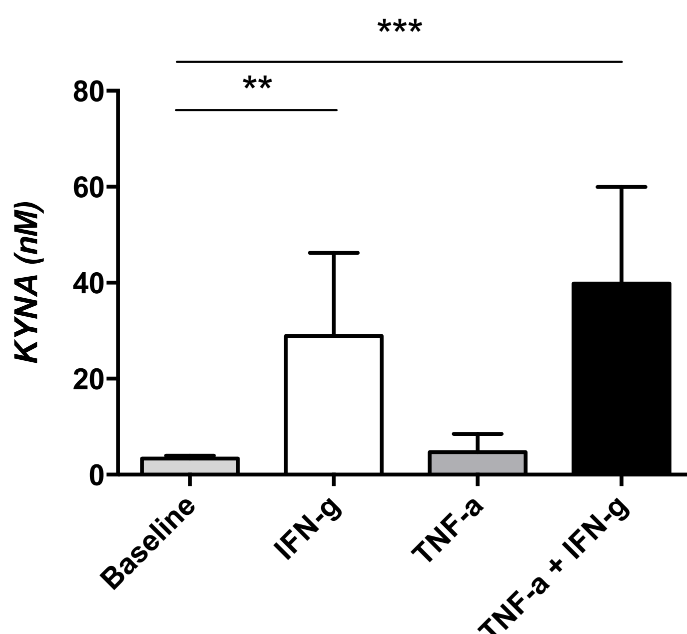

Figure 13. Levels of KYNA at baseline and following stimulation with IFN-γ and TNFα, alone or in combination. KYNA was significantly increased following stimulation with IFN-γ (27.2 ± 18 nM, P=0.01), as well as following stimulation with a combination of IFN-γ and TNF-α (39.8 ± 20.1 nM; P=0.0003) compared to baseline levels (3.4 ± 0.6 nM). All P-values are two sided. Statistical significance set to $P<0.05$. *$P<0.05$, **$P<0.01$, ***$P<0.001$

4.4 PAPER IV

4.4.1 Differences in CSF metabolites and cytokine levels between MZ and DZ twins

No difference was observed in the variance of any metabolite between MZ and DZ pairs (Table 4A). There was no difference in any of the investigated metabolites between probands and co-twins (Table 4B). There was however a trend towards higher levels of CSF KYNA, adjusted for smoking, in probands than in co-twins

(*P*=0.059). We could not detect any systematic difference in CSF metabolite levels between twin pairs in the MZ group compared to the DZ group (Figure 14).

The lack of systematic differences might be attributed to the fact that the sample is too small to detect any such differences. However, the relatively large difference in several CSF metabolites observed in individual MZ twin pairs, principally indicate that regulation of these metabolites partly is a result of environmental components. Hence, our findings are in consonance with the general view that the pathophysiological underpinnings of psychotic disorders also reflect a functional environmental constituent. Thus, the present results may contribute to our knowledge on gene-environment interactions in psychotic disorders.

	A			**B**		
	MZ Mean difference	**DZ Mean difference**	***P***	**Proband n=10**	**Co-twin n=13**	***P***
KYNA (nM)	2.1±0.6	5.0±2.8	0.690	5.8±1.6	2.9±0.4	0.115
QUIN (nM)	29.3±11.0	8.6±2.3	0.690	32.1±6.3	31.2±8.6	0.693
TRP (μM)	0.4±0.2	1.3±0.7	0.310	2.6±0.4	1.9±0.1	0.166
5-HIAA (nM)	41.9±16.1	36.8±20.2	0.548	94.1±12.6	81.6±10.4	0.446
HVA (nM)	39.2±15.2	55.2±25.2	1.000	125.5±18.8	101.1±12.8	0.284
IL-6 (pg/ml)	1.3±0.82	0.3±0.04	0.548	1.22±0.16	1.46±0.43	0.927
IL-8 (pg/ml)	8.0±5.6	6.9±1.7	0.421	28.2±4.9	29.7±6.0	0.879
TNF (pg/ml)	0.08±0.05	0.06±0.02	0.548	0.25±0.03	0.24±0.05	0.232

Table 4 A) Difference of CSF metabolite and cytokine levels between proband and co-twin in 5 MZ and 5 DZ pairs using Mann-Whitney U-test. Values represent absolute difference between proband and co-twin and are presented as mean ± SEM. B) CSF levels of metabolites and cytokines in probands and co-twins computed using Mann Whitney.

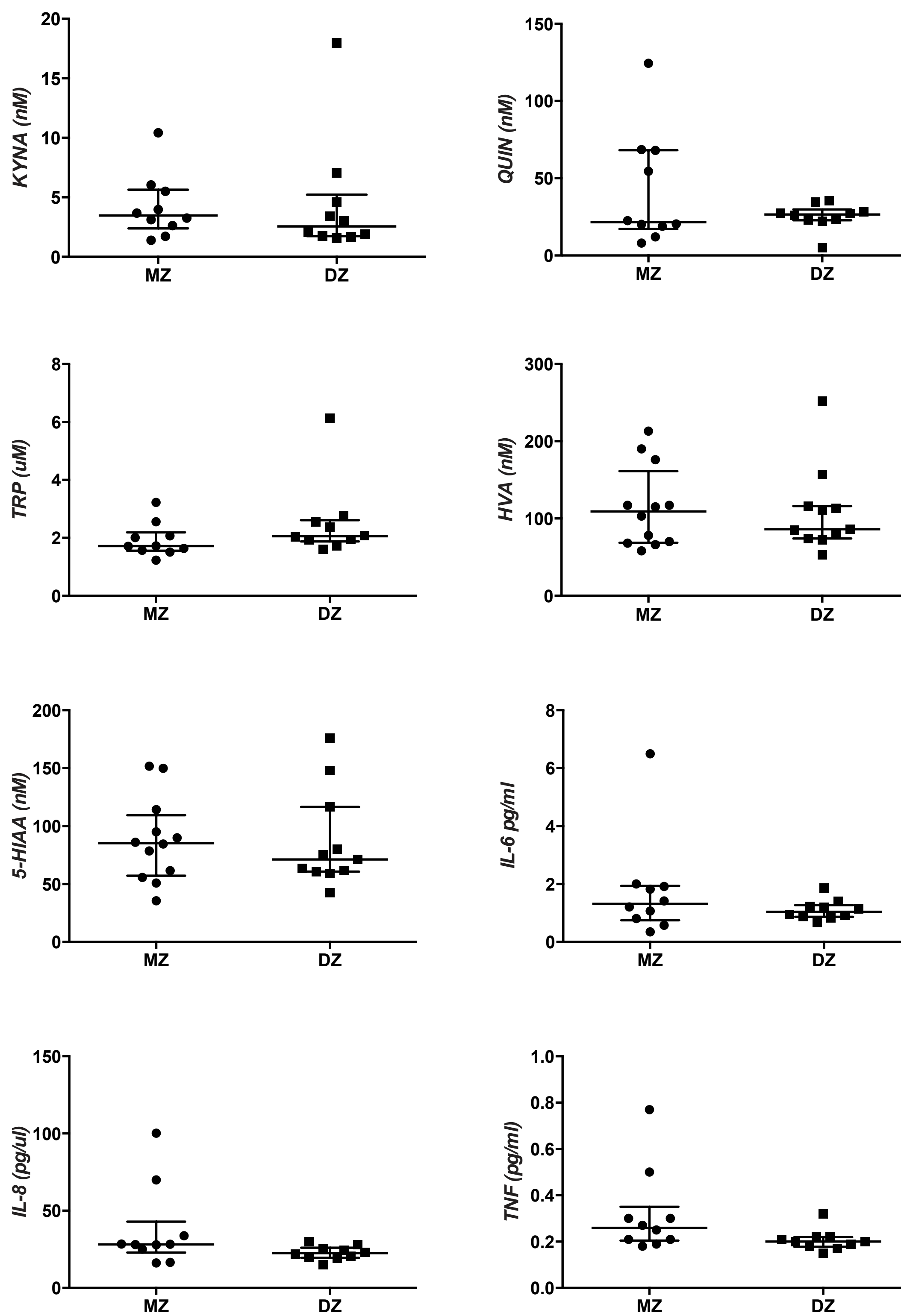

Figure 14. CSF levels of KYNA, QUIN, TRP, HVA, 5-HIAA, IL-6, IL-8 and TNF-α in probands and co-twins displayed for MZ and DZ pairs.

4.4.2 Correlations between CSF metabolites and cytokines

There was a correlation between CSF levels of IL-8 and CSF QUIN (rho=0.481, *P*=0.020), CSF IL-8 and CSF TRP (rho=-0.617, *P*=0.002), CSF KYNA and CSF HVA (rho=0.577, *P*=0.004), CSF KYNA and CSF 5-HIAA (rho=0.660, *P*=0.001), and a nearly significant correlation between CSF TRP and TNF-a (rho=-0.41, *P*=0.051).

These findings that are in line with previous data showing correlations between KYNA, HVA and 5-HIAA, (Nilsson-Todd et al., 2007; Nilsson et al., 2007), as well as a link between inflammatory signaling and the kynurenine pathway (Mándi and Vécsei, 2012), are illustrating the notion that immune signaling, tryptophan breakdown pathways and dopamine neurotransmission are linked. All of these components are also separately (and in combination) implicated in psychotic disorders.

4.4.3 Relation of levels of CSF metabolites and cytokines to psychometric rating scales

Analyzing the levels of the individual metabolites in relation to the psychiatric rating scales revealed an association of CSF KYNA, adjusted for smoking, to total SCID-II score (*P*=0.018) and to total SPQ-B score (*P*=0.049). CSF HVA, adjusted for neuroleptic use, was associated to total SCID-II score (*P*=0.007), to total SAPS score (*P*=0.003) and to total SANS score (*P*=0.024). 5-HIAA, adjusted for use of antidepressant medication, was associated to total SCID-II score (*P*=0.002) and to total SPQ-B score (*P*=0.010), total SANS score (*P*<0.001) and total SAPS score (*P*=0.001). CSF QUIN, when adjusted for CSF albumin levels, was associated to total SPQ-B score (*P*=0.027). No other kynurenines or cytokines were associated to any of the rating scales (See Table 5).

4.4.4 KYNA, HVA and 5-HIAA associations with sub-ratings

Since the total score for SCID-II in our analysis consisted of the sub-ratings for paranoid, schizoid and schizotypal personality disorder (cluster A), an analysis of the sub-ratings revealed an association of CSF KYNA with schizotypal personality (*P*=0.013) but not schizoid (*P*=0.103). There was a trend towards an association between CSF KYNA and the paranoid sub-scale (*P*=0.062). The associations between levels of CSF HVA and sub-ratings of SCID-II were significant for all three parts, i.e. paranoid (*P*=0.013), schizoid (*P*=0.006) and schizotypal (*P*=0.020). 5-HIAA was associated to the subrating for schizotypal (*P*=0.035), but not for the paranoid and schizoid personality disorders.

	SPQ-B		SCID-II		
	Estimate (95%CI)	***P***	**Estimate (95%CI)**	***P***	**Co-factor**
KYNA	1.048 (1.000-1.099)	0.049*	1.082(1.015-1.153)	0.018*	Smoking
QUIN	1.051 (1.006-1.098)	0.027*	1.001 (0.928-1.079)	0.982	CSF Albumin
TRP	1.007 (0.980-1.034)	0.606	1.008 (0.978-1.039)	0.588	None
5-HIAA	1.043 (1.012-1.075)	0.010*	1.083 (1.036-1.113)	0.002**	Antidepressant
HVA	1.033 (0.998-1.069)	0.066	1.066 (1.020-1.114)	0.007**	Neuroleptic
IL-6	0.977 (0.925-1.031)	0.377	0.994 (0.925-1.070)	0.872	Snuff
IL-8	0.979 (0.956-1.002)	0.067	0.983 (0.956-1.011)	0.193	Neuroleptic
TNF	0.985 (0.966-1.005)	0.127	0.985 (0.959-1.012)	0.241	Neuroleptic

	SANS		SAPS		
	Estimate (95%CI)	***P***	**Estimate (95%CI)**	***P***	**Co-factor**
KYNA	1.010 (0.998-1.023)	0.562	1.019 (0.998-1.042)	0.075	Smoking
QUIN	1.008 (0.995-1.021)	0.223	1.004 (0.984-1.025)	0.647	CSF Albumin
TRP	0.997 (0.991-1.003)	0.346	0.996 (0.984-1.007)	0.455	None
5-HIAA	1.014 (1.007-1.020)	<0.001*	1.022 (1.010-1.033)	0.001**	Antidepressant
HVA	1.014 (1.006-1.023)	0.003**	1.020 (1.003-1.037)	0.024*	Neuroleptic
IL-6	0.998 (0.984-1.012)	0.734	1.001 (0.986-1.015)	0.935	Snuff
IL-8	0.994 (0.986-1.003)	0.187	0.999 (0.987-1.012)	0.867	Neuroleptic
TNF	0.996 (0.989-1.003)	0.278	0.992 (0.983-1.000)	0.057	Neuroleptic

Table 5. Linear mixed model analysis of KYNA, QUIN, tryptophan, HVA, 5-HIAA, IL-6, IL-8 and TNF levels against SPQ-B, SCID-II, SANS and SAPS.

The presently found associations between KYNA, HVA and 5-HIAA with measures of schizotypal traits in the SCID-II and SPQ-B ratings are in line with previous experimental and clinical observations. KYNA is thus tightly linked to dopamine signaling in the brain and modulates midbrain dopaminergic firing (Erhardt and Engberg, 2002; Linderholm et al., 2007; Olsson et al., 2009; Schwieler et al., 2004).

In addition experimental studies show an increased dopamine release and increased locomotor activity following an amphetamine challenge in rodents with centrally elevated KYNA levels (Olsson et al., 2009, 2012b). The levels of CSF KYNA have also previously been found to correlate to CSF levels of the monoamine metabolites HVA and 5-HIAA; (Nilsson-Todd et al., 2007; Nilsson et al., 2007). These findings contribute to the notion that KYNA, as well as dopamine, plays a role in psychotic disorders, in this context extended to sub-clinical features captured by the SCID-II and SPQ-B ratings.

In the present study, there was a trend towards an association between KYNA and SAPS. All the patients were however, well medicated, and had a relatively high level of functioning, which might explain that the association to the SAPS score did not reach significance. This indicates that measures of schizotypal features might be more effective in detecting the traits shared between patients with psychotic disorder and their first-degree relatives, and thus reflect a genetic vulnerability to psychotic disorder. Indeed, a shared genetic basis of schizophrenia and schizotypal personality traits has previously been suggested (see Siever and Davis, 2004 for review).

The positive association between HVA and SANS detected here has to our knowledge not been reported previously. A limitation of the present study is that our cohort is small and highly heterogeneous, with a proportion of patients with major depression. Since the SANS might also pick up traits of depressive character, we cannot exclude that the correlation reflects other pathogenic processes although the total SANS score in our sample was not high in depressed patients. On the contrary, CSF 5-HIAA levels, in this sample also associated to both SANS and SAPS, have previously been associated with suicidal behavior (Jimenez-Treviño et al., 2011). It would be of value to investigate the relation of specific metabolites or cytokines on individual items; however, in the present study such an approach was hampered by the sample size. Studying the similarities and differences in the associations between KYNA, HVA and 5-HIAA to the investigated rating scales, might reflect distinct disease processes connected to different neurochemical aberrations. Thus, specific symptoms of psychotic disorders may be reflected by distinct pathophysiological mechanisms.

KYNA has previously been shown to be associated to manic and psychotic features in individuals with bipolar disorder (Olsson et al., 2012a). In our study, the trend towards an association to positive symptoms using SAPS did not reach significance, tentatively related to lack of power as well as to the fact that all patients were well-medicated.

None of the cytokines analyzed was found to correlate to any psychometric scale. This may be related to the fact that the cytokine analysis was made on previously thawed samples. Indeed, freeze-thaw cycles are known to affect the concentration of cytokines in human samples(de Jager et al., 2009; Lindqvist et al., 2009; Schwieler et al., 2014).

In conclusion, the present results indicate that the lack of a systematic differences between MZ-, and DZ pairs, as well as the relatively large variability within the MZ group, reflect a strong environmental effect upon the levels of these markers. In addition the present result show that CSF KYNA, CSF HVA and CSF 5-HIAA are associated to ratings of schizotypal personality traits in a cohort of twins with and without psychotic morbidity, a finding that constitutes another piece of evidence of the importance of KYNA in psychotic symptomatology.

5 GENERAL DISCUSSION

Experimental and clinical evidence for an immunological component in psychotic disorders is amassing. Clinical research investigating neuroinflammation and immune signaling in psychotic disorders show elevated levels of the cytokines IL-1β and IL-6 in patients with schizophrenia and bipolar disorder (Garver et al., 2003; Sasayama et al., 2013; Schwieler et al., 2014; Söderlund et al., 2009, 2011). GWAS efforts in schizophrenia contribute to this picture by repeatedly reporting hits in the MHC region (Jia et al., 2011; Ripke et al., 2014; Stefansson et al., 2009). The activation of the kynurenine pathway, being critically regulated by the immune system, may directly convey immune signaling to glutamatergic, cholinergic and dopaminergic neurotransmission, hereby linking the view of an inflammatory component of schizophrenia with the well established dopamine- and glutamate hypotheses of the disease (see Müller et al., 2011 for review). Results from this thesis further strengthen the view that KYNA is a pivotal compound along the mechanistic pathways leading to psychosis. By performing causal inference analyses, we were able to confirm the association of KYNA with positive psychotic symptoms, likely induced by increased dopamine signaling. Furthermore, in bipolar disorder we found a genome-wide significant gene variant, rs10158645, which might impact the levels of KYNA allegedly by a previously unknown route; a decreased function of SNX7 leading to increased signaling through caspase-8/IL-1β. We also found that rs10158645, as well as both KYNA and IL-1β, associated with cognitive flexibility in the set-shifting paradigm, with higher levels of IL-1β and KYNA in the group with poor performance. The results from this thesis hence confirm prior data proposing a role for KYNA in psychosis and cognition, and more importantly, they also extend our knowledge on the signaling pathways involved (see Figure 15).

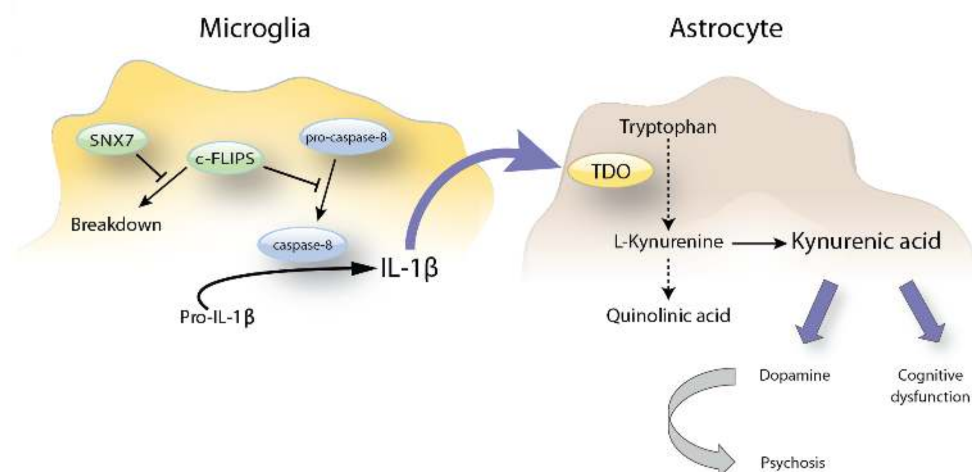

Figure 15. SNX7 mediated induction of the kynurenine pathway in bipolar disorder

The kynurenic acid hypothesis of schizophrenia states that levels of KYNA are elevated in patients with schizophrenia. QUIN, representing the other, microglial, branch of the kynurenine pathway has been assumed to be in the range of healthy controls (Schwarcz et al., 1988). Whether there is a specific activation of the KYNA branch without activation of the microglial branch of tryptophan degradation in patients with psychotic disorders has been repeatedly questioned. *In vitro* studies using pro-inflammatory cytokines, e.g. IFN-γ often show an equal activation of both branches of the pathway, and in certain disorders both QUIN and KYNA are elevated (see introduction). We, therefore, measured CSF levels of QUIN in patients with schizophrenia and in healthy controls and could confirm the picture with a selective activation of the KYNA branch of the kynurenine pathway. QUIN levels in patients were hence in the same range as in the healthy controls, and, as a result, the QUIN/KYNA ratio was higher in controls compared to patients with schizophrenia. There are several rational explanations for a specific increase in KYNA in psychotic disorders. Firstly, the increased activity of the kynurenine pathway might be attributed to increased activity of TDO, induced by IL-1β as observed in Paper I of this thesis. Secondly, the predominance of the KYNA branch in psychotic disorders might be attributed to the differences in activity of the KAT and the KMO enzymes. In contrast to the KAT enzymes, displaying *Km* values in the millimolar range (Okuno et al., 1991) the KMO enzyme gets saturated at relatively low concentrations ($Km \approx 20$ μM; Bender and McCreanor, 1985), and can, therefore, act as a second rate-limiting step in the synthesis of QUIN. In a situation were levels of kynurenine are elevated, a

comparatively larger proportion of kynurenine is converted to KYNA, unless downstream enzymes, leading to QUIN, are induced as well. In psychotic disorders this is of particular relevance since a suboptimal function of the KMO enzyme, in conjunct with elevated levels of KYNA, are reported (Holtze et al., 2011; Lavebratt et al., 2014; Sathyasaikumar et al., 2011). The notion of KMO being indirectly responsible for KYNA levels is supported by experimental studies where elevated KYNA levels are seen in *Kmo* knockout mice as well as following administration of pharmacological inhibitors of KMO (Erhardt et al., 2001b; Giorgini et al., 2013; Speciale et al., 1996; Zwilling et al., 2011). However, the activity of other enzymes of the kynurenine pathway might also contribute to the clinically observed imbalance in kynurenine metabolism. For example, a disruptive mutation in KYNU, the enzyme converting 3-HK to 3-HAA (see Figure 1) was recently identified in a study investigating rare mutations constituting a polygenic burden in schizophrenia (Purcell et al., 2014).

In this thesis, cell culture studies were employed to study specific biological processes. The use of simplified *in vitro* models in the studies of psychiatric disorders might be viewed upon with skepticism, but are an invaluable compliment to clinically oriented research. This is specifically the case in fields where direct tissue observation possibilities are limited, for instance in studies involving the brain. As technical advancements in brain imaging are gaining new ground, such techniques hold a promise of large contributions to future psychiatric research. *In vitro* techniques can however provide answers that will not be accessible by other methods for decades to come. In this thesis, cell cultures were employed to study the kynurenine pathway, namely the enzymes and metabolites produced along its path. In study I, we were able to show that astrocytic TDO is specifically upregulated in response to IL-1β, leading to an increased production of KYNA. TDO, in contrast to IDO, has for long been considered not to be regulated by immunological stimuli. Albeit the evidence for a direct involvement of IDO in psychotic disorders has been scarce, IDO is the enzyme most frequently cited in the context of kynurenine pathway induction in psychiatric disorders. The reason for the negligible interest in TDO is the general view that TDO is not regulated by inflammatory stimuli. This assumption is however made based on a too general interpretation of data. TDO is hence neither induced in response to stimulation with the prototypical IDO inducer IFN-γ or in response to a general immune stimulation with endotoxins (Saito et al., 1991, 1992; Takikawa et al., 1986).

However, it turned out during the work with this thesis, that TDO is indeed up-regulated in response to IL-1β. This finding is consistent with the fact that TDO-, but not IDO expression, is elevated in the brains of patients with schizophrenia and bipolar disorder (Miller et al., 2004, 2006).

A drawback of our *in vitro* studies is that fetal astrocytes were used. Some studies point to the fact that fetal astrocyte populations might differ from adult populations in the type of surface receptors they express, and thus their responses to external stimuli (Lafortune et al., 1996). However, also adult astrocytes do not constitute a single homogenous population as it is proposed that several different subtypes of astrocytes exist (Álvarez et al., 2014; Castejón, 1999; Hochstim et al., 2008; Svensson and Brodin, 2010). Adult human astrocytes are however not easily accessible. Therefore, a high validity model of the kynurenine pathway, based on peripheral cells such as dermal fibroblasts would have a large impact on the possibilities to perform biological research in psychiatric disorders. During the work with this thesis, the prospect to use dermal fibroblasts for the study of the kynurenine pathway was hence investigated.
Dermal fibroblasts are not only relatively easy to collect from adults, but can also easily be assembled from different individuals, with and without psychotic disorders. This introduces the opportunity to investigate differences in molecular genetics between patients and controls using cell culture experiments. The data presented in this thesis confirm that fibroblasts indeed express the entire kynurenine pathway and that the cells are sensitive to stimulation with proinflammatory cytokines in order to increase the activity of the pathway. However, many questions remain to be answered. Most importantly, does the response to specific cytokines resemble responses in glial cells? Are the relative increases in expression of specific enzymes in agreement with what we know from studies of microglia or astrocytes? The fibroblast model has been further investigated in patients with schizophrenia and bipolar disorder (Johansson et al., 2013), but needs further validation before it can be considered a reliable model. Nevertheless, the results from this thesis support the view that also peripheral cell systems are valuable tools for research in psychiatric disorders.

Based on the above mentioned complexities of cell culture experiments, it is crucial to take the entire context of immune signaling into consideration when designing a study investigating the impact of immune signaling on metabolic systems or

neurotransmission; the cellular source of the cytokines, the type of target cells, and synergistic effects by combinations of cytokines may all affect the observed results. Hence, results from studies using an approach with endotoxin or viral exposure will likely differ from studies using single cytokines as the former will illicit a general immune response composed of the whole array of immune components. The observed result will, therefore, be the sum of all these mechanistic sub-components. Studying single cytokines instead allows narrowing down on specific mechanisms. Both approaches have their strengths and weaknesses and will undoubtedly model different aspects of immune-brain interactions.

In this thesis, data on a number of kynurenine metabolites and cytokines in twins with severe psychiatric disorders are presented for the first time. Twin studies have been an invaluable resource for investigating the impact of genes on specific phenotypes. They have aided in assessing heredity rates for disorders, such as schizophrenia and bipolar disorder, and are as valuable in exploring heredity of less complex phenotypes, such as levels of kynurenine metabolites. In this study we found evidence of an environmental impact on the levels of TRP, KYNA, QUIN, HVA, 5-HIAA, IL-6, IL-8 and TNF-α.

Results presented in paper IV, further complement and support the findings of a link between KYNA and psychosis, in this case extended to subclinical schizotypal features also present in first-degree relatives of patients. These data, therefore, contribute to the notion that brain KYNA is a potential endophenotype for psychotic disorders. Data presented in this thesis indicate that non-affected family members tend to have higher CSF levels of KYNA than previously observed in healthy controls (Erhardt et al., 2001a; Linderholm et al., 2012; Nilsson et al., 2007). A higher rate of the phenotype in non-affected family members compared to the general population is one of the criteria in order to consider a phenotype an endophenotype (Gottesman and Gould, 2003). Further studies of inheritance patterns and the expression of the phenotype in relatives of patients with psychotic disorders are, therefore, warranted.

In all the clinical studies in this thesis, patients were treated with antipsychotic medication. This is indeed a problem inherent to almost all clinical research of psychotic disorders. The use of statistical models where medication is added as an explanatory variable might in part compensate for this, hence reducing the risk that

findings are due to medication effects. Nevertheless, in the present studies it is difficult to fully disregard effects of medication, and several studies show effects of antipsychotic medication on immune signaling (Müller et al., 1997; see Pollmächer et al., 2000 for review). In order to dispel any prevailing doubts about the authenticity of these findings further studies employing drug-naïve, and drug-free patients are needed.

According to the results of this thesis, several possible pathological mechanisms leading to the development of psychosis may exist. It is known that a genetic vulnerability is the strongest risk factor for both schizophrenia and bipolar disorder. Culprit genes may encompass the regulation of immune signaling as was observed in paper I, where rs10158645 was associated with a reduced expression of *SNX7*. Disrupted immune signaling, in the form of elevated levels of specific cytokines, may induce TDO, hence increasing the production of KYNA. Brain KYNA then modulates glutamatergic, cholinergic, and, indirectly, dopaminergic neurotransmission. All these neurotransmitter system are implicated in the development of psychotic behavior and cognitive functions.

The results of the present thesis add new, substantial information regarding pathological mechanisms leading to the development of psychotic disorders. We suggest a chain of immune signaling events, originating in a genetic aberration, affecting tryptophan metabolism and dopamine neurotransmission. This molecular pathway is here implicated in core symptoms of psychotic disorders like cognitive impairment and psychosis. Clearly, the presently revealed dysfunction of immune signaling may open up for novel strategies in the treatment of psychotic disorders.
Intracellular signaling pathways are still sparsely investigated in psychotic research. Clinically observable changes in neurotransmission and immune signaling are however accompanied by, or even based on alterations in intracellular signaling pathways. The study of such pathways hence provides new directions in which to focus our research, as well as targets for new and better pharmacological interventions.

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