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Glutamatergic Mechanisms in Schizophrenia: Role of Endogenous Kynurenic Acid

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ABSTRACT

Kynurenic acid, a tryptohan metabolite synthesised in astrocytes, is an endogenous antagonist at glutamate receptors, in particular it blocks the glycine site of the (NMDA)-receptor, and at the α7* nicotinic receptor. The compound has been found to be elevated in the cerebrospinal fluid (CSF) as well as in the postmortem prefrontal cortex of patients with schizophrenia. Experimental data have shown that acute elevation of brain kynurenic acid is associated with an increased neuronal activity of ventral tegmental area (VTA) dopamine neurons as well as disrupted prepulse inhibition (PPI). The aim of the present thesis was to study the involvement of endogenous kynurenic acid in the pathophysiology of schizophrenia. Thus, the impact of subchronically elevated levels of kynurenic acid on PPI and on the spontaneous firing of VTA dopamine neurons was investigated. Furthermore, a putative interaction between endogenous kynurenic acid and the antipsychotic drugs clozapine and haloperidol on noradrenergic locus coeruleus (LC) neurons was analysed. Finally, kynurenic acid concentration in CSF from healthy controls and male patients with schizophrenia was analysed and CSF kynurenic acid concentration was correlated to the CSF concentrations of monoamine metabolites.

To subchronically elevate endogenous brain kynurenic acid, rats were exposed to kynurenine (the precursor of kynurenic acid; 20 mg/kg/day) and probenecid (a compound that prevents the efflux of kynurenic acid from the brain; 10 mg/kg/day) via subcutaneously implanted osmotic pumps, for 14 days. This treatment increased neuronal firing of VTA dopamine neurons, changed the response of these neurons to systemically administered nicotine (3-400 μg/kg, i.v.) and tended to disrupt PPI.

Clozapine (1.25-10 mg/kg, i.v.) and haloperidol (0.05-0. 8 mg/kg, i.v.) was found to increase the firing rate of LC noradrenergic neurons in control rats. A 2-fold increase in rat brain kynurenic acid levels, by pretreatment with the kynurenine 3-hydroxylase inhibitor PNU 156561A (40 mg/kg, i.v., 3 h), prevented the increase in firing rate of LC noradrenaline neurons induced by haloperidol and clozapine in high doses (2.5-10 mg/kg, i.v.). However, the excitatory action of the lowest dose of clozapine (1.25 mg/kg, i.v.) was not affected by elevated levels of brain kynurenic acid. Furthermore, pretreatment with L-701,324 (4 mg/kg, i.v.) a selective antagonist at the glycine site of the NMDA receptor, prevented the excitatory effects of both clozapine and haloperidol. Our results suggest that the excitation of LC noradrenaline neurons by haloperidol and clozapine involves a glutamatergic component.

Analysis of CSF confirmed that kynurenic acid concentration is elevated in male patients with schizophrenia. Positive correlations were found between kynurenic acid concentration and concentrations of the monoamine metabolites, homovanillic acid (HVA) and 5-hydroxy-indoleacetic acid (5-HIAA), which suggest that increased kynurenic acid formation is associated with an increased dopamine and serotonin turnover.

The results of the present thesis suggest that endogenous kynurenic acid acts a biologically important modulator of glutamatergic neurotransmission within the brain, and lend further support to the hypothesis that endogenous kynurenic acid participates in the pathophysiology of schizophrenia.

LIST OF PUBLICATIONS

I. Nilsson LK, Schwieler L, Engberg G, Linderholm KR, Erhardt S (2005). Activation of noradrenergic locus coeruleus neurons by clozapine and haloperidol: involvement of glutamatergic mechanisms. *International Journal of Neuropsychopharmacology* 8:329-339.

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V. Nilsson LK, Nordin C, Jönsson EG, Skogh E, Erhardt S (2005). Cerebrospinal fluid kynurenic acid in male patients with schizophrenia – correlation with monoamine metabolites. Submitted manuscript.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
cf.	compare (*confer* lat.)
CNS	central nervous system
CSF	cerebrospinal fluid
EAA	excitatory amino acid
e.g.	for example (*exempli gratia* lat.)
GABA	γ-aminobutyric acid
HPLC	high performance liquid chromatography
IDO	indoleamine 2,3-dioxygenase
i.e.	that is (*id est* lat.)
i.p.	intraperitoneal
i.v.	intravenous
KAT	kynurenine amino transferase
mGluR	metabotropic glutamate receptors
LC	locus coeruleus
LP	lumbar puncture
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
PPI	prepulse inhibition
SEM	standard error of the mean
TDO	tryptophan 2,3-dioxygenase
VTA	ventral tegmental area

1 INTRODUCTION

1.1 Schizophrenia

1.1.1 The disease – symptoms

Schizophrenia is a dramatic and disabling psychiatric disorder, almost always of chronic character and with devastating social and psychological impact. Suicide is a major contributor to morbidity and mortality associated with the disease and accounts for approximately 10% of the deaths in patients with schizophrenia (Black and Fisher 1992; see Meltzer et al. 2002). The annual incidence of schizophrenia is 0.2 - 0.4 per 1000, unrelated to gender, with a lifetime prevalence of approximately 1% in the general population (see Carpenter and Buchanan 1994; see Jablensky 1999). The risk among first-degree relatives is approximately 10-fold greater than in the general population (McGue and Gottesman 1991; see Harrison and Owen 2003). This risk is increased to nearly 50% when both parents are affected (McGuffin et al. 1995), and to 60-84% when a monozygotic twin is affected (Cardno et al. 1999). The lack of complete concordance in monozygotic twins indicates that environmental and epigenetic factors also play a role for development of the disease. Thus, although the incidence of schizophrenia is generally claimed to be independent of geographic, ethical and socioeconomic variables (see Carpenter and Buchanan 1994; see Jablensky 1999), it has recently been suggested that poverty and lower social class as well as upbringing in urban areas increases the risk of schizophrenia (Bruce et al. 1991; Peen and Dekker 1997). It also has been found that the rates of schizophrenia are increased in some ethnic minority populations such as second generation of Afro-Caribbean people in the UK (Boydell et al. 2001), Dutch Antillean and Surinamese immigrants in Holland (Selten et al. 1997), and African-American people (Rabkin 1979). Furthermore, the risk of developing schizophrenia is increased by prenatal and perinatal events, including maternal influenza, rubella, malnutrition, diabetes mellitus, and smoking during pregnancy as well as obstetric complications (Susser and Lin 1992; Takei et al. 1996; Thomas et al. 2001).

Schizophrenia is characterised by a multiplicity of symptoms arising from almost all domains of mental function, e.g. language, emotion, reasoning, motor activity and perception. These symptoms vary between patients, thus creating very diverse profiles. The symptoms are commonly divided into three broad types: positive symptoms, negative symptoms and cognitive impairment (see Andreasen 1995). Psychotic (positive) symptoms tend to be episodic over time, often necessitating temporary hospitalisation, while negative

Symptoms of schizophrenia

Positive symptoms:

- Hallucinations (perceptual experiences not shared by others)
- Thought disorder (often manifested as disorganised speech)
- Delusions (false beliefs, e.g. that others can interfere with your thought or that you are Jesus Christ)
- Bizarre behaviour

Negative symptoms:

- Avolition (impaired goal-directed behaviours)
- Flattened affect (e.g. immobile facial expression, monotonous voice)
- Anhedonia (loss of the ability to experience pleasure)
- Alogia (reduced quantity or content of speech)
- Social withdrawal

Cognitive impairments:

- Deficit in attention and concentration
- Deficit in learning and memory
- Lack of insight, judgement
- Lack of executive functions (e.g. abstract thinking, problem solving)

(see Andreasen et al. 1995; see Cutting, 2003; see Fuller et al. 2003; see Goldberg et al. 2003)

symptoms and cognitive problems tend to be more stable over time and contribute significantly to functional impairment, including difficulties managing community living and work (Green et al. 2000).

Schizophrenia typically emerges during adolescence or early adulthood. The disorder usually has a gradual, insidious onset that proceeds over an average of 5 years. A common course is that negative and depressive symptoms appear first, closely followed by cognitive and social impairments, which are then followed by the emergence of psychotic symptoms and first psychiatric contact several years later (Häfner et al. 1999; Häfner et al. 2003).

1.1.2 Antipsychotic drugs

The first widely used therapeutically effective drug in psychiatry was morphine, which was subcutaneously administered to patients in psychiatric hospitals during the 1860s for the rapid control of agitation and aggression (see Ban 2001). Other early pharmacological treatments for schizophrenia include administration of potassium bromide, chloral hydrate, cocaine, manganese, or castor oil or the injection of sulfur oil to induce fever (see Ban 2001). Sleep therapy and insulin coma, which dominated the treatment of schizophrenia from the late 1930s to the mid 1950s, were other strategies (see Ban 2001). Antipsychotic drugs of today have one major property, namely that they interfere with dopaminergic systems of the brain, most commonly by exerting competitive antagonism of brain dopamine receptors, especially at the D_2 receptor (see Carlsson 1988). The antipsychotic actions of the first antipsychotic drug, chlorpromazine, were discovered in

the early 1950s when the drug was tested on patients with schizophrenia in Paris, France (Delay et al. 1952). At about the same time the antihypertensive drug reserpine was introduced as a potential antipsychotic agent in North America (Noce et al. 1954, 1955). These novel possibilities to alleviate psychotic symptoms started a revolution in psychiatric treatment and soon antipsychotic drugs (named neuroleptics for their cataleptic effects in animals) were being administered to patients with schizophrenia all around the world. Approximately a decade after the discovery of the antipsychotic actions of chlorpromazine, the clinical efficacy of antipsychotic drugs was shown to be due to their ability to block dopamine D_2 receptors in the central nervous system (CNS; Carlsson and Lindqvist 1963; Seeman and Lee 1975; Creese et al. 1976). In the late 1950s the compound haloperidol was developed (see Janssen 1970). Haloperidol is often mentioned as an example of traditional antipsychotic drug, and has until recently been the most widely used antipsychotic in the treatment of schizophrenia. Haloperidol has high affinity for the D_2 receptor family of dopamine receptors (D_2, D_3, and D_4, see section 1.4.3), and for the opioid sigma site. Furthermore, it possesses measurable affinity for the 5-HT_{2A}-serotonergic and α_1-noradrenergic receptors, though this is probably not relevant at doses used clinically (Su 1982; Tam and Cook 1984; see Brunello et al. 1995; see Tamminga 2004). Although positive symptoms are reduced in the majority of patients treated with traditional antipsychotics, e.g. haloperidol, these drugs have little or no effect against negative and cognitive symptoms – indeed, they can even cause or exacerbate these symptoms (Delay et al. 1952; Seeman and Lee 1975; Creese et al. 1976; Palao et al. 1994; see King 1998). Moreover, the traditional antipsychotics are associated with problematic extrapyramidal side-effects (EPS), thought to be directly related to D_2 receptor blockade in nigrostriatal pathways (see section 1.4.3).

Extrapyramidal side-effects

- Parkinsonian symptoms
  (eg, muscle stiffness and tremor)
- Akathisia
  (motor restlessness, inability to sit still)
- Dystonia
  (prolonged muscle contraction and disordered muscle tone resulting in twisting body motions)
- Tardive dyskinesia
  (involuntary movements in the extremities, or oral-facial region)

(see Hansen et al 1997)

Clozapine was introduced as an antipsychotic drug in the 1960s (see Hippius 1999; see Capuano et al. 2002), but was removed from the market in 1975 because of its association with fatal agranulocytosis (Idänpään-Heikkilä et al 1977; see Krupp and Barnes 1992). However, following the discovery that clozapine displays efficacy against treatment-

resistant schizophrenia (Claghorn et al. 1987; Kane et al. 1988), and of new technological advancements in haematological monitoring, clozapine was re-introduced to the market in 1990. Comparative studies have since demonstrated the superiority of clozapine over traditional antipsychotic drugs and placebo in alleviating both positive and negative symptoms as well as cognitive deficits (see Baldessarini and Frankenburg 1991; see King 1998; see Markowitz et al. 1999; see Meltzer and McGurk 1999), without producing EPS (see Claghorn et al. 1987; Kane et al. 1988; see Coward et al. 1989; see Lieberman et al. 1991). This unique therapeutic profile of clozapine classifies it as an "atypical" antipsychotic drug and has stimulated the development of novel antipsychotic drugs. However, these novel antipsychotics have other serious side-effects, ranging from weight gain and diabetes to cardiotoxicity (see Gardner et al 2005), and none of them has proven as effective as clozapine. In fact, a recent study on patients with schizophrenia revealed that a majority of the patients (74%) discontinued their prescribed treatment with an antipsychotic drug (not clozapine) within 18 months, due to inefficacy or intolerable side-effects (Lieberman et al. 2005). Such discouraging observations demonstrate the necessity of developing more efficacious treatment for millions of patients suffering from this debilitating illness.

Although many attempts have been made during the past few years to explain the pharmacological mechanism underlying the superior antipsychotic action of clozapine, our knowledge still remains fragmentary. So far it has been shown that clozapine not only exhibits affinity for a variety of dopamine receptors (highest affinity for the D_4 receptor; van Tol et al. 1991), but also for other metabotropic receptors, e.g. those for serotonin (preferentially 5-$HT_{2A,\ 2C}$ and 5-HT_6 5-HT_7) noradrenaline (mainly α_1), acetylcholine and histamine (H_1; see Ashby and Wang 1996). Moreover, previous studies suggest an interaction with some ionotropic receptors, for example, the NMDA receptor (Arvanov et al. 1997; Ossowska et al. 1999; Ninan and Wang 2003) and the $GABA_A$ receptor (Squires and Saederup 1998). In addition, recent findings point to an interaction between clozapine and the glycine site of the NMDA receptor (Schwieler and Erhardt 2003; Schwieler et al. 2004; Javitt et al. 2005). All typical and atypical antipsychotic drugs act as D_2 receptor antagonists, although the symptoms of schizophrenia are hypothesised to arise from a combination of hyperactivity and hypoactivity in dopaminegic pathways (see section 1.6). In view of this imbalance, compounds with partial agonistic actions may offer therapeutic advantages since they can act either as functional agonist or antagonists, depending on the synaptic levels of the endogenous neurotransmitter. In line with this, a recently introduced drug with partial agonist activity on both D_2 and 5-HT_{1A} receptors, aripiprazole (Burris et

al. 2002; Jordan et al. 2002), has shown efficacy against both positive and negative symptoms of schizophrenia as well as low risk of EPS and metabolic and cardiac side-effects (see Lieberman 2004; see Naber and Lambert 2004).

1.2 Excitatory amino acids – glutamate

The most common neurotransmitters in the brain are amino acids, though at first they were not fully accepted as neurotransmitters since they seemed to lack the regional specificity expected for a chemical signalling agent. The major compounds mediating synaptic excitation in the CNS are glutamate and aspartate and they are thus called excitatory amino acids (EAA; see Orrego and Villanueva 1993). Glutamate and aspartate are nonessential amino acids: they can be readily synthesized by the body and are therefore not required in the diet. The glutamate neurons operate in close cooperation with neighbouring astrocytes, which are thought to control extracellular glutamate levels. Thus, once glutamate is released into the synaptic cleft, its clearance is ensured both by diffusion and by uptake through highly specific transporters located on both neurons and astrocytes (see Robinson 1998; see Danbolt et al. 2001). The astrocytes take up extracellular glutamate, convert it into glutamine for temporary storage, and then release it by diffusion to be taken up by the nerve endings and reconverted to glutamate inside the nerve cells (see Danbolt 2001). This trafficking of glutamate and glutamine between astrocytes and neurons has been proposed to be a major pathway by which glutamate is recycled and it is commonly referred to as the glutamine–glutamate cycle (Nicklas et al. 1987). Clearing of glutamate from the synaptic cleft is essential: extracellular glutamate levels must be kept low to ensure a high signal-to-noise (background) ratio and, moreover, excessive activation of glutamate receptors leads to death of central neurons (see Meldrum and Garthwaite 1990; see Choi 1992). Glutamine, in contrast to glutamate, is nontoxic and does not activate glutamate receptors and is therefore thought to serve as a convenient storage reservoir for glutamate. In the brain, glutamate is very likely used by a number of descending pathways and is thought to be involved in numerous functions including cognition, memory, emotions and learning (see Fonnum 1984).

At least four subtypes of excitatory amino acid receptors have been identified, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), kainite and metabotropic receptors (see Ozawa et al. 1998). NMDA, AMPA and kainate receptors (named after their pharmacological agonists) are all ionotropic receptors, which means that they are ligand gated ion channel receptors (figure 1), whereas the metabotropic receptors are coupled to G-proteins and operate through

second messenger systems (see Seeburg 1993; see Ozawa et al. 1998). So far eight different metabotropic glutamate receptors (mGluR) have been discovered in brain (mGluR1 through mGluR8; Schoepp 1994). It is generally presumed that glutamate/aspartate is the endogenous ligand for all of these receptors; however, alternatives such as cysteine and homocysteic acid have also been proposed. Insterestingly, in the late 1980s a naturally occurring antagonist for glutamate receptors, kynurenic acid (see section 1.3), was discovered in the human brain (Moroni et al. 1988a; Turski et al. 1988). In light of the major role of glutamatergic pathways in the modulation of mood, cognition and motor behaviour as well as their reciprocal interactions with monoaminergic networks and their dense innervation of corticolimbic structures, the glutamate system is very likely involved in the pathophysiology of schizophrenia.

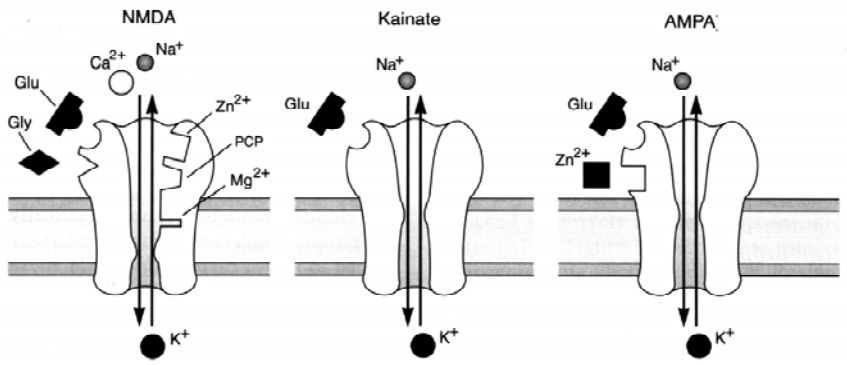


Figure 1. Schematic drawing of the ionotropic excitatory amino acid receptors (Adapted from Kandel et al. 1991).

1.2.1 NMDA receptors

NMDA receptors are present throughout the brain at excitatory synapses and have been implicated in the induction of long term potentiation (LTP), a process associated with learning and memory (see Ozawa et al. 1998). Specialised characteristics of the NMDA receptor include voltage-dependent block by Mg^{2+} (Mayer et al. 1984; Nowak et al. 1984), Ca^{2+} permeability (MacDermott et al. 1986; Mayer and Westbrook 1987) and slow deactivation kinetics (Lester et al. 1990; see McBain and Mayer 1994; see Edmonds et al. 1995). In addition to Ca^{2+}, the NMDA receptor ionchannel is also permeable to Na^{+} and K^{+} (figure. 1; Zarei and Dani 1994). Furthermore, extracellular Zn^{2+} and polyamines act on the receptor to modify its functions (see Danysz and Parsons 1998).

Over the past decade, a variety of NMDA receptor subunits have been identified, including NR1, NR2 (having at least four variants, A, B, C and D) and NR3 (Moriyoshi

et al. 1991; see Hollmann and Heinemann 1994; see Dingledine et al. 1999). All NMDA receptors appear to function as heteromeric complexes composed of multiple NR1 subunits in combination with at least one type of NR2, where the glutamate binding site is localised (see Hollmann and Heinemann 1994; Laube et al. 1997; Anson et al. 1998). The NR3 subunit does not form functional receptors alone, but can co-assemble with NR1/NR2 complexes (Perez-Otano et al. 2001). At resting membrane potential the channel of the NMDA receptor is blocked by Mg^{2+}, which is removed upon depolarisation (see McBain and Mayer 1994). Activation of the NMDA receptor occurs only when glutamate release is combined with postsynaptic depolarisation, to remove Mg^{2+}, and it also requires the presence of glycine or D-serine, which thus function as obligatory NMDA receptor co-agonists (Johnson and Ascher 1987; Kleckner and Dingledine 1988; Matsui et al. 1995; see Snyder and Kim 2000). Glycine and/or D-serine bind to a "strychnine-insensitive" glycine modulatory site on the NMDA receptor (subunit NR1; Hirai et al. 1996), which must be occupied in order for glutamate to open the channel (see Danysz and Parsons 1998). Electrophysiological studies in acute slice preparations from the rat hippocampus and the frontal cortex indicate that the glycine site is not saturated by glycine/D-serine under physiological conditions (Berger et al. 1998; Bergeron et al. 1998; Chen et al 2003; see Millan 2005) and thus, availability of glycine/D-serine at the glycine site plays a critical role in optimal NMDA receptor functioning.

1.3 Kynurenic acid

Kynurenic acid is one of the first identified metabolic products of tryptophan (Ellinger 1904; Homer 1914), which is an amino acid required by all forms of life for protein synthesis and other important metabolic functions. Humans and animals do not possess the enzymatic machinery to synthesise tryptophan and thus, they must ingest it in the form of proteins that can be hydrolysed into the constituent amino acid in the digestive system. The generation of kynurenic acid from tryptophan is part of a sequence of enzymatic steps that was later named the kynurenine pathway (figure 2), after the key compound kynurenine (Beadle et al. 1947; see Heidelberger et al. 1949). Several years after the delineation of this pathway, two independent research groups discovered the presence of kynurenic acid in the human brain (Moroni et al. 1988a; Turski et al. 1988).

1.3.1 Synthesis of kynurenic acid – the kynurenine pathway

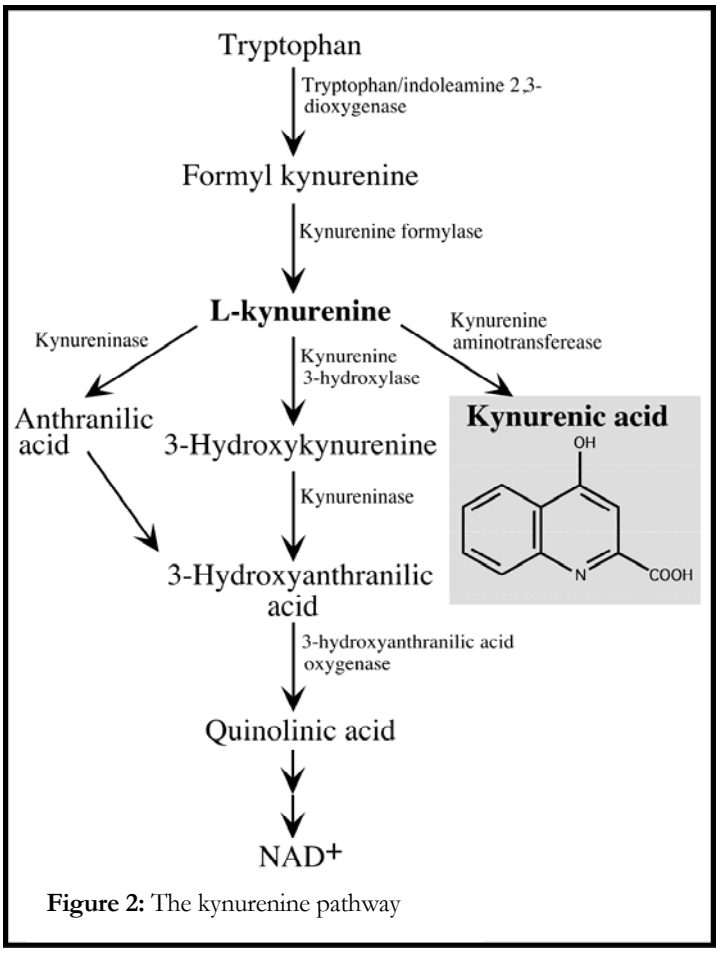


Figure 2: The kynurenine pathway

The first and regulatory step of the kynurenine pathway, oxidative ring opening of tryptophan, is controlled by the two enzymes tryptophan-2,3-dioxygenase (TDO; Hayaishi et al. 1957) and indoleamine-2,3-dioxygenase (IDO; Hayaishi 1976). The product of this reaction is formyl kynurenine, which is rapidly and almost completely converted to L-kynurenine by kynurenine formylase (Mehler and Knox 1950; Gál and Sherman 1978). Kynurenine can be further metabolised by three different enzymes: A. kynurenine 3-hydroxylase which forms 3-hydroxykynurenine, B. kynureninase which forms anthranilic acid and C. kynurenine aminotransferase (KAT) which forms kynurenic acid (see Moroni 1999). Kynurenine 3-hydroxylase has the highest affinity for kynurenine, suggesting that, under normal conditions, it metabolises most of the available kynurenine (Bender and McCreanor 1982; see Moroni 1999). Anthranilic acid and 3-hydroxykynurenine can be metabolised to 3-hydroxyanthranilic acid that can subsequently be converted to quinolinic acid, an excitotoxic NMDA receptor agonist (Stone and Perkins 1981), by 3-hydroxyanthranilic acid oxygenase. Further metabolism along the kynurenine pathway (not shown in the figure) either results in total oxidation of tryptophan, with the generation of adenosine triphosphate and CO_2, or synthesis of nicotinamide adenine dinucleotide (NAD+; see Moffett and Namboodiri 2003). These metabolic processes are catalysed by enzymes only known to be present in hepatocytes. The transamination reaction, which forms kynurenic acid, is irreversible and there is no enzyme that further metabolises the compound. In the mid 1950s, it was suggested that kynurenic acid is further metabolised to quinaldic acid (Takahashi et al. 1956); however, this finding has never been confirmed.

All of the enzymes required for the synthesis of kynurenic acid are present in the brain (Swartz et al. 1990; Guidetti et al. 1995) but their cerebral activity is much lower than in peripheral organs such as the liver and the kidney (see Stone 1993; see Schwarcz and Pellicciari 2002). The kynurenine pathway enzymes kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilic acid oxygenase in the brain have been confirmed to be identical with those expressed in the periphery, using specific antibodies and molecular cloning, (see Schwarcz and Pellicciari, 2002). Comparison of peripheral and central KATs reveals a more complex picture since peripheral organs contain several aminotransferases capable of forming kynurenic acid from kynurenine (Okuno and Kido 1991), whereas only two distinct KATs have been identified in both rat and human brain (Okuno et al 1991a,b; Buchli et al. 1995; Guidetti et al. 1997). These two enzymes, KAT I and KAT II, differ with regard to their pH optimum and substrate specificity. KAT I has a pH optimum of 9.5 to 10 and shows relatively little substrate specificity, whereas KAT II operates best at physiological pH and preferentially recognises kynurenine as a substrate (Schmidt et al. 1993). Thus, at a physiological concentration of kynurenine (i.e. in the low micromolar range) in the brain, KAT II is primarily responsible for its transamination to kynurenic acid (Guidetti et al. 1997). Preliminary results from a recent study provide evidence for a third kynurenic acid-producing enzyme, glutamate-oxaloacetate transaminase, in the mammalian brain (Amori et al. 2005). Whether this enzyme plays a role in cerebral synthesis of kynurenic acid under physiological and/or pathophysiological conditions is still to be investigated.

In the brain, all enzymes of the kynurenine pathway are primarily expressed in astrocytes and/or microglial cells and infiltrating macrophages (Okuno et al. 1991b; Du et al. 1992; Heyes et al. 1996; Guillemin et al. 2001; Kiss et al. 2003; Miller et al. 2004). Formation of kynurenic acid and quinolinic acid seem to be spatially separated: astrocytes do not appear to contain kynurenine 3-hydroxylase and therefore favour the synthesis of kynurenic acid (Guilllemin et al. 2001; Kiss et al. 2003), whereas microglial cells possess very little KAT activity and preferentially form intermediates of the quinolinic acid branch of the pathway (Guillemin et al. 2001; Lehrmann et al. 2001). So far no catabolic enzymes or re-uptake mechanism for kynurenic acid has been detected in glial cells or neurons. Thus, after kynurenic acid has been released from the astrocytes it appears that the only mechanism clearing it from the extracellular space is a probenecid-sensitive carrier system that transports kynurenic acid out of the brain (Moroni et al. 1988a). In accordance, probenecid administration to rats is associated with elevated concentration of endogenous kynurenic acid in brain (e.g. Miller et al. 1992; Russi et al. 1992; Erhardt

et al. 2002a). Furthermore, rapid renal excretion seems to be the single most important mechanism of brain kynurenic acid disposition in the rat (Turski and Schwarcz 1988).

Due to its polar structure, kynurenic acid can only pass the blood-brain-barrier to a limited extent under normal physiological conditions (Fukui et al. 1991; Scharfman and Goodman 1998; see Scharfman et al. 2000), suggesting that peripheral sources do not contribute significantly to brain content of the compound. In contrast, the precursor L-kynurenine can access the brain via the large neutral amino acid transporter (Smith et al. 1987; Fukui et al. 1991), and upon entry into the brain L-kynurenine is transported into astrocytes by a similar Na^+-independent concentrative process (see Christensen 1984; Speciale et al. 1989; Kiss et al. 2003). In fact, although L-kynurenine can be produced in the brain, since both IDO and TDO are present (Gál 1974; Haber et al. 1993; Guillemin et al. 2001; Miller et al. 2004), the cerebral kynurenine pathway is driven mainly by blood-borne L-kynurenine synthesised in peripheral tissues (Gál and Sherman 1978; see Stone 1993). Accordingly, systemic administration of kynurenine results in elevated levels of brain kynurenic acid in both rats (e.g. Swartz et al. 1990; Wu et al. 1992a; Erhardt et al. 2004) and monkeys (Jauch et al. 1993).

Other pharmacological tools to elevate endogenous concentration of kynurenic acid are compounds that inhibit the enzyme kynurenine 3-hydroxylase, e.g. PNU 156561A and Ro 61-8048 (Connick et al. 1992; Russi et al. 1992; Chiarugi et al. 1996; Speciale et al. 1996; Cozzi et al. 1999) also drugs that inhibit cyclooxygenase (COX)-1 (Schwieler et al. 2005a,b). By blocking the major branch of the kynurenine pathway, these compounds increase the availability of kynurenine for transamination to kynurenic acid. In addition, endogenous extracellular kynurenic acid levels rise rapidly and substantially following induced seizures in animal models of epilepsy (Wu and Schwarcz 1996), or in the early phases after an excitotoxic insult (Ceresoli-Borroni et al. 1999). It is also possible to manipulate cerebral kynurenic acid levels in the other direction. Aminooxyacetic acid has been found to reduce brain concentration of kynurenic acid by approximately 50% by inhibiting KAT enzymes (Speciale et al. 1990; Swartz et al. 1990; Wu et al. 1992b; Guidetti et al. 1997). In addition, recent findings demonstrate that decreased concentration of rat brain kynurenic acid can be achieved following systemic administration of drugs that inhibit COX-2, such as parecoxib and meloxicam (Schwieler et al. 2005a,b).

1.3.2 Mechanism of action – physiological significance

At low concentration kynurenic acid acts as a non-competitive receptor antagonist, blocking the strychnine-insensitive glycine recognition site of the NMDA receptor ion channel complex (IC_{50}≈8 μM; Ganong and Cotman 1986; Birch et al. 1988; Kessler et al. 1989; Parson et al. 1997). Moreover, kynurenic acid is able to block the agonist recognition site on the NMDA receptor (IC_{50}=200-500 μM; Kessler et al. 1989), and at even higher concentrations (IC_{50} in the mM range) the compound acts as a competitive antagonist at AMPA/kainite receptors (see Stone and Connick 1985; Bertolino et al. 1989; Kessler et al. 1989). A recent study also shows that kynurenic acid non-competitively blocks the α7* nicotinic receptor with the same IC_{50} value as for the glycine site of the NMDA receptor (Hilmas et al. 2001). The concentration of endogenous kynurenic acid in brain tissue varies significantly between species, reaching approximately 1 μM in humans (Moroni et al. 1988a; Turski et al. 1988) but only 20 nM in rats (Moroni et al. 1988b, Connick et al. 1992, Erhardt et al. 2001b; Erhardt and Engberg 2002; see Erhardt et al. 2003; Erhardt et al. 2004). Thus, control rats as well as rats treated with drugs affecting brain kynurenic acid formation display brain kynurenic acid concentrations in the nM range, which is far below those required to affect NMDA or nicotinic receptors *in vitro*. Unfortunately, at first these observations raised the debate about whether kynurenic acid was of any physiological significance for glutamatergic/nicotinergic neurotransmission. In support of a physiological significance of endogenous kynurenic acid, a moderate elevation of brain kynurenic acid concentration is associated with dramatic effects on the neuronal activity of midbrain dopamine neurons in rats. Thus, previous studies have shown that acutely elevated levels of endogenous kynurenic acid in the rat brain (4-fold, i.e. ≈80 nM) are associated with increased firing of midbrain dopamine neurons (Erhardt et al. 2001b; Erhardt and Engberg 2002; see Erhardt et al. 2003). Moreover, it was recently found that decreased brain kynurenic acid concentration dampens neuronal activity of midbrain dopamine neurons (Schwieler et al. 2005b), implying that kynurenic acid tonically modulates firing of these neurons. Thus, these results demonstrate a physiological role of the compound. Since reduction in brain kynurenic acid levels dampens neuronal activity of VTA dopamine neurons it stands clear that synaptic levels of endogenous kynurenic acid are sufficient to antagonise central glutamatergic receptors in spite of the low endogenous levels of the compound in rat whole-brain (Schwieler et al. 2005b). A tentative explanation for this paradox could be that kynurenic acid is synthesised in and released from astrocytes (Curatolo et al. 1996; Guillemin et al. 2001; Kiss et al. 2003) that surround glutamatergic synapses in a highly intimate manner (Harris and Rosenberg 1993; Roberts et al. 1995; see

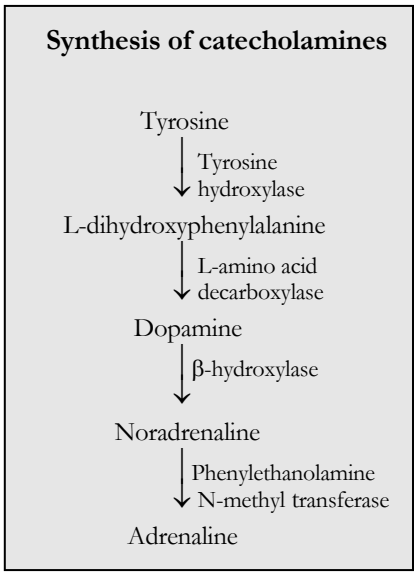
Coyle and Schwarcz 2000; see Newman 2003). Thus, released kynurenic acid might be encapsulated inside the synaptic cleft resulting in substantially higher concentration in this limited compartment of the brain.

Kynurenic acid displays anticonvulsant and neuroprotective properties (Foster et al. 1984; Hicks et al. 1994; Harris et al. 1998; Cozzi et al. 1999; see Moroni 1999), actions probably related to its interaction with glutamatergic receptors. In addition, an abnormal kynurenic acid metabolism has been suggested in a variety of human diseases such as amyotrophic lateral sclerosis (ALS, where kynurenic acid levels in cerebrospinal fluid (CSF) are decreased; Ilzecka et al. 2003), neonatal asphyxia (rat brain kynurenic acid decreased; Ceresoli-Borroni and Schwarcz 2001), chronic inflammatory bowel disease (serum kynurenic acid increased; Forrest et al. 2002), Alzheimer`s disease (brain kynurenic acid increased; Baran et al. 1999), Down`s syndrome (cerebral cortex kynurenic acid increased; Baran et al. 1996), eating disorder (reduced CSF kynurenic acid; Demitrack et al. 1995), HVA-1 (brain kynurenic acid increased; Baran et al. 2000) and schizophrenia (CSF kynurenic acid increased; Erhardt et al. 2001, postmortem cerebral cortex kynurenic acid increased; Schwarcz et al. 2001).

1.4 Catecholamines

1.4.1 Background

Catecholamines are neurotransmitters (dopamine, noradrenaline and adrenaline) named after their chemical structure, i.e. they all contain a nucleus of catechol (a benzene ring possessing two adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives. Noradrenaline was identified in 1946 by Ulf von Euler; it was found in the brain a few years later (Holtz 1950), and was thereafter suggested to be a neurotransmitter within central neurons (Vogt 1954). That dopamine acts as an independent neurotransmitter, and is not merely an intermediate in the biosynthesis of noradrenaline and adrenaline, was discovered in the late 1950s (Carlsson et al. 1957, 1958; Carlsson 1959). By the 1960s, Hillarp and co-workers had developed histofluorescence staining techniques that allowed the visualisation of catecholamine-containing cell bodies and enabled mapping of catecholaminergic pathways



throughout the brain (Falck et al. 1962; see Carlsson 1987). Once mapped, the various noradrenergic and dopaminergic nuclei were designated with the letter codes A1-A7 and A8-A15, respectively (Dahlström and Fuxe 1964).

1.4.2 Noradrenergic systems

Axons form noradrenaline-containing neurons are distributed widely throughout the CNS, from spinal cord to neocortex, suggesting a prominent role of this neurotransmitter in central brain functions (Fuxe 1965; Ungerstedt 1971; Lindvall and Björklund 1974; Swanson and Hartman 1975). The noradrenergic cell bodies, however, are restricted to a number of small clusters (A1-A7) in the pons and medulla (Dahlström and Fuxe 1964), of which the majority are concentrated in the nucleus locus coeruleus (LC; A6). This small cell group is located adjacent to the fourth ventricle in the grey matter of the pons, and contains on each side of the brain approximately 12,000 neurons in humans and 1,500 neurons in the rat (Dahlström and Fuxe 1964). The LC has long been implicated in the regulation of states of alertness and in enhancing the ability of the individual to react adaptively to sensory environmental stimuli (Foote et al. 1991). Single cell activity of the noradrenergic LC neurons is well correlated with vigilance and is highest in alert or attentive waking state, moderate in drowsy wakefulness, low in slow-wave sleep state, and falls to zero during rapid eye movement sleep (Hobson et al. 1975; Foot et al. 1980; Aston-Jones and Bloom 1981a). The LC densely innervates the prefrontal cortex and is the primary source of an extensive, yet regionally specialised, noradrenergic innervation of the forebrain (see Berridge and Waterhouse 2003). Interestingly, the LC provides the sole source of noradrenaline to hippocampus and neocortex, two regions of importance for higher cognitive and affective processes (see Berridge and Waterhouse 2003) and has been suggested to be involved in task-related cognitive processes (Usher et al. 1999).

The receptors for noradrenaline (and adrenaline) are called adrenergic receptors (or alternatively adrenoceptors) and belong to the G-protein coupled receptor family. Adrenergic receptors are widely distributed in the CNS (see Nicholas et al. 1996; Day et al. 1997) and are divided into two classes, α and β receptors (Ahlquist 1948), which are further subdivided into $\alpha_{1A,B,D}$, $\alpha_{2A,B,C}$ and β_{1-3} subtypes (see Bylund 1988; see Docherty 1998). Certain functional characteristics of the noradrenergic system in the brain, especially its involvement in cognition, vigilance and attention, make it interesting in relation to schizophrenia, and thus, noradrenaline is suggested to play an important role for the symptoms and cognitive deficits of the disease (Fields et al. 1988; van Kammen and Kelly 1991; Breier 1999; see Arnsten 2004; see Yamamoto and Hornykiewicz 2004).

Anatomical and physiological studies have shown that the core of the LC receives its major afferent projection from the nucleus paragiganto-cellularis and nucleus prepositus hypoglossi, whereas the shell appears to be innervated by a variety of extrinsic afferent inputs (Aston-Jones et al. 1986; see Ennis et al. 1998). The innervation of the LC from nucleus paragiganto-cellularis is predominantly excitatory and these effects are mediated via glutamate (Ennis and Aston-Jones 1986, 1988; Chen and Engberg 1989), whereas the afferentiation from nucleus prepositus hypoglossi is of pure inhibitory, γ-aminobutyric acid (GABA)-ergic origin (Chen and Engberg 1989; Ennis and Aston-Jones 1989). The typical response of LC neurons to toe pinch, i.e. an instantaneous, short lasting excitation followed by a quiescent interval (Korf et al. 1974), is mediated via release of excitatory amino acids from nucleus paragiganto-cellularis nerve terminals (Ennis and Aston-Jones 1988). Moreover, anatomical and electrophysiological studies have revealed that the prefrontal cortex is a major source of afferent drive to the LC and it activates the LC via release of glutamate (Arnsten and Goldman-Rakic 1984, Jodo and Aston-Jones 1997, Jodo et al. 1998). The existence of this connection links circuits involved in higher cognitive and affective processes with the LC-efferent pathways. It also has been shown that the basal activity of the LC noradrenergic neurons is controlled by local α_2 receptors, probably via noradrenergic, recurrent axon collaterals within the LC (Aghajanian et al. 1977; Engberg 1981).

In both anaesthetised and awake animals, noradrenaline-containing LC neurons are characterised electrophysiologically by slow, spontaneous firing rates (1-5 Hz), broad action potential waveforms (1-2 ms) and burst firing activity that is followed by a prolonged period of quiescence or decreased firing activity (Aghajanian et al. 1977). A typical characteristic is the response to a noxious stimulus by a burst of activity followed by a quiescent interval. In a state of awakeness, firing comprised of a brief burst of 2-3 action potentials (15-70 ms in rat) followed by a more prolonged period of suppression of firing activity (approximately 300-700 ms) has been observed. This activity mode is associated with overt attending to novel stimulus and with sustained attention in tests of vigilance using conditioned stimuli (Aston-Jones et al. 1994; see Berridge and Waterhouse 2003).

1.4.3 Dopaminergic systems

In contrast to the widespread noradrenergic systems of the brain, the dopaminergic systems are restricted to three major ascending pathways. The cell bodies of these dopamine pathways are located in the substantia nigra (SN; A9), the ventral tegmental area

(VTA; A10) and in the arcuate and periventricular hypothalamic nuclei (A12, A14; Dahlström and Fuxe 1964) The neurons of the substantia nigra project, via the median forebrain bundle and then the internal capsule, to the dorsal part of the striatum, i.e. the caudate and the putamen, and hence this projection is referred to as the nigrostriatal dopamine pathway (Andén et al. 1964). This pathway is of critical importance for motor function, and degeneration of this system is the underlying cause of Parkinson`s disease (Carlsson 1959; Ehringer and Hornkiewicz 1960). The dopamine neurons of the VTA project, via the median forebrain bundle, to both subcortical (limbic) and cortical areas, forming a mesolimbocortical dopamine pathway. Based on the different projections the mesolimbocortical pathway is further subdivided into the mesolimbic dopamine system, projecting to the amygdaloid complex, the nucleus accumbens, the olfactory tubercle and the septal area, and into the mesocortical dopamine system, projecting to areas in the prefrontal cortex, such as prelimbic, infralimbic, and cingule cortices (Dahlström and Fuxe 1964; Andén et al. 1966; Ungerstedt 1971; see Moore and Bloom 1978; Björklund and Lindvall 1984). Several processes, for example motivation, control of emotions (e.g. reward and reinforcement), and cognition are attributed to the mesocorticolimbic dopamine system (see Fibiger and Phillips 1988; see Le Moal and Simon 1991; Schultz et al. 1993; see Schultz 1998). The tuberohypophyseal dopamine pathway is involved in endocrine control. This pathway originates in the hypothalamus, and projects to the median eminence and the intermediate and posterior lobes of the pituitary (see Moore and Bloom 1978).

Based on their pharmacology and coupling to adenylyl cyclase, the dopamine receptors are divided into D_1–like receptors and D_2–like receptors. All dopamine receptors belong to the family of G-protein coupled transmembrane receptors (Kebabian and Calne 1979; Bunzow et al. 1988). The D_1 family, which consists of D_1 and D_5 receptors, stimulates adenylyl cyclase, whereas the D_2 family, which consists of D_2, D_3 and D_4 receptors, inhibits adenylyl cyclase (see Jaber et al. 1996). Both D_1 and D_2 like receptors are localised pre- and postsynaptically. The presynaptic autoreceptors belong to the D_2-receptor family (see Stoof and Kebabian 1984; White and Wang 1984). It appears that D_2 receptors are more predominantly expressed in areas associated with motor control, while D_3- and D_4 receptors are preferentially located in areas where the dopamine systems are though to serve a role in modulating emotion and cognition. In addition, the D_1 receptors have been attributed a major role in cognitive functions (see Goldman-Rakic et al. 2004).

Spontaneous firing of midbrain dopamine neurons in the anaesthetised as well as in the freely moving rat is characterised by triphasic and broad action potential

waveforms (>2.0 ms), with an inflection in the rising phase. There are two basic modes of firing of these neurons, single spike firing and burst firing (see Wang 1981; Grace and Bunney 1984a, b; Clark and Chiodo 1988). Single spike firing is relatively regular and occurs at a low frequency (1-10 Hz), while burst firing is characterised by a high-frequency discharge of multiple action potentials with short interspike intervals. Spikes within the bursts exhibit progressively decreasing spike amplitude as well as increased spike duration. Furthermore, the burst is followed by a quiescent period before spiking re-starts (Wang 1981; Grace and Bunney 1984b; Clark and Chiodo 1988; Grenhoff et al. 1988). According to the conventional criteria (e.g. those of Grace and Bunney 1984b; Clark and Chiodo, 1988) up to 50% of spontaneously active midbrain dopamine neurons in the anaesthetised rat exhibit this burst firing pattern, but in a study of freely moving rats over 90% of the VTA dopamine neurons were found to exhibit burst firing (Freeman and Bunney 1987). This discrepancy suggests that bursting is related to either the degree of sensory stimulation (which should be greater in the freely moving rat), or to an interaction between movement and stimulation. The firing pattern of the midbrain dopamine neurons appears to be fundamental for transmitter release in terminal areas, where burst firing activity might be seen as the major phasic response and single spike firing may represent a basal endogenous dopaminergic tone (see Gonon 1988; Bean and Roth 1991; Manley et al. 1992). Thus, a switch from single spike mode to burst firing mode is associated with a massive release of dopamine in terminal areas (Gonon 1988) and when burst firing activity is dampened the release will decrease as well (Nissbrandt et al. 1994). Furthermore, burst firing of midbrain dopamine neurons is induced by primary food and fluid rewards as well as reward-predicting stimuli in behaving monkeys (Schultz et al. 1993; see Schultz 1998). There are significant differences between spontaneous firing of VTA dopamine neurons *in vivo* and *in vitro*. For example, burst firing is not seen in slice preparations. This suggests that afferent inputs play a role in modulating the activity, particularly the firing pattern, of these neurons (Sanghera et al. 1984; Grace and Onn 1989; Seutin et al. 1990; Johnson et al. 1992).

The firing of VTA dopamine neurons appears to be physiologically regulated mainly by simultaneous inhibitory GABAergic and stimulatory glutamatergic inputs to the VTA. The GABAergic input to the dopamine neurons in the VTA originates from either intrinsic interneurons within the VTA (Beart and McDonald 1980; see Kalivas 1993; Van Bockstaele and Pickel 1995) or from extrinsic sources, so called long-loop GABAergic systems, originating from rostral areas including the nucleus accumbens and the ventral pallidum (figure 3; Walaas and Fonnum 1980; Yim and Mogenson 1980;

Kalivas et al. 1993). The intrinsic GABAergic neurons synapse to dopamine neurons in the VTA (O'Brien and White 1987; Bayer and Pickel 1991) and in addition send axon projections to the nucleus accumbens and to the prefrontal cortex (Thierry et al. 1980; see Kalivas 1993; Van Bockstaele and Pickel 1995; Steffensen et al. 1998; Carr and Sesack 2000a). The influence of GABA on the dopaminergic neurons is exerted via $GABA_A$ and $GABA_B$ receptors, both expressed on VTA dopamine neurons (e.g. Churchill et al. 1992; Wirtshafter and Sheppard 2001).

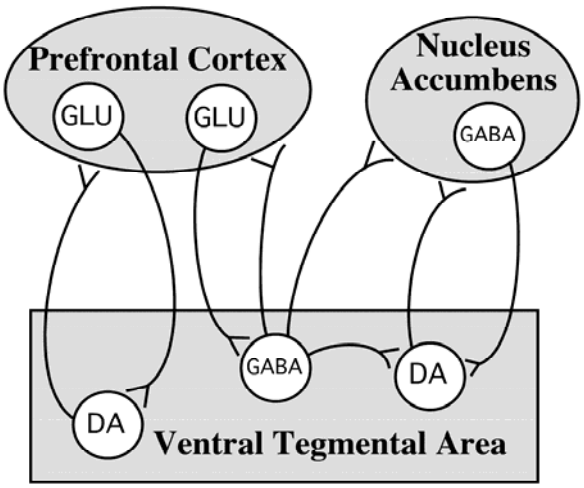


Figure 3. Schematic drawing of the connections between the prefrontal cortex, nucleus accumbens and the VTA. DA=dopamine, GLU=glutamate.

In vivo electrophysiological studies on midbrain (VTA and substantia nigra) dopamine neurons have made the paradoxical observation that systemically administered $GABA_A$ agonists stimulate cell firing (MacNeil et al. 1978; Grace and Bunney 1979; Waszczak and Walters 1980; Waszczak et al. 1980; Erhardt and Engberg 2000). Since systemic administration of $GABA_A$ agonists is also associated with a decrease in firing frequency of non-dopamine cells, presumably GABAergic, their excitatory actions on dopamine neurons have previously been attributed to disinhibition of these cells (Grace and Bunney 1979; Waszczak et al. 1980). However, it has recently been suggested that the excitatory actions of $GABA_A$ agonists on midbrain dopamine neurons are mediated via release of glutamate (Erhardt and Engberg 2000). Furthermore, systemic administration of $GABA_B$ receptor antagonists has been shown to increase the activity of midbrain dopamine neurons, whereas systemic administration of the $GABA_B$ agonist baclofen is associated with a cessation of firing rate and burst firing activity of midbrain dopamine neurons (Erhardt et al. 1998, 1999, 2002b). In addition, local administration of baclofen into VTA is associated with a reduction in dopamine cell firing (see Lacey 1993), and subsequently decreased somatodendritic (Klitenick et al. 1992; Yoshida et al. 1994; Giorgetti et al. 2002) as well as terminal (Westerink et al. 1996; Westerink et al. 1998; Xi and Stein 1999) dopamine release. This inhibitory action of baclofen may be accounted for by stimulation of $GABA_B$ receptors located on dopamine and/or glutamate neurons (Wu et al. 1999). The intra-VTA perfusion of $GABA_B$ receptor

antagonists appears to increase local dopamine release (Giorgetti et al. 2002), which suggests that these receptors exert a tonic inhibitory control over somatodendritic release of dopamine.

The glutamatergic innervation of the VTA originates mainly from the prefrontal cortex (Carr and Sesack 2000b; figure 3) but also from the pedunculopontine nucleus (Charara et al. 1996). In accordance, stimulation of the prefrontal cortex increases burst firing activity of VTA dopamine neurons (Gariano and Groves 1988; Murase et al. 1993a; Tong et al. 1996), whereas inactivation of the prefrontal cortex decreases burst firing activity (Svensson and Tung 1989; Murase et al. 1993a). These effects might be mediated by a monosynaptic projection from the prefrontal cortex to the dopamine neurons in the VTA (Sesack and Pickel 1992). Furthermore, it has been shown that excitatory glutamatergic inputs from the prefrontal cortex to the VTA synapse not only on dopaminergic cells that project back to the prefrontal cortex, but also onto GABA interneurons and on GABAergic cells that project to the nucleus accumbens (figure 3; Bonci and Malenka 1999; Carr and Sesack 2000b). Hereby, the prefrontal cortex may alter the neuronal activity of VTA dopamine neurons both directly, by synapsing on dopamine neurons projecting back to the prefrontal cortex, and indirectly, via influence on GABAergic interneurons that directly or via contact with other dopamine cells project to the nucleus accumbens.

The excitatory effects of glutamate can be meditated by ionotropic as well as metabotropic glutamate receptors (see section 1.2), which are present in the VTA (Seutin et al. 1990; Albin et al. 1992; Paquet et al. 1997). The activity of the dopamine neurons in the VTA can also be modulated by noradrenergic afferents originating in the LC (Phillipson 1979; Simon et al. 1979; Hervé et al. 1982; Grenhoff et al. 1993, 1995 Grenhoff and Svensson 1993) and by serotonergic input from the median and dorsal raphe nuclei (Phillipson 1979; Hervé et al. 1987; see Broderick and Phelix 1997; Vertes et al. 1999). There is also an additional excitatory cholinergic input, arising from the pedunculopontine and laterodorsal tegmental nuclei (Clarke et al. 1987; Oakman et al. 1995; Blaha et al. 1996), acting on muscarinic and nicotinic acetylcholinergic receptors within the VTA.

1.5 Human cerebrospinal fluid studies

1.5.1 General aspects

The living human brain is not readily accessible for functional investigations. One of the few alternative approaches is to study the CSF. Before the development of modern methods, e.g. positron emission tomography (PET) and single photon emission computerized tomography (SPECT), lumbar puncture (LP) was the only method for studying the working brain. The first publications describing puncture of the human lumbar sac are from the late eighteen hundreds and the purpose of these experiments was to find means to reduce abnormally elevated cerebrospinal pressure (Quincke 1891; Wynter 1891). In modern everyday medicine LP is a diagnostic tool and mainly used for investigations of infectious and neurological diseases of the brain and spinal cord.

CSF is a transparent, colourless liquid consisting of 99% water; however, over 100 compounds have been identified in the remaining 1% (Geigy Scientific Tables 1981). The total volume of CSF in the cerebrospinal system is estimated to approximately 140 mL and the rate of formation to about 500 mL every 24 h (Geigy Scientific Tables 1981; Kandel et al. 2000), which gives a turnover of 3-4 times a day. Although the production of CSF has been reported to decrease with age (May et al. 1990) and to be reduced in patients with Alzheimer's disease (Silverberg et al. 2001), very few studies have investigated CSF production and turnover in relation to age and disease in humans.

Several confounding factors may influence the concentration of a measured compound in CSF, including age and the individual´s characteristics (e.g. body height, back length and body weight; Wode-Helgodt and Sedvall 1978; Nordin et al. 1987, 1993, 1995; Blennow et al 1993; Eklundh et al. 1996; Jönsson et al. 1996) as well as methodological factors and external conditions at time of LP (see Eklundh 2000). The expression "back length", also known as the neuroaxis distance, is used for the distance measured between the site of puncture and the external occipital protuberance (Eklundh et al. 1996; Eklundh et al. 2001). The use of the back length parameter is an attempt to describe a more relevant measure of the length of the spinal compartment than body height, which to a great extent stems from the length of the legs, and less from the head and the trunk (Wode-Helgodt and Sedvall 1978).

A limitation of studies requiring invasive experiments such as LP is the availability of healthy volunteers, and thus some investigators use patients with neurological and medical complaints as control subjects. Furthermore, judging from many studies of human CSF, it could be questioned to what extent "healthy volunteers" represent a cross-section of the

population, since invasive experiments may attract certain personalities (Gustavsson et al. 1997). Considering all the inconsistent results from CSF studies some attempts have been made to create a standardised method for the LP procedure (Bertilsson and Åsberg 1984).

1.5.2 Monoamine metabolites and glutamate in the cerebrospinal fluid in schizophrenia

In order to investigate the pathophysiology of schizophrenia, CSF from patients with schizophrenia has been extensively analysed. To study dysregulations of the monoamine system a common approach is to analyse CSF concentrations of the monoamine metabolites, since the actual neurotransmitters are present in extremely low concentration in the CSF and therefore difficult to measure (see e.g. Scheinin 1985). The most frequently studied metabolites in psychiatric disorders are homovanillic acid (HVA), the main metabolite of dopamine; 5-hydroxy-indoleacetic acid (5-HIAA), the main metabolite of 5-hydroxytryptamine (5-HT; serotonin); and 4-hydroxy-3-methoxyphenylglycol (HMPG), the main metabolite of noradrenaline. However, investigations regarding CSF monoamine metabolites in patients with schizophrenia have shown mixed results and thus not revealed any consistent aberration from levels found in control groups (Persson and Roos, 1969; Post et al. 1975; Berger et al. 1980; Nybäck et al. 1983; Bjerkenstedt et al. 1985; Lindström 1985; Hsiao et al. 1993; Wieselgren and Lindström 1998).

In search of evidence for the involvement of glutamate in schizophrenia, several investigators have also examined the concentration of this compound in CSF. An initial report by Kim and co-workers (1980) show lower concentration of glutamate in 20 patients with schizophrenia compared to 44 control subjects. This studie was among the first to propose that schizophrenia might involve a glutamatergic deficit. Limitations of this study include the fact that the patients used a variety of psychotic medications and that most of the patients were women (18 of 20), whereas the control group consisted of equal numbers of male and female subjects. In addition, the control group contained a heterogeneous sample of individuals, not only healthy volunteers but also people with neurological and medical complaints. Taken together, these circumstances make it difficult to appreciate the significance of the difference between patients with schizophrenia and control subjects that was observed in the study. Following this initial study several investigators have tried to replicate these findings but have so far been unable to detect a reduced glutamate concentration in CSF from patients with schizophrenia compared to healthy control subjects (Gattaz et al. 1982; Korpi et al. 1987; Perry et al. 1992) Rather, an inverse correlation between the severity of schizophrenia and CSF concentration of glutamate has been found (Faustman et al. 1999). Interestingly though, it has been shown

that the ratio of D-serine (the co-agonist for the NMDA receptor) to total serine in CSF from first episode, drug naïve, patients with schizophrenia is significantly decreased as compared to healthy controls (Hashimoto et al. 2005). Altogether, the few findings connecting changes in CSF glutamate or monoamine concentrations to psychiatric disorders have been replicated, and their clinical implications have thus been restricted.

1.5.3 Kynurenic acid in the cerebrospinal fluid

The concentration of kynurenic acid in human CSF has not been investigated to any great extent, partly because its presence in the human brain was not discovered until the late 1980s (Moroni et a. 1988a; Turski et al. 1988). What has been shown so far is that the kynurenic acid concentration in CSF is elevated in patients with amyotrophic lateral sclerosis (ALS; Ilzecka et al. 2003), decreased in CSF of patients with relapsing-onset multiple sclerosis (Rejdak et al. 2002) as well as in patients with eating disorder (Demitrack et al. 1995). A previous study from our group revealed that KYNA concentration is elevated in CSF of patients with schizophrenia, compared to healthy controls (Erhardt et al. 2001a). This study included 17 control subjects and 28 patients with schizophrenia, where the majority were drug naïve, first episode patients, but also patients treated with antipsychotic drugs were included.

1.6 Hypotheses of schizophrenia

A large body of evidence suggests that alterations in several neurotransmitter systems are involved in pathophysiological processes leading to the development of schizophrenic symptoms. Among these, the dopamine and glutamate systems have received most attention (see Javitt and Zukin 1991; see Jentsch and Roth 1999; see Carlsson et al. 2001), although other systems such as GABAergic, serotonergic, cholinergic or opioid systems have also been implicated. Although there have been impressive discoveries in the field of schizophrenia research over the past decades, the etiology and fundamental pathophysiology of the disease remains unclear. Thus, research to gain increased knowledge about neurochemical processes responsible for symptom manifestation is of great importance.

1.6.1 The dopamine hypothesis

The implication of dopamine in the pathophysiology of schizophrenia is originally based on elucidation of the pharmacological mechanisms of agents known to alleviate symptoms

of the disease. Findings that the clinical potency of antipsychotic drugs was positively correlated to their ability to block dopamine D_2 receptors (Seeman and Lee 1975; Creese et al. 1976) and that dopamine-releasing drugs can induce psychosis (Snyder 1973; see Angrist and van Kammen 1984) led to the association between striatal dopaminergic hyperactivity and positive symptoms, or "the dopamine hypothesis of schizophrenia" (Carlsson and Lindqvist 1963; see Carlsson 1988, see Carlsson et al. 2001). In recent years, data from brain imaging studies have provided further support for an abnormal striatal dopamine activity in schizophrenia. Thus, increased striatal dopamine synthesis (Reith et al. 1994; Lindström et al. 1999) and baseline dopamine release, at least during phases of exacerbation of psychosis (Abi-Dargham et al. 2000), have been found in patients with schizophrenia compared to control subjects. In addition, it has been shown that the change in release of dopamine after amphetamine administration is greater in patients with schizophrenia than in controls (Laruelle et al. 1996; Breier et al. 1997; Abi-Dargham et al. 1998; see Abi-Dargham and Laruelle 2005).

Over the years, clinical observations have pointed to both the strength and the limitations of the dopamine hypothesis of schizophrenia. For a subgroup of patients, especially those with pronounced positive symptoms, the hypothesis has proven to be sound concerning symptoms and treatment. For other patients – those with prominent negative symptoms and impaired cognitive functioning – the hypothesis is not as applicable and these patients improve little, if at all, when treated with traditional antipsychotic drugs (Breier 1999; King 1998). During the last few years, such clinical observations have been an incitement to reformulate the original dopamine hypothesis as a dopamine imbalance theory (see Abi-Dargham and Laruelle 2005). The dopamine imbalance hypothesis proposes that schizophrenia is associated with a persistent deficit in prefrontal cortical dopamine functions, which results in decreased signalling via D_1 receptors in this area (contributing to the negative symptoms and cognitive impairments), and an intermittent excess of subcortical dopamine functions involving D_2 receptors (contributing to the positive symptoms; see Davis et al. 1991; see Jentsch and Roth 1999; see Abi-Dargham and Laruelle 2005). Today it is generally accepted that the symptoms of schizophrenia are related to dopamine; nevertheless the primary cause of the disease remains to be reveled.

1.6.2 The glutamate deficiency theory

In recent years progress in the field of schizophrenia research has led to the suggestion that dopamine only plays an intermediary role in the pathophysiology of the disease and

that the main abnormalities of the disease lie elsewhere (see Carlsson et al. 2001). In particular an attenuated glutamatergic neurotransmission is believed to participate in the pathogenesis of schizophrenia (see Javitt and Zukin 1991; see Jentsch and Roth 1999). The glutamate deficiency hypothesis of schizophrenia arose from clinical observations that dissociative anaesthetics e.g. phencyclidine (PCP) and ketamine produce symptoms clinically indistinguishable from schizophrenia (Luby et al. 1959; Itil et al. 1967; Adler et al. 1999). PCP was first developed as a surgical anaesthetic in the late 1950s (Greifenstein et al. 1958; Johnstone et al. 1959) but despite its useful efficacy, widespread use was limited because, after surgery, the patients experienced hallucinations, disordered speech, delirium, agitation and disorientatation (see Pradhan 1984). In spite of the observations that PCP induces psychosis in humans, it became a popular drug of abuse within certain subcultures by the early 1960s and, subsequently, chronic PCP abusers have commonly been misdiagnosed as being schizophrenic. At subanaesthetic doses (0.05-0.1 mg/kg), PCP is a non-competitive NMDA receptor antagonist (Vincent et al. 1979; Zukin and Zukin 1979; Anis et al. 1983; Thomson et al. 1985), whereas at higher and lethal doses it also inhibits reuptake of dopamine and blocks the □igma opioid receptor. Several studies also have revealed that other non-competitive NMDA receptor antagonists, e.g. ketamine, can actually induce positive and negative symptoms of schizophrenia as well as cognitive dysfunction in healthy humans (see Javitt and Zukin 1991; Krystal et al. 1994; see Tamminga 1998; see Jentsch and Roth 1999) and, furthermore, exacerbate both positive and negative symptoms in patients with schizophrenia (Luby et al. 1959; Itil et al. 1967; Malhotra et al. 1997; see Jentsch and Roth 1999). These non-competitive NMDA receptor antagonists are so called open channel blockers that act on a binding site within the NMDA receptor ion channel (see Javitt and Zukin 1991). Furthermore, it has been shown that also competitive NMDA receptor antagonists as well as blockers of the glycine site can induce schizophrenia-like symptoms (Kristensen et al. 1992; Grotta et al. 1995; Yenari et al. 1998; Albers et al. 1999). In electrophysiological experiments in rats, systemic administration of non-competitive NMDA receptor antagonists is associated with an increase in firing rate and burst firing activity of midbrain dopamine neurons (French et al. 1993; Murase et al. 1993b; French 1994; Erhardt and Engberg 2000; Schwieler et al. 2004). This paradoxical activation is probably mediated via inhibition of tonic GABAergic inputs (Zhang et al. 1993), since GABAergic neurons, as compared to glutamatergic pyramidal neurons, seem to display a particular vulnerability to NMDA receptor antagonism (Grunze et al. 1996; Li et al. 2002). Interestingly, subchronic administration of PCP to rats has been shown to produce increased

dopamine release in mesolimbic areas, such as the nucleus accumbens (Jentsch et al. 1998a; see Jentsch and Roth 1999), and a reduced release in the frontal cortex (Jentsch et al. 1997, 1998b,c, 1999; see Jentsch and Roth 1999). Since the symptoms of schizophrenia are suggested to originate from dopaminegic imbalance, i.e. deficit in prefrontal cortical dopamine and excess of subcortical dopamine, subchronic administration of PCP might serve as a pharmacological model for schizophrenia. In support of a glutamate deficiency in schizophrenia, an initial report by Kim and co-workers (1980) shows lower concentration of glutamate in the CSF of patients with schizophrenia compared to controls, although subsequent studies have not been unable to replicate these findings (see section 1.5.2). Thus, direct evidence for a glutamate/NMDA receptor dysfunction in schizophrenia is still missing, and the lack of adequate radioligands to visualise the glutamate system in the living brain is a major obstacle to investigations of the glutamate deficiency theory of the disease. However, indirect support for the hypothesis is that adjunctive treatment with glycine or D-serine has beneficial effects particularly on the negative symptoms of schizophrenia (see Tuominen 2005).

1.6.3 The kynurenic acid hypothesis

Because kynurenic acid is an endogenous NMDA receptor antagonist, its role in the pathophysiology of schizophrenia has in recent years gained increased attention. The kynurenic acid hypothesis of schizophrenia is partly based on the findings that patients with schizophrenia display elevated levels of kynurenic acid in the CSF (approximately 1.67 nM vs. 0.97 nM in controls; Erhardt et al. 2001a) and in the postmortem prefrontal cortex (2.9 pmol/mg protein vs. 1.9 pmol/mg protein in controls; Schwarcz et al. 2001). An explanation for these findings might be that fact that the expression of the enzyme TDO, which catalyses the first step of the kynurenine pathway, is increased in the postmortem prefrontal cortex from patients with schizophrenia (Miller et al. 2004).

Furthermore, rat studies have shown that an acute increase in the levels of kynurenic acid in the brain (4-fold) disrupts prepulse inhibition (PPI; Erhardt et al. 2004), in similarity to administration of NMDA receptor antagonists (e.g. PCP, MK 801, ketamine; see Geyer et al. 2001), thus mimicking the deficits observed in patients with schizophrenia (Mansbach and Geyer 1989, 1991, see Geyer et al. 2001). Additionally, previous studies have shown that acutely elevated levels of brain kynurenic acid (4-fold) produce electrophysiological effects on midbrain dopamine neurons (Erhardt et al. 2001b; Erhardt and Engberg 2002; see Erhardt et al. 2003) similar to those produced by

systemic administration of NMDA receptor antagonists, i.e. increased firing rate and burst firing activity (French et al. 1993; French 1994). Thus, since kynurenic acid blocks glutamate receptors, preferably NMDA receptors, and increases brain dopaminergic activity, the kynurenic acid hypothesis is in agreement with states of deficit in glutamatergic systems as well as increased dopaminergic activity in schizophrenia.

At low concentration kynurenic acid specifically blocks the glycine site of the NMDA receptor complex (Ganong and Cotman 1986; Birch et al. 1988; Kessler et al. 1989; Parson et al. 1997). Interestingly, compounds that block the glycine site of the NMDA receptor has been shown to induce schizophrenia-like symptoms in humans (Albers et al. 1999). Clinical studies in patients with schizophrenia also have revealed beneficial treatment effects on negative symptoms of the disease when glycine or D-serine is added to the antipsychotic treatment (see Touminen 2005), findings pointing towards a deficit in NMDA/glycine site activation in schizophrenia. However, no beneficial effects of glycine or D-serine are observed in patients treated with the atypical antipsychotic drug clozapine. Interestingly, it was recently shown that clozapine interacts with the NMDA receptor complex in the VTA, probably via the glycine site of the NMDA receptor (Schwieler and Erhardt 2003; Schwieler et al. 2004), and that clozapine inhibits rat brain synaptosomal glycine transport (Javitt et al. 2005). Taken together, these clinical and preclinical observations make it tempting to speculate that an ability of clozapine to *per se* modulate activation of the glycine site might be one of the pharmacological characteristics that contribute to its superior efficacy in alleviating schizophrenic symptoms. It also has been found that COX-2 inhibitors, which decrease rat brain kynurenic acid concentration and dopaminergic activity (Schwieler et al. 2005a,b), display beneficial antipsychotic effects when added to conventional antipsychotic treatment in patients with schizophrenia (Müller et al. 2002). In contrast, COX-1 inhibitors are associated with an increase in brain kynurenic acid concentration, and subsequent increase in dopaminergic activity (Schwieler et al. 2005a,b), and have been reported to induce psychotic side effects (see Hoppmann et al. 1991; see Jiang and Chang 1999; Tharumaratnam et al. 2000; Clunie et al. 2003).

2 SPECIFIC AIMS OF THE STUDY

1. To study whether the antipsychotic drugs clozapine and haloperidol affect the neuronal activity of the locus coeruleus by interfering with glutamatergic mechanisms.

2. To investigate the impact of subchronically elevated levels of endogenous kynurenic acid on dopamine cell firing and behaviour.

3. To analyse kynurenic acid in the cerebrospinal fluid from patients with schizophrenia as well as from healthy controls, and furthermore to correlate cerebrospinal fluid kynurenic acid concentration with concentrations of monoamine metabolites.

3 MATERIALS AND METHODS

3.1 Animals

Animal experiments were performed on male Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden). The rats were housed in groups of three to five in each cage and free access to food (R34 rat chow) and water was provided. Environmental conditions were checked daily and 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 experiments the rats were housed in a daily cycle of lights on at 06.00 AM and lights off at 06.00 PM. For behavioural experiments the rats were housed in a room with a regulated, reversed 12-h light/dark cycle with lights off at 07.00 AM and lights on at 07.00 PM. The rats were kept in their respective light/dark conditions at least one week prior experiments to adjust their diurnal rhythm. In Study I rats weighed 180-300 g on the day of the experiments. The rats in Study II weighed at least 180 g (on the day of surgery) and at most 350 g (on the day of the experiment). Experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm, Sweden, and all efforts were made to minimize the number of animals used and their suffering.

3.2 Drugs and chemicals

The following drugs were used: chloral hydrate and Tris (hydroxymethyl)-aminomethane (Merck, Darmstadt, Germany). (-)nicotine tartrate (doses of nicotine are expressed as the salt; ICN Pharmaceuticals, Plainview, NY, USA). Kynurenine, probenecid, zinc acetate (Sigma, St. Louis, MO, USA). Sodium acetate (Riedel-de Haen, Germany). Perchloric acid, sodium bicarbonate (Kebo Lab, Stockholm, Sweden). Acetonitrile (Labasco, Partille, Sweden). PNU 156561A [(R,S)-2-amino-4-oxo-4(3´-f´-dichlorophenyl) butanoic acid] (kindly donated by Dr. C. Speciale, Pharmacia & Upjohn, Milano, Italy). β-cyclodextrin, clozapine, D-cycloserine (Sigma, St. Louis, MO, USA). L-701,324 (Tocris, Bio Nuclear AB, Bromma, Sweden). Haloperidol (Janssen Pharmaceutical, N.V, 2340 Beerse, Belgium). Chemicals used for the detection of monoamine metabolites in CSF are listed in Swahn et al. 1976.

3.3 Elevation of endogenous kynurenic acid

3.3.1 Pretreatment with PNU156561A

To acutely elevate levels of endogenous brain kynurenic acid, rats were pretreated with PNU 156561A (40 mg/kg, dissolved in 10% β-cyclodextrin) intravenously 3 h before electrophysiological experiments. For the 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 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 x 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 was inserted to the needle and approximately 1 mL was injected carefully into the animal. After drug administration, the cannula was removed and the rats were placed individually in a Plexiglas cage.

3.3.2 Kynurenine and probenecid administration

To subchronically elevate endogenous brain kynurenic acid concentration rats were administered kynurenine and probenecid for 14 days via subcutaneously implanted osmotic pumps (2ML2 Alzet, USA), with a volume of 2 mL and a continuous flow rate of 5 μL/h. The osmotic pumps were filled under aseptic conditions, using a 5 mL plastic syringe and a blunt-tipped filling tube, with either vehicle (0.1 M Tris in deionised water; pH adjusted to 7.4 with acetic acid), kynurenine (dissolved in deionised water; pH adjusted to 3.5 with sodium bicarbonate) in a concentration equivalent to approximately 20 mg/kg/day at day of surgery, or probenecid (dissolved in 0.1 M Tris buffer; pH 8.0) in a concentration equivalent to approximately 10 mg/kg/day at the day of surgery. Both drugs were dissolved in the highest possible concentration as permitted in the osmotic pumps (volume: 2 mL) and all solutions were filtered through a sterile filter (Acrodisc Syringe Filter 0.2 μm Supor Membrane) before the pumps were filled.

3.4 Surgery for subchronic exposure

The osmotic pumps were inserted through an incision in the neck and placed subcutaneously on the back of the rats during chloral hydrate anaesthesia (400 mg/kg, via intraperitoneal (i.p.) injection). The implantations were performed under aseptic

conditions, and all the surgical implements and the site of the incision were cleaned with 70% ethanol solution. The control rats were implanted with one osmotic pump containing 0.1 M buffer and the drug-treated rats with two osmotic pumps containing kynurenine and probenecid, respectively. After surgery the incision was closed with 3-5 stitches and rats were placed in single cages to recover for 24 hours in the regular animal facilitiesbefore reunited in groups of 3-4 per cage. The rats were checked daily for any signs of infection or deviant behaviour, and occasionally massaged around the pumps to adjust their positions. After 7 days the rats designated for behavioural experiments were run in a testsession of PPI and after 14 days of treatment main behavioural experiments or, in another subset of animals, electrophysiological experiments were performed. In a smaller selection of animals the osmotic pumps were removed after 4 days, without any further experiments, for subsequent analysis of brain kynurenic acid.

3.5 *In vivo* electrophysiology

3.5.1 Anaesthesia and surgery

Rats were weighed and thereafter anaesthetised with 8% chloral hydrate (400 mg/kg, i.p.). For best anaesthesia, the rats were left to fall asleep in a quiet environment for approximately 10 minutes before anything else was done. If the degree of anaesthesia was not satisfactory by the end of this period an additional 0.5 mL of the chloral hydrate was administered i.p. The rat was placed onto a heating pad to maintain its body temperature at 37°. 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. For i.v. administrations a cannula was inserted into a lateral tail vein. Following successful insertion into the tail vein, the tail and the needle were secured with strips of plaster. A syringe containing 0.9% NaCl was connected to the cannula and 0.5-1 mL was injected into the rat to assure that no resistance to the injection was felt. Additional chloral hydrate was administered though the tail vein when required to maintain a stable level of surgical anaesthesia. The level of anaesthesia was determined from the response following hind paw pinching and by observing the breathing pattern. 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. For recordings from the LC a 3 mm diameter hole was drilled

posterior 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.

3.5.2 Preparation of recording electrode

Single barrel recording electrodes were prepared from glass capillaries (Harvard Apparatus, inner diameter 1.16 mm) pulled in a vertical electrode puller (Narishige, Japan) set at 14.5 amperes. The electrodes were 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. The optimal impedance for LC-recordings was generally 4-6 MΩ and for VTA-recordings 6-8 MΩ measured at 135 Hz in 0.9% saline.

3.5.3 Extracellular single cell recording

The recording electrode was secured onto a hydraulic microdrive (David Kopf Instruments, Tujunga, CA, USA) mounted to the stereotaxic instrument. The coordinates for lambda were visually estimated, placing the tip of the electrode just above lambda without touching the skull, and the coordinates for VTA and LC were estimated 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 and LC 1.1 mm posterior to lambda and 1.2 mm lateral to the midline. The recording electrode was vertically lowered until its tip touched the brain surface, which caused a signal on the oscilloscope. The microdrive depth was set to zero and the electrode was lowered into the brain to a depth of approximately 7 mm for recordings from VTA and 4.5 mm for recordings from LC, which placed the tip of the electrode just above the respective brain region. From this point the electrode was lowered slowly, using the hydrolic microdrve, into VTA or LC, where VTA dopamine neurons were found 7.5-8.5 mm from the brain surface and LC noradrenaline neurons were found 5.0-6.5 mm from the brain surface. Identified dopamine/noradrenaline neuron single unit potentials 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 audiomonitor and on a strip chart recorder (Gould).

After each experiment the rat was killed with an overdose of chloral hydrate and the brain was rapidly removed. In the subchronically treated rats also the liver was

removed and blood samples collected. Brains, livers and blood were stored at –30°C until subsequent kynurenic acid analysis. The recording sites of the electrophysiological experiments included in this thesis were not verified histologically (since the brains were used for identification of kynurenic acid). Thus, the noradrenergic and dopaminergic neurons were mainly identified based upon their typical neurophysiological characteristics (see below).

3.5.4 Electrophysiological characteristics of noradrenergic neurons

The electrophysiological characteristics of noradrenergic neurons in the LC were identical to those previously described for these neurons in rat (Aghajanian et al. 1977), including the characteristic rhythm and firing rate (1-5 Hz) and the typical response to noxious stimuli by a burst of activity followed by a quiescent interval. Furthermore, the LC was found just medial to the large cells of the mesencephalic nucleus of the fifth cranial nerve, which typically respond to gentle movements of the jaw by bursts of activity. The localization of the LC just below the fourth ventricle, a zone of electrical silence, was also observed. The effects of incremental doses of clozapine or haloperidol were studied on one noradrenaline neuron in each rat, naïve or with acutely elevated levels of kynurenic acid.

3.5.5 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 1984a, b) including:

1. A biphasic (positive-negative) or triphasic (positive-negative-positive) waveform, often with a prominent inflection in the initial phase.
2. An action potential of long duration (2.5-4.0 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.

Basal electrophysiological feature of three to fourteen dopamine neurons were recorded in each rat subchronically treated with kynurenine and probenecid or vehicle (0.1 M Tris

buffer). In addition, on the last neuron recorded from in each rat, the actions of nicotine were studied.

3.5.6 Drug administration

All drugs administered during the electrophysiological experiments were injected i.v. via the lateral tail vein. Before drug administration the basal activity was recorded for approximately 3 minutes. From the basal activity median values of firing rate, percentage of spikes fired in bursts and variation coefficience were estimated and considered 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 coefficience following each incremental dose administered.

3.5.7 Influence of anaesthesia

The influence of anaesthetics as a confounding factor on neuronal activity and afferent responsiveness remains unclear and care must be taken in the interpretation of results obtained in anaesthetised animals to infer a normal function. The firing pattern of dopamine neurons in anaesthetised, paralysed 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 anaesthetised and paralysed rats (Freeman and Bunney 1987; see Overton and Clark 1997; Hyland et al. 2002). The characteristics of noradrenaline neurons in the LC in anaesthetised rats (Aghajanian et al. 1977) seem to correspond rather well to freely-moving rats (Aston-Jones and Bloom 1981a,b) and awake head-restrained rats (Souliére et al. 2000), showing their classical response to a non-noxious environmental stimulus. Previous reports have shown that the primary metabolite of chloral hydrate, trichloroethanol (presumable 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 noradrenaline and dopamine cell firing. However, since all rats were anaesthetised with chloral hydrate using the same routines throughout the experiments the differences found between control rats and rats with manipulated kynurenine systems should not be attributed to the anaesthesia.

3.5.8 Data analysis

The distribution of spikes was analysed 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 analyse 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 artefacts in the sampling procedure, the spike analyser ignored time intervals below 20 ms. The onset of a burst was determined as an interspike interval shorter than 80 ms and the termination of a burst by the next interval longer than 160 ms (Grace and Bunney 1984a, b). The 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 analysed with regards 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 coefficience (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.

3.6 Prepulse inhibition

3.6.1 Apparatus

Two startle chambers were used for measuring the startle response (SR-LAB, San Diego Instruments, San Diego, CA). Each chamber consisted of a Plexiglas cylinder (9 cm diameter) mounted on a frame, housed within a ventilated chamber (39 x 38 x 58 cm). Sudden movements within the cylinder were detected by a piezoelectric accelerometer attached below the cylinder. A loudspeaker (Radio Shack Supertweeter) mounted 24 cm above the cylinder provided the broadband background noise and acoustic stimuli. Presentations of the acoustic stimuli were controlled by the SR-LAB software and interface system, which also rectified, digitized (0-4095), and recorded responses from the accelerometer. 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.

3.6.2 Experimental protocol

On postoperative day 14, animals pretreated with kynurenine + probenecid or vehicle were tested in the startle chambers. Equal numbers of animals from both the subchronically treated groups were tested in each of the two startle chambers to control for any differences in the apparatus. Seven days prior to the main experiment, animals were pre-exposed to the chambers and a testsession where the purpose was to acclimatize the animals to the testing chambers and startle/prepulse stimuli. In the main experimental session on postoperative day 14, a background noise (65 dB) was presented alone for 5 min and then continued throughout the remainder of the session. The following acoustic PPI session 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 50 trials (twelve "pulse-alone" trials, ten each of the "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 acustic 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 acustic session were also excluded. An average of 15 s (ranging from 9 to 21 s) separated consecutive trials. The startle response to the 120 dB pulse for each "pulse-alone" and "prepulse + pulse trial" was recorded. Startle magnitude and PPI were then calculated from these data for each animal. The acustic session, excluding the five "pulse-alone" trials at the beginning and in the end, was divided into two trial blocks. The testsession used on postoperative day 7 to familiarise 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).

3.7 Sampling of human cerebrospinal fluid

3.7.1 Ethical aspects

The work described in the present study was carried out in accordance with "The code of ethics of the world medical association (Declaration of Helsinki) for experiments including humans: http://www.wma.net/e/policy/b3.htm". The studies were approved by the

Ethical Committees of the University Hospital in Linköping, Karolinska Institutet, Göteborgs Forskningskommitté Östra and the medical faculty of Uppsala Universitet. All subjects participating gave informed consent after written and verbal information about the procedure and the purpose of the study.

3.7.2 Subjects

Cerebrospinal fluid samples from patients with schizophrenia, with a DSM-III-R (American Psychiatric Association 1987) or Research Diagnostic Criteria (RDC; Spitzer et al. 1978) verified schizophrenia, and from healthy control subjects were obtained from different hospitals in Sweden (University Hospital in Linköping, Karolinska Hospital, Sahlgrenska University Hospital in Göteborg and Västerås Central Hospital). The samples were collected over a long period of time (1979-2004). At the hospitals, patients with schizophrenia were invited to participate in the study and none of them were subjected to involuntary treatment. Informed consent was obtained from patients and volunteers after written and verbal information about the procedure and the purpose of the study. All patients included in the study were competent to give informed consent. Due to a limited number of CSF samples, female patients with schizophrenia were not included in the study. As healthy controls, mainly students and hospital staff members were recruited. All healthy controls were in good physical health and were taking no regular medication. Furthermore, all subjects were found to be free from current signs of psychiatric morbidity or difficulties in social adjustment at the time of sampling and had no family history of major psychosis or suicide in first or second-degree relatives.

Study III includes CSF samples from 90 male patients with schizophrenia (mean age 29.9 years ±0.9; range 18-55 years) and 49 healthy male controls (mean age 27.0 years ±0.8; range 19-44 years). Thirty-seven of the patients were first episodes and drug-naïve, i.e. they had never been treated with antipsychotic drugs (mean age 27.5 years ±1.4; range 18-55 years). Nineteen patients were drug-free (mean age 31.3 years ±1.6; range 20-46 years) at the time of CSF sampling; however, these patients had previously at some point been treated with antipsychotic drugs. These patients had been drug free from 21 days up to one year before the lumbar puncture and were readmitted to psychiatric care due to a new episode of acute psychosis. Thirty-four patients were undergoing antipsychotic treatment with either traditional or atypical antipsychotic drugs (mean age 31.8 years ±1.5; range 20-48 years), at time of CSF sampling.

In Study IV CSF kynurenic acid concentration from 56 healthy volunteers (43 males and 13 females), mean age 26.8 years ±0.73; range 19-44, was correlated to age, back

length and body height. In 43 of the healthy volunteers (30 males and 13 females) CSF content of three monoamine metabolites, HVA, 5-HIAA and HMPG was analysed and correlated to CSF kynurenic acid concentration.

Study V included 53 male patients with schizophrenia. Their mean age was 31.5 years ±1.0; range 20-48 years. In these patients, CSF content of kynurenic acid was correlted to conecentrations fo HVA, 5-HIAA and HMPG. Four of them were drug-naïve first episode patients. Nineteen were drug-free at the time of CSF sampling, but had previously been treated with antipsychotic drugs. Thirty patients were undergoing antipsychotic treatment with either traditional or atypical antipsychotic drugs, at time of CSF sampling.

The monoamine data in Study IV and V are part of a large cohort of CSF samples in which the mean concentrations of HVA, 5-HIAA and HMPG (Härnryd et al. 1984; Oxenstierna et al. 1984; Oxenstierna et al. 1996; Jönsson 1997) have been previously evaluated. The kynurenic acid data from male subjects included in Study IV and V are all included in Study III as well, whereas the healthy female controls are only presented in study IV.

3.7.3 Lumbar puncture

CSF was obtained by lumbar puncture (L4-L5) performed between 8 and 9 am with the subjects in erect, sitting, recumbent or lying position. The subjects had had at least 8 h of supervised bedrest in the hospital, abstaining from food and smoking. Samples of 10-18 mL CSF were drawn, in one or in consecutive fractions, according to a standardized sampling procedure (Sedvall et al. 1980; Bertilsson and Åsberg 1984). The samples were carefully mixed and centrifuged and thereafter immediately frozen, coded and sent blindly to Karolinska Institutet.

3.8 Analysis of kynurenic acid

3.8.1 Sample preparation

Immediately after each animal experiment, electrophysiological or behavioural, the rats were killed by decapitation. The brains were rapidly taken out and stored at -70°C for subsequent analysis of kynurenic acid. In the rats subchronically treated with kynurenine and probenecid blood was also collected and the liver was removed after the electrophysiological experiment, for subsequent analysis of kynurenic acid. The brains and the livers 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 80 μL perchloric acid (70%) was added to the supernatant. Thereafter the supernatant was centrifuged twice and stored at -18°C. Blood samples were centrifuged two times at 20,000 g for 5 min and 100 μL perchloric acid (70%) was added to the supernatant. Thereafter the supernatant was centrifuged three times and stored at -30°C. Before analysis, all samples were defrosted and centrifuged at 20,000 g for 5 min.

3.8.2 High performance liquid chromatography

Kynurenic acid is a stable compound and is not degraded even by repeated thawing (Heyes and Quearry 1990). It was analysed 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 x 150 mm, Dr Maisch GmbH, Ammerbuch, Germany) and a fluorescence detector (Jasco Ltd, Hachioji City, Japan) with an excitation wavelength of 344 nm and an emission wavelength of 398 nm (18 nm bandwidth). A mobile phase of 50 mM sodium acetate pH 6.20 (adjusted with acetic acid) and 7.0% acetonitrile was pumped through the reversed-phase column at a flow rate of 0.5 mL/min. Samples of 25 μl were manually injected (ECOM, Prague, Czech Republic). 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 0.10 mL/min. The signals from the fluorescence detector were transferred to a computer for analysis with Datalys Azur (Grenoble, France). The retention time of kynurenic acid was approximately 13 min. 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.

3.9 Analysis of monoamine metabolites

5-HIAA, HVA and HMPG concentrations were measured by mass fragmentography with deuterium labelled internal standards (Swahn et al. 1976). For determination of CSF concentrations of 5-HIAA, HVA and HMPG 2 mL of CSF was used. Before any further processing the following amounts of standards were added: 2800 pmoles of HVA-d_2, 2300 pmoles of 5-HIAA-d_2, and 950 pmoles of HMPG-d_2. Standard solutions were prepared containing the mentioned amounts of standards and a known amount of the authentic

substances that ranged between 0-1600 pmoles for HVA, 0-800 pmoles for 5-HIAA, and 0-500 pmoles for HMPG. The pH of the samples was adjusted to about 2 with 4 M formic acid. NaCl as added to saturate the solutions before extraction with 3 portions of diethyl ether (4 mL). The diethyl ether was removed and evaporated under a stream of nitrogen. The residue was transferred to a small conical test tube with 2 portions of methanol (0.3 mL), which was subsequently removed with nitrogen. When the solvent was completely removed, derivatives were prepared by the addition of a mixture of purified pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluoropropan-1-ol (PFPOH; 4:1; 50 μL). The tubes were sealed with a ground glass stopper and allowed to react for 15 minutes at 75ºC. The reagent was again evaporated and the residue was dissolved in ethyl acetate containing 1% PFPA (30 μl). About 2 μL of the final solution was used for analysis.

The analysis was performed on a Finnigan 3200 GC-MS system. The instrumental conditions were as follows. The gas chromatograph was equipped with an OV-17 column (1.5 m x 2 mm ID), operated at about 150ºC. The injector temperature was 180 ºC. The flow of helium was about 30 mL/min. From the mass spectra of the derivatives, the following fragment pairs were selected to be monitored: 458; 461; (HMPG), 460; 462 (HVA), and 438; 440 (5-HIAA). The separator temperature was 275ºC and the electron energy 50 eV. For the simultaneous recording of the metabolites the programmable multiple ion monitor unit was run in the current amplifier mode, with a sample time of 100 ms, filters on 0.05 Hz, and the preamplifier sensitivity at 10^{-8}. The other settings were adjusted for optimal resolution and sensitivity.

3.10 Statistical analysis

3.10.1 Electrophysiology

All data are expressed as mean ±SEM. Statistically significant differences regarding firing rate, variation coefficient and estimation of kynurenic acid, concentration were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test. Significance was assumed for all values where $p<0.05$.

Study I: Differences in per cent spikes fired in bursts were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test. For comparisons between groups a two-way analysis of variance (ANOVA) followed by Dunnett´s test as a post-hoc was used.

Study II: Burst firing activity was analysed with Kruskal-Wallis analysis of variance followed by the non-parametric Wilcoxon signed rank test. For comparisons between groups Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test was used. Statistical analyses were preformd using GraphPad Prism and Statistica StatSoft.

3.10.2 Prepulse inhibition

Startle magnitudes were calculated as the average response to the "pulse-alone" trials within the two blocks and analysed with mixed-design analysis of variance (ANOVA), with blocks as the repeated measure and subchronic treatment groups as categorical factors. Second, the amount of PPI was calculated as a percentage score for each "prepulse + pulse" trial type: % PPI = 100 – ([(startle response for "prepulse + pulse" trial)/(startle response for pulse-alone trial)] x 100). All data were analysed in a repeated measure ANOVA including blocks (first and second halves of the session) and trials types as repeated measures and treatment groups (vehicle versus kynurenine + probenecid) as categorical factors. The main effect of prepulse intensity was always significant and is not reported specifically. All data in the figures are expressed as mean ±SEM of block 1 and 2. Significance was assumed for all values where $p<0.05$. Statistical analyses were preformd using Statistica StatSoft.

3.10.3 Cerebrospinal fluid studies

All values are given as mean ±SEM. Differences regarding kynurenic acid CSF levels between controls and patients with schizophrenia as well as between male and female controls were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test. Linear regression analysis was performed, using the least-squares method, to study interrelationships between CSF concentration of kynurenic acid, HVA, 5-HIAA and HMPG and also to study correlations of the monoamine metabolites and kynurenic acid, with age, body height and back length. Significance was assumed for all values where $p<0.05$. Statistical analyses were preformd using GraphPad Prism.

4 RESULTS AND DISCUSSION

4.1 Effects of antipsychotic drugs on locus coeruleus noradrenergic neurons in rats with acute elevation of brain kynurenic acid levels (Study I)

Certain functional characteristics of the noradrenergic system in the brain make it interesting in relation to schizophrenia, especially its proposed involvement in cognition, vigilance and attention (Fields et al. 1988; see King 1998; see van Kammen and Kelley 1991; see Arnsten 2004). Recent studies from our laboratory suggest that the atypical antipsychotic drug clozapine interferes with the glycine site of the NMDA receptor. Thus, pharmacologically elevated levels of kynurenic acid as well as systemic administration of the selective glycine/NMDA receptor antagonist L-701,324 prevent and even reverse the excitatory actions of clozapine on VTA dopamine neurons (Schwieler and Erhardt 2003; Schwieler et al. 2004). The purpose of this *in vivo* electrophysiological study was to investigate whether the traditional antipsychotic drug haloperidol or clozapine affects the neuronal activity of the LC by interfering with glutamatergic mechanisms.

I.v. administration of clozapine (1.25-10 mg/kg, n=11) was associated with a robust increase in firing rate of LC noradrenergic neurons (figure 4, 5), whereas haloperidol (0.05-0.8 mg/kg, n=8) increased the firing rate only to a minor extent (figure 6). These findings are in accordance with previous electrophysiological results (Souto et al. 1979; Dinan and Aston-Jones 1984; Ramirez and Wang 1986; Rasmussen and Aghajanian 1988; Dawe et al. 2001). When kynurenic acid levels were elevated pharmacologically (2-fold), by pretreatment with PNU 156561A (40 mg/kg, i.v., 3 h), the excitatory actions of high doses of clozapine (2.5-10 mg/kg, n=5; figure 4b, 5) or haloperidol (0.05-0.8 mg/kg, n=4; figure 6) were clearly antagonised. However, the excitatory response following the lowest dose of clozapine (1.25 mg/kg) was not affected by elevated levels of endogenous kynurenic acid (figure 4b, 5). Administration of L-701,324 (4 mg/kg, i.v., 2-5 min, n=13), a specific antagonist at the glycine site of the NMDA receptor, did not by itself affect the firing rate (figure 4c). However, this pretreatment was found to prevent the excitatory actions of both clozapine (1.25-10 mg/kg, n=7; figure 4c, 5) and haloperidol (0.05-0.8 mg/kg, n=4; figure 6).

Recently, kynurenic acid was shown to block the α7* nicotinic receptor with the same IC_{50} value as for the glycine site of the NMDA-receptor (Hilmas et al. 2001). Although clozapine has been shown to stimulate α7* nicotinic receptors (Simosky et al. 2003), such an action of the drug seems unrelated to its activation of LC since this effect

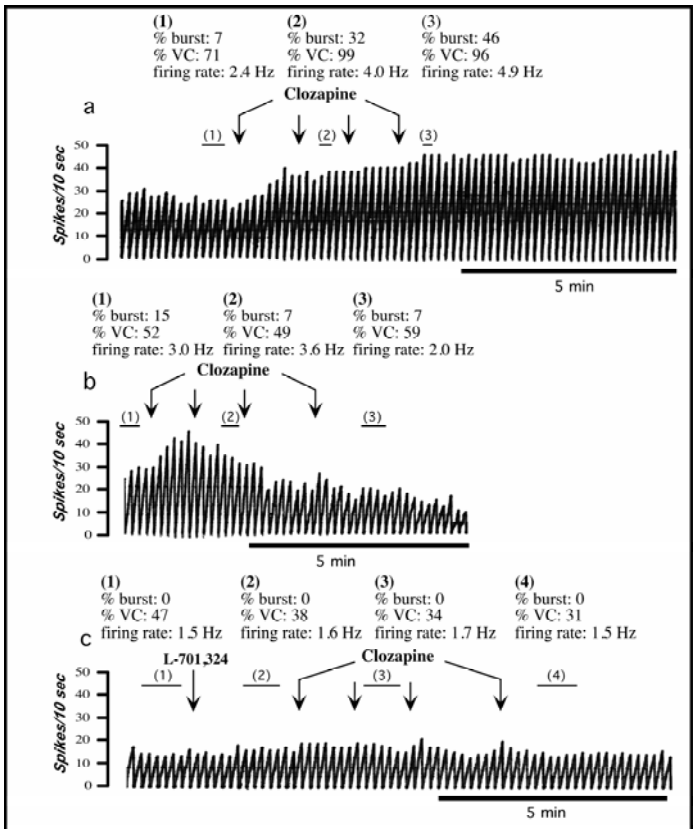


Figure 4. Extracellular recordings from three LC noradrenergic neurons following i.v. administration of clozapine (1.25+1.25 +2.5+5 mg/kg, injected at arrows). **a)** Excitatory effects of clozapine in a control rat. **b)** Antagonism of clozapine-induced excitation following elevated levels of kynurenic acid, as induced by pretreatment with PNU 156561A (40 mg/kg, i.v., 3 h). **c)** Prevention of the excitatory effects of clozapine following administration of L-701,324 (4 mg/kg, i.v.).

was completely blocked by L-701,324. Rather, the present results suggest that the excitation of LC noradreline neurons by haloperidol and clozapine involves a glutamatergic component. In support of this, it has recently been shown that clozapine interacts with the NMDA receptor complex in the VTA, probably with the glycine site of the NMDA receptor (Schwieler and Erhardt 2003; Schwieler et al. 2004). Moreover, clozapine inhibits rat brain synaptosomal glycine transport (Javitt et al. 2005). Interestingly, also haloperidol has been shown to interact with the glycine site of the NMDA receptor as revealed from voltage-clamped hippocampal neurons (Fletcher and MacDonald 1993). In the present study though, the partial agonist of the glycine/NMDA receptor, D-cycloserine (2-64 mg/kg, i.v., n=3), was not associated with an excitation of LC noradrenergic neurons, suggesting that activation of the glycine/NMDA receptor do not solely explain the excitatory actions on LC noradrenergic neurons induced by haloperidol and clozapine. Both antipsychotic compounds have previously been shown to produce an increase in extracellular concentrations of glutamate (Daly and Moghaddam 1993; See and Chapman 1994; See and Lynch 1995, Yamamoto and Cooperman 1994), suggesting that a release of glutamate, tentatively in combination with an activation of the glycine site of the NMDA receptor, might be responsible for the activation of LC noradrenergic neurons induced by clozapine and haloperidol. Although present results do not ascertain a putative release of glutamate by clozapine and haloperidol, such an effect could be mediated via presynaptic mechanisms, e.g. interactions with dopaminergic, serotonergic, adrenergic

and/or muscarinic receptors located on the glutamatergic afferents. The difference in efficacy between haloperidol and clozapine in increasing LC noradrenergic firing might be explained by a more effective release of glutamate (Daly and Moghaddam 1993; Yamamoto and Cooperman 1994) as well as a more pronounced glycine/NMDA receptor activation by clozapine (Schwieler and Erhardt 2003). The more effective action by L-701,324, as compared to administration of PNU 156561A, to antagonize the excitation of LC noradrenergic neurons by the lowest dose of clozapine is intriguing but might be related to the exceptionally slow dissociation of L-701,324 from the receptor (Priestley et al. 1996).

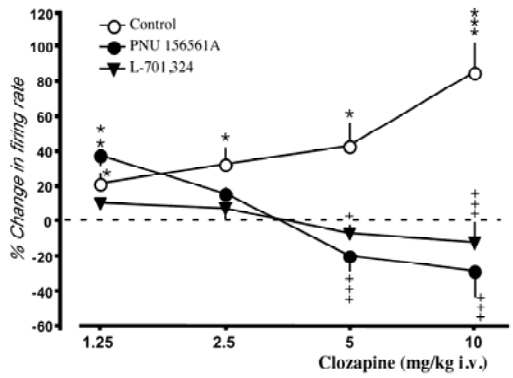


Figure 5. Effects of intravenously administered clozapine (1.25-10 mg/kg) on firing activity of noradrenergic neurons in the LC in control rats, in rats pretreated with PNU 156561A (40 mg/kg, i.v., 3 h) and in rats pretreated with L-701,324 (4 mg/kg, i.v., 2-5 min). Each value represent mean ±SEM from 4-11 LC noradrenergic neurons.*p<0.05 vs corresponding predrug values (Mann-Whitney U-test). $^{+}$p<0.05 vs corresponding control value (two-way analysis of variance followed by Dunnett´s test as a post-hoc).

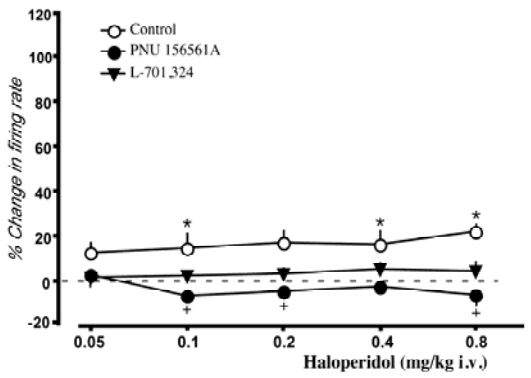


Figure 6. Effects of intravenously administered haloperidol on firing activity of noradrenergic neurons in the LC in control rats, in rats pretreated with PNU 156561A (40 mg/kg, i.v., 3 h) and in rats pretreated with L-701,324 (4 mg/kg, i.v., 2-5 min). Each value represent mean ±SEM from 3-8 LC noradrenergic neurons. *p<0.05 vs corresponding predrug values (Mann-Whitney U-test). $^{+}$p<0.05 vs corresponding control value (two-way analysis of variance followed by Dunnett´s test as a post-hoc).

Taken together, the present results suggest that clozapine (and haloperidol) activates LC noradrenergic neurons via glutamaterg mechanisms. The excitatory action on LC noradrenaline neurons of the lowest dose of clozapine in a situation of elevated brain kynurenic acid, tentatively mimicking a condition occurring in patients with schizophrenia (Erhardt et al. 2001a; Schwarcz et al. 2001), is of particular interest since this dose seems to correspond to a clinically therapeutic dose. With regard to this it is tempting to speculate whether this action of clozapine contributes to its efficacy against negative symptoms and cognitive deficits.

4.2 Effects of subchronic kynurenine and probenecid administration (Study II)

Previous studies have shown that acute elevation of rat brain kynurenic acid concentration is associated with increased firing of midbrain dopamine neurons (Erhardt et al. 2001b; Erhardt and Engberg 2002; see Erhardt et al. 2003) as well as a disrupted PPI (Erhardt et al. 2004), effects also observed after systemic administration of NMDA receptor antagonists (Mansbach and Geyer 1989, 1991; French el al. 1993; French 1994; see Geyer et al. 2001). However, if elevated levels of kynurenic acid in brain are of biological importance for schizophrenia, this should be a more or less enduring condition in the brains of the patients. Thus, the purpose of the present study was to investigate the effects of subchronically increased kynurenic acid levels in the brain on basal activity of VTA dopamine neurons and to study the impact of such pharmacological manipulation on PPI. To subchronically increase endogenous brain kynurenic acid concentration, rats were treated with kynurenine (the precursor of kynurenic acid; 20 mg/kg/day, s.c.) in combination with probenecid (which prevents efflux of kynurenic acid through the blood-brain-barrier; 10 mg/kg/day, s.c.) via osmotic pumps for 14 days.

The results of the present study revealed that subchronic exposure to kynurenine and probenecid had profound effects on basal firing activity of VTA dopamine neurons, including increase in firing rate and percent burst firing. The electrophysiological characteristics of 124 VTA dopamine neurons from vehicle-treated rats (0.1 M Tris buffer, s.c., n=14) and 111 VTA dopamine neurons from rats subchronically treated with kynurenine and probenecid (n=10) are summarised in table 1. According to the conventional criteria of burst firing activity (e.g. those of Grace and Bunney 1984a, b) 77% of the VTA dopamine neurons in the vehicle-treated group and 93% of the VTA dopamine neurons in the kynurenine + probenecid-treated group were classified as bursting. Thus, subchronic treatment with kynurenine and probenecid was associated with an increased number of bursts during a 100-spike sampling period, an increased number of spikes within a burst as well as an increase in cells found per track (3.1 neurons/track vs. 2.0 neurons/track in controls).

Table 1. Effects of subchronic kynurenine (20 mg/kg/day, s.c.) and probenecid (10 mg/kg/day, s.c.) on the firing rate and spike distribution of dopamine neurons in the VTA.

	Controls (0.1M Tris buffer)		Kynurenine+Probenecid	
	All neurons (n=124)	Bursting neurons (n=95)	All neurons (n=111)	Bursting neurons (n=103)
Firing rate (Hz)	4.4 ±0.2	5.0 ±0.2	5.4 ±0.2***	5.6 ± 0.2*
Variation coefficient (%)	78.2 ±2.6	82.1 ±3.1	81.5 ±2.6	82.0 ±2.7
Per cent burst firing (%)	30.0 ±2.8	39.0 ±3.0	48.1 ±2.8***	51.8 ±2.7**
Mean number of bursts[a]	8.0 ±0.7	10.4 ±0.7	10.6 ±0.6**	11.4 ±0.6
Mean spikes per burst	2.9 ±0.3	3.8 ±0.3	4.8 ±0.4***	5.2 ±0.4***

Values represent mean ±SEM from 14 control rats and 10 rats treated with kynurenine + probenecid. *$p<0.05$ vs. controls (Mann Whitney U-test); [a]during a 100-spike sampling period.

In naïve control rats (n=3), acute i.v. administration of kynurenine (20 mg/kg) in combination with probenecid (10 mg/kg) did not influence the firing rate, percentage of spikes fired in bursts or variation coefficient of VTA dopamine neurons within fifteen minutes of administration (data not shown), which argues against a direct effect of the drugs *per se*. Since kynurenine is the precursor of kynurenic acid and quinolinic acid, and both compounds are extruded via the same probenecid-sensitive carrier (Morrison et al. 1999), it is believed that the brain concentrations of both kynurenic acid and quinolinic acid would increase followig this treatment. Thus, the effects of subchronic kynurenine and probenecid treatment on VTA dopamine cell firing should be related to increased formation of quinolinic acid and/or kynurenic acid in the brain (Wu et al. 2000). In support of a predominating role of quinolinic acid in this regard is the fact that quinolinic acid is an NMDA receptor agonist (Stone and Perkins 1981) and it is well known that microiontophoretically applied NMDA or glutamate increases midbrain dopaminergic firing (Zhang et al. 1997). However, the effects of subchronic treatment with kynurenine and probenecid are remarkably similar to the firing characteristics observed when brain kynurenic acid concentration is acutely elevated by pretreatment with the kynurenine 3-hydroxylase inhibitor PNU 156561A (Erhardt et al. 2001b; Erhardt and Engberg 2002; see Erhardt et al. 2003; Schwieler and Erhardt 2003), or following administration of NMDA receptor antagonists like PCP or MK 801 (French et al. 1993; French 1994). Moreover, the reversal of the excitatory effects by nicotine into an inhibitory response on VTA dopamine firing following subchronic administration of kynurenine and probenecid (see below), is also seen following acute elevation of kynurenic acid (Erhardt et al. 2002a). These data point towards an involvement of

kynurenic acid in the activation of VTA dopamine neurons by subchronic treatment with kynurenine and probenecid, rather than an involvement of quinolinic acid.

To investigate putative adaptive changes in receptor sensitivity following subchronic treatment with kynurenine (20 mg/kg/day, s.c.) and probenecid (10 mg/kg/day, s.c.) the response of VTA dopamine neurons to nicotine was analysed. Previous studies have shown that systemically administered nicotine activates VTA dopamine neurons by a glutamate releasing action (Grillner and Svensson 2000; Erhardt et al 2002a; Schilström et al. 2003). In the present study, nicotine (3–400 μg/kg, n=12) produced an increase in firing rate and in percentage of spikes fired in bursts of VTA dopamine neurons in control rats (figure 7). This excitation was often preceded by an instantaneous (within seconds after injection) but brief reduction of the firing rate, especially pronounced when higher doses were administered. The decrease in firing rate lasted less than 120 sec and during this period no burst firing activity seemed to occur. This inhibitory action of nicotine is probably mediated via a release of GABA, which occurs almost

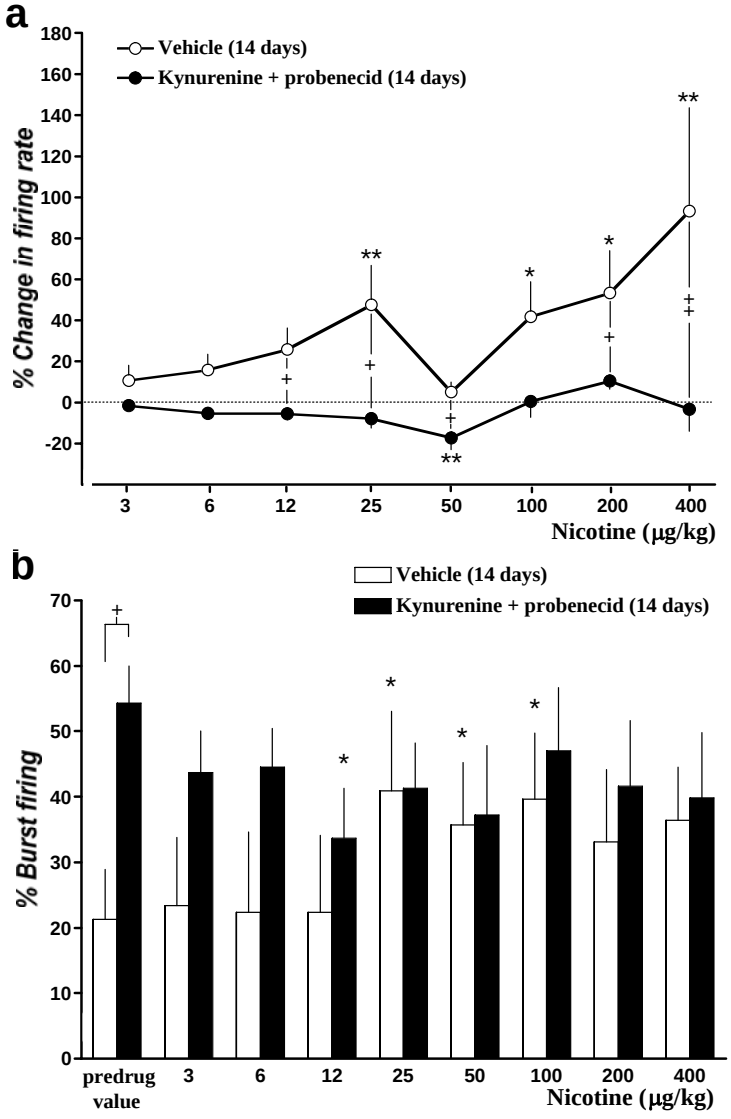


Fig 7: Effects of incremental doses of i.v. administered nicotine (3-400 □ g/kg) on **a)** firing rate, *p<0.05 vs. corresponding predrug values and $^{+}$p<0.05 vs. corresponding vehicle value (Mann-Whitney U-test) and **b)** the percentage of burst firing activity, *p<0.05 vs. corresponding predrug values (Wilcoxon signed rank test) and $^{+}$p<0.05 vs. corresponding vehicle value (Mann-Whitney U-test) in control rats (subchronically treated with vehicle (0.1 M Tris buffer, s.c.)) and in rats subchronically treated with kynurenine (20 mg/kg/day, s.c.) and probenecid (10 mg/kg/day, s.c.). Each value represent mean ±SEM from 5-6 VTA dopamine neurons.

simultaneously with its glutamate releasing effect (Erhardt et al. 2002a). Pretreatment with kynurenine and probenecid was found not only to antagonise the increase in firing rate and burst firing activity of VTA dopaminergic neurons induced by nicotine (3–400 μg/kg, i.v., n=7) but also to reverse the action of nicotine, leading to a decrease in neuronal activity (figure 7). These results are in excellent agreement with a previous study analysing the effects of nicotine in rats with acutely elevated levels of kynurenic acid (Erhardt et al. 2002a).

In summary, the effect on the spontaneous activity of VTA dopamine neurons as well as the change in their response to nicotine in rats subchronically treated with kynurenine and probenecid should be attributed to a blockade of glutamatergic and/or nicotinergic receptors by kynurenic acid. A putative contribution of an α7* nicotinic receptor blockade by kynurenic acid appears unlikely to account for the hyperactivity of VTA dopamine neurons, in view of the finding that the α7* nicotinic receptor agonist AR-R17779 is associated with an increase in VTA dopamine burst firing (Schilström et al. 2003). In addition, blockade of the α7* nicotinic receptor with MLA does not affect firing rate or burst firing activity of VTA dopamine nerons (Schilström et al. 2003; Schwieler et al. 2004). Nicotine induced increase of VTA dopamine cell firing in control rats (Grillner and Svensson 2000; Erhardt et al 2002a; Schilström et al. 2003) has previously been suggested to be the result of glutamate release via activation of α7* and α4β2 nicotinic receptors located on glutamatergic afferents (Toth et al. 1992; McGehee et al. 1995; Schilström et al. 2000a, 2003). It has previously been reported that prolonged exposure to kynurenic acid or chronic blockade of the α7* nicotinic receptor causes upregulation of α7* and α4β2 nicotinic receptors (Molinari et al. 1998; Hilmas et al. 2001; see Pereira et al. 2002). However, since the presently observed change in response to nicotine is of the same magnitude as that seen following acutely elevated levels of kynurenic acid, it is suggested that no upregulation of major importance for the nicotinic effect occurs following subchronic treatment with kynurenine and probenecid.

Whereas a single dose of kynurenine and probenecid (corresponding to a daily dose of the subchronic treatment) as well as a four-day treatment with the compounds significantly increased brain kynurenic acid concentration, the subchronic treatment (14 days) did not produce elevated whole brain levels (table 2). The lack of increase in whole brain kynurenic acid levels at day 14 may per se point to a development of tolerance in the conversion of kynurenine into kynurenic acid with subchronic kynurenine and probenecid treatment. However, this appears unlikely since the subchronic treatment produced effects on spontaneous VTA dopamine cell firing identical in magnitude to

those observed following acute elevation of brain kynurenic acid. Rather, the present effects of subchronic treatment with kynurenine and probenecid should be related to an increased turnover of kynurenic acid involving increased release to, and elimination from glutamatergic boutons (Swartz et al. 1990; Curatolo et al. 1996; Guillemin et al. 2001; Kiss et al. 2003). The increased turnover would thus be enough to allow receptor interaction (Turski et al. 1989), but insufficient to produce a detectable increase in whole brain kynurenic acid concentration. In this regard our results are in analogy with previous findings where e.g. subchronic L-DOPA treatment is found to produce motor sensitisation without any observable increases in striatal dopamine levels (Carey, 1991, 1993).

Table 2. Concentrations of kynurenic acid (nM)

	Controls (0.1M Tris buffer, s.c.)14 days	Kynurenine (20 mg/kg/day, s.c.) + Probenecid (10 mg/kg/day, s.c.) 14 days	Kynurenine (20 mg/kg/day, s.c.) + Probenecid (10 mg/kg/day, s.c.) 4 days	Kynurenine (20 mg/kg, i.v.) + Probenecid (10 mg/kg, i.v.) acute administration 1h
Whole brain	18.1 ±2.3 (n=33)	15.8 ±1.9 (n=30)	30.7 ±3.4* (n=4)	119.0 ±20.8*** (n=5)
Liver	93.1 ±11.6 (n=11)	115.1 ±18.1 (n=7)	n.t.	n.t.
Blood	76.3 ±11.8 (n=14)	80.6 ±21.3 (n=10)	n.t.	n.t.

Values represent mean ±SEM, n.t. not tested. *p<0.05 vs. controls (Mann Whitney U-test).

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 (see Gayer et al. 2001). PPI is a model that reflects such sensory gating deficits in patients with schizophrenia (see Braff et al. 2001). Administration of NMDA receptor antagonists has been found to disrupt PPI in rodents and infrahuman primates, mimicking the

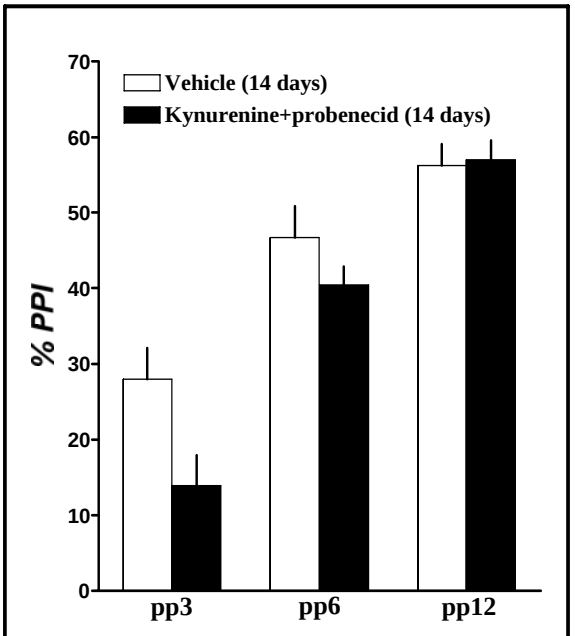


Figure 8: Effects of subchronic administration of vehicle (0.1 M Tris buffer, s.c., n=18) or kynurenine (20 mg/kg/day, s.c.) and probenecid (10 mg/kg/day, s.c., n=20) on PPI. Values represent mean ±SEM for each group

similar deficits observed in patients with schizophrenia (see Gayer et al. 2001) In addition, acute elevation of rat brain kynurenic acid also disrupts PPI (Erhardt et al. 2004). Thus, the present study examined the effects on PPI following subchronic administration of kynurenine and probenecid. A tendency towards a diminished PPI was found in kynurenine and probenecid treated rats (n=20) compared to vehicle treated rats (n=18; $F(1,36)=2.88$, $p=0.098$; figure 8). In addition, such treatment had no significant effect on startle magnitude compared to rats treated with vehicle (0.1 M Tris buffer).

4.3 Kynurenic acid in human cerebrospinal fluid (Study III, IV and V)

4.3.1 Elevated concentration of cerebrospinal fluid kynurenic acid in male patients with schizophrenia (Study III)

A previous report has revealed that the endogenous concentration of kynurenic acid is elevated in the CSF of male patients with schizophrenia when compared to male healthy controls (Erhardt et al. 2001a). In that initial study the patients with schizophrenia were pooled into one group, including both drug-naïve, first-episode patients and patients on antipsychotic treatment. In this study we further investigated a putative involvement of endogenous kynurenic acid in the pathophysiology of schizophrenia by analysing the concentration of the compound in CSF from a large cohort of patients. Thus, here we investigated the concentration of kynurenic acid in different subgroups of patients, i.e. drug-naïve, first episode patients with schizophrenia, drug-free patients and patients undergoing antipsychotic treatment. The results of the analysis of CSF kynurenic acid in 90 male patients with schizophrenia and 49 healthy male controls are summarised in figure 9. Data from all patients with schizophrenia (n=90) revealed

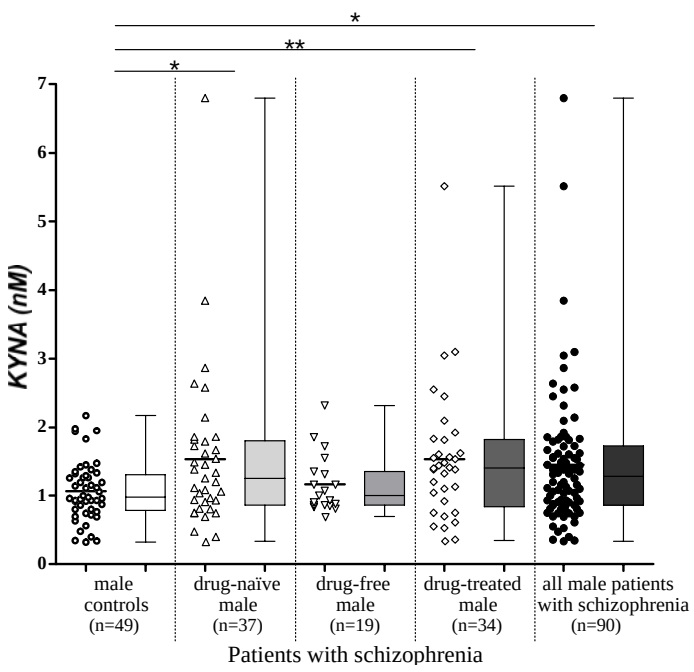


Figure 9. Kynurenic acid in human CSF. Each point represents the concentration of kynurenic acid in a single CSF sample in units of nM together with the mean for all groups. Corresponding box-plots illustrates median, range and quartiles for each group. *p<0.05, p**<0.01 (Mann-Whitney U-test).

that kynurenic acid levels in CSF were significantly elevated compared to healthy male controls (n=49), thus confirming previous findings (Erhardt et al. 2001a). When the patients with schizophrenia were subdivided into different groups, we found that CSF kynurenic acid levels in drug-naïve, first-episode patients (n=37) as well as in patients undergoing antipsychotic treatment (n=34) were significantly higher than in healthy male controls. In drug-free patients with schizophrenia (n=19), i.e. patients previously treated with antipsychotic medications but drug-free at time of sampling, we found no difference in CSF kynurenic acid concentration as compared to controls. Although no definitive conclusions regarding the effects of antipsychotic treatment on CSF kynurenic acid levels in man can be made from present results, a previous study has demonstrated that chronic treatment with the antipsychotic drug haloperidol does not result in increased levels of kynurenic acid content in the rat brain, but rather tended to decrease the levels of kynurenic acid (Schwarcz et al. 2001).

Drug-free patients with schizophrenia might represent a subgroup with a different disease pattern, tentatively with a better outcome, which might explain the lack of elevated CSF kynurenic acid levels in this group. Thus, although not applicable in the present study (due to lack of information regarding the symptoms in most of the patients), it is desirable to correlate CSF kynurenic acid levels to the severity of schizophrenia as well as to different symptoms in future studies.

Moreover, a positive correlation was found between CSF kynurenic acid levels and age in the male patients with schizophrenia (range 18-55 years) but not in healthy male controls (range 19-44 years; figure 10). Previous studies have shown an age-related increase of endogenous brain kynurenic acid in rats (Gramsbergen et al. 1992, Moroni et al. 1988b) and in male patients with schizophrenia (Erhardt et al. 2001a) as well as in patients with acute headache (Kepplinger et al. 2005). However, no such correlation has been found in CSF of healthy controls (Erhardt et al. 2001a). In the present study, the absence of correlation between CSF kynurenic acid and age might be explained by the relatively narrow age range in the control group. It is also

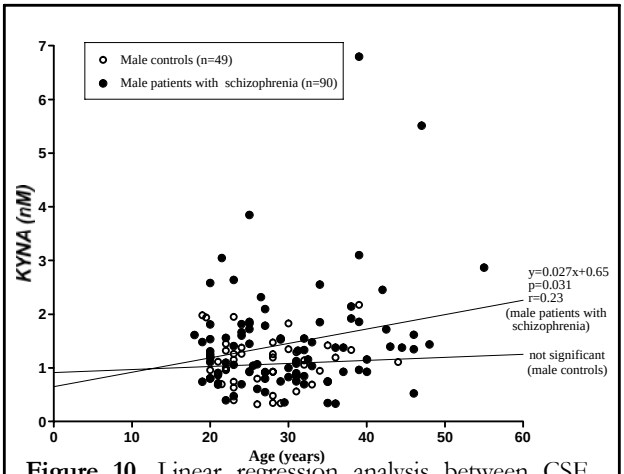


Figure 10. Linear regression analysis between CSF kynurenic acid concentration (nM) and age (years) in male controls and male patients with schizophrenia. A significant correlation between kynurenic acid and age was observed in male patients with schizophrenia

possible that age-related changes could not be detected at these young ages in healthy controls, or that diseases in the CNS accentuate age-induced changes or induce a premature ageing of the CSF system.

4.3.2 Cerebrospinal fluid kynurenic acid – correlation with monoamine metabolites and influence of confounding factors (Study IV and V)

The mean concentrations (±SEM) of kynurenic acid and the monoamine metabolites (HVA, 5-HIAA and HMPG) in CSF from healthy male and female controls and from male patients with schizophrenia are summarised in table 3.

Table 3. CSF concentrations of kynurenic acid, HVA, 5-HIAA and HMPG (nM).

Subjects	KYNA	HVA	5-HIAA	HMPG
Male controls	1.06 ±0.06 (n=49)[III]			
	1.06 ±0.07 (n=43)[IV]	138.9 ±12.6 (n=30)[a,b]	74.8 ±5.9 (n=30)[a,b]	43.4 ±1.2 (n=30)[a,b]
Female controls	1.91 ±0.20* (n=13)[IV]	181.3 ±21.9 (n=13)[IV]	93.7 ±11.4 (n=13)[IV]	39.2 ±2.0* (n=13)[IV]
Male patients with schizophrenia	1.45 ±0.10 (n=90)[III]			
	1.33 ±0.09 (n=53)[V]	165.1 ±10.3 (n=53)[a]	80.12 ±3.89 (n=53)[a]	41.33 ±1.1 (n=53)[a]

Values represent mean ±SEM. *p<0.05 vs. male controls (Mann Whitney U-test). Roman numerals refer to the study in which analysis were performed.
[a] Samples from Härnryd et al. 1984; Oxenstierna et al. 1984; 1996, [b] Samples from Jönsson 1997

Our investigations revealed a positive inter-correlation between kynurenic acid, HVA and 5-HIAA concentrations in CSF in both male (n=30) and female (n=13) healthy controls (figure 11), while HMPG content in the CSF did not correlate with CSF kynurenic acid, HVA or 5-HIAA (data not shown). Among control subjects (n=55), a negative correlation was found between kynurenic acid CSF concentration and back length (figure 12a). However, when males (n=43) and females (n=12) were analysed separately the correlation was only significant in female controls. Also CSF levels of HVA and 5-HIAA were negatively correlated with back length in the healthy controls (n=42; data not shown), but when divided into male (n=30) and female (n=12) the correlation was only significant in females. CSF concentration of HMPG did not correlate with back length in the healthy controls (data not shown). A negative correlation was observed between CSF kynurenic acid and body height among all healthy controls (n=56; figure 12b) but not in males (n=43) and females (n=13) when analysed separately. None of the monoamine metabolites were found to correlate with body height in the healthy controls (data not shown). With regard to age, no correlation was found with CSF kynurenic acid concentration in the controls (male; n=43, female; n=13;

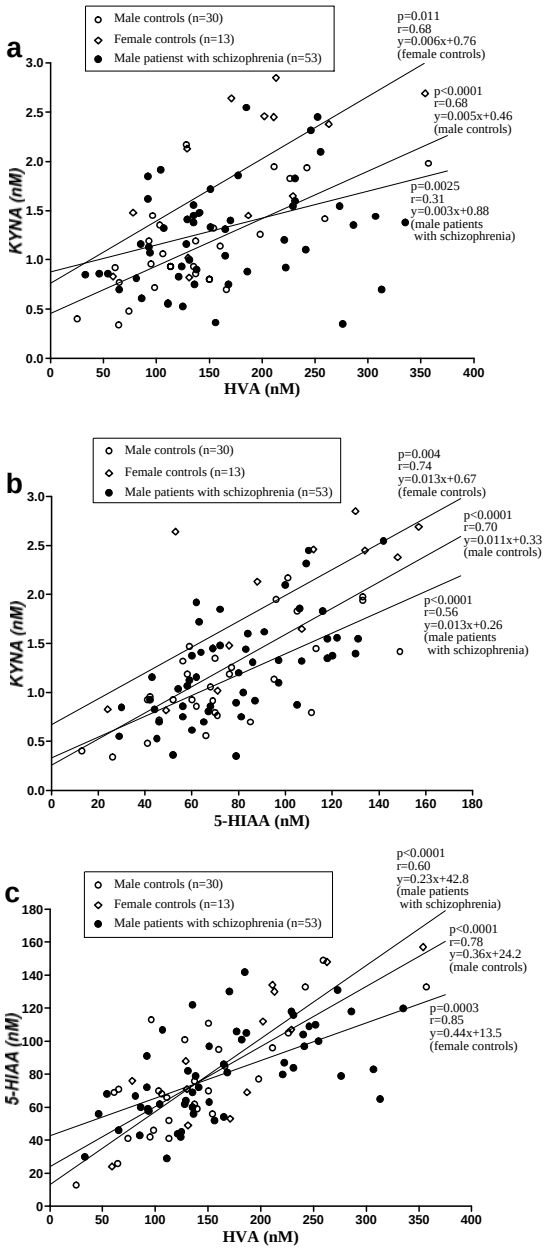


Figure 11. Linear regression analysis between CSF concentration (nM) of **a)** kynurenic acid vs. HVA **b)** kynurenic acid vs. 5-HIAA and **c)** 5-HIAA vs. HVA in healthy male and female controls and male patients with schizophrenia.

data not shown). Furthermore, female controls were found to have significantly higher mean values of kynurenic acid and lower levels of HMPG than male controls, while mean levels of HVA and 5-HIAA did not differ significantly between men and women. The increased concentration of kynurenic acid found in CSF from females might be related to the fact that women in general have a shorter back (and body height) compared to men, which could result in a smaller dilution gradient. This gender difference should be taken in consideration when analysing CSF kynurenic acid in mixed gender groups.

In agreement with results from control subjects, a positive inter-correlation between kynurenic acid, HVA and 5-HIAA concentrations in CSF was also found in male patients with schizophrenia (n=53, figure 11), while HMPG content in the CSF did not correlate with CSF kynurenic acid, HVA or 5-HIAA (data not shown).

In summary, the present findings suggest that increased kynurenic acid formation is associated with an increased turnover of dopamine and serotonin. In support of this, recent preclinical studies demonstrate that kynurenic acid acts as a neuromodulator that tonically controls glutamatergic neurotransmission and hereby indirectly interacts with the dopaminergic systems. Thus, acute elevation or decrease of endogenous levels of kynurenic acid in the rat brain is associated with increased or reduced activity, respectively, of midbrain dopamine neurons (Erhardt et al. 2001b; Erhardt and Engberg 2002; Schwieler et al. 2005b). In

patients with schizophrenia, striatal dopamine (see Abi-Dargham and Laruelle 2005) and postmortem cortical (Schwarcz et al. 2001) as well as CSF (Erhardt et al. 2001a) kynurenic acid content is increased. This makes it tempting to speculate about a possible link between increased kynurenic acid formation and hyperdopaminergic activity in these patients. A direct physiological interaction between kynurenic acid and the serotonergic system has not yet been investigated; however, the correlation between CSF kynurenic acid and CSF 5-HIAA might be related to tryptophan, which is the common precursor for both serotonin and kynurenic acid.

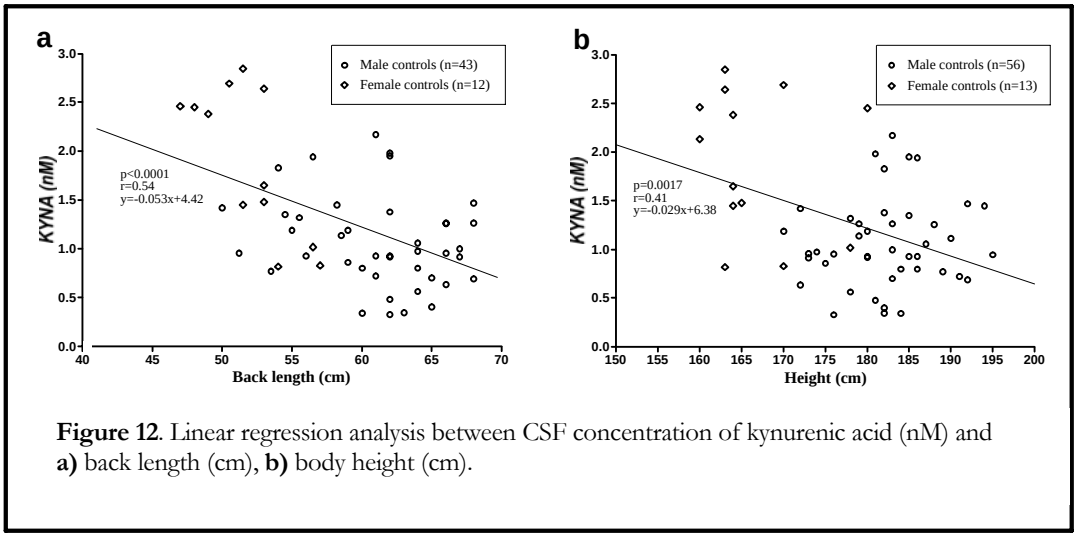


Figure 12. Linear regression analysis between CSF concentration of kynurenic acid (nM) and **a)** back length (cm), **b)** body height (cm).

5 GENERAL DISCUSSION

The dopamine hypothesis has for decades dominated thinking about the pathophysiology of schizophrenia and it is generally believed that many symptoms associated with the disease are mediated via the mesolimbic and mesocortical dopamine systems. In the past few years though, it has become clear that dopamine is just part of the story and that the main abnormalities lie elsewhere (see Carlsson et al. 2001). In this regard the model of glutamatergic dysfunction has attracted growing interest and the origin of this theory lies in the discovery that the NMDA receptor antagonist PCP produces both positive and negative symptoms in man (see Javitt and Zukin 1991; cf. Introduction). Given the fundamental role of glutamate in neurodevelopment and neuroplasticity this theory may be even more attractive in view of the neuroarchitectural aberrations that are associated with schizophrenia, such as abnormal migration and synaptic connections (Akbarian et al. 1993a,b; see McGlashan and Hoffman 2000). Multiple lines of evidence from animal experiments and clinical studies using agonists at the glycine site of the NMDA receptor further link schizophrenia to aberrations in glutamatergic mechanisms (see Coyle and Tsai 2004). However, evidence with regard to specific NMDA receptor function, e.g. from ligand binding studies and from receptor expression studies have been surprisingly few and inconsistent. In addition, data on glutamate (and glutamine) as measured biochemically in the CSF or by proton magnetic resonance spectroscopy are relatively sparse and difficult to interpret. Thus, the reduced glutamate concentration in the CSF from patients with schizophrenia (Kim et al. 1980) has so far not been confirmed in subsequent studies. Furthermore, Théberge and co-workers (2002, 2003) reported reduced levels of glutamate and glutamine in the anterior cingulum of chronic patients with schizophrenia, while first-episode patients showed increased levels of glutamine in this region, findings partly confirmed by Ohrmann et al. (2005).

The results of the present thesis show that subchronic exposure to kynurenine and probenecid in rats is associated with the same profound effects on basal activity of VTA dopamine neurons as after acute pretreatment with these compounds, or with the kynurenine 3-hydroxylase inhibitor PNU 156561A, effects most likely related to increased formation of kynurenic acid in the brain (cf. Results and Discussion). However, the disruption of PPI that is observed following acute elevation of kynurenic acid levels was not detectable in the rats subchronically treated with kynurenine and probenecid. Furthermore, our findings suggest that brain endogenous kynurenic acid in

the brain is an important modulator of the effects of the antipsychotic drugs clozapine and haloperidol as well as of nicotine. Finally, the pathophysiological role of kynurenic acid in schizophrenia was further strengthened by the the finding that kynurenic acid was increased in the CSF from male patients with schizophrenia, A dysfunction in the formation of kynurenic acid in schizophrenia, as proposed in the present thesis and forming part of the model of glutamatergic dysfunction in the disease, is consistent with the dopamine hypothesis of schizophrenia given the profound modulatory role of the compound in the control of VTA dopaminergic activity. Thus, the present results suggest that endogenous kynurenic acid acts as a biologically important modulator of glutamatergic neurotransmission within the brain, and lend further support to the hypothesis that endogenous kynurenic acid participates in the pathophysiology of schizophrenia.

In addition to blocking the glycine site of the NMDA receptor, kynurenic acid is also able to antagonise α7* nicotinic receptor (see Introduction, section 1.3.2). However, the contribution of the latter action in the effect of kynurenic acid on VTA dopamine neurons appears to be of minor importance. Previous electrophysiological studies have shown that specific blockade of the NMDA/glycine receptor, with the selective antagonist L-701,324, is associated with an increased firing rate and burst firing activity (Schwieler et al. 2004), whereas blockade of the α7* nicotinic receptor (with MLA) does not affect the activity of VTA dopmine neurons (Schilström et al. 2003; Schwieler et al. 2004). Rather, stimulation of α7* nicotinic receptors is associated with an increase in burst firing of VTA dopamine neurons (Schilström et al. 2003). Furthermore, the excitatory actions on VTA dopamine firing following pharmacologically elevated brain kynurenic acid concentration have previously been shown to be reversed by systemic administration of the partial NMDA/glycine receptor agonist D-cycloserine, pointing to an interaction of kynurenic acid at the NMDA/glycine site (Erhardt et al. 2002a; Schwieler et al. 2005b). The paradoxical increase in firing of midbrain dopamine neurons following increased brain kynurenic acid levels or systemic administration of NMDA receptor antagonists (PCP, MK 801; French et al. 1993; French 1994) has previously been suggested to be related to inhibition of a tonic GABAergic input that normally dampens activity of midbrain dopamine neurons (Erhardt et al. 2002b). In support of this view, GABAergic interneurons, as compared to glutamatergic pyramidal neurons, display a particular vulnerability to NMDA receptor antagonists (Grunze et al. 1996; Li et al. 2002). Thus, endogenous kynurenic acid (like PCP or MK 801) may primarily reduce the activity of GABAergic projections to the VTA, thereby disinhibiting the VTA

dopamine neurons, resulting in an increased neuronal activity, as observed in the present subchronic study.

The response of VTA dopamine neurons to nicotine was analysed to investigate putative adaptive changes in receptor sensitivity following subchronic treatment with kynurenine and probenecid. It has previously been reported that prolonged exposure to kynurenic acid or chronic blockade of the α7* nicotinic receptor *in vitro* causes upregulation of α7* and α4β2 nicotinic receptors (Molinari et al. 1998; Hilmas et al. 2001; see Pereira et al. 2002). Several studies have also shown that chronic PCP or MK-801 exposure alters glutamate receptor subunits and, in particular, upregulate the expression of the NR1 subunit (Wang et al. 1999; Oh et al. 2001; Lindahl and Keifer 2004), a critical component of the NMDA receptor. To the extent that such upregulations occur it would mitigate the impact of subchronic exposure to kynurenine and probenecid. However, since the presently observed effects on basal firing of VTA dopamine neurons and the change in response to nicotine are of the same magnitude as those observed following acute elevation of levels of kynurenic acid in the brain (Erhardt et al. 2002a), it is suggested that no adaptive changes occur following subchronic treatment with kynurenine and probenecid. Rather, if any compensatory mechanisms develop following this treatment, they might be related to an increased elimination of kynurenic acid, since elevated brain levels of kynurenic acid were not detected following the subchronic treatment. Notably, the lack of adaptive changes, as revealed from the response to nicotine on VTA dopamine neurons, following subchronic treatment with kynurenine and probenecid is in line with studies in postmortem schizophrenic brains showing no consistent alterations in NMDA receptor ligand binding (see Meador-Woodruff and Healy 2000).

Several studies carried out over the past decade have reported that patients with schizophrenia smoke more than other psychiatric patients and much more than the general population (see Dalack et al. 1998). The prevalence of smoking in the general population in industrial countries is 20-50% (Collishaw and Lopez 1996), whereas the prevalence in patients with schizophrenia is commonly found to be over 80% (O'Farrell et al. 1983; Hughes et al. 1986; Goff et al. 1992; de Leon et al. 1995; Kelly and McCreadie 1999; Cantor-Graae et al. 2001). In addition, patients with schizophrenia seem to inhale more deeply than other smokers, thus extracting more nicotine per cigarette (Olincy et al. 1997), and they frequently smoke the cigarettes down to the very end, where the highest concentration of nicotine is found (Masterson and O'Shea 1984). A number of explanations for the extensive co-occurrence of smoking and

schizophrenia have been proposed but many of these ideas are speculative, since no study has yet determined exactly what impact smoking has on the disease. For instance, it has been suggested that smoking might be an attempt to self-medicate symptoms of the disease or side-effects of the antipsychotic treatment (Armitage et al. 1968; O'Farrell et al. 1983; Hughes et al. 1986; see Svensson et al. 1990; Goff et al. 1992). The finding that the prevalence of smoking among first-episode patients is as high as that in patients chronically treated with antipsychotic drugs (McEvoy and Brown 1999) suggests that it is the disease rather than the treatment that leads to the high prevalence. One specific reason for this high rate of smoking may be alleviation of cognitive dysfunction (see Dalack et al. 1998), since nicotine improves many aspects of cognitive performance in normal human subjects as well as in rodents (see Wesnes and Warburton 1983; see Levin and Simon 1998; see Rezvani and Levin 2001). Indeed, some studies provide evidence that smoking may improve cognition in patients with schizophrenia, plausibly by increasing dopamine release in the prefrontal areas of the brain (Adler et al. 1993; Taiminen et al. 1998, George et al. 2002). In addition, also beneficial effects on positive symptoms have been suggested to contribute to the intense drive to smoke among patients with schizophrenia (Glynn and Sussman 1990; see Lohr and Flynn 1992). Animal studies have revealed that nicotine administration is associated with an increase in activity of VTA dopamine neurons in rats (Grenhoff et al. 1986; Mereu et al. 1987; Nisell et al. 1996; Grillner and Svensson 2000; Erhardt et al. 2002a; Schilström et al. 2003) and a subsequent increase in dopamine utilization in terminal areas, e.g. the nucleus accumbens and the prefrontal cortex (Imperato et al. 1986; Damsma et al. 1989; Nisell et al. 1994, 1996). In view of the presumable hypofunctionality of cortical dopamine systems proposed in schizophrenia (see Davis et al. 1991; see Jentsch and Roth 1999; see Abi-Dargham and Laruelle 2005), this action of nicotine might contribute to ameliorating negative symptoms as well as cognitive deficits in patients with schizophrenia. However, it also has been shown that the excitatory effect of nicotine of VTA dopamine neurons is preceded by an instantaneous (within seconds after injection) but brief reduction of the firing rate (as also found in the present thesis), probably of GABAergic origin (Erhardt et al 2002a). This inhibitory response, together with the well-documented excitatory action of nicotine, may explain how nicotine can act both as a depressant and as a stimulant. Here we found that after subchronic exposure to kynurenine and probenecid, which was accompanied by increased dopaminergic activity of VTA dopamine neurons, the actions of nicotine on VTA dopamine neurons were predominantly inhibitory. These findings are identical with those from a previous study

in rats with acutely elevated levels of kynurenic acid in the brain (Erhardt et al 2002a). As the firing of VTA dopamine neurons appears to be physiologically regulated mainly by simultaneous inhibitory GABAergic and stimulatory glutamatergic inputs, nicotine, by releasing glutamate and GABA, may normalise/stabilise the activity of VTA dopamine neurons. Thus, in a situation of increased endogenous concentration of kynurenic acid in the brain, that blocks the action of glutamate and induce dopaminergic hyperactivity, the action of GABA released by nicotine, may prevail. Correspondingly, under normal conditions, nicotine-induced glutamate release would prevail and serve to increase dopaminergic activity. Hence, nicotine may modulate human behaviour by either facilitating or inhibiting dopaminergic neurotransmission, depending on the concentration of endogenous kynurenic acid in the brain. Midbrain dopamine neurons may play an important role in generating positive symptoms in schizophrenia where increased phasic release of dopamine (see Grace 1991), induced by burst firing activity of these neurons, may mediate the excess of subcortical dopamine functions (see Davis et al. 1991; see Jentsch and Roth 1999; see Abi-Dargham and Laruelle 2005). Thus, the increased firing rate and burst firing activity of midbrain dopamine neurons following subchronic administration of kynurenine and probenecid, might represent a pathophysiological condition similar to that seen in patients with schizophrenia. Thereby, the ability of nicotine to inhibit burst firing activity of VTA dopamine neurons, and probably subsequent dopamine release, in states of dopaminergic hyperactivity in the brain, suggest that patients with schizophrenia smoke not only for the cognitive enhancing effects of nicotine but also to alleviate positive symptoms of the disease. In fact, cross-sectional studies have shown that the patients with schizophrenia who smoke also exhibit high rates of positive symptoms (Goff et al. 1992; Ziedonis et al. 1994), which might be their underlying drive to smoke.

Previous studies have shown that specific blockade of the NMDA/glycine receptor (with L-701,324) does not reduce PPI (Bristow et al. 1995; Depoortere et al. 1999), whereas NMDA receptor antagonists (competitive or non-competitive) cause deficits in PPI (Mansbach and Geyer 1989, 1991; Bakshi et al. 1999, see Geyer et al. 2001). Thus, acutely elevated levels of brain kynurenic acid (4-fold in whole brain) may plausibly disrupt PPI via blockade of the glutamate recognition site of the NMDA receptor (Erhardt et al. 2004). Studies regarding the α7* nicotinic receptor have shown some conflicting results concerning PPI. A reduction of hippocampal α7* nicotinic receptor, as measured by α-bungarotoxin binding, has been shown to reduce PPI in mice (Stevens et al. 2001), whereas the α7* nicotinic receptor antagonist MLA has no

effect on PPI in rats (Suemaru et al. 2004). Furthermore, it has also been reported that acoustic startle and PPI are normal in α7* null mutant mice (Paylor et al. 1998; see also Stevens et al. 2001). Taken together, present findings suggest that the concentration of kynurenic acid in the synapses after subchronic administration of kynurenine and probenecid is high enough to block the glycine site of the NMDA receptor (thus increasing firing of VTA dopamine neurons) but too low to interact with the glutamate recognition site of the NMDA receptor.

Judging from receptor occupancy data measured by ex vivo autoradiography (Leysen et al. 1993) or in-vivo receptor binding (Sumiyoshi et al. 1994), the lowest dose of clozapine used in Study I seems to correspond with clinically therapeutic doses with regard to occupancy of 5-HT_{2A} and dopamine D_2 receptors (Farde et al. 1988, 1992; Nordström et al. 1993a, 1993b, Schotte et al. 1996). This is of particular interest since this dose still produced excitatory actions on LC noradrenaline neurons in a situation of elevated brain kynurenic acid, tentatively mimicking a condition occurring in patients with schizophrenia (Erhardt et al. 2001a; Schwarcz et al. 2001), whereas higher doses of clozapine and all doses of haloperidol failed to increase LC activity. In view of the hypothesis that negative and cognitive symptoms of schizophrenia stem from reduced levels of dopamine in the prefrontal cortex (see Jentsch and Roth 1999), it is interesting that a recent study demonstrates that clozapine co-releases dopamine and noradrenaline from noradrenergic terminals in the cerebral cortex (Devoto et al. 2003). With regard to this latter finding it is tempting to speculate whether clozapine's efficacy against negative symptoms and cognitive deficits in schizophrenia might be related to its excitatory action on LC noradrenaline neurons, and subsequent co-release of dopamine and noradrenaline in cerebral cortex from noradrenergic neurons originating in the LC. Furthermore, the inability of haloperidol to produce excitation of LC noradrenergic neurons, in states of elevated brain levels of kynurenic acid, might underlie the poor efficacy of the drug in alleviating negative and cognitive symptoms in schizophrenia. However, the mechanisms behind this action of a lower, but not higher, dose of clozapine are still elusive. With regard to VTA dopamine neurons, it has previously been shown that all excitatory actions of clozapine are antagonised and even reversed into an inhibitory response in rats with elevated levels of kynurenic acid, whereas the excitatory actions of haloperidol are even more pronounced (Schwieler and Erhardt 2003). Taken together, these specific features of clozapine in modulating the activity of noradrenergic and dopaminergic systems in a situation of elevated levels of kynurenic acid in the brain might contribute to its superior efficacy in the treatment of schizophrenia.

The finding that the CSF concentration of kynurenic acid was elevated in a large cohort of male patients with schizophrenia further strengthen an implication of endogenous kynurenic acid in the pathophysiology of schizophrenia. Furthermore, the positive correlation between CSF kynurenic acid and HVA suggests that increased kynurenic acid formation is associated with increased dopamine transmission and/or turnover, or vice versa. This is in line with experimental data from the rat, which have demonstrated that elevated or reduced levels of brain kynurenic acid are associated with an excitation or inhibition of midbrain dopamine neurons, respectively (Erhardt et al. 2001b; Erhardt and Engberg 2002; Schwieler et al. 2005b). Thus, it is tantalizing to speculate whether the increased CSF concentration of kynurenic acid accounts for the hyperdopaminergic activity observed in patients with schizophrenia. Thus, increased dopaminergic activity as a consequence of high levels of endogenous brain kynurenic acid may participate in generating symptoms of the disease.

Why kynurenic acid concentration is elevated in male patients with schizophrenia is not known. Recently though, it was found that the expression of the enzyme tryptophan 2,3-dioxygenase, which catalyses the first step in the synthesis of kynurenic acid from tryptophan, is increased in the postmortem prefrontal cortex of patients with schizophrenia (Miller et al. 2004), thus pointing towards an increased kynurenic acid formation in schizophrenia. Since kynurenic acid is synthesised in and released from astrocytes (Curatolo et al. 1996; Guillemin et al. 2001; Kiss et al. 2003), one possibility is that the increased kynurenic acid formation in schizophrenia is related to a disturbed astrocytic functioning. The postmortem findings that patients with schizophrenia do not show astrogliosis (Roberts et al. 1986, 1987; Arnold et al. 1996, 1998; Falkai et al. 1999) might suggest that there is an increase in astrocyte activity rather than the astrocyte number in schizophrenia. In support of this theory, several studies have demonstrated an elevation of the protein S100B, a biological marker of astrocytic activity, in serum (Lara et al. 2001; Rothermundt et al. 2001, 2004a,b; Schroeter et al. 2003; Schmitt et al. 2005) and in CSF (Rothermundt et al. 2004b) from patients with schizophrenia.

Since schizophrenia is associated with a dysfunction of dopaminergic systems (see Abi-Dargham and Laruelle 2005) which is tentatively induced by increased brain kynurenic acid concentration (Erhardt et al. 2001a; Schwarcz et al. 2001), future therapeutic strategies in the treatment of the disease are suggested to be directed towards brain kynurenic acid formation. In support of that, it has been shown that when conventional antipsychotic treatment is supplemented with COX-2 inhibitors, which decrease rat brain kynurenic acid concentration and as a result decrease dopaminergic

activity (Schwieler et al. 2004b, 2005), there are beneficial effects with regard to both positive and negative symptoms in patients with schizophrenia (Müller et al. 2002, 2004).

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