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Kynurenic acid in psychiatric disorders
– studies on the mechanisms of action

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ABSTRACT

Kynurenic acid, a metabolite of tryptophan along the kynurenine pathway, is an endogenous antagonist at NMDA- and α7* nicotinic acetylcholine- (α7*nACh) receptors. Previous studies have shown that elevated levels of endogenous kynurenic acid are associated with an increased firing of midbrain dopamine neurons as well as a reduced prepulse inhibition (PPI), a behavioral model for sensorimotor gating. Furthermore, kynurenic acid is elevated in the CSF and in the postmortem prefrontal cortex of patients with schizophrenia. The aim of this thesis was to investigate the mechanisms of action of kynurenic acid with regard to its action on midbrain dopamine firing and PPI; to analyze the interaction between the antipsychotic drug clozapine and kynurenic acid, and finally; to further study the involvement of kynurenic acid in psychiatric disorders.

The excitation of ventral tegmental area (VTA) dopamine neurons observed in rats with elevated levels of kynurenic acid were mimicked by pretreatment with 4-chlorokynurenine (4-Cl-KYN). Administration of SDZ 220-581 was also found to increase firing of VTA dopamine neurons. However, administration of methyllycaconitine (MLA) decreased firing of these neurons. These results demonstrate that the increased firing of VTA dopamine neurons following elevation of brain kynurenic acid is primarily mediated through glutamatergic rather than by cholinergic mechanisms.

Administration of SDZ 220–581 or CGS 19755 was associated with a robust reduction in PPI, whereas L-701,324, 4-Cl-KYN or MLA failed to alter PPI. Kynurenine increased brain kynurenic acid levels 5-fold and tended to decrease PPI. These results suggest that neither antagonism of the glycine site of the NMDA receptor nor antagonism of the α7*nACh receptor disrupts PPI. Rather, blockade of the glutamate recognition site is necessary to reduce PPI.

Clozapine increased firing of VTA dopamine neurons in control rats. Pretreatment with indomethacin was found to elevate brain kynurenic acid levels and to reverse the excitatory action of clozapine into an inhibitory response. In contrast, pretreatment with parecoxib decreased brain kynurenic acid formation and clearly potentiated the excitatory effect of clozapine. These results show that endogenous levels of brain kynurenic acid are of importance for the response of clozapine on VTA dopamine neurons and we propose that clozapine is able to interact as a partial agonist with the glycine site of the NMDA receptor.

CSF kynurenic acid and kynurenine was found to be elevated in patients with schizophrenia compared to controls. Violent male suicide attempters and male suicide attempters with a diagnosis of major depression had higher CSF kynurenic acid than controls. Furthermore, CSF levels of kynurenic acid correlated with CSF MIP-1β in all subjects, with CSF eotaxin-3 in controls and tended to correlate with IL-6 in all subjects.

An up-regulated kynurenine pathway may be a consequence of an activated immune system as studies have shown that immunological agents induce the first and rate-limiting enzymes in the production of kynurenic acid, thus indicating that kynurenic acid is a marker of immune-activation. Elevation of endogenous kynurenic acid may be a potential mechanism by which the immune system initiate psychiatric symptoms. This theory is supported by the close correlation between cytokines/chemokines and kynurenic acid in the CSF of suicide attempters observed in the present thesis. Altogether the present results suggest that kynurenic acid, with its unique receptor profile, may be the link between an activated immune system and the alterations in glutamatergic, cholinergic and dopaminergic neurotransmission proposed to occur in psychiatric disorders.

LIST OF PUBLICATIONS

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III. Schwieler L, LINDERHOLM KR, Nilsson-Todd LK, Erhardt S, Engberg G. Clozapine interacts with the glycine site of the NMDA receptor: Electrophysiological studies of dopamine neurons in the rat ventral tegmental area. *Life Sciences* 2008;83:170-175.

IV. LINDERHOLM KR, Skogh E, Olsson S, Dahl M-L, Holtze M, Samuelsson M, Lundberg KM, Erhardt S. Increased levels of kynurenine and kynurenic acid in the CSF of patients with schizophrenia. *Submitted manuscript*.

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LIST OF ABBREVIATIONS

4-Cl-KYN	4-chloro-kynurenine
7-Cl-KYNA	7-chloro-kynurenic acid
α7*nACh	α7*-nicotinic acetylcholine receptor
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	analysis of variance
COX	cyclooxygenase
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assay
EPS	extrapyramidal side effects
GABA	γ-aminobutyric acid
HPLC	high-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
IDO	indoleamine-2,3-dioxygenase
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
KAT	kynurenineaminotransferase
LTP	long term potentiation
MDD	major depressive disorder
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
PPI	prepulse inhibition
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
TDO	tryptophan-2,3-dioxygenase
TNF-α	tumor necrosis factor-α
VTA	ventral tegmental area

1 INTRODUCTION

1.1 Schizophrenia

Schizophrenia is a severe mental illness characterized by abnormalities in perception or expression of reality. The disorder has a major impact on social functioning and is almost always of a chronic character. The global prevalence is approximately 0.55 to 0.85% (Carpenter and Buchanan, 1994; Goldner et al., 2002). The onset of symptoms typically occurs in young adulthood and usually occurs a few years earlier in men than in women. Average life expectancy of people with schizophrenia is 10 to 12 years less than those without (Brown et al, 2000). This is due to increased physical health problems and a higher suicide rate, accounting for approximately 5-10% of the deaths in patients with the disorder (Black and Fisher, 1992; Meltzer et al., 2002; Palmer et al., 2005). Patients with schizophrenia also have an increased risk for psychiatric comorbidity, including major depressive disorder (MDD), anxiety disorders and substance abuse (Sim et al., 2006). As a result, schizophrenia is not only associated with substantial human suffering, but also with high costs to society in terms of healthcare and loss in productivity.

Symptoms of schizophrenia most commonly manifest as hallucinations, delusions or disorganized speech and thinking. There is also often an observable pattern of emotional difficulties as well as impairment in social cognition leading to isolation and social withdrawal, although the individual expression of symptoms may vary. To guide clinicians and investigators the symptoms are often divided into three broad clusters: positive symptoms, negative symptoms and cognitive impairments (Andreasen, 1995). Positive symptoms refer to symptoms that most individuals do not normally experience, such as paranoid or bizarre delusions, auditory hallucinations and thought disorder. These are typically regarded as manifestations of psychosis. Negative symptoms reflect absence or loss of abilities normally found in healthy persons. Common negative symptoms include blunted affect and emotion, anhedonia, poverty of speech (alogia), lack of motivation and asociality. Cognitive impairments seen in patients with schizophrenia include deficits in memory, attention and executive functions, lack of judgment and insight. The positive (psychotic) symptoms tend to be episodic over time, often necessitating temporary hospitalization. In contrast, negative

symptoms and cognitive impairments tend to be more stable but are suggested to contribute more to functional impairment and disability as well as poor quality of life (Fenton and McGlashan, 1991; Green et al., 2000).

The causes of schizophrenia have been the subject of much debate and the complexity of the disorder has made the search for a pathophysiological explanation extremely difficult. Family, twin and adoption studies provide evidence indicating genetic factors contributing to the disease. Thus, the risk among first-degree relatives is approximately 10-fold higher than in the general population and adoption studies indicate an increased risk in those with a biological parent with schizophrenia (Kessler, 1980; McGue and Gottesman, 1991; Harrison and Owen, 2003). Despite studies reporting concordance rates between monozygotic twins as high as 50 – 84% (Farmer et al., 1987; Cardno et al., 1999), the lack of complete concordance indicates that neurodevelopmental and environmental factors also contribute to the cause of the disease. Thus, it has been suggested that the primary pathological process of schizophrenia occurs during brain development, e.g. by obstetric complications, maternal stress, malnutrition or smoking during pregnancy (Mortensen et al., 1999; Susser and Lin, 1992). Furthermore, environmental factors associated with schizophrenia include psychosocial factors such as urban upbringing, social isolation, migration and drug abuse (Susser, 2002). Recent epidemiological studies suggest that an immunological component may play a part in the etiology as well (Sperner-Unterweger, 2005). For instance, prenatal infections during the second trimester are associated with an increased risk of schizophrenia (Brown, 2006). In support of an immunological component in schizophrenia, we recently reported that the pro-inflammatory cytokine interleukin (IL)-1β is markedly elevated in the CSF of first-episode patients with schizophrenia (Söderlund et al., 2009).

1.1.1 Antipsychotic drugs, clozapine

Schizophrenia is associated with positive, negative and cognitive symptoms (see 1.1). The beneficial effects of classic antipsychotic drugs (e.g. haloperidol) in ameliorating the positive symptoms of schizophrenia are thought to be related to a reduced dopaminergic neurotransmission within the limbic region of the brain (Carlsson et al, 2001). However, reduced dopaminergic neurotransmission is also associated with severe side effects, such as extrapyramidal side effects (EPS; Farde et al., 1992;

Gerlach, 2002). The antipsychotic drug clozapine has very low incidence of EPS and has thus been classified as an atypical antipsychotic drug (Claghorn et al., 1987; Coward et al., 1989). Furthermore, clozapine has a superior efficacy in treatment-resistant schizophrenia as well as in ameliorating negative symptoms (Hagger et al., 1993; Breier et al., 1994) and cognitive deficits (Kane et al., 1988). Clozapine is therefore considered the most effective antipsychotic drug used today (Kane et al., 1988; Pickar et al., 1992). The efficacy is suggested to be related to a relatively weak binding potential to dopamine D_2 receptors (Kapur and Seeman, 2001) and/or to a preferential action on the mesolimbic dopamine system (Anden and Stock, 1973; Bartholini, 1976; Chiodo and Bunney, 1983; 1985; Moghaddam and Bunney, 1990). Moreover, it is established that clozapine not only interacts with a variety of dopamine receptors, but also with receptors for serotonin (5-HT; Meltzer and Huang, 2008), acetylcholine (Snyder et al., 1974; Bymaster et al., 2003), noradrenaline (Coward, 1992) and histamine (Brunello et al., 1995; Coward, 1992). Studies also show interaction with the N-methyl-D-aspartate (NMDA) receptor (Arvanov et al., 1997; Lidsky et al., 1993; Ninan and Wang, 2003; Ossowska et al., 1999; Jardemark et al., 2003, Schwieler and Erhardt, 2003, Schwieler et al., 2004) and the γ-aminobutyric $acid_A$ ($GABA_A$) receptor (Squires and Saederup, 1998). Unfortunately, clozapine is associated with a risk of severe side effects, especially potentially fatal agranulocytosis (Idänpään-Heikkilä et al., 1977; Krupp and Barns, 1992). Nevertheless, the superiority of clozapine as an antipsychotic drug has led to the development of a new generation of antipsychotic drugs, e.g. olanzapine, risperidone, quetiapine and ziprasidone, nowadays widely used in the treatment of schizophrenia. However, also these newly developed drugs are associated with side effects, most prominently weight gain and altered glucose and lipid metabolism, resulting in increased risk of cardiovascular diseases (Gardner et al., 2005). Thus, the need for novel, more effective and tolerable drugs in the treatment of schizophrenia is substantial.

1.2 Hypotheses of schizophrenia

The field of schizophrenia research has shifted dramatically over the years. Today most evidence indicates that the pathophysiological processes leading to the development of schizophrenia are related to alterations in several neurotransmitters, especially dopamine and glutamate (Javitt and Zukin, 1991; Jentsch and Roth, 1999; Carlsson et al., 2001; Javitt, 2007).

1.2.1 The dopamine hypothesis of schizophrenia

The dopamine hypothesis originally proposed a hyperactivity of the dopamine system as the underlying cause of psychotic symptoms in patients with schizophrenia (Carlsson and Lindqvist, 1963). For several decades this was the predominant theory and originated from findings that antipsychotic drugs reverse the symptoms (by blocking D_2 receptors) and that dopamine-releasing drugs (e.g. amphetamine) can induce a state of psychosis (Snyder, 1973; Angrist and van Kammen, 1984, Carlsson et al., 2001). The first compound to be used in the treatment of schizophrenia was chlorpromazine (Delay and Denicker, 1952) and since then several antipsychotic drugs have been developed with the common feature of antagonizing the dopamine D_2 receptor. However, over the years more attention has been focused on the negative and cognitive symptoms and the resistance they show to dopamine D_2 receptor antagonists (King, 1998; Breier, 1999). These symptoms have been suggested to be caused by persistent deficits in dopamine transmission at dopamine D_1 receptors in the prefrontal cortex, whereas an excess of subcortical dopamine functions, involving activation of dopamine D_2 receptors, contribute to the positive symptoms (Davis et al., 1991; Jentsch and Roth, 1999; Abi-Dargham and Laurelle, 2005). These findings have led to a reformulation of the original dopamine hypothesis to a dopamine imbalance theory of schizophrenia (Abi-Dargham and Laurelle, 2005). Thus, simultaneous occurrence of both both hypo- and hyperdopaminergic states is nowadays generally accepted as a theory for the cause of the symptoms. In recent years, data from brain imaging studies have provided further support for an abnormal dopamine activity in patients with schizophrenia. Studies have shown increased baseline dopamine release during phases of exacerbation of psychosis, and an increased release of dopamine after amphetamine administration, in patients with schizophrenia compared to control subjects (Abi-Dargham et al., 1998; 2000; Laruelle et al., 1996; Breier et al, 1997; Abi-Dargham and Laruelle, 2005). The dopamine imbalance theory for schizophrenia is also supported by studies showing reduced central dopamine output in chronic patients (Karoum et al., 1987). However, the underlying mechanisms behind such an imbalance in the dopamine systems remains to be revealed.

1.2.2 The glutamate deficiency theory of schizophrenia

About half a century ago, clinical observations of symptoms indistinguishable from schizophrenia were made following administration of a dissociative anaesthetic (Luby et al., 1959; Itil et al., 1967). The drug used, phencyclidine (PCP), was developed and introduced as an "ideal" anaesthetic, but was later found to be associated with severe side effects, e.g. hallucinations, paranoia, delusions and disorganized speech during anaesthetic recovery (Pradhan, 1984). Furthermore, a significant number of patients developed psychosis, which persisted long after the recovery from anaesthesia, and consequently the drug was withdrawn from the market and human use in 1965 (Pradhan, 1984; Domino and Luby, 1981). Over a decade later, it was found that in subanaesthetic doses (0.05-0.1 mg/kg) PCP acts as a non-competitive NMDA receptor antagonist (Zukin and Zukin, 1979; Vincent et al., 1979). The glutamate deficiency theory of schizophrenia was hence formulated, based on these findings together with a report showing low levels of glutamate in the CSF of patients with schizophrenia (Kim et al., 1980). Furthermore, several studies have also revealed that other non-competitive NMDA receptor antagonists, e.g. ketamine, exacerbate both positive and negative symptoms in patients with schizophrenia (Luby et al., 1959; Itil et al., 1967; Malhotra et al., 1997; Jentsch and Roth, 1999). Also, these compounds can induce cognitive dysfunctions as well as positive and negative symptoms of schizophrenia in healthy controls (Javitt and Zukin, 1991; Krystal et al., 1994; Tamminga, 1998; Jentsch and Roth, 1999). Hereby, PCP-induced psychosis in healthy volunteers is indistinguishable from an acute episode of schizophrenia (Yesavage and Freman, 1978; Erard et al., 1980). When further investigated, it was found that not only non-competitive NMDA receptor antagonists, but also competitive NMDA receptor antagonists as well as antagonists of the glycine site of the NMDA receptor are able to induce symptoms of schizophrenia (Kristensen et al., 1992; Grotta et al., 1995; Yenari et al., 1998; Albers et al., 1999). Furthermore, adjunctive treatment with glycine or D-serine shows beneficial effects on schizophrenia, particularly with regard to negative symptoms, indirectly supporting the glutamate deficiency theory (Tuominen, 2005).

In electrophysiological experiments, systemic administration of NMDA receptor antagonists in rats is associated with an increase in firing rate and burst firing activity of midbrain dopamine neurons (French et al., 1993; French, 1994; Erhardt et al., 2001; Erhardt and Engberg, 2002; Schwieler et al, 2004). This paradoxical activation is

suggested to be caused by an inhibition of tonic GABAergic input to the midbrain dopamine neurons (Zhang et al., 1993). Furthermore, studies show that subchronic administration of PCP to rats not only increases dopamine release in mesolimbic areas, i.e. the nucleus accumbens (Jentsch et al., 1998; Jentsch and Roth, 1999), but also reduces dopamine release in the prefrontal cortex (Jentsch et al., 1997; 1998a; b). As previously mentioned (see 1.2.1), hypofunction in the prefrontal dopamine system has been suggested to result in negative and cognitive symptoms (Weinberger et al., 1988; Daniel et al., 1989; 1991; Dolan et al., 1995). Thus, chronic PCP administration is suggested to produce effects most consistent with schizophrenia, mimicking both dopaminergic hypofunction in the prefrontal cortex as well as a hyperdopaminergic state in the nucleus accumbens (Jentsch and Roth, 1999). Altogether, increasing evidence suggests that an altered dopamine function in schizophrenia may be a consequence of NMDA receptor hypofunction, although the reason for such a deficiency remains to be determined.

1.2.3 The kynurenic acid hypothesis of schizophrenia

In the early 2000s it was discovered that patients with schizophrenia display elevated levels of kynurenic acid in the cerebrospinal fluid (CSF; Erhardt et al., 2001a) and in the prefrontal cortex in postmortem patients (Schwarcz et al., 2001). These findings were later confirmed in a large cohort of patients with schizophrenia, including both drug-naïve, first-episode patients as well as patients on antipsychotic treatment (Nilsson et al., 2005). In line with higher concentrations of kynurenic acid in the post mortem brain of patients with schizophrenia, it has been shown that the levels of kynurenine are also elevated in the post mortem brain of these patients (Miller et al., 2006; 2008). Elevated kynurenine and kynurenic acid levels in patients with schizophrenia may arise from an increased synthesis of kynurenine from tryptophan, or alternatively, a decreased synthesis of 3-hydroxykynurenine from kynurenine. An increased synthesis of kynurenine might result from an induction of tryptophan-2,3-dioxygenase (TDO) and/or indoleamine-2,3-dioxygenase (IDO), enzymes known to be induced during infections or immune-activation (Holtze et al., 2008; Asp et al., 2009; Schmidt et al., 2009) and numerous studies suggest that kynurenic acid is a biological marker of neuroinflammation (King and Thomas 2007; Dantzer et al., 2008). Supporting an activation of the brain immune system in schizophrenia, it was recently found that the

CSF concentration of the pro-inflammatory cytokine IL-1β is elevated in first-episode patients (Söderlund et al., 2009). Indeed, gene expression of TDO as well as the density of TDO-immunopositive cells is found to be elevated in post mortem brain of patients with schizophrenia, whereas IDO expression appears to be unaffected (Miller et al., 2004; 2006). A decreased synthesis of 3-hydroxykynurenine from kynurenine, resulting in increased kynurenine levels, is supported by experimental as well as genetic studies. It is well known that pharmacological blockade of the enzyme converting kynurenine to 3-hydroxykynurenine, kynurenine 3-monooxygenase (KMO), results in elevated brain kynurenic acid levels (Erhardt et al., 2009). In addition, we recently reported that a non-synonymous polymorphism in the KMO gene results in higher CSF kynurenic acid levels in both healthy volunteers and patients with schizophrenia (Holtze et al., 2010). Also, a post mortem study shows decreased KMO gene expression as well as decreased KMO enzyme activity in individuals with schizophrenia (Sathyasaikumar et al., 2009).

The unique properties of kynurenic acid, i.e. being an antagonist of both NMDA receptors and the α7*nicotinic acetylcholine receptor (α7*nACh), is particularly interesting given that hypoglutamatergia, possibly induced by NMDA receptor hypofunction, is widely accepted as part of the pathophysiology of schizophrenia (Javitt et al., 2007). In addition, the importance of intact α7*nACh receptor signaling in cognitive functions has been suggested in numerous studies during the last decade (Albuquerque et al., 2009). In further support of the kynurenic acid hypothesis of schizophrenia, animal studies show that pharmacologically elevated levels of brain kynurenic acid impair contextual learning and working memory (Chess and Bucci 2006; Chess et al., 2007; 2009) and disrupt prepulse inhibition (PPI; Erhardt et al., 2004), a behavioral model measuring sensory motor gating, domains well-known to be affected in patients with schizophrenia. Furthermore, rats with acutely as well as subchronically elevated levels of kynurenic acid in the brain display increased midbrain dopamine firing activity, mimicking the effect seen following administration of psychotomimetic NMDA receptors antagonists (Erhardt and Engberg, 2002; Erhardt et al., 2003; Nilsson et al., 2006; French et al., 1993; 1994). Indeed, midbrain dopamine neurons are suggested to play an important role in generating positive symptoms in schizophrenia, where an increased phasic release of dopamine (Grace, 1991) induced by burst-firing activity of these neurons, may mediate the excess of subcortical

dopamine (Davis et al., 1991; Jentsch and Roth 1999). Therefore, the increased firing of rat midbrain dopamine neurons following chronically elevated levels of kynurenic acid (Nilsson et al., 2006; Olsson et al., 2009) may represent a pathophysiological condition similar to that seen in patients with schizophrenia. Indeed, in rats with subchronically elevated levels of KYNA, dopamine release in the nucleus accumbens is clearly enhanced following an amphetamine challenge (Olsson et al., 2009), a finding in agreement with the increased striatal dopamine release by amphetamine, as observed by brain imaging studies in patients with schizophrenia (Laruelle et al., 1999).

Interestingly, cyclo-oxygenase (COX)-2 inhibitors, e.g. celecoxib and parecoxib, which decrease rat brain kynurenic acid concentration and dopaminergic activity (Schwieler et al. 2005; 2006), display beneficial antipsychotic effects when added to conventional antipsychotic treatment in patients with schizophrenia (Müller et al. 2002). In contrast, COX-1 inhibitors, e.g. diclofenac and indomethacin, are associated with an increase in brain kynurenic acid concentration and subsequent increase in dopaminergic activity (Schwieler et al. 2005; 2006), and such drugs have been reported to induce psychotic side effects (see Hoppmann et al. 1991; Tharumaratnam et al. 2000; Clunie et al. 2003).

1.3 Suicide

Suicide accounts for approximately one million deaths annually worldwide and the frequency of suicide attempts is approximately 20 times that of completed suicides (WHO). The rates of suicide have increased by 60% in the past half century, mainly in developing countries (WHO). The prevalence varies between countries but the highest rates are found in Asia and Eastern and Northern Europe, including Sweden (WHO). However in some regions the reported number of suicides might be extraordinary low due to social, religious and political factors and demonstrate the stigmatization and taboo suicide is associated with. In Sweden, as well as in many other countries, suicide is the leading cause of death between the ages 15 and 44, whereas most suicides are committed by people over the age of 45 (NASP). Men are more likely to commit suicide but women attempt suicide three times as often (WHO; NASP). Studies show an incidence of mental disorder between 80 and 98% in all suicide victims and suicide attempters, most commonly mood disorders, followed by substance abuse and schizophrenia (Arsenault-Lapierre et al, 2004; Bertolote et al, 2004). MDD is the most prevalent psychiatric diagnosis among suicide victims as well as attempters.

Schizophrenia is the third most common psychiatric disorder in suicide victims and suicide accounts for approximately 5-10% of all deaths in this patient category (see 1.1; Black and Fisher, 1992; Palmer et al., 2005).

1.3.1 Immunological aspects

In many ways depressive symptoms resemble ”sickness behavior”, i.e. fatigue, anorexia, loss of interest in social activities, failure to concentrate, anhedonia and exacerbation of symptoms by stressors (Charlton, 2000). Interestingly, sickness behavior is induced by pro-inflammatory cytokines as an inflammatory response of the immune system (Charlton, 2000). Already in the early 1990s it was suggested that the immune system could be involved in depressive disorders (Smith, 1991). Since then the field of psychoneuroimmunology has grown enormously and our knowledge on the immune-to-brain communication and the interference between the central nervous system (CNS) and the immune system has increased. Indeed, the potential role of immune mediators in psychiatric disease has recently become widely recognized (Dantzer et al, 2008). Several studies show increased blood levels of pro-inflammatory cytokines in depressed patients, e.g. interleukin-6 (IL-6; Berk et al., 1997), IL-1β (Thomas et al., 2005) and tumor necrosis factor-α (TNF-α; Hestad et al., 2003; Mikova et al., 2001), as well as in suicide attempters (Mendlovic et al., 1999; Kim et al., 2008). In line with this notion, it is shown that immunotherapy with cytokines, e.g. interferon-γ, induces depressive symptoms approximately one month after start of medication (Raison et al., 2009; Wichers and Maes 2004; Wichers et al., 2005). Correspondingly, the symptoms are well-treated with antidepressant drugs (Capuron et al, 2002). Furthermore, current antidepressant drugs may alleviate symptoms of depression via anti-inflammatory actions (Kulmatycki and Jamali, 2006), and a small-scale study using COX-2 inhibitors has demonstrated positive results in treatment-resistant depression (Müller et al 2006). However, there is presently very limited knowledge about the details of the interactions between neurobiological and immunological factors, and the link to specific psychiatric symptoms. However recently, it was shown that CSF IL-6 is higher in individuals with MDD that had made a suicide attempt than in healthy volunteers. Patients who performed violent suicide attempts displayed the highest levels of IL-6, and IL-6 correlated positively with the severity of depressive symptoms (Lindqvist et al., 2009).

1.4 The kynurenine pathway

Kynurenic acid was first identified 1853 in canine urine (Liebig, 1853). Following the discovery of tryptophan (Hopkins and Cole, 1901), an amino acid required by all life forms for protein synthesis, the compound was recognized as a tryptophan metabolite (Ellinger, 1904). Later on, the sequence of enzymatic steps converting tryptophan to kynurenines, including kynurenic acid, was named the ”kynurenine pathway” (Beadle et al., 1947; fig. 1). This pathway produces three neuroactive metabolites, i.e. kynurenic acid, an anticonvulsant and neuroprotectant, quinolinic acid, an excitotoxin, and 3-hydroxykynurenine, a free-radical generator (Stone et al., 1993). Of these compounds, kynurenic acid and quinolinic acid are suggested to participate in brain physiological and pathophysiological processes (Erhardt et al., 2009; Chen and Guillemin, 2009).

In mammals, the kynurenine pathway metabolizes over 95% of all dietary tryptophan (Wolf, 1974; fig. 1) whereas only a minor fraction is metabolized to the neurotransmitter serotonin (Peters, 1991). The first and regulatory step in the pathway is controlled by the two enzymes TDO (Hayaishi et al., 1993) and IDO (Hayaishi, 1976), both inducing the oxidative opening of the indole ring of tryptophan. The product, formyl kynurenine, is rapidly converted to the key metabolite L-kynurenine by kynurenine formylase (Mehler and Knox, 1950; Gál and Sherman, 1978). Kynurenine serves as

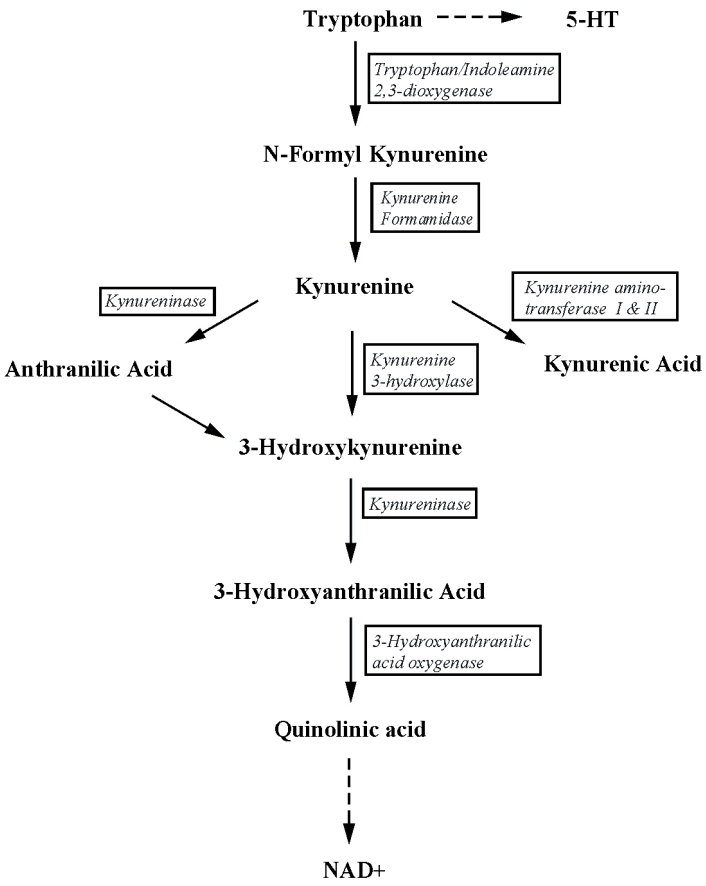

Figure 1. The kynurenine pathway

substrate for three different enzymes: I) kynureninase, which forms anthranilic acid, II) kynurenine 3-hydroxylase, which forms 3-hydroxy-kynurenine, and III) kynurenine aminotransferase (KAT), which forms kynurenic acid (Moroni, 1999). The enzymes of the kynurenine pathway in the brain are primarily expressed in astrocytes, microglial cells and infiltrating macrophages (Okuno et al., 1991b; Du et al., 1992; Heyes et al., 1996; Guillemin et al., 2001; Kiss et al., 2003; Miller et al., 2004). However, degradation of kynurenine seems to be spatially separated as the enzymes responsible for the synthesis of kynurenic acid or quinolinic acid are mainly expressed in astrocytes or microglia, respectively. Astrocytes appear to preferentially synthesize kynurenic acid, as they contain very little kynurenine 3-hydroxylase, whereas microglia favor the formation of quinolinic acid due to their very low expression of KAT (Guillemin et al., 2001; Kiss et al., 2003; Lehrmann et al., 2001). In fact, astrocytes seem more capable of degrading quinolinic acid than producing the compound (Guillemin et al., 2001). Under normal conditions, it is suggested that most kynurenine is metabolized by kynurenine 3-hydroxylase, having the highest enzymatic affinity to kynurenine (Bender and McCreanor, 1982; Moroni, 1999). Subsequently, anthranilic acid and 3-hydroxykynurenine can be metabolized to 3-hydroxyanthranilic acid and then to the neurotoxic compound quinolinic acid (Stone and Perkins, 1981). Further metabolism along this branch of the kynurenine pathway results in either total oxidation (generating adenosine triphosphate and CO_2) or synthesis of nicotinamide adenine dinucleotide (NAD^+; Hayaishi, 1993).

1.4.1 Kynurenic acid

In 1988, two independent research groups discovered the presence of kynurenic acid in the human brain (Moroni et al., 1988; Turski et al., 1988). In line with this finding, it was later discovered that all enzymes required for the formation of kynurenic acid from tryptophan are present in the brain (Swartz et al., 1990; Guidetti et al., 1995), although the activity of these enzymes seems to be much higher in peripheral organs, i.e. the liver and the kidneys (Stone, 1993; Schwarcz and Pellicciari, 2002). In the periphery several aminotransferases responsible for the conversion of kynurenine to kynurenic acid have been identified, and a few have been recognized in both the human and the rat brain. The first to be identified were KAT I and KAT II, which are preferentially expressed in glial cells. (Okuno et al., 1991a, b; Buchli et al., 1995; Guidetti et al., 1997). KAT I and KAT II differ from each other with regard to pH optimum and

substrate specificity, where KAT II operates best at physiological pH, shows the highest specificity to kynurenine and is primarily responsible for kynurenic acid formation in the brain (Schmidt et al., 1993; Guidetti et al., 1997). Thus, studies suggest KAT II accounts for >70% of the KYNA production in the rat (Guidetti et al., 1997). However, both KAT I and KAT II have Km in the millimolar range, suggesting that the availability of kynurenine is the rate limiting step of kynurenic acid synthesis. Two additional enzymes converting kynurenine to kynurenic acid have been discovered in the mammalian brain, KAT III and mitochondrial aspartate aminotransferase, but whether these enzymes play a significant role in synthesis of kynurenic acid remains to be clarified (Yu et al., 2006; Guidetti et al., 2007). The transamination of kynurenine by KATs, forming kynurenic acid, is irreversible and no further enzymatic steps appear to occur. Importantly, kynurenic acid, due to its polar structure, is unable to pass the blood brain barrier (Fukui et al., 1991). Thus, after release from astrocytes into the extracellular space, the only clearing alternative for kynurenic acid is via a probenecid-sensitive transporter out of the brain (Moroni et al., 1988a). Once kynurenic acid reaches the blood, it is rapidly excreted through the renal filtration (Turski and Schwarcz, 1988).

Kynurenic acid is an antagonist of glutamate receptors in the human brain; in low doses, the compound blocks the glycine site of the NMDA receptor (IC_{50} ~8-15 μM; Ganong and Cotman, 1986; Kessler et al., 1989; Parsons et al., 1997; Birch et al., 1988) as well as the α7*nACh receptors (IC_{50} ~7 μM; Hilmas et al., 2001). At higher concentrations also the glutamate recognition site of the NMDA receptor (IC_{50} = 200-500 μM; Kessler et al., 1989) and the alpha-amino-3-hydroxy-5-methylisoxazole propionate (AMPA)/kainate receptors are blocked (IC_{50} in the millimolar range; Bertolino et al., 1989; Kessler et al., 1989). Furthermore, kynurenic acid is also an endogenous agonist of the G protein-coupled receptor 35 (GPR35; EC_{50} = 39 μM for human). GPR35 is predominantly detected in immune cells and the gastrointestinal tract whereas its expression is limited in the brain (Wang et al., 2006). Recently, it was also reported that low levels of kynurenic acid facilitate AMPA receptor responses in Xenopus oocytes but not in rat hippocampal slices (Prescott et al., 2006).

1.4.2 Regulation of synthesis

The synthesis of kynurenic acid is mainly driven by the intracellular concentration of L-kynurenine (Schwarcz and Pellicciari, 2002). Thus, the concentration of kynurenic acid in the brain increases following systemic injections of kynurenine in both rats and primates (Swartz et al., 1990; Wu et al., 1992; Jauch et al., 1993). Furthermore, levels of kynurenic acid also increase by inhibiting the conversion of kynurenine to 3-hydroxykynurenine (Russi et al., 1992; Speciale et al., 1996; Erhardt et al., 2009), i.e. by drugs blocking kynurenine 3-hydroxylase. Hereby, the metabolism of kynurenine is driven towards the other branch of the kynurenine pathway, i.e. to the formation of kynurenic acid.

Amino acids, e.g. glutamine and phenylalanine, are competitive substrates of KAT I and KAT II, and thus, intracellular concentrations of these compound are suggested to control the production of kynurenic acid (Chang et al., 1997). Furthermore, the efflux of kynurenic acid from the brain is dependent on large amino acid carriers, and inhibition of this process, e.g. with probenecid, results in increased kynurenic acid levels in the brain (Moroni et al., 1988). Studies have also shown that inhibition of COX-1, e.g. by administration of indomethacin or diclofenac, is associated with increased levels of kynurenic acid in the brain (Edwards et al., 2000; Schwieler et al., 2005; 2006). Furthermore, systemic administration of COX-2 inhibitors, e.g. parecoxib and meloxicam, decrease brain levels of kynurenic acid (Schwieler et al., 2005; 2006). Moreover, the rate limiting enzymes in the kynurenine pathway, i.e. IDO and TDO (see 1.4), are induced by immunological agents, e.g. pro-inflammatory cytokines like interferon-γ, that induce the entire cascade of the kynurenine pathway (Yoshida et al., 1986; Carlin et al., 1987). Also, the expression of KAT I and KAT II is induced by interferon-γ, hence increasing the synthesis and release of kynurenic acid by astrocytes (Guillemin et al., 2001). The biological significance of the induction of the kynurenine pathway by immunological agents is not fully understood. However, it has been suggested that increased tryptophan breakdown serves as a defense mechanism for reducing the supply of tryptophan needed by pathogens (Pfefferkorn, 1984; Moffet and Namboodiri, 2003).

1.5 The glutamate system of the brain

Glutamate is one of the ordinary 20 amino acids in the body and one of the primary neurotransmitters in the mammalian brain. When first investigated it was not considered to fulfill the criteria as a neurotransmitter, but today glutamate is classified as one of the so called excitatory amino acid (EAA) and known to be involved in most aspects of brain function, e.g. cognition, memory and learning (McEntee and Crook, 1993). Thus, it is well known that glutamate accounts for most of the fast excitatory signaling in the vertebrate nervous system (Orrego and Villanueva, 1993). However, massive activation of glutamate receptors may lead to neuronal damage and even to cell death, so-called excitotoxicity (Meldrum and Garthwaite, 1990; Choi, 1992). Therefore, astrocytes that surround the synaptic cleft are of major importance for regulating the extracellular concentration of glutamate. Clearance of glutamate is ensured by diffusion and by specific transporters located on both neurons and astrocytes. In astrocytes, accumulated glutamate is converted to glutamine, released again and converted back to glutamate by neurons. This process is referred to as the glutamate-glutamine cycle and enables glutamate to be recycled and transported back to neurons in an inactive and non-toxic form (Danbolt et al., 2001).

The receptors mediating the effects of glutamate are divided into four subtypes, NMDA, AMPA, kainate and metabotropic glutamate receptors (mGluR; Ozawa et al. 1998). NMDA, AMPA and kainate are ionotropic receptors, i.e. ligand gated ion channel receptors, whereas the mGluRs are linked to G proteins and act through a second messenger (Seeburg, 1993; Ozawa et al., 1998). The endogenous agonists for all these receptors are generally believed to be glutamate and/or aspartate and the only known naturally occurring antagonist of the ionotropic EAA receptors is kynurenic acid (see 1.4.1).

1.5.1 The NMDA receptor

The NMDA receptor is widely distributed throughout the brain and known to play a critical role in synaptic plasticity, a cellular mechanism for learning and memory. Several subunits of the NMDA receptor have been identified; NR1, NR2 and NR3 (Paoletti and Neyton, 2007). All NMDA receptors appear to function as heteromeric complexes and require the co-expression of at least one NR1 and one NR2 subunit

(Paoletti and Neyton, 2007). Furthermore, the subunits have distinct binding sites and thus the NMDA receptor is a quite complex receptor. At resting membrane potential the channel of the NMDA receptor is blocked by Mg^{2+}, which is removed upon depolarization of the cell, e.g. by AMPA receptor activation (McBain and Mayer, 1994). In addition, the NMDA receptor requires the presence of a co-agonist, glycine or D-serine, on the NR1 subunit to function (fig. 2; Paoletti and Neyton, 2007; Hirai et al., 1996). Thus, the NMDA receptor is unique in that it requires membrane depolarization, removing the Mg^{2+}, and also simultaneous co-activation by two ligands, glutamate/aspartate and glycine/D-serine, to be activated. In addition, the function of the receptor can also be modified by extracellular Zn^{2+} and polyamines (Danysz and Parsons, 1998). It has previously been claimed that the glycine/D-serine site might be constantly occupied under normal conditions (Obrenovitch et al., 1997), however, several studies have demonstrated that this site is not saturated *in vivo* (Danysz and Parsons, 1998). Thus, the availability of glycine and/or D-serine appears of critical importance for optimal NMDA receptor functioning. The activated NMDA receptor is permeable for both K^+ and Na^+, but has particular high permeability for Ca^{2+}, which acts as a second messenger in the cell. The increased Ca^{2+}, due to high glutamatergic activity, results in a permanent increase in synaptic efficacy. This process is known as long term potentiation (LTP; Lynch, 2004) and is of critical importance for learning and memory. However, there is a gentle balance and excessive Ca^{2+} leads to excitotoxicity (see 1.5).

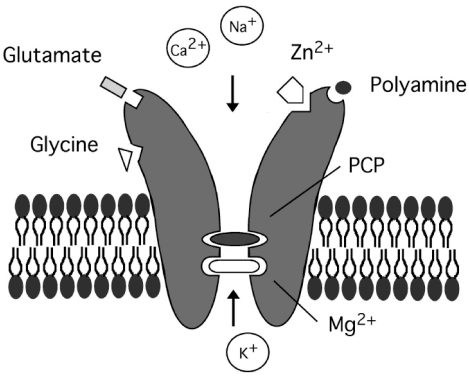

Figure 2. The NMDA receptor.

1.6 The dopamine system

Dopamine was recognized as a neurotransmitter of its own rights in the late 1950s (Carlsson et al., 1957; 1958; Carlsson, 1959). Prior to this point dopamine was considered only as an intermediate in the synthesis of noradrenaline and adrenaline. Today we know that dopamine, noradrenaline and adrenaline are metabolites of the amino acid tyrosine. Due to their mutual chemical structure, i.e. a nucleus of catechol and a side chain of ethylamine or one of its derivates, these compounds are described as

catecholamines. The dopamine system has been extensively investigated and the transmitter is considered a key modulator in numerous functions of the brain, e.g. behavior, cognition, voluntary movement and reward. The effects of dopamine are mediated through dopamine receptors, subgrouped into dopamine D_1-like receptors and dopamine D_2-like receptors (Jaber et al., 1996). The receptors in both of the subgroups are localized pre- as well as postsynaptically, where presynaptic dopamine D_2 receptors act as autoreceptors, providing important inhibitory feedback to the dopamine neuron (Stoof and Kebabian, 1984; White and Wang, 1984).

1.6.1 Dopamine pathways

Studies of the dopamine system have identified three major dopamine pathways. The cell bodies of these projections are located in the substantia nigra, ventral tegmental area (VTA) and in the arcuate and periventricular hypothalamic nuclei (Dahlström and Fuxe, 1964). The dopamine neurons of substantia nigra project to the dorsal part of the striatum, i.e. the caudate and the putamen, via the medial forebrain and the internal capsule. This projection is referred to as the nigrostriatal dopamine pathway and of critical importance for motor function (Carlsson, 1959; Ehringer and Hornkiewicz, 1960; Andén et al., 1964). Degeneration of these neurons causes Parkinson´s disease and inhibition of this system, e.g. by antipsychotic drugs, is associated with EPS (see 1.1.1). From the VTA, dopamine neurons project, via the medial forebrain bundle, to cortical and subcortical (limbic) areas, thus forming the mesolimbocortical pathway. This pathway is further subdivided into the mesolimbic dopamine system, projecting to amygdaloid complex, nucleus accumbens, olfactory tubercle and septal area, and the mesocortical dopamine system, projecting to areas in the prefrontal cortex, such as prelimbic, infralimbic and cingule cortices (Dahlström and Fuxe, 1964; Andén et al., 1966; Ungerstedt, 1971; Moore and Bloom, 1978; Björklund and Lindvall, 1984). These pathways are crucial for processes such as motivation, reward and cognition (Fibiger and Phillips, 1988; LeMoal and Simon, 1991; Schultz et al., 1993; Schultz, 1998), domains frequently disturbed in patients with schizophrenia (see 1.1). The tuberohypophysial dopamine system originates in the hypothalamus and projects to the median eminence and the intermediate and posterior lobes of the pituitary. This pathway is involved in the control of endocrine functions and thus responsible for

endocrine-related side effects of antipsychotic drugs, e.g. sexual dysfunction, infertility, gynecomastia and galactorrhea.

1.6.2 Electrophysiology of midbrain dopamine neurons

Firing of dopamine neurons has been thoroughly investigated in electrophysiological studies ranging from *in vitro* recordings of isolated neurons to activity recordings of freely moving primates. These studies have revealed that dopamine neurons alternate between spontaneous firing, at frequencies of 1-10 Hz, and states of silence (Grace and Bunney, 1984a). Furthermore, there are two basic modes of firing of these neurons: an irregular and relatively slow single spike firing or a relatively rapid burst firing (Wang, 1981; Grace and Bunney, 1984a; b; Clark and Chiodo, 1988). When firing in burst mode, spikes within the burst exhibit progressively decreasing spike amplitude as well as increased spike duration. The different firing patterns of midbrain dopamine neurons appear fundamental for transmitter release in terminal areas, as a switch from single spike firing to burst firing is associated with a massive release of dopamine, and accordingly, when burst firing is dampened, the release decreases (Gonon, 1988; Nissbrandt et al., 1994). In freely moving rats over 90% of VTA dopamine neurons exhibit burst firing (Freeman and Bunney, 1987), whereas up to 50% of spontaneously active midbrain dopamine neurons fire in bursts in anaesthetized rats (Grace and Bunney, 1984a; Clark and Chiodo, 1988). This discrepancy is suggested to be due to the degree of sensory stimulation or to the interaction between movement and stimulation. Supporting this notion, many experiments show that burst firing of midbrain dopamine neurons is induced by food and fluid rewards as well as reward-related tasks in monkeys (Schultz et al., 1993; Schultz, 1998).

1.6.3 Afferent regulation of dopamine neurons in ventral tegmental area

There are significant differences between spontaneous firing of VTA dopamine neurons *in vivo* and *in vitro*. Most prominent is the fact that burst firing is not seen in slice preparation, suggesting that afferent inputs drive the firing of dopamine neurons (Sanghera et al., 1984; Grace and Onn, 1989; Seutin et al., 1990; Johnson et al., 1992). Most important in this regard are inhibitory GABAergic and stimulatory glutamatergic inputs. Extrinsic GABAergic neurons project to the VTA from areas such as nucleus accumbens and frontal cortex (Walaas and Fonnum, 1980; Yim and Mogenson, 1980; Kalivas et al., 1993). Furthermore, in the VTA approximately 15-20% of the total

neuronal population consists of intrinsic GABAergic interneurons (Beart and McDonald, 1980; Kalivas et al., 1993; Van Bockstaele and Pickle, 1995). Interestingly, these neurons not only synapse on dopamine neurons but also project to the nucleus accumbens and the prefrontal cortex (Thierry et al., 1980; Kalivas, 1993; Van Bockstaele and Pickel, 1995; Steffensen et al., 1998; Carr and Sesack, 2000a).

The effects of GABA, the main inhibitory neurotransmitter in the brain, on dopamine neurons is exerted via $GABA_A$ and $GABA_B$ receptors, both expressed on VTA dopamine neurons (Churchill et al., 1992; Wirtshafter and Sheppard, 2001). Thus, *in vivo* electrophysiological studies have shown that stimulation of $GABA_B$ receptors by baclofen is associated with a reduction of midbrain dopamine cell firing (Engberg et al., 1993; Erhardt et al., 1998; 2002). Furthermore, systemic administration of a selective $GABA_B$ receptor antagonist has been shown to increase dopamine cell firing, suggesting a tonic inhibitory control of midbrain dopamine neurons by these receptors (Erhardt et al., 1999; 2002). However, when administrating $GABA_A$ receptor agonists, in vivo electrophysiological studies have shown a paradoxical increase in firing activity of VTA dopamine neurons (Waszczak and Walters, 1980). This excitatory effect of $GABA_A$ agonists is suggested to be related to disinhibition of dopamine neurons (Grace and Bunney, 1979; Waszczak et al., 1980). Furthermore, it has also been suggested that the excitatory actions of $GABA_A$ agonists on VTA dopamine neurons are mediated via release of the excitatory transmitter glutamate (Erhardt and Engberg, 2002).

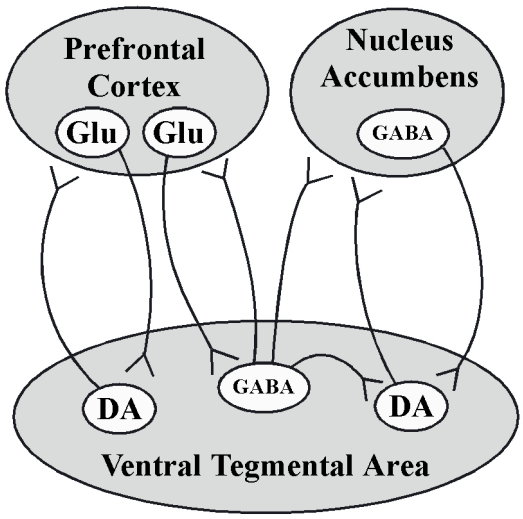

Figure 3. Schematic drawing of the connections between the prefrontal cortex, nucleus accumbens and the VTA. DA = dopamine, Glu = glutamate

The major glutamatergic input to the VTA originates from the prefrontal cortex and the pedunculopontine nucleus, and is important for the induction of burst firing activity (Carr and Sesack, 2000b; Charara et al., 1996). Thus, stimulation or inactivation of the prefrontal cortex increases or decreases burst firing activity of VTA dopamine neurons,

respectively (Murase et al., 1993). The glutamatergic afferents are connected with both GABAergic interneurons and dopaminergic cells, projecting back to the prefrontal cortex (fig. 3; Bonci and Malenka, 1999; Carr and Sesack, 2000b). Thus, the activity of VTA dopamine neurons by glutamatergic afferents is regulated both directly, by afferents synapsing on dopamine neurons that project to the prefrontal cortex, and indirectly, by glutamatergic afferents synapsing on GABAergic interneurons. The GABAergic interneurons in turn synapse on dopamine neurons projecting to the nucleus accumbens (fig. 3; Carr and Sesack, 2000b). The effects of glutamate in this regard can be mediated by both metabotropic and ionotropic receptors (see 1.5), which are shown to be present in the VTA (Seutin et al., 1990; Albin et al., 1992; Paquet et al., 1997). However, the ionotropic NMDA receptors have gained the highest level off attention over the years, and studies have shown that systemic administration of non-competitive NMDA receptor antagonists, e.g. PCP and MK-801, inhibit GABAergic interneurons (Zhang et al., 1993). This effect appears to cause a disinhibition of VTA dopamine neurons as systemic administration of non-competitive NMDA receptor antagonists is associated with increased burst firing of VTA dopamine neurons as well as increased dopamine release in the nucleus accumbens (French et al., 1993; Murase et al., 1993a; French, 1994; Schmidt et al., 1996; Yan et al., 1997; Kretschmer, 1999). Interestingly, NMDA receptor antagonists are known to induce psychotomimetic effects in humans and thus provide profound evidence for the glutamate deficiency theory of schizophrenia (see 1.2.2).

2 SPECIFIC AIMS OF THE STUDY

1. To study the mechanism involved in the excitatory action of elevated levels of endogenous kynurenic acid on VTA dopamine firing as well as in its disruptive effects on PPI.

2. To examine the actions of clozapine on glutamatergic neurotransmission by investigating its effect on VTA dopamine neurons following manipulation of endogenous levels of brain kynurenic acid.

3. To analyze kynurenic acid and its precursors, tryptophan and kynurenine, in the CSF of patients with schizophrenia and healthy volunteers.

4. To analyze levels of kynurenic acid and its relation to pro-inflammatory cytokines and chemokines in the CSF of healthy male volunteers and male suicide attempters with well-defined clinical psychiatric profiles.

3 MATERIALS AND METHODS

3.1 Animals

In all animal experiments male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden; weighing between 200 and 330 g) were used. The animals were housed in groups of five with free access to food and water. Environmental conditions were checked daily and maintained under constant temperature (25^{o}C) and 40-60% humidity with a regulated 12-h light/dark cycle. For electrophysiological experiments (paper I and III) rats were housed in a daily cycle of lights on at 06.00 AM and lights off at 06.00 PM. For behavioral experiments (paper II) the rats were housed in a room with a regulated, reversed 12-h light/dark cycle, with lights off at 07.00 AM and lights on at 07.00 PM. Rats were kept in this room for two weeks prior to experiments to adjust diurnal rhythm. In the behavioral experiments, the animals were handled at least 2 days preceding the experiments to reduce any subsequent handling stress. Experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm, Sweden, and all efforts were made to minimize the number of animals used and their suffering.

3.2 Drugs

The following drugs were used: 4-chloro-kynurenine (4-Cl-KYN), precursor to the selective NMDA glycine site antagonist 7-chloro-kynurenic acid (7-Cl-KYNA), kindly supplied by Vistagen Therapeutics, South San Francisco, CA, USA; L-kynurenine, precursor to kynurenic acid, Sigma, St. Louis, MO, USA; CGS 19755, competitive NMDA receptor antagonist, Tocris, Avonmouth, UK; SDZ 220-581, competitive NMDA receptor antagonist, Tocris, Avonmouth, UK; L-701,324, selective NMDA glycine site antagonist, Sigma, St. Louis, MO, USA; methyllycaconitine (MLA), α7*nACh receptor antagonist, Sigma, St. Louis, MO, USA; parecoxib, selective COX-2 inhibitor, Pharmacia, Buckinghamshire, Great Britain; indomethacin, COX-1 > COX-2 inhibitor, A/S Dumex Ltd. Copenhagen, Denmark; clozapine, atypical antipsychotic drug, Sigma, St. Louis, MO, USA; chloral hydrate, general anesthetic drug, Merck, Darmstadt, Germany; isoflurane, general anesthetic drug, Abbott Scandinavia, Solna, Sweden.

3.3 In vivo electrophysiology

3.3.1 Anesthesia and surgery

Rats were weighed and thereafter anesthetized with 8% chloral hydrate (400 mg/kg, i.p.). For best induction of general anesthesia, rats were left in a quiet environment for approximately 10-15 min. If the degree of anesthesia was not satisfactory by the end of this period an additional 8% chloral hydrate (200 mg/kg, i.p.) was administered. The rat was then placed on a heating pad to maintain body temperature at 37°C and subsequently mounted to the ear-bars of a conventional stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was set in a horizontal position and the nose was secured using a clamp at the front of the frame. For i.v. administration a cannula was inserted into a lateral tail vein and secured with strips of adhesive tape. A syringe containing 0.9% NaCl was connected to the cannula and approximately 0.5 mL was injected to assure a proper position of the cannula. An incision was made with a scalpel from the nose bridge along the center of the head to its base and the skull surface was exposed. For recordings from the VTA a burr hole of approximately 3 mm in diameter was drilled immediately anterior to lambda and lateral to the midline on the right side of the skull. The dura was carefully removed using a needle and a pair of tweezers. Additional chloral hydrate was administered through the tail vein when required to maintain a stable level of anesthesia. The level of anesthesia during the experiments was determined from the response following a hind paw pinching and by observing the breathing pattern. When required, additional 8% chloral hydrate was administered through the tail vein to maintain a stable level of surgical anesthesia.

3.3.2 Preparation of recording electrode

Single barrel recording electrodes were prepared from glass capillaries (Harvard Apparatus, inner diameter 1.16 mm) pulled in a vertical electrode puller (Narishige, Japan) set at 14.5 amperes. The electrode was filled with 0.5 M sodium acetate (Riedel-de Haen, Germany) saturated with Pontamine Sky Blue (BDH Laboratory Supplies, UK) and the tip was broken under a microscope to a diameter of approximately 1-2 μm. The *in vitro* impedance of the electrode was measured in a microelectrode tester. The impedance was typically 5-8 MΩ measured at 135 Hz in 0.9% saline.

3.3.3 Extracellular single cell recording

The recording electrode was secured onto a hydraulic microdrive (David Kopf Instruments, Tujunga, CA, USA) mounted to the stereotaxic instrument. The coordinates for lambda were visually estimated, placing the tip of the electrode just above lambda without touching the skull and the coordinates for VTA were set in relation to that. According to the stereotaxic coordinates from the atlas of Paxinos and Watson (1998) VTA was set to approximately 3.0 mm anterior to lambda and 0.7 mm lateral to the midline. The recording electrode was vertically lowered until its tip touched the brain surface, which caused a signal on the oscilloscope. The microdrive counter was set to zero and the electrode was lowered into the brain to a depth of approximately 7.0 mm. From this point the electrode was lowered slowly, using the hydraulic microdrive, into VTA where dopamine neurons were found 7.5-8.5 mm from the brain surface. Single unit potentials from electrophysiologically identified dopamine neurons were passed through a high input impedance amplifier and filters. The impulses were discriminated from background noise and fed into a computer, simultaneously displayed on a digital storage oscilloscope, monitored on an audio monitor and on a strip chart recorder (Gould). After each experiment the rat was killed with an overdose of chloral hydrate and the brain was rapidly removed and stored at -70°C until subsequent analysis. The recording sites of the experiments were not verified histologically since the brains were used for identification of kynurenic acid or 7-Cl-KYNA. Instead, dopaminergic neurons were mainly identified based upon their typical neurophysiological characteristics (see below) and, when appropiate, the inhibitory action of a single dose of the dopamine agonist apomorphine at the end of the experiments.

3.3.4 Electrophysiological characteristics of dopaminergic neurons

Dopamine neurons were identified using the electrophysiological characteristics previously described for midbrain dopamine neurons (Wang, 1981; Grace and Bunney, 1984a, b) including:

1. A biphasic (positive – negative) or triphasic (positive – negative – positive) waveform, often with a prominent inflection in the initial phase.
2. An action potential of long duration (2.5-4.0 ms), giving a characteristic low pitched sound on the audio monitor.

3. A slow, irregular firing pattern with a frequency between 1 and 9 Hz, sometimes alternating with bursts of high frequency spike activity and decreasing amplitude of spikes within each burst.

3.3.5 Drug administration and experimental protocol

Drugs were administered via the lateral tail vein or i.p. into the abdomen before or during the electrophysiological experiments. The basal activity, i.e. firing rate and percentage of spikes fired in bursts, was recorded for approximately 3 min before drug administration. The effect of a drug on firing was assessed by comparing the basal activity with the firing rate and percentage of spikes fired in bursts following each incremental dose administered. The firing activity was recorded from only one VTA dopamine neuron in each rat in all experiments in paper III and in the analysis of the response to MLA and SDZ 220-581 in paper I. In rats pretreated with kynurenine or 4-Cl-KYN in paper I, an average of 7 neurons were analyzed in each rat to estimate electrophysiological characteristics following 1-3.5 h drug pretreatment. In these experiments, the electrode was moved 0.1 mm between each track in a pattern that ensured that cells were recorded from only once. The number of spontaneously firing dopamine cells in VTA was calculated from the number of cells found per track. In paper III, rats were pretreated with a COX-1 inhibitor (indomethacin, 50 mg/kg, i.p., 1-3.5 h, n = 9) or COX-2 inhibitor (parecoxib, 25 mg/kg, i.v., 1-1.5 h, n = 12) in order to increase or decrease endogenous brain levels of kynurenic acid, respectively. Previous studies have shown that such treatment with indomethacin is associated with 153% elevation of brain kynurenic acid levels as well as an increase in firing rate and percentage of spikes fired in bursts in midbrain dopamine neurons (Schwieler et al., 2005, 2006). Additionally, pretreatment with parecoxib has shown 52% decrease in brain levels of kynurenic acid as well as a decrease in dopamine firing activity (Schwieler et al., 2006). Therefore, efforts were made to avoid recording of dopamine neurons with very high or very low frequency and burst firing activity. Furthermore, in line with a previous study (Erhardt and Engberg, 2002) 25% of all VTA dopamine neurons in rats with elevated levels of kynurenic acid occasionally showed depolarization-block characteristics. This effect was also seen occasionally in rats with elevated brain levels of 7-Cl-KYNA and such neurons were excluded from further analysis.

3.3.6 Influence of anesthesia

The influence of anesthetics as a confounding factor on neuronal activity and afferent responsiveness remains unclear and care must be taken in the interpretation of results obtained in anesthetized animals to infer normal function. The firing patterns of dopamine neurons in anesthetized, paralyzed and freely moving rats are similar in some respects but different in others; for example, higher burst frequencies are found in freely moving rats compared to anesthetized and paralyzed rats (Freeman and Bunney, 1987; Overton and Clark, 1997; Hyland et al., 2002). Previous reports have shown that the primary metabolite of chloral hydrate, trichloroethanol (presumably responsible for the depressant effects of chloral hydrate in the nervous system, Breimer, 1977), impairs NMDA receptor activation (Peoples and Weight, 1998; Scheibler et al., 1999). Furthermore, it has been shown that chloral hydrate decreases extracellular levels of synaptically released glutamate in the striatum (Kreuter et al., 2004). These influences on the glutamatergic system may interact with our experiments and also with the effects of elevated levels of kynurenic acid or 7-Cl-KYNA on dopamine cell firing. However, since all rats were anesthetized with chloral hydrate using the same routines the experiments' differences in firing activity found between control rats and drug treated rats should not be attributable to the anesthesia.

3.3.7 Data analysis

The distribution of spikes in paper III was analyzed on-line with a Macintosh computer. The software used for the analysis of firing was written in-house using high levels object oriented programming language called "G" (Lab VIEW; National Instruments, Austin, TX, USA). In paper I, the distribution of spikes was analyzed utilizing a Spike II software program (Cambridge Electronic Design, Cambridge, England) on a Hewlett-Packard Compaq computer. Both software programs were designed to sample and analyze the intervals of an arbitrary number of TTL pulses (corresponding to spikes passing through the discriminating filter) using a time resolution of one ms. An interspike interval was designated as the time (in ms) elapsed between the rising edges of two sequential TTL (transistor-transistor logic) pulses. In order to avoid artifacts in the sampling procedure, the spike analyzers were set to ignore time intervals below 20 ms. The onset of a burst was determined as an interspike interval shorter than 80 ms and the termination of a burst by the next interval longer than 160 ms (Grace and

Bunney, 1984a, b). The intervals were analyzed with regard to the number of bursts that occurred during a sampling of 100-500 spikes along with calculation of the percentage of spikes fired in bursts. Cells were considered to be bursting if at least one interspike time interval of 100 recorded spikes was below 80 ms.

3.4 Microdialysis

Male rats, weighing between 200 and 250 g, were anesthetized in a Plexiglas chamber filled continuously with 4.8% isoflurane (Forene®, Abbott Scandinavia, Solna, Sweden) in air using a vaporizer (Univentor 400, Malta). For the best induction of general anesthesia, rats were left in a quiet environment for approximately 5 min. Thereafter, the rat was mounted in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) so that the skull was set in a horizontal position. Anesthesia was maintained using a nose cone delivering 2.4% isoflurane throughout the surgery. A hole was drilled in the skull and following careful removal of the dura, a guide cannula (AgnTho's, Lidingö, Sweden) was directed to the region of nucleus accumbens and fixated with Dentalon® (AgnTho's, Lidingö, Sweden). Day one after surgery the guide cannula was exchanged with a microdialysis probe and experiments was carried out in unanesthetized freely moving rats. The probe was perfused with perfusion fluid (NaC1, 147.0; KC1, 2.7; $CaC1_2$, 1.2; and $MgC1_2$, 0.85 mM; CMA Microdialysis AB, Solna, Sweden) at a flow rate of 1 mL/min (infusion pump, Univentor 864, Malta). For analysis of kynurenic acid and 7-Cl-KYNA acid, forty- and thirty-minute fractions were collected (Univentor 820 Microsampler, Malta), respectively, and injected into an HPLC column. When the experiment was terminated, the rat was decapitated. Coronal sections (50 mm thick) were made using a cryostat (SLEE Medical GmbH, Mainz, Germany), and correct dialysis-probe placement was verified according to the atlas of Paxinos and Watson (1998). Stereotaxic coordinates of the implantation were 1.6 mm anterior and 1.4 mm lateral with reference to the bregma and lowered 8.2 mm from the brain surface. No distinction could be made between the core and the shell of the nucleus accumbens.

3.5 Prepulse inhibition

3.5.1 Apparatus

For measuring the startle response two startle chambers were used (SR-LAB, San Diego Instruments, San Diego, CA, USA). Each chamber consisted of a Plexiglas cylinder (9 cm diameter) mounted on a frame, housed within a ventilated chamber (39 x 38 x 58 cm). Sudden movements within the cylinder were detected by a piezoelectric accelerometer attached below the cylinder. A loudspeaker (Radio Shack Supertweeter) mounted 24 cm above the cylinder provided the broadband background noise and acoustic stimuli. Presentations of the acoustic stimuli were controlled by the SR-LAB software and interface system, which also rectified, digitized (0-4095) and recorded responses from the accelerometer. Sound levels [dB(A) scale] and accelerometer sensitivities within each chamber were calibrated regularly, as described earlier (Mansbach et al., 1988), and found to remain constant over the test period.

3.5.2 Experimental protocol

To elevate levels of endogenous brain kynurenic acid, rats (n = 14) were pretreated with kynurenine (200 mg/kg) i.p. 60 min before testing. Control rats (n = 13) received vehicle i.p. 60 min before testing for comparison with animals treated with kynurenine. In order to block the glutamate recognition site of the NMDA receptor, rats were pretreated with SDZ 220-581 (2.5 mg/kg, n = 12) s.c. 30 min before testing or CGS 19755 (10 mg/kg, n = 12) s.c. 45 min before testing. For these experiments, rats receiving saline (n = 12) s.c. 30 min before testing, were used as controls. In a third experiment, rats were treated with drugs blocking the glycine-site of the NMDA receptor or the α7*nACh receptor. In order to block the glycine-site of the NMDA receptor, *in situ* produced 7-Cl-KYNA or pretreatment with L-701,324 (1 mg/kg, n = 13 or 4 mg/kg, n = 17) i.p. 15 min before testing were used. To produce 7-Cl-KYNA *in situ*, rats were pretreated with 4-Cl-KYN (25 mg/kg, n = 15; 50 mg/kg, n = 14; or 100 mg/kg, n = 10) i.p. 60 min before testing. For selective blocking of the α7*nACh receptor, rats were treated with MLA (6 mg/kg, n = 15) i.p. 10 min before testing. Controls in this study (n = 18) received saline i.p. 15 min before testing. All drug combinations were balanced across the two startle chambers. The experimental session

consisted of a 5 min acclimatization period to a 65-dB background noise (continuous throughout the session), followed by a 20 min acoustic PPI test session. Seven days before any drug testing, animals were pre-exposed to the chambers and the testing session. The purpose of the pre-exposure was to acclimatize the animals to the testing chambers and startle/prepulse stimuli and to baseline-match the groups for subsequent testing (groups were matched for equivalent mean startle magnitude and percent PPI, as defined below). In the test session, a background noise (65 dB) was presented alone for 5 min and then continued throughout the remainder of the session. The test session used in all of the experiments contained five different trial types and had a duration of 20 min: a "pulse-alone" trial, in which a 40-ms 120-dB broadband burst was presented; three "prepulse-pulse" trials, in which 20-ms stimuli that were either 3, 6, or 12 dB above the background noise were presented 100 ms before the onset of the 120-dB pulse; and a "no stimulus" trial, which included only the background noise. All trial types were presented several times in a pseudorandom order for 60 trials (12 pulse-alone trials, 10 each of the remaining prepulse trial types, and eight no-stimulus trials). Five pulse-alone trials, which were not included in the calculation of PPI values, were presented at the beginning of the test session to achieve a relatively stable level of startle reactivity for the remainder of the session (based on the observation that the most rapid habituation of the startle reflex occurs within the first few presentations of the startling stimulus (Geyer et al., 1990)). In addition, five pulse-alone trials occurred at the end of the session to assess startle habituation but were not included in the calculation of PPI. An average of 15 sec (range 9-21 sec) separated consecutive trials. The whole session lasted approximately 24 min. The brief baseline session used to familiarize rats with the testing procedure and match groups for pharmacological studies consisted of 24 trials (18 120-dB pulse-alone and six prepulse-pulse trials with a 12-dB prepulse intensity).

3.6 Sampling of human cerebrospinal fluid

3.6.1 Ethical aspects

The work described in the studies was carried out in accordance with "The code of ethics of the World Medical Association" (Declaration of Helsinki) for experiments

including humans: http://www.wma.net/e/policy/b3.htm. All enrolled patients consented orally and in writing to participate in the study. The studies were approved by the Regional Ethical Committee in Linköping, Lund and Malmö.

3.6.2 Subjects

In paper IV, 24 patients, aged 36.8 years ± 7.9 (mean ± SD), range 23-49 years, with a DSM-IV (fourth edition, American Psychiatric Association 1994) verified schizophrenia were enrolled in the study. Patients consisted of 16 males, aged 36.8 years ± 7.9 (mean ± SD), range 23-49 years, and 8 females, aged 35.9 years ± 6.6 (mean ± SD), range 26-46 years. All patients were prescribed and taking olanzapine (2.5-25 mg/day) as the only antipsychotic drug. 44 healthy volunteers (29 male, 15 female), aged 24.9 years ± 6.2 (mean ±SD), range 18-51 years, mainly recruited among medical students, hospital staff members and their relatives, via the University Hospital in Linköping, Sweden, were used as controls.

Patients in paper V were enrolled in the study at Lund University Hospital after a suicide attempt between the years of 1987 and 2001. Patients with diabetes, current antibiotic treatment as well as all patients who received antidepressant or neuroleptic drugs during a wash-out period of 16 ± 7 (mean ± SD) days after the suicide attempt were excluded from analyses in the current study. Fifty-four male suicide attempters, with a mean age of 36 ± 11 (mean ± SD) years, range 19-61 years, and without traces of antidepressant or neuroleptic medication in their blood samples were found to be suitable for the present study. Occasional doses of benzodiazepines were allowed during the wash-out period, but no tranquilizers were given during the night before the lumbar puncture. After the wash-out period, a lumbar puncture and a psychiatric evaluation were carried out, as described below. Patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, third edition, revised (DSM III-R; American Psychiatric Association 1987). In order to identify subjects with potential infections at the time of the lumbar punctures, blood samples were taken from all patients and healthy volunteers included in this study for analysis of inflammatory and hematological markers (white blood cell count, erythrocyte sedimentation rate or C-reactive protein, hemoglobin, glucose or glycosylated hemoglobin, thyroid stimulating hormone, liver enzymes and creatinine). Furthermore, all subjects were

checked for fever. No signs of ongoing infection was found in any of the patients or healthy volunteers. The healthy volunteers in paper V were recruited via the Neuropsychiatric and Psychiatric Clinics at the University Hospitals in Linköping and Stockholm, Sweden. They consisted of 66 males, aged 26 ± 6 (mean ± SD) years, range 20-51 years.

In both studies, all healthy volunteers were thoroughly checked for psychiatric co-morbidity by an evaluating psychiatrist and using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I; First et al., 1997a). The healthy volunteers also completed the SCID-II questionnaire for personality disorders (First et al., 1997b). All controls were physically healthy and considered to be eligible for the studies with respect to the clinical interview and SCID results. All were considered healthy by the psychiatrist performing the examinations, showed no signs of psychiatric or somatic illness, or had any laboratory test outside standardized reference values. None of them had a family history of major psychosis, suicide in first- or second-degree relatives or difficulties in social adjustment at the time of sampling.

3.6.3 Lumbar Puncture

Lumbar punctures were performed in the morning between 8 and 11 AM, after a night of fasting and bed rest, and CSF was drawn from the L4–L5 interspace using a standardized protocol with the subject in the right decubitus position. In paper IV, a volume of 12 mL CSF was collected in two fractions. Samples were inverted to avoid gradient effects, divided into aliquots and frozen at -70°C until assayed. CSF from the second fraction (7-12 mL) was used for analysis through out the study. In paper V, two samples of 12 mL (portion 1) and 6 mL (portion 2) were taken and thereafter stored in aliquots and frozen in -70°C until analysis. Aliquots from portion 1 were used in this study.

3.7 Analysis of kynurenic acid and 7-chloro-kynurenic acid

3.7.1 Sample preparation

Immediately after each electrophysiological or behavioral experiment the rats were

killed by decapitation. The brains were rapidly taken out and stored at -70°C. The brains were sonicated with an equal weight of sonication solution (perchloric acid 0.4 M, $Na_2S_2O_5$ 0.1%, and ethylenediaminetetra-acetate (EDTA) 0.05%). Thereafter samples were centrifuged at 20,000 g for 5 min and approximately 40 μL perchloric acid (70%) was added to the supernatant. Subsequently the samples were centrifuged twice at 20,000 g for 5 min and stored at -70°C. Before analysis, all samples were thawed and centrifuged at 20,000 g for 5 min.

3.7.2 High performance liquid chromatography

Kynurenic acid and 7-Cl-KYNA were analysed with an isocratic reversed-phase high-performance liquid chromatography system (HPLC), including a dual piston, high liquid delivery pump (Bischoff, Leonberg, Germany), a ReproSil-Pur C18 column (4 x 150 mm. Dr Maisch Gmbh, Ammerbuch, Germany) and a fluorescence detector (Jasco Ltd, Hachioji City, Japan) with an excitation wavelength of 344 nm and an emission wavelength of 398 nm (18 nm bandwidth). A mobile phase of 50 mM sodium acetate pH 6.20 (adjusted with acetic acid) and 7.0 or 10.0% acetonitrile for kynurenic acid or 7-Cl-KYNA, respectively, was pumped through the reversed-phase column at a flow-rate of 0.5 mL/min. Samples of 30 or 50 μL were manually injected (ECOM, Prague, Czech Republic). Zinc acetate (0.5 M, not pH adjusted) was delivered post-column by a peristaltic pump (P-500, Pharmacia, Uppsala, Sweden) at a flow rate of 0.10 mL/min. The signals from the fluorescence detector were transferred to a computer for analysis with Datalys Azur (Grenoble, France). The retention time of kynurenic acid or 7-Cl-KYNA was about 7-8 or 16 min, respectively. Initially, the sensitivity of the system was verified by analysis of a standard mixture of kynurenic acid with concentrations from 0.5 to 30 nM or 7-Cl-KYNA with concentrations from 1 to 100 nM, resulting in a linear standard plot. The standard concentrations were used to relate the height of the peaks in the chromatogram to the correct concentration of kynurenic acid or 7-Cl-KYNA in the samples. Kynurenic acid is a stable compound and is not degraded even by repeated thawing (Heyes and Quearry, 1990).

3.7.3 Chemicals

The chemicals used for sample preparation were perchloric acid (Kebo Lab,

Stockholm, Sweden), $Na_2S_2O_5$ (Sigma, St. Louis, MO, USA) and ethylenediaminetetra-acetate (EDTA; Sigma, St. Louis, MO, USA). For HPLC, zinc acetate and acetic acid (Sigma, St. Louis, MO, USA); sodium acetate (Riedel-de Haen, Germany); acetonitrile (Labasco, Partille, Sweden); 7-Cl-KYNA (Tocris, Avonmouth, UK) and kynurenic acid (Sigma, St. Louis, MO, USA) were used.

3.8 Analysis of tryptophan and kynurenine

3.8.1 High performance liquid chromatography

Tryptophan and kynurenine were analyzed with an HPLC system. Separation of kynurenine and tryptophan was achieved by reversed-phase liquid chromatography using a 20 mM NaH_2PO_4 buffer (not pH adjusted) with 5.0% acetonitrile. The mobile phase was delivered by an HPLC pump (Bischoff Chromatography, Leonberg, Germany) through a ReproSil-Pur C18 column (4 x 150 mm, Dr Maisch GmbH, Ammerbuch, Germany) at a rate of 0.5 mL/min. Samples of 50 μL were thawed at +4°C and manually injected immediately (Rheodyne, Cotati, CA, USA). Following separation, the analysate was first passed through a guard cell with an oxidizing potential of 50 mV. Samples were then quantified by sequential oxidation and reduction in a high-sensitivity analytical cell (ESA 5011; ESA Inc., Chelmsford, MA, USA) controlled by a potentiostat (Coulochem III; ESA Inc.) with an applied potential of 600 mV for detection of kynurenine and tryptophan. The signals from the detector were transferred to a computer for analysis (Datalys Azur, Grenoble, France). The retention time of kynurenine was approximately 8-9 min and 15-16 min for tryptophan. The sensitivity of the system was verified by analysis of standard mixtures of kynurenine, with concentrations from 5 to 100 nM, and tryptophan, with concentrations from 0.5 to 5 μM, resulting in a linear standard plot. The heights of the peaks in the chromatogram were used to relate the concentrations of the samples to the standard plot.

3.8.2 Chemicals

For HPLC, NaH_2PO_4 (Sigma, St. Louis, MO, USA); acetonitrile (Labasco, Partille, Sweden); L-kynurenine (Sigma, St. Louis, MO, USA) and tryptophan (Sigma, St. Louis, MO, USA) were used.

3.9 Analysis of cytokines and chemokines

IL-1β, IL-6, eotaxin, eotaxin-3 and macrophage inflammatory protein (MIP)-1β were quantified in CSF using multiplex sandwich enzyme-linked immunosorbent assays (ELISAs) with electrochemoluminescence in the quantifying step. We employed MesoScale Discovery assays (MesoScale, Gaithersburg, Maryland) as per the manufacturer´s protocol. CSF samples were analyzed on a SECTOR 6000 instrument (www.mesoscale.com). The respective detection limits in our analysis were IL-6: 0.1 pg/mL; IL-1β: 0.2 pg/mL; eotaxin, eotaxin-3 and MIP-1β. The cytokine analyses were undertaken in 2008, 10 ± 5 (mean ± SD) years after lumbar puncture.

3.10 Statistical analysis

3.10.1 Electrophysiology

All data are expressed as mean ± SEM. Statistically significant differences regarding firing rate were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney *U*-test. In experiments in paper III, the analysis of variance was followed by Mann-Whitney *U*-test with Bonferroni correction. Differences in percent spikes fired in bursts were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney *U*-test or Wilcoxon signed rank test. Significance was assumed for all values where $p < 0.05$.

3.10.2 Prepulse inhibition

For each pulse-alone and prepulse-pulse trial, the startle response to the 120-dB burst was recorded. Two measures were then calculated from these data for each animal. First, startle magnitudes were calculated as the average response to the pulse-alone trials within each of the four blocks and analyzed with mixed-design analyses of variance (ANOVAs), with block as the repeated measure and pretreatment and/or treatment as between-subject factors. Data from the first and last blocks of five pulse-alone trials are not presented, since the startle data from the middle two blocks when PPI was assessed were representative of the treatment effects, and no reliable effects on startle habituation were observed. Second, the amount of PPI was calculated as a percentage score for each prepulse + pulse trial type: %PPI = 100 - ([(startle response

for prepulse + pulse trial) / (startle response for pulse-alone trial)] x 100). All data were first analyzed in a three-factor ANOVA with blocks (first and second halves of the session) and prepulse as within subject factors and treatment as a between subject factor. When the block factor did not interact with another factor, only the two-factor ANOVA (treatment and prepulse intensity) are reported. The main effect of prepulse intensity was always significant and is not reported specifically. Post hoc comparisons of means were carried out with Tukey's test. Each experiment was analyzed separately. All data are presented as mean ±SEM. Significance was assumed for all values where $p < 0.05$.

3.10.3 Human cerebrospinal fluid studies

Paper V: The Statistical Package for the Social Sciences (SPSS) program version 15.00 for Windows was used. For non-normally distributed variables, values were transformed into natural logarithms (ln) before analysis. Linear regression correcting for age was used to identify significant predictors of kynurenic acid levels. We used Pearson´s R correcting for age (partial correlation) for correlation analyses. The cytokines and chemokines were measured in duplicates and the mean value was used for all analyses.

Paper IV: Statistical analyses were performed using GraphPad Prism® 4.0b (GraphPad Software Inc., San Diego, CA, USA). Differences regarding CSF concentrations were established using Kruskal-Wallis analysis of variance followed by Mann–Whitney *U*-test. Significance was assumed for all values where $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 Studying the mode of action of kynurenic acid – effects of pharmacologically elevated endogenous levels on midbrain dopamine neuron firing and prepulse inhibition (paper I and II)

As previously described (see 1.1.3), kynurenic acid is able to interact with several receptors. Among these, the compound antagonizes ionotropic glutamate receptors with a preferential selectivity at the glycine site of the NMDA receptor (IC_{50} ~8-15 μM; Parsons et al., 1997). In similar concentrations (IC_{50} ~7 μM; Hilmas et al., 2001) also the α7*nACh receptor is blocked. At higher concentrations the compound acts as a competitive antagonist at the glutamate recognition site of the NMDA receptor (IC_{50} = 200-500 μM; Kessler et al., 1989). In paper I and II, we have focused on the receptor-blocking effect of kynurenic acid at these sites, with regard to neurotransmission of glutamate and dopamine in the brain and possible involvement of the compound in psychiatric disorders.

Previous studies have shown that acute and chronic pharmacological elevation of brain kynurenic acid is associated with increased firing of rat midbrain dopamine neurons and disruption of PPI in the rat (Erhardt et al., 2001b, 2004; Nilsson et al., 2006; Olsson et al., 2009). Thus, elevated levels of endogenous brain kynurenic acid are associated with increased firing rate and burst firing activity of rat VTA dopamine neurons (Erhardt et al., 2001b; Erhardt and Engberg, 2002; Nilsson et al., 2006; Schwieler et al., 2006). Accordingly, lowering of kynurenic acid brain concentration decreases firing rate and burst firing activity of VTA dopamine neurons, indicating a tonic modulatory control of these neurons by kynurenic acid (Schwieler et al., 2006). A previous study (Erhardt and Engberg, 2002) suggest that the activation of midbrain dopamine neurons by pharmacologically elevated levels of endogenous kynurenic acid (by the kynurenine-3-hydroxylase inhibitor PNU 156561A) is executed via blockade of the glycine site of the NMDA receptor since the action of kynurenic acid is reversed by D-cycloserine. Furthermore, recent studies have shown that local administration of kynurenic acid in the rat striatum produces a decrease in terminal dopamine release via specific blockade of α7*nACh receptors (Rassoulpour et al., 2005).

Study I and II aimed at verifying that the activation of midbrain dopamine neurons is executed via the glycine site of the NMDA receptor and furthermore to analyze the specific receptor involved in the ability of kynurenic acid to reduce PPI. For this purpose we administered drugs selectively blocking the different receptor-sites known to be blocked by kynurenic acid; Methyllycaconitine (MLA), a selective antagonist at the α7*nACh receptor; SDZ 220-581, a selective blocker of the glutamate recognition site of the NMDA receptor; 4-Cl-KYN, which *in situ* is transformed to 7-Cl-KYNA (Hokari et al., 1996), a highly selective antagonist at the glycine site of the NMDA receptor (Kleckner and Dingledine, 1989), was given to selectively block the glycine site of the NMDA receptor. In paper II also the effects on PPI following administration of CGS 19755, a selective blocker of the glutamate recognition site of the NMDA receptor or L-701,324, a selective blocker of the NMDA glycine site, were analyzed.

4.1.1 Effects on midbrain dopamine cell firing following selective blockade of receptors known to be antagonized by kynurenic acid (paper I)

Study I describes a series of electrophysiological experiments aiming to reveal the specific receptor involved in the excitation of VTA dopamine neurons following pharmacologically elevated levels of endogenous kynurenic acid. Thus, dopaminergic response following administration of antagonists at different kynurenic acid-inhibiting sites was explored. In particular, the effects of 4-Cl-KYN, MLA and SDZ 220-581 were analyzed with regard to firing rate and burst firing activity of VTA dopamine neurons.

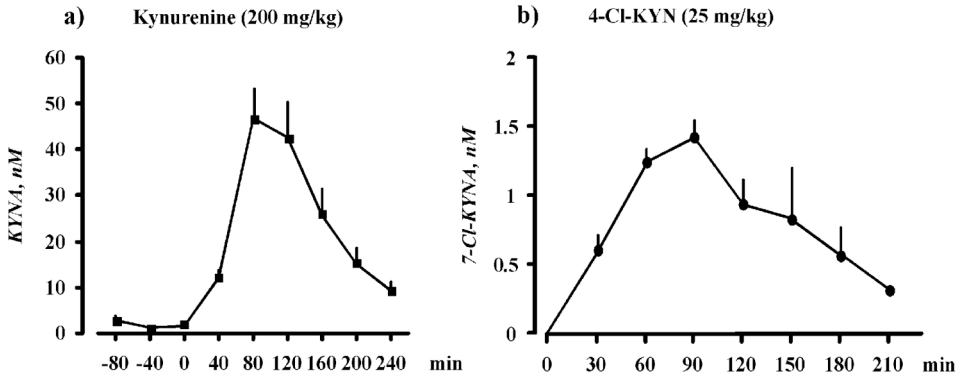

Figure 4. Concentrations of *in situ* produced (**a**) kynurenic acid (KYNA) and (**b**) 7-Cl-KYNA in nucleus accumbens following systemic treatment in rats with kynurenine (200 mg/kg, s.c., n = 6-10) or 4-Cl-KYN (25 mg/kg, s.c., n = 4-6), respectively. Each value represents mean ± SEM.

Utilizing microdialysis techniques, concentrations of *in situ*-produced kynurenic acid and 7-Cl-KYNA were measured in nucleus accumbens after systemic administration of kynurenine (200 mg/kg, s.c., n = 10) or 4-Cl-KYN (25 mg/kg, s.c., n = 6; fig. 4). Based on their respective time-response curve, firing of VTA dopamine neurons were monitored 1 – 3.5 h after administration of kynurenine (200 mg/kg, i.p., 60 neurons in 9 rats) or 4-Cl-KYN (25 mg/kg, i.p., 105 neurons in 8 rats) and compared to the dopamine firing observed in saline treated controls (71 neurons in 13 rats).

Systemic administration of kynurenine is associated with an increase in firing rate and burst firing activity of VTA dopamine neurons. Furthermore, it is associated with an almost two-fold increase in the number of dopamine neurons detected per track, indicating that elevated brain kynurenic acid concentrations increased the number of spontaneously active dopamine neurons. Also, the excitatory effects on VTA dopamine neurons observed in rats with pharmacologically elevated levels of endogenous kynurenic acid were mimicked by pretreatment with 4-Cl-KYN, which *in situ* is transformed to 7-Cl-KYNA, a selective antagonist at the glycine site of the NMDA receptor (table 1).

Table 1. Firing rate and spike distribution of dopamine neurons in the VTA following intravenous pretreatment (1-3.5 h) with kynurenine (200 mg/kg, i.p.) or 4-Cl-Kyn (25 mg/kg, i.p.).

	Controls (71 neurons)	**kynurenine** (60 neurons)	**4-chlorokynurenine** (105 neurons)
Firing rate, Hz	4.0 ±0.2	5.0 ±0.2**	5.1 ±0.2**
Mean % spikes in burst	26.7 ±2.8	40.2 ±4.0^{++}	43.1 ±3.0^{+++}
Cells found per track	1.5 ±0.2	2.1 ±0.2	2.8 ±0.4*

Values represent means ± SEM from control rats (n=13) and rats treated with kynurenine (n=9) or 4-Cl-KYN (n=8). *P<0.05, **P<0.01 vs. corresponding control value (Mann-Whitney U-test). $^{++}$P<0.01, $^{+++}$P<0.001 vs. corresponding control value (Wilcoxon signed rank test).

Our results are in consonance with previous electrophysiological studies showing that pharmacologically elevated levels of endogenous brain kynurenic acid are associated with increase in firing of midbrain dopamine neurons (Erhardt et al., 2001a; Erhardt and Engberg, 2002; Nilsson et al., 2006; Schwieler et al., 2006; Olsson et al., 2009) and

that blockade of the glycine site of the NMDA receptor per se could induce this effect. Furthermore, administration of SDZ 220-581 (n = 7; 10 mg/kg, i.v.), a selective and centrally active competitive NMDA receptor antagonist at the glutamate recognition site (Urwyler et al., 1996a, b), was associated with an increase in firing rate and burst firing activity of VTA dopamine neurons. However, the effect was typically not observed until 5 min after administration and also not seen following administration of the drug in a lower dose (5 mg/kg, i.v.). These discrepancies might be explained in terms of potency or problems of the drug crossing the blood-brain barrier.

The excitatory actions on VTA dopamine neurons by pharmacologically elevated levels of endogenous kynurenic acid are thus in accordance with the effects on these neurons by non-competitive NMDA receptor antagonists, i.e. PCP, MK 801 (French et al., 1991, 1993; Zhang et al., 1992), as well as by antagonists of the glycine site of the NMDA receptor, i.e. L-701,324 (Schwieler et al., 2004). Notably, kynurenic acid blocks the glutamate recognition site of the NMDA receptor although the IC_{50} value of kynurenic acid for this site is considerably higher than that of the glycine site of the NMDA receptor. Thus, the possibility that blockade of also glutamate recognition site may contribute to the observed excitation of VTA dopamine neurons by pharmacologically elevated levels of endogenous kynurenic acid cannot be disregarded.

Intravenous administration of MLA, an α7*nACh receptor antagonist (0.5-4 mg/kg, n = 6-12) failed to alter firing rate or burst firing activity of VTA dopamine neurons. However, when the drug was injected i.p. in a relatively high dose (6 mg/kg, n = 12), a significant decrease in firing was observed. The effects on VTA dopamine firing following low doses (0.5-4 mg/kg) of MLA on VTA dopamine neurons are in line with a previous study (Wang et al., 2006). Furthermore, the significant decrease in firing by a high dose of MLA (6 mg/kg), are in consonance with the increased firing activity observed following administration of galantamine, an α7*nACh receptor agonist (Schilström et al., 2007).

Altogether, present results suggest that the excitatory effect on VTA dopamine neurons by pharmacologically elevated levels of endogenous kynurenic acid in the brain is mediated via blockade of the NMDA receptor.

4.1.2 Effects on prepulse inhibition following selective blockade of receptors known to be antagonized by kynurenic acid (paper II)

PPI is a behavioral model for sensorimotor gating which is found to be reduced in schizophrenia. Disruption of sensorimotor gating is considered to reflect dysfunctions in the ability to filter out extraneous stimuli that might interfere with information processing and attention. Interestingly, non-competetive NMDA receptor antagonists, e.g. PCP, ketamine or MK 801, disrupt PPI in rodents. These effects on PPI are also in line with the NMDA receptor hypofunction hypothesis of schizophrenia, based on the finding that NMDA receptor antagonists cause psychotic symptoms in healthy volunteers and exacerbate clinical symptoms in patients with schizophrenia. In line with these findings, a previous study has also shown that pharmacologically elevated levels of endogenous kynurenic acid disrupt PPI in the rat. However, the specific receptor mechanism involved was not ascertained even though it is suggested to be mediated, at least partly, via blockade of the glutamate recognition site of the NMDA receptor (Erhardt et al., 2004). In paper II, we investigated which receptor(s) mediate the effects of pharmacologically elevated levels of endogenous kynurenic acid on PPI. For this purpose we administered drugs selectively blocking the receptors known to be blocked by kynurenic acid; MLA, a selective α7*nACh receptor antagonist; SDZ 220-581 and CGS 19755, selective blockers of the glutamate recognition site of the NMDA receptor; L-701,324 and 4-Cl-KYN, *in situ* converted to 7-Cl-KYNA, was given to selectively block the glycine site of the NMDA receptor.

The results of this study show that rats administered kynurenine (200 mg/kg) displayed a five-fold increase in whole brain kynurenic acid levels (123.1 nM ± 18.8, n = 14) compared to controls 23.3 nM ± 2.7, n = 13). This increase was associated with a tendency to disrupt PPI ($F(1,25) = 2.56$, $p = 0.12$: fig. 5). Treatment with drugs blocking the glutamate recognition site of the NMDA receptor, i.e. SDZ 220-581 (2.5 mg/kg; n = 12; $F(1,22)=12.33$, $p < 0.01$) or CGS 19755 (10 mg/kg; n = 12; $F(1,22)=16.47$, $p < 0.001$), was found to clearly reduce PPI (fig. 6) and our results thus confirm that blockade of the glutamate recognition site of the NMDA receptor is associated with a disrupted PPI (Bakshi et al., 1999; Depoortere et al., 1999). In

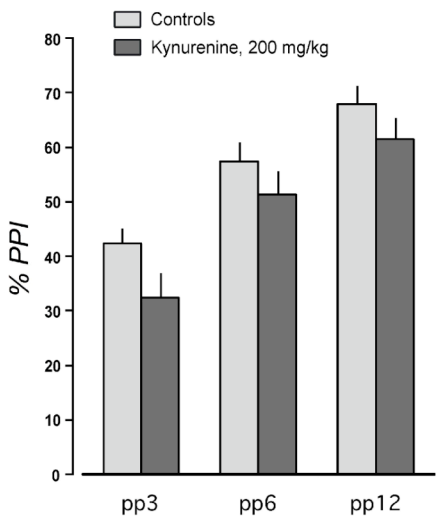

Figure 5. Effects of kynurenine (200 mg/kg, i.p., 60 min, n = 14) or vehicle (i.p. 60 min, n = 13) on prepulse inhibition (PPI). Values represent mean ± SEM for each group.

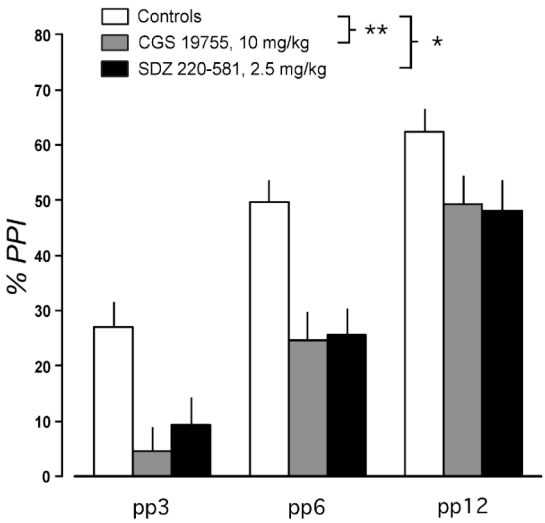

Figure 6. Effects of CGS 19755 (10 mg/kg, s.c., 45 min, n = 12), SDZ 220-581 (2.5 mg/kg, s.c., 30 min, n = 12) or saline (n = 12) on PPI. Values represent mean ± SEM for each group. Statistics: *p < 0.01 vs. saline, **p < 0.001 vs. saline.

contrast, blockade of either the α7*nACh receptor with MLA (6 mg/kg; n = 15); the NMDA glycine site with L-701,324 (1 mg/kg; n = 13; or 4 mg/kg; n = 17) or 4-Cl-KYN (25; 50 or 100 mg/kg; n = 15, n = 14 and n = 10, respectively) failed to reduce PPI. The effects of L-701,324 and 4-Cl-KYN are in line with previous studies showing that systemic administration of antagonists of the NMDA glycine site does not affect PPI (Depoortere et al., 1999; Bristow et al., 1995). However, 7-Cl-KYNA when locally injected intracerebroventricularly (i.c.v.), or into the nucleus accumbens, has been found to reduce PPI (Furuya et al., 1997; Kretschmer et al., 1997, 1998). A benefit of using systemic administration of 4-Cl-KYN is the *in situ* production of 7-Cl-KYNA. Hence, 4-Cl-KYN utilizes the same enzymatic machinery as kynurenine and hence 7-Cl-KYNA will be produced in the same regions and micro-compartments as kynurenic acid is produced. Thus, the absence of an effect on PPI following pretreatment of 4-Cl-KYN reliably suggests that the glycine site of the NMDA receptor is not primarily involved in the modulation of PPI. Furthermore, the tendency toward an increased PPI in this study following administration of MLA, is consistent with a previous study by Schreiber et al (2002), and by the finding that PPI is normal in α7* null mutant mice (Paylor et al., 1998). The results of the present study is however, not in line with other reports, as it has been shown that administration of α-bungarotoxin, another α7*nACh receptor antagonist, or removal of hippocampal cholinergic afferents, actually disrupts PPI (Bickford et al., 1995; Luntz-Leybman et al., 1992; Stevens, et al., 2001).

Altogether, the results suggests that neither antagonism of the glycine site of the NMDA receptor nor antagonism of the α7*nACh receptor is associated with the ability of kynurenic acid to disrupt PPI. Rather, with regard to the effects of kynurenic acid, blockade of the glutamate recognition site is necessary to reduce PPI.

4.2 Altered levels of kynurenic acid change the response of clozapine on midbrain dopamine neuron firing (paper III)

Previous electrophysiological studies show that all antipsychotic drugs, including clozapine, when acutely administered increase the firing rate and burst firing activity of VTA dopamine neurons (Gessa et al, 2000; Tung et al, 1991; White and Wang, 1983). However, previous studies show that the response of VTA dopamine neurons is changed from an excitatory action in control rats to a pure inhibitory response in rats with pharmacologically elevated levels of brain kynurenic acid (Schwieler and Erhardt, 2003; Schwieler et al., 2004). Furthermore, *in vivo* electrophysiological experiments have shown a modulatory role of endogenous kynurenic acid in regulating neuronal activity of VTA dopamine firing. Thus, pharmacological elevation of endogenous brain kynurenic acid levels is associated with increased firing rate and burst firing activity of rat midbrain dopamine neurons (Erhardt et al., 2001a; Erhardt and Engberg, 2002; Nilsson et al., 2006; Schwieler et al., 2006; Olsson et al., 2009). Accordingly, lowering of endogenous brain kynurenic acid concentration decreases firing rate and burst firing activity of VTA dopamine neurons, demonstrating a tonic modulatory control of these neurons by kynurenic acid (Schwieler et al., 2006). In the present study the response to clozapine on firing activity of VTA dopamine neurons was investigated in rats with increased or decreased levels of endogenous brain kynurenic acid. Rat brain kynurenic acid concentrations were pharmacologically manipulated by administration of a preferential cyclooxygenase 1 (COX-1) inhibitor (indomethacin) or a selective COX-2 inhibitor (parecoxib), drugs previously shown to increase or decrease brain kynurenic acid, respectively (Schwieler et al., 2005, 2006).

Present results show that in control rats, intravenous administration of clozapine (0.078-10 mg/kg, n = 7) produced a dose-dependent increase in firing activity (fig. 7) and in percent spikes fired within bursts, in line with previous studies (White and Wang, 1983; Tung et al., 1991; Gessa et al., 2000; Schwieler and Erhardt, 2003;

Schwieler et al., 2004). Furthermore, our results confirm that pretreatment with indomethacin (50 mg/kg, i.p., n = 9) or parecoxib (25 mg/kg, i.v., n = 12), increase or decrease endogenous levels of kynurenic acid, respectively (253% and 48% of brain kynurenic acid levels detected in controls). In line with a previous study (Schwieler et al., 2006), the elevated or lowered kynurenic acid concentration was associated with increased or decreased VTA dopamine firing activity, respectively. Moreover, the elevated brain kynurenic acid levels converted the excitatory effects of clozapine into a pure inhibitory response (fig. 7). In contrast, the reduction in brain kynurenic acid concentration by parecoxib clearly potentiated the excitatory actions of clozapine; i.e. considerably lower doses were required to produce a significant increase in firing activity (fig. 7).

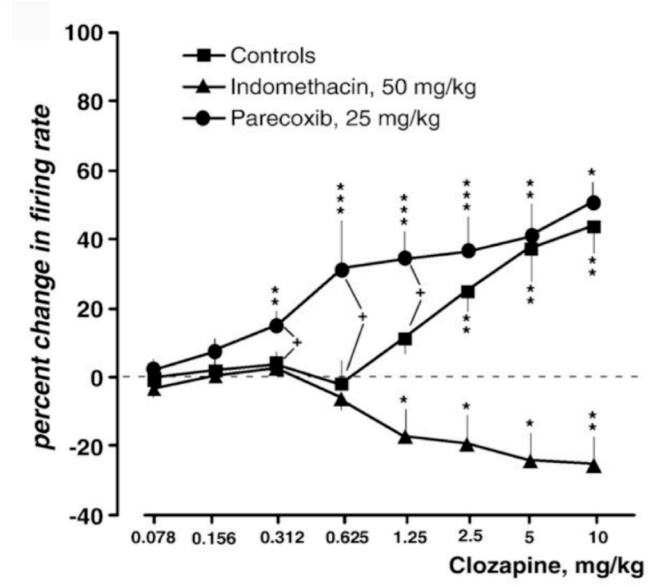

Figure 7. Effects of incremental doses of intravenously administered clozapine (0.078-10 mg/kg) in control rats and rats pretreated with indomethacin (50 mg/kg, i.p., 1-3.5 h) or parecoxib (25 mg/kg, i.v.,1-1.5 h) on the firing rate (statistics: *p < 0.05, **p < 0.01, ***p < 0.001 vs. corresponding predrug value and +p < 0.05 vs. corresponding control value, Mann-Whitney U-test with Bonferroni correction). Each value represents mean ± SEM from 4-12 VTA DA neurons.

Present results are in line with previous studies showing that elevated brain levels of kynurenic acid or systemic administration of a selective NMDA glycine site antagonist, i.e. L-701,324, reverses the excitatory actions of clozapine into an inhibitory response (Schwieler and Erhardt, 2003; Schwieler et al., 2004). Notably, a clear-cut inhibitory effect of clozapine is observed already at a dose of 1.25 mg/kg. Such doses in rats correspond with clinically therapeutic doses according to brain imaging studies in humans (Nordström et al., 1993a, 1993b; Schotte et al., 1996; Farde et al., 1997). This is of particular interest since this dose did not produce a significant activation of VTA dopamine neurons in control rats but clearly inhibited the same neurons in a situation where endogenous brain kynurenic acid was elevated. The activation of midbrain dopamine neurons by antipsychotics, including clozapine, has generally been attributed

to a blockade of somatodendritic dopamine D_2 receptors (Pucak and Grace, 1996; Gessa et al., 2000). However, clozapine displays a weak dopamine D_2-receptor occupancy (Mukherjee et al., 2001) and other dopamine antagonists, e.g. haloperidol or sulpiride, do not affect midbrain dopamine firing in doses equipotent to those of clozapine with regard to dopamine D_2-receptor occupancy (Pucak and Grace, 1996; Schwieler and Erhardt, 2003). Thus, antagonism of somatodendritic dopamine D_2 receptors by clozapine should not solely account for the activation of VTA dopamine neurons. Rather, the presently observed interaction of endogenous kynurenic acid with clozapine strongly suggests that the activation of VTA dopamine neurons by the drug involves an action on glutamatergic neurotransmission. Thus, clozapine has been shown to modulate the response of NMDA in frontal cortex slices *in vitro* (Arvanov et al.,1997; Chen and Yang, 2002; Jardemark et al., 2003) and to prevent NMDA receptor hyperactivity seen after repeated PCP treatment in vivo (Arvanov and Wang, 1999). Further, clozapine has been found to inhibit the System A transport which, together with glycine transporters, is responsible for homeostasis of glycine (Javitt et al., 2005). Studies from our laboratory also point to a direct interaction between clozapine and the glycine site of the NMDA receptor (Schwieler and Erhardt, 2003; Schwieler et al., 2004). Indeed, the activation of VTA dopamine neurons by clozapine resembles that observed by i.v. administration of L-701,324 (Schwieler et al., 2004) or by elevated endogenous levels of kynurenic acid (Erhardt and Engberg, 2002). These actions are thought to be induced by blockade of the glycine site of the NMDA receptor on inhibitory γ-aminobutyric acid (GABA) afferents controlling activity of midbrain dopamine neurons. The enhanced response of VTA dopamine neurons to clozapine seen following lowered kynurenic acid is what should be expected from a partial NMDA glycine site agonist (Tsai et al., 1999; Coyle and Tsai, 2004). Thus, under a condition where the NMDA glycine site may be saturated by the endogenous ligands (glycine or D-serine) an antagonistic action of clozapine would prevail leading to increased firing activity. In contrast, when a high degree of blockade of the NMDA glycine site is present (due to elevated kynurenic acid concentration) an agonistic action of clozapine would be more prominent, hereby decreasing impulse activity. Thus, clozapine appears to stabilize dopamine neurons, dampening both hyper- and hypoactivity via an agonistic and antagonistic action at the NMDA glycine site, respectively. Such an effect of clozapine is also proposed to occur with respect to prefrontal cortex neurons (Homayoun and Moghaddam, 2007). Indeed, the presently

revealed effects of clozapine on VTA dopamine neurons bear a striking similarity to the actions on these neurons by systemic administration of D-cycloserine, a partial NMDA/glycine site agonist. Thus, administration of D-cycloserine increases VTA dopamine firing in control rats and decreases firing in rats with elevated brain levels of kynurenic acid (Erhardt and Engberg, 2002). A partial agonistic action at the NMDA glycine site of clozapine is also supported by clinical data. Thus, D-cycloserine in combination with traditional or second generation antipsychotics improves symptoms of schizophrenia. A unique interaction between clozapine and the glycine site of the NMDA receptor might explain the paradoxical worsening in negative symptoms when D-cycloserine is added to clozapine in patients with schizophrenia (Goff et al., 1999).

4.3 Kynurenic acid in human cerebrospinal fluid from patients with psychiatric disorders (paper IV and V)

Previous studies have shown that kynurenic acid is elevated in the CSF and in the post mortem brain of patients with schizophrenia (Erhardt et al., 2001a, Schwarcz et al., 2001, Nilsson et al., 2005). Furthermore, a recent study also showed increased levels of kynurenic acid in the CSF from patients with bipolar disorder (Olsson et al., 2010) and an up-regulated kynurenine pathway has been associated with depression (Myint et al., 2007; Wichers et al., 2005; Wichers and Maes, 2004; Raison et al., 2009). An increased synthesis of kynurenic acid might result from an induction of IDO and/or TDO, enzymes responsible for the rate-limiting step of the kynurenine pathway. Notably, these enzymes are induced during infections or immune-activation (Asp et al., 2009; Holtze et al., 2008; Schmidt et al., 2009) and numerous studies suggest that kynurenic acid is a biological marker of neuroinflammation (Dantzer et al., 2008; King et al., 2008). Increasing evidence also suggests that immunological processes are involved in the etiology of depression (Charlton, 2000; Hart, 1988; Dantzer et al., 1989) and a recent study show increased concentration of the pro-inflammatory cytokine interleukin-1β in first-episode patients with schizophrenia (Söderlund et al., 2009). Thus, in paper IV we investigate levels of key metabolites in the kynurenine pathway in the CSF of patients with schizophrenia and in paper V levels of kynurenic acid were measured in suicide attempters and compared to inflammatory markers and diagnostic groups.

4.3.1 Analysis of kynurenic acid and its precursors kynurenine and tryptophan in the cerebrospinal fluid of patients with schizophrenia (paper IV)

Kynurenic acid has an exceptional receptor binding profile as it blocks both glutamatergic and cholinergic receptors. This is particularly interesting in schizophrenia, given that hypoglutamatergia, possibly induced by NMDA receptor hypofunction, is widely accepted as part of the pathophysiology. In addition, the importance of an intact α7*nACh receptor signaling in cognitive functions has been suggested in numerous studies over the last decade (Albuquerque et al., 2009). In further support of the kynurenic acid hypothesis of schizophrenia are studies showing that pharmacologically elevated levels of kynurenic acid disrupt cognitive functions such as sensory motor gating, contextual learning and working memory (Erhardt et al., 2004, Chess and Bucci 2006, Chess et al 2007; 2009), domains to a large extent affected in patients with schizophrenia.

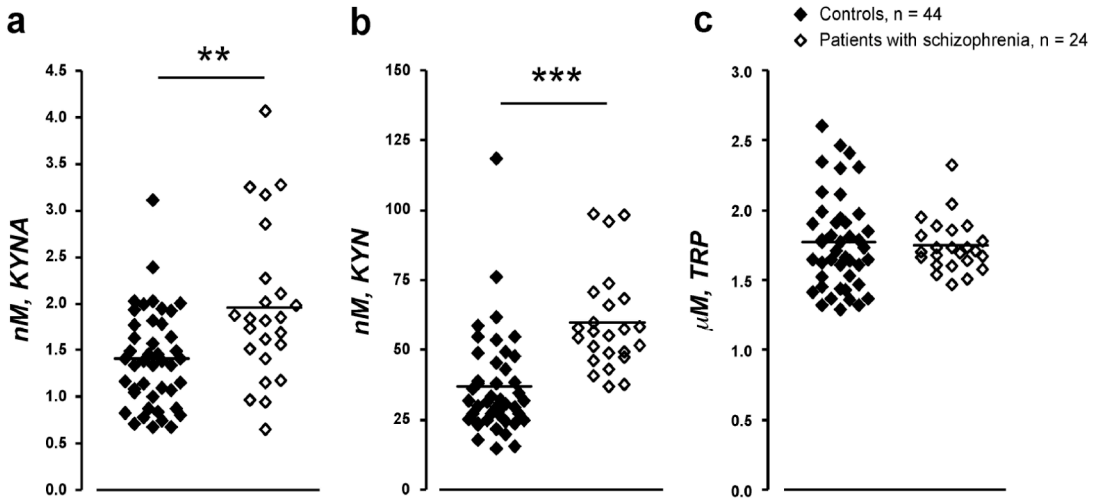

Figure 8. (**a**) Kynurenic acid (KYNA), (**b**) kynurenine (KYN) and (**c**) tryptophan (TRP) in cerebrospinal fluid (CSF) of healthy volunteers and patients with schizophrenia. Each point represents the concentration in a single CSF sample together with the mean for the two groups. Statistics: **p<0.01, ***p <0.001 (Mann-Whitney U-test).

In the present study, kynurenic acid and its precursors kynurenine and tryptophan were measured in the CSF of male (n = 16) and female patients (n = 8) with schizophrenia and in a group of healthy male (n = 29) and female (15) volunteers. All patients were prescribed and taking the antipsychotic drug olanzapine at the time of lumbar puncture. The results show that CSF kynurenic acid is elevated ($p < 0.01$) in patients with schizophrenia (1.97 nM ± 0.17, n = 24; fig. 8) compared to healthy volunteers (1.42 nM

± 0.08, n = 44). Furthermore, we found elevated levels ($p < 0.001$) of kynurenine, the immediate kynurenic acid precursor (59.9 nM ± 3.60, n = 24; fig. 8), but not of tryptophan (1.75 μM ± 0.04, n = 24; fig. 8), in the CSF of patients with schizophrenia compared to healthy volunteers (37.2 nM ± 2.77, 1.78 μM ± 0.05, respectively, n = 44). When analyzed with regard to gender, we were able to confirm increased levels ($p < 0.01$) of CSF kynurenic acid in male patients with schizophrenia (2.03 nM ± 0.23 vs. 1.36 nM ± 0.08 in healthy male volunteers). Additionally, we here report increased levels ($p < 0.001$) of kynurenine in male patients with schizophrenia (60.7 nM ± 4.37 vs. 28.6 nM ± 1.44 in healthy male volunteers). Levels of CSF tryptophan in male patients with schizophrenia were found to be within the same range as in healthy male volunteers. In a smaller cohort of female patients with schizophrenia (n = 8), levels of kynurenic acid, kynurenine and tryptophan were unchanged compared to healthy female volunteers (n = 15). Present results are thus in line with previous studies showing increased CSF kynurenic acid in first-episode, drug-naive patients or patients receiving various antipsychotic treatments (Erhardt et al., 2001a; Nilsson et al., 2005), further supporting a role of kynurenic acid of in the pathophysiology of schizophrenia. The cause of the difference between male and female patients is unclear, however this may be related to the relatively low number of female patients and volunteers analyzed.

Kynurenine was markedly elevated in male patients with schizophrenia. This is a novel finding in line with previous post mortem studies (Miller et al., 2006; 2008) further supporting an induction of the kynurenine pathway in schizophrenia. The elevated CSF kynurenine levels may arise from an increased synthesis of kynurenine from tryptophan, or alternatively, a decreased synthesis of 3-hydroxykynurenine from kynurenine. An increased synthesis of kynurenine might result from an induction of IDO/TDO, enzymes responsible for the rate-limiting step of the kynurenine pathway. These enzymes are induced during infections or immune-activation (Schmidt et al., 2009; Asp et al., 2009; Holtze et al., 2008) and gene expression of TDO is found to be elevated in post mortem brain of patients with schizophrenia (Miller et al., 2004). Alternatively, a decreased synthesis of 3-hydroxykynurenine from kynurenine, resulting in increased kynurenine levels, is supported by experimental as well as genetic studies (Speciale et al., 1996; Erhardt et al., 2000; Erhardt and Engberg 2000; Schwieler and Erhardt, 2003; Rassoulpour et al., 2005; Sathyasaikumar et al., 2009; Holtze et al., 2010). In conclusion, present results confirm increased levels of kynurenic

acid, and additionally, we found increased levels of kynurenine, in the CSF of patients with schizophrenia.

4.3.2 Kynurenic acid in the cerebrospinal fluid of male suicide attempters (paper V)

A recent study shows increased levels of IL-6 in the CSF of suicide attempters and the highest levels were found in patients who performed violent suicide attempts and/or had a diagnosis of MDD (Lindqvist et al., 2009). In this study, CSF concentration of kynurenic acid and the correlation to CSF levels of pro-inflammatory cytokines and chemokines were analyzed in 54 male suicide attempters and 66 healthy male volunteers. The suicide attempters with well-defined clinical psychiatric profiles were lumbar punctured after a drug wash-out period of approximately 2 weeks after the suicide attempt. CSF kynurenic acid concentrations displayed a skewness above 2 in all 120 subjects and all values were subsequently transformed into normal distribution, using their natural logaritms, which were used for all calculations. Also, CSF kynurenic acid levels were adjusted for age in all analyses. The results show that a violent suicide attempt predicts a significantly increased kynurenic acid level (1.80 nM ± 0.37, n = 20; $p < 0.05$; fig. 9) compared to healthy volunteers (1.14 nM ± 0.06, n = 66). Moreover, a diagnosis of MDD (n = 14) is clearly associated with increased CSF kynurenic acid levels (1.93 nM ± 0.46, $p = 0.013$; fig. 9) whereas a diagnosis of substance abuse (n = 15) is associated with lower levels of CSF kynurenic acid (0.95 nM ± 0.19; $p < 0.05$). In all subjects, MIP-1β was significantly correlated with kynurenic acid, and in healthy controls, a correlation was found between CSF kynurenic acid and eotaxin-3.

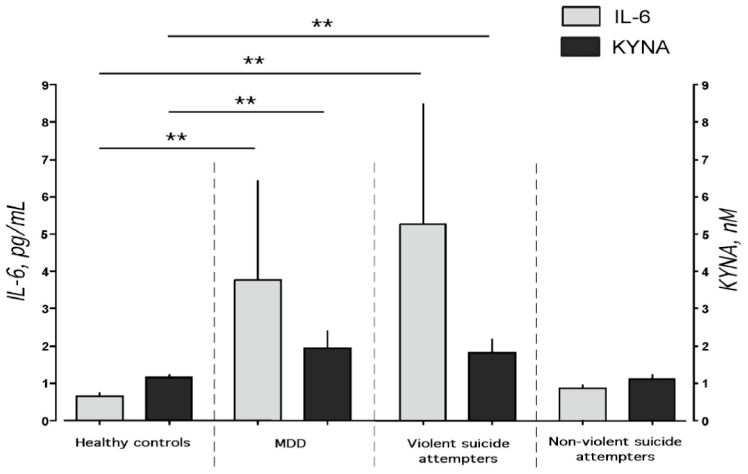

Figure 9. Levels of IL-6 and kynurenic acid (KYNA) in the CSF of healthy volunteers and in suicide attempters, divided into different categories, i.e. patients with MDD, and a violent / non-violent suicide attempt. Statistics: ** $p < 0.01$ (Mann-Whitney U-test).

Furthermore, a trend towards a correlation ($p = 0.08$) between ln-IL-6 and ln-kynurenic acid was observed in a smaller cohort of patients ($n = 33$), indicating that the elevated CSF kynurenic acid levels in patients with MDD might causally be related to the increased levels of IL-6 (Lindqvist et al. 2009).

Taken together, present results support the notion that immune activation is associated with increased levels of kynurenic acid. Thus, cytokine-induced induction of IDO/TDO resulting in elevated brain levels of kynurenic acid might be a potential mechanism by which the immune system contributes to the development of psychiatric disorders and manifestations of psychiatric symptoms.

In conclusion, the present study demonstrates that immunologically active compounds, e.g. kynurenic acid and IL-6, are elevated in the CSF of violent suicide attempters and especially in those patients suffering from MDD, whereas a diagnosis of substance abuse is associated with lower levels of CSF kynurenic acid.

5 GENERAL DISCUSSION

The physiological role of kynurenic acid has been questioned ever since its presence was first discovered in the human brain (Moroni et al., 1988; Turski et al., 1988). Thus, the concentration of the compound found in human post mortem and CSF studies or in animal whole brain and extracellular fluid is below that required to affect either the NMDA glycine site (IC_{50} = 8-15 μM; Parsons et al., 1997) or the α7*nACh receptor (IC_{50} = 7 μM; Hilmas et al., 2001). However, a variety of animal studies have clearly confirmed that very low, i.e. nM concentrations, of kynurenic acid play an important physiological role in the brain (Amori et al., 2009; Rassoulpour et al., 2005; Sapko et al., 2006; Shephard et al., 2003; Wu et al., 2009). Furthermore, pharmacologically elevated levels of kynurenic acid increase midbrain dopamine firing (Erhardt et al., 2001b; Erhardt and Engberg 2002; Nilsson et al., 2006; Schwieler et al., 2006; 2008; Linderholm et al., 2007; Olsson et al., 2009) whereas lowered levels of kynurenic acid dampen the activity of these neurons (Schwieler et al., 2006; 2008). The discrepancy between *in vivo* and *in vitro* studies might be related to the production and release of kynurenic acid by astrocytes, known to be closely connected to the synapse and there adjoining glutamatergic boutons (Araque et al., 1999). Such an appropriate location puts the newly produced kynurenic acid in an excellent position to interact with glutamatergic and cholinergic receptors. Thus, although the levels of CSF kynurenic acid found in the patients with schizophrenia and the suicide attempters in the present thesis are relatively low, the real concentration at critical sites of action, i.e. within the synapses, should be sufficient to interact with glutamatergic/cholinergic receptors. Interestingly, both NMDA receptor and α7*nACh receptor hypofunctions are suggested to be widely implicated in the pathophysiology of psychiatric disorders (Javitt, 2007; Albuquerque et al., 2009). Besides elevated levels of kynurenic acid in patients with schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005; present thesis), patients with bipolar disorder also display elevated CSF kynurenic acid levels (Olsson et al., 2010). Interestingly, animal studies point to a close relationship between kynurenic acid and cognitive functions and it is thus tempting to speculate that dysfunctions in cognitive domains, which are common in almost all psychiatric disorders, is related to elevated brain levels of kynurenic acid (Wonodi and Schwarcz, 2010).

Although the physiological significance of brain kynurenic acid has been demonstrated in a number of studies during the last decade (see Erhardt et al., 2009), it is unclear what type of receptor(s) participate in the various effects of kynurenic acid in the brain. A previous study suggests that the increased firing of rat midbrain dopamine neurons following pharmacological elevation of brain kynurenic acid is mediated via blockade of the glycine site of the NMDA receptor (Erhardt and Engberg 2002). However, local administration of kynurenic acid in the rat striatum decreases terminal dopamine release via specific blockade of α7*nACh receptors (Rassoulpour et al., 2005). In addition, a previous study has suggested that blockade of the glutamate recognition site is responsible for the disrupted PPI following pharmacologically elevated levels of kynurenic acid in the rat (Erhardt et al., 2004). In the present thesis, we show that 7-Cl-KYNA, a selective antagonist at the glycine site of the NMDA without blocking the glutamate recognition site or the α7*nACh receptor, produces the same magnitude of response with regard to firing of VTA dopamine neurons as kynurenic acid at equipotent concentrations. This finding provides strong evidence that kynurenic acid increases VTA dopamine firing by blocking the glycine site of the NMDA receptor. The excitatory action on VTA dopamine neurons by SDZ 220-581, a competitive antagonist at the glutamate recognition site of the NMDA receptor, principally indicates that the ability of kynurenic acid to block also this site could participate in its excitatory effects on VTA dopamine neurons, although relatively high concentrations of endogenous brain KYNA would be required. Blockade of the α7*nACh receptor, by MLA in high dose, were associated with a reduced firing of the VTA dopamine neurons. This finding is in line with the excitatory action of galantamine, an α7*nACh receptor agonist (Schilström et al., 2007) on VTA dopamine neurons, making it unlikely that blockade of the α7*nACh receptor contributes to the increased firing of VTA dopamine neurons following elevated levels of kynurenic acid. The present thesis also suggests that neither antagonism of the glycine site of the NMDA receptor nor antagonism of the αα7*nACh receptor disrupt PPI. Rather, with regard to the effects of kynurenic acid, blockade of the glutamate recognition site is necessary to reduce PPI.

Although the present thesis reveals that blockade of the NMDA receptor accounts for the excitatory action on VTA dopamine neurons as well as the disrupted PPI by elevated levels of endogenous kynurenic acid, the antagonizing action at the

αα7*nACh receptor by kynurenic acid should not be disregarded, especially with respect to the functional effects in other brain areas, e.g. on striatal dopamine release (Rassoulpour et al., 2005). Indeed, in recent years it has been suggested that symptoms of schizophrenia might be attenuated by administration of drugs activating the glycine site of the NMDA receptor (e.g. by serine or glycine) or the αα7*nACh receptor (e.g. by galantamine). Given the proposed role of endogenous kynurenic acid in the pathophysiology of schizophrenia, the beneficial effects of such treatment might rationally be explained in terms of displacement of kynurenic acid at the levels of the NMDA receptor and/or the αα7*nACh receptor.

In the present thesis we also found that the response on VTA dopamine firing by clozapine is dependent on brain levels of endogenous kynurenic acid. Elevation of rat brain levels of kynurenic acid may rationally mimic the hypoglutamatergic and hypocholinergic condition proposed in patients with schizophrenia (see Erhardt et al., 2009; Wonodi and Schwarcz 2010). Notably, in such a situation, clozapine produces an inhibitory action on VTA dopamine neurons. This effect is probably induced via an agonistic action at the NMDA glycine site and is most prominent in the hypoglutamatergic brain. On the contrary, when the NMDA glycine site is saturated by endogenous ligands as a result of decreased kynurenic acid concentration, an antagonistic action of clozapine is associated with an activation of VTA dopamine neurons. This pharmacological profile, i.e. partial agonism of the glycine site of the NMDA receptor, may contribute to the unique efficacy of clozapine as an antipsychotic drug.

Increasing evidence suggests that immunological processes may contribute to the etiology of psychiatric disorders (Dantzer, 2008; Sperner-Unterweger, 2005). Indeed, it was recently found that the CSF levels of IL-1β are elevated in first-episode patients with schizophrenia (Söderlund et al., 2009). Furthermore, elevated CSF levels of IL-6 were recently reported in suicide attempters (Lindqvist et al., 2009). An up-regulated kynurenine pathway may be a consequence of an activated immune system as studies have shown that immunological agents induce TDO/IDO (Asp et al., 2009; Babcock and Carlin 2000; Guilleman et al, 2001; Holtze et al., 2008; Schmidt et al., 2009; Shirey et al., 2006; Takikawa et al., 1988), the rate-limiting enzymes in the production of kynurenic acid. Interestingly, numerous studies suggest that kynurenic acid may serve

as a marker of immune-activation (Eastman et al 1994; Schwarcz and Hunter 2007; Silva et al., 2002; Holtze et al., 2008; Asp et al., 2009). Elevation of endogenous kynurenic acid might thus, be a potential mechanism by which the immune system contributes to the development of psychiatric disorders and manifestations of psychiatric symptoms. This theory is supported by the close correlation between cytokines/chemokines and kynurenic acid in the CSF of suicide attempters observed in the present thesis. Indeed, kynurenic acid, with its unique receptor profile, may be the link between an activated immune system and the alterations in glutamatergic, cholinergic and dopaminergic neurotransmission proposed to occur in psychiatric disorders.

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