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# THE KYNURENINE PATHWAY AND DEVELOPMENT OF SCHIZOPHRENIA – IMMUNOLOGICAL AND GENETIC ASPECTS

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# ABSTRACT

Schizophrenia is a complex disorder with symptoms ranging from hallucinations to poor social functioning and cognitive deficits. Despite decades of research, the etiology of the disease remains puzzling. Genetic aberrations as well as exposure to infection during early life are associated with an increased risk for development of the disease. Recent studies also describe an immune activation in schizophrenia. The immune-related and neuroactive compound kynurenic acid (KYNA), is implicated in the pathophysiology of the disease. KYNA is an astrocyte-derived end product of the kynurenine pathway, the main metabolic route of tryptophan degradation. The aim of the present thesis was to evaluate if disturbances – genetic or caused by early-life infection – in the kynurenine pathway, in particular with regard to KYNA, could contribute to the development of neuropsychiatric disorders, such as schizophrenia. Our results show that infection with the neurotropic influenza A/WSN/33 virus generates a profound induction of the kynurenine pathway *in vitro* in mouse cell cultures of cortical glial cells and hippocampal neurons, including a robust increase of transcripts encoding IDO and TDO, the enzymes regulating the first and rate-limiting reaction of KYNA production. A systemic injection of the influenza A/WSN/33 virus to wild-type and immune-deficient mice (*Tap1*$^{-/-}$), that lack CD8$^{+}$ T cells, at postnatal day (P) 3 or 4 showed that the infection stimulated the kynurenine pathway in early life. The *Tap1*$^{-/-}$ mice showed a more persistent induction of the kynurenine pathway enzymes. At P13, infiltration of T cells was observed in the brains of infected wild-type mice, accompanied by a transient elevation of brain KYNA, which was also observed in the *Tap1*$^{-/-}$ mice. When investigating the long-term behavioral effects of the neonatal infection, we found that *Tap1*$^{-/-}$, but not wild-type mice, displayed impaired prepulse inhibition (PPI) in adult life. In adult wild-type mice, the neonatal virus infection was associated with a potentiated D-amphetamine-induced increase in horizontal activity, a behavioral response proposed to reflect schizophrenia. To assess the specific role of brain KYNA in the behavioral abnormalities seen following neonatal infection, brain KYNA was elevated in wild-type mice at P7-16 by the administration of its immediate precursor L-kynurenine. Similar to infected *Tap1*$^{-/-}$ mice, these mice showed a mild disruption in PPI in adulthood. Furthermore, these mice showed a tendency of hyper-responsiveness to D-amphetamine in locomotor activity. These results indicate that induction of the kynurenine pathway, involving a transient accumulation of brain KYNA in early life, could contribute to behavioral aberrations in adulthood related to schizophrenia. Thus, elevated levels of brain KYNA during a critical period in neurodevelopment might offer a molecular basis of infection as a risk factor for schizophrenia. Furthermore, this thesis reveals that elevated levels of CSF KYNA in human subjects of a Swedish population are associated with a missense single nucleotide polymorphism in the *KMO* gene, rs1053230. This genetic deficit could result in a dysfunctional KMO enzyme, thereby shunting kynurenine metabolism to KYNA, in line with the increased levels of kynurenine and KYNA observed in patients with schizophrenia.

*Altogether, this thesis suggests that an early-life CNS induction and/or a genetic deficit of the kynurenine pathway could predispose for development of schizophrenia through the elevation of brain KYNA.*

# LIST OF PUBLICATIONS

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACMSD | 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase |
| ALS | amyotrophic lateral sclerosis |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole propionate |
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| α7nACh | α7 nicotinic acetylcholine |
| BBB | blood-brain barrier |
| BDV | Borna disease virus |
| CNS | central nervous system |
| CNV | copy number variation |
| COX | cyclooxygenase |
| CSF | cerebrospinal fluid |
| Ct | cycle threshold |
| DSM | Diagnostic and Statistical Manual |
| e.g. | for example (*exempli gratia* lat.) |
| GABA | γ-aminobutyric acid |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GPR35 | G protein-coupled receptor 35 |
| GWAS | genome-wide association studies |
| HAAO | 3-hydroxyanthranilate 3,4-dioxygenase |
| HIV | human immunodeficiency virus |
| HPLC | high-performance liquid chromatography |
| HSV | herpes simplex virus |
| ICD | International Classification of Diseases |
| IDO | indoleamine-pyrrole 2,3-dioxygenase |
| i.e. | that is (*id est* lat.) |
| IFN | interferon |
| IL | interleukin |
| i.p. | intraperitoneal |
| ISI | interstimulus interval |

| | |
|---|---|
| KAT | kynurenine aminotransferase |
| $K_m$ | Michaelis-Menten constant |
| KMO | kynurenine 3-monooxygenase (kynurenine 3-hydroxylase) |
| KYNA | kynurenic acid |
| KYNU | kynureninase |
| LD | linkage disequilibrium |
| LPS | lipopolysaccharide |
| MHC | major histocompatibility complex |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NMDA | *N*-methyl-D-aspartate |
| P | Postnatal day |
| PA | picolinic acid |
| PCP | phencyclidine |
| PCR | polymerase chain reaction |
| PPI | prepulse inhibition |
| QPRT | quinolinate phosphoribosyltransferase |
| QUIN | quinolinic acid |
| SEM | standard error of the mean |
| SNP | single nucleotide polymorphism |
| TAP | transporter associated with antigen processing |
| TBE | tick-borne encephalitis |
| TDO | tryptophan 2,3-dioxygenase |
| TLR | Toll-like receptors |
| TNF | tumor necrosis factor |
| 3-HK | 3-hydroxykynurenine |
| 3-HAA | 3-hydroxyanthranilic acid |

# 1 INTRODUCTION

## 1.1 SCHIZOPHRENIA

Schizophrenia is a devastating and disabling mental disorder that emerges independently of sex, ethnical origin or social/economic status, with a global lifetime prevalence estimated to 0.55-0.87% (Goldner et al., 2002; Perälä et al., 2007). The disease is commonly characterized by an abnormal reality perception, often with a sentiment of lost identity and mental capacity, impairing life dramatically by affecting both social and mental functioning.

Schizophrenia develops insidiously and gradually, typically in late adolescence with a delay of 3-4 years for females (Hafner, 1998). The disease shows a complex and diverse profile, and the symptoms may vary between patients and also within individuals over time. The symptoms are generally divided into three broad categories: Positive (also denoted as psychotic) symptoms, negative symptoms and cognitive dysfunctions (Andreasen, 1995). Positive symptoms refer to those features that are additive to normal behavior, including auditory hallucinations and bizarre delusions as well as disorganized or monotonic speech. Loss of normal functioning, such as social and emotional withdrawal, as well as loss of ambition or will, and a reduced ability to experience pleasure are classified as negative symptoms. Cognitive dysfunctions include a broad range of features where the patient has difficulties in problem solving and abstract thinking. Poor judgment and deficits in attention and memory are also examples of cognitive dysfunctions observed in patients with schizophrenia. As a uniform guideline for clinicians worldwide, schizophrenia diagnosis can be established with either of the two international diagnostic criteria systems: The 4$^{th}$ edition of the American Psychiatric Association's Diagnostic and Statistical Manual of mental disorders (DSM-IV) or the 10$^{th}$ International Classification of Disease (ICD-10).

The onset of schizophrenia (typically presented as an acute psychosis) is often preceded by non-psychotic premorbid signs of neurological and behavioral problems, reviewed in (Jones, 1997). Already during the first years of life, delayed development of speech and motor functions (standing, walking etc.) has been observed in children destined to develop schizophrenia (Jones et al., 1994; Rosso et al., 2000; Isohanni et al., 2001).

Social withdrawal and isolation as well as lower IQ scores have also been reported for children that later develop the disease (Aylward et al., 1984; Jones et al., 1994; Woodberry et al., 2008). The observations in early life, before the characteristic symptoms appear, suggest a neurodevelopmental influence on schizophrenia pathogenesis, see (Lewis and Levitt, 2002). Such potential disturbances of brain maturation could be a consequence of either genetic or environmental insults, or a combination of the two factors.

## 1.2 RISK FACTORS FOR THE DEVELOPMENT OF SCHIZOPHRENIA

Over the years, a wide range of factors have been suggested to display a specific risk for development of schizophrenia. These factors include environmental influences throughout the life span, such as *Toxoplasma gondii* exposure and cannabis use (Semple et al., 2005; Yolken et al., 2009), as well as genetic susceptibility to the disease. Though these factors are often considered individually, it is not unlikely that environmental exposure acts in conjunction with a genetic vulnerability (van Os and Kapur, 2009; Owen et al., 2011).

### 1.2.1 Environmental influences

Substantial evidence from numerous studies, not least those reporting a seasonal variation in the number of births of individuals later diagnosed with schizophrenia, reviewed in (Torrey et al., 1997), suggests that factors related to fetal, neonatal and/or early life environment are involved in the etiology of schizophrenia. In line with this view, previous studies have reported an increased risk for the disease that is associated with factors such as complications during pregnancy and delivery, exposure to infection during early life, migration or ethnical minority status during childhood, or urban upbringing. Stress, social adversity or drug abuse, e.g. cannabis, during adolescent or early adult life also influences the risk of developing schizophrenia, although these factors may be more related to the onset of the disease, reviewed in (Cannon and Clarke, 2005; Brown, 2011). Already at the time of the initial description of schizophrenia by Kraepelin and Bleuler in the early 1900s, pathological characteristics prior to the disease diagnosis were noted. In 1987, a neurodevelopmental hypothesis of

schizophrenia was presented in two influential studies (Murray and Lewis, 1987; Weinberger, 1987). The hypothesis is based on the observation of structural brain changes in patients with schizophrenia, seen already at the onset of the disease, and occurrence of premorbid signs at young age in individuals destined to develop schizophrenia. Also, the notion that neonatal lesions in the primate brain could result in delayed behavioral disturbances contributed to this theory. Thus, disturbances in the central nervous system (CNS) during pre- or postnatal life may result in disturbed development of the nervous system, for example involving perturbed maturation of brain circuits, reviewed in (Lewis and Levitt, 2002). Such disturbances might involve exposure to infectious agents in early life.

#### *1.2.1.1 Infections in early life*

Infections during pregnancy, induced for example by herpes simplex virus (HSV), human immunodeficiency virus-1 (HIV-1), varicella zoster virus or the protozoan parasite *Toxoplasma gondii* can cause neurological sequelae in the offspring (Johnson, 1994). However, long-term consequences on behavior and cognitive functions may be more subtle, without any explicit signs of malformation. The 1957 influenza A2 pandemic in Finland became an influential target of studies where infection in early life was associated with schizophrenia development. It was reported that the offspring of mothers who were exposed to influenza virus infection during the second trimester of pregnancy had an increased risk of being hospitalized with schizophrenia diagnosis as adults (Mednick et al., 1988). However, this study had several limitations as the results were based on hospital records, and there were no evidence that the mothers were actually infected during this time-point. In a prospective study, maternal respiratory infections during the second trimester were also reported to increase the risk of schizophrenia spectrum disorders in the offspring (Brown et al., 2000b). These data included different infectious agents, indicating that the respiratory infectious risk may be wide-ranging, rather than influenza-specific. Some years later, Brown and colleagues examined serological data which implied a seven-fold increased risk of schizophrenia development in individuals exposed to influenza virus during the first trimester of prenatal life (Brown et al., 2004a).

Also other neonatal infections have similarly been associated with the later development of schizophrenia, reviewed in (Karlsson, 2003; Yolken and Torrey, 2008). Serological studies report increased levels of HSV type 2 antibodies in mothers who gave birth to offspring later developing schizophrenia or other psychotic illnesses (Buka et al., 2001a; Buka et al., 2008; Mortensen et al., 2010), and serologically documented exposure of rubella virus during fetal life has also been reported to increase the risk of non-affective psychosis in adulthood (Brown et al., 2000a). Maternal exposure to *Toxoplasma gondii* additionally appears to be a risk factor for schizophrenia. An association between elevated levels of antibodies to *Toxoplasma gondii* in maternal sera and risk of schizophrenia or schizophrenia spectrum disorder development in the offspring has been presented, although the result did not reach the threshold for statistical significance (Brown et al., 2005). This finding was supported by the report of increased *Toxoplasma gondii* antibody levels in infant blood of individuals who later developed schizophrenia (Mortensen et al., 2007). In addition, maternal infection with *Toxoplasma gondii*, as evidenced by increased levels of antibodies to the type I strain, was associated with increased risk of psychosis in the offspring (Xiao et al., 2009). Moreover, viral or bacterial CNS infections during childhood have been suggested to increase the risk for this disease (Koponen et al., 2004; Abrahao et al., 2005). In a recent epidemiologic study of a Swedish cohort including 1.2 million individuals, Dalman and coworkers presented a link between viral CNS infections (particularly with mumps virus or cytomegalovirus) during childhood and later development of non-affective psychosis (Dalman et al., 2008). Although contradicting results also have been presented (Suvisaari et al., 2003; Weiser et al., 2010), overall, these results indicate an infectious etiology of schizophrenia.

Numerous translational models are utilized to approach a better understanding of the etiology and pathophysiology of schizophrenia (Powell, 2010). Several experimental studies have suggested that infections in early life have persistent effects, supporting the role of the neurodevelopmental hypothesis of schizophrenia. For instance, respiratory exposure to a mouse-adapted influenza A virus (NWS/33CHINI) in pregnant mice, at gestational day 9, has been shown to cause neuroanatomic abnormalities, as well as changes in gene and protein expression, in the developing brains of their newborn pups (Fatemi et al., 1998a; Fatemi et al., 1998b; Fatemi et al., 2002). In adulthood, these mice showed behavioral changes regarding open-field,

novel object, and social interaction tests, as well as deficits in sensory motor gating (Shi et al., 2003). Furthermore, long-term effects have been observed following intranasal infection with another mouse-adapted influenza A virus (WSN/33) in pregnant mice at gestational day 14. These effects included changes in gene expression in the adult mice brains, but no behavioral changes were observed (Asp et al., 2005). In immunodeficient mice, a systemic infection with influenza A/WSN/33 virus on postnatal day (P) 3 (thus mimicking the hematogenous route of infection from mother to fetus) resulted in a triggered transcriptional activation in the brain as well as behavioral changes in adulthood (Asp et al., 2009).

In addition to influenza virus infections, other microbes have also been utilized to model the neurodevelopmental theory of schizophrenia by exposure during periods corresponding to the late 2$^{nd}$ to early 3$^{rd}$ trimester of human pregnancy. For example, subcutaneous injection of the neuroinvasive agent HSV type 1 in neonatal mice pups increased hyperactivity in adulthood, concomitant with deficits in learning (Crnic and Pizer, 1988). Likewise, neonatal rats intracerebrally inoculated with HSV type 1 showed deficits in sensorimotor gating in adult life (Engel et al., 2000). Studies using Borna disease virus (BDV), a highly neurotropic agent that can induce selective damage to the cerebellum, have also been presented. For instance, newborn rat pups inoculated intracranially with BDV displayed behavioral abnormalities in adulthood, including deficits in sensorimotor gating and in spatial learning and memory, altered social interactions, as well as increased hyper-reactivity to aversive stimuli (Pletnikov et al., 1999; Rubin et al., 1999; Pletnikov et al., 2002; Lancaster et al., 2007). Moreover, impaired sensorimotor gating was observed in adult rats born to dams treated with the bacterial endotoxin lipopolysaccharide (LPS) during pregnancy (Borrell et al., 2002).

In light of these neonatal infection models, further investigations have focused on exposure to cytokines, the modulating molecules which are part of the immune system. Subcutaneous administration of various cytokines, such as epidermal growth factor (EGF) or interleukin (IL)-1α, to neonatal rodents induces schizophrenia-associated behavioral abnormalities during post-pubertal life, including increased rearing activity, impaired startle and sensorimotor gating as well as altered social interactions, reviewed in (Watanabe et al., 2010). Similarly, a single injection of IL-6 to pregnant mice

resulted in behavioral abnormalities, including deficits in prepulse inhibition (PPI, reflecting impaired sensorimotor gating) and in latent inhibition (reflecting the ability to ignore irrelevant stimuli) in the adult offspring (Smith et al., 2007). Cytokines, particularly the IL-6 family, are also suggested to display important functions during CNS development (Bauer et al., 2007; Deverman and Patterson, 2009). These functions involve for example regulating the self-renewal process of neuroepithelial cells, i.e. the precursors of neurons, astrocytes and oligodendrocytes, as well as serveing as neurotropic factors, thus promoting cell survival, and as apoptotic triggering signals during development. In addition, chemokines (i.e. chemotactic cytokines) may serve as migration signals for *de novo* generated neurons and glial cells. Cytokine imbalance during CNS development, for example resulting from an early life infection, might thus have crucial consequences for the maturation of the brain. Indeed, an excess of pro-inflammatory cytokines in the fetal brain is hypothesized to underlie the schizophrenia-related pathology observed experimentally following prenatal maternal infections, reviewed in (Meyer et al., 2009). With regard to this view, it is notable that elevated levels of tumor necrosis factor (TNF) and IL-8 in maternal blood has been associated with increased risk of psychotic illness or schizophrenia spectrum disorder in the offspring (Buka et al., 2001b; Brown et al., 2004b).

#### *1.2.1.2 NMDA receptor hypofunction in early life*

Disturbance of excitatory transmitter signaling in early life, in particular as regards acetylcholine and glutamate, may affect maturation of the brain's circuitry. Thus, a hypofunction of the glutamatergic *N*-methyl-D-aspartate (NMDA) transmitter system during neurodevelopment has been proposed as one potential mechanism in the pathogenesis of schizophrenia (Olney and Farber, 1995; Olney et al., 1999; Coyle, 2006). Neurons are extremely sensitive to disturbances in excitatory transmitter signaling during the brain growth spurt period, i.e. the developmental period of synaptogenesis, and abnormal inhibition or activation of the NMDA receptor at that time triggers neuronal apoptosis or exitotoxic neurodegeneration, respectively (Olney, 2002). In accordance, a short-lasting pharmacological blockade of the NMDA receptors during a few hours in neonatal life has been found to trigger apoptotic neurodegeneration in the developing rat brain with the absence of gliosis (Ikonomidou

et al., 1999; Young et al., 2005). The developmental period of synapsogenesis initiates during the third trimester and spans through the first years of life in humans, whereas in rodents the brain growth spurt occurs in the postnatal period, ranging approximately from the first day of life to 14 days after birth (Dobbing and Sands, 1979; Rice and Barone, 2000). However, this vulnerable period does not only differ between species, but also varies between specific brain regions. In accordance with the NMDA receptor hypofunction theory, mice that are born with a reduced number of the essential NR1 subunit of the NMDA receptors display behavioral aberrations similar to those seen in pharmacologically induced animal models of schizophrenia (Mohn et al., 1999). Furthermore, administration of the NMDA receptor antagonist MK-801 to seven-day-old rats was associated with impaired sensorimotor gating and increased locomotor activity in adulthood (Harris et al., 2003). Similar effects were observed following postnatal treatment with the NMDA receptor antagonist phencyclidine (PCP), where sensorimotor gating impairments and delayed spatial attention were observed in adult rats (Wang et al., 2001). Sub-chronic blockade of the NMDA receptors by increasing concentrations of the competitive antagonist CGP 40116 in rats during postnatal life has also been shown to provoke a wide range of schizophrenia-associated behaviors in adulthood (Wedzony et al., 2008).

Interestingly, kynurenic acid (KYNA), an end product of the kynurenine pathway, is the only known endogenous antagonist that blocks both the NMDA and α7 nicotinic acetylcholine (α7nACh) receptors. In the late gestational period of rodents, levels of the kynurenine pathway metabolites KYNA and 3-hydroxykynurenine (3-HK), as well as the pivotal metabolite kynurenine, are reported to be extremely high in the fetal brain. After birth, however, the concentration of these metabolites dramatically decreases within a few hours. With regard to KYNA, this effect is thought to reflect a standby defense mechanism against a possible delivery-related hypoxic-ischemic insult (Ceresoli-Borroni and Schwarcz, 2000). However, the effect of an abnormal kynurenine pathway induction in the neonatal brain, particularly during the critical periods of neurodevelopment, is not well-known. This also motivates elaboration of whether a neonatal CNS infection could have an impact on the kynurenine pathway in the brain and if this could mediate any long-term effects.

### 1.2.2 Genetic aspects

Given the large amount of evidence, there is not much doubt that genetic vulnerability plays a part in schizophrenia etiology, see (Cardno and Gottesman, 2000; Cannon et al., 2003). Family history is by far the most established risk factor for schizophrenia, and children with both parents affected are estimated to have an 89% risk of developing the disease (Lichtenstein et al., 2006). In addition, monozygotic twins show a much higher concordance rate of schizophrenia development than dizygotic twins, with the risk of being affected if the other twin has the disease being 30-65% and 0-28% respectively (Cardno and Gottesman, 2000; Kringlen, 2000). Despite many years of research, a causal gene for development of the disease has not been identified. This has led to speculations such as whether one may consider critical genes for different symptoms or other phenotypes (observed characteristics) rather than investigating susceptibility genes for schizophrenia *per se*. It is also likely that several gene polymorphisms synergistically influence the generation of schizophrenia, possibly also interacting with environmental factors (Harrison and Owen, 2003; van Os and Kapur, 2009).

#### *1.2.2.1 Single nucleotide polymorphisms*

The genomes of two unrelated individuals are identical in 99.9%. The remaining 0.1% contains variations in the DNA sequence and is thus what makes each of us unique. An uncommon variatiant of an allele at a specific locus (a certain DNA-site) that is displayed in over 1% of a given population is termed a polymorphism. The most common polymorphisms in humans are the single nucleotide polymorphisms (SNPs) that are spread out through the genome (Sherry et al., 2001). As the name implies, a SNP is a DNA-site in the genome where only one single nucleotide base differs between individuals. Therefore, SNPs are utilized as gene markers of DNA variations within populations. The functional consequence of a SNP depends on where in the DNA it is located (Burton et al., 2005). If the SNP is positioned in an exon, i.e. a gene-encoding region, the base-variants may result in a change of the codon (three bases that code for an amino acid) that will have another translation and is therefore termed non-synonymous. Either this new codon translates to a change in amino acids (missense SNP) or a premature stop-codon (nonsense SNP), which alters the length of the protein.

Synonymous SNPs on the other hand do not affect the result of the coding sequence. A SNP that is positioned in a non-coding region may nonetheless influence the gene product. For example if the SNP is located in the promoter site, where transcript regulation occur, it might affect the gene expression. Also, a SNP in an intron may affect the mRNA splicing process, forming mRNA from preRNA that contains both introns and exons.

#### *1.2.2.2 Genetic linkage and association studies*

There are many strategies for finding genomic variations related to different diseases. An initial approach usually involves an analysis termed linkage study, where the entire genome can be scanned for shared genome region variations between affected members within a family (Dawn Teare and Barrett, 2005). These studies may provide a first trace of large genome regions that could contain a disease-causing gene. To investigate the influence of a specific gene, association studies may be utilized (Cordell and Clayton, 2005). In these studies, SNPs are used as gene markers, and the aim is to find shared alleles between affected unrelated individuals. Several databases are provided for the selection of which SNPs to include in an association study; the largest one being the dbSNP database established by the National Centre of Biotechnology Information (NCBI). This database receives information from many other databases, including the one of the International HapMap project. The HapMap project strives to create a haplotype map of the human genome. A haplotype is the combination of a specific set of alleles displayed on a single chromosome, and the HapMap database assesses linkage disequilibrium (LD) to determine these haplotypes. Two genomic regions that are located closely on the same chromosome are often inherited together, thus no recombination has occurred at this site during meiosis (the formation of sperm and egg cells). Two genetic markers are in LD if they are inherited together more frequently than can be expected by chance, and accordingly the occurrence of one of them can predict the other (Dawn Teare and Barrett, 2005). The software Haploview further provides the identification of LD-blocks (also termed haplotype-blocks), i.e. SNPs that are in strong LD and thus often inherited together. TagSNPs, i.e. SNPs that represent different haplotype-blocks, can also be identified using the HapMap database. Thus, tagSNPs capture genetic variations across large regions of the gene, without having to genotype all SNPs. To assess if the control sample in an association study provides an

accurate representation of the whole population regarding the specific genotype and allele frequencies, analysis of Hardy-Weinberg equilibrium is performed. Hardy-Weinberg equilibrium indicates that the control sample is sufficiently large and randomly mating, and that there is no population stratification (i.e. subpopulations with different descent).

#### *1.2.2.3 Risk genes for schizophrenia*

Several minor risk alleles have been presented, including variances in genes that are implicated in dopamine or glutamatergic signaling (Harrison and Weinberger, 2005; Allen et al., 2008; Lisman et al., 2008), which are the neurotransmitter systems predominantly associated with schizophrenia pathophysiology (see section 1.5). In particular, risk alleles have been found in genes encoding the catecholamine degrading enzyme (*COMT*), the dopamine D1 and D2 receptors (*DRD1* and *DRD2*), the regulator of G-protein signaling 4 (*RGS4*), and the metabotropic glutamate receptor 3 (*GRM3*). In agreement, reports of genetic linkage and association with genes encoding enzymes that reduce the availability of the NMDA receptor ligands glutamate or D-serine have been presented (Harrison and Owen, 2003; Detera-Wadleigh and McMahon, 2006; Morita et al., 2007), further supporting a hypofunction of the NMDA receptors in patients with schizophrenia. Particular evidence has been reported for the gene encoding dysbindin (*DTNBP1*), a modulator of synaptic glutamate release, as this gene has been associated in independent samples with both negative and cognitive symptoms (Fanous and Kendler, 2008). Also the gene encoding neuregulin (*NRG1*), a neurotropic factor implicated in glutamatergic, nicotinic and GABAergic transmission (Falls, 2003), as well as the gene encoding the D-serine degrading enzyme D-amino acid oxidase (*DAAO*), are suggested as candidate genes for schizophrenia vulnerability, reviewed in (Lisman et al., 2008). Furthermore, substantial studies implicate *DISC1* in schizophrenia. This gene is potentially involved in neurodevelopment (Ozeki et al., 2003). Interestingly, genes encoding inflammatory proteins, e.g. the cytokines TNF and IL-1β, as well as the promoter site of IL-10, have also been reported to be associated with schizophrenia susceptibility (Schwab et al., 2003; Yu et al., 2004; Shirts et al., 2006; Ozbey et al., 2009; Sasayama et al., 2011; Zhong et al., 2011). Contradicting results have however been presented in different populations, for example regarding a tentative IL-2 or IL-4 association to schizophrenia vulnerability (Schwarz et al., 2006; Watanabe et al., 2008).

In the last few years, genome wide-association studies (GWAS) have provided the latest technology of relatively fast large-scale genetic analyses that may include several hundred thousand single nucleotide polymorphisms (SNPs) across the genome. Interestingly, one of the first published GWAS of schizophrenia presented evidence of disease association with polymorphisms in the gene encoding the cytokine receptor IL-3Rα, which is implicated in the regulation of granulocyte and macrophage activity (Lencz et al., 2007). Since then, these studies have also revealed associations between schizophrenia and several genes involved in neurodevelopmental processes, see (Lee et al., 2011). For example, recent results from a large GWAS showed association with markers in the regions of the major histocompatibility complex (MHC), as well as in two genes involved in brain development, memory and cognition (*NRGN* and *TCF4*) (Stefansson et al., 2009). MHC is a cluster of genes encoding proteins that serve as antigen-presenting molecules expressed on the cell surface. Association between schizophrenia and polymorphisms in the MHC was confirmed by the International Schizophrenia Consortium (Purcell et al., 2009). The development of microarray technology (where multiple genome regions can be analyzed simultaneously) has further provided genome-wide studies of copy number variations (CNVs), i.e. segments of DNA, ranging from one kilobase to several megabases, that are duplicated, deleted or rearranged in the genome. The CNVs may interfere with regulatory or coding regions and can thus affect gene functions as well as gene dosage, reviewed in (Freeman et al., 2006; Cook and Scherer, 2008). Many neuropsychiatric disorders including schizophrenia, Parkinson's disease, bipolar disorder and autism, have been associated with specific CNVs. Interestingly, a genetic overlap has been observed between CNVs in schizophrenia and neurodevelopmental disorders, such as autism and attention deficit hyperactivity disorder (ADHD) (O'Donovan et al., 2008; Owen et al., 2011). The common regions include a deletion in the location of the α7nACh receptor gene (*CHRANA7*), which is a receptor implicated in both neuregulin and glutamate signaling (Stefansson et al., 2008; Burbach and van der Zwaag, 2009). Strong evidence for a genetic overlap between schizophrenia and bipolar disorder, has also been shown: In a GWAS from the International Schizophrenia Consortium, including 6,909 individuals, a combined polygenetic contribution of several alleles of minor effect, involving different domains of brain development, was associated with increased risk of both diseases (Purcell et al., 2009).

No single gene encoding any enzymes within the kynurenine pathway (see section 1.3) had alone been associated to schizophrenia susceptibility prior to the studies included in this thesis. A complex genotype of several risk genes, including one SNP in the gene encoding tryptophan 2,3-dioxygenase (TDO), has however been associated with the disease (Miller et al., 2009). A polymorphism in this gene has furthermore been associated with vulnerability of autism (Nabi et al., 2004). It is however uncertain if an abnormal variance of this gene would result in increased levels of kynurenine and KYNA, as seen in patients with schizophrenia (Linderholm et al., 2010; Sathyasaikumar et al., 2010). Rather, a decrease may be expected. On the contrary, these metabolites are likely to be increased as a result of a polymorphism in the gene encoding kynurenine 3-monooxygenase (KMO). Indeed, this gene is maped to 1q42-44, a chromosome region that has shown strong linkage to schizophrenia. Such an alignment was the focus of a Japanese study, but no association to schizophrenia was concluded (Aoyama et al., 2006).

## 1.3 THE KYNURENINE PATHWAY

Tryptophan is an essential amino acid that is necessary for protein synthesis and several metabolic functions, for example because it is the precursor of serotonin, melatonin and niacin (vitamin $B_3$). In mammalians, tryptophan is metabolized vastly through the kynurenine pathway (**Figure 1**), named after the central degradation product kynurenine (Beadle et al., 1947; Peters, 1991). Induction of the kynurenine pathway can be triggered by several different infections (see section 1.3.2), and subsequently generate neuroactive and/or immune modulatory kynurenine metabolites (Stone, 1993; Moffett and Namboodiri, 2003; Mandi and Vecsei, 2011). Interestingly, dysfunctions of the kynurenine pathway, reflected for example by elevated levels of the neuromodulatory end product KYNA, are associated with schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001), a disease recently also linked to immune activation of the brain (Söderlund et al., 2009).

### 1.3.1 Enzymatic steps

The first and rate-limiting enzymatic step of tryptophan degradation along the kynurenine pathway is the oxidative opening of the indole ring, either by indoleamine 2,3-dioxygenase (IDO) or by TDO, forming N-formyl kynurenine. Although both enzymes are found in the brain, TDO appears preferentially expressed in the liver. N-formyl kynurenine is rapidly hydrolyzed by kynurenine formamidase to form kynurenine, a central metabolite in the pathway that is able to cross the blood-brain barrier (BBB) via the large neutral amino acid carrier (Fukui et al., 1991). Kynurenine is then further catabolized – the compound serves as a substrate for three distinct enzymes: (*i*) kynurenine aminotransferases (KAT 1, KAT 2, KAT 3 or mitAAT), forming KYNA, (*ii*) kynureninase (KYNU), generating antranilic acid, and (*iii*) KMO, resulting in 3-HK. The activity of the KAT enzymes may be influenced by additional molecular factors, such as stimulating co-substrates. Furthermore, other substrates may compete with the binding of kynurenine, thus making the regulation of KYNA production a complex machinery (Schwarcz and Pellicciari, 2002). The metabolites anthranilic acid and 3-HK are metabolized to 3-hydroxyanthranilic acid (3-HAA). 3-HK may also be metabolized into the side product xanthurenic acid by the KAT enzymes. 3-hydroxyanthranilate 3,4-dioxygenase (HAAO) subsequently catabolizes 3-HAA into 2-amino-3-carboxymuconic acid semialdehyde, which in turn is converted into either picolinic acid (PA) or quinolinic acid (QUIN). Additionally, 2-amino-3-carboxymuconic acid semialdehyde can undergo a total oxidation via 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD), forming $CO_2$ and adenosine triphosphate (ATP). QUIN is finally metabolized by quinolinate phosphoribosyltransferase (QPRT), which participates in synthesis of nicotinamide adenine dinucleotide ($NAD^+$) (Stone, 1993; Moroni, 1999; Guillemin et al., 2001b; Schwarcz and Pellicciari, 2002; Guillemin et al., 2003b; Guidetti et al., 2007; Guillemin et al., 2007).

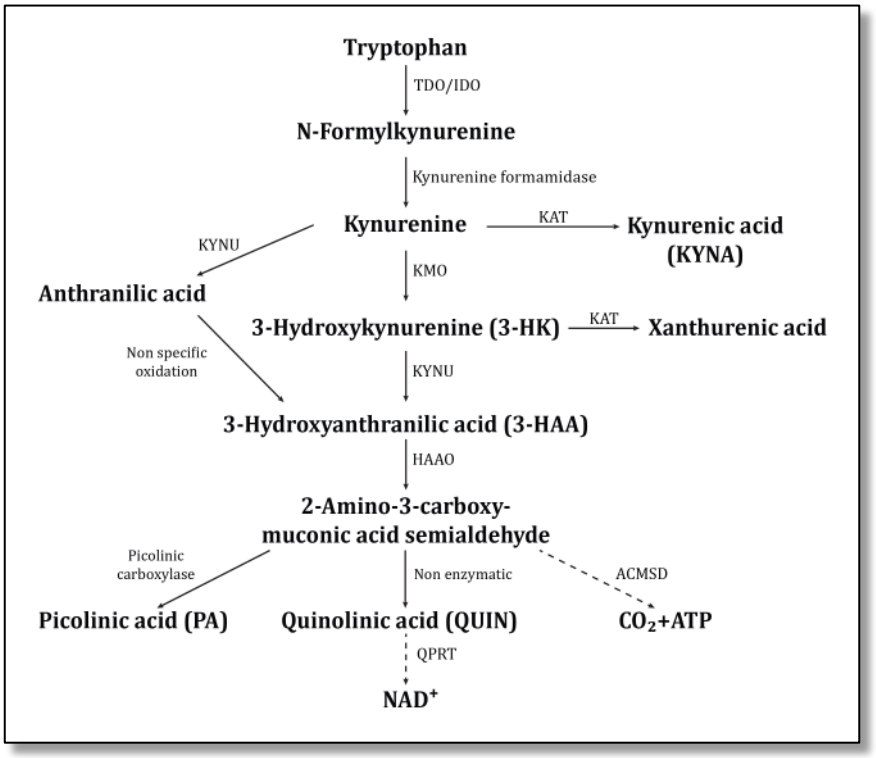


**Figure 1.** The kynurenine pathway

The enzymes of the kynurenine pathway are found both in the CNS and in the periphery of mammalians. Although differences regarding the activity of the kynurenine pathway enzymes have been reported between rodent species (Heyes et al., 1997b; Allegri et al., 2003), it is assumed that the mechanisms of metabolite synthesis in the rodent and human brain are relatively similar (Guidetti et al., 1995; Schwarcz and Pellicciari, 2002; Kiss et al., 2003). In rats and gerbils, 60-78% of the brain kynurenine is reported to derive from the periphery (Gal and Sherman, 1978; Kita et al., 2002), a result that ought to be reflected in other mammalians as well. Nevertheless, IDO and TDO is expressed in a variety of cell types within the brain, including astrocytes, neurons and endothelial cells of the BBB (Guillemin et al., 2001b; Schroten et al., 2001; Miller et al., 2004; Guillemin et al., 2005b; Miller et al., 2006; Guillemin et al., 2007; Owe-Young et al., 2008). IDO has also been observed in microglia and macrophages, where only low expression of TDO is found (Heyes et al., 1996; Guillemin et al., 2003b). However, the course of kynurenine degradation within the CNS appears to be spatially separated, as the expressions of enzymes in the different pathway branches differ between specific cell types. Thus, since the KMO and KYNU enzymes are predominantly expressed in microglia and macrophages, these cells produce substantial amounts of the neurotoxic metabolite QUIN (Lehrmann et al.,

2001; Guillemin et al., 2003b). The KAT enzymes are, however, mainly expressed in astrocytes (Roberts et al., 1992; Kiss et al., 2003), although these enzymes have also been observed in oligodendrocytes, macrophages and microglia, as well as in neurons (Du et al., 1992; Guillemin et al., 2003b; Wejksza et al., 2005; Guillemin et al., 2007). As astrocytes lack the KMO enzyme (i.e. 3-HK is not generated) and produce very little KYNU enzyme, synthesis of the neuroprotective metabolite KYNA is favored in these cells (Heyes et al., 1997a; Guillemin et al., 2001b). KYNA passes the BBB poorly (Fukui et al., 1991); the metabolite is removed from the brain by a probenecid-dependent transporting process (Moroni et al., 1988), and further eliminated in the periphery by urinary excretion (Turski and Schwarcz, 1988). Notably, as the QPRT enzyme appears to be highly expressed in astrocytes, it is proposed that extracellular QUIN may be taken up and catabolized by these cells (Guillemin et al., 2001b), or removed from the brain by a probenecid-dependent transport process and eliminated via urinary excretion (Schwarcz and Pellicciari, 2002).

### 1.3.2 Kynurenine pathway induction following immune activation

Many infections are associated with an induction of tryptophan degradation along the kynurenine pathway. In humans, a number of kynurenine pathway metabolites have been reported to be elevated during infections or inflammatory diseases of the CNS (Heyes et al., 1992b). For instance, the metabolite QUIN is increased in the cerebrospinal fluid (CSF) of patients infected with *Borrelia burgdorferi*, causing Lyme disease (Halperin and Heyes, 1992), or with HIV-1 (Achim et al., 1993), including patients with AIDS dementia complex (Guillemin et al., 2005a). Increased levels of both PA and QUIN have also been reported in patients with severe malaria (Dobbie et al., 2000; Medana et al., 2002). Other reports show an association of CSF KYNA with infectious diseases affecting the CNS. For example, elevated CSF concentrations of this metabolite have been reported in individuals infected with tick-borne encephalitis (TBE) and in young children with cerebral malaria (Medana et al., 2003; Holtze et al., 2011). Also, patients infected with HIV-1 display elevated levels of both kynurenine and KYNA in the CSF and brain (Bara et al., 2000; Atlas et al., 2007). Interestingly, high levels of CSF KYNA in the HIV-1 infected patients were associated with psychotic symptoms (Atlas et al., 2007).

Several experimental studies have also demonstrated a relationship between CNS infectious diseases and induction of the kynurenine pathway. For example, studies on CNS inflammation in rhesus macaques infected either with neurotropic simian immunodeficiency virus (SIV, the simian analog of HIV-1) or with poliovirus, reported elevated levels of the metabolites QUIN and KYNA in the CSF, as well as increased activity of the enzymes IDO, KMO and KYNU, but not KAT, in the spinal cord (Heyes et al., 1992a; Heyes et al., 1992c; Heyes et al., 1993). The levels of KYNA were increased to a lesser degree compared with QUIN, and IDO activity was increased to a higher extent than the other enzymes. Similar results were obtained in neurotropic measles virus-infected hamsters, where the infection increased the activity of HAAO (the enzyme that generates QUIN) in the brain, while no change in KAT activity was observed (Eastman et al., 1994). Increased activity of IDO, as well as increased concentration of QUIN, was also observed in the spinal cord of mice with HSV encephalitis (Reinhard, 1998). Furthermore, mice infected with the parasite *Toxoplasma gondii* displayed increased IDO activity and corresponding mRNA transcripts in the brain, as well as increased brain kynurenine and KYNA concentrations, probably mediated by stimulation of the cytokine IFN-γ (Fujigaki et al., 2002; Silva et al., 2002; Schwarcz and Hunter, 2007). Altogether, a large body of both clinical and experimental studies suggests a significant role of the kynurenine pathway in brain immune activation.

The concept of IDO induction by immune activation is widely accepted, and occurs in a variety of cell types both in the periphery and the CNS. Already in the 1970s it was shown that the bacterial endotoxin LPS could induce IDO (Hayaishi and Yoshida, 1978). A few years later it was also shown that cytokines, the signal substances of the immune system, could induce IDO activity (Yoshida et al., 1981), and block the growth of *Toxoplasma gondii* (Pfefferkorn, 1984). These findings raised the idea that tryptophan starvation may serve as a defense mechanism of the host by reducing the local supply of this essential amino acid to intracellular pathogens. Ten years later, researchers were able to show that increased IDO activity prevents a maternal T cell-mediated rejection of the developing fetus (Munn et al., 1998) (Mellor and Munn, 1999). These experiments suggested that increased IDO activity has an immunotolerant function by inhibiting T-cells. Two different theories have emerged to explain the mechanism by which the T cell suppressive effect appear (Moffett and Namboodiri,

2003). Munn and Mellor originally proposed that depletion of tryptophan by IDO induction supress T-cells – *the tryptophan depletion theory*. Later on, new compelling data signified immune functions of the tryptophan degradation metabolites – *the tryptophan utilization theory*. This view is originally based on the observation that several metabolites of the kynurenine pathway display immunoregulatory properties (see section 1.3.3), including proliferative inhibition of T cells, B cells, and natural killer cells (Frumento et al., 2002; Terness et al., 2002). The physiological functions of IDO are also widely studied in the cancer research field, were IDO induction is related to the prevention of an immune attack from the host (Belladonna et al., 2007; Gonzalez et al., 2008). Thus, IDO shows a multifunctional role, including involvement of acute immune responses, as well as having an important function in mediating tolerance of both tumor cells and the intra-uterine fetus.

The predominant and most potent IDO-inducing cytokine is IFN-γ, although other cytokines, such as IFN-β, TNF, IL-6 and IL-1β, also have this ability (Guillemin et al., 2001a; Guillemin et al., 2005b; Fujigaki et al., 2006; O'Connor et al., 2009; Kegel et al., 2011). In line with the observations of an induction of IDO following immune activation, the production of QUIN by microglia, and to a lesser extent by macrophages, as well as the production of KYNA by astrocytes, also increase following immune stimuli with IFN-γ (Guillemin et al., 2001b; Guillemin et al., 2003b). In addition, levels of the metabolite PA are increased in pericytes of the BBB following a combined stimulus with IFN-γ and TNF (Owe-Young et al., 2008).

Contrary to IDO, the tryptophan-breakdown enzyme TDO is not traditionally considered to be induced by immune activation (Saito et al., 1992; Saito et al., 1993; Heyes et al., 1998). However, a recent study from our laboratory demonstrates that IL-1β stimulation induces gene expression of both IDO and TDO in human astrocytes, concomitant with an increased KYNA concentration (Kegel et al., 2011). In addition, induction of TDO gene expression has been reported in the placenta during bacterial intra-uterine infections, as well as in cell cultures exposed to LPS (Manuelpillai et al., 2003; Manuelpillai et al., 2005). This immunofacilitation of TDO have also been implied in a tetracycline-inducible eukaryotic system (Schmidt et al., 2009).

### 1.3.3 Physiological significance of the kynurenine pathway

The kynurenine pathway generates several metabolites suggested to be neuroactive and/or immunomodulatory, see (Stone, 1993; Moroni, 1999; Moffett and Namboodiri, 2003; Mandi and Vecsei, 2011). Most attention has been on KYNA and QUIN, but 3-HK, 3-HAA, and PA should also be included in this category. However, also the pivotal metabolite kynurenine exerts physiological functions, such as regulation of vascular tone (Wang et al., 2010), an important and profound mechanism in inflammatory responses. Kynurenine was also recently suggested to directly mediate both a tumor promoting and a T cell suppressive effect (Opitz et al., 2011). Although the kynurenine pathway metabolites are generally found in lower concentrations in the brain, CSF and spinal cord than in the periphery, their presence in the CNS is undeniably relevant for physiological and pathologic conditions.

**KYNA** was originally detected in canine urine (Liebig, 1853). More than a century later, KYNA was found in the mammalian brain (Moroni et al., 1988; Turski et al., 1988) and has since then been widely studied as a neuroactive compound. In the brain, KYNA has a unique receptor profile. At low concentrations, KYNA antagonizes the co-agonist glycine site of the NMDA receptor ($IC_{50} \approx$ 8-15 μM, i.e. the ligand concentration that yields 50% receptor inhibition) (Stone, 1993; Parsons et al., 1997), and the α7nAch ($IC_{50} \approx 7$ μM) (Hilmas et al., 2001). At higher concentrations, the glutamate recognition site of the NMDA receptor ($IC_{50} \approx$ 200-500 μM) and the α-amino-3-hydroxy-5-methy-4-isoxazole propionate (AMPA)/kainate receptors ($IC_{50}$ in the millimolar range) are also blocked (Kessler et al., 1989). As the level of KYNA in the rodent brain is in the nM range, far below the levels required for antagonizing the receptors, the physiological significance has been under debate. However, astrocytes play an important role in regulating synaptic transmission and accordingly, these glial cells have been shown to intimately surround glutamatergic synapses (Newman, 2003). Since KYNA is produced in astrocytes, the local concentration within the synapse should be considerably higher compared to that received from analysis of whole brain tissues. Experimental evidence show that endogenous concentrations of KYNA tonically modulate firing of dopamine neurons and affect glutamate release (Erhardt et al., 2009; Konradsson-Geuken et al., 2010; Pocivavsek et al., 2011b). Importantly, KYNA is implicated in the pathophysiology of several psychiatric

disorders (see section 1.5.3), and increased concentration of KYNA is found in the CNS of patients with schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005; Linderholm et al., 2010; Sathyasaikumar et al., 2010), bipolar disorder (Olsson et al., 2010), and suicide attempters with major depression (Linderholm, 2010), as well as in patients with neurodegenerative disorders like Alzheimer's disease or amyotrophic lateral sclerosis (ALS) (Baran et al., 1999; Ilzecka et al., 2003). Notably, cognitive impairments are recognized in all of these disorders (Andreasen, 1995; Martinez-Aran et al., 2004; Hinkelmann et al., 2009; Ballard et al., 2011; Giordana et al., 2011). A physiological role of KYNA elevation might be related to the neuroprotective effects of the compound. At very least, this could be the case regarding the neurodegenerative disorders where KYNA may balance neurotoxic events. The neuroprotective property of KYNA is probably mediated by blockade of the NMDA receptors and possibly also via decreased glutamate release by antagonizing the α7nACh receptors (Foster et al., 1984; Stone, 1993; Urenjak and Obrenovitch, 2000; Zwilling et al., 2011).

Interestingly, KYNA is also reported to encompass immunomodulatory properties, including an anti-inflammatory effect, reviewed in (Mandi and Vecsei, 2011). In the periphery, for example in mononuclear cells and in blood monocytes, KYNA has been found to attenuate the production of TNF (Wang et al., 2006; Tiszlavicz et al., 2011). This cytokine is a mediator of early pro-inflammatory immune responses, thus promoting activation of several other cytokines, including IL-1, IL-6 and IL-8 (Bradley, 2008). As TNF also can be released from astrocytes and microglia (Chung and Benveniste, 1990), and is associated with potentiated glutamate-induced neuronal cell death (Zou and Crews, 2005), it is suggested that inhibition of TNF production could contribute to the neuroprotective properties of KYNA (Mandi and Vecsei, 2011). This effect of KYNA is possibly mediated via a blockade of α7nACh receptors. However, recent reports reveal that KYNA also serves as an agonist on the G protein-coupled receptor 35 (GPR35), a receptor found in several immune cells with high expression in monocytes, T cells, neutrophils, and dendritic cells (Wang et al., 2006; Fallarini et al., 2010). The immunomodulatory effects of KYNA may thus include affinity to α7nACh and/or GPR35 receptors.

The kynurenine pathway metabolite **QUIN** has been frequently studied in the mammalian brain. Ever since the discovery of its ability to produce seizures in the mouse (Lapin, 1978), QUIN has been regarded as a neuroactive compound. In the brain, QUIN is an NMDA receptor agonist with excitatory actions, but also with neurotoxic and convulsive properties (Stone and Perkins, 1981; Schwarcz et al., 1983; Foster et al., 1984). Thus, potential neurological damage by QUIN is suggested to impact the pathogenesis of neurological disorders. Increased levels of CNS QUIN are indeed found in several neurodegenerative disorders, including Huntington's disease, Alzheimer's disease, ALS and AIDS dementia complex (Guillemin and Brew, 2002; Guidetti et al., 2004; Guillemin et al., 2005a; Chen et al., 2010). In the brains of patients with Huntington's disease, levels of the QUIN precursor **3-HK** are also reported to be elevated (Guidetti et al., 2004). Notably though, CSF QUIN in patients with inflammatory diseases are found in substantially higher concentrations than those observed in patients with neurodegenerative disorders (Heyes et al., 1992b). Indeed, QUIN is also involved in important immunomodulatory functions, reviewed in (Stone, 2001; Mandi and Vecsei, 2011). In particular, the suppression of T cells related to IDO activation (see section 1.3.2) might reflect the actions of QUIN and its precursor **3-HAA**, as these kynurenine pathway metabolites induce apoptosis of T cells (Fallarino et al., 2002). The latter metabolite, in analogy with 3-HK and kynurenine, is also reported to modulate the cytokine balance (Molano et al., 2008). Cytokine imbalance has also been observed in patients with schizophrenia (Muller and Schwarz, 2010). However, this is not reported to occur in the CNS and the importance of an imbalance in the periphery might be difficult to interpret in regard to a neuropsychological disease like schizophrenia. Another immune-related function of QUIN is the ability to serve as a substrate for production of the ubiquitous co-enzyme $NAD^+$ that is essential for many cellular reactions, including ATP synthesis and DNA repair, reviewed in (Moffett and Namboodiri, 2003). While QUIN is rapidly converted to $NAD^+$ in the liver, QUIN is stored in several types of immune cells (Moffett et al., 1993) and may potentially serve to prevent $NAD^+$ depletion following oxidative DNA damage springing from an immune response, see (Stone, 2001; Moffett and Namboodiri, 2003). Indeed, whereas microglia and macrophages are able to produce some QUIN under normal conditions, immune activation greatly promotes the synthesis of this kynurenine metabolite. In the brain, this effect is especially attributed to infiltrating macrophages, as these cells are suggested to synthesize the major proportion of QUIN during brain inflammatory

diseases (Guillemin et al., 2003b). Furthermore, the QUIN precursors of the kynurenine pathway, 3-HK and 3-HAA, are both found to be neurotoxic, including apoptotic properties (Okuda et al., 1998; Morita et al., 2001). Although the neurotoxic mechanism is not fully established for 3-HAA, 3-HK is a generator of toxic free radicals (Eastman and Guilarte, 1989).

The chelating metal ion agent **PA** was the first kynurenine pathway metabolite to be recognized as an immune modulator by being able to stimulate chemokine release of macrophages (Ruffmann et al., 1984; Bosco et al., 2000). Subsequently, it was shown that PA is involved in several immunological functions, for instance having an antiviral effect against HIV-1 and human HSV type 2 (Fernandez-Pol et al., 2001) as well as exhibiting an antimicrobial effect against *Mycobacterium avium* complex (MAC) organisms by potentiating macrophage activity (Cai et al., 2006). The capacity to induce expression of nitrite and nitric oxide synthase in macrophages has further implicated PA in inflammatory actions. PA has also been implied as a neuroactive metabolite of the kynurenine pathway (Melillo et al., 1993; Melillo et al., 1994). In this regard, PA effectively blocks QUIN neurotoxicity, although its neuroprotective effect differs from that of KYNA (Jhamandas et al., 1990; Beninger et al., 1994). However, PA seems to be less potent than KYNA in antagonizing the QUIN-induced neurotoxicity (Jhamandas et al., 1990). Although the immediate precursor enzyme of PA has only been found in low levels in the human brain (Fukuoka et al., 2002; Pucci et al., 2007), elevated levels of CSF PA have been detected in patients with infectious diseases, such as cerebral malaria infection (Medana et al., 2002; Medana et al., 2003). Furthermore – in addition to macrophages – astrocytes, neurons and pericytes of the BBB are able to produce PA (Guillemin et al., 2001b; Guillemin et al., 2007; Owe-Young et al., 2008), indicating that this tryptophan metabolite is an immunomodulating and/or neuroactive mediator also in the brain.

## 1.4 VIRUS INFECTIONS AND CNS IMMUNE RESPONSES

The CNS was previously considered to have an immune-privileged status. This conservative view was based on the findings in the mid-1900s of an absent immune reaction following allogeneic transplants. The absence or low expression of MHC

molecules on resident cells of the CNS, the absence of a proper lymphatic system, and the presence of the BBB further supported this notion. Today, this view is somewhat modified and it is now clear that the CNS is not completely isolated from the peripheral immune system. A complex and bi-directional communication does indeed exist between these systems, reviewed in (Dantzer et al., 2000; Quan and Banks, 2007; Dantzer et al., 2008; Wilson et al., 2010; Trakhtenberg and Goldberg, 2011). Examples are communication via the vagus nerve or the circumventricular organs (i.e. structures in the brain that lack a BBB), where cytokines and other circulating molecules are able to directly interact with the CNS. Interactions between the two systems can also occur through cytokine transport across the BBB as well as through secretion of prostaglandins by the BBB cells. Although immune activation of the CNS is rigorously controlled under normal conditions, for example through restriction of MHC molecule expression and suppression of T cell proliferation, reviewed in (Griffin, 2003), it involves a complex network of innate immune cells, such as brain resident microglial cells that are able to scan the brain parenchyma for injury, tumors or infection. Also, BBB-guarding macrophages ensure immediate detection of a pathogenic attack (Kreutzberg, 1996), reviewed in (McGavern and Kang, 2011). The blood-CSF barrier (formed by the CSF-producing choroid plexus and the arachnoid membrane) is also protected by macrophages. In the event of a pathogen invasion, macrophages can enter the CNS and be recruited to the site of infection (Raivich and Banati, 2004). Astrocytes are also important mediators of the innate immune system within the CNS, for example by expressing many receptors involved in innate immune responses, such as Toll-like receptors (TLR) and scavenger receptors, and by releasing cytokines following immune stimulation, reviewed in (Farina et al., 2007). Essentially though, one must bear in mind that the CNS immune system is a fairly novel field of research and the interpreted actions may still be ambiguous.

The nature of the immune responses in early life may not fully reflect the events in adulthood, as the immune system develops throughout the first years of life in humans, reviewed in (Ygberg and Nilsson, 2011). Furthermore, the BBB might be compromised during neonatal life, for example indicated by an efficient infiltration into the rodent brain of systemically administered cytokines, reviewed in (Watanabe et al., 2010). Even though recent studies demonstrated that the formation of a functional BBB occurs

already during embryogenesis in rodents (Daneman et al., 2010), the maturation phase of the BBB seems to occur in postnatal life (Liebner et al., 2011).

Viruses rarely access the CNS directly. The first immune response to virus infections, including neurotropic viruses, therefore usually occurs in the periphery. Thus, neurotropic viruses normally induce infections through the gastrointestinal or respiratory tracts. Although rare in the mature brain, viruses may pass the CNS barriers using different strategies, reviewed in (McGavern and Kang, 2011). Following a viral invasion the expression of type I interferons (IFN-α/β) is stimulated, promoting an anti-viral effect. A fast production of these cytokines is important for host survival as it limits virus spread and replication. In the brain, the type I IFNs can be released from astrocytes and microglia, and to some extent also from neurons, reviewed in (Paul et al., 2007). Infected neurons are also likely to produce different chemokines and cytokines such as IFN-γ and IL-6, thus mediating activation signals to microglia and astrocytes (Maciejewski-Lenoir et al., 1999; Neumann, 2001), reviewed in (Griffin, 2003). Together with perivascular macrophages, astrocytes and microglia serve as important components of the innate immune response in the CNS. By expressing for example TLR, astrocytes and microglia are able to recognize conserved structures on invading pathogens, such as viral nucleic acids (Paul et al., 2007). The TLR signaling further induces the release of a symphony of inflammatory mediators, which is specific to the virus infection but commonly includes the cytokines IL-1, IL-6, IL-12, TNF, as well as different chemokines, see (Falsig et al., 2008). An innate immune response to viral infections may also include the expression of inducible nitric oxide synthase (iNOS), which is the inducible form of the enzyme responsible for nitric oxide (NO) production. This mechanism is stimulated by pro-inflammatory cytokines such as IFN-γ, IL-1β and TNF (Saha and Pahan, 2006).

Following activation of the innate immune system, peripheral leukocytes enter the CNS across the BBB and are recruited to the site of infection, see (Raivich and Banati, 2004; Becher et al., 2006). However, initial induction of the adaptive (i.e. antigen-specific) immune response, such as activation of T cells and B cells, is thought to occur outside the CNS. It is also notable that during a peripheral immune response, the permeability of the BBB is enhanced (e.g. mediated by TNF), and even in the absence of a CNS infection, activated T cells cross the BBB to screen for any possible pathogen

infiltrations at this site, reviewed in (Hickey, 2001). Cells in the CNS do not normally express MHC molecules. However, during a viral CNS immune response, the expression of MHC class I molecules (which present antigens to $CD8^+$ T cells) and class II molecules (which present antigens to $CD4^+$ T cells) is induced (Pope et al., 1998; Kimura and Griffin, 2000). Although several cell types in the CNS are able to produce MHC molecules during an infection, microglia are the most efficient antigen-presenting cells (Neumann, 2001). As neurons are non-renewable and thus fairly resistant to apoptosis, viral clearance from these cells may occur in a non-cytolytic manner. This process is generally dependent on antibody responses and includes the inhibition of intracellular viral replication (Griffin, 2010). In addition to antibodies, both $CD4^+$ and $CD8^+$ T cells contribute to the non-cytolytic viral clearance via IFN-γ expression, mediating an antiviral effect (Binder and Griffin, 2001), see (Guidotti and Chisari, 2001). For example, a more rapid reduction of viral RNA from neurons is observed in the presence of $CD8^+$ T cells (Kimura and Griffin, 2000).

## 1.5 HYPOTHESES OF SCHIZOPHRENIA PATHOPHYSIOLOGY

The complexity of schizophrenia, including the heterogeneous subset of symptoms, has during the years of schizophrenia research resulted in a number of theories regarding the disease pathophysiology encompassing many neurotransmitter systems in the brain. The most established hypothesis suggest alterations predominantly in the dopamine and glutamate systems of the brain, see (Carlsson et al., 2004; Javitt, 2007).

### 1.5.1 Dopamine in schizophrenia

#### *1.5.1.1 Brain dopamine pathways and receptors*

The mammalian brain contains four major dopaminergic systems (Moore and Bloom, 1978; Le Moal and Simon, 1991). The nigrostriatal dopamine pathway, projecting from substantia nigra in the midbrain and terminating in the dorsal part of the striatum, is an important regulator of motor functions. Degeneration of the dopamine neurons in the nigrostriatal dopamine system underlies the pathophysiology of Parkinson's disease (Hornykiewicz and Kish, 1987), and this pathway is also associated with the extrapyramidal side effects caused by antipsychotic drugs. The mesocorticolimbic

dopamine pathway originates in the ventral tegmental area in the midbrain and terminates in either subcortical (limbic) areas, i.e. the mesolimbic dopamine pathway, or the prefrontal cortex, i.e. the mesocortical dopamine pathway. The mesolimbic dopamine pathway is involved in emotional control, motivation and reward, as well as locomotor behavior in rodents. The mesocortical dopamine pathway regulates working memory, executive planning, organization, attention, and social behavior. Finally, the tuberoinfundibular dopamine pathway originates in the hypothalamus and projects to the pituitary, and is involved in endocrine control.

Dopamine neurotransmission is mediated by five dopamine receptors, divided into two receptor families based on their pharmacological and biochemical properties: The $D_1$-like and $D_2$-like receptors. The $D_1$-like receptors are subdivided into $D_1$ and $D_5$ receptors, and the $D_2$-like receptor family includes $D_2$, $D_3$ and $D_4$ receptors (Jaber et al., 1996). The dopamine receptors are suggested to be involved in different functions related to a number of brain disorders, including not only schizophrenia and Parkinson's disease, but also drug addiction, attention deficit disorders, psychosis and mood disorders.

#### *1.5.1.2 The dopamine hypothesis of schizophrenia*

The dopamine hypothesis, presented almost 50 years ago, originally proposed that a general hyper-dopaminergic activity was the underlying cause of psychotic symptoms in patients with schizophrenia (Carlsson and Lindqvist, 1963). This theory was initially supported only by pharmacological evidence. In particular, by the fact that therapeutic effectiveness of antipsychotic drugs is attributed to a specific blockade of $D_2$ receptors (Seeman and Lee, 1975; Creese et al., 1976; Seeman et al., 1976), and by the observation that dopamine-releasing drugs, such as amphetamine, may induce a psychotic state in healthy individuals and exacerbate psychotic symptoms in patients with schizophrenia (Angrist et al., 1974; Snyder et al., 1974). In the past decades, brain imaging techniques have revealed a hyper-reactive state of dopaminergic neurotransmission in patients with schizophrenia, as reflected by an increased dopamine release in the striatum (Abi-Dargham et al., 1998; Lindstrom et al., 1999) and a potentiated striatal release following amphetamine administration (Laruelle et al., 1996; Breier et al., 1997; Abi-Dargham et al., 1998; Laruelle and Abi-Dargham, 1999).

Antipsychotic drugs used today block dopamine $D_2$ receptors. In most patients typical antipsychotic drugs (i.e. classical neuroleptics), with pronounced $D_2$ receptor antagonism, are not sufficient treatment, since these therapeutics do not improve cognitive functioning or affect negative symptoms (King, 1998; Breier, 1999; Meltzer et al., 1999). These observations led to a reformulation of the dopamine hypothesis to encompass impairment in $D_1$ receptor-mediated signaling in the prefrontal cortex. Accordingly, the dopamine imbalance hypothesis of schizophrenia was presented, suggesting that the cognitive dysfunctions and negative symptoms are mainly due to a persistent deficit in prefrontal cortical dopamine functions, whereas the positive symptoms may arise from an intermittent facilitation of subcortical dopamine transmission in the limbic system via $D_2$ receptors (Weinberger et al., 1986), see (Davis et al., 1991; Svensson, 2003). Although this might explain the episodic psychotic states and the more stable negative symptoms and cognitive impaired functions (Green et al., 2000), the etiology of this putative imbalance in dopamine neurotransmission still remains elusive.

### 1.5.2 Glutamate in schizophrenia

#### *1.5.2.1 Brain glutamate system and receptors*

The amino acid glutamate is the major excitatory neurotransmitter of the mammalian brain. It is found in almost every brain region and is involved in most aspects of brain functions. Glutamate is crucial for synaptic plasticity, memory and learning, and thus of great interest for schizophrenia pathophysiology (McEntee and Crook, 1993). Two glutamate receptor families have been described: The ionotropic glutamate receptors (NMDA, AMPA and kainate receptors), which are ligand-gated ion channel receptors, and the metabotropic glutamate receptors (mGluR), which are G-protein coupled receptors (Ozawa et al., 1998). The NMDA receptor, which is of particular interest for schizophrenia pathophysiology, is a complex receptor that is both ion- and voltage-gated. Thus, receptor activation requires not only agonist binding and depolarization of the cell, but also D-serine or glycine binding at the co-agonistic allosteric site, concomitant with several additional regulatory agents, resulting in removal of the physical $Mg^{2+}$ block within the ion channel. The NMDA receptors are arranged in heteromeric complexes composed of the subunits NR1, NR2 and NR3, the glycine site

being located on the NR1 subunit. As the receptor subunits express distinctive ligand-binding sites, and as different subunits are able to modify the receptor properties, the composition of these complexes is crucial for NMDA receptor functioning. Activation of the NMDA receptor may cause a long-lasting enhancement of the synaptic efficacy, a process that is thought to regulate functions of memory and learning (Lynch, 2004). An overstimulation of the NMDA receptor may, however, cause exitotoxicity, probably as a result of an excessive $Ca^{2+}$ influx into the cell, and thus lead to neuronal damage and even cell death (Meldrum and Garthwaite, 1990).

#### *1.5.2.2 The glutamate deficiency theory of schizophrenia*

In the mid-twentieth century, clinicians observed that administration of the dissociative anesthetic drug PCP produced severe side effects as it induced a psychotic state in mentally healthy patients (Luby et al., 1959; Bakker and Amini, 1961; Ban et al., 1961). In the 1970s PCP became a drug of abuse, and intoxicated patients with long-term usage were often indistinguishable from patients with schizophrenia (Yesavage and Freman, 1978). It was however not until 1985 that PCP was recognized as an NMDA receptor antagonist (Snell and Johnson, 1985). This action of PCP came to support the glutamate deficiency theory of schizophrenia – suggesting a hypofunction in glutamatergic neurotransmission to underlie the pathophysiology. The hypothesis was initially formulated by Kim and co-workers, who reported low glutamate levels in the CSF of patients with schizophrenia (Kim et al., 1980). Mounting evidence supporting this theory has since then been presented. In particular, non-competitive inhibition of the NMDA receptor, e.g. by PCP or ketamine, has been shown to induce positive and negative symptoms of schizophrenia as well as cognitive dysfunctions (Javitt and Zukin, 1991; Krystal et al., 1994; Jentsch and Roth, 1999). These drugs may furthermore worsen or re-awaken symptoms in patients with schizophrenia (Itil et al., 1967; Malhotra et al., 1997; Lahti et al., 2001). In addition, several studies report that competitive NMDA receptor blockade, as well as antagonism of the glycine site of the NMDA receptor, may induce psychomimetic effects (Kristensen et al., 1992; Grotta et al., 1995; Yenari et al., 1998). Furthermore, experimental studies in rodents have shown that systemic administration of PCP is associated with increased activity of midbrain dopamine neurons (French et al., 1993). Subchronic blockade of the NMDA receptor through PCP treatment of rats has been reported to increase mesolimbic dopamine release, and also to reduce dopamine release in the prefrontal cortex (Jentsch et al.,

1997; Jentsch et al., 1998; Jentsch and Roth, 1999). A relationship between the glutamate deficiency theory and the dopamine hypothesis of schizophrenia was subsequently demonstrated through imaging studies in humans. Thus, in line with observations in patients with schizophrenia (see section 1.5.1.2), ketamine administration to healthy volunteers increases dopamine levels in the striatum and potentiates amphetamine-induced dopamine release (Breier et al., 1998; Kegeles et al., 2000). Over the years, it has become increasingly evident that the imbalance of dopamine neurotransmission in schizophrenia may be secondary to NMDA receptor hypofunction, see (Svensson, 2000; Laruelle et al., 2003). Still, the underlying cause of this brain dysfunction remains to be determined.

### 1.5.3 The kynurenic acid hypothesis of schizophrenia

Since the initial discovery in 2001 of increased KYNA levels in the CSF and the prefrontal cortex of patients with schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001), these findings have been confirmed in several clinical studies, including drug-naïve, first episode patients and patients treated with antipsychotic drugs (Nilsson et al., 2005; Erhardt et al., 2007; Erhardt et al., 2009; Linderholm et al., 2010; Sathyasaikumar et al., 2010). The clinical findings have formed the basis of the kynurenic acid hypothesis of schizophrenia. KYNA, a tryptophan metabolite (**Figure 1**), shows a unique receptor-binding profile by blocking the co-agonistic glycine site of the NMDA receptor and the α7nACh receptor (see section 1.3.3), thus partly sharing pharmacological properties with the psychotomimetic drugs PCP and ketamine. Accordingly, the compound may induce deficits in both glutamatergic and cholinergic neurotransmission, features commonly implicated in schizophrenia.

During the past decade, the kynurenic acid hypothesis has been strengthened by a large number of experimental studies, giving robust support for a physiological significance of KYNA. Electrophysiological studies *in vivo* show that KYNA affect dopamine neurotransmission. Thus, elevated endogenous concentration of the compound in the rat brain is associated with increased midbrain dopamine firing (Erhardt et al., 2001b; Erhardt and Engberg, 2002; Nilsson et al., 2006; Schwieler et al., 2006; Linderholm et al., 2007; Olsson et al., 2009), in analogy with systemically administered NMDA

receptor antagonists, such as MK-801 and PCP (French et al., 1993; Zhang et al., 1993). The somewhat paradoxical increase in firing rate following administration of an NMDA receptor antagonist may be explained by a disinhibition of dopamine neurons via blockade of GABAergic interneurons (Zhang et al., 1993; Erhardt and Engberg, 2002). Hyperactivity in the mesolimbic dopamine pathway may cause psychosis in patients with schizophrenia and it is possible that elevation of brain KYNA contributes to these symptoms. A reduction in endogenous brain KYNA, by administration of selective cyclooxygenase (COX)-2 inhibitors, is associated with a dampened activity of midbrain dopamine neurons (Schwieler et al., 2006; Schwieler et al., 2008). Such results demonstrate that brain KYNA tonically modulate dopamine neurotransmission. In this context it could be worth mentioning that inhibition of COX-2 has been associated with beneficial antipsychotic effects when added to conventional antipsychotic drugs (Muller et al., 2002). Notably, the antipsychotic drug clozapine, known to be superior in the treatment of schizophrenia, have been shown to display a partial agonistic action at the glycine site of the NMDA receptor (Schwieler et al., 2008). Thus, a hypoglutamatergic state, induced by elevated levels of KYNA, would be restored thanks to the agonistic properties of clozapine. If accurate, such an effect may form part of the therapeutic action of clozapine.

Additional support for the KYNA hypothesis is provided by the observation that sub-chronic elevation of KYNA in adult rodents potentiates amphetamine-induced dopamine release in the nucleus accumbens (Olsson et al., 2009). This is in consonance with the hyper-responsiveness to D-amphetamine observed in patients with schizophrenia (Laruelle and Abi-Dargham, 1999). Furthermore, KYNA is associated with a number of functional domains implicated in schizophrenia. A large body of studies shows the involvement of NMDA or α7nACh receptor in cognitive functions, see (Robbins and Murphy, 2006; Albuquerque et al., 2009). Thus, blockade of these receptors, e.g. by KYNA, may induce the cognitive deficits that make up the core symptoms of schizophrenia. Pharmacologically increased brain KYNA in rats disrupt sensory motor gating and auditory sensory gating, as well as impair cognitive functions, such as contextual processing and learning and working memory (Shepard et al., 2003; Erhardt et al., 2004; Chess and Bucci, 2006; Chess et al., 2007; Chess et al., 2009). Local hippocampal infusions of kynurenine, which increased extracellular KYNA, also impaired cognitive performance of the rat in the Morris Water Maze test (Pocivavsek et

al., 2011b). In line with these studies, mice with a targeted deletion of KAT 2 (the enzyme that converts kynurenine to KYNA) display reduced levels of brain KYNA, and enhanced cognitive performance, including object exploration and recognition, passive avoidance, and spatial learning (Potter et al., 2010). Similar cognitive improvements were recently reported following local infusion in the hippocampus of a pharmacological KAT 2 inhibitor (Pocivavsek et al., 2011b).

The synthesis of KYNA is mainly driven by the concentration of its precursor kynurenine (Schwarcz and Pellicciari, 2002). The IDO/TDO enzymes, and subsequently the availability of kynurenine, are thus suggested to be rate limiting for the production of KYNA. Furthermore, KYNA synthesis is indirectly controlled by KMO since a reduced activity of this enzyme would shunt the metabolism of kynurenine towards KYNA. Indeed, patients with schizophrenia have elevated concentrations of kynurenine in the CSF and in brain cortical regions (Miller et al., 2006; Miller et al., 2008; Linderholm et al., 2010; Sathyasaikumar et al., 2010). Thus, the increased brain KYNA levels observed in schizophrenia may possibly be a consequence of increased brain IDO/TDO expression and/or reduced activity of KMO. Indeed, elevated levels of TDO at the mRNA and protein level have been observed in schizophrenia (Miller et al., 2004; Miller et al., 2006). Importantly, activation of IDO following immune activation is frequently reported, see (Widner et al., 2000; Puccetti, 2007). Induction of the kynurenine pathway, as displayed by increased metabolite levels in the CSF, has furthermore been reported in infectious diseases involving the brain (Heyes et al., 1992b; Medana et al., 2002; Atlas et al., 2007; Holtze et al., 2011). An involvement of brain immune activation in schizophrenia was recently demonstrated where the pro-inflammatory cytokine IL-1β was elevated in CSF from first episode patients (Söderlund et al., 2009). Recent data from our laboratory interestingly show that this cytokine induces gene expression of both IDO and TDO, as well as increase levels of KYNA in human astrocytes (Kegel et al., 2011). The underlying cause of the deficits in the kynurenine pathway in patients with schizophrenia might thus involve a pathological cytokine expression in the CNS, possibly paralleled by genetic implications.

# 2 AIMS OF THE THESIS

The overall aim of this thesis was to evaluate if disturbances in the kynurenine pathway may contribute to the development of neuropsychiatric disorders. First, we investigated if an abnormal activity in the kynurenine pathway could serve as a possible link between CNS infection in early life and later development of schizophrenia. Second, we investigated if development of schizophrenia could involve a genetic variation in the kynurenine pathway.

The specific aims are:

1) To analyze if the mouse-adapted neurotropic strain of influenza A virus (WSN/33) could induce the kynurenine pathway *in vitro* and *in vivo* in wild-type and *Tap1*$^{-/-}$ mice (lacking CD8$^{+}$ T-cells).

2) To investigate potential long-term effects of the neonatal influenza A/WSN/33 virus infection on sensorimotor gating and on D-amphetamine-induced locomotor activity in adult mice.

3) To investigate if transiently elevated levels of endogenous brain KYNA in postnatal life could mimic the long-term behavioral effects of the neonatal influenza A/WSN/33 virus infection observed in adult mice.

4) To examine genetic variations in the kynurenine pathway by studying a possible association between polymorphisms in the *KMO* gene and schizophrenia.

5) To examine a potential association between polymorphisms in the *KMO* gene and levels of CSF KYNA.

# 3 METHODOLOGICAL CONSIDERATIONS

A detailed description of the experimental protocol can be found in each separate paper. Here, only methodological aspects are presented.

## 3.1 ANIMALS

All animal experiments were approved by the ethical committee of Northern Stockholm, Sweden, and performed in accordance with the accepted protocol. Every effort was made to minimize both the number of animals utilized and animal suffering.

Wild-type C57BL/6 mice used in **paper I** were bred at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. In **paper II and III** the wild-type C57BL/6 mice were obtained from Scanbur AB, Sweden. The influenza A virus-infected mice and their respective controls (**paper II and III**) were bred at the Department of Neuroscience, Karolinska Institutet, Sweden. Mice treated with L-kynurenine and their corresponding saline treated controls (**paper III)** were bred at the Department of Physiology and Pharmacology, Karolinska Institutet, Sweden.

In **paper II** mice with a targeted disruption of the gene encoding "Transporter associated with antigen processing 1" (*Tap1*$^{-/-}$) generated on C57BL/6 background were obtained from The Jackson Laboratory, USA and bred at the Department of Neuroscience, Karolinska Institutet, Sweden. The transporter associated with antigen processing (TAP) is required for the intracellular transport of peptides to MHC class I molecules in the endoplasmatic reticulum (Van Kaer et al., 1992). Mice with a null mutation in the *Tap1* gene thus express reduced levels of MHC class I molecules. This results in a lack of functional $CD8^+$ T-cells. These cells are considered important for viral clearance in adult mice, including clearance of virus from the brain parenchyma (Stevenson et al., 1997a; Stevenson et al., 1997b). Indeed, in adult mice, influenza A virus infection is cleared from the brain more rapidly in wild-type C57BL/6 mice than in *Tap1*$^{-/-}$ mice (Mori et al., 1999; Aronsson et al., 2001). We included the immunodeficient *Tap1*$^{-/-}$ mice in our studies to further investigate the outcome of influenza A virus infection, as previous reports indicated a more pronounced innate immune response in these mice, as well as behavioral deficiencies in contrast to wild-type mice (Asp et al., 2009).

All mice were housed under standard, pathogen-free, laboratory conditions with free access to food pellets and tap water in a light-controlled room (12 h light/dark cycle, light on at 6 a.m.). Pregnant mice were kept in a single cage and the male pups (utilized in behavior assessments) were separated after a weaning period of three weeks.

## 3.2 DRUGS

To increase the concentration of endogenous brain KYNA, the precursor kynurenine was administered to mice in **paper III**. L-kynurenine sulfate salt (Sigma Aldrich) was dissolved in distilled $H_2O$ and adjusted with NaOH to approximately pH 8.2. D-amphetamine was used to investigate behavioral aberrations, similar to those seen in schizophrenia, in L-kynurenine-treated or infected mice (**paper III**). D-amphetamine hemisulfate salt (Sigma Aldrich) was dissolved in 0.9% NaCl. This psychostimulant drug increases dopamine efflux and causes behavioral hyperactivity in animals.

## 3.3 NEONATAL TREATMENT

In our studies, two types of neonatal treatments were carried out in separate sets of mice: Influenza A virus infection at P3 or P4 and L-kynurenine treatment at P7-P16.

### 3.3.1 Influenza A/WSN/33 virus infection of mice

In **paper I, II and III**, influenza A/WSN/33 virus, obtained from Dr. S. Nakajima (The Institute of Public Health, Tokyo, Japan), was utilized to model a viral CNS infection in mice. This mouse-adapted and neurotropic strain of influenza virus is developed from the human influenza A virus, originally isolated in 1933 by Wilson Smith and coworkers (Smith et al., 1933; Francis and Moore, 1940). Injection of this strain of the influenza A virus into the olfactory bulb of young adolescent mice has previously been reported to decrease anxiety and impair spatial learning in adulthood, as well as altering the expression of genes involved in the regulation of synaptic activities (Beraki et al., 2005). Also, when administered in such manner the virus targets regions in the brain parenchyma that are implicated in neuropsychiatric disturbances (Mori et al., 1999). In this thesis, the influenza A virus was injected intraperitonatally (i.p.) to mimic a hematogenous route of the infection from the mother to the fetus. This method was

based on the neurodevelopmental theory of schizophrenia and particular the hypothesis that maternal infection is a risk factor for developing the disease (see section 1.2.1). In line with this theory, the time-point selected for the influenza A virus infection, P3 or P4, corresponds to approximately late 2nd/early 3rd trimester of human gestation with regard to brain development (Rice and Barone, 2000). Due to the neurotropic property of the A/WSN/33 strain of influenza virus, a peripheral infection targets the brain and elicits an immune response. The dosage of the influenza A virus (i.e. 2,400 plaque-forming units suspended in 30 μL) was based on pilot studies assessed by experimenters at the Department of Neuroscience, Karolinska Institutet. The mice were monitored daily following the infection. Signs of severe infection symptoms, such as reduced weight gain, resulted in immediate sacrificing and exclusion from the study, according to institutional guidelines.

### 3.3.2 L-kynurenine treatment of mice

To mimic a transient elevation of brain KYNA in neonatal mice, the precursor L-kynurenine was injected i.p. daily from P7 to P16, every 12th hour (**paper III**). Pilot studies demonstrated a two-fold elevation of brain KYNA concentrations following this treatment compared with brain concentrations in control mice injected with saline in a similar manner.

## 3.4 INFLUENZA A/WSN/33 VIRUS INFECTION OF PRIMARY CELL CULTURES

In **paper I**, primary cell cultures of neurons or glial cells were established from mouse brains. The cultures were infected with influenza A/WSN/33 virus. The choice of primary cell cultures instead of cell lines was based on the fact that immortalized cell lines, often derived from cancerous tissue, may not have the same characteristics as normal cells, for example regarding gene expression. Thus, primary cell cultures may be more likely than cell lines to reflect *in vivo* cell properties. However, one must bear in mind that these cells grow in an environment detached from physiological events in the brain. In our study, hippocampal neuron cultures and cerebral cortex glial cell cultures were prepared from C57BL/6 mice embryos sampled at embryotic day (E) 16 (day of vaginal plug = E0). Hippocampus and cortex were dissected, collected and dissociated in trypsin, which degrades the extracellular matrix. Neurons were cultured

in Neurobasal medium (optimal for prenatal and embryonic neuronal cells growth) with B-27 Serum-Free Supplement, which reduces glial cell growth. The cells were able to differentiate for seven days in the culture before the experiment, resulting in a nearly pure neuron culture with less than 2.5% glial cells, and were denoted hippocampal neuron cultures. Cerebral cortex cells were cultured in Dulbecco's Modified Eagle's Medium/F-12 (1:1) medium with 10% serum and G-5 Supplement, designed for glial cell growth (astrocytes). The cortical cultures were allowed 2-3 passages prior to the experiment. At this point, the majority of these proliferating cells stained positive for the astrocyte marker glial fibrillary acidic protein (GFAP) (Nygård, 2007), and were denoted cerebral cortex glial cell cultures.

Prior to the infection, the cell cultures were washed twice with Modified Eagle's Medium. Influenza A/WSN/33 virus was diluted in Modified Eagle's Medium and added to the cultures in 0.5 multiples of infection, i.e. one virus particle per two cells. The cultures were carefully agitated every 10 min to evenly distribute the virus in the cell cultures. Incubation temperature was 37 °C. Virus supernatants were removed after one hour and complete cell culture medium was added. The infection in cerebral cortex glial cell cultures and hippocampal neuron cultures was allowed to proceed for 24 hours and 48 hours, respectively, before the cells were harvested.

## 3.5 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The high-performance liquid chromatography (HPLC) system is used for identification, quantification and separation of compounds in liquid samples using a two-phase system. In **paper I, II, III and V** an isocratic reversed-phase HPLC system was used to analyze endogenous KYNA and/or kynurenine. Samples of brain and spleen tissue were homogenized in perchloric acid, sodium hydrogensulfite, ethylenediaminetetra-acetic acid (EDTA) prior to the HPLC analyses (**paper I, II and III**). CSF samples were analyzed unprocessed (**paper V**).

Separate samples was injected into the HPLC apparatus and mixed with a mobile phase of acetonitrile, used as a moderately polar solvent of the samples. In reversed-phase HPLC, a constant pH is necessary to stabilize the hydrophobicity of the substance to be analyzed. Therefore, the mobile phase also included a buffer solution of sodium acetate with pH 6.2. The solution is pumped through the system and when it reaches the

stationary phase of porous silica beads within the column, the molecules bind to the non-polar beads. Depending on the polarity of the molecules, the time they take passing through the column will differ, generating separation by explicit retention time. Retention time is longer for less polar molecules. The HPLC system was connected to a fluorescence detector with an excitation wavelength of 344 nm and an emission wavelength of 398 nm for KYNA analysis, and to a UV-VIS detector operating at 365 nm for kynurenine analysis. The eluate of the column was mixed with zinc acetate, forming a zinc complex required for the fluorescence/UV-VIS detection. Identification and quantification of KYNA or kynurenine was achieved by performing a scatter plot of the standard solution at different concentrations, relating the peak altitudes in the chromatogram to the respectively standard solution concentrations. These plots were subsequently used to obtain the concentration of KYNA or kynurenine in the samples.

## 3.6 IMMUNOFLUORESCENT LABELING OF T CELLS

Immunofluorescence is a type of immunohistochemistry that uses fluorescence to visualize the distribution of small molecules in biological tissue. In **paper I** this method was used to investigate the putative invasion of T cells in infected mouse brains. Coronal sections of fresh frozen hemispheres were incubated at 4 °C with either rat anti-mouse CD4 antibodies or rat anti-mouse CD8a antibodies for 24 hours. A secondary donkey anti-rat IgG antibody conjugated to the fluorophore rhodamine red was thereafter applied at room temperature for 60 min. Photomicrographs were taken with a fluorescent microscope. As positive control, sections of mouse thymus were handled correspondingly, and as negative control only secondary antibody on mouse brain sections was used (Asp, 2009).

## 3.7 RNA ISOLATION AND REAL-TIME PCR

Real-time polymerase chain reaction (PCR) was performed to analyze gene expression, i.e. mRNA transcripts, in tissue or cell samples (**paper I and II**). Prior to the gene expression analyses, total RNA from the samples was purified with Qiagen RNeasy Mini kit. Residual genomic DNA was eliminated from the RNA using DNase I (Invitrogen). The isolated total RNA, including viral RNA and 1-5% mRNA, was

thereafter converted to complementary DNA (cDNA) by using the reverse transcriptase Superscript II and random hexamer primers (Invitrogen).

With the PCR method it is possible to selectively amplify a specific DNA sequence from a very limited starting material. Real-time PCR allows detection of the product as it is being generated, i.e. in real time. This amplification process employs thermal cycles of repeated heating and cooling. The reaction mix consists of two oligonucleotide primers designed to hybridize with the forward or reverse strand of the sequence to be amplified, deoxynucleoside triphosphate nucleotides (dATP, dCTP, dGTP, dTTP), and a thermostable DNA polymerase synthesizing the novel DNA strand. For every thermal cycle the product amount is doubled.

In this thesis, two types of fluorescent detection chemistries were applied: TaqMan® and SYBR® Green. The TaqMan assay was only used to analyze the expression of the endogenous control gene *Gapdh* (encoding glyceraldehyde-3-phosphate dehydrogenase, GAPDH). For all other gene expression levels, SYBR® Green was used. The TaqMan technique depends on the correct hybridization of both primers and a dye-labeled probe to emit the fluorescent signal, while SYBR Green binds to all double stranded DNA generated in the PCR amplification. Thus, a more specific binding to the target sequence is achieved when using the TaqMan assay. The limitation of SYBR Green assay can however be compensated for by observing the melting temperature for the target sequence, as well as by cloning and sequencing the PCR product, thus verifying that the primers bind to the correct target.

In our studies, the levels of transcripts are presented as relative changes obtained by using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The absolute transcript copy number was not analyzed. Explained briefly, the cycle threshold (Ct) refers to the number of thermal cycles required for a certain DNA sequence to reach an arbitrary threshold for the fluorescent signal. The delta Ct-value (ΔCt) is obtained by the difference in Ct-numbers between the target sequence and an endogenous control sequence, presumably not affected by the experimental treatment. This compensates for (or normalizes) any differences in the amount of cDNA that was synthesized or in the amount of cDNA that was added to the PCR reaction. For all gene expression analyses included in this thesis, transcripts encoding GAPDH were verified as valid endogenous

controls. The delta delta Ct-value (ΔΔCt) is the difference in ΔCt between separate samples, for instance between virus-infected and non-infected samples. The change in expression levels of a target gene was thus first normalized to the endogenous control *Gapdh* and then presented as a fold change relative to the untreated control sample.

## 3.8 BEHAVIORAL TESTS

The behavioral tests were performed in adult mice to investigate potential long-term behavioral effects similar to schizophrenia characteristics, following a CNS disturbance in neonatal life induced by virus infection or by elevated brain KYNA as described above. Behavioral tests were performed in **paper II** (PPI) and **paper III** (PPI and locomotor activity).

To avoid potential confounds when performing animal behavioral experiments, various issues need to be considered regarding handling of the animals, circadian rhythm, hormonal cycles, body weight, and interfering external noise or smell, etc. In our studies, all mice were handled by a small number of persons, including the operator performing the actual behavioral experiments. Environmental factors such as temperature and humidity in colony rooms, light/dark cycle, and testing time of day were kept constant throughout the course of the studies. All comparative mice (treated vs. controls) were maintained under the same laboratory conditions. Only male mice were included in these studies, and all comparisons were made within genotypes.

### 3.8.1 Prepulse inhibition

PPI of an acoustic startle reflex is a cross-species translational model widely used in the fields of behavioral neuroscience and neuropsychopharmacology. It is an operational measure of sensorimotor gating, i.e. the filtering of environmental inputs, and is defined as the ability of a weak non-startling stimulus (the prepulse) to reduce the response of a subsequent startling stimulus (the pulse) presented 30 to 500 ms after the prepulse (Hoffman and Ison, 1980). Although considerable evidence has established that patients with schizophrenia have deficiencies in sensorimotor gating (McGhie and Chapman, 1961), as measured by PPI (Braff et al., 2001; Swerdlow et al., 2008), the

model is not a tool for schizophrenia diagnosis. PPI deficiencies are observed in many other disorders such as obsessive-compulsive disorder, Huntington's disease, Tourette's syndrome and bipolar disorder (Braff et al., 2001; Perry et al., 2001). Nevertheless, the PPI test in rodents serves as a robust and reliable animal model representing the pre-attentive gating deficits in schizophrenia due to its demonstrated face, predictive, and construct validity (Swerdlow et al., 1994).

One of the core characteristics in patients with schizophrenia is difficulty in sustained attention. This aspect of information processing has been suggested to be closely associated with working memory (Awh et al., 2006), an acknowledged measure of cognition. In rodents, however, PPI does not reveal any information about higher cognitive functions. Rather, the translational PPI test of mice and rats is a useful tool for evaluating animal models that are relevant to schizophrenia (Swerdlow et al., 2008). In our studies, PPI was used to assess neonatal CNS virus infection in the mouse as a model for development of schizophrenia.

For startle and PPI testing in our studies, the mouse was placed in a Plexiglas cylinder (3.7 cm in diameter), inside a ventilated and sound-attenuating startle chamber (35 x 33 x 33 cm; San Diego Instruments, San Diego, CA). Two chambers were used simultaneously, allowing one treated mouse and one control mouse to be tested at the same time. A loudspeaker placed inside the chamber provided a broadband background noise of 65 dB as well as the acoustic stimuli, controlled by a standard computer. Sudden movements by the mouse were detected by a piezoelectric accelerometer attached below the cylinder, and registered by the Startle software.

The session used in our studies was designed to fully characterize the startle and PPI phenotype based on previous reports of isolation-reared C57BL/6 mice (Varty et al., 2006). Briefly described, the session consisted of a five-minute acclimatization period to the background noise, which was continuous throughout the session, followed by three different test blocks (**Table 1**): A variable stimulus intensity block (to measure the startle threshold), a variable prepulse intensity block, and a variable interstimulus interval (ISI) block.

To obtain percent acoustic PPI from the variable prepulse intensity block and the variable ISI block, the following formula was used: 100 - ((average startle of PREPULSE + PULSE trial) / (average startle of PULSE ALONE trial)) x 100

**Table 1.** Overview of the test session build-up. Throughout the session, the trial types were presented in a pseudo-random order with an average inter-trial interval of 15 seconds.

| BLOCK | TRIALS | PRESENTATION OF TRIALS |
|---|---|---|
| **Variable stimulus intensity block** | 40-ms startle pulse of 80, 90, 100, 110 and 120 dB (**PULSE ALONE**) | 4 times each |
| **Variable prepulse intensity block** | 40-ms startle pulse of 120 dB (**PULSE ALONE**) | 12 times |
| | 20-ms of 69, 73 or 81 dB followed 100-ms later by 120 dB (**PREPULSE + PULSE**) | 10 times each |
| **Variable interstimulus interval block** | 40-ms startle pulse of 120 dB (**PULSE ALONE**) | 8 times |
| | 20-ms of 73 dB followed 25-ms, 50-ms, 100-ms, 200-ms or 500-ms later by 120 dB (**PREPULSE + PULSE**) | 4 times each |

### 3.8.2 Open field locomotor activity

The open field arena is commonly used to study both basal and drug-induced locomotor activity in rodents. Acute administration of amphetamine enhances locomotor activity. Amphetamine stimulates extracellular dopamine release, and may also display psychomimetic effects in humans. Patients with schizophrenia show a hyper-responsiveness to this drug, by demonstrating an excessive striatal dopamine release, and the amount of abnormal dopamine release correlates with the positive symptoms (Laruelle et al., 1996; Breier et al., 1997; Abi-Dargham et al., 1998). The potentiated increase of locomotor response to acute administration of D-amphetamine in mice has therefore been used to model schizophrenia.

The open field arena (50 cm x 50 cm) was surrounded by 21.6 cm high walls of Plexiglas, enclosed in a solid and sound-dampened chamber. To detect movement of the mouse, the boxes were equipped with photocell beams. In our studies horizontal activity, defined as total counts of crossed photocells (computer recorded, collected in five-minute blocks), was analyzed. All mice were habituated during three sessions of

60 minutes, 24 hours apart. Basal horizontal activity was assessed by the last habituation session (0-60 min). D-amphetamine or vehicle was thereafter immediately injected i.p., followed by a test session of 90 minutes (60-150 min). For statistical analyses of the test session, the last recorded count of the basal horizontal activity (55-60 min) was set to represent baseline activity.

## 3.9 HUMAN SAMPLES

In **paper IV and V** human samples originating from Sweden, Denmark and Norway were analyzed. These studies was approved by the local ethics committees including the Danish Scientific Committees, the Danish Data Protection Agency, the Norwegian Scientific-Ethical Committees, the Norwegian Data Protection Agency, the Ethical Committee of the Karolinska Hospital, the Stockholm Regional Ethical Committee and the Swedish Data Inspection Board (Jönsson et al., 2009). All participants had given informed consent prior to inclusion in the studies, in line with the Declaration of Helsinki.

The patients included in our studies were recruited from psychiatric departments in the local regions of Stockholm, Oslo and Copenhagen, and were clinically diagnosed according to the DSM-III-R/DSM-IV or the ICD-10. Healthy control subjects were included based on statements that they felt completely healthy or on interviews asserting that they were not suffering from any severe psychiatric disorder. All participants were Caucasians. In **paper IV**, patients with schizophrenia, schizoaffective disorder or schizophreniform disorder were compared with unrelated, age-matched controls. In **paper V**, only patients with schizophrenia (drug-naïve, drug-free or drug-treated) were included as affected individuals. A more detailed description of the participants included in these case-control association studies has been summarized in Jönsson et al. 2009.

## 3.10 GENOTYPING OF HUMAN DNA SAMPLES

Genomic DNA was isolated from whole blood, and the genotyping of SNPs were performed by the SNP technology platform in Uppsala, Sweden, using the Illumina

BeadStation 500GX and the 1536-plex Illumina Golden Gate assay (Illumina Inc.). Briefly explained, this bead array technology depends on allele-specific primers together with a locus-specific probe that both hybridize to the genomic DNA (immobilized on solid support). The fluorescent signal was then obtained from additional allele-specific labeled primers (www.genotyping.se). Since the genotyping was performed as contract research, further presentation of this method is not given.

In the association studies included in this thesis, polymorphisms in the *KMO* gene were investigated by genotyping 15 SNPs. These SNPs were selected in 2007 during my degree project from the Caucasian database of the International HapMap Project (www.hapmap.org) and the non-public RAVEN database (searching SNPs at potential transcription factor binding sites). LD-blocks were obtained with Haploview 4.1, and SNPs were selected within and between the gene LD-blocks, including non-synonymous SNPs as well as tagSNPs representing a haplotype frequency > 5%. Ten of the 15 polymorphisms were genotyped in HapMap Caucasian samples (release 27) linked to the variation of an additional 40 of the 63 *KMO* HapMap SNPs genotyped within the region that was analyzed in our studies ($R^2 > 0.85$ Haploview 4.1). Thus, the selected SNPs represented at least 79% of the total *KMO* SNPs. Prior to the analyses, the SNP genotype frequencies in the control sample were tested for Hardy-Weinberg equilibrium.

# 4 RESULTS AND DISCUSSION

## 4.1 INFLUENZA VIRUS INFECTION OF MICE *IN VITRO* AND *IN VIVO*: INDUCTION OF THE KYNURENINE PATHWAY

Disturbances during development of the human brain are suggested to contribute to the pathogenesis of schizophrenia (Murray and Lewis, 1987; Weinberger, 1987). As a potential mechanism in this regard, early-life hypofunction in glutamatergic NMDA receptor signaling has been suggested (Olney et al., 1999; Coyle, 2006). Accordingly, neonatal blockade of the NMDA receptor causes deficits in sensorimotor gating and increases locomotor activity in adult rats (Harris et al., 2003). Furthermore, mice expressing very low levels of the essential NR1 subunit of the NMDA receptor display behavioral aberrations that are proposed to model schizophrenia (Mohn et al., 1999). These results raise the possibility that blockade of NMDA receptors through elevated levels of endogenous KYNA in early life may induce neurodevelopmental impairments. Tryptophan breakdown via the kynurenine pathway is induced by various infections of the CNS (see section 1.3.2), and can be considered as part of the host innate immune response (King and Thomas, 2007). Indeed, early-life infections are recognized as a risk factor for the development of psychotic illness, including schizophrenia, later in life (Brown et al., 2000a; Buka et al., 2001a; Mortensen et al., 2007; Dalman et al., 2008).

In our studies (**paper I-III**), the impact of a mouse-adapted neurotropic strain of influenza A virus infection (WSN/33) on the kynurenine pathway during early life was studied *in vitro* and *in vivo*. With the developmental theory of schizophrenia in mind, long-term behavioral effects of the neonatal infection or kynurenine pathway induction were investigated in adult life.

### 4.1.1 Effects of the virus infection in mouse brain cell cultures

In **paper I**, primary cultures of hippocampal neurons or cortical glial cells were infected with influenza A/WSN/33 virus. The effects on gene expression of the virus infection *in vitro* are shown in **Figure 2**. In both types of cultures, the virus infection

induced robust increases in the levels of transcripts from the genes encoding IDO and TDO, the enzymes metabolizing tryptophan in the first step of the kynurenine pathway. With regard to transcripts encoding IDO, this effect is potentially mediated by pro-inflammatory cytokines produced by the infected cells (Ronni et al., 1997; Guillemin et al., 2001a; Guillemin et al., 2005b), or alternatively by a direct effect of viral proteins (Boasso et al., 2007). Notably, IDO has been suggested to be an important immune-controlling enzyme (King and Thomas, 2007). However, the gene encoding TDO (Tdo2) has not been considered to be regulated by inflammatory stimuli (Saito et al., 1992; Saito et al., 1993; Heyes et al., 1998), although TDO induction take place in placental tissue during intrauterine bacterial infections (Manuelpillai et al., 2003).

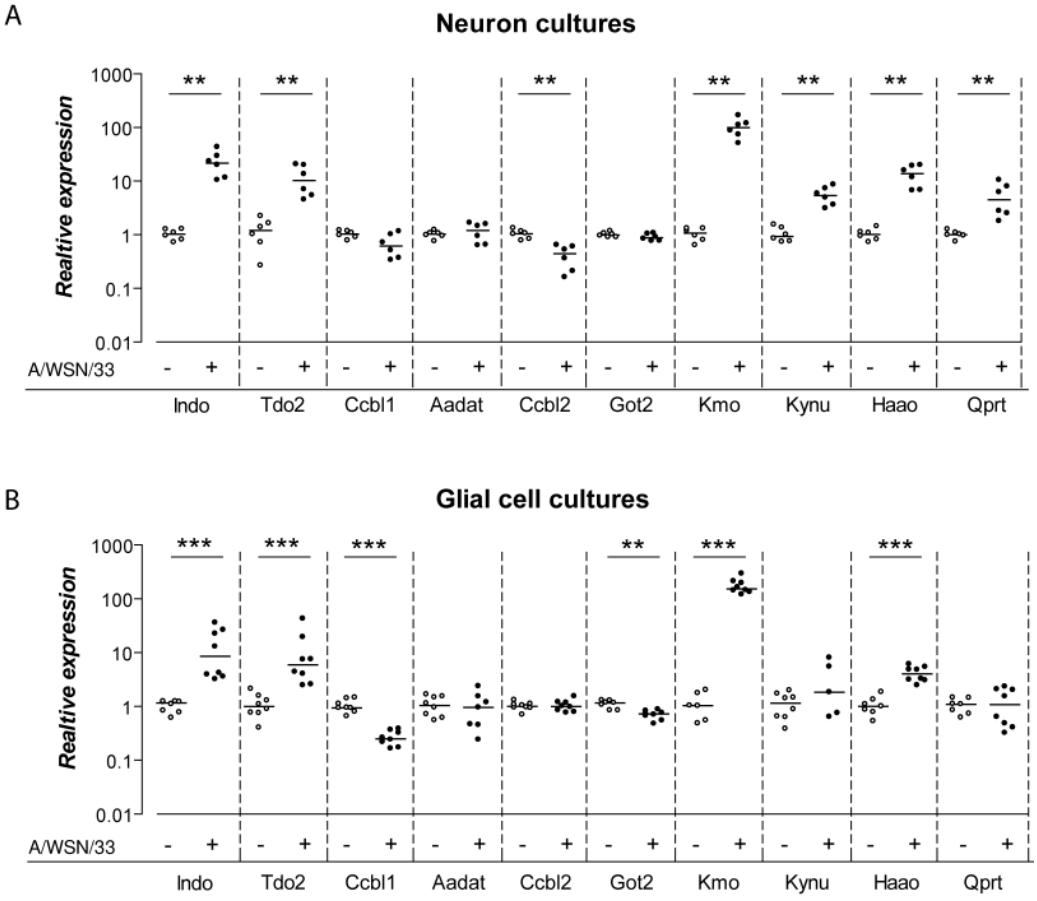


**Figure 2.** Levels of transcripts in hippocampal neuron cultures and cortical glial cell cultures. Dots represent biological replicates from two separate experiments. The levels of transcripts from the genes *Indo* (encoding IDO), *Tdo2* (encoding TDO), *Ccbl1* (encoding KAT 1), *Aadat* (encoding KAT 2), *Ccbl2* (encoding KAT 3), *Got2* (encoding mitAAT), *Kmo* (encoding KMO), *Kynu* (encoding KYNU), *Haao* (encoding HAAO) and *Qprt* (encoding QPRT) in primary hippocampal neuron cultures (**A**) and primary cortical glial cell cultures (**B**). The levels of transcripts in influenza A/WSN/33 virus-infected cultures (+) are relative to those observed in control cultures (-). The horizontal lines indicate median values. ** $p < 0.01$, *** $p < 0.001$. Mann Whitney *U* test.

Large relative increases in the levels of transcripts encoding KMO, the enzyme converting kynurenine towards the neurotoxin QUIN, were observed in both hippocampal neuron cultures and cortical glial cell cultures following infection. The two cell culture types appeared diversified in their response to infection with regard to transcripts encoding KYNU and QPRT, as these transcript levels were only induced in the neuronal cultures. This finding is in line with previous reports (Heyes et al., 1997a; Guillemin et al., 2005b) and supports the notion that production of various metabolites in the kynurenine pathway is dependent on specific cell types. In light of the recently proposed link between the levels of transcripts encoding KYNU and KMO and the capacity to produce QUIN (Guillemin et al., 2007), our findings suggest further metabolism of kynurenine towards QUIN, at least in infected neuronal cultures. However, measurements of other metabolites downstream kynurenine in the pathway were not performed here. With regard to the kynurenine metabolism into KYNA, four KAT enzymes was analyzed. Mitochondrial aspartate aminotransferase (mitAAT, identical to glutamate oxaloacetate transaminase 2 encoded by the gene Got2) was included in our analyses, as it was recently suggested to account for a significant proportion of total brain KAT activity in mice (Guidetti et al., 2007). However, none of the transcripts encoding the KAT enzymes in the two cultures was elevated following virus infection. The levels of transcripts encoding KAT 3 were lower in the infected hippocampal neuron cultures as compared with controls, and in glial cell cultures, levels of transcripts encoding KAT 1 and mitAAT were reduced in the infected cultures compared with controls. It should be noted that the standard culture conditions employed here may account for some of the differences between the two cultures and thus further studies are needed to address such issues.

*In summary, our results from studies of cortical glial cell or hippocampal neuron cultures in vitro demonstrate that a virus infection induces the kynurenine pathway, involving a robust increase in levels of the transcripts encoding IDO and TDO, enzymes regulating the first and rate-limiting reaction of KYNA production. These observations further emphasis a role of the kynurenine pathway in immune responses of the brain.*

### 4.1.2 Early-life effects of neonatal virus infection in wild-type and *Tap1*$^{-/-}$ mouse brain

In the studies *in vivo*, mice were infected neonatally at P3 or P4 and the brains and spleens were harvested at P7, P13 or P24. In the brains of infected wild-type mice (**paper I**), the highest levels of viral RNA were observed at P7, and at P24 the viral RNA was detected at low levels in all but one animal. The levels of viral RNA in the brains far exceeded those observed in spleens (10- to 1000-fold) at all time-points investigated, and the virus was cleared from the spleens more rapidly than from the brains. Invasion of $CD4^+$ and $CD8^+$ T cells, both important mediators of virus clearance from infected brains (Stevenson et al., 1997a), was detected in the brains of infected mice at P13, but not at P7. At P24, the number of infiltrating T cells had decreased considerably.

The effects on gene expression by the neonatal infection of the wild-type mice are shown in **Figure 3**. Already at P7, activation of tryptophan degradation was indicated by altered expression of several of the genes in the kynurenine pathway. Based on our results *in vitro* and on other reports (Guillemin et al., 2001b; Guillemin et al., 2007), see (Moffett and Namboodiri, 2003), it is likely that the response *in vivo* observed here involves several cell types including neurons, astrocytes and microglia. The finding that gene expression was induced at P7 although no infiltrating T cells were observed at this time-point may suggest that the activation of tryptophan degradation along the kynurenine pathway contributes to a limitation of viral replication, as initially proposed by Pfefferkorn (1984). However, several kynurenine pathway metabolites have previously been reported to suppress T cell proliferation, see (Moffett and Namboodiri, 2003), which may be particularly important in the brain with its limited regenerative capacity. In support of this latter view, our results showed that the infiltration of T cells at P13 was concomitant with a sustained elevation of transcripts encoding enzymes metabolizing kynurenine towards the synthesis of QUIN, i.e. KMO, KYNU, and HAAO, and the additional induction of the gene encoding QPRT. Present data does not ascertain whether this gene expression response is mediated by the invading T cells themselves or by the brain parenchyma to limit the actions of potentially destructive T cells.

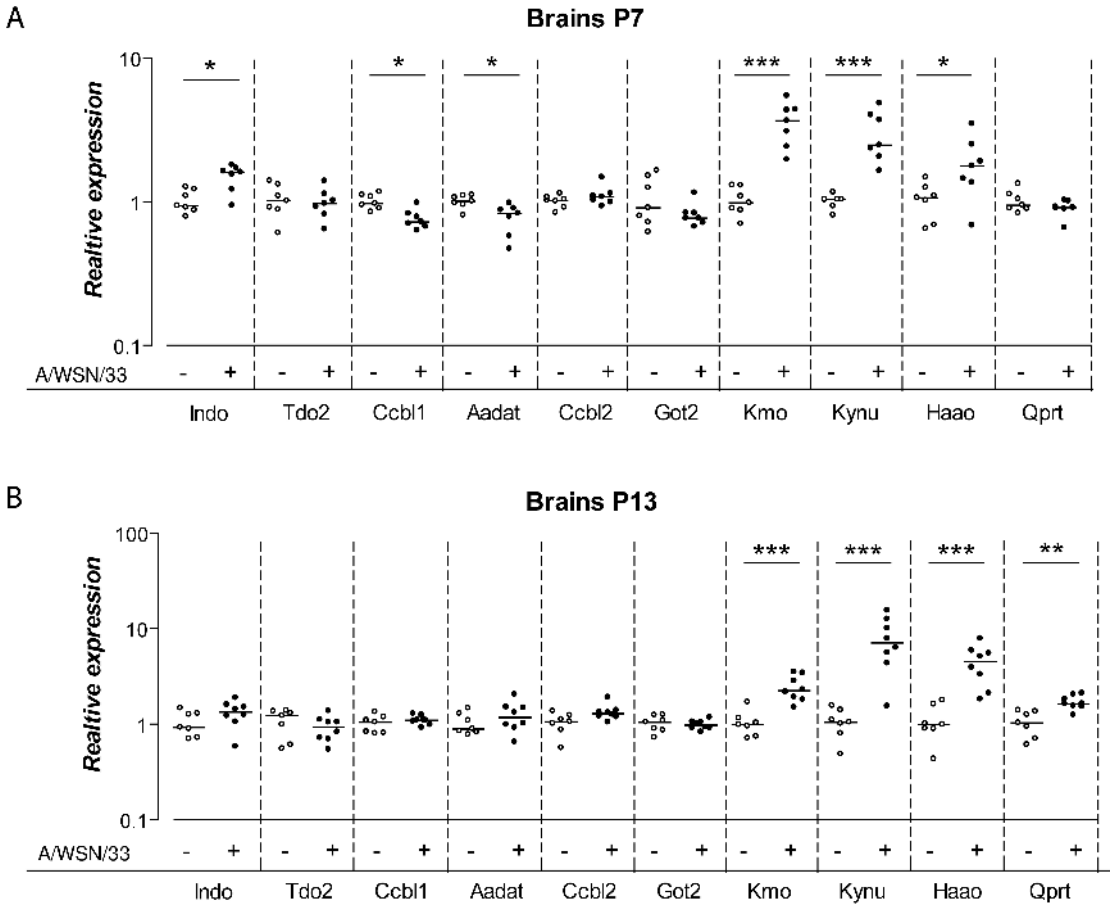


**Figure 3.** Levels of transcripts in mouse brain. Dots represent individual mice from two separate experiments. The levels of transcripts from the genes *Indo* (encoding IDO), *Tdo2* (encoding TDO), *Ccbl1* (encoding KAT 1), *Aadat* (encoding KAT 2), *Ccbl2* (encoding KAT 3), *Got2* (encoding mitAAT), *Kmo* (encoding KMO), *Kynu* (encoding KYNU), *Haao* (encoding HAAO) and *Qprt* (encoding QPRT) in brains at P7 (**A**) and at P13 (**B**) following intraperitoneal injection with 2,400 plaque-forming units of influenza A/WSN/33 virus (+) or vehicle (-) on P3 or P4. The levels of transcripts in infected brains are relative to those observed in controls. The horizontal lines indicate median values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mann Whitney *U* test.

Induction of the kynurenine pathway was accompanied by a transient increase in brain KYNA concentration, significantly elevated at P13 (**Figure 4**). Neither levels of transcripts encoding the enzymes IDO/TDO nor those encoding the KAT enzymes were elevated at this time-point. Thus, induction of the genes encoding the KAT enzymes did not appear to be necessary for increased KYNA formation. This is in line with previous experimental studies where poliovirus-infected rhesus macaques showed increased CSF KYNA despite no changes in KAT activity (Heyes et al., 1993). Indeed, recent studies investigating the activities of enzymes in the kynurenine pathway in the brains of patients with schizophrenia found unchanged activity of KAT, even though

brain KYNA was elevated (Sathyasaikumar et al., 2010). The elevated levels of mice brain KYNA in the present study may be related to increased levels of transcripts encoding IDO at earlier time-points (as indicated by increased levels of these transcripts at P7). A slight, but non-significant, increase of these transcripts was observed at P13, possibly contributing to the KYNA elevation. One might also speculate if the elevation of brain KYNA could be related to an increased synthesis of peripheral kynurenine, which readily crosses the BBB (Fukui et al., 1991). Indeed, systemic administration of L-kynurenine in rats is associated with a marked elevation in brain KYNA (Erhardt and Engberg, 2002; Linderholm et al., 2007). Analyses of spleens from the infected mice did not show elevation of TDO/IDO transcripts or of kynurenine or KYNA concentrations at any of the measured time-points. However, a trend towards increased levels of IDO transcripts at P7 and P13, as well as of kynurenine at these time-pionts, was observer, but without statistical significance. Thus, increased concentration of peripheral kynurenine should not be disregarded in contributing to the elevated KYNA concentration in the mouse brain following the influenza infection. At P24, none of the transcripts that were elevated at P13, i.e. those encoding KMO, KYNU, HAAO and QPRT, were altered.

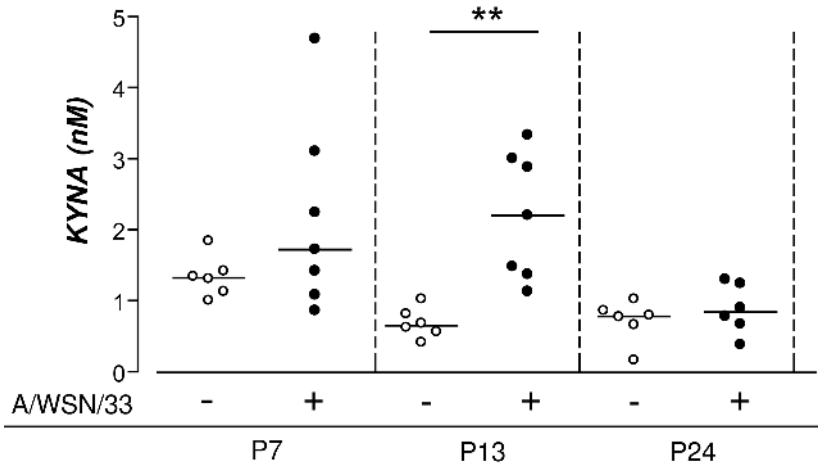


**Figure 4.** Levels of KYNA in mouse brain at P7, P13 and P24 following intraperitoneal injection with 2,400 plaque-forming units of influenza A/WSN/33 virus (+) or with phosphate buffered saline (-) on P3-4. Dots represent individual mice from two separate experiments. The horizontal lines indicate median values. ** $p < 0.01$. Mann Whitney *U* test.

In **paper II**, neonatally infected *Tap1*$^{-/-}$ mice, with reduced expression of MHC class I molecules and thus lacking functional CD8$^{+}$ T cells (Van Kaer et al., 1992), were used. In a previous report employing the same infection model as used in our studies, an increased expression of glial markers in the brain parenchyma was observed at P24 in *Tap1*$^{-/-}$, but not in wild-type mice, following the neonatal virus infection (Asp et al., 2009). Thus, a more pronounced innate immune response in these immunodeficient mice was proposed. As compared with wild-type mice, the absence of invading CD8$^{+}$ T cells did however not affect virus replication, distribution or rates of clearance in these mice. Nor did the survival rate between the two strains differ (Asp et al., 2009).

Following virus infection of neonatal *Tap1*$^{-/-}$ mice, the transcript levels of several enzymes of the kynurenine pathway were altered at all measured time-points, i.e. P7, P13 and P24 (**Figure 5**). The changes in gene expression were accompanied by a transient increase in the levels of KYNA in the brain parenchyma at P13 (**Figure 6**). Thus, increased tryptophan degradation via induction of the kynurenine pathway in the *Tap1*$^{-/-}$ mice brains is largely in agreement with our previous findings in wild-type mice. A notable difference, however, was the consistent elevation of transcripts encoding IDO in the *Tap1*$^{-/-}$ mice brains throughout all time-points investigated. Levels of transcripts encoding mitAAT were not altered in virus-infected mice at any of the investigated time-points. Decreased levels of transcripts encoding KAT 2 (at P7 and P13), and increased levels of transcripts encoding KAT 3 (at P13) were observed. However, as indicated by the results in **paper I**, the influence of a transcriptional changes of the KAT enzymes seems to be of minor importance for the formation of KYNA. Rather, the availability of kynurenine should determine KYNA concentrations. As was also observed in our studies *in vitro*, the results *in vivo* indicate further metabolism of kynurenine into 3-HK and other downstreem metabolites of the kynurenine pathway. However, measurements of these metabolites were not performed in our studies.

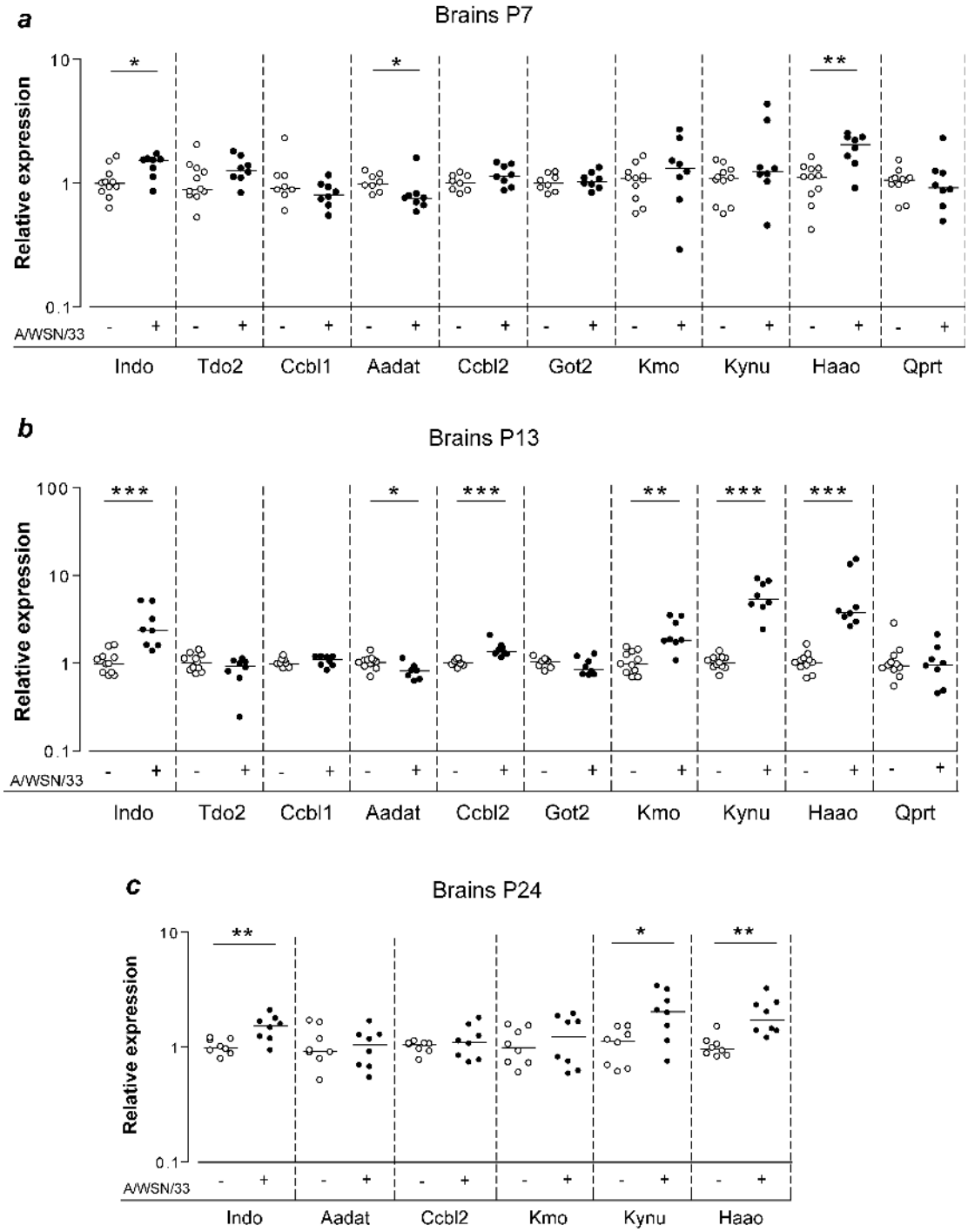


**Figure 5.** Levels of transcripts in brains of *Tap1*$^{-/-}$ mice. Dots represent individual mice. The levels of transcripts from the genes *Indo* (encoding IDO), *Tdo2* (encoding TDO), *Ccbl1* (encoding KAT 1), *Aadat* (encoding KAT 2), *Ccbl2* (encoding KAT 3), *Got2* (encoding mitAAT), *Kmo* (encoding KMO), *Kynu* (encoding KYNU), *Haao* (encoding HAAO), and *Qprt* (encoding QPRT) in brains at P7 (***a***), at P13 (***b***), and at P24 (***c***) following intraperitoneal injection with 2,400 plaque-forming units of influenza A/WSN/33 virus (+) or phosphate buffered saline (-) on P3 or P4. The levels of transcripts in virus-infected brains are relative to those observed in uninfected brains. The horizontal lines indicate median values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mann Whitney *U* test.

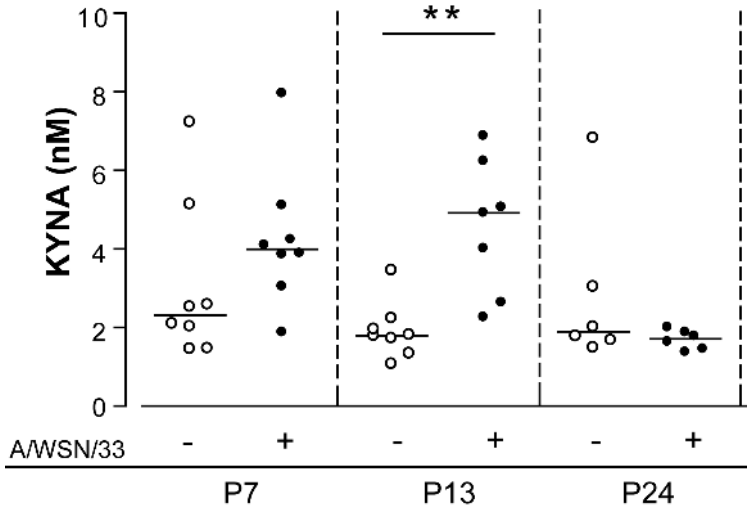


**Figure 6.** Levels of KYNA in brains of *Tap1*$^{-/-}$ mice at P7, P13 and P24 following intraperitoneal injection with 2,400 plaque-forming units of influenza A/WSN/33 virus (+) or phosphate buffered saline (-) on P3 or P4. Dots represent individual mice. The horizontal lines indicate median values. ** $p < 0.01$. Mann Whitney *U* test.

In **paper I**, no evidence of an activated brain kynurenine pathway was observed at P24 in infected wild-type mice. The increased levels of transcripts encoding IDO, KYNU, and HAAO observed in the *Tap1*$^{-/-}$ mice at this time-point (**paper II**), suggest a more persistent induction of the kynurenine pathway in these mice. Although the cellular origins of these transcripts were not further investigated, IDO, KYNU, and HAAO can all be expressed by cells in the brain parenchyma, such as astrocytes, microglia and neurons, as well as by invading cells, such as macrophages (Alberati-Giani et al., 1996; Guillemin et al., 2001b; Guillemin et al., 2003b; Guillemin et al., 2007). Our observations thus provide further support for a more pronounced innate immune response in the brains of *Tap1*$^{-/-}$ mice than in wild-type mice following a neonatal virus infection, as also proposed by Asp et al. (2009). The difference in innate immune response between the two genotypes may be related to the absence of functional $CD8^+$ T cells in the *Tap1*$^{-/-}$ mice. $CD8^+$ and $CD4^+$ T cells have been reported to be critically important in down-regulating the systemic innate immune response also in young mice (Zhao et al., 2008).

*In summary, the studies on wild-type and Tap1$^{-/-}$ mice reveal that a neonatal systemic infection targeting the brain stimulates tryptophan degradation via the kynurenine pathway in early life. The latter group of genetically vulnerable mice appeared to be more sensitive to the CNS infection, as they showed a more persistent induction. As the kynurenine pathway induction also included elevated levels of the NMDA receptor antagonist KYNA, these early-life disturbances might disrupt glutamatergic signaling in the developing brain, a condition proposed to occur in schizophrenia.*

### 4.1.3 Behavioral effects in adult mice following neonatal treatment

Frequent animal studies show an association between early-life infections and persistent behavioral effects related to both cognitive and emotional domains, as well as disrupted sensorimotor gating in adult animals (see section 1.2.1.1). To assess long-term behavioral effects following the neonatal infection and the subsequent kynurenine pathway induction, we analyzed putative behavioral deficiencies in adult mice by utilizing the PPI test (wild-type and *Tap1*$^{-/-}$ mice) and by evaluating locomotor activity (wild-type mice only), following a challenge with D-amphetamine (5 mg/kg). Separate sets of mice were treated with L-kynurenine 200 mg/kg twice a day i.p. at P7-16. This treatment resulted in a two-fold elevation of brain KYNA at the end of treatment, thus mimicking the induction of the kynurenine pathway and the transient accumulation of KYNA in early life following a neonatal influenza infection. Neonatally L-kynurenine-treated mice were subsequently assessed in adulthood with regard to PPI and locomotor activity. No statistical analyses were performed between the different sets of mice (i.e. influenza virus-infected wild-type mice, influenza virus-infected *Tap1*$^{-/-}$ mice and L-kynurenine-treated mice). **Table 2** shows the whole brain KYNA concentrations in adult mice, measured after the behavior assessments.

**Table 2.** Whole brain KYNA concentration in adult C57BL/6 mice.

| Neonatal treatment | KYNA (mean ± SEM) | Significance difference |
|---|---|---|
| Influenza A virus, WT (n = 8) | 2.03 ± 0.20 nM | $p = 0.17$ |
| Uninfected, WT (n = 10) | 1.69 ± 0.16 nM | |
| Influenza A virus, TAP (n = 10) | 4.20 ± 1.74 nM | $p = 0.54$ |
| Uninfected, TAP (n = 14) | 2.98 ± 0.53 nM | |
| L-kynurenine (n = 8) | 5.37 ± 1.10 nM | $p = 0.37$ |
| Control (n = 9) | 4.06 ± 0.51 nM | |

WT: wild-type mice, TAP: *Tap1*$^{-/-}$ mice.

#### *4.1.3.1 Prepulse inhibition*

Sensorimotor gating was assessed in adult animals by performing the PPI test on neonatally infected wild-type and *Tap1*$^{-/-}$ mice as well as on neonatally L-kynurenine-treated mice (**paper II and III**).

Wild-type mice neonatally infected at P3 or P4 with the neurotropic influenza A/WSN/33 virus did not differ from uninfected mice with regard to PPI in any of the test blocks performed.

In contrast, virus-infected *Tap1*$^{-/-}$ mice displayed impaired PPI in the varied ISI block of the test session, as indicated by a main effect of virus infection ($F(1,26) = 5.67$, $p < 0.05$; **Figure 7a**) without any interaction between virus infection and ISI. The *post hoc* test revealed a decrease in PPI in virus-infected mice at the 200 ms ISI ($p < 0.05$). Startle magnitude to the P120 trials in the varied ISI blocks did not differ between the groups (**Figure 7b**). In the varied prepulse intensity block, there was no difference between virus-infected and uninfected *Tap1*$^{-/-}$ mice. There was, however, a trend towards a higher startle threshold response in the virus-infected *Tap1*$^{-/-}$ mice compared with uninfected *Tap1*$^{-/-}$ mice ($F(1,26) = 3.81$, $p = 0.062$). Since no interaction between acoustic stimuli and virus infection was obtained, the groups should not differ in their threshold for startle response.

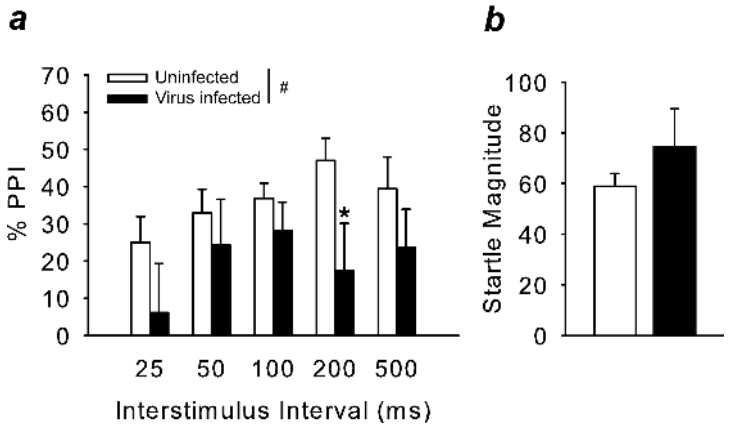


**Figure 7.** PPI (***a***) and startle magnitude (***b***) in uninfected (n = 16) and influenza A/WSN/33 virus-infected (n = 12) *Tap1*$^{-/-}$ mice at 5-6 months of age, during the varied ISI block of the startle session. # $p < 0.05$, main effect of virus infection on PPI during the varied ISI block. * $p < 0.05$, statistically different from respective uninfected mice, Tukey's *post hoc* comparison. Data are presented as mean + SEM.

The finding of a reduced PPI in adult *Tap1*$^{-/-}$ mice, but not in adult wild-type mice, is in line with a previous study, employing the same infection model as used in the present studies, reporting deficits in working memory and increased anxiety in adult *Tap1*$^{-/-}$ mice, but not in adult wild-type mice (Asp et al., 2009). Thus, an interaction between the *Tap1* gene and neonatal influenza infection is indicated. In humans, genetic polymorphisms in *TAP1* have been suggested to associate with susceptibility to virus infection (Xu et al., 2007), and possibly with schizophrenia (Fellerhoff and Wank, 2009).

Mice subjected to a subchronic elevation of endogenous brain KYNA in early life, displayed a subtle PPI impairment in the ISI-block as indicated by an interaction between L-kynurenine treatment and ISI ($F(4,64) = 3.37$, $p < 0.05$; **Figure 8A**). However, no main effect of neonatal L-kynurenine treatment was observed. *Post hoc* analysis revealed a significant effect of L-kynurenine treatment at the 500 ms ISI ($p < 0.01$). No difference was observed in startle magnitude to the P120 trials in this block between the groups (**Figure 8B**). In the varied prepulse intensity block, there was no main effect on L-kynurenine treatment or any interaction between L-kynurenine

treatment and prepulse intensity. No difference in startle threshold response was obtained between the two groups.

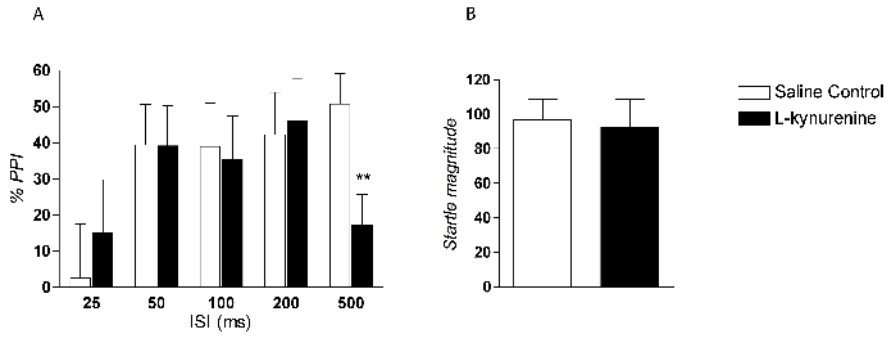


**Figure 8.** (**A**) PPI and (**B**) startle magnitude during the varied ISI block of the startle session in 3-4-months-old mice, neonatally injected with L-kynurenine (2 x 200 mg/kg/day, n = 8) or saline at P7-16 (n = 10). Statistical analysis was performed using a one-factor ANOVA, followed by a Tukey's *post hoc* multiple comparison test. $F(4,64) = 3.37$, $p < 0.05$, interaction between L-kynurenine treatment and ISI. ** $p < 0.01$, statistically different from saline control mice. Data are presented as mean + SEM.

Acutely increased brain KYNA in adult rats has previously been associated with disruptions in PPI. Based on this, brain KYNA was proposed as an endogenous modulator of sensorimotor gating (Erhardt et al., 2004). Notably though, in the present study we did not detect any significant elevation in whole brain KYNA concentrations in infected mice or in L-kynurenine-treated mice at the time of PPI testing, although KYNA levels might have been increased in specific brain regions. Given the persistent effects of neonatally administered NMDA receptor antagonists, e.g. PCP or MK-801, on sensorimotor gating in rats (Wang et al., 2001; Harris et al., 2003) it is tempting to suggest that also the transient elevation of KYNA in early life might have contributed to the deficits in PPI observed in the adult *Tap1*$^{-/-}$ and L-kynurenine-treated mice. Notably though, the neonatal virus infection induced similar transient increases in brain KYNA in wild-type as in *Tap1*$^{-/-}$ mice. The discrepancy in PPI response between these genotypes may reflect a general behavioural vulnerability of *Tap1*$^{-/-}$ mice due to their reduced expression of MHC class I molecules, which are suggested to be of importance for synaptic plasticity and regeneration (Oliveira et al., 2004; Goddard et al., 2007). Also, *Tap1*$^{-/-}$ mice showed a more persistent induction of the kynurenine pathway in

early life compared with the wild-type mice. In consonance, compared with the virus-infection in wild-type mice, the L-kynurenine treatment is likely to result in a more stable and prolonged increase in brain KYNA concentration in early life. The data on PPI in the neonatal L-kynurenine-treated mice should be interpreted with caution, however, since the only statistically significant decrease in PPI was observed at the 500 ms ISI, without a main effect on L-kynurenine treatment.

### *4.1.3.2 Locomotor activity*

An open field arena was used to assess spontaneous and D-amphetamine-induced locomotor activity in adult life of neonatally virus-infected wild-type mice and neonatally L-kynurenine-treated mice, and their respective controls (**paper III**).

Evaluation of spontaneous horizontal activity in adult wild-type mice did not reveal any differences between influenza virus-infected mice and their uninfected controls (**Figure 9A**). Acute administration of D-amphetamine was associated with an increased horizontal activity in both virus-infected and uninfected control mice. However, in the virus-infected mice, a potentiated D-amphetamine-induced increase in horizontal activity was observed compared with the uninfected controls, as obtained during the 10-50 minutes of the test period (Mann Whitney *U* test, $p = 0.04$, **Figure 9B**).

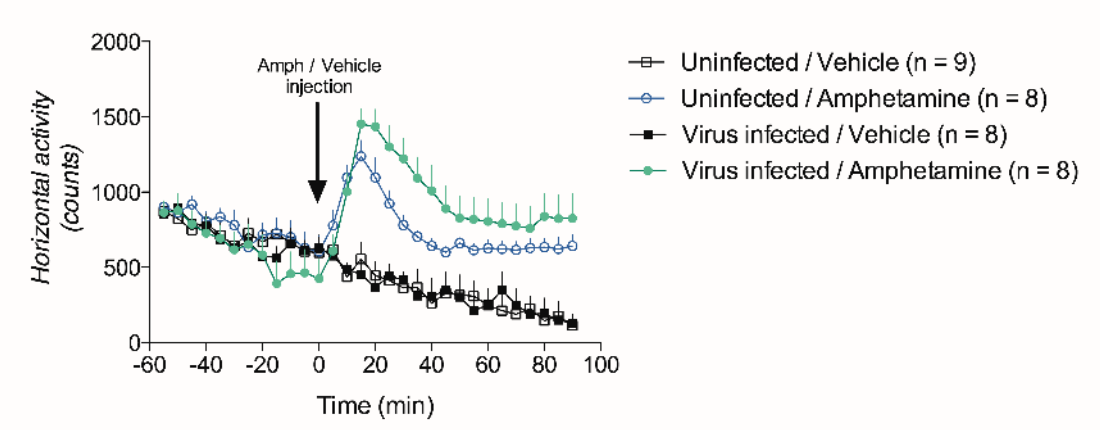


**Figure 9A.** Horizontal activity during habituation three and following acutely administered D-amphetamine (5 mg/kg) or vehicle to 5-6-month-old mice, injected with influenza A virus (2,400 plaque-forming units) or phosphate buffered saline (PBS) at P3 or P4. Each point represents the mean + SEM of counts recorded during five-minute intervals.

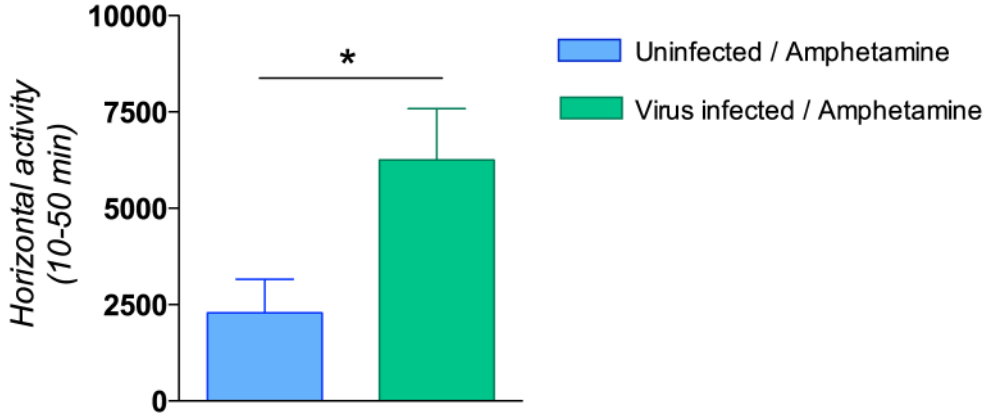


**Figure 9B.** Assessment of D-amphetamine induced increase of horizontal activity in influenza A/WSN/33 virus-infected (n = 8) and uninfected mice (n = 8). Bars represent mean + SEM of the accumulated number of counts (adjusted for baseline value, i.e. the last five min counts of habituation three) during 10-50 min following drug administration. Mann Whitney *U* test, * $p < 0.05$.

To investigate if this behavioral aberration in infected mice is related to the elevated brain KYNA in early life as observed in the infected animals, the adult L-kynurenine-treated mice were evaluated accordingly. The spontaneous horizontal activity in these mice did not differ from their respective saline controls (**Figure 10A**). A trend towards a potentiatiated D-amphetamine-induced increase in horizontal activity was observed in these mice. Unfortunately, this experiment included too few animals to achieve statistical significance (**Figure 10B**). Additional experiments are needed to confirm whether neonatal brain KYNA has a substantial effect on mice behavior in adult life.

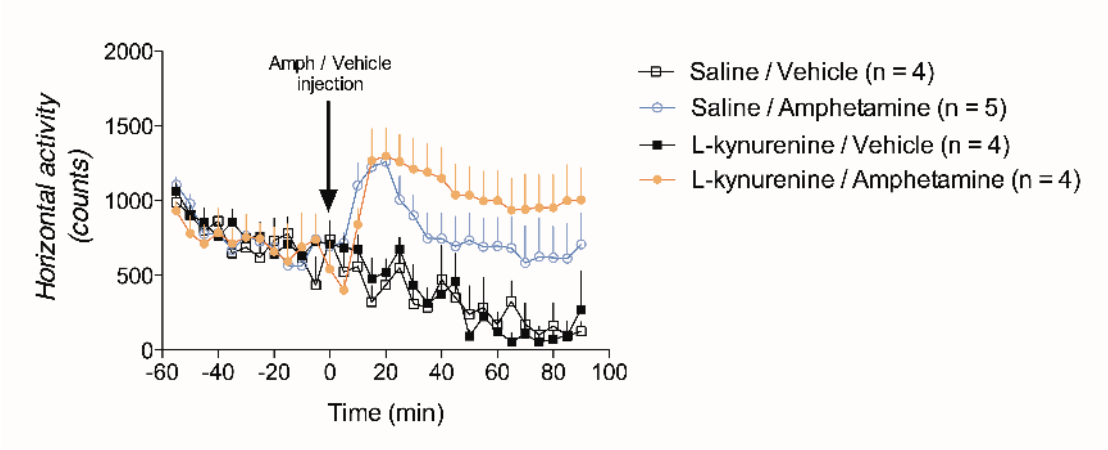


**Figure 10A.** Horizontal activity during habituation three and following acutely administered D-amphetamine (5 mg/kg) or vehicle to 3-4-month-old-mice, injected with L-kynurenine (2 x 200 mg/kg/day) or saline at P7-16. Each point represents the mean + SEM of counts recorded during five-minute intervals.

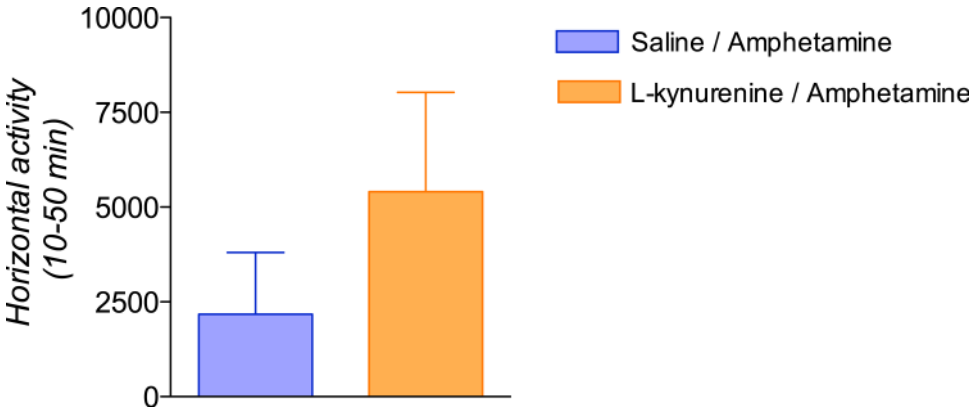


**Figure 10B.** Assessment of D-amphetamine induced increase of horizontal activity in L-kynurenine-treated mice (n = 4) and saline controls (n = 5). Bars represent mean + SEM of the accumulated number of counts (adjusted for baseline value, i.e. the last five min counts of habituation three) during 10-50 min following drug administration. Mann Whitney *U* test, $p = 0.41$.

Subchronic elevation of brain KYNA in adult rodents, possibly mimicking a physiological condition of patients with psychiatric disorders (Erhardt et al., 2001b; Linderholm et al., 2010; Olsson et al., 2010), has previously been shown to potentiate the D-amphetamine-induced increase in brain dopamine efflux (Olsson et al., 2009) as well as to exaggerate D-amphetamine-induced hyperlocomotion (Olsson et al., 2011). Present results are in line with the D-amphetamine-enhancement of striatal dopamine release observed in brain imaging studies in patients with schizophrenia (see section 1.5.1.2), although no significant elevation in brain KYNA concentration was found at the time of locomotor assessments in either infected or L-kynurenine-treated mice. A temporary elevation of brain KYNA in neonatal life could be associated with structural changes in the brain as a result of dysfunctions in the glutamatergic/ dopaminergic/cholinergic neurotransmission during a critical period of the neurodevelopment. Such a view is concomitant with the theory that an early-life NMDA receptor hypofunction may cause irreversible morphological changes in the developing brain (Olney and Farber, 1995).

*In summary, the present behavioral studies show that a neonatal infection with the neurotropic influenza A/WSN/33 virus is associated with disturbances in adult wild-type mice as reflected by increased sensitivity to D-amphetamine-induced increase in locomotor activity. Adult mice subjected to a subchronic elevation of endogenous brain*

*KYNA in early life showed a similar trend of hyper-responsiveness to D-amphetamine. Although no long-term effects of the neonatal CNS infection were observed in wild-type mice when tested for PPI in adult life, such deficiencies were observed in the genetically immunodeficient mice. Furthermore, pharmacologically elevated brain KYNA in early life tended to affect sensorimotor gating in adulthood. These results indicate that induction of the kynurenine pathway, including a transient accumulation of brain KYNA in early life, could contribute to behavioral deficits related to schizophrenia in adulthood. Thus, elevated levels of brain KYNA during a critical period in neurodevelopment might offer a molecular basis of infection as a risk factor for schizophrenia.*

## 4.2 HUMAN GENETIC STUDIES

As a large body of evidence depicts a genetic involvement in the etiology of schizophrenia (see section 1.2.2), the last studies presented in this thesis address genetic aspects on the kynurenine pathway and psychotic disorders. During the past decade, the kynurenine pathway metabolite KYNA has gained increased attention as a potential keystone in dopamine-related psychiatric disorders, including schizophrenia and bipolar disorder (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005; Linderholm et al., 2010; Olsson et al., 2010; Sathyasaikumar et al., 2010). Since the availability of kynurenine appears to be a limiting component for KYNA synthesis (Moroni, 1999), the formation of this end product indirectly depends on the activity of KMO, the enzyme converting kynurenine into 3-HK (Moroni, 1999) (**Figure 1**). Accordingly, a pharmacological inhibition of KMO, e.g. by PNU 156561A or m-nitrobenzoyl-alanine (mNBA), will shunt the metabolism of kynurenine towards KYNA (Carpenedo et al., 1994; Speciale et al., 1996; Wu et al., 2000). A functional polymorphism of the gene encoding KMO, resulting in a reduction of its gene expression and/or enzyme activity, might thus participate in the elevation of KYNA concentration in the CSF and in the *post mortem* brains of patients with schizophrenia. In the studies presented here we analyze a possible association between *KMO* gene variations and broad-spectrum schizophrenia (including schizoaffective and schizophreniform disorders) in a combined case-control sample from the Scandinavian population (**paper IV**). Furthermore, CSF KYNA concentrations in a subset of the

Swedish population of patients with schizophrenia and healthy controls were analyzed with respect to *KMO* gene variation (**paper V**). In both studies, polymorphisms in *KMO* were assessed by analyzing 15 SNPs, covering at least 79% of the total variation.

In the first study of the combined Scandinavian sample, no single marker (**Table 3**) or haplotype (data not shown) was associated with the disease. One SNP, rs2065799, showed evidence of association heterogeneity with respect to country of origin (**Table 3**): The minor allele was over-represented among Norwegian patients ($p$ = 0.01; odds ratio (OR) = 2.3, 95% confidence interval ($CI^{95}$) 1.2 - 4.4), but no association was found in the Danish or Swedish samples ($p$-values = 0.66 and 0.16, respectively). After adjustment for multiple testing, the association between the marker rs2065799 and the disease in the Norwegian sample did not reach the threshold for global significance ($p$ = 0.14). No population stratification was evident between the healthy controls ($F_{ST}$ = 0.001 ± 0.001; 95% bootstrap confidence interval). The combined Scandinavian sample was well-powered to detect nominally significant allele differences of modest effects; for an allele OR = 1.4 the power varied between 1 and 0.72 (minor allele frequency [MAF] = 0.2 and MAF = 0.05, respectively). However, the power to detect an OR of 1.2 was limited for alleles with a MAF of 0.1 or lower (power < 0.45). The present results are in line with a previous report of a Japanese population (Aoyama et al., 2006), which could indicate worldwide population consensus.

**Table 3.** Allele and genotype association between SNPs in *KMO* and schizophrenia.

| | | | HW[c] | Case/Ctrl | | | Test of association (p-value) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SNP | Base(1/2)[a] | MAF[b] | (p-value) | 1 1 | 1 2 | 2 2 | Allele | Country[d] | Genotype | Country[d] |
| RS10926508 | A/G | 0.03 | 0.30 | 785/1393 | 50/77 | 0/2 | 0.85 | 0.80 | 0.68 | 0.73 |
| RS2992642 | A/C | 0.26 | 0.89 | 421/773 | 311/537 | 61/95 | 0.15 | 0.72 | 0.35 | 0.80 |
| RS3014572 | T/C | 0.27 | 0.39 | 419/774 | 350/595 | 64/101 | 0.17 | 0.81 | 0.39 | 0.71 |
| RS2050513 | C/A | 0.16 | 0.18 | 598/1036 | 213/381 | 22/45 | 0.32 | 0.83 | 0.59 | 0.85 |
| RS3014569 | T/C | 0.14 | 1.00 | 620/1090 | 199/341 | 11/26 | 0.94 | 0.47 | 0.77 | 0.71 |
| RS10926513 | A/T | 0.40 | 0.78 | 307/529 | 392/707 | 137/228 | 0.95 | 0.29 | 0.69 | 0.30 |
| RS6661244 | C/T | 0.33 | 1.00 | 375/666 | 367/648 | 94/158 | 0.54 | 0.36 | 0.82 | 0.57 |
| RS6689793 | G/C | 0.09 | 0.15 | 687/1222 | 140/234 | 8/17 | 0.95 | 0.68 | 0.88 | 0.88 |
| RS3007737 | C/T | 0.43 | 0.46 | 255/486 | 432/704 | 147/277 | 0.58 | 0.76 | 0.23 | 0.69 |
| RS2065799 | C/T | 0.07 | 0.13 | 713/1268 | 116/193 | 6/12 | 0.66 | 0.04* | 0.49 | 0.03* |
| RS3765806 | C/G | 0.33 | 0.28 | 372/680 | 376/627 | 85/164 | 0.72 | 0.98 | 0.53 | 0.98 |
| RS12139441 | A/G | 0.18 | 0.42 | 564/998 | 244/421 | 26/51 | 0.95 | 0.52 | 0.83 | 0.52 |
| RS4660103 | G/A | 0.28 | 0.56 | 432/769 | 336/597 | 67/106 | 0.86 | 0.88 | 0.78 | 0.97 |
| RS850678 | A/T | 0.25 | 0.37 | 453/829 | 327/541 | 53/100 | 0.52 | 0.25 | 0.49 | 0.49 |
| RS1053230 | C/T | 0.23 | 0.46 | 520/873 | 281/528 | 34/71 | 0.23 | 0.45 | 0.48 | 0.43 |

[a] Major/Minor allele

[b] Minor allele frequency in controls

[c] Test of Hardy-Weinberg equilibrium

[d] Test of association heterogeneity

* Indicate nominal significance ($p < 0.05$)

In the second study, we found an association between the *KMO* polymorphism rs1053230 and KYNA concentrations in CSF (Likelihood ratio chi square = 10.0, df = 1, $p = 0.0015$). The additive value was 1.1 (95% confidence interval between 0.34 and 1.79) and a copy of the T-allele was associated with a 45% increase in KYNA CSF concentrations (least square mean for individuals with the CC and CT genotypes were 1.0 nM, and 1.49 nM, respectively; **Figure 11**). The association was found in both patients and controls and was significant after correction for multiple testing (adjusted $p = 0.023$, empirical 5% quantile = 0.003). Although there was a tendency towards a stronger association in affected individuals, this difference was not statistically significant ($p$-value for affection state as modifier was 0.73). The rs1053230 is located on exon 15 and results in a shift of the amino acid sequence from arginine to cysteine. These results indicate that the different alleles of the *KMO* polymorphism rs1053230 predict KYNA concentrations. At the time of CSF collection, all patients with schizophrenia received or had previously received antipsychotics. Such drugs have been reported to decrease KYNA content in the rat brain (Ceresoli-Borroni et al., 2006), thereby limiting the functional consequence of the *KMO* (rs1053230) T allele.

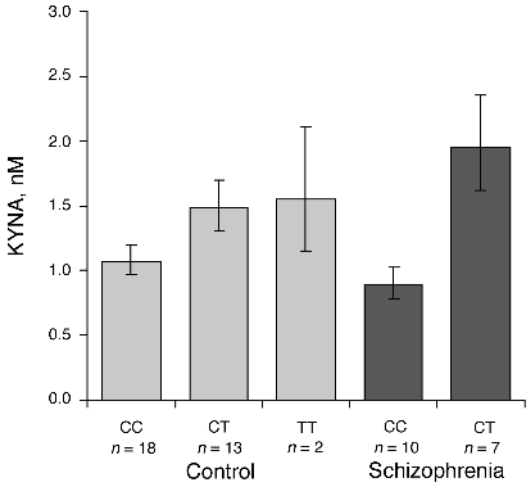


**Figure 11.** The CSF concentrations of KYNA increase with the T-allele of rs1053230 ($p$ = 0.023 after correction for multiple testing). Least square means and standard errors are given for control and patients with schizophrenia with the CC, CT and TT genotypes, respectively, adjusting for effects of back length, age and sex.

This is the first study showing an association between a *KMO* polymorphism and a proposed phenotype for schizophrenia, i.e. elevated levels of CSF KYNA concentrations. The KMO enzyme is located on the outer membrane of mitochondria (Okamoto et al., 1967). According to computerized protein structure predictions, large parts of the protein are located inside the membrane (www.predictprotein.org). Interestingly, in this prediction, the *KMO* polymorphism rs1053230 is located in the part of the gene sequence coding for protein positions outside of the mitochondria membrane, likely to be the site for substrate interaction. An exchange of amino acids in this part of the enzyme may also influence substrate binding, for example by changeing the hydropathy index from -4.5 (arginine, the most hydrophilic amino acid) to 2.5 (cysteine, a moderate hydrophobic amino acid) (Kyte and Doolittle, 1982). The increased levels of KYNA, seen in individuals with the mutant T allele, may thus reflect a reduction of kynurenine binding to KMO. The metabolism of kynurenine would thereby be shunted towards KYNA, in analogy with administration of pharmacological compounds blocking KMO.

Similar disease frequencies in carriers and non-carriers of the rs1053230 mutant allele suggest that elevated KYNA concentrations in patients suffering from schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005) are more likely to reflect the pathophysiology of the disease than to represent a mechanism of disease susceptibility. It should be noted that the participants in the CSF study represent a small fraction of patients and control subjects (included in the previously published report Nilsson et al., 2005). The lack of a difference in CSF KYNA concentrations between patients and controls presented in our study is thus possibly related to the restricted number of samples analyzed. It is of interest to note that, despite this limitation, the *KMO* (rs1053230) T allele association with an increase in KYNA concentration tended to be stronger in patients with schizophrenia compared with healthy controls. This might reflect an up-regulated turnover of the kynurenine pathway in patients with schizophrenia. Indeed, previous studies have shown that levels of kynurenine, the precursor of KYNA, are increased in patients with schizophrenia (Linderholm et al., 2010). Thus, a change in the *KMO* codon sequence from arginine to cysteine in combination with increased kynurenine turnover may predispose for elevated KYNA levels as seen in patients with schizophrenia.

*In summary, the present association studies analyzing genetic variations in the KMO gene reveal that increased levels of CSF KYNA, as previously reported in patients with schizophrenia, are associated with a functional missense polymorphism in the KMO gene. However, the KMO gene polymorphisms investigated are not likely to confer major susceptibility to schizophrenia per se.*

# 5 GENERAL DISCUSSION

Accumulating evidence suggests that schizophrenia is associated with immunological processes in the brain. Direct evidence for a central immune activation is relatively sparse, and conceivably, the most prominent studies in this regard show elevated levels of IL-1β and IL-6 (Garver et al., 2003; Söderlund et al., 2009; Schwieler et al., 2011) as well as KYNA in the CNS of affected patients (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005; Linderholm et al., 2010; Sathyasaikumar et al., 2010). Indeed, not only cytokines, but also kynurenine pathway metabolites are suggested as regulatory mediators of the innate and adaptive immune responses, reviewed in (King and Thomas, 2007; Gonzalez et al., 2008). Recent results from our laboratory show that IL-1β induces genes encoding the enzymes IDO and TDO, resulting in increased concentration of KYNA (Kegel et al., 2011). These findings may link together the elevation of IL-1β, KYNA and TDO activity seen in the CNS of patients with schizophrenia into a common pathophysiological assembly underlying the disease (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005; Miller et al., 2006; Söderlund et al., 2009; Linderholm et al., 2010; Sathyasaikumar et al., 2010). Further support for an immunological involvement in schizophrenia is presented by the finding of mRNA sequences of the human endogenous retrovirus (HERV) in the CSF of affected patients (Karlsson et al., 2001). Also, increased mRNA expression of genes related to various immune responses has been found in the brain of patients with schizophrenia (Saetre et al., 2007). Clearly, infection, particularly in early-life is considered a risk factor for development of the disease. Thus, early-life exposure to a variety of infectious pathogens, such as *Toxoplasma gondii*, mumps virus, rubella virus, HSV type 2, cytomegalovirus and influenza viruses, is associated with development of psychotic illness, including schizophrenia (Brown et al., 2000a; Buka et al., 2001a; Mortensen et al., 2007; Dalman et al., 2008). However, since no specific infectious agent appears responsible for the development of schizophrenia, this implies a common mechanism of immune activation in the disease, possibly including an abnormal expression of specific cytokines in early brain development (Meyer et al., 2009). Indeed, cytokines are also suggested to influence neurodevelopment (Bauer et al., 2007; Deverman and Patterson, 2009), and could underlie abnormal cytoarchitectonics of the brain as postulated by the neurodevelopmental hypothesis of schizophrenia, reviewed in (Marenco and Weinberger, 2000). The findings of the

present thesis show that a neonatal virus infection is associated with an early-life induction of the kynurenine pathway and with schizophrenia-like behavior in the adult mouse, hereby merging together the neurodevelopmental hypothesis of schizophrenia with the view of an immune activation in the disease.

In addition to an immune-mediated induction of the kynurenine pathway, impairments of the KMO enzyme might contribute to the increased KYNA concentration observed in patients with schizophrenia. KMO converts kynurenine to 3-HK and experimental studies show that pharmacological inhibition of this enzyme leads to increased brain KYNA concentration (Speciale et al., 1996). In the present thesis, an association was found between the non-synonymous missense *KMO* SNP rs1053230 (changing the amino acid codon) and CSF KYNA concentration. Given the position of the SNP in the gene region, this genetic variation is likely to affect the interaction between the KMO enzyme and its substrate kynurenine. Thus, this SNP seems particularly relevant for kynurenine metabolism. It was recently shown that *KMO* gene expression as well as the enzyme activity is down-regulated in the *post mortem* brain of patients with schizophrenia (Wonodi et al., 2011). In that study, another *KMO* SNP (rs2275163) was associated with neurocognitive endophenotypic deficits (Wonodi et al., 2011). Overall, these findings support a malfunctioning KMO enzyme in schizophrenia. Interestingly, the presently shown association between the minor (T) allele of the *KMO* SNP rs1053230 and increased CSF KYNA concentrations tended to be stronger in patients with schizophrenia, suggesting that the activity of up-stream enzymes in the kynurenine pathway (i.e. IDO or TDO) is concomitantly increased. Kynurenine concentration is indeed elevated in the brain and the CSF of patients with schizophrenia (Linderholm et al., 2010; Sathyasaikumar et al., 2010), and the kynurenine availability is shown to be essential for the production of KYNA (Moroni, 1999). Notably, the *KMO* gene maps to the chromosome region 1q42-q44, a block that shows a strong linkage to schizophrenia (Owen et al., 2004) making analysis of this gene particularly relevant for the disease etiology. In line with a Japanese study (Aoyama et al., 2006) we could not report a schizophrenia association with any *KMO* SNP investigated. However, in both our and the Japanese study, an initial association was found. Thus, in the Norwegian population, the *KMO* SNP rs1053230 was nominally associated with schizophrenia. In this aspect, the strategy of analyzing possible associations with phenotypes, such as

CSF KYNA, rather than the disease *per se*, is probably a more constructive genetic approach for complex diseases like schizophrenia, as reviewed in (Meyer-Lindenberg and Weinberger, 2006).

The present thesis, comprising studies both *in vitro* and *in vivo*, depicts a prominent effect of the neurotropic influenza A/WSN/33 virus in activating the kynurenine pathway, as revealed by the transcript induction of enzymes along the pathway and by elevated levels of KYNA. This is in line with clinical findings showing that infections trigger the kynurenine pathway, as reflected by elevated concentrations of various kynurenine metabolites, including for example KYNA and QUIN, in the CSF or brain of patients with cerebral infections such as HIV-1, TBE and cerebral malaria as well as other CNS inflammatory neurological diseases (Heyes et al., 1992b; Medana et al., 2002; Atlas et al., 2007; Holtze et al., 2011). Indeed, several metabolites of the kynurenine pathway have immunomodulatory functions, and may be implicated in the host defense of invading pathogens. Thus, although metabolites such as 3-HK and QUIN were not measured in the present thesis, elevated transcript levels of KMO, KYNU, HAAO and QPRT indicate that the synthesis of these metabolites is increased. One might speculate that exposure to infections in early life results in elevated levels of various kynurenine pathway metabolites that all contribute to a disturbed immune balance in the developing brain. However, the physiological consequences of elevated levels of these metabolites are ambiguous since elevated levels of KYNA are shown to dampen the effects of some of these compounds. Thus, KYNA is able to scavenge free radicals generated by 3-HK (Goda et al., 1999; Lugo-Huitron et al., 2011). With regard to QUIN, a large body of evidence shows that KYNA blocks the excitotoxic effects of this NMDA receptor agonist (Foster et al., 1984; Sas et al., 2007). Furthermore, endogenous KYNA has been demonstrated to control the vulnerability to QUIN neurotoxicity in the mouse brain (Sapko et al., 2006), and a reduced concentration of KYNA is required for a toxic effect of QUIN.

In the present thesis, neonatal infection with a neurotropic strain of influenza A virus increased brain KYNA concentrations in both wild-type mice and in immunodeficient *Tap1*$^{-/-}$ mice. Interestingly, the neonatal infection was associated with deficits in PPI in the adult immunodeficient mice and a hyper-responsiveness to D-amphetamine in locomotor response in the adult wild-type mice. To investigate if the elevated levels

of endogenous KYNA seen in the infected neonatal mice could account for the observed long-lasting behavioral disturbances, we investigated behavioral effects in adult mice neonatally treated with L-kynurenine, the BBB-crossing precursor of KYNA. Indeed, pharmacologically elevated levels of endogenous brain KYNA in neonatal mice tended to result in a hyper-responsiveness to D-amphetamine, and was associated with mild reductions in PPI in adulthood. These results are in line with brain imaging studies in patients with schizophrenia reporting an enhanced striatal dopamine release by D-amphetamine (Laruelle et al., 1996; Breier et al., 1997; Abi-Dargham et al., 1998; Laruelle and Abi-Dargham, 1999), and the frequently observed PPI deficits in these patients (Braff et al., 2001). Kynurenine is preferentially metabolized to 3-HK under normal physiological conditions. However, with regard to the biochemical kinetics of KMO and KYNU, these enzymes should be saturated more rapidly at rising kynurenine concentrations than the KAT enzymes. Therefore, synthesis of KYNA ought to be favored when L-kynurenine is systemically administered. Indeed, focal infusion of $^{3}$H-kynurenine in the rat brain has been shown to increase KYNA to a higher extent than QUIN, with a ratio of 1 to 0.2 (Guidetti et al., 1995; Amori et al., 2009). In all probability the effects of systemically administered L-kynurenine in the present study are related to increased levels of brain KYNA rather than any other kynurenine pathway metabolite. Thus, as the neonatal virus-infection and the neonatal L-kynurenine-treatment showed similar behavioral aberrations, the long-term effects of the neonatal virus infection may specifically be related to the increase in endogenous brain KYNA concentration.

The findings of the present studies are in line with the neurodevelopmental hypothesis of schizophrenia, suggesting early life as a particularly vulnerable time for environmental insults (Olney et al., 1999; Marenco and Weinberger, 2000). Indeed, administration of NMDA receptor antagonists during a critical period of brain development has been shown to result in apoptotic neurodegeneration (Rudin et al., 2005; Young et al., 2005; Ikonomidou, 2009), as well as in persistent behavioral deficits in rodents (Harris et al., 2003; Fredriksson et al., 2007; Mouri et al., 2007). In addition, the α7nACh receptor has been implicated in neurodevelopmental processes (Ross et al., 2010). Interestingly, long-term behavioral effects of elevated brain KYNA are also implied in two recent studies were a subchronic elevation of brain KYNA concentration during adolescence was associated with cognitive dysfunctions as well as

decreased social interaction in the young adult rat (Akagbosu et al., 2010; Trecartin and Bucci, 2011). Given the unique receptor-binding profile of KYNA, present findings further emphasize a significant role of KYNA as a mediator of the behavioral alterations observed in adult mice. A temporary elevation of brain KYNA in neonatal life could be associated with persistent dysfunctions in glutamatergic/cholinergic/ dopaminergic neurotransmission, hereby affecting neuronal circuits that are important for brain development. Alternatively, an increased turnover in brain KYNA may be long-lasting and persist throughout the time period for behavioral evaluation. In this regard, it is notable that the neonatal influenza virus-infection in both immunodeficient *Tap1*$^{-/-}$ and wild-type mice, as well as the neonatal L-kynurenine treatment, tended to be associated with a small increase in whole brain KYNA content in adult life, hereby indicating a persistently increased turnover of brain KYNA (**Table 2**: Infected *Tap1*$^{-/-}$ 1.4-fold increase, infected wild-type 1.2-fold increase, L-kynurenine-treated 1.3-fold increase). This is in line with a recent microdialysis study showing increased extracellular KYNA levels in adulthood following perinatal treatment with L-kynurenine (Pocivavsek et al., 2011a). Indeed, subchronic elevation of brain KYNA in adult rodents, mimicking a pathophysiological condition in patients with psychotic disorders, has been shown to potentiate the D-amphetamine-induced increase in brain dopamine efflux and in locomotor activity (Olsson et al., 2009; Olsson et al., 2011). Furthermore, acutely increased brain KYNA in rats is associated with impaired sensorimotor gating (Erhardt et al., 2004). Thus, a slight but long-lasting increase in KYNA turnover might, by itself or in symphony with neurodevelopmental effects, contribute to the presently observed behavioral aberrations in adult life.

The results of the present thesis, derived from both experimental studies and genetic association studies, offer substantial support to the view that KYNA participates in the etiology of schizophrenia. During the past few years it has become increasingly evident that this tryptophan-derived neuroactive metabolite serves as a biomarker of immune activation in the brain. The complicated interplay between the kynurenine pathway and the more traditional mediators of the immune system, e.g. cytokines and prostaglandins, is a venue for future investigations. For example, not only are cytokines able to induce the kynurenine pathway, but also kynurenine metabolites are shown to induce cytokine release (Guillemin et al., 2003a; Wang et al., 2006; Fallarini

et al., 2010; Kegel et al., 2010). Moreover, although the kynurenine pathway is the major route of KYNA production, another pathway for the synthesis of KYNA has been suggested: a stress-induced oxidative pathway mediated though indol-3-pyruvic acid (Bartolini et al., 2003). Investigations of this pathway with regard to immune activation and behavioral abnormalities as well as genetic variations might offer additional insight into the pathophysiology/etiology of schizophrenia.

In summary, the present thesis shows that a neurotropic virus infection in early life is associated with an induction of the kynurenine pathway followed by behavioral aberrations in the adult mouse, and that a genetic deficit in this pathway is associated with increased CSF KYNA in patients with schizophrenia and in healthy controls. Indeed, a genetic disturbance in patients with schizophrenia is essentially what was proposed in the initial neurodevelopmental hypothesis of this disease. However, it has become increasingly apparent that genetic aberrations only partly contribute to the etiology of this disease. In this regard, an early-life exposure to infection could have a more substantial effect on an already genetically vulnerable neuronal system (Cannon et al., 2003). This view is in line with our findings of a more pronounced induction of the kynurenine pathway in early life in the immunodeficient *Tap1*$^{-/-}$ mice. Hereby, our results further support a gene-environmental interaction as suggested in the development of schizophrenia. Thus, a genetic deficit of the *KMO* gene concomitantly with an immune-mediated induction of IDO or TDO activity should trigger the production of brain KYNA as observed in patients with schizophrenia. Altogether, this thesis suggests that an early-life CNS induction and/or a genetic deficit of the kynurenine pathway could predispose for development of schizophrenia through the elevation of brain KYNA.

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