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ENDOGENOUS KYNURENIC ACID AND SCHIZOPHRENIA – PHYSIOLOGICAL AND PHARMACOLOGICAL ASPECTS

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To my surprise but to Kristina Ikelbergs wisdom

ABSTRACT

Kynurenic acid is a glutamate receptor antagonist with a preferential action at the glycine/D-serine site of the N-methyl-D-aspartic acid (NMDA) receptor. The compound is a metabolite of tryptophan and is synthesized in astrocytes. Previous studies have shown increased levels of kynurenic acid in the CSF and post mortem in the prefrontal cortex of patients with schizophrenia. The aim of the present thesis was to further investigate in the rat the physiological significance of kynurenic acid as well as its importance for the pathophysiology of schizophrenia. Studies include a disclosure of a prostaglandin-mediated regulation of kynurenic acid synthesis and analysis of the role of the compound in the regulation of firing activity of dopamine neurons in ventral tegmental area (VTA). Furthermore, an interaction of the antipsychotic drug clozapine with endogenous kynurenic acid is analyzed and the significance of kynurenic acid for behavior is studied using prepulse inhibition (PPI) methodology.

Systemic administration of diclofenac and indomethacin, inhibitors with a preferential selectivity for cyclooxygenase (COX)-1, was associated with an increased formation of kynurenic acid in brain, whereas meloxicam and parecoxib, selective COX-2 inhibitors, decreased brain kynurenic acid formation. Both the elevation and the lowering in brain kynurenic acid levels following administration of the COX inhibitors were effectively prevented by the prostaglandin E_1/E_2 analog misoprostol. Pharmacological manipulation of kynurenic acid synthesis with COX inhibitors thus enabled us to study a role of the compound in the control of firing of midbrain dopamine neurons. An increase in brain kynurenic acid concentration (by 150-300%), induced by indomethacin, increased firing rate and burst firing activity of VTA dopamine neurons whereas a reduction in brain kynurenic acid concentration (by 39-44%) elicited by parecoxib, was associated with a clear-cut reduction in firing activity of these neurons. Thus, endogenous brain concentrations of kynurenic acid appear to be of critical physiological importance for maintaining neuronal activity of VTA dopamine neurons.

In the next series of experiments we investigated the effects of clozapine and haloperidol on VTA dopamine neurons in rats with attenuated NMDA receptor function induced by increased levels of endogenous brain kynurenic acid. Here, clozapine, in contrast to haloperidol, was found to interact with the NMDA receptor complex. We propose a novel mechanism of action of the atypical antipsychotic drug clozapine, i.e. stimulation of the glycine/D-serine site of the NMDA receptor.

Elevated levels of endogenous kynurenic acid induced by systemic administration of kynurenine or PNU 156561A were associated with a disruption in PPI, an effect that could be reversed by antipsychotic drugs. It is proposed that kynurenic acid acts as an endogenous modulator of the PPI response.

Taken together, the results of the present thesis suggest that endogenous kynurenic acid in the brain is involved in the physiological regulation of glutamate neurotransmission. Hereby, this endogenous NMDA receptor antagonist may participate in the pathophysiology of schizophrenia.

LIST OF PUBLICATIONS

I. Schwieler L, Erhardt S, Erhardt C, Engberg G (2005). Prostaglandin-mediated control of rat brain kynurenic acid synthesis – opposite action by COX-1 and COX-2 isoforms. *Journal of Neural Transmission* 112;863-872.

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LIST OF ABBREVIATIONS

AMPA	amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analyses of variance
COX	cyclooxygenase
CNS	central nervous system
EPS	extrapyramidal side effects
GABA	γ-aminobutyric acid
HIV	human immunodeficiency virus
IDO	indoleamine 2,3-dioxygenase
ISH	interspike time interval histogram
KAT	kynurenine aminotransferase
LTP	long term potentiation
mGluR	metabotropic glutamate receptor
NAD	nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NSAID	nonsteroidal anti-inflammatory drugs
PCP	phencyclidine
PET	positron emission tomography
PG	prostaglandin
PPI	prepulse inhibition
TDO	tryptophan 2,3-dioxygenase
VTA	ventral tegmental area

1 INTRODUCTION

1.1 Schizophrenia

Schizophrenia is a serious mental illness which impairs both functioning and personality, and therefore impacts almost every aspect of life. This mental disease, which affects approximately 1% of the world's population (Carpenter and Buchanan, 1994; Jablensky, 1997) strikes people early in life just when they are about to achieve adulthood (Almeida et al., 1995). Even though the outcome is variable and some patients do well, the majority will experience a lifetime of disability and about 10% of the affected individuals will eventually commit suicide (Black and Fisher, 1992; Meltzer, 2002). As a result, schizophrenia is not only associated with substantial emotional burden for the families of those affected but is also a tremendous economic cost to society in terms of healthcare and lost productivity.

There are currently no biological markers for schizophrenia and therefore its diagnosis relies solely upon clinical assessment of each patient's symptoms. However, the clinical presentation of schizophrenia is very complex with numerous different symptoms that vary from patient to patient. To guide clinicians and investigators to survey signs and symptoms in a standardized manner, criterion-based systems for diagnosing have been developed during the past several decades. One of the most widely used is the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-IV). In this criterion-based system symptoms of schizophrenia are divided into three broad clusters: positive symptoms, negative symptoms and cognitive impairment (Andreasen, 1995). The *positive symptoms* (or psychotic symptoms) involve the loss of contact with reality, including false beliefs (delusions), perceptual experience not shared by others (hallucinations), bizarre behavior and disorganized speech. The manifestation of psychotic symptoms is not constant in presence or intensity, but tends to fluctuate over time (Green et al., 2000). The *negative symptoms* reflect diminishment or absence of emotional and behavioral processes. Common negative symptoms are lack of pleasure (anhedonia), withdrawal from social contacts, amotivation, apathy, and reduced quantity or content of speech (alogia). Negative symptoms are associated with poor psychosocial functioning (Tek et al., 2001) and are usually more pervasive and fluctuate less over time than psychotic symptoms (Fenton and McGlashan, 1991). Patients with schizophrenia also suffer from c*ognitive impairments* such as impaired attention, memory and executive functions (e.g. the ability to plan, initiate and regulate goal directed behavior). The majority

of the patients show a reduction in cognitive abilities at the initial onset of psychoses (Saykin et al., 1994).

The complexity of schizophrenia has made the exploration of the underlying cause of the disease extremely difficult. However, data from family and twin studies show that the rates of schizophrenia are higher among relatives of people with the disease than in the general population, which indicates that genetic factors contribute to the disease. Although several putative susceptibility genes for schizophrenia have been identified, the risk conferred by any single gene seems to be small. In addition, the degree of concordance for schizophrenia among monozygotic twins (sharing 100% of the same genes) is approximately 50% (Farmer et al., 1987; Cardno et al., 1999), indicating that epigenetic and environmental factors may also be important for development of the disease. Environmental risks that have been associated with schizophrenia include biological and psychosocial factors such as urban upbringing, social isolation, migration, drug abuse, season of birth, and obstetric complications (Susser, 2002). Furthermore, recent epidemiological studies reveal that an immunologic component may also play a part in the etiology of schizophrenia (Sperner-Unterweger, 2005). For instance, prenatal infections during the second trimester are associated with an increased risk of schizophrenia (see Brown, 2006). This includes infection with influenza virus (Mednick et al., 1988; Cannon et al., 1996; Limosin et al., 2003; Brown et al., 2004), rubella (Brown et al., 2001) poliovirus (Suvisaari et al., 1999) and herpes simplex virus (Buka et al., 2001). It has also been postulated that retroviruses may be important in the etiopathogenesis in schizophrenia since retroviral sequences (HERV-W) have been found in CSF from these patients (Karlsson et al., 2001). These results may indicate that several types of infections may increase the risk of schizophrenia, tentatively through some common immunological mechanism.

1.2 Pharmacological treatment of schizophrenia

The first useful treatment for schizophrenia was discovered by the French suregon Henri Laborit in the beginning of the 1950s. When searching for an antihistaminic that could calm patients before surgery, he found by serendipity a new compound with an exceptional tranquilizing property. Laborit hypothesized that a drug that calmed patients approaching surgery might also be useful for patients with psychiatric symptoms. Acting on this suggestion, John Delay and Pierre Deniker found within a year that the compound, chlorpromazine, had remarkable beneficial effects in patients suffering from psychotic

illnesses (Delay et al., 1952). Chlorpromazine revolutionized psychiatry and in particular it represented a major breakthrough in the treatment of schizophrenia. However, it soon became clear that chlorpromazine and other drugs that followed its introduction, like haloperidol, induced extrapyramidal side effects (EPS) e.g. abnormal involuntary movements. Furthermore, although these drugs were useful against positive symptoms, they were found to have little or no effect against negative and cognitive symptoms (Delay et al., 1952; Seeman and Lee, 1975; Creese et al., 1976; Palao et al., 1994; see King 1998). The mechanism of action of these classical antipsychotic drugs was at this time unknown and it took almost a decade before Carlsson and Lindqvist suggested that haloperidol and chlorpromazine block monoamine receptors (Carlsson and Lindqvist, 1963). A few years later, the clinical efficacy of typical antipsychotics was shown to be due to their ability to block dopamine D_2 receptors in the central nervous system (CNS; Seeman and Lee 1975; Creese et al., 1976). The introduction of positron emission tomography (PET) made visualization and quantification of radiolabelled D_2 receptors possible and the exact receptor occupancy following antipsychotic treatment could be estimated. These studies revealed that, for typical antipsychotics, receptor occupancy in the striatum of at least 70% was required for antipsychotic efficacy while occupancy above 80% potentially increased the risk of EPS (Farde et al., 1988; 1992; Farde and Nordström, 1993; Nordström et al 1993; Kapur et al., 1996).

One of the most effective antipsychotic drugs used today is clozapine, which has a remarkable efficacy in treatment-resistant schizophrenia (Kane et al., 1988; 2001; Pickar et al., 1992). It is classified as an atypical antipsychotic drug due to its very low incidence of EPS associated with its use (Claghorn et al., 1987; Coward et al., 1989). Clozapine has also been shown to be superior in ameliorating both negative and cognitive symptoms, in comparison to classical antipsychotics (Kane et al., 1988; see Baldessarini and Frankenburg, 1991; see Meltzer and McGurk, 1999). Despite these advantages, clinical use of the drug has been limited by a low but significant risk of potentially fatal agranulocytosis (Idänpään-Heikkilä et al., 1977; see Krupp and Barnes, 1992). The receptor pharmacology of clozapine is complex since the compound binds to many receptors in the brain. Clozapine interacts not only with all dopamine receptors (the D_4 subtype showing the highest affinity: 19 nM, Van Tol et al., 1991), but also with other metabotropic receptors e.g. those for serotonin ($5HT_1$ and $5HT_2$, Canton et al., 1990), acetylcholine (Snyder et al., 1974), noradrenaline (see Coward, 1992), and histamine (see Coward, 1992; see Brunello et al., 1995). Various pharmacological explanations have been put forward in an attempt to explain the unique clinical profile of clozapine. These include a relative dopamine D_4

selectivity (Seeman et al., 1998) or a preferential action of clozapine on the mesolimbic dopamine system as compared to the nigrostriatal dopamine system (Andén and Stock, 1973; Bartholini, 1976; Chiodo and Bunney, 1983; 1985; Moghaddam and Bunney, 1990). Moreover, since clozapine is a potent serotonin 5-HT_2-receptor antagonist, it has been suggested that concurrent 5-HT_2 and dopamine D_2 receptor antagonism may contribute to its atypical profile (see Deutch et al., 1991; Meltzer and Nash, 1991; Ichikawa et al., 2001). Another potential explanation for the preferential efficacy of clozapine might be its ability to potentiate glutamatergic neurotransmission. Thus, clozapine has been shown to modulate the response to N-methyl-D-aspartate (NMDA) in frontal cortex slices *in vitro* (Arvanov et al., 1997; Chen et al., 2002, Jardemark et al., 2003) and to prevent phencyclidine (PCP)-induced NMDA receptor hyperactivity *in vivo* (Arvanov and Wang, 1999). Furthermore, studies utilizing microdialysis indicate that the systemic administration of clozapine increases extracellular levels of glutamate (Daly and Moghaddam, 1993; Yamamoto et al., 1994). However, the pharmacology underlying the unique antipsychotic action of clozapine is still obscure.

The superiority of clozapine over classic antipsychotic drugs has stimulated researchers to develop novel compounds with similar therapeutic profiles but without clozapine's severe side effects. This has resulted in a new generation of drugs (e.g. olanzapine, quetiapine, risperidone, sertindole) that are widely used in the treatment of schizophrenia. However, the safety advantage of the atypical drugs has been questioned because of their ability to induce potentially important adverse side effects such as weight gain and altered glucose and lipid metabolism, resulting in increased risk of cardiovascular dysfunction (see Gardner et al., 2005). Furthermore, several studies have shown that the majority of patients discontinue treatment due to inefficacy or intolerable side effects (Lieberman et al., 2005). Although the new generation of antipsychotic drugs represents a step forward, they are far from ideal. Obviously, further research is required in order to develop more effective, safe and well-tolerated drugs for the treatment of schizophrenia.

1.3 The dopamine system of the brain

1.3.1 Dopamine pathways

The dopamine system constitutes a very small fraction of the several billions of neurons in the brain (see Williams and Herrup, 1988; see Prakash and Wurst, 2006). Nevertheless, this system has been shown to be a key modulator in an astonishing array of human behaviors.

This includes physical movement, motivation and control of emotions (e.g. reward and reinforcement), cognitive functions and endocrine regulation.

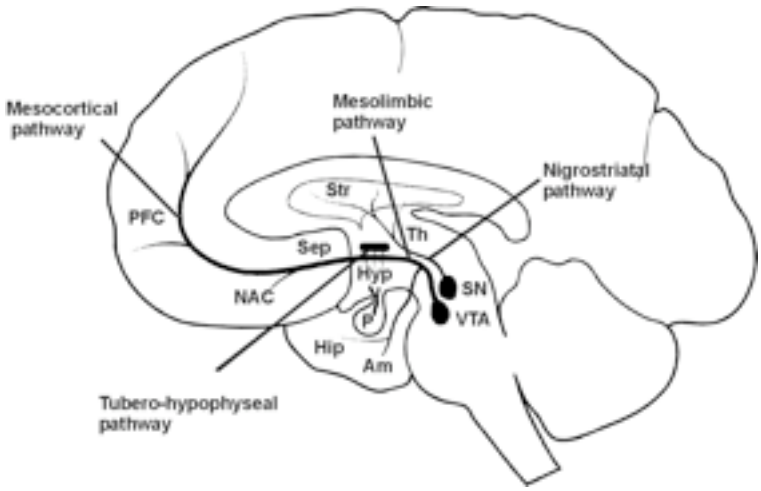

Figure 1. Dopaminergic pathways in the human brain. Abbreviations: Am= amygdala; Hip= hippocampus; Hyp= hypothalamus; NAC= nucleus accumbens; P= pituitary gland; PFC= prefrontal cortex; Sep= septum; SN= substantia nigra; Str=striatum; Th=thalamus; VTA= ventral tegmental area (Modified from Rang et al., 1999)

Extensive research has revealed that several clusters of cells in the midbrain form long nerve fibers. On the basis of their efferent projections, these have been divided into three major dopamine pathways (Fig. 1). The cell bodies of these dopamine pathways are located in the substantia nigra (A9), the ventral tegmental area of Tsai (VTA; A10) and in the arcuate and periventricular hypothalamic nuclei (A12, A14, see also Dahlström and Fuxe, 1964). The dopamine neurons of the substantia nigra primarily innervate the dorsal part of striatum i.e. the caudate and the putamen, comprising the *nigrostriatal* dopamine system. This pathway is important for regulation of movements, and degeneration of these neurons causes Parkinson's disease. In addition, inhibition of this system by antipsychotic drugs is associated with EPS. The efferent projections from the VTA have a more diverse distribution, innervating both cortical and subcortical structures of the brain. Based on the different projections this system is further subdivided into the *mesolimbic* and the *mesocortical* dopamine systems. The mesolimbic pathway projects to several limbic areas of the brain, including the nucleus accumbens, the amygdala, the septal area, and the olfactory tubercle, whereas the mesocortical dopamine system most densely projects to the prefrontal cortex (Dahlström and Fuxe, 1964; Andén et al., 1966; Ungerstedt, 1971; Björklund and Lindvall, 1984). The mesolimbic and mesocortical dopamine systems are crucial for several important behavioral functions such as motivation, control of emotions, and cognition, functions that are frequently disturbed in patients suffering from schizophrenia. Finally, the *tuberohypophysial* dopamine system, which is involved in endocrine control, originates in

the hypothalamus and projects to the pituitary stalk. Inhibition of this system by antipsychotic drugs causes an increased prolactin secretion, which is responsible for side effects such as sexual dysfunction, infertility, gynecomastia, and galactorrhea.

The effects of dopamine are mediated through dopamine receptors that are divided into two main subgroups. These subgroups are classified as D1-like (D1 and D5 receptors) that stimulate adenylyl cyclase, and D2-like (D2, D3, D4 receptors) which inhibit adenylyl cyclase (see Jaber et al., 1996). The dopamine receptors differ in their pharmacological characteristics as well as in their anatomical distribution in the human brain. The D1 and D2-like receptors can also be located both pre- and postsynaptically. Presynaptic D2-like receptors function as autoreceptors that provide an important inhibitory feedback mechanism for dopamine neurons. Thus, activation of these autoreceptors leads to increased potassium conductance that ultimately hyperpolarizes the plasma membrane of dopaminergic neurons, making the cell less excitable and reducing dopamine release (West and Grace, 2002). However, this inhibitory feedback mechanism can be disrupted by agents that block D2 receptors. Blockade of these autoreceptors will thus lead to increased dopamine synthesis and release, which is followed by a compensatory increase in the firing rate of the dopaminergic neurons (see Cooper et al., 2003).

1.3.2 Electrophysiology of midbrain dopamine neurons

The electrophysiological analysis of dopamine neurons started with a paper from 1973 where the physiological, neurochemical, and histochemical characteristics of catecholaminergic neurons were described (Coyle, 1973). Since then, substantial progress has been made in which the electrophysiology of these neurons has been dissected in preparations ranging from *in vitro* recordings of isolated neurons to activity recordings in freely behaving primates. These studies have provided substantial insight into the firing characteristics of dopamine neurons and knowledge about their different firing patterns. Dopamine neurons displays two basic modes of firing: a relatively slow irregular single spike firing mode or, alternatively, a burst firing mode with short interspike intervals where the spike amplitude progressively decreases and the spike duration increases (see Wang, 1981; Grace and Bunney, 1984, 1984a; Clark and Chiodo, 1988). Burst firing has been shown to be of crucial importance for transmitter release. Thus, a change from single spike mode to burst firing mode has been associated with an increased release of dopamine in terminal areas (Gonon, 1988), whereas extermination of burst firing will reduce the

dopamine terminal efflux (Nissbrandt et al., 1994). The functional relevance of burst firing is not fully understood but studies have shown a correlation between burst firing and specific behaviors. For instance, increased burst firing of dopamine neurons has been associated with the presentation of a novel visual or auditory stimulus to rats (Freeman et al., 1985). Furthermore, experiments with monkeys show that short phasic alternation in firing of dopamine neurons is coupled to reward-related tasks (Schultz et al., 1993; Schultz, 1998; Schultz et al., 2000). In freely moving rats it has been shown that over 90% of VTA dopamine neurons exhibit burst firing (Freeman and Bunney, 1987). However, sensory input is not a determinant factor for burst firing of dopamine neurons, since 50 to 73% of midbrain dopamine neurons emit bursts in anesthetized rats (Grace and Bunney, 1984a; Grenhoff et al., 1988; Clark and Chiodo, 1988). This indicates that the bursting mechanism is intact also when sensory input is limited (e.g. under anesthesia).

1.3.3 *Afferent regulation of dopamine neurons in Ventral Tegmental Area*

Spontaneous burst firing does not occur in brain slice preparations, where most sources of organized synaptic input are lacking. It is therefore generally believed that the firing pattern of dopamine neurons is driven by afferent inputs (Sanghera et al., 1984; Grace and Onn, 1989; Seutin et al., 1990; Johnson et al., 1992). Several different afferents project to the VTA dopamine neurons, and they utilize a variety of neurotransmitters, including glutamate, γ-aminobutyric acid (GABA), serotonin, acetylcholine, and noradrenalin. Inhibitory GABAergic and stimulatory glutamatergic inputs to the VTA seem to be of major importance for the regulation of dopaminergic VTA neurons. Thus, in the VTA approximately 15 to 20% of the total neuronal population is the intrinsic GABAergic interneurons (Beart and McDonald, 1980; see Kalivas et al., 1993; Van Bockstaele and Pickle, 1995). Also extrinsic GABAergic sources, including neurons that project from areas such as nucleus accumbens and frontal cortex have been demonstrated (Walaas and Fonnum, 1980; Yim and Mogenson, 1980; see Kalivas et al., 1993). The intrinsic GABAergic neurons of the VTA not only synapse to dopamine neurons in the VTA (O'Brien and White, 1987; Bayer and Pickel, 1991) but also send afferent fibers to the prefrontal cortex and the nucleus accumbens (Fig. 2, see Kalivas et al., 1993; Carr and Sesack, 2000). The influence of GABA on dopamineric neurons is exerted via both $GABA_A$ and $GABA_B$ receptors. *In vivo* electrophysiological studies have shown that stimulation of $GABA_B$ receptors by baclofen is associated with a reduction of midbrain dopamine cell firing (Erhardt et al., 1998; 2002). In accordance, local application of

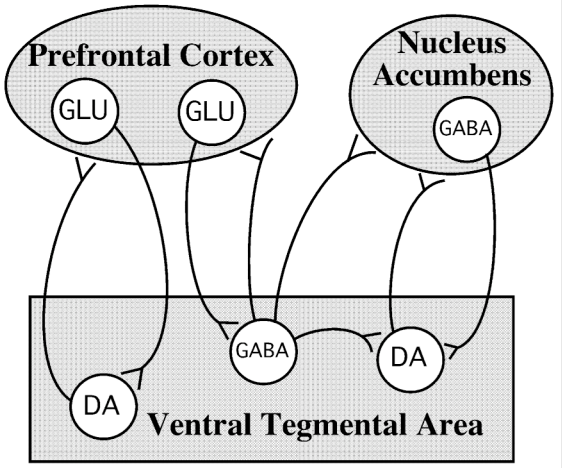

Figure 2. Schematic drawing of the connections between the prefrontal cortex, nucleus accumbens and the VTA. DA=dopamine, GLU=glutamate.

baclofen into the VTA induces a cessation of dopaminergic firing activity (see Lacey, 1993), which results in a decreased release of dopamine (Klitenick et al., 1992; Yoshida et al., 1994; Westernick et al., 1996; 1998; Xi and Stein, 1999; Giorgetti et al., 2002). Furthermore, systemic administration of a selective $GABA_B$ receptor antagonist (SCH 50911) has been shown to stimulate dopamine cell firing (Erhardt et al., 1999). These results are in concordance with a recent study where local administration of a $GABA_B$ receptor antagonist increased local dopamine release (Giorgetti et al., 2002), suggesting a tonic inhibitory control of VTA dopamine neurons by these receptors.

With regard to the $GABA_A$ receptors, several *in vivo* electrophysiological studies have shown that agonists acting at these receptors induce a paradoxical increase of firing activity of midbrain dopamine neurons (MacNeil et al., 1978; Grace and Bunney, 1979; Waszczak and Walters, 1980; Erhardt and Engberg, 2002). The excitatory effect of $GABA_A$ agonists has been suggested to be due to a disinhibition of dopamine neurons (Grace and Bunney, 1979; Waszczak et al., 1980). However, a recent study proposed that the excitatory action of $GABA_A$ antagonists on midbrain dopamine neurons is mediated via release of the excitatory transmitter glutamate (Erhardt and Engberg, 2000).

The major excitatory input to the VTA stems from glutamatergic innervations from prefrontal cortex (Carr and Sesack, 2000) and the pedunculopontine nucleus (Charara et al., 1996). These glutamatergic afferents have been found to be of critical importance for the induction of burst firing activity. 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 from prefrontal cortex establish contacts with GABA interneurons and with GABAergic cells that project to the nucleus accumbens. Furthermore, prefrontal cortex afferents also synapse with dopaminergic cells in VTA that project back to prefrontal cortex (Carr and Sesack, 2000a). Prefrontal cortex may thus affect the activity of VTA dopamine neurons both directly, by the monosynaptic projections onto dopamine neurons in VTA, and indirectly, by influencing GABAergic interneurons that synapse to dopamine neurons that in turn

project to the nucleus accumbens (Fig. 2). The excitatory effects of glutamate can be mediated by both ionotropic and metabotropic glutamate receptors (see section 1.4). However, during the past few years, extensive research has been focusing on the significance of the ionotropic glutamate NMDA receptor for regulation of dopamine neurons. Stimulation of these receptors has been shown to profoundly induce burst firing of midbrain dopamine neurons (Johnson et al., 1992; Chergui et al., 1993). Furthermore, the systemic administration of non-competitive NMDA receptor antagonist, e.g. MK-801 or PCP, is associated with an increased burst firing of midbrain dopamine neurons (French et al., 1993; Murase et al., 1993a; French, 1994) as well as increased release of dopamine (Schmidt et al., 1996; Yan et al., 1997; Kretschmer, 1999). This paradoxical activation has been suggested to be due to a disinhibition of dopamine neurons via GABAergic interneurons (see Adell and Artigas, 2004) since systemic administration of PCP and MK-801 inhibits the activity of these neurons (Zhang et al., 1993).

In summary, burst firing activity of midbrain dopamine neurons, seems to be regulated by both excitatory glutamate- and inhibitory GABA-containing afferents where concomitant $GABA_B$- and NMDA receptor stimulation provides important contribution for a particularly effective regulation of firing *in vivo*.

1.3.4 The dopamine hypothesis of schizophrenia

The dopamine hypothesis of schizophrenia has for several decades been the cornerstone of schizophrenia research. As first formulated, this hypothesis proposed that a hyperactivity of the dopamine system was responsible for the psychotic symptoms displayed by these patients (Carlsson and Lindqvist, 1963). This idea originated from the findings that the effect of antipsychotic drugs correlated with the degree of D_2 receptor occupancy (Seeman and Lee, 1975; Creese et al., 1976) and that dopamine-releasing drugs can induce a state of psychosis (Snyder 1973; see Angrist and van Kammen, 1984). This indirect pharmacological evidence has recently found further support in studies where new imaging techniques have been used (see Laruelle et al., 2005). Thus, studies have reported that dopamine synthesis in striatum is increased when patients are experiencing psychotic symptoms (Reith et al., 1994; Lindström et al., 1999) and that the synaptic level of dopamine at baseline is higher in patients experiencing their first episode of schizophrenia and during illness exacerbation (Abi-Dargham et al., 2000). It has also been shown that amphetamine-induced release of dopamine is increased in schizophrenic patients compared with healthy controls (Laruelle et al., 1996; Breier et al., 1997; Abi-Dargham et al., 1998).

Upon closer examination, the simplest form of the dopamine hypothesis has proven to have shortcomings and cannot fully account for the pathophysiology of this complex disorder. More recently, increased attention focused on the negative and cognitive symptoms in schizophrenia and their resistance to D_2 receptor antagonism (King, 1998; Breier, 1999) has led to a reformulation of the classical dopamine hypothesis. These symptoms are suggested to arise from a deficit in dopamine transmission at D_1 receptors in the prefrontal cortex (see Davis et al., 1991). Thus, both hypo and hyperfunctioning of brain dopamine systems might occur simultaneously, although in different brain regions. The positive symptoms of schizophrenia may in this regard be due to an excess of subcortical dopamine functions involving D_2 receptors whereas the negative and cognitive symptoms are likely related to a deficit of dopamine in prefrontal cortex. However, the mechanism(s) by which such a dopamine imbalance might emerge in the brain of patients with schizophrenia remains unknown.

1.4 The glutamatergic system

Glutamate is one of the ordinary 20 amino acids that take parts in typical metabolic functions like energy production and protein synthesis. It was therefore hard to believe that a compound with so many functions and present virtually everywhere in the body could play a critical role as neurotransmitter. Today it is widely acknowledged that glutamate is one of the primary excitatory neurotransmitters in the mammalian brain. Glutamate is involved in most aspects of normal brain function including cognition, memory and learning. However, massive activation of glutamate receptors may result in cell dysfunction and even cell death. From this it follows that glutamate is essential for brain functioning but at the same time also highly neurotoxic and must therefore be strictly controlled. In the brain, astrocytes, which surround glutamatergic neurons, are thought to be of major importance for regulating the extracellular concentration of glutamate. Thus, astrocytes are capable of taking up glutamate and storing it as glutamine, which then cycles back to the nerve terminals where it can be converted back to glutamate (see Danbolt, 2001). This process is referred to as the glutamate-glutamine cycle, and is important because it allows glutamate to be inactivated by astrocytes and transported back to neurons in an inactive (non-toxic) form.

The receptors that mediate the effects of glutamate are divided into two broad families named metabotropic and ionotropic receptors. The metabotropic glutamate receptors (mGluR) are linked to G proteins and operate through a second messenger

system. To date, eight receptors from this family have been cloned (mGluR1 through mGluR8); they are divided into three groups based on second messenger coupling and ligand sensitivity. The ionotropic receptors, which are ligand-gated ion channel receptors, are differentiated into three groups originally named after reasonably selective agonists: amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and NMDA. The AMPA receptor is widely expressed in the brain and appears to serve as synaptic receptor for fast excitatory synaptic transmission mediated by glutamate. AMPA receptors are composed of a combination of GluR1-4 subunits and work often in concert with NMDA receptors. The kainate receptor is comprised of combinations of GluR5–7 and KA1–2 subunits and has a much more restricted distribution in the brain compared to AMPA and NMDA receptors. The endogenous agonists for all glutamate receptors are generally believed to be glutamate and/or aspartate, but other alternatives such as cysteic and homocysteic acid have also been proposed.

1.4.1 NMDA receptors

The NMDA receptor, which is widely distributed throughout the entire brain, is comprised of seven subunits, NR1, NR2 A-D and NR3 A-B, which are all products of separate genes. Electrophysiological studies have demonstrated that the minimal requirement for a functional NMDA receptor is likely to be a tetramer composed of two NR1 (incorporating a strychnine-insensitive glycine-binding site) and two NR2 subunits (incorporating the glutamate-recognition site). In some receptors an NR3 subunit can substitute one of the NR2 subunits. Thus, the NMDA receptor is a quite complex receptor, which has several distinct binding-sites for endogenous ligands (Fig. 3). A unique property of the NMDA receptor is that opening of the channel requires that both glutamate and glycine act in concert for receptor activation. In addition, recent studies strongly support a role for D-serine as an endogenous co-agonist at the glycine site of the

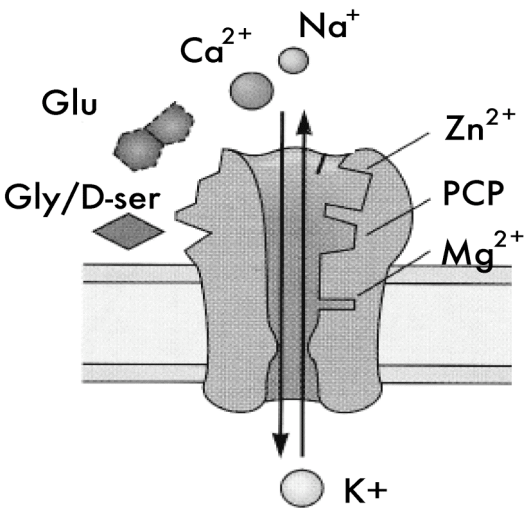

Figure 3. Schematic drawing of the NMDA receptor. Abbreviations: Glu=glutamate; Gly/D-ser= Glycine/D-serine; Mg2+=magnesium; Zn= zinc; PCP= phencyclidin.

NMDA receptor *in vivo* (Snyder et al., 2000; Mothetet al., 2000). It has been claimed that the glycine/D-serine site might be constantly occupied and therefore physiologically silent (Obrenovitch et al., 1997). However, this view has been reconsidered since several studies have demonstrated that this site is not saturated *in vivo* (see Danysz and Parsons, 1998). The availability of glycine and D-serine is thus of critical importance for optimal NMDA receptor functioning. Activation of the NMDA receptor is not only dependent on ligand stimulation but is also restricted by a voltage-dependent Mg^{2+}-block. This means that NMDA receptors are activated only after depolarization of the cell membrane, e.g. by AMPA receptor activation, which relieves the Mg^{2+}-blockade. The ion channel of the NMDA receptor is permeable to both K^+ and Na^+ but has a particular high permeability for Ca^{2+}, which acts as a second messenger to activate intracellular signaling cascades. Thus, increased Ca^{2+} influx due to high glutamatergic activity on NMDA receptors results in a permanent increase in synaptic efficacy known as long term potentiation (LTP, see Lynch, 2004), a process associated with learning and memory. However, excessive Ca^{2+} influx due to activation of extrasynaptic NMDA receptors causes neural injury and ultimately death through a pathologic process known as excitotoxcity (see Arundine et al., 2003).

1.4.2 The glutamate deficiency theory of schizophrenia

In 1980 Kim and colleagues reported that schizophrenic patients had low levels of glutamate in the CSF (Kim et al., 1980). Based on their findings they introduced the hypothesis that there is a dysfunction of glutamatergic neurons in schizophrenia. This new theory was not well received since the findings could not be replicated in subsequent studies, and because the knowledge of the glutamatergic system was to limited at this time. However, since then evidence has been accumulating which supports the idea that schizophrenia might be associated with a persistent dysfunction of glutamate transmission involving NMDA receptors. The strongest line of evidence for a NMDA receptor hypofunction in schizophrenia comes from clinical observations that NMDA receptor antagonists, e.g. PCP (angel dust) or ketamine, have psychotomimetic properties (Luby et al., 1959; Itil et al., 1967; Adler et al., 1999). Thus, these drugs (collectively known as "dissociative anesthetics") induce both positive and negative symptoms in healthy subjects and schizophrenic patients. Furthermore, untreated patients with schizophrenia show greater sensitivity than normal individuals to the psychotomimetic properties (Lahti et al., 2001), which may indicate that these drugs affect a system that is already vulnerable in

patients with schizophrenia. Both PCP and ketamine antagonize the NMDA receptor non-competitively by binding to a site within the NMDA receptor ion channel (Fig. 3). However, different compounds that antagonize other ligand-binding sites of the NMDA receptor, such as the glycine site or the glutamate recognition site, also have psychotomimetic properties (Kristense et al., 1992; Grotta et al., 1995; Yenari et al., 1998; Albers et al., 1999). The mechanism by which NMDA receptor antagonists induce schizophrenia-like symptoms is not well understood. However, a growing body of animal and human studies suggests that NMDA receptor hypofunction might be related to the dopaminergic imbalance suggested to account for schizophrenia symptoms (see section 1.3.4). Thus, electrophysiological studies have shown that systemic administration of NMDA receptor antagonists is associated with increased firing rate and burst firing activity of midbrain dopamine neurons (French et al., 1993; Murase et al., 1993a; French, 1994; Erhardt et al., 2001; Erhardt and Engberg, 2002) as well as an enhancement of amphetamine-induced dopamine release in striatum (Miller and Abercrombie, 1996; Kegeles et al., 2000; see Laruelle et al., 2005). Furthermore, subchronic administration of PCP to rats has been found not only to increase dopamine release in mesolimbic areas (Jentsch et al., 1998; see Jentsch and Roth 1999) but also to reduce dopamine release in the prefrontal cortex (Jentsch et al., 1997; 1998a, b). Notably, chronic recreational ketamine users display a selective up-regulation of D_1 receptors in the dorsolateral prefrontal cortex (Narendran et al., 2005). Since dopamine transmission at D_1 receptors has been shown to be important for optimal prefrontal cortex performance (see Goldman-Rakic et al., 2000) the up-regulation of these receptors is suggested to be secondary to a drug-induced deficit in prefrontal dopamine functioning.

Altogether, mounting evidence suggests that an altered dopamine function in schizophrenia may be a consequence of a NMDA receptor hypofunction. However, at present there is no generally accepted explanation for how as NMDA dysfunction in schizophrenia may arise.

1.5 Kynurenic acid

The amino acid tryptophan was discovered in Cambridge, England by Hopkins and Cole in 1901. Only three years later Ellinger was able to isolate one of its major metabolites, kynurenic acid, from the urine of two cocker spaniel dogs that had been on a strict tryptophan diet (Ellinger, 1904). Kynurenic acid was one of the first tryptophan metabolites to be reported. A substantial body of research has since then revealed that the

generation of kynurenic acid from tryptophan involves a cascade of enzymatic steps, which together have been named the "kynurenine pathway" after the key compound kynurenine. Several metabolites of this pathway, including kynurenic acid, have later been shown to be involved in many diverse physiological and pathological processes.

1.5.1 The kynurenine pathway

In mammals, most of the tryptophan ingested is metabolized via the kynurenine pathway (major enzymes are illustrated in Fig. 4) leading to either the biosynthesis of nicotinamide adenine dinucleotide (NAD) or a complete oxidation of the amino acid (see Hayaishi, 1993). It is well known that more than 95% of tryptophan from food is metabolized along this pathway, whereas only a tiny amount of dietary trypto-phan is converted to the well-known neurotransmitter serotonin (Wolf, 1974; Peters, 1991). The kynurenine pathway is initiated by the oxidative opening of the indole ring of tryptophan by indoleamine 2,3-dioxygenase (IDO, Hayaishi, 1976) or the more specific enzyme tryptophan 2,3-dioxygenase (TDO, most active in the liver, Hayaishi et al., 1957). This enzymatic step converts tryptophan to formyl kynurenine, which is rapidly and almost completely degraded to L-kynurenine by kynurenine formylase (Mehler and Knox, 1950; Gál and Scherman, 1978). L-kynurenine in turn serves as a substrate of several distinct enzymes: **I)** kynureninase (yields anthranilic acid), **II)** kynurenine 3-hydroxylase (yields 3-hydroxykynurenine), and **III)** kynurenine aminotransferase (KAT; yields kynurenic acid, see Moroni, 1999). Since kynurenine 3-

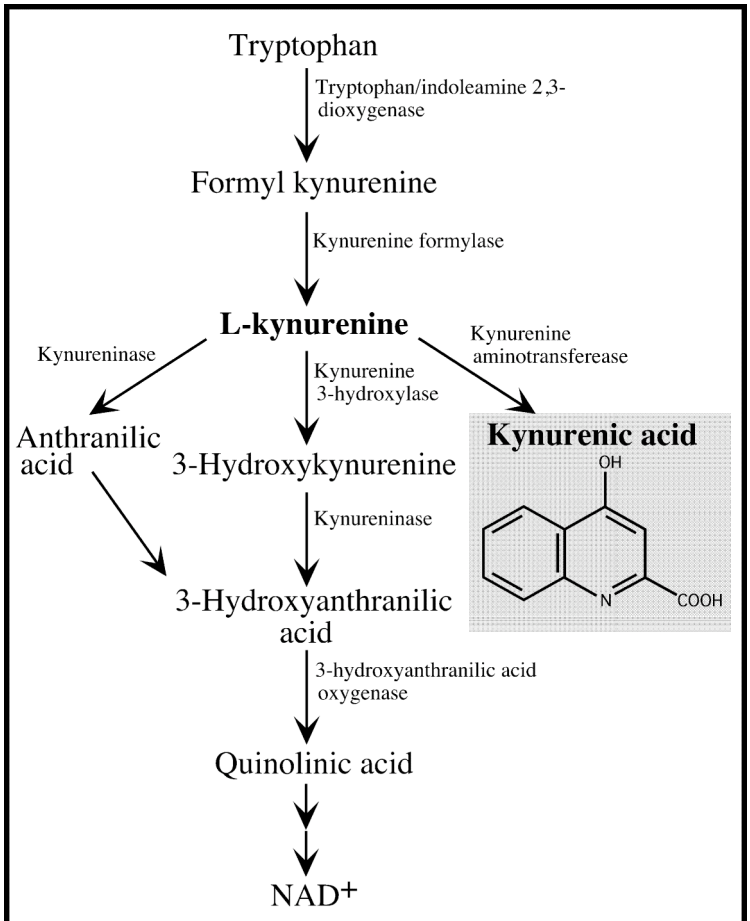

Figure 4. The kynurenine pathway

hydroxylase has the highest affinity for kynurenine it is suggested that this enzyme under normal conditions metabolizes most of the available kynurenine (Bender and McCreanor, 1982; see Moroni 1999). Both anthranilic acid and 3-hydroxykynurenine can be metabolized to 3-hydroxyanthranilic acid that subsequently can be converted to the neurotoxic compound quinolinic acid (Stone and Perkins 1981). Finally, quinolinic acid is then metabolized through several steps with adenosine triphosphate or NAD as end products (see Hayaishi, 1993).

All enzymes involved in the peripheral degradation of tryptophan to kynurenic acid were already known and well characterized in the early 1970s. However, the cellular localization of the enzymes of the kynurenine pathway in the brain was only investigated once the neurobiological significance of the metabolites from this pathway was recognized. Thus, all enzymes of the catabolic cascade are present in the brain (Swartz et al., 1990; Guidetti et al., 1995) but their cerebral activity is much lower than in the peripheral organs (see Stone, 1993). Almost all of the enzymes in brain have been confirmed to be identical with those in peripheral organs except for the enzymes that yield kynurenic acid. Compared to peripheral organs, which express several enzymes for the metabolism of kynurenic acid, only two such enzymes appear to exist in the brain; these are termed KAT I and KAT II (Okuno et al., 1991; Buchli et al., 1995; Guidetti et al., 1997). In the brain KAT II has been found to be responsible for most of the *de novo* formation of kynurenic acid (Guidetti et al., 1997). This makes sense since KAT II operates best at physiological pH and preferentially recognizes L-kynurenine as a substrate whereas KAT I has a much higher pH optimum (9.9 to 10) and shows relatively little substrate specificity (Schmidt et al., 1993). Recently, a third member of the KAT family was found in mammals and is suggested to belong to the same subfamily as KAT I (Yu et al., 2006). KAT III shares several features with KAT I including similar genomic structure and expression in multiple tissues such as brain, kidney, liver, heart, and lung. The KAT III enzyme has been shown to be up-regulated in KAT II knock-out mice but further studies are needed to clarify its biological functions in mammals.

The cerebral metabolism of kynurenic acid is driven mainly by blood-borne L-kynurenine, which easily enters the brain via the large neutral amino acid transporter (Fukui et al., 1991). In contrast, peripheral sources of kynurenic acid do not significantly contribute to the cerebral contents of the compound since kynurenic acid, due to its polar structure, is almost completely unable to pass the blood brain barrier under normal physiological conditions (Fukui et al., 1991; see Scharfman et al., 2000). L-kynurenine that reaches the brain is rapidly taken up by astrocytes and microglia. A small amount of L-

kynurenine is also actively transported into neurons by a Na^+ dependent mechanism (Speciale and Schwarcz, 1990). The L-kynurenine degradation process is functionally different in astrocytes and microglia. Thus, astrocytes do not appear to contain kynurenine 3-hydroxylase and therefore favor synthesis of kynurenic acid (Guillemine et al., 2001; Kiss et al., 2003), whereas microglia cells harbor very little KAT activity and preferentially form metabolites of the quinolinic acid branch of the pathway (Guillemine et al., 2001; Lehrman et al., 2001). In fact, astrocytes have been found to be more capable of catabolizing quinolinic acid than producing it (Guillemine et al., 2001). Thus, astrocytes have been suggested to protect neurons by degrading quinolinic acid and by producing kynurenic acid that antagonizes the neurotoxic effect induced by quinolinic acid. Accumulated kynurenic acid in the astrocytes is readily liberated into the extracellular milieu (Swartz et al., 1990; Gramsbergen et al., 1997). So far, no catabolic enzyme or re-uptake mechanism for kynurenic acid has been detected in glial cells or neurons. Thus, the only known mechanism for clearing it from the extracellular space is the probenecid-sensitive carrier system that transports kynurenic acid out of the brain (Moroni et al., 1988). In accordance, inhibition of this system by probenecid is associated with a substantial elevation of brain kynurenic acid concentration in rats (Miller et al., 1992).

1.5.2 Regulation of kynurenic acid synthesis

The formation of kynurenic acid is preferentially determined by the intracellular concentration of L-kynurenine (see Schwarcz and Pellicciari, 2002). Thus, systemic injections of kynurenine results in elevated levels of brain kynurenic acid in both rats (Swartz et al., 1990; Wu et al., 1992) and primates (Jauch et al., 1993). Furthermore, the generation of kynurenic acid is critically influenced by additional modulatory factors. For example, in the brain, the extracellular concentration of kynurenic acid is decreased by depolarizing agents like K^+ or veratridine (Gramsbergen et al., 1991; Wu et al., 1992). A reduction of kynurenic acid is also seen during compromised cellular energy metabolism (hypoglycemia, Gramsbergen et al., 1997) an effect probably related to inhibition of the uptake of kynurenine into astrocytes (Hodgkins and Schwarcz, 1998). This effect is reversed by administration of e.g. pyruvate, which stimulates cellular energy metabolism (Hodgkins and Schwarcz, 1998). In addition, pyruvate and 2-oxoglutarate serve as co-substrates for KAT and subsequently stimulate the production of kynurenic acid (Hodgkins et al., 1999). Furthermore, the activity of KAT is probably influenced by the intracellular concentration of amino acids, such as glutamine and phenylalanine, which are

competitive substrates of KAT I, and KAT II (Urbanska et al., 1997; Battaglia et al., 2000). Taken together, all these regulating mechanisms indicate that intricate machinery has evolved to regulate the extracellular levels of kynurenic acid.

1.5.3 Mechanism of action and physiological significance

More than twenty years ago Perkins and Stone (1982) described kynurenic acid as a neuroinhibitory compound that affects NMDA receptors. Subsequent studies have revealed that kynurenic acid in low concentrations acts as a non-competitive antagonist of the NMDA receptor by binding to the strychnine-insensitive glycine recognition site (IC_{50}~8-15 μM; Ganong and Cotman, 1986; Birch et al., 1988; Kessler et al., 1989; Parsons et al., 1997). At higher concentration the compound is able to block the agonist recognition site of all ionotropic glutamate receptors (NMDA, IC_{50} ~200-500 μM; AMPA/Kainate, IC_{50} mM; see Stone 1993). A recent study showed that low concentrations of kynurenic acid also block α7* nicotinic receptors (IC_{50}~7 μM; Hilmas et al., 2001).

The physiological role of kynurenic acid in brain has long been a matter of controversy since whole brain kynurenic acid concentrations in rodents are far below those required to affect NMDA receptors, a fact shown by *in vitro* studies (Stone, 1993; Parsons et al., 1997; Urenjak and Obrenovitch, 2000). Research over the past decade, however, has provided mounting evidence that moderately increased levels of brain kynurenic acid are sufficient to inhibit excitatory synaptic function. In fact, elevation of brain kynurenic acid concentration is associated with a number of physiological effects, such as sensory perception, control of seizures, and prevention of ischemic or excitotoxic neural degeneration (Hajos and Engberg, 1990, 1990a; Nozaki and Beal, 1992; Carpenedo et al., 1994; Wu et al., 2000). Furthermore, it has been found that when the levels of endogenous kynurenic acid in the brain are elevated pharmacologically, the result is a pronounced excitation of midbrain dopamine neurons (Erhardt et al., 2001, 2003; Erhardt and Engberg, 2002), an effect that mimics the action of other NMDA receptor antagonists, e.g. phencyclidine (French et al., 1993; French, 1994) and MK-801 (Zhang et al., 1992). Altogether, these studies show that a moderate increase of endogenous brain kynurenic acid is sufficient to reduce the activity of excitatory synapses in spite of the low concentration of the compound in rat whole-brain. One possible explanation for this discrepancy could be that kynurenic acid is synthesized in and released from astrocytes (Guillemin et al., 2001; Kiss et al., 2003), which are known to communicate dynamically

with glutamatergic neurons via intimate synaptic junctions (Coyle and Schwarcz, 2000; Newman, 2003; Hertz and Zielke, 2004; Araque and Perea, 2004). Thus, the concentration of kynurenic acid within the synaptic cleft is likely to be substantially higher than is inferred from the total concentration in homogenates of brain tissue.

1.5.4 The kynurenine pathway is activated by immune stimulation

In the late 1970s Hayaishi and co-workers discovered that immune stimulating agents such as influenza virus induced *de novo* synthesis of IDO (Hayaishi and Yoshida, 1978), the first regulatory step of the kynurenine pathway (Hayaishi, 1976). Subsequently it was found that interferon-γ and other pro-inflammatory cytokines induce tryptophan breakdown via the kynurenine pathway (Yoshida et al., 1986, Carlin et al., 1987). The biological significance of this response to immune stimulation is not fully understood. However, it is suggested to be a biostatic defense mechanism for reducing the local supply of tryptophan needed for replication of intracellular pathogens (Pfefferkorn, 1984; see Moffet and Namboodiri, 2003). Furthermore, kynurenine metabolites have been found to possess remarkable immunomodulatory properties. Thus, several metabolites of the quinolinic branch of the kynurenine pathway suppress the proliferation of activated T cells, hereby affecting the organism's tolerance to non-harmful antigens (see Schwarcz, 2004; see Mellor, 2005). The immune-activated metabolism of tryptophan has been shown to accelerate the entire enzymatic cascade of the kynurenine pathway. In line with this, interferon-γ induces increased expression of KAT I and KAT II and this increase correlates with an increased synthesis and release of kynurenic acid by astrocytes (Guillemine et al., 2001). One example of a disease with sustained immunoactivation and subsequently increased IDO expression is human immunodeficiency virus (HIV). Recent studies have revealed an increased accumulation of kynurenic acid in cerebrospinal fluid (CSF, Atlas et al., 2006) and brain tissue from these patients (Baran et al., 2000) as well as increased activity of both KAT I and KAT II (Baran et al., 2000). In addition, elevated kynurenic acid metabolism has been observed in diseases such as chronic inflammatory bowel disease (Forrest et al., 2002), Alzheimer's disease (Baran et al., 1999), and schizophrenia (Erhardt et al., 2001a; Schwarcz et al., 2001; Nilsson et al., 2005).

1.6 Cyclooxygenases and the central nervous system

Cyclooxygenase (COX) is the key enzyme in the conversion of arachidonic acid to prostaglandins (PGs). Three isoforms of COX have been identified, all of which are present in the brain (see Smith et al., 2000; Chandrasekharan et al., 2002). The first of the COX isoforms to be isolated, named COX-1, is constitutively expressed in most tissues and responsible for the production of PGs with general "housekeeping" functions, whereas COX-2 is an inducible enzyme responsible for the synthesis of PGs during inflammation. In the brain, however, both isoforms appears to have constitutive functions (Yamagata et al., 1993). A third form, COX-3, has recently been described and is a product of the COX-1 gene (Chandrasekharan et al., 2002). Of the three isoforms, COX-3 is the one most expressed in cerebral cortex but the functional role of this isoform is still relatively unknown. The synthesis of PG by COX is interrupted by agents collectively known as nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs are historically divided into two groups; **I)** classical NSAIDs (introduced before 1995) inhibit both COX-1 and -2, but in general bind more tightly to COX-1, and **II)** the newly developed selective COX-2 inhibitors (see Smith et al., 2000). NSAIDs are frequently used to reduce inflammation, pain, and fever. Furthermore, long-term use of these drugs has been found to effectively reduce the risk of fatal thrombotic events, as well as development of Alzheimer's disease (see Kaufmann et al., 1997).

Experiments with selective COX inhibitors (see DeWitt, 1999), as well as studies of knockout mice that lack either COX-1 or -2 (see Langenbach et al., 1999) have established that each COX isoform in brain is responsible for its own particular physiological functions. Furthermore, synthesis of prostaglandins via COX-2 has been observed in cells that also express COX-1 (Shinohara et al., 1999; see Smith et al., 2000), indicating that the activity of COX-1 and -2 are segregated within cells. Factors that have been suggested to permit the two COX isoforms to signal independently are their differential intracellular localization (Morita et al., 1995; see Kaufmann et al., 1997) and expression pattern (Smith et al., 1996). In addition, different kinetic properties permit COX-2 to compete more effectively for newly released arachidonic acid when the both isoforms are expressed in the same cell (Swinney et al., 1997; Chen et al., 1999). The potential role of the different COX isoforms in brain has been extensively studied in recent years and these studies have revealed that COX is involved in both normal and abnormal nerve activity. Thus, COX-2 has been suggested to affect NMDA receptor dependent synaptic activity such as neural plasticity and memory consolidation (see

Minghetti, 2004). Furthermore, COX-2 is rapidly up-regulated after seizures or ischemia and growing evidence indicates that this enzyme mediates parts of the glutamate neurotoxicity following activation of NMDA receptors (Planas et al., 1995; Nogawa et al., 1997; Nakayama et al., 1998). Previous studies also support a link between COX activity and the dopamine system. Thus, COX-1 inhibitors antagonize catalepsy induced by dopamine receptor antagonists in rodents (Ono et al., 1992; Naidu and Kulkarni, 2002; Ross et al., 2002) and, in addition, the NSAID indomethacin is shown to potentiate the stimulatory action of morphine on dopaminergic neuronal activity (Melis et al., 2000).

1.7 The kynurenic acid hypothesis of schizophrenia

Multiple lines of clinical and experimental evidence indicate that the core symptoms of schizophrenia result from hypofunction of the NMDA receptor (see section 1.4.3). However, there is at present no generally accepted explanation for how this NMDA dysfunction may arise. Several findings indicate, though, that kynurenic acid may be of pathophysiological significance for the induction of schizophrenic symptoms. Thus, patients with schizophrenia display elevated levels of kynurenic acid in the CSF (Erhardt et al., 2001a, Nilsson et al., 2005) and in the prefrontal cortex postmortem (Schwarcz et al., 2001). Furthermore, it was recently shown that the expression of TDO, which is the first regulatory step in the kynurenine pathway (Hayaishi, 1976), is increased in the postmortem prefrontal cortex from patients with schizophrenia (Miller et al., 2004). At low concentrations kynurenic acid preferentially blocks the glycine/D-serine site of the NMDA receptor (Ganong and Cotman, 1986; Birch et al., 1988; Kessler et al., 1989; Parsons et al., 1997). Interestingly, other compounds that block this site have been shown to induce schizophrenia-like symptoms in humans (Albers et al., 1999). Moreover, clinical studies in patients with schizophrenia have also revealed that glycine or D-serine, which stimulate the glycine/D-serine site of the NMDA receptor, ameliorate negative symptoms when added to conventional antipsychotics (see Touminen et al., 2005), findings pointing towards a dysfunction of the NMDA receptor in this disease.

According to several *in vivo* electrophysiological studies, kynurenic acid might have physiological significance in the brain. Thus, a moderate (4 fold) increase of whole brain kynurenic acid is associated with a marked activation of rat midbrain dopamine neurons, including increased firing rate and burst firing activity (Erhardt et al., 2001, 2003; Erhardt and Engberg, 2002). The actions of elevated levels of kynurenic acid on midbrain dopamine neurons show in this regard striking similarities to the actions of systemic

administration of other NMDA receptor antagonists, e.g. PCP (French et al., 1993; French, 1994) and MK-801 (Zhang et al., 1992) on these neurons. Elevated levels of kynurenic acid might thus be responsible for the dysfunctioning of the dopamine system that has been suggested to elicit the symptoms of schizophrenia.

2 SPECIFIC AIMS OF THE STUDY

1. To study a prostaglandin-mediated control of kynurenic acid synthesis in rat brain.

2. To examine the physiological significance of endogenous brain kynurenic acid for neuronal activity of dopamine neurons in ventral tegmental area.

3. To study whether the antipsychotic drugs clozapine and haloperidol affect the neuronal activity of dopamine neurons in the ventral tegmental area by interfering with glutamatergic mechanisms.

4. To examine behavioral effects of elevated brain levels of kynurenic acid in rats.

3 MATERIALS AND METHODS

3.1 Animals

Experiments were performed on male Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden). The animals were housed in groups of five or four, and were given free access to food (R34 rat chow) and water. Environmental conditions were checked daily. The rats were maintained under constant temperature (25°C), and 40-60% humidity in a room with a regulated 12 h light/dark cycle. For electrophysiological (paper II-IV) and biochemical (paper I) experiments the rats were housed in a daily cycle of lights on at 06.00 PM. For behavioral experiments (paper V) the rats were housed in a room with regulated, reversed 12-h light/dark cycle with lights off at 07.00 AM and lights on at 07.00 PM. The rats were kept in their respective light/dark conditions for two weeks prior to experiments to adjust their diurnal rhythm. In the behavioral experiments, rats were handled once daily for the 2 weeks preceding the experiments to reduce any subsequent handling stress. The rats in paper I-IV weighed 200-330 g on the day of the experiments whereas the rats in paper V weighed 250-500 g. Experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm, Sweden, and every effort was made to minimize the number of animals used and their suffering.

3.2 Drugs

Cyclooxygenase inhibitors

Diclofenac	COX-1>COX-2 inhibitor, Novartis, Sweden.
Indomethacin	COX-1>COX-2 inhibitor A/S Dumex Ltd. Copenhagen, Denmark.
Ketorolac	Selective COX-1 inhibitor, Hoffmann-La Roche AG, Basel, Switzerland.
Meloxicam	Selective COX-2 inhibitor, Sigma, St. Louis, MO, USA.
Parecoxib	Selective COX-2 inhibitor, Pharmacia & Upjohn, Sweden, and Pharmacia, Buckinghamshire, Great Britain.

Glutamatergic drugs

D-cycloserine	Partial NMDA/glycine site agonist, Sigma, St. Louis, MO, USA.
L-701,324	Selective NMDA/glycine site antagonist, Sigma, St. Louis, MO, USA.
MK 801	Non-competitive NMDA receptor antagonist, Merck Sharp & Dohme, West Point, PA, USA.

Antipsychotic drugs

Clozapine	Atypical antipsychotic drug, Sigma, St. Louis, MO, USA.
Haloperidol	Traditional antipsychotic drug, Janssen Pharmaceutical, Beerse, Belgium.

Other drugs

Apomorphine	Dopamine agonist, Merck, Darmstadt, Germany.
β-cyclodextrin	Sigma, St. Louis, MO, USA.
Chloral hydrate	General anesthetic drug, Merck, Darmstadt, Germany.
L-kynurenine	Precursor of kynurenic acid, Sigma, St. Louis, MO, USA.
Misoprostol	Prostaglandin E_1/E_2 agonist kindly donated by Pharmacia & Upjohn, Sweden.
MLA	α7* nicotinic receptor antagonist, Sigma, St. Louis, MO, USA.
PNU 156561A	[(R,S)-2-amino-4-oxo-4(3´-f´-dichlorophenyl)butanoic acid], kynurenine 3-hydroxylase inhibitor, kindly donated by Dr. C. Speciale, Pharmacia & Upjohn, Milano, Italy.

3.3 Pretreatment with PNU 156561A (paper III and V)

To elevate levels of endogenous brain kynurenic acid, rats were pretreated with PNU 156561A (dissolved in 10% b-cyclodextrin) i.v. 5 h before electrophysiological experiments (40 mg/kg) and 4 h before behavioral studies (10 mg/kg). This drug inhibit the enzyme kynurenine 3-hydroxylase and thereby shunts the synthesis of kynurenine towards kynurenic acid. For intravenous (i.v.) injection, the rat was placed in an adjustable restrainer consisting of a Plexiglas tube with a sliding piston to adjust the space for the rat (rat from 180 g up to 320 g). An elastic rubber band was clamped to the base of the tail to

restrict venous blood flow, using a pair of curved forceps, and the tail was immersed in a beaker of tempered water to dilate the veins. A temporary cannula (0.5 × 1.6 mm needle) was inserted into a lateral tail vein, a successful insertion was recognized when blood leaked out through the cannula, and the rubber band was removed. A syringe containing PNU 156561A or vehicle was inserted to the needle and approximately 1 mL was carefully injected. After drug administration, the cannula was removed and the rats were placed individually in a Plexiglas cage.

3.4 *In vivo* electrophysiology

3.4.1 Anesthesia and surgery

Rats were weighed and thereafter anesthetized with 8% chloral hydrate intraperitoneally (400 mg/kg, i.p.). For the best induction of anesthesia, the rats were left to fall asleep in a quiet environment for approximately 10 min. If the degree of anesthesia was not satisfactory by the end of this period additional 8% chloral hydrate (200 mg/kg, i.p.) was administered. The rat was placed onto a heating pad to maintain its body temperature at 37°C. It was subsequently mounted onto the earbars of a conventional stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), so that the skull was set in a horizontal plane, and the nose was secured using a clamp at the front of the frame. An elastic rubber band was clamped to the base of the tail to restrict venous blood flow, using a pair of curved forceps, and the tail was immersed in a beaker of tempered water to dilate the veins. A cannula was inserted into a lateral tail vein and secured with strips of adhesive. A syringe containing 0.9% NaCl was connected to the cannula and 0.5-1 mL was injected into the rat to assure that the cannula was in a proper position. An incision was made with a scalpel from the nose bridge along the centre 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.

3.4.2 Maintenance of general 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. In the first series of experiments in paper II, where the effects of COX inhibitors on firing were recorded during a period of 45 min, 8% chloral hydrate (20 mg/kg, i.p.) was regularly administrated every fifth minute to maintain a stable level of anesthesia.

3.4.3 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 saturated with Pontamine Sky Blue 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. For VTA-recordings, the impedances were typically 5-8 MΩ measured at 135 Hz in 0.9% saline.

3.4.4 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 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. Using the stereotaxic instrument the electrode was lowered into the brain to a depth of approximately 7 mm. From this point the electrode was lowered slowly, using the hydraulic micro drive, into VTA where dopamine neurons were found 7.5-8.5 mm from the brain surface. Single unit potentials from 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, and simultaneously displayed on a digital storage oscilloscope, monitored on an audio monitor and on a strip chart recorder (Gould).

3.4.5 Identification of VTA dopamine neurons

After each experiment the recording site was histologically verified (paper III, IV) except in those experiments where brains were used for the analysis of kynurenic acid (paper II). In the experiments where histological verification was not performed, the dopaminergic neurons were mainly identified based upon their typical neurophysiological characteristics (see below). To further confirm that recordings had been made on dopamine neurons only, the inhibitory action of a single dose of the dopamine agonist apomorphine was verified at the end of the experiments, when appropriate.

3.4.6 Electrophysiological characteristics of dopaminergic neurons

The dopamine neurons were identified using the electrophysiological characteristics previously described for midbrain dopamine neurons (Wang, 1981; Grace and Bunney, 1984, 1984a) 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 msec), 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. A decreasing amplitude of spikes within each burst was also observed.

3.4.7 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 was recorded for approximately 3 min before drug administration. From the basal activity median values of firing rate, percentage of spikes fired in bursts and variation coefficients (see section 3.4.9) were estimated and used as the control/predrug activity. The effect of a drug on firing was assessed by comparing the median basal activity with the median values of firing rate, percentage of spikes fired in bursts and variation coefficients following each incremental dose administered. The firing activity was recorded from only one VTA dopamine neuron in each rat in all studies with exception of the second series of

experiments in paper II. Here, three to thirteen cells were analyzed in each rat to estimate basal electrophysiological characteristics following 3-4 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 numbers of cells found per track.

In the third paper, rats were pretreated with PNU 156561A (40mg/kg, i.v., 5-7h) in order to increase endogenous levels of kynurenic acid. Previous studies have shown that such treatment is associated with a 3-5-fold elevation of brain kynurenic acid concentrations (Erhardt and Engberg, 2000, 2002; Erhardt et al., 2000, 2001, b, 2002a), as well as an increase in firing rate and percentage of spikes fired in bursts of midbrain dopamine neurons (Erhardt and Engberg, 2002; Erhardt et al., 2001). In this study, efforts were made to avoid recording of dopamine neurons with very high frequency and burst firing activity, i.e. basal firing rate over 10 Hz and basal percentage of spikes fired in bursts over 90%. 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. Such neurons were excluded from pharmacological analyses.

3.4.8 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 a 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; see Overton and Clark, 1997; Hyland et al., 2002). Previous reports have shown that the primary metabolite of chloral hydrate, trichloroethanol (presumably responsibly 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 recently 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 on dopamine cell firing. However, since all rats were anesthetized with chloral hydrate using the same routines throughout the

experiments the differences found between control rats and drug treated rats should not be attributable to the anesthesia.

3.4.9 Data analysis

The distribution of spikes was analyzed on-line with a Macintosh computer. The software used for the analysis of firing was written in-house using a high level object orientated programming language called "G" (Lab VIEW; National Instruments, Austin, TX, USA). The software was 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 pulses. In order to avoid artifacts in the sampling procedure, the spike analyzer was 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, 1984, 1984a). The software program also sorted the intervals of recorded spikes and divided them into 3-ms bins and displayed the results as an interspike time interval histogram (ISH) with regard to the number of intervals corresponding to each bin. The intervals were analyzed with regard to the number of bursts that occurred during a 100-spike sampling period along with a calculation of the percentage of spikes fired in bursts. Firing rate, percentage of spikes fired in bursts and variation coefficients (calculated as the ratio between the standard deviation and the mean interval of an ISH and used as a measure of the regularity of firing (Werner and Mountcastle, 1963)) were expressed as the median of at least three consecutive ISHs.

3.5 Prepulse inhibition

3.5.1 Apparatus

Two startle chambers were used for measuring the startle response (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 × 38 × 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. As described previously (Mansbach et al., 1988), sound levels [dB(A) scale] and accelerometer sensitivities within each chamber were calibrated regularly and found to remain constant over the test period.

3.5.2 Experimental protocol

To elevate levels of endogenous brain kynurenic acid, rats were pretreated with PNU 156561A (dissolved in 10% β-cyclodextrin) i.v. 4 h before testing or kynurenine (100 mg/kg, i.p.) 60 min before testing. Control rats received vehicle either i.v. 4 h or i.p. 60 min before testing. Animals pretreated with kynurenine or vehicle were treated with an injection of saline, clozapine (7.5 kg/mg, i.p) or haloperidol (0.2 mg/kg, i.p.) 30 min before testing in the startle chambers. 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 prepulse inhibition (PPI) test session. Two days before any drug testing, animals were pre-exposed to the chambers and a testing session. The purpose of the pre-exposure was to acclimatize the animals to the testing chambers and startle/prepulse stimuli and to base-line match the groups for subsequent testing (groups were matched for equivalent mean startle magnitude and percent PPI, as defined below). In the main experimental 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 pulses 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 (twelve "pulse-alone" trials, ten each of the remaining "prepulse + pulse" trials 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 at the end of the session were also excluded. An average of 15 s (ranging from 9 to 21 s) separated consecutive trials. The whole session lasted approximately 24 min. The baseline session used to familiarize rats with the testing procedure consisted of 24 trials (eighteen 120 dB "pulse-alone" and six "prepulse + pulse" trials with a 12 dB prepulse intensity).

3.6 Analysis of kynurenic acid

3.6.1 Sample preparation

Immediately after each electrophysiological or behavioral experiment the rats were killed by decapitation. In paper I, the rats were killed by asphyxiation with CO_2 before decapitation. The brains were then rapidly taken out and stored at -70°C for subsequent analysis of kynurenic acid. The brains were sonicated with an equal weight of homogenization medium (perchloric acid 0.4 M, $Na_2S_2O_5$ 0.1%, and ethylenediaminetetra-acetate (EDTA, 0.05%). The samples were centrifuged at 20,000 g for 5 min and approximately 40 μL perchloric acid (70%) was added to the supernatant. Thereafter the supernatant was centrifuged twice and stored at -70°C. Before analysis, all samples were defrosted and centrifuged at 20,000 g for 5 min.

3.6.2 High performance liquid chromatography

Kynurenic acid is a stable compound and is not degraded even by repeated thawing (Heyes and Quearry, 1990). It was analyzed with an isocratic reversed-phase high-performance liquid chromatography (HPLC) system, including a dual piston, high liquid delivery pump (Bischoff, Leonberg, Germany), a ReproSil-Pur C18 column (4 × 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% acetonitrile was pumped through the reversed-phase column at a flow rate of 0.5 mL/min. Samples of 30 μl or 50 μl were manually injected (Rheodyne, Rhonert Park, CA, USA). Zinc acetate (0.5 M, not pH adjusted) was delivered postcolumn by a peristaltic pump (P-500, Pharmacia, Uppsala, Sweden) at a flow rate of 10 mL/h. The signals from the fluorescence detector were transferred to a computer for analysis with

Datalys Azur (Grenoble, France). The retention time of kynurenic acid was approximately 13 min, and the detection limit of the method was approximately 0.125 pmol (signal: noise ratio 5:1; see Fig. 5). Initially the sensitivity of the fluorescence method was evaluated by injection of a standard mixture of kynurenic acid, with concentrations from 1.25 nM to 60 nM. This resulted in a standard plot, which was used to relate the heights of the peaks in the chromatogram to the correct concentration of kynurenic acid in the samples.

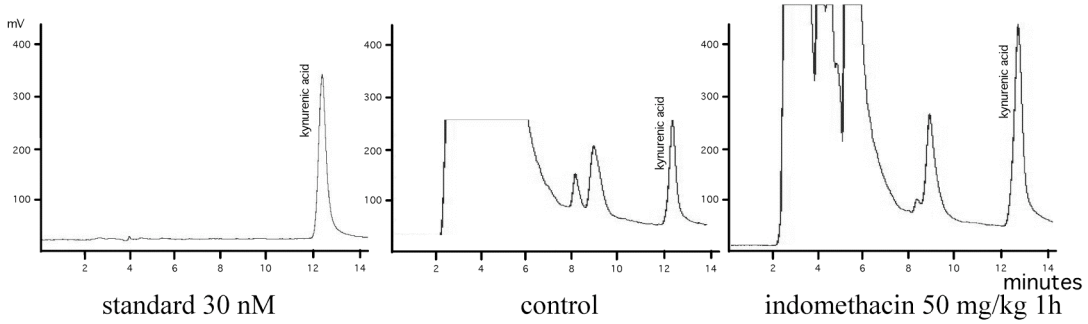

Figure 5. Chromatograms illustrating the measurement of kynurenic acid in rat whole brain

3.6.3 Chemicals

Zinc acetate and kynurenic acid, Sigma, St. Louis, MO, USA. Sodium acetate, Riedel-de Haen, Germany. Perchloric acid, Kebo Lab, Stockholm, Sweden. Acetonitrile, Labasco, Partille, Sweden.

3.7 Statistical analysis

3.7.1 Biochemical and electrophysiological studies (paper I-IV)

Firing rate, variation coefficient, burst firing activity and estimation of kynurenic acid levels are expressed as mean ±SEM. Significance was assumed for all values where $p<0.05$. Due to the relatively few observations in each group no evidence of a normal distribution of measurements was obtained. Therefore, statistically significant differences were established using the following nonparametric tests: Kruskal-Wallis analysis of variance followed by Wilcoxon signed rank test or Mann-Whitney U-test. Adjusted Bonferroni Comparison was

occasionally used to correct for the α level due to multiple tests. Statistical analyses were performed using GraphPad Prism® version 4.

3.7.2 Behavioral studies, PPI (paper V)

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. For simplicity of presentation, data from the first and last blocks of five "pulse-alone" trials are not presented, because 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)] × 100). All data were first analyzed in a four-factor ANOVA including blocks (first and second halves of the session) and trial types as repeated measures and pretreatment and treatment as between-subject factors. When the blocks factor did not interact with another factor, only the three-factor ANOVA (pretreatment, treatment, and trial types) is 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. Statistically significant differences regarding estimation of kynurenic acid 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 Effects of cyclooxygenase inhibitors on kynurenic acid synthesis and on neuronal activity of midbrain dopamine neurons (paper I and II)

In the initial studies, a putative prostaglandin-mediated control of kynurenic acid synthesis was examined. For this purpose, the effects of drugs with different selectivity for COX-1 and COX-2 on endogenous levels of brain kynurenic acid as well as on firing activity of midbrain dopamine neurons were investigated in rats.

4.1.1 Effects of cyclooxygenase inhibitors on kynurenic acid formation in the rat brain (Paper I)

COX is the key enzyme in the conversion of arachidonic acid to prostaglandins. Three isoforms of COX have been identified, all of which are present in the brain (see Smith et al., 2000; Chandrasekharan et al., 2002). Recently, the NSAID diclofenac, which interacts with both COX-1 and COX-2, was found to increase rat brain kynurenic acid formation (Edwards et al., 2000). In this study we analyzed whether this previously reported effect of diclofenac is related to prostaglandins and how drugs with various selectivity for different COX isoforms modulate the synthesis of rat brain kynurenic acid.

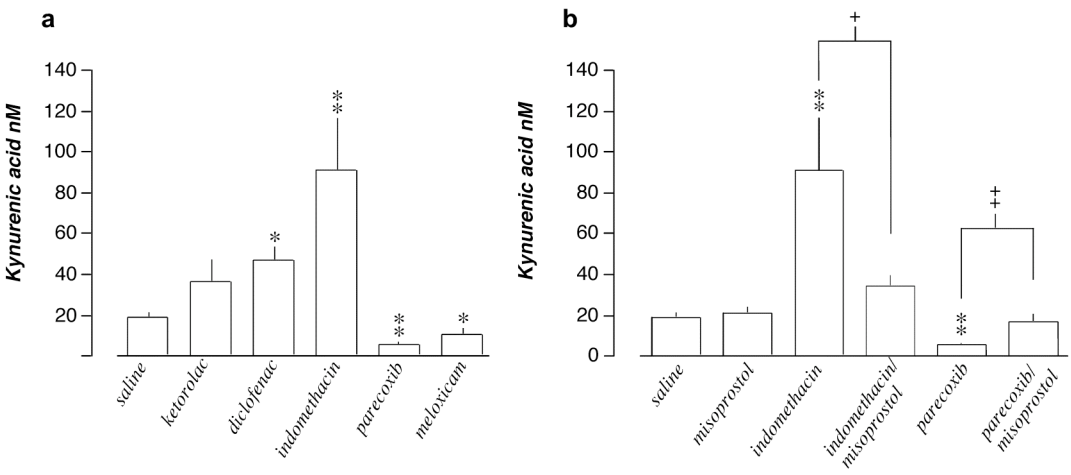

Fig. 6. Concentrations of whole rat brain KYNA in **a)** controls, ketorolac (10 mg/kg), diclofenac (50 mg/kg), indomethacin (50 mg/kg), parecoxib (25), meloxicam (5 mg/kg) and **b)** in controls, misoprostol (500 + 500 µg/kg), indomethacin (50 mg/kg), indomethacin (50 mg/kg) + misoprostol (500 + 500 µg/kg, s.c.), parecoxib (25 mg/kg), parecoxib (25 mg/kg) + misoprostol (500 + 500 µg/kg). Due to the relatively short half-life of misoprostol, the drug was administered (500 µg/kg) at two occasions, 3 h and 1.5 h before decapitation. All drugs were given intraperitoneally 3.5 hours before decapitation. Each value represent mean ± S.E.M. from 6-10 rats. +P<0.05, ++P<0.01, *P<0.05, **P<0.01 vs. controls (Mann-Whitney U-test).

We found that administration of the non-selective COX-inhibitor diclofenac (50 mg/kg, i.p., 3.5 h; n=7) or administration of indomethacin (50 mg/kg, i.p., 3.5 h; n=7), a drug with preferential selectivity for COX-1 (Warner et al., 1999), was associated with an increased formation of brain kynurenic acid (Fig. 6a). Ketorolac (10 mg/kg, i.p., 3.5 h; n=7), a highly selective COX-1 inhibitor (Warner et al., 1999) which essentially lacks COX-2 inhibitory effects, showed only a tendency to increase concentrations of brain kynurenic acid (Fig. 6a). However, the discrepancy between ketolorac and indomethacin in this regard may tentatively be ascribed to ketorolac's poor ability to pass the blood brain barrier (Jett et al., 1999). Alternatively, our results may indicate that inhibition of both COX-1 and COX-2 is a prerequisite to elevate brain kynurenic acid. Compared to non-selective COX inhibitors, COX-2 selective inhibitors showed an opposite action on rat brain kynurenic acid formation. Thus, both parecoxib (25 mg/kg, i.p., 3.5 h; n=6) and meloxicam (5 mg/kg, i.p., 3.5 h; n=7) significantly decreased brain kynurenic acid concentration (Fig. 6a).

The detailed mechanisms by which COX inhibitors modulate the synthesis of brain kynurenic acid is ambiguous, especially in view of the diverse effects of NSAIDs with COX-1 inhibitory actions and selective COX-2 inhibitors. Since both the elevation and the lowering of brain kynurenic acid levels following administration of the COX inhibitors (indomethacin 50 mg/kg, i.p., 3.5 h; n=10 or parecoxib 25 mg/kg, i.p., 3.5 h; n=7), were effectively prevented by pretreatment with the prostaglandin E_1/E_2 analog misoprostol (500+500 μg/kg, s.c., 1.5 and 3 h, Fig. 6b) it appears that prostaglandins tonically modulate kynurenic acid metabolism in a complicated counteractive way depending on the COX isoforms involved. However, elevation of brain kynurenic acid by kynurenine (200 mg/kg, i.p., 2 h; n=6, 126.5 ±12.2 nM), the immediate precursor of kynurenic acid, was not prevented by co-administration of misoprostol (500+500 μg/kg, s.c., 1.5 and 3 h; n=6, 127.7 ±28.4 nM). This indicates *per se* that the prostaglandin mediated control of brain kynurenic acid synthesis is not executed at the level of KAT, the enzyme responsible for the conversion of kynurenine to kynurenic acid. Rather, prostaglandins tonically modulate brain kynurenic acid metabolism by controlling, directly or indirectly, the synthesis of kynurenine. Indeed, previous studies have shown that NSAIDs increase activity of the first and rate-limiting enzyme for the kynurenine pathway, tryptophan-2,3-dioxygenase in rats (Franzone et al., 1980; see McCormack, 1994). Given the opposite actions on brain kynurenic acid levels by NSAIDs with different selectivity for COX-1 and COX-2 one may suggest that the two forms of COX are modulating kynurenic acid synthesis at distinctly different levels of the kynurenine pathway. Thus,

although both forms of COX are functionally similar and catalyze transformation of arachidonic acid to the same inflammatory mediators, prostaglandins may tonically induce a facilitatory or inhibitory action on the synthesis of brain kynurenic acid depending on the metabolic step of the kynurenine metabolism that is affected.

4.1.2 Effects of cyclooxygenase-1 and cyclooxygenase-2 inhibitors on neuronal firing of rat midbrain dopaminergic neurons (Paper II)

In the present *in vivo* electrophysiological study we investigate whether alteration of endogenous levels of rat brain kynurenic acid, induced by drugs with various selectivity for the COX isoforms, influences firing characteristics of VTA dopamine neurons. For this purpose, the COX-1 inhibitor indomethacin and the selective COX-2 inhibitor parecoxib were used, which increase and decrease brain kynurenic acid concentration, respectively.

In the first series of experiments, the activity of VTA dopamine neurons was monitored before, during and after administration of the COX inhibitors. These results reveal that i.v. administration of the selective COX-2 inhibitor parecoxib (25 mg/kg, n=9), which decreased the concentration of whole brain kynurenic acid (by 39%, table 1), was associated with an inhibition of VTA dopamine neurons. Thus, firing rate and burst firing activity were significantly decreased within 10-20 min after administration and continued to decrease time-dependently throughout the 45 minute period of observation (Fig. 7).

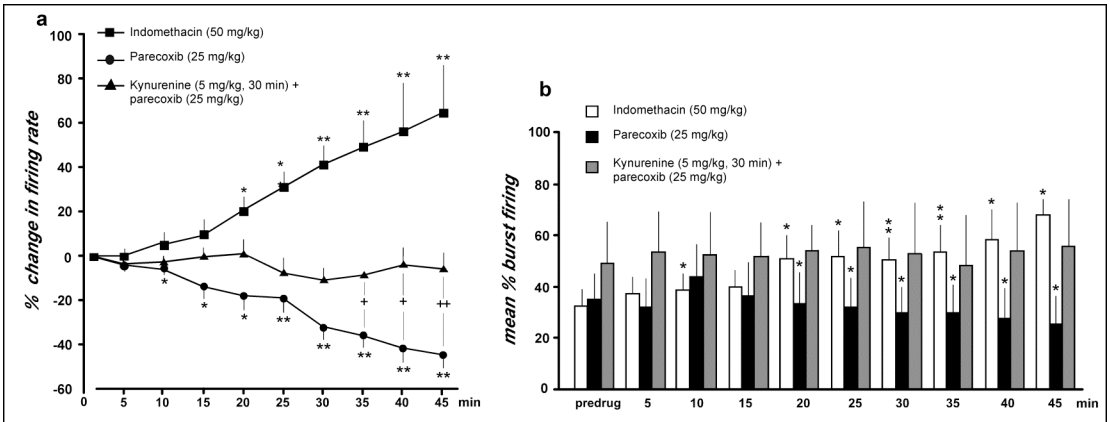

Fig. 7. Time-response curves depicting the action of parecoxib (25 mg/kg, i.v.), indomethacin (50 mg/kg i.v.) or parecoxib (25 mg/kg, i.v.) in rats pretreated with kynurenine (5 mg/kg, i.p., 30 min) on **(a)** the firing rate (statistics: *P<0.05, **P<0.01, Mann Whitney U-test with Bonferroni correction, vs. corresponding predrug value and +P<0.05, ++P<0.01 Mann Whitney U-test with Bonferroni correction between parecoxib and parecoxib/kynurenine) and **(b)** the percentage of burst firing activity (statistics: *P<0.05, **P<0.01 Wilcoxon signed rank test vs. corresponding predrug value). Each value represents mean ±SEM from 5-13 VTA dopamine neurons.

In analogy, intravenous administration of indomethacin, a COX-1 preferential inhibitor (50 mg/kg, n=13) which increased brain kynurenic acid concentration (by 150%, table 1), produced the opposite effects on the firing of VTA dopamine neurons as compared with parecoxib. Firing rate and burst firing activity were significantly increased within 10-20 min

after administration and continued to increase time-dependently during the 45 minute observation period (Fig.7).

Table 1. Effects of parecoxib or indomethacin on whole brain kynurenic acid concentrations

	Kynurenic acid (nM)
Controls (n=10)	20.3 ±2.3
parecoxib, i.v., 1 h (n=9)	12.4 ±1.5**
parecoxib, i.v., 1 h + L-701, 324, i.v., 10 –15 min (n=7)	10.2 ±3.9*
parecoxib, i.p., 3-4 h (n=13)	11.3 ±2.7*
kynurenine, i.p., 1.5 h + parecoxib, i.v., 1 h (n=6)	40.7 ±5.8*
indomethacin, i.v., 1 h (n=13)	50.6 ±7.0**
indomethacin, i.v., 1 h + D-cycloserine, i.v., 10 –15 min (n=9)	44.8 ±8.8***
indomethacin, i.p., 3-4 h (n=10)	84.1 ±20.7*

Values represent means ±SEM from controls, rats treated with kynurenine (5mg/kg), parecoxib (25 mg/kg), indomethacin (50 mg/kg) and D-cycloserine (32 mg/kg). Statistics: *P<0.05, **P<0.01, *** 0.001 vs control value (Mann-Whitney U-test).

In the second series of experiments the basal firing characteristics of VTA dopamine neurons were analyzed after intraperitoneal pretreatment with parecoxib or indomethacin by recording from a number of cells in each rat. The electrophysiological characteristics of 78 VTA dopamine neurons in saline treated rats (n=10), 64 VTA dopamine neurons in rats pretreated with parecoxib (25 mg/kg i.p., 3-4 h; n=13) and 89 VTA dopamine neurons in rats pretreated with indomethacin (50 mg/kg i.p., 3-4 h; n=10) are summarized in table 2.

Table 2. Firing rate and spike distribution of dopamine neurons in the VTA following intraperitoneal treatment with parecoxib or indomethacin.

	Controls (78 neurons)	**Parecoxib** (64 neurons)	**Indomethacin** (89 neurons)
Firing rate, Hz	4.5 ±0.2	3.9 ±0.2*	6.1 ±0.2***
Mean % spikes in burst	32.2 ±3.1	26.4 ±3.4	54.3 ±2.8***
Mean number of bursts[a]	9.3 ±0.9	8.5 ±1.1	12.5 ±0.7**
Mean spikes per burst	2.6 ±0.3	2.1 ±0.3	3.9 ±0.5***
Cells found per track	1.9 ±0.2	0.7 ±0.1*	2.9 ±0.3*
Mean % variation coefficient	83.5 ±3.3	76.4 ±3.7	76.8 ±2.8

Values represent means ±SEM from control rats (n=10), rats pretreated with parecoxib (25 mg/kg, i.p., 3-4 h; n=13) or indomethacin (50 mg/kg, i.p., 3-4 h; n=10). Statistics: *P<0.05, **P<0.01, *** 0.001 vs corresponding control value (Mann-Whitney U-test).
[a]during a 100-spike sampling period.

Results from these experiments show that pretreatment with the selective COX-2 inhibitor parecoxib decreased the firing rate and tended to decrease the burst firing activity of VTA dopamine neurons. In addition, the number of spontaneously active VTA dopamine neurons decreased after pretreatment with parecoxib, as shown by the decreased number of cells found per track. The opposite effect on the basal firing activity was found in rats pretreated with indomethacin. Thus, mean firing rate and mean percent spikes fired in bursts of VTA dopamine neurons were significantly increased. In addition, this pretreatment also increased the number of spontaneously active VTA dopamine neurons. Intraperitoneal administration of indomethacin and parecoxib in these experiments resulted in an increase and a decrease in whole brain kynurenic acid concentrations, respectively (table 1).

4.1.3 Involvement of endogenous kynurenic acid in the effects of cyclooxygenase inhibitors on dopamine firing (paper II)

In the following experiments the mechanism underlying the effects of COX inhibitors on the neuronal firing of midbrain dopamine neurons were more thoroughly investigated. The excitatory and inhibitory actions on midbrain dopamine neurons exerted by indomethacin and parecoxib, respectively, were correlated to their opposite effects on the formation of the NMDA/glycine receptor antagonist kynurenic acid. Such a relationship *per se* strongly supports an involvement of kynurenic acid in the effects of COX-inhibitors on VTA dopamine firing. In further support, the excitation of midbrain dopamine neurons observed after systemic administration of indomethacin strikingly resembles the excitatory action induced by acute or chronic elevation of rat brain kynurenic acid (Erhardt et al., 2001; Erhardt and Engberg, 2002; Nilsson et al., 2005a). We therefore investigated if drugs acting selectively at the NMDA/glycine receptor interact with the actions of indomethacin and parecoxib on VTA dopamine neurons.

Systemic administration of the partial NMDA/glycine receptor agonist D-cycloserine (2-32 mg/kg; n=9) was *per se* associated with a slight dose-dependent increase in firing rate and burst firing activity. In rats pretreated with indomethacin (50 mg/kg, i.v., 1 h; n=9) systemic administration of D-cycloserine (2-32 mg/kg), significantly decreased the firing rate and percent spikes fired in burst. This result resembles that of a previous study where increased levels of brain kynurenic acid, induced by PNU 156651A, converted the excitatory action of D-cycloserine into a pure inhibitory response (Erhardt and Engberg, 2002).

Systemic administration of the selective NMDA/glycine site antagonist L-701,324 (Grimwood et al., 1995) was *per se* associated with a dose-dependent increase in firing rate and burst firing activity. Somewhat surprisingly, parecoxib pretreatment (25 mg/kg i.v., 1 h), enhanced the excitatory action of L-701,324 (0.06–2 mg/kg, i.v; n=7). Although the mechanism behind this phenomenon is obscure it may be related to a reduced occupancy of NMDA/glycine sites due to the decrease in endogenous kynurenic acid concentration. Even more convincing results for an involvement of kynurenic acid in the action of parecoxib on VTA dopamine neurons are the experiments that used kynurenine, the immediate precursor of kynurenic acid. Thus, pretreatment with kynurenine (5 mg/kg i.p., 30 min before parecoxib administration; n=5), prevented the reduction of brain kynurenic acid levels by parecoxib (table 1), and simultaneously antagonized the inhibitory action of the drug on the neuronal firing of the VTA dopamine neurons (Fig. 7). This effect of kynurenine should conclusively link the action of parecoxib on VTA dopamine neurons to the kynurenine pathway.

Altogether, the findings of the present study suggest that the actions of COX-inhibitors on neuronal firing of VTA dopamine neurons are linked to their effects on the synthesis of endogenous brain kynurenic acid. Furthermore, such a regulatory effect by kynurenic acid on VTA dopamine neurons should be attributed to its antagonism at the glycine site of the NMDA receptor as also indicated by the interaction with D-cycloserine and L-701,324. Recent studies though show that kynurenic acid, apart from being an antagonist at the NMDA/glycine-site, also blocks the α7* nicotinic receptor (Hilmas et al., 2001). Such an action of kynurenic acid appears, however, unlikely to account for the presently observed effects on VTA dopamine neurons, since administration of the selective α7* nicotinic receptor antagonist, MLA was previously shown not to influence the firing of VTA dopamine neurons (Schilström et al., 2003; see paper IV).

In conclusion, this study reveals that endogenous brain concentrations of kynurenic acid are of critical importance for the tonic control of neuronal activity of midbrain dopamine neurons. Thus, a reduction in brain kynurenic acid levels is associated with a decrease in activity of VTA dopamine neurons and it stands clear that endogenous kynurenic acid serves to maintain the firing activity of these neurons.

4.2 The atypical antipsychotic drug clozapine affects the neuronal activity of midbrain dopamine neurons by interfering with glutamatergic mechanisms (Paper III and IV).

4.2.1 Effects of antipsychotic drugs on midbrain dopamine firing in rats with elevated levels of endogenous brain kynurenic acid (paper III)

In this *in vivo* electrophysiological study, we investigate the effects of the atypical antipsychotic drug clozapine and the classic antipsychotic drug haloperidol on VTA dopamine neurons following increased levels of endogenous brain kynurenic acid. Brain kynurenic acid concentration was pharmacologically elevated by administration of the kynurenine 3-hydroxylase inhibitor PNU 156561 A. This compound blocks an alternative pathway for the precursor kynurenine, and thereby shunts the synthesis of kynurenine towards kynurenic acid (see section 3.3). This treatment is associated with hyperdopaminergia (Erhardt et al., 2001; Erhardt and Engberg, 2002) that may tentatively mimic a condition similar to that occurring in schizophrenia.

Acutely administered haloperidol (0.05-0.8 mg/kg, i.v.; n=7) or clozapine (1.25-10 mg/kg, i.v.; n=11) in control rats was associated with increased firing rate and burst firing activity of VTA dopamine neurons. These findings are in agreement with previous *in vivo* electrophysiological studies (White and Wang, 1983; Tung et al., 1991; Gessa et al., 2000) and it is generally accepted that antipsychotic drugs increase dopamine cell firing rate by blockade of somatodendritic dopamine autoreceptors (Pucak and Grace, 1994; 1996). However, pretreatment with PNU 156561A (40mg/kg, i.v., n=8, 5-7 h) was found not only to antagonize the increase in firing rate and percentage of spikes fired in burst of VTA dopamine neurons induced by clozapine (1.25-10 mg/kg,

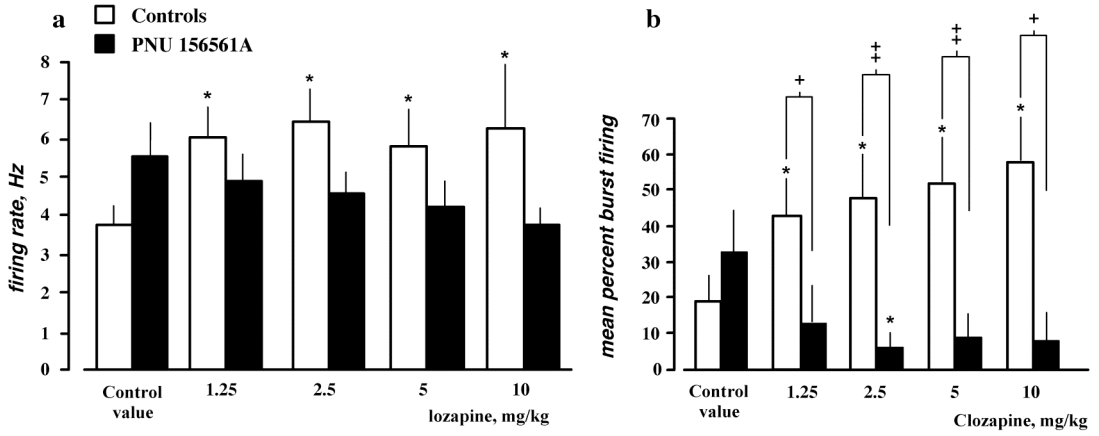

Fig. 9. Effects of incremental doses of intravenously administered clozapine (1.25-10 mg/kg) in control rats and rats pretreated with PNU 156561A (40 mg/kg, i.v., 5-7 h) on **a)** the firing rate, **b)** the percent burst firing activity . Each value represents mean + SEM from 6-11 VTA DA neurons. Statistic: *P<0.05 (Wilcoxon signed rank test) vs. corresponding predrug value and +P<0.05, ++P<0.01vs corresponding control value (Mann Whitney U-test).

i.v.), but also to reverse the action of clozapine, leading to a significant decrease in percentage of spikes fired in burst and a tendency to reduced firing rate (Fig. 8a,b). This reduction in burst firing activity was observed in seven of eight dopamine neurons, irrespective of their predrug basal characteristics. Furthermore, five of eight spontaneously bursting neurons were converted to non-bursting neurons by clozapine.

The effects of haloperidol on VTA dopamine neurons were clearly potentiated after pretreatment with PNU 156561A, in contrast to those of clozapine, since haloperidol forced all dopamine neurons recorded from into a depolarization block already after administration of 0.1 mg/kg haloperidol (n=4).

We suggest that an increased dopaminergic transmission, as induced by elevated kynurenic acid levels, may promote haloperidol to induce depolarization block by its potent antagonism at somatodendritic dopamine D_2 receptors. In contrast to haloperidol, the inhibitory action of clozapine could not be explained in terms of antagonism of dopamine receptors but rather by an interaction with the glycine site of NMDA receptor or, alternatively, of the α7* nicotinic receptor. Thus, the present data do not allow any definitive conclusion regarding the mechanism behind the inhibitory action of clozapine on VTA dopamine neurons. Hypothetically, the effect could be mediated by **I)** activation of presynaptic α7* nicotinic receptors located on glutamatergic afferents (McGehee et al., 1995; Wonnacott et al., 2000) leading to a potentially increased glutamate release by clozapine, **II)** activation of postsynaptic α7* nicotinic receptors, located on cell soma or dendrites of VTA dopamine neurons, or **III)** displacement of kynurenic acid at postsynaptic NMDA receptors. In paper IV these questions were more thoroughly investigated by utilizing the NMDA receptor antagonist MK 801, the antagonist at the glycine site of the NMDA receptor L-701,324 and the α7* nicotinic receptor antagonist MLA.

4.2.2 Clozapine modulates midbrain dopamine neurons via interaction with the NMDA receptor (paper IV)

Administration of the NMDA receptor antagonist MK 801 alone (1 mg/kg, i.v., n = 8) increased the firing rate (57%) and tended to increase the percentage of spikes fired in bursts (from 16% to 32%) of VTA dopamine neurons. This treatment antagonized the excitatory action of clozapine (1.25-10 mg/kg, i.v; n=8, Fig. 9a,b) pointing to an interaction between clozapine and glutamatergic NMDA receptors. In line with this view, pretreatment with L-701,324 (1-8 mg/kg, i.v; n=8) which *per se* was associated with

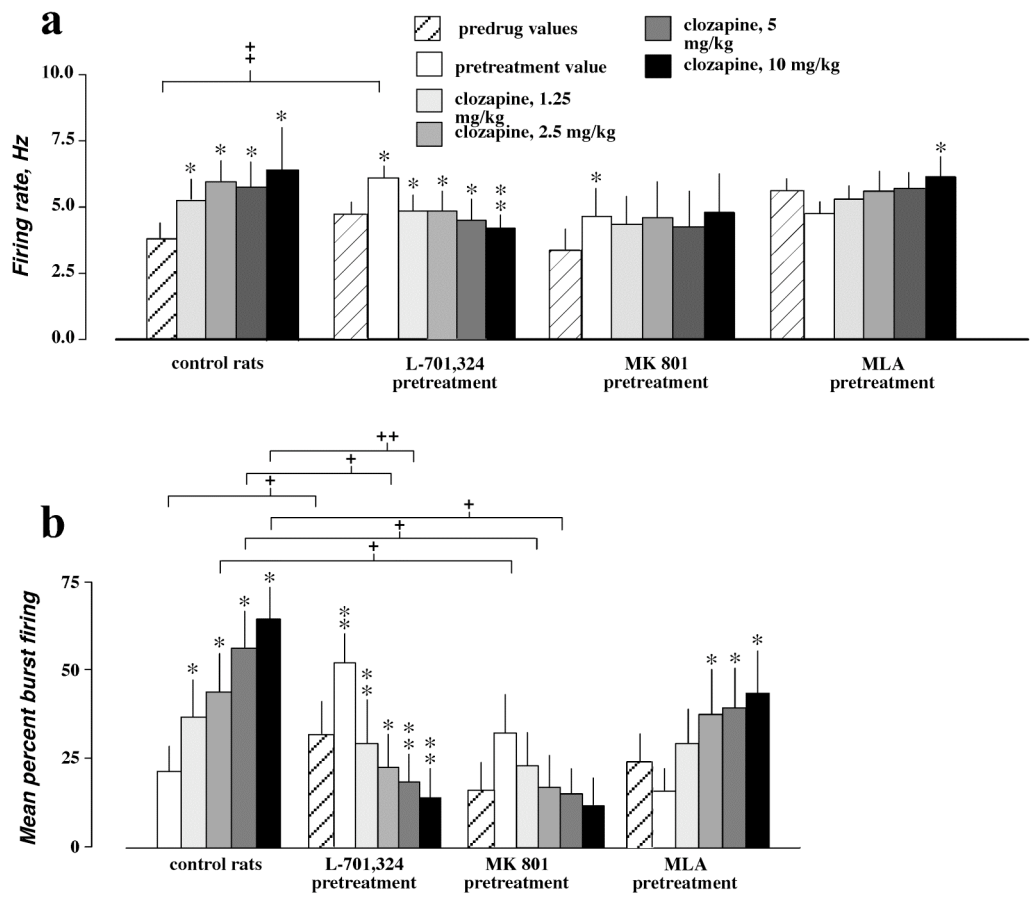

Fig. 9. Effects of incremental doses of intravenously administered clozapine (1.25-10 mg/kg) in control rats and rats pretreated with L-701,324 (8 mg/kg, i.v.), MLA (6 mg/kg, i.p.) or MK 801 (1 mg/kg, i.v) on **a)** the firing rate **b)** the percent burst firing activity. Each value represents mean ± SEM from 6-11 VTA DA neurons. Statistic: *P<0.05 (Wilcoxon signed rank test) vs. corresponding predrug value and +P<0.05, ++P<0.01vs corresponding control value (Mann Whitney U-test).

increased firing rate and percentage of spikes fired in burst, not only antagonized, but also reversed the excitatory action of clozapine on VTA dopamine neurons, leading to a decrease in firing rate and in burst firing activity (Fig. 9a,b and 10). These results are indicative of an interaction of clozapine with the glycine site of the NMDA receptor. Pretreatment with the α7* nicotinic receptor antagonist MLA (6 mg/ kg, i.p., 10 min; n=7), did not affect spont-aneous firing of VTA dopamine neurons nor the ability of clozapine to increase burst firing activity of the same neurons. However, MLA slightly antagonized the increase in firing rate by clozapine (Fig. 9a,b) as observed in control rats. Therefore, one cannot

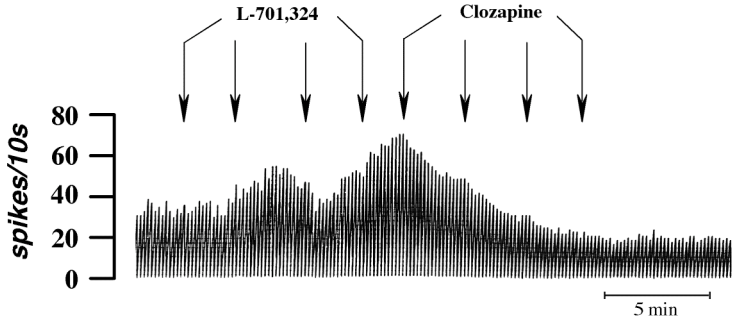

Fig. 10. Extracellular recording from a spontaneously bursting DA neuron in the VTA depicting the effect of intravenous administration of clozapine following pretreatment with L-701,324.

fully exclude that α7* nicotinic receptors may play a role in the inhibition in firing rate of VTA dopamine neurons by clozapine.

Altogether, these data provide pharmacological evidence that clozapine interferes with the NMDA receptor. Thus, not only the regulation of basal firing of VTA dopamine neurons is related to blockade of the glycine site of the NMDA receptor, it also appears that the interaction between elevated levels of kynurenic acid and the response of these neurons to clozapine is related to the same site. Tentatively, clozapine could be an agonist at the glycine site of the NMDA receptor or an inhibitor of the glycine transporter, resulting in increased synaptic levels of glycine. In support of the latter theory it was recently shown that clozapine inhibits rat brain synaptosomal glycine transport (Javitt et al., 2005). A putative interaction with the glycine site of the NMDA receptor may account for clozapine's unique clinical efficacy and suggest the glycine site of the NMDA receptor as a target for novel antipsychotic drugs.

4.3 Increased levels of endogenous kynurenic acid disrupt prepulse inhibition - a behavioral model for schizophrenia (paper V)

Previous studies have shown that a moderate elevation of endogenous brain kynurenic acid dramatically increases the firing activity of midbrain dopamine neurons (Erhardt et al., 2001; Erhardt and Engberg, 2002), thereby mimicking the dopaminergic hyperactivity believed to be one underlying cause of schizophrenia. Obviously, an important question is whether increased levels of endogenous brain kynurenic acid are able to induce symptoms similar to those observed in schizophrenia. Patients with schizophrenia have long been considered to have an impaired ability to filter out extraneous stimuli in the environment that would interfere with attention and information processing (Venables, 1960; McGhei and Chapman, 1961; Freedman et al., 1987; Geyer and Braff, 1987). Prepulse inhibition is a behavioral model that reflects such sensory gating deficits in these patients (c.f., introduction). We therefore examined the effects of elevated endogenous brain kynurenic acid on PPI in rats.

Elevation of endogenous brain kynurenic acid concentration was achieved through administration of kynurenine (100 mg/kg, i.p., 1 h; n=13), the precursor of kynurenic acid, or by administration of the kynurenine 3-hydroxylase inhibitor PNU 156561A (10 mg/kg, i.v., 4 h; n=9, table 3). A four-fold increase in brain kynurenic acid levels by these drugs was found to significantly reduce PPI compared to saline-treated control rats (n=28, Fig. 11).

Table 3. Mean whole brain concentration of kynurenic acid	**Kynurenic acid (nM)**
Controls	27 ±5 nM, n =28
PNU 156561A (10 mg/kg, i.v., 4 h)	106 ±38 nM**, n = 9
Kynurenine (100 mg/kg, i.p., 60 min)	124 ±12 nM***, n = 13
Saline + Haloperidol (Saline + 0.2 mg/kg, i.p., 30 min)	19 ±3 nM***, n = 11
Saline + Clozapine (Saline + 7.5 mg/kg, i.p., 30 min)	20 ±3 nM***, n = 12
Kynurenine + Haloperidol (100 mg/kg, i.p., 60 min + 0.2 mg/kg, i.p., 30 min)	183 ±26 nM***, n = 8
Kynurenine + Clozapine (100 mg/kg, i.p., 60 min + 7.5 mg/kg, i.p., 30 min)	236 ±40 nM***, n = 12
Kynurenine + Probenecid (100 mg/kg, i.p., 60 min + 200 mg/kg, i.p., 120 min)	1691 ±44 nM***, n = 7

Statistics: **P<0.01, *** 0.001 vs control value (Mann-Whitney U-test).

The second finding of the present study is that the reduced PPI as induced by elevated brain levels of kynurenic acid can be restored by administration of the antipsychotic drugs haloperidol (0.2 mg/kg, i.p., 30 min; n=8) or clozapine (7.5 mg/kg, i.p., 30 min; n=12).

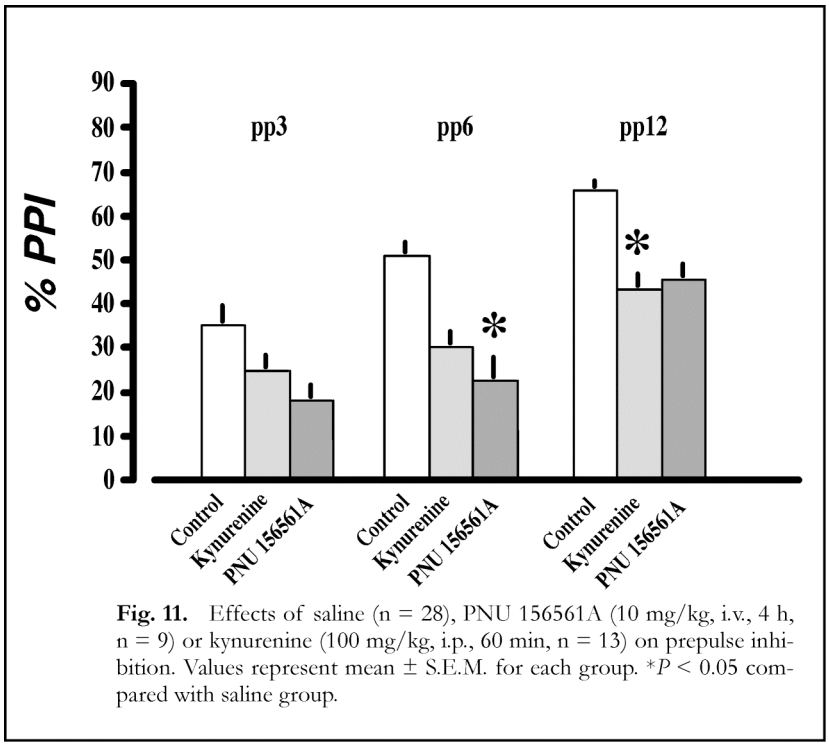

Fig. 11. Effects of saline (n = 28), PNU 156561A (10 mg/kg, i.v., 4 h, n = 9) or kynurenine (100 mg/kg, i.p., 60 min, n = 13) on prepulse inhibition. Values represent mean ± S.E.M. for each group. *$P < 0.05$ compared with saline group.

The present findings, that increaseed brain kynurenic acid disrupts PPI, are in line with previous studies in which 7-chlorokynurenic acid, a potent synthetic analog of kynurenic acid (Le-hmann et al., 1993; Wu et al., 1997), reduced PPI when administered locally into the nucleus accumbens (Kretschmer and Koch, 1997; 1998). In a previous study, it was also shown that pharmacological elevation of hippocampal kynurenic acid concentration disrupts auditory sensory gating in rats, another measure of gating that is

deficient in patients with schizophrenia (Freedman et al., 1987). Since kynurenic acid may interact with both nicotinic and glutamatergic receptors, the ability of kynurenic acid to modulate PPI could be due to interaction with any of these systems. However, the present results do not allow any definitive conclusion regarding the mechanism by which kynurenic acid disrupts PPI.

In conclusion, this study shows that elevated levels of endogenous kynurenic acid are associated with a disruption in PPI, an effect that can be reversed by antipsychotic drugs. These findings not only reveal that kynurenic acid serves as an endogenous modulator of PPI, they also provide further support for the notion that the compound participates in the pathophysiology of schizophrenia.

5 GENERAL DISCUSSION

It is generally accepted that the mesolimbic and mesocortical dopamine systems of the brain are involved in the mediation of symptoms in schizophrenia. However, growing evidence suggests that the underlying cause of the disease lies elsewhere and is related to a glutamatergic hypofunction. This view is largely based on the observation that NMDA receptor antagonists such as PCP and ketamine produce symptoms that resemble both positive and negative symptoms of schizophrenia (Luby et al., 1959; Itil et al., 1967; Adler et al., 1999). Indeed, multiple lines of evidence from experimental and clinical studies using agonists acting at the modulatory glycine/D-serine site of NMDA receptor further link schizophrenia with an attenuated glutamatergic neurotransmission (see Coyle and Tsai, 2004). However, direct evidence for a reduced functioning of the NMDA receptor, e.g. from ligand binding studies, is surprisingly sparse and even inconsistent. Recent studies from our laboratory suggest that elevated levels of kynurenic acid would account for the glutamatergic hypofunction that is related to schizophrenia. Thus, this endogenous antagonist at the glycine/D-serine site of the NMDA receptor has been shown to be elevated in the CSF (Erhardt et al., 2001a; Nilsson et al., 2005) as well as in the post mortem brain (Schwarcz et al., 2001) from patients with schizophrenia. In addition, a recent study shows that the expression of tryptophan 2,3-dioxygenase is increased in the frontal cortex of schizophrenics (Miller et al., 2004), further indicating a disturbance in the kynurenine pathway in these patients. In line with a putative role of kynurenic acid in schizophrenia electrophysiological studies show that pharmacologically elevated levels of the compound are associated with hyperactivity of midbrain dopaminergic neurons (Erhardt et al., 2001; Erhardt and Engberg 2002). The results of the present thesis add further evidence to the hypothesis that kynurenic acid is involved in the pathophysiology of schizophrenia. Thus, a decrease in brain kynurenic acid levels is associated with a reduced dopaminergic activity, offering indisputable evidence that the compound is a significant modulator of glutamatergic neurotransmission. In addition, utilizing the PPI technique, we offer behavioral support for a role of kynurenic acid in sensory gating, a phenomenon that has been proposed to underlie the disturbances in filtering sensory information seen in patients with schizophrenia. Finally, we propose a novel mechanism of action of the atypical antipsychotic drug clozapine, i.e. stimulation of the glycine/D-serine site of the NMDA receptor. Such an action of the drug would directly interfere with a modulation of the NMDA receptor by kynurenic acid.

The present thesis also describes a prostaglandin-mediated regulation of kynurenic acid formation. Thus, administration of COX-1 inhibitors was associated with an increased formation of kynurenic acid in brain. Unexpectedly, COX-2 selective inhibitors had the opposite action on rat brain kynurenic acid. The disparate effects of COX-1 and COX-2 inhibitors in this regard are puzzling; however, one might suggest that the two COX isoforms modulate kynurenic acid synthesis at distinctly different steps in the kynurenine pathway. A regulation of kynurenic acid by inflammatory mediators is interesting given the possible involvement of immunological mechanisms in schizophrenia, as suggested by several epidemiological studies (c.f. Introduction). Indeed, it is well established that immune stimulation profoundly increases the metabolism of tryptophan via the kynurenine pathway (see Moffet and Namboodiri, 2003). Thus, interferon-γ and other pro-inflammatory cytokines have been shown to increase kynurenine, the immediate precursor of kynurenic acid, by inducing IDO expression (Yoshida et al., 1986; Carlin et al., 1987). Notably, a recent study from our laboratory shows that acute psychotic symptoms in HIV-1 infected patients are associated with increased levels of kynurenic acid in CSF (Atlas et al., 2006). Thus, it is possible that kynurenic acid in this regard serves as a functional link between immunological processes and development of psychiatric symptoms.

The physiological role of brain kynurenic acid has long been a matter of controversy since whole brain kynurenic acid concentrations in rodents (nM range) are far below those required to affect NMDA receptors (μM range), as shown by *in vitro* studies (Stone, 1993; Parsons et al., 1997; Urenjak and Obrenovitch, 2000). One possible explanation for this discrepancy could be that kynurenic acid is synthesized and released from astrocytes (Guillemin et al., 2001; Kiss et al., 2003), which are known to communicate dynamically with glutamatergic neurons via intimate synaptic junctions (Coyle and Schwarcz, 2000; Newman, 2003; Hertz and Zielke, 2004; Araque and Perea, 2004). Thus, the concentration of kynurenic acid within the synaptic cleft could be substantially higher than is inferred from the total concentration in homogenates of brain tissue. This is also supported by our electrophysiological studies utilizing COX inhibitors: a decreased endogenous brain kynurenic acid concentration, as induced by a selective COX-2 inhibitor, was associated with a clear-cut reduction in firing rate and burst firing activity of VTA dopamine neurons. Thus, endogenous brain concentrations of kynurenic acid appear to be of critical importance for maintaining firing activity of VTA dopamine neurons. Such a modulatory function of the compound should not only be of major importance for the functioning of the mesocorticolimbic dopamine pathway but also for glutamatergic neurotransmission in general. Hence, one might speculate that the

compound affects a broad range of fundamental processes related to NMDA receptor functions such as neurodevelopment and synaptic plasticity as well as cognition, behavior and sensory perception.

The activation of midbrain dopamine neurons by kynurenic acid is in all probability related to the glycine/D-serine site of the NMDA receptor. The increase in firing rate and burst firing activity of midbrain dopamine neurons induced by elevated levels of endogenous kynurenic acid or systemic administration of other NMDA receptor antagonists (French et al., 1992, 1993; Zhang et al., 1992) has previously been suggested to be due to an inhibition of a tonic GABAergic input that normally dampens neuronal activity of midbrain dopamine neurons (Erhardt et al., 2002). In support of this view, GABAergic interneurons, as compared to glutamatergic neurons, display a particular vulnerability to NMDA receptor antagonists (Grunze et al., 1996; Li et al., 2002). Thus, the action of COX-1 inhibitors on VTA dopamine neurons would be mediated via the same mechanism. In analogy, decreasing the kynurenic acid concentration, by use of the selective COX-2 inhibitor, should decrease firing of VTA dopamine neurons by promoting GABAergic transmission. Recently, kynurenic acid was suggested to antagonize also α7* nicotinic receptors at low concentrations (Hilmas et al., 2001). However, it is unlikely that such an action of kynurenic acid could account for changes in firing rate of midbrain dopamine neurons observed in the present study. Thus, blockade of the α7* nicotinic receptor with MLA does not affect the activity of VTA dopamine neurons (Schilström et al., 2003; paper IV). Moreover, stimulation of α7* nicotinic receptor is, if anything, associated with an increase of burst firing of VTA dopamine neurons (Schilström et al., 2003).

From our experiments we could see that endogenous brain kynurenic acid is of critical importance for firing activity of midbrain dopamine neurons. Conversely, several studies have shown that stimulation of the dopamine system changes the levels of kynurenic acid in brain. Thus, agents that elevate synaptic dopamine concentration (e.g. amphetamine, L-dopa) as well as drugs that stimulate dopamine receptors (D1 and D2), significantly decrease the levels of kynurenic acid in rat brain (Poeggeler et al., 1998; Rassoulpour et al., 1998; Wu et al., 2001). The mechanism by which dopamine down-regulates this compound is not fully clarified but it has been proposed that the decrease in kynurenic acid levels may be caused by stimulation of astrocytic dopamine receptors (Wu et al., 2002). Such a direct regulation of kynurenic acid metabolism by dopamine may tentatively indicate the existence of a feedback mechanism aiming at lowering levels of kynurenic acid, which in turn would dampen dopaminergic activity and further dopamine release.

An important question is whether increased levels of kynurenic acid are able to induce symptoms similar to those observed in schizophrenia. Patients with schizophrenia have long been considered to have an impaired ability to filter out extraneous stimuli in the environment that would interfere with attention and information processing (Venables, 1960; McGhei and Chapman, 1961; Freedman et al., 1987; Geyer and Braff, 1987). Prepulse inhibition is a behavioral model that reflects such sensory gating deficits in these patients. The cross-species nature of PPI enables the use of animal models to investigate behavioral phenomena that are similar to the deficits seen in schizophrenia. As shown by such studies, administration of NMDA receptor antagonists (e.g. PCP, MK 801, ketamine) can disrupt PPI in rodents (Geyer et al., 1990; Mansbach and Geyer, 1988) and in primates (Javitt and Lindsley, 2001; Linn and Javitt, 2001). In the present thesis we found that elevated levels of endogenous kynurenic acid are associated with a disruption in PPI, an effect that could be reversed by antipsychotic drugs. In line with our results, a previous study has shown that pharmacological elevation of hippocampal kynurenic acid concentration disrupts auditory sensory gating in rats, another measure of gating that is deficient in patients with schizophrenia (Freedman et al., 1987). Since kynurenic acid interacts with both cholinergic and glutamatergic receptors, the ability of kynurenic acid to modulate PPI could theoretically be due to interaction with any of these system. Although the present results do not allow any definitive conclusion regarding the mechanism by which kynurenic acid disrupts PPI we suggest that this compound serves as an endogenous modulator of PPI.

If NMDA receptor hypofunction is involved in the pathophysiology of schizophrenia, then interventions directed at enhancing NMDA receptor function should reduce symptoms. However, it is unlikely that agents that stimulate the recognition site of the NMDA receptor would be suitable candidates in this regard, because of their tendency to produce excessive excitotoxicity (c.f. Introduction). Instead, clinical trials have focused on agents that stimulate the modulatory glycine/D-serine site of the NMDA receptor. Interestingly, clinical treatment augmentation studies have shown that agents acting at the glycine site of the NMDA receptor, such as glycine, D-serine and the partial agonist D-cycloserine, further ameliorate symptoms when used in combination with conventional antipsychotic drugs (Javitt et al., 1994; Tsai et al., 1998; Heresco-Levy et al., 1998, 1999; Evins et al., 2002; see Coyle and Tsai, 2004), but not when added to clozapine (Goff et al., 1996, 1999). Indeed, when D-cycloserine was added to the treatment regimen of patients maintained on clozapine, a significant dose-related exacerbation of negative symptoms was revealed (Goff et al., 1996, 1999). This may be related to the ability of clozapine itself to

increase the NMDA receptor activity to a level that prevents further enhancement by D-serine and glycine treatment. Such a profile of clozapine would also explain why co-administration of D-cycloserine even produces exacerbation of the negative symptoms due to its partial antagonistic action. Indeed, the findings of the present thesis suggest that administration of clozapine leads to a stimulation of the modulatory glycine/D-serine site of the NMDA receptor. Tentatively, clozapine could either directly stimulate the glycine/D-serine of the NMDA receptor or act as an inhibitor of the glycine transporter, resulting in increased synaptic levels of glycine. In support of the latter theory, recent experimental studies have shown that clozapine, but neither haloperidol nor risperidone, inhibits glycine transporters in cortical synaptosomes (Javitt et al., 2005). A stimulatory action of clozapine on NMDA receptors may explain the clinical profile of the drug, i.e. a therapeutic action also on the negative symptoms of the disease since these symptoms also are induced by NMDA receptor antagonists (c.f. Introduction).

Altogether, the present thesis provides evidence that endogenous brain kynurenic acid participates in the pathophysiology of schizophrenia. Further support for this view comes from clinical reports on the side effects of NSAIDs. Thus, several studies have reported that traditional NSAIDs with an inhibitory action on COX-1 and that increase kynurenic acid levels (especially indomethacin), induce psychiatric side effects such as psychosis, delusions and cognitive dysfunctions (see Hoppmann et al., 1991; see Jiang and Chang, 1999; Tharumaratnam et al., 2000; Clunie et al., 2003). If the psychiatric side effects of NSAIDs that interfere with COX-1 could be attributed to increased levels of kynurenic acid, then selective COX-2 inhibitors producing decreased brain kynurenic acid levels would be beneficial in the treatment of schizophrenia. Actually, the selective COX-2 inhibitor celecoxib was recently shown to display antipsychotic effects in patients with an acute exacerbation of schizophrenia with regard to positive, negative (Müller et al., 2002) as well as some cognitive symptoms (Müller et al., 2004) when added to conventional antipsychotic treatment. Thus, the diverse effects on brain kynurenic acid metabolism exerted by COX inhibitors with selectivity for different COX isoforms may provide a rational explanation not only for the psychiatric side effects induced by non-selective COX inhibitors but also for the antipsychotic properties of the COX-2 selective agent celecoxib.

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