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Importance of Endogenous Kynurenic Acid in Brain Catecholaminergic Processes and in the Pathophysiology of Schizophrenia

By

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Abstract

Kynurenic acid is a metabolite of tryptophan and the only known naturally occurring N-Methyl- D-Aspartic acid (NMDA)-receptor antagonist (at the co-agonist glycine site) in the human brain.

The aim of the present work was to investigate the physiological and pharmacological significance of endogenous kynurenic acid as well as its putative pathophysiological implications. For this purpose *in vivo* extracellular single unit recording from rat brain catecholamine neurons were used. An inhibitor of kynurenine 3-hydroxylase (PNU 156561A) was administered i.v. (40 mg/kg; 3-9 hours before the electrophysiological experiment) to cause an elevation of endogenous kynurenic acid levels. This effect was mediated by blockade of an alternative pathway for the precursor of kynurenine, thereby promoting the synthesis of kynurenic acid. After each experiment, the brains were rapidly taken out and analysis of kynurenic acid were performed using an isocratic reversed-phase HPLC system.

Pharmacologically induced elevation (3-5 fold) of endogenous kynurenic acid levels was associated with increased firing rate and burst activity and a decreased regularity of firing of midbrain dopamine (DA) neurons. These actions were reversed by treatment with D-cycloserine (128 mg/kg i.v., 5 min), an agonist at the glycine site of the NMDA receptor. The effects of PNU 156561A on the neuronal activity of midbrain DA neurons are in all probability induced by a blockade of NMDA receptors by kynurenic acid and mediated via inhibition of GABAergic afferents. The $GABA_A$-agonist muscimol produces a paradoxical hyperactivity of substantia nigra (SN) DA neurons. Pretreatment with PNU 156561A, producing an increase in endogenous kynurenic acid, as well as systemically administered MK-801 or intracerebroventricular administration of kynurenic acid clearly antagonized this excitatory action of muscimol. However, in rats with elevated levels of endogenous kynurenic acid muscimol´s excitatory action was reversed into a pure inhibitory response. Our results suggest an excitatory glutamatergic component in the actions of muscimol on SN DA neurons.

Previous studies have shown that systemically administered nicotine is associated with an activation of rat midbrain DA neurons and noradrenergic (NA) neurons in the locus coeruleus (LC). Elevation of endogenous kynurenic acid by PNU 156561A was found to antagonize the nicotine-induced (25-200 µg/kg i.v.) increase in firing rate of LC NA neurons. Similarly, the increase in firing rate and burst firing activity of ventral tegmental area (VTA) DA neurons elicited by nicotine (1.5-400 µg/kg i.v.) was antagonized but also reversed into an inhibitory response by elevated levels of kynurenic acid. These antagonistic actions induced by PNU 156561A pretreatment were prevented by administration of D-cycloserine (128 mg/kg i.v., 5 min). A novel finding in this study is that the activation of VTA DA neurons by nicotine was preceded by a short-lasting inhibition in firing rate. This inhibitory action of nicotine was antagonized by pretreatment with the selective $GABA_B$ receptor antagonist CGP 35348 (200 mg/kg i.v., 3 min). In addition, CGP 35348 also facilitated the nicotine-induced increase in burst firing activity of VTA DA neurons. We propose that nicotine exerts both excitatory and inhibitory actions of VTA DA neurons and that these effects are related to release of glutamate and GABA, respectively.

Present experimental data support a contribution of endogenous kynurenic acid in brain glutamatergic neurotransmission. Further, our findings demonstrate that a moderate elevation in brain kynurenic acid levels produces dysregulation of neuronal firing of midbrain DA neurons, similarly to the effects of systemic administration of the NMDA receptor antagonists phencyclidine (PCP, angel dust) or ketamine. Schizophrenia is thought to be mediated, at least in part, by dysregulation of the midbrain DA pathways and interestingly, both PCP and ketamine induce schizophrenia-like positive and negative symptoms as well as cognitive dysfunction in healthy volunteers, and furthermore, aggravate psychotic symptoms in schizophrenic patients. Based upon these findings we investigated the endogenous levels of kynurenic acid in cerebrospinal fluid (CSF) in patients with schizophrenia. CSF levels of kynurenic acid from 28 schizophrenic patients (25 of whom were drug-naive) and 17 healthy volunteers were analyzed. Kynurenic acid was detected in all controls, with little inter-individual variation (0.97 ±0.07 nM). In patients suffering from schizophrenia, CSF levels of kynurenic acid levels were higher than in volunteers (1.67 ±0.27 nM; $p=0.038$, Mann-Whitney U-test) and the variation between individuals was larger, with a maximum value of 6.8 nM. These results indicate a contribution of kynurenic acid in the pathophysiology of schizophrenia.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
cf,	compare (*confer* lat.)
CNS	central nervous system
CSF	cerebro spinal fluid
EAA	excitatory amino acid
e.g.	for example (*exempli gratia* lat.)
GABA	γ-aminobutyric acid
HPLC	high performance liquid chromatograph
i.c.v.	intracerebroventricular
i.e.	that is (*id est* lat.)
i.p.	intraperitoneal
i.v.	intravenous
KAT	kynurenine amino transferase
LC	locus coeruleus
L-DOPA	L-dihydroxyphenylalanine
mGluR	metabotropic glutamate receptor
nAChR	nicotinic acetylcholine receptor
NMDA	*N*-methyl-D-aspartic acid
PCP	phencyclidine; angel dust)
PET	positron emission tomography
SEM	standard error mean
SN	substantia nigra
SN-ZC	substantia nigra zona compacta
VTA	ventral tegmental area

Schizophrenia is among the most severe and debilitating psychiatric disorders and one of the world's most important public health problems. It is a tragic and devastating mental illness that typically strikes maturing people just when they are entering into adulthood. The lifetime prevalence is approximately one per cent worldwide and it appears relatively independent of geographic, cultural, and socioeconomic variables (Carpenter & Buchanan 1994). Both morbidity and mortality are high (the suicide rate is 10 per cent), leading not only to human suffering but also to high costs to society in terms of lost productivity as well as cost of healthcare. In Sweden, the cost of healthcare for schizophrenic patients is estimated at four billion SEK per year (2000)*.

The symptoms and signs of schizophrenia are very complex and diverse, and they encompass abnormalities in perception (hallucinations), inferential thinking (delusions), language (disorganized speech), social and motor behavior, attention, emotional expression, and executive ability (see Andreasen 1995). These symptoms are commonly divided into three different clusters (see Andreasen et al. 1996). The positive symptoms of schizophrenia include hallucinations (usually in form of voices, and often exhortatory in their message), delusions (often paranoid in nature), bizarre behavior and perceptual distortions, whereas the negative symptoms are characterized by a decrease or loss of normal functions. These symptoms are loss of speech (alogia – literally an absence of words), and withdrawal from social contacts, amotivation, apathy, anhedonia (loss of the ability to experience pleasure) and blunting of emotions (emotions are either absent or inappropriate to the situation). Patients with schizophrenia also have impairment in many different cognitive systems, such as deficit in memory and attention as well as lack of insight, judgment, and executive functions (see Green et al. 2000).

Although family, twin and adoption studies provide evidence of a genetic component in the cause of schizophrenia (Asherson et al. 1996), the etiology as well as the pathogenesis of the disease is yet unknown. The most typical age of onset of schizophrenia is during the late teens and early 20s, a time when brain maturation is reaching completion (Armstrong et al. 1995). Thus, it has been suggested that the pathogenesis of the disease must involve a neurodevelopmental process related to the final stages of "brain sculpturing", such as neuronal pruning or activity-dependent changes (i.e. psychological experiences that affect brain plas-

*** http://www.hubin.org/literature/texts/med_vet_4_2000_se.html**

ticity; Woods 1998). In addition, it has been suggested that the primary pathological process of schizophrenia occurs during brain development long before the illness becomes clinically evident (Weinberger 1987). Such primary cerebral insults have been suggested to derive from e.g. obstetric complications, maternal stress during pregnancy or infections during pregnancy or childhood (Mortensen et al. 1999). Psychological factors, such as stress, may precipitate acute episodes, but do not appear to be the underlying cause.

All clinically used drugs for schizophrenia have one major property in common, namely that they all target the dopaminergic systems of the brain (Meltzer 1987), and in particular, they antagonize central dopamine receptors, especially the D_2-receptor (see Carlsson 1988). However, the response to treatment with antidopaminergic drugs is seldom entirely satisfactory; a significant proportion of schizophrenic patients do not respond to this treatment and most patients experience an incomplete therapeutic efficacy (see Brenner et al. 1990). Moreover, due to side effects, especially endocrine and extrapyramidal motor disturbances (see Meltzer 1985, Hansen et al. 1997), the patient compliance for these drugs is often affected.

In recent years the ”dopamine hypothesis of schizophrenia”, originally formulated nearly forty years ago (Carlsson & Lindquist 1963), has been modified into a more diversified view where an attenuated glutamatergic neurotransmission is believed to participate in the pathogenesis of the disease (see Carlsson et al. 2000). Thus, the changes in dopaminergic functions may be secondary to aberrations elsewhere, and are perhaps partly compensatory mechanisms (see Carlsson et al. 2000).

Excitatory amino acids

Glutamate and aspartate are the major neurotransmitters mediating synaptic excitation in central nervous system (CNS). Both these so called excitatory amino acids (EAA) are non-essential amino acids, which means that the body easily synthesizes them and they are not required in the diet. During the late 1950s and early 1960s detailed analyses of EAA were conducted. However, EAAs were not considered to fulfill the criteria as neurotransmitters because they lacked the regional specificity required of a chemically signaling agent. Today it is widely acknowledged that glutamate is a *bona fide* transmitter accounting for fast excitatory signaling in the vertebrate nervous system (Orrego & Villanueva 1993). Interestingly, glutamate neurons, in contrast to other neurons in the brain, seem to operate in close cooperation with neighboring astrocytes, which accumulate glutamate, store it as glutamine, and then release it for conversion back to glutamate by the nerve cells. Released glutamate exerts its excitatory action via activation of EAA receptors, probably located on virtually all neurons in the vertebrate CNS. EAA receptors are divided into at least four different subtypes, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), kainate, and metabotropic receptors. While metabotropic receptors are coupled to G-proteins and operate through second messenger systems, NMDA-, AMPA-, and kainate-receptors, named according to their specific agonists, are all ionotropic, which means that they are ligand-gated ion channel receptors (see Fig 1). It is generally accepted that the endogenous ligands for all of these receptors (ionotropic and metabotropic) are glutamate and/or aspartate, but other alternatives, such as cysteic and homocysteic acid, have also been proposed. The only known naturally occurring antagonist of

Synthesis and metabolism of glutamate

- Most glutamate is synthesized during the normal oxidative metabolism of glucose (glycolysis). These processes involve the breakdown of glucose to pyruvate, which is converted to acetyl coenzyme A (acetyl CoA). Acetyl CoA is then incorporated in the citric acid cycle in which α-ketoglutarate is a key constituent. By transamination, α-ketoglutarate is converted to glutamate.
- Another pathway for glutamate synthesis is the formation of glutamate from glutamine. This process involves a phosphate-activated glutaminase, a mitochondrial enzyme that requires ATP for its activity. Glutamine is formed from released glutamate and stored in astrocytes.
- Catabolism of glutamate is closely related to its synthesis including formation of glutamine and conversion of glutamate to α-ketoglutarate.
- Released glutamate is rapidly inactivated through an uptake mechanism.

The NMDA receptor complex consists of several distinct recognition sites.
1) A primary neurotransmitter site that binds aspartate, glutamate, and NMDA.
2) A cation channel that permits the conductance of calcium and sodium.
3) A voltage -dependent site that binds magnesium and at resting membrane potential blocks channel conductance.
4) A co-agonist site that binds glycine.
5) An inhibitory site that binds phencyclidine, ketamine, MK-801, and other non-competitive antagonists.
6) An allosteric modulatory site that recognizes polyamines.
7) Some evidence exists for a site that binds zinc.

the ionotropic EAA receptors is kynurenic acid, which is also synthesized in astrocytes.

The AMPA as well as the kainate receptors, often referred to as non-NMDA receptors, are associated with a cation channel that is nonselective with respect to Na^+- and K^+- ions. The AMPA receptor is widely distributed in the brain and is considered the most important receptor for fast excitatory signaling in the CNS (Seeburg 1993). Sixteen different subunits for ionotropic glutamate receptors have been cloned (Hollmann & Heinemann 1994) and four of these subunits, termed GluR1-GluR4, form the AMPA subtype (Bettler & Mulle 1995). Each of these AMPA-related subunits occurs in two forms, called ”flip” and ”flop”, which arise from alternative mRNA splicing (Sommer et al. 1990). Kainate receptors are formed from five different subunits (GluR5, GluR6, GluR7, KA1 and KA2; Seeburg 1993). These receptors have restricted distribution in the mammal brain and their physiological functions are not clear.

If less is known about the kainate receptor, more is known with regard to the NMDA receptor, which is widely distributed throughout the entire brain. The molecular basis of the NMDA receptor diversity has been clarified with the cloning of the NMDA receptor genes. Two main subunits designated NR1 and NR2 have been identified, with the NR2 having at least four variants labeled 2A-2D. Several splice variants of the NR1 subunit have also been described. The native receptor is composed of heteropentametric subunit protein complex, in which each subunit possesses four transmembrane segments. Although the NMDA receptors, like the non-NMDA-receptors, allow permeability to both Na^+ and K^+ ions, only NMDA-receptors also permit a pronounced Ca^{2+} influx, and they operate over a much slower time scale. A unique feature of the NMDA-receptors is that their channels require the simultaneous binding of glutamate and local membrane depo-

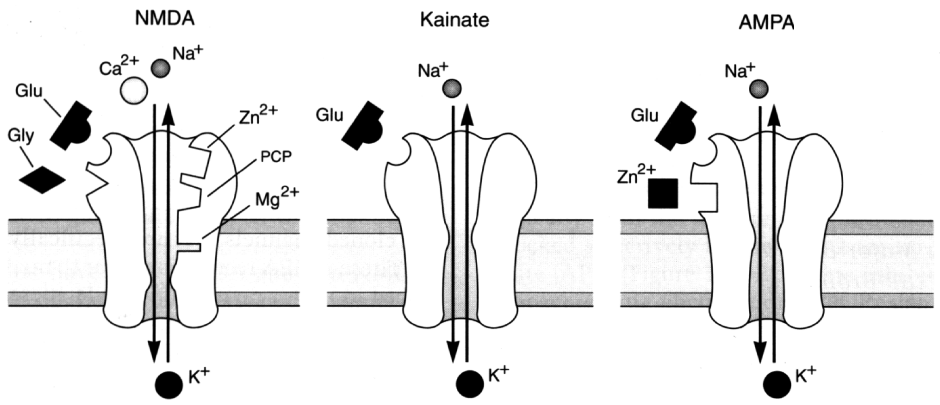

Fig. 1. Schematic drawing of the ionotropic excitatory amino acid receptors (Adapted from Kandel et al. 1991).

dependent manner by physiological concentrations of Mg^{2+}. Thus, ion current flow through open unblocked channels occurs only when presynaptic transmitter (glutamate) release is combined with postsynaptic depolarization. In addition, the channel is able to function efficiently only in the presence of glycine, which functions as an obligatory NMDA receptor co-agonist.

Metabotropic glutamate receptors are coupled to G proteins and operate through second messenger systems. Eight different metabotropic receptors, named mGluR1 through mGluR8 have been identified, and these are divided into three groups based on similarity of amino acid sequence, pharmacology, and second messenger coupling (Schoepp 1994). The first group consists of mGluR1 and mGluR5 and is positively coupled to the phosphoinositide second messenger system. The second group (mGluR2 and mGluR3) as well as the third group (mGluR4, mGluR6, mGluR7 and mGluR8) is negatively coupled to adenylyl cyclase. Both ionotropic and metabotropic receptors are expressed in the ventral tegmental area (VTA) and substantia nigra (SN) as well as in the locus coeruleus (LC).

Catecholaminergic systems in the brain: anatomy and function

The development of the fluorescent histochemical method by Hillarp and coworkers, by making brain monoaminergic neurons visible (Falck et al. 1962), enabled a detailed mapping of monoaminergic pathways. This method also made it possible for Dahlström and Fuxe (1964) to classify brain monoaminergic cell groups, and subsequently designate the various noradrenergic and dopaminergic

nuclei with the letter A (A1-A7 and A8-A15, respectively), the serotonergic nuclei with the letter B (B1-B9), and the adrenergic nuclei with the letter C (C1-C3).

Noradrenaline

The noradrenergic neurons are restricted to a number of small clusters (A1-A7) in the pons and medulla (Dahlström & Fuxe 1964); of these, the most prominent nucleus is the locus coeruleus (LC; A6 and A4), which is located in the gray matter of the pons. In man, this very small cell group contains only approximately 12,000 neurons and, in the rat about 1,500 neurons, on each side of the brain. However, the fibers of these neurons form five major noradrenergic tracts, mainly in the medial forebrain bundle, and thus innervate large areas of the brain including the cortex, the hippocampus, the cerebellum, and the spinal cord (Ungerstedt 1971, Lindvall & Björklund 1974). The LC has long been thought to regulate states of alertness and to enhance the ability of the individual to react adaptively to sensory environmental stimuli (Foote et al. 1991). Thus, activation of these neurons is associated with an increased arousal, attention, and vigilance, and accordingly, during sleep, especially during rapid eye movement (REM) sleep, they seem to be almost completely silent (Aston-Jones & Bloom 1981). Moreover, foreign or threatening stimuli excite these neurons much more effectively than familiar ones. Studies that are more recent also indicate an even closer relationship between LC activity and behavioral performance; for example, the nucleus is suggested to play an important role in task-related cognitive processes (Usher et al. 1999). Anatomic and physiologic studies have shown that the core of the LC receives its major afferent projection from the nucleus paragigantocellularis and nucleus prepositus hypoglossi, whereas the shell appears to be innervated by a variety of extrinsic afferent inputs (see Ennis et al. 1998). The innervation of the LC from nucleus paragiganto-cellularis is predominantly excitatory (Ennis & Aston-Jones 1986, 1988) and mediated via release of EAA (Ennis & Aston-Jones 1988, Chen & Engberg 1989), whereas the afferentiation from nucleus pre-

Synthesis of Catecholamines

All synthesis of catecholamines begins with the amino acid tyrosine, which is transported across the blood-brain barrier into the catecholaminergic neurons (Pardridge & Oldendorf 1977). The first step, which is also rate-limiting, is the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. This enzyme is expressed in all neurons that synthesize catecholamines. L-DOPA is then decarboxylated to dopamine, a reaction catalyzed by L-amino acid decarboxylase. Dopamine is subsequently converted to noradrenaline by dopamine β-hydoxylase. Phenylethanolamine N-methyl transferase catalyzes the *N*-methylation of noradrenaline to adrenaline.

positus hypoglossi is of pure inhibitory origin (Ennis & Aston-Jones 1989, Chen & Engberg 1989). The typical electrophysiological response of LC neurons to toe pinch or foot pad stimuli, i.e., an instantaneous, short-lasting excitation accompanied by a quiescent interval (Korf et al. 1974), is mediated via release of EAA from nucleus paragiganto-cellularis nerve terminals (Ennis & Aston-Jones 1988). In addition, it has also been shown that local adrenergic receptors (α_2) control the activity of the LC noradrenergic neurons, probably via noradrenergic, recurrent axon collaterals within the LC (see Aghajanian 1977, Engberg 1981). According to the close relationship between mood and states of arousal (depressed people may respond very poorly to external stimuli), it has been suggested that depression results from a functional deficiency of noradrenaline (concomitant with serotonin) in the brain, whereas mania could result from an excess (see Harro & Oreland 1996, Anand & Charney 2000).

Uptake and Degradation of Catecholamines

Once released, reuptake of catecholamines is the main mechanism for disposal of released transmitter. Thus, released catecholamines are transported back into the neuron via a high-affinity uptake carrier. Noradrenaline and dopamine are metabolized by two enzymes, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). MAO occurs mainly in the mitochondria of the catecholaminergic neurons and converts catecholamines to their corresponding aldehydes. Thus, noradrenaline becomes dihydroxymandelic acid (DOMA) and dopamine becomes dihydroxyphenyl acetic acid (DOPAC). The other metabolic pathway for catecholamines involves methylation of one of the catechol-OH groups. Here the main final metbolite of noradrenaline will be 3-methoxy-4-hydroxymandelic acid (VMA) and for dopamine 3-methoxy-tyramine (3-MT) and homovanillic acid (HVA).

Dopamine

Until the late 1950s, before Carlsson and coworkers proposed a biological role for dopamine (Carlsson et al. 1957, 1958, Carlsson 1959), dopamine was exclusively considered an intermediate in the biosynthesis of noradrenaline and adrenaline. Since Carlsson first discovered that dopamine is a neurotransmitter in its own right, its anatomical distribution in the brain as well as its functional role has been thoroughly studied.

By combining, Hillarp's fluorescent histochemical method with anatomical lesions and biochemical determinations, the major ascending dopamine pathways in the brain were evaluated. Thus, dopamine neurons located in the SN-zona compacta (SN-ZC; A9), the ventral tegmental area of Tsai (VTA; A10) and the retrorubral nucleus (A8) constitute the origin of the mesostriatal dopamine system. The dorsal part of this pathway projects, via the medial forebrain bundle and then the internal capsule, to the dorsal part of the striatum (principally the cau-

date nucleus and putamen) and hence it is named the nigrostriatal dopamine system (Andén et al. 1964). The nigrostriatal pathway exerts a powerful influence upon motor function and deficits in this system are the underlying pathophysiological cause of Parkinson's disease (Carlsson 1959, Ehringer & Hornykiewicz 1960). Another major dopaminergic pathway is named the mesolimbocortical dopamine system. This system originates in the VTA and projects via the medial forebrain bundle to both cortical and subcortical (limbic) structures. For this reason, the mesolimbocortical dopamine system is often subdivided into the mesolimbic dopamine system - projecting to the amygdaloid complex, the nucleus accumbens, the olfactory tubercle and the septal area - and the mesocortical dopamine system, innervating structures in the prefrontal cortex, such as prelimbic, infralimbic, and cingulate cortices (Andén et al. 1966, Ungerstedt 1971, Björklund & Lindvall 1984). Several processes, for example motivation, control of emotions such as reward and reinforcement, and cognition are attributed to the mesocorticolimbic dopamine system (Fibiger & Phillips 1988, LeMoal & Simon 1991, Schultz et al. 1993, Schultz 1998). Interestingly, all of these processes are implicated in the signs and symptoms of schizophrenia.

Electrophysiology of brain dopaminergic neurons

The electrophysiological properties of midbrain dopamine neurons have been thoroughly studied both *in vivo* and *in vitro* (Aghajanian & Bunney 1973, Grace & Bunney 1983, 1984a, 1984b, Grace & Onn 1989, Johnson & North 1992). Thus, dopamine neurons can alternate between spontaneous activity and states of silence, probably due to temporary hyperpolarization or depolarization block (Grace & Bunney 1984a). As revealed in both intra- and extra-cellular studies as many as 40-50% of all cat midbrain dopamine neurons are inactive (Romo et al. 1986). In the anesthetized as well as in the freely moving rat, active dopamine neurons spontaneously fire in at least two major modes: an irregular and relatively slow single spike firing mode or, alternatively, a relatively rapid burst firing mode, riding on a depolarizing wave and resulting in decreasing spike amplitude (5-15 mV) and increasing spike width within each burst (Grace & Bunney 1984a, b). According to the conventional criteria (e.g. those of Grace & Bunney 1984a, b), up to 50% of spontaneously active midbrain dopaminergic cells show burst firing (Grace & Bunney 1984a, Clark & Chiodo 1988). In general, dopamine neurons exhibit a firing rate of 1-10 spikes per second (mean 4.5 spikes per second: Grace & Bunney 1984a). The action potentials of the midbrain dopamine neurons have a characteristic shape with

a long duration (> 2 ms), and with an inflection in the rising phase (Aghajanian & Bunney 1973, Grace & Bunney 1984a, b).

Firing pattern of dopamine neurons appears fundamental for transmitter release in terminal areas (see Gonon 1988, Bean & Roth 1991, Manley et al. 1992). Thus, a switch from a single spike mode of firing to burst firing activity leads to a massive release of dopamine from the terminals (as much as a six-fold increase has been observed (Gonon 1988)), thereby affecting dopamine-receptors at post-synaptic target neurons, as assessed e.g. by the expression of several immediate-early genes (Chergui et al. 1996, 1997). In consonance, pharmacological obliteration of burst firing in nigral dopamine neurons has been shown to reduce the dopamine terminal efflux (Nissbrandt et al. 1994). Spontaneous burst firing appears to be the result of tonically active afferent innervations of the dopamine neurons, since this mode of firing is absent in the midbrain dopamine slice preparation, where the afferents to the dopamine neurons have been cut (Sanghera et al. 1984, Grace & Onn 1989, Seutin et al. 1990, Johnson et al. 1992). Furthermore, bursting activity of midbrain dopamine neurons in behaving monkeys is found to relate to primary food and fluid rewards and to conditioned incentive stimuli predicting reward (Schultz et al. 1993, see Schultz 1998).

Afferent control of dopaminergic firing activity

Midbrain dopamine neurons receive neuronal inputs from a variety of sources that utilize a variety of neurotransmitters, e.g. glutamate, γ-aminobutyric acid (GABA), acetylcholine, serotonine and noradrenaline. Furthermore, the firing activity of dopamine neurons is controlled by autoinhibitory somatodendritic dopamine D_2 receptors.

Substantia nigra

Changes in firing pattern of nigral dopamine neurons, i.e. a conversion from a single firing mode to a bursting mode are suggested to be a result of activation of glutamatergic projections to the SN from e.g. the prefrontal cortex (Carter 1982, Naito & Kita 1994) and the subthalamic nucleus (Kita & Kitai 1987). The SN-ZC dopamine neurons are also closely interacting with and controlled by GABA neurons originating in the SN-zona reticulata (Hajos & Greenfield 1994, Tepper et al. 1995) or in the striatum (see Engberg et al. 1997). Thus, systemic administration of the $GABA_B$-receptor agonist baclofen is associated with a dose-dependent regularization of the firing pattern, concomitant with a cessation of

burst firing (Engberg et al. 1993, Erhardt et al. 1998). Firing activity of SN-ZC dopamine neurons is also inhibited by somatodendritic $GABA_A$-receptors (Johnson & North 1992, Tepper et al. 1995), but, paradoxically, systemic administration of $GABA_A$-receptor agonists is associated with an activation of these neurons (Waszczak et al. 1980, Engberg et al. 1993).

Ventral tegmental area

EAA-containing afferents, which originate in the prefrontal cortex (Christie et al. 1985, Sesack et al. 1989), the subthalamic nucleus (Phillipson 1979, Robledo & Féger 1990), the pedunculopontine nucleus (Di Loreto et al. 1992), the mesopontine nuclei (Clements & Grant 1990, Cornwall et al. 1990) and the habenula (Bayer et al. 1990) have been found to play an essential role in the genesis of burst firing activity of VTA dopamine neurons. Thus, stimulation of the prefrontal cortex increases burst firing of VTA dopamine neurons (Gariano & Groves 1988, Murase et al. 1993a, Tong et al. 1996), whereas inactivation of the prefrontal cortex produces the opposite effect (Svensson & Tung 1989, Murase et al. 1993a). These effects may be mediated by a monosynaptic projection from the prefrontal cortex to the dopamine neurons in the VTA (Sesack & Pickel 1992). However, the quantitatively largest input to the VTA dopamine neurons arises from GABAergic afferents. These afferents either come from intrinsic GABAergic interneurons in the VTA (Di Chiara et al. 1979, Stanford & Lacey 1996, Fisher et al. 1998, Steffensen et al. 1998, Carr & Sesack 2000) or from GABAergic neurons with extensive axon collaterals originating from rostral areas such as the nucleus accumbens, the striatum, the globus pallidus, and the ventral pallidum (Fonnum et al. 1978, Ribak et al. 1980, Walaas & Fonnum 1980, Kalivas et al. 1993). In addition, these latter GABAergic neurons form part of long-loop systems. $GABA_A$- and $GABA_B$- receptors are present on both dopaminergic neurons and on GABAergic interneurons. As far as the VTA is concerned, there is evidence that the $GABA_A$ –mediated input comes from intrinsic interneurons, and the $GABA_B$–mediated input comes from the long-loop control afferents (Johnson & North 1992).

Recently, a GABAergic component in the induction of burst firing of midbrain dopamine neurons was demonstrated, since $GABA_B$-receptor antagonists were shown to both convert non-bursting neurons to bursting neurons and to increase the mean percent bursting of VTA and SN dopamine neurons (Erhardt et al. 1999, Erhardt et al. 2001c). Consequently, burst firing of midbrain dopamine neurons seems to be driven by both EAA- and GABA- containing afferents where

concomitant $GABA_B$- and NMDA-receptor stimulation provides a machinery for a particularly effective initiation of burst firing *in vivo*. In addition, during the last years a substantial proportion of studies suggest that VTA dopamine neurons projecting to the prefrontal cortex and nucleus accumbens, respectively, are differentially innervated (Murase et al. 1993b, Carr & Sesack 2000, Takahata & Moghaddam 2000). Thus, Carr and Sesack (2000) have shown that prefrontal cortex terminals synapse onto GABA interneurons within the VTA, onto GABA neurons projecting to the nucleus accumbens and onto dopamine neurons that directly project back to the prefrontal cortex. Hereby, the prefrontal cortex may alter the neuronal activity of VTA dopamine neurons both directly, by synapsing onto dopamine neurons projecting back to the prefrontal cortex and indirectly, by influencing GABAergic interneurons that have contact with dopamine neurons that in turn project to the nucleus accumbens (Fig. 2). Moreover, it has been suggested that dopamine projections to the prefrontal cortex are under tonic excitatory regulation of AMPA receptors in the VTA, whereas dopamine projections to the nucleus accumbens are under indirect tonic inhibitory control of AMPA receptors (Takahata & Moghaddam 2000). These findings may have important implications for understanding the pathophysiology of schizophrenia since several studies suggest that this disease is associated with both overactivity in the mesolimbic dopamine system and deficits in the mesocortical dopamine system (cf. Weinberger 1987, Svensson et al. 1995 and see below).

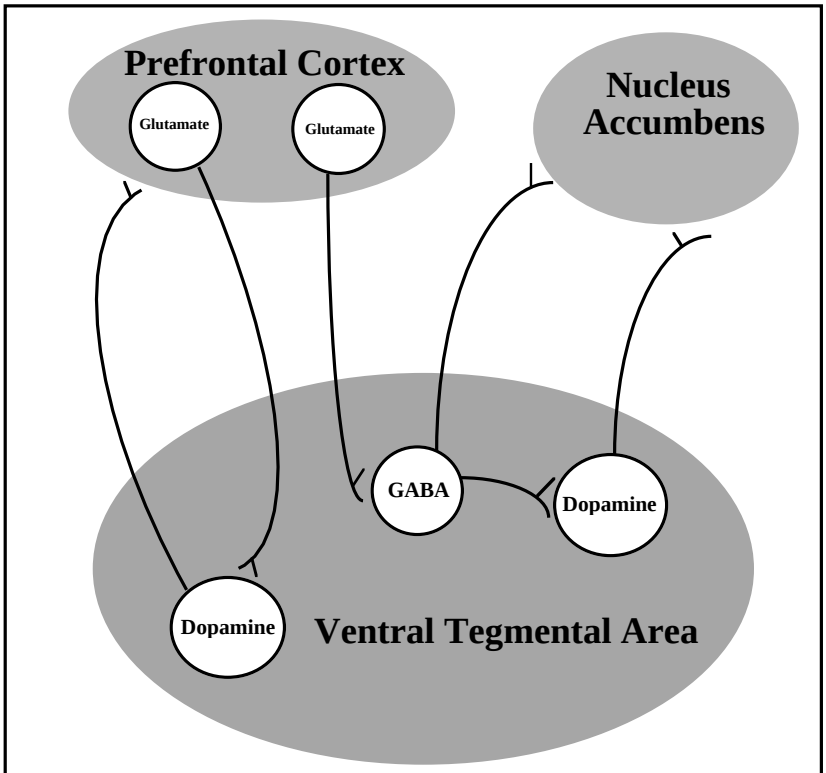

Fig. 2. Schematic drawing of the relationship between prefontal cortex terminals and mesoprefrontal and mesoaccumbens projecting neurons of the VTA.

The dopamine hypothesis of schizophrenia

For the past four decades, the predominant biological theory has been that dopaminergic hyperactivity in the brain is the underlying cause of schizophrenia (Carlsson & Lindqvist 1963). For many years, this idea was only supported by indirect, pharmacological evidence and the roots of the hypothesis lie in the discovery that dopamine antagonists reverse the symptoms of schizophrenia. The first compound to be used in the treatment of schizophrenia was chlorpromazine (Delay & Denicker 1952). Since then several structurally dissimilar antipsychotic drugs have been produced and all these compounds have one common feature, i.e. they all antagonize the dopamine D_2 receptor. However, it is well known that neuroleptics do not cure schizophrenia: they only dull the symptoms.

The ability of drugs to induce psychosis by increasing the dopaminergic tone constitutes a second line of evidence supporting the dopamine hypothesis of schizophrenia. Thus, patients who have Parkinson's disease and are treated with L-DOPA, the precursor of dopamine, can exhibit periods of psychotic symptoms (see Cummings 1992), and amphetamine, which releases dopamine in the brain, can induce a psychosis-like state in healthy controls (Snyder 1973) as well as exacerbate positive symptoms in schizophrenic patients (Angrist et al. 1974).

While clinical studies have long failed to distinguish a hyperactivity of dopamine among schizophrenic patients (see Farde et al. 1990, see Davis et al. 1991), recent positron emission tomography (PET) studies demonstrate an excessive, abnormal dopamine release in this disorder (Laruelle et al. 1996, Abi-Dargham et al. 2000). However, the notion that amphetamine-induced psychosis is devoid of negative symptoms, as well as the failure of classical neuroleptics to attenuate negative symptoms in schizophrenic patients, suggest that other mechanisms than dopamine hyperactivity could be implicated in the pathophysiology of schizophrenia.

The negative symptoms, e.g. hypofrontality including lack of goal-oriented behavior, affective flattening, and emotional indifference to normally rewarding stimuli are rather suggestive of a dopaminergic hypofunction. This theory is supported by the findings of a reduced cerebral blood flow in the frontal cortex in schizophrenics (Ingvar & Franzén 1974, Weinberger et al. 1986), and a study showing reduced central dopamine output in chronic schizophrenics (Karoum et al. 1987). Thus, both hypo- and hyperfunctioning of brain dopamine systems in schizophrenia have been proposed, and because of different innervations of VTA dopamine neurons projecting to the prefrontal cortex and nucleus accumbens (see above, Murase et al. 1993b, Carr & Sesack 2000, Takahata & Moghaddam 2000),

these phenomena might even occur simultaneously. A distorted firing pattern, in particular a change in the burst firing mode, leading to a reduced signal-to-noise ratio, has been suggested to be responsible for both the increased stimulation of D_2 receptors by dopamine in schizophrenia (Grace 1991, Abi-Dargham et al. 2000) and the poor capacity to learn novel tasks that characterizes schizophrenia (Svensson 2000). The mechanism underlying the changes in phasic activity of dopamine neurons remains elusive, and thus the classical dopamine hypothesis of schizophrenia is now yielding to a more diversified view, where other neurotransmitters, e.g. glutamate, are believed to play a part in the pathophysiology of the disease (Carlsson et al. 2000, 2001).

Nicotine

Nicotine (1-methyl-2-[3-pyridyl]pyrrolidine) is one of many substances found in tobacco leaves, but nicotine comprises only approximately 5% of the weight of the dry plant leaves (Posselt & Reimann 1828). When Columbus at the end of the fifteenth century came to what is now known as America, he and his crewmembers observed the indigenous Americans smoking tobacco leaves for hedonistic, ritual, or magical purposes. Jean Nicot (1530-1600) believed that tobacco chewing had medical effects and hence he introduced tobacco chewing to Catherine de Medici, the French queen at that time (who used it as treatment against her headache), and promoted the import and cultivation of the plant. Therefore, the Swedish botanist Carolus Linnaeus named the plant *Nicotina rustica* or *Nicotina tabacum* to honor him.

When the leaves are smoked, chewed, or snorted (as snuff), nicotine is absorbed into the blood and exerts a variety of physiological and pharmacological effects. The central actions of nicotine appear complex and cannot easily be described in terms of stimulation or inhibition. At the cellular level, its actions are mediated via activation of nicotinic acetylcholine receptors (nAChRs), located both pre- and postsynaptically.

Whereas nicotine appears to be the only psychopharmacologically active substance present in tobacco, it is other substances from cigarettes, e.g. tar, carbon monoxide, and carcinogenic hydrocarbons, that are mainly responsible for smoking-related morbidity. Thus, the habit of tobacco smoking constitutes a major health problem that strongly contributes to the morbidity and mortality around the world (see Morb Mortal Wkly Rep 1993; 42:645-649).

Nicotine-induced excitation of LC noradrenaline neurons (Hajos & Engberg 1988, Engberg 1989, Engberg 1992, Engberg & Hajos 1994) and the increased noradrenaline release (Mitchell et al. 1992) are thought to contribute to its effects on arousal and cognition. The addictive effects of tobacco have for the last decades been attributed to nicotine and to its activating properties on midbrain dopamine neurons. The mechanisms behind these effects are still controversial, some studies suggest that nicotine activates dopamine neurons through activation of somatodendritic located nAChRs (Calabresi et al. 1989, Pidoplichko et al. 1997, Picciotto et al. 1998), whereas others suggest that nicotine acts presynaptically on glutamate receptors and hence promotes release of glutamate (Toth et al. 1992, Schilström et al. 1998, 2000, Grillner & Svensson 2000, Erhardt et al. 2001a). Conceivably, both mechanisms may be at work simultaneously: the firing rate of the dopamine neuron is increased through activation of somatodendritic $\alpha4\beta2$ nAChRs, while presynaptically located $\alpha7$* nAChRs induce glutamate release that accounts for the increase in burst firing activity (Svensson et al. 2000). A large number of studies have emphasized the role of nicotine in the reinforcing effects of tobacco use. For example, if mecamylamine (a nicotinic receptor antagonist) is administered to smokers, they will smoke more heavily, smoking more cigarettes as well as increasing the number and depth of inhalations (Stolerman et al. 1973), possibly in an attempt to overcome the actions of the antagonist. Furthermore, smokers who smoke "light" cigarettes compensate the low concentration of nicotine by smoking more cigarettes (Hill et al. 1983, McMorrow & Foxx 1983, see Scherer 1999). Animal studies show that nicotine is self-administered just as other addictive drugs, such as cocaine and amphetamine (Corrigall 1999).

Some Physiological and Pharmacological Effects of Nicotine

Peripheral effects

- Increase heart rate and blood pressure by stimulating the sympathetic ganglia and the adrenal glands to release noradrenaline and adrenaline.
- Stimulation of chemoreceptors in the aorta and carotid arteries, leading to vasoconstriction, tachycardia and increased blood pressure.
- Actions on parasympathetic autonomic ganglia increase hydrochloric acid (HCl) secretion in the stomach and increase motor activity in the bowel.

CNS effects

- Enhanced learning and memory as well as increased motivation and attention.
- At high doses it can produce tremors and convulsions, at lower doses it increases respiration rate and causes the release of antidiuretic hormone from the pituitary gland.

Smoking and schizophrenia

The prevalence of smoking in the general population in the industrial world is 20-50% (Collishaw & Lopez 1996), whereas the rate of smoking among patients suffering from mood disorders is approximately 40-70%. For patients with schizophrenia, the prevalence is extremely high, almost 90% (Hughes et al. 1986, Goff et al. 1992, Ziedonis et al. 1994, de Leon et al. 1995, Kelly et al. 1999, Cantor-Graae et al. 2001) and it has been proposed that smoking is a form of self-medication for these patients (Armitage et al. 1968, Hughes et al. 1986, Svensson et al. 1990). Some studies also report that these patients prefer high-nicotine cigarettes, and often smoke them down to the filter, where the concentrations of nicotine are highest (O'Farrell et al. 1983, Masterson & O´Shea 1984). The reason for the high prevalence of smoking among these patients remains elusive; many ideas are speculative, as there has been no study to determine exactly what effects smoking has on psychological or psychopathological symptoms in schizophrenic patients. Influenced by psychoanalytical theories, one of the earliest explanations for the excessive smoking among schizophrenics suggested that the cigarette (by producing pleasant or irritating sensations in or around the mouth) would soothe the schizophrenics' fixation at the oral stage of development (Brill 1922, Bergler 1953). Yet another theory suggests that since many schizophrenics are unemployed, they seem to "have the time" for smoking or smoke just because they are bored (Hughes et al. 1986). However, even after taking into consideration factors such as socioeconomic status or lifestyle, the prevalence of smoking is still extremely high among the schizophrenic population (Hughes et al. 1986, de Leon et al. 1995). Another idea concerning the prevalence of smoking in this group comes from the facts that polycyclic hydrocarbons in cigarette smoke stimulate the hepatic microsomal system, inducing liver enzymes to increase the metabolism of psychotropic medications (Benowitz 1988, Ziedonis et al. 1997), and that renal excretion of neuroleptics is increased by smoking (Miller et al. 1977, Jann et al. 1986). However, long before the introduction of neuroleptics in the 1960s it was well recognized among psychiatrists that at least institutionalized schizophrenics were heavy smokers, and a recent study also show that first-episode patients smoke at the same prevalence rate as chronic patients (McEvoy & Brown 1999), making this "schizophrenics smoke to off-set the effects of neuroleptics- idea" less attractive. A study from de Leon and coworkers (1995), utilizing a logistic regression analysis, also stated that the diagnosis of schizophrenia, but not neuroleptic treatment, was associated with heavy smoking. Other studies suggest that the illness itself may lead patients to

smoke. Thus, Freedman et al. (1995) and Leonard et al. (2000) proposed a disease-related dysfunction in nicotinic receptors and further, Adler et al (1998) suggested cholinergic sensory gating deficits to account for smoking among schizophrenics. Patients with schizophrenia may self-medicate with nicotine to alleviate both positive and negative symptoms (Glynn & Sussman 1990, Lohr & Flynn 1992, Kelly et al. 1999) as well as to improve cognition (Adler et al. 1993, Taiminen et al. 1998). It has also been reported that cessation of smoking, and subsequent nicotine withdrawal, worsens psychotic symptoms (O´Farrell et al. 1983, Glynn & Sussman 1990, Greeman & McClellan 1991, Dalack & Meador-Woodruff 1996). These putative beneficial effects of nicotine may be mediated through the regulation of the mesocorticolimbic dopamine system. Chronic administration of nicotine increases the activity of the VTA dopamine neurons, leading to excessive release of dopamine, especially in the prefrontal cortex (Vezina et al. 1992). Since a reduction in neuronal activity in the prefrontal cortex is believed to be responsible for the negative symptoms and the deficits in cognition (Weinberger et al. 1986, Paulman et al. 1990, Svensson et al. 1990), enhancement of the activity in this region, as induced by nicotine in cigarettes, may serve to alleviate or improve these symptoms. The mechanism underlying nicotine's alleviation of positive symptoms and psychotic states is, however, poorly understood.

Kynurenic acid

Kynurenic acid was first identified as early as 1853 when it was found in canine urine (Liebig 1853), and half a century later the compound was recognized as a biproduct of tryptophan metabolism (Ellinger 1904, Homer 1914). This route of conversion of tryptophan was in 1947 named the ”kynurenine pathway” (Beadle et al. 1947, see Heidelberger et al. 1949; Fig. 3). Interestingly, over 95 % of all dietary tryptophan is metabolized to kynurenines (Wolf 1974) and in peripheral tissues, only 1 % is converted to serotonin (5-HT). In peripheral tissues, in particular the liver, the indole ring of tryptophan is opened by either tryptophan dioxygenase or indoleamine 2,3-dioxygenase, which results in the formation of formylkynurenine. Kynurenine formylase then rapidly and almost complete converts formylkynurenine to L-kynurenine, the key compound of the ”kynurenine pathway” (Mehler & Knox 1950). L-kynurenine is present in low concentrations in the blood, the brain and in peripheral organs and it can easily cross the blood-brain barrier through the large neutral amino acid carrier. L-kynurenine can be metabolized by three different enzymes in mammalian tissues: I) kynurenine 3-hydroxylase which

form 3-hydroxy-kynurenine; II) kynureninease which form anthranilic acid and III) kynurenine aminotransferase (KAT) which causes the formation of kynurenic acid. Kynurenine 3-hydroxylase has the highest affinity for kynurenine, suggesting that under normal conditions, it metabolizes most of the available kynurenine (Bender & McCreanor 1982, see Moroni 1999). In the end, quinolinic acid is formed through this mechanism. In the late 1980s, two groups independently discovered the presence of kynurenic acid in the human brain (Moroni et al. 1988a, Turski et al. 1988). In the brain, as in the periphery, kynurenic acid is formed by irreversible transamination of kynurenine. In mammalian peripheral organs several rather unspecific aminotransferases have been identified. For example in the rat liver, four such enzymes have been characterized (Kido 1984); these catalyze the conversion of kynurenine to kynurenic acid by using a variety of oxoacids as cofactors (Noguchi et al. 1973, 1975, Harada et al. 1978, Okuno et al. 1980, Hodgkins et al. 1999). However, in the rat and human brain, two distinct enzymes responsible for the conversion of kynurenine to kynurenic acid have been characterized, i.e. KAT I and KAT II (Okuno et al. 1991a, Schmidt et al. 1993, Guidetti et al. 1997). Both these enzymes are preferentially localized in glial cells (Ceresoli-Boroni et al. 1999a) and a substantial proportion of both types of KAT are present in the mitochondria of the astrocytes (Okuno et al. 1991b, Du et al. 1992). Pharmacological studies as well as lesioning experiments

The large amino acid carrier

The cerebrovascular large amino acid carrier is a sodium independent, equilibrative mechanism that facilitates the exchange of 10 or more neutral amino acids between the plasma and the brain. Transport affinity is greatest for those amino acids with large, neutral side chains, such as leucin, tryptophan, and L-phenylalanine (Smith et al. 1987). L-kynurenine is an α-amino acid, that has the free carboxyl group and unsubstituted α-amino group that are required for significant L-system activity (Smith et al. 1989). Further, the side chain amino group (pKa ca 4-5) would be expected to be primarily uncharged at physiological pH (7.4), which is also necessary for significant affinity (see Fukui et al. 1991).

Kynurenine aminotransferase

KAT I is a soluble enzyme that prefers pyruvate as a cosubstrate and it has a pH optimum at 9.5-10 (Schmidt et al. 1993). It is potently inhibited by the competing substrates tryptophan, phenylalanine, and glutamine (Baran et al. 1994). Antibodies against this enzyme have demonstrated its preferential localization in astrocytes (Du et al. 1992, Roberts et al. 1992).

KAT II is localized in the soluble cytoplasm of the astrocytes (Okuno et al. 1991b, Ceresoli-Boroni et al. 1999a) and prefers oxoglutarate as a cosubstrate and is selectively inhibited by quisqualic acid (Guidetti et al. 1997). Its presence in the brain has been confirmed with Northern blot hybridization (Buchli et al. 1995) and it displays a pH optimum in the physiological range (Schmidt et al. 1993).

suggest that under physiological conditions KAT II accounts for >70 % of the production of kynurenic acid in the rat (Guidetti et al. 1997). This is also most likely since this enzyme displays a pH optimum in the physiological range (Schmidt et al. 1993). However, both KAT I and II enzymes have K_m in the millimolar range, suggesting that the kynurenine availability is the rate limiting step for kynurenic acid biosynthesis.

Because of its polar structure (Fukui et al. 1991), kynurenic acid is almost completely unable to pass the blood brain barrier (unless there are exceedingly high concentrations in the periphery; Scharfman et al. 2000). Thus, whereas kynurenine easily enters the brain, influx rates of kynurenic acid from blood, calculated from reported plasma concentrations and from measured K_{in} values, were only 0.0023 nmol/h/g (Fukui et al. 1991), which suggests that peripheral sources of kynurenic acid normally have only limited effects on cerebral concentration of kynurenic acid. However, the ability of kynurenic acid to penetrate into the brain might be enhanced during conditions when the blood brain barrier is compromised (Scharfman et al. 2000). Upon entry into the brain, the precursor, kynurenine, is rapidly transported into astrocytes by a Na^+ -independent concentrative process, similar to the large neutral amino acid carrier that transports kynurenine through the blood brain barrier (Christensen 1984, Speciale et al. 1989). Furthermore, it seems that once formed in the astrocytes, kynurenic acid is readily liberated into the extracellular milieu (Swartz et al. 1990, Gramsbergen et al. 1997). In addition, a less efficient, sodium-dependent mechanism also transports kynurenine into neurons (Speciale & Schwarcz 1990).

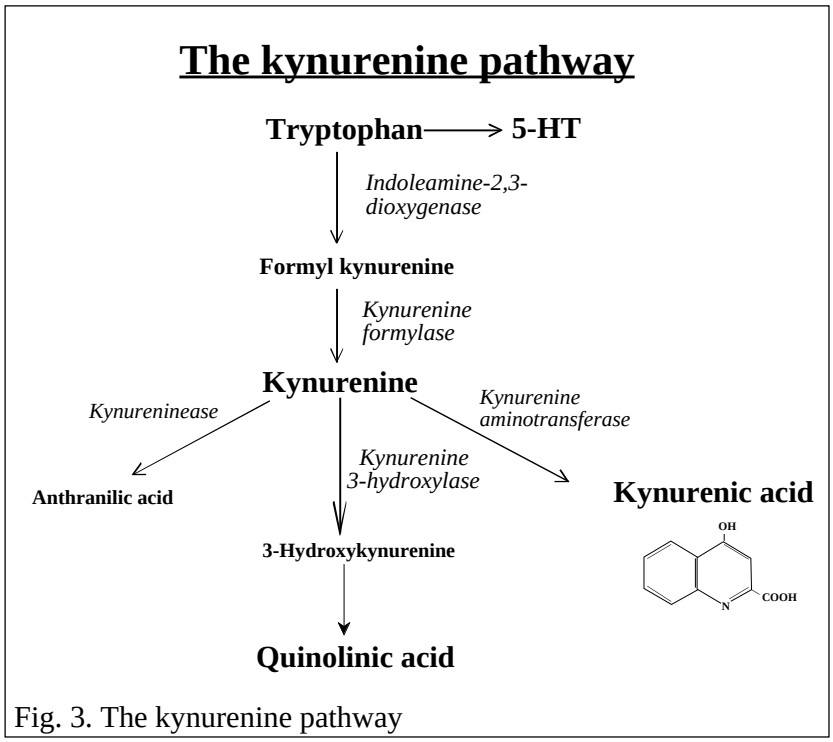

Fig. 3. The kynurenine pathway

No catabolic enzymes or re-uptake mechanisms for kynurenic acid have been detected so far, and although quinaldic acid was suggested in the mid

fifties to be a metabolite of kynurenic acid (Takahashi et al. 1956), this finding has never been confirmed (Turski & Schwarcz 1988). Furthermore, it has been shown that kynurenic acid can be enzymatically degraded by micro-organisms (Horibata et al. 1961). Under normal conditions though, the only alternative for kynurenic acid to be eliminated from the brain is via a probenecid-sensitive transporter (Moroni et al. 1988a). Therefore, rapid renal excretion seems to constitute the single most prominent mechanism of brain kynurenic acid disposition in the rat (Turski & Schwarcz 1988).

Regulation of the synthesis of kynurenic acid

Several distinct mechanisms that regulate the synthesis of kynurenic acid from its precursor kynurenine have been demonstrated in animal models. Some are brain-specific, whereas others can be observed both in brain and in peripheral organs. Thus, administration of kynurenine to a rat brain slice preparation enhances the production of kynurenic acid (Turski et al. 1989), and systemic injections of kynurenine to rats raise whole brain kynurenic acid (Wu et al. 1992). Increased brain kynurenic acid concentration is observed following acute seizures (Wu & Schwarcz 1996) and because of neuronal loss (Hodgkins & Schwarcz 1998a). Furthermore, production of kynurenic acid in the brain is probably also controlled by intracellular concentrations of amino acids, such as glutamine, phenylalanine and L-α-aminoadipate, which are competitive substrates of KAT I and KAT II (Chang et al. 1997). Several ways to increase the brain levels of endogenous kynurenic acid have been discovered. One example is administration of probenecid (Miller et al. 1992, Russi et al. 1992); this drug inhibits the efflux of kynurenic acid from the brain via a probenecid-sensitive carrier (Mo-

Mechanisms affecting synthesis of kynurenic acid in the brain

- Hypoglycemia reduces the synthesis of kynurenic acid (Gramsbergen et al. 1997) via inhibition of the uptake of kynurenine into glial cells (Hodgkins & Schwarcz 1998b). Lactate and pyruvate reverse this effect (Hodgkins & Schwarcz 1998b), lactate probably substitutes for glucose either by initiating gluconeogenesis (Dringen et al. 1993, Wiesinger et al. 1997) or by conversion to pyruvate and subsequent stimulation of cellular energy metabolism. Pyruvate is also able to serve as a cosubstrate of KAT (Guidetti et al. 1997).
- Extracellular concentrations of kynurenic acid change with the ionic milieu (Turski et al. 1989) and decrease in the presence of depolarizing agents such as veratridine (a commonly used tool for probing Na^+-channels) and high potassium concentrations (Gramsbergen et al. 1991, Wu et al. 1992) or after tissue exposure to L-α-aminoadipate (Wu et al. 1995).
- An increased dopaminergic transmission has been found to reduce the brain concentrations of kynurenic acid (Rassoulpour et al. 1998a, Poeggeler et al. 1998, Wu et al. 2000).

roni et al. 1988a). These experiments provide evidence that this transport mechanism for kynurenic acid is extremely efficient and probably functionally important for the clearance of kynurenic acid from the extracellular spaces of the brain. In addition, systemic administration of kynurenine causes a large and persistent increase in brain kynurenic acid levels in both rats (Swartz et al. 1990) and monkeys (Jauch et al. 1993). Further, the importance of drugs inhibiting the conversion of kynurenine towards quinolinic acid has been emphasized. Since these drugs, e.g. PNU 156561A and Ro 61-8048, inhibit an alternative pathway for kynurenine, the synthesis of kynurenic acid is promoted (Connick et al. 1992, Russi et al. 1992,

Mechanisms affecting synthesis both in brain and in peripheral organs

- Pyruvate and other 2-oxoacids augment the production of kynurenic acid primarily due to their ability to facilitate the transamination of kynurenine to kynurenic acid, by acting as co-substrates (Hodgkins et al. 1999).

- Some glutamate receptor ligands, e.g. L-glutamate, L-aspartate and quisqualate, reduce the formation of kynurenic acid in the brain and in liver slices (Urbanska et al. 1997, Battaglia et al. 2000), whereas NMDA, AMPA or kainate are unable to diminish the production of kynurenic acid (Urbanska et al. 1997). These findings indicate that activation of metabotropic glutamate receptors reduces the synthesis of kynurenic acid. Battaglia and coworkers conclude that some ligands of metabotropic glutamate receptors act intracellularly and inhibit KAT II activity.

Chiarugi et al. 1996, Speciale et al. 1996, Cozzi et al. 1999). Aminooxyacetic acid has been found to reduce brain concentrations of kynurenic acid by approximately 50% by inhibiting the KAT enzymes (Speciale et al. 1990, Russi et al. 1992). However, since aminooxyacetic acid was administered through a microdialysis probe in either the hippocampus (Russi et al. 1992) or the striatum (Speciale et al. 1990) its role in regulating also peripheral kynurenic acid is still unknown. In addition, the concentration of kynurenic acid seems to increase with age (Moroni et al. 1988b, Gramsbergen et al. 1992), probably due to increases in the amount of KAT per astrocyte, rather than a larger number of KAT-containing neurons (Gramsbergen et al. 1997).

Mechanism of action of kynurenic acid

Kynurenic acid is generally referred to as a broad spectrum EAA receptor antagonist (Perkins & Stone 1982, see Stone 1993), although at low concentrations the compound is a non-competitive antagonist of the NMDA-receptor ion channel complex, acting on the strychnine-insensitive glycine recognition site (Birch et al. 1988) with an IC_{50} value in the low micromolar range (~8-15 µM; Ganong & Cot-

man 1986, Kessler et al. 1989, Parsons et al. 1997). However, the compound is also an antagonist at the agonist recognition site of the NMDA receptor (IC_{50} 200-500 µM; Kessler et al. 1989). At higher concentrations (IC_{50} in the millimolar range), it is able to interact as a competitive antagonist at AMPA and kainate receptors (Bertolino et al. 1989, see Stone & Connick 1985, Kessler et al. 1989). The specificity of kynurenic acid as an EAA receptor antagonist has been under investigation during the past few years. Some studies reveal that kynurenic acid does not interfere with nicotinic receptors (Bijak et al. 1991, Bertolino et al. 1997) but a recent communication shows that kynurenic acid blocks the α7* nicotinic receptor with the same IC_{50} as for NMDA receptors (Hilmas et al. 2000). In a recent study though, kynurenic acid failed to affect nicotine-induced convulsions in mice, which may indicate that α7* nicotinic receptor-mediated events play no role in seizure activity produced by nicotine (Kis et al. 2000).

Physiological significance

Kynurenic acid is an anticonvulsant and displays neuroprotective properties (Foster et al. 1984; see Moroni 1999), actions that in all probability are related to its interaction with excitotoxic EAA neurotransmitters. Although, kynurenic acid has been suggested to be involved in the pathophysiology of several brain disorders, including e.g. Parkinson's disease (Ogawa et al. 1992; Miranda et al. 1999), Huntingson's disease (Beal et al. 1992; Guidetti et al. 2000), Alzheimer's disease (Baran et al. 1999), epilepsy (Yamamoto et al. 1995), and eating disorders (Demitrack et al. 1995), our knowledge about a potential physiological significance of the compound is limited. Thus, one of the outstanding debates in the kynurenine field is whether manipulation of the "kynurenine pathway" can modify the levels of quinolinic acid and kynurenic acid sufficiently to activate or antagonize EAA receptors, respectively (see Schwarcz et al. 1992a, see Stone 2001). In general, the concentration of kynurenic acid required to block glutamate receptors lies in the range 10-1000 µM (Bertolino et al. 1989, Stone & Connick 1985, Ganong & Cotman 1986, Kessler et al. 1989, Parsons et al. 1997). The concentration of kynurenic acid in brain tissues varies substantially between species, ranging from approximately 6 nM in mice (Moroni et al. 1988a) to approximately 1 mM in humans (Moroni et al. 1988a, Turski et al. 1988), and in the rat brain the concentration is approximately 20 nM (Moroni et al. 1988a, Connick et al. 1992). However, since several neuroanatomical studies reveal a production of kynurenic acid in astrocytes in close apposition to glutamatergic synapses (Roberts et al. 1995) higher concentrations of kynurenic

acid may be reached in the immediate vicinity of or inside the synaptic cleft and hence, released kynurenic acid is in an excellent position to influence NMDA receptor function. Therefore, most studies showing submicromolar levels of brain kynurenic acid, measured in whole brain or with microdialysis techniques, do not take into account that diffusion and dilution from site of release occurs due to either tissue homogenization or sampling of the extracellular compartment distant from the synapse. Thus, many studies performed hitherto may have underestimated the concentration of kynurenic acid at the site of action (i.e. the synapse).

The glutamate deficiency theory of schizophrenia – phencyclidine psychosis

Phencyclidine (phenylcyclohexylpiperidine; PCP; angel dust) was introduced in the 1950s as an "ideal" anesthetic drug, since it had little influence on the cardiovascular or the respiratory systems (Greifenstein et al. 1958, Johnstone et al. 1959). However, this drug had severe side effects: a significant number of patients developed psychosis, which persisted beyond recovery from anesthesia (see Pradhan 1984). Therefore, it was withdrawn from human use in 1965 (see Domino & Luby 1981). Interestingly, administration of subanesthetic doses of PCP induces symptoms resembling the positive symptoms of schizophrenia, including not only hallucinations and paranoia, but also delusions, sensations of disturbance from one's environment, violent behavior, and thought disorders. Furthermore, a unique feature of PCP is its ability to produce also negative symptoms similar to those seen in schizophrenia, such as social withdrawal and apathy. In addition, the drug induces cognitive dysfunctions (Luby et al. 1959). Thus, PCP-induced psychosis in normal individuals is apparently indistinguishable from an acute episode of schizophrenia (Yesavage & Freman 1978, Erard et al. 1980).

In subanestethic, psychotomimetic doses (0.05-0.1 mg/kg i.v.), PCP is a non-competitive NMDA-receptor antagonist (Zukin & Zukin 1979, Vincent et al. 1979, Thomson et al. 1985, Anis et al. 1983); in higher doses - doses sublethal or even lethal to humans - it also inhibits reuptake of dopamine and blocks an opiate receptor (the σ-receptor). Interestingly, congeners of PCP, e.g. the anesthetic agent ketamine and MK-801 (dizocilpine), compounds that also acts as non-competitive NMDA receptor antagonists, are able to induce schizophrenia-like symptoms when administered to healthy controls (see Javitt & Zukin 1991, Krystal et al. 1994, Malhotra et al. 1996, Jentsch & Roth 1999). In addition, when administered to schizophrenic patients, PCP and ketamine aggravate psychotic symptoms (Luby et al. 1959, Lahti et al. 1995a, b, Malhotra et al. 1997). In fact, PCP was reported to reproduce primary, "pathology-specific" perceptual and cognitive abnormalities (Luby et al. 1959, Domino & Luby 1981). It has also been reported that competi-

tive NMDA-receptor antagonists as well as antagonists at the glycine site of the NMDA-receptor display psychotomimetic properties when administered to healthy controls or neurosurgical patients (Kristensen et al. 1992, Grotta et al. 1995, Yenari et al. 1998, Albers et al. 1999).

In electrophysiological experiments, administration of PCP, ketamine, or MK-801 is associated with an increase in firing rate and in burst firing activity of VTA dopamine neurons (French et al. 1993, French 1994). This activation is suggested to involve GABAergic receptor mechanisms since intravenous administration of PCP and MK-801 inhibit the activity of putative GABA-containing midbrain interneurons (Zhang et al. 1993). Acute administration of PCP also profoundly increases dopamine release both in the nucleus accumbens (Carboni et al. 1989) and in the prefrontal cortex (Hondo et al. 1994, Hertel et al. 1995), when measured with microdialysis techniques. Interestingly, subchronic exposure to PCP shows a somewhat different picture compared to that of acute administration. Thus, three weeks after subchronic PCP administration, increased dopamine release in mesolimbic regions was still observed (Jentsch et al. 1998a, see Jentsch & Roth 1999), whereas in the prefrontal cortex a reduced release of dopamine was demonstrated (Jentsch et al. 1997, 1998b, c, 1999, see Jentsch & Roth 1999). As previously mentioned, negative symptoms in schizophrenia, especially hypofrontality and some cognitive deficits, are suggested to be the results of a dysfunction in prefrontal dopamine transmission (Weinberger et al. 1988, Daniel et al. 1989, 1991, Dolan et al. 1995). Therefore, chronic PCP administration, resulting in a reduced dopamine release in the prefrontal cortex and increased dopamine release in nucleus accumbens, conceivably produces effects that are most consistent with schizophrenia (see Jentsch & Roth 1999). In addition, using prepulse inhibition, a model reflecting sensory gating deficits in schizophrenia, administration of PCP to rats has been found to disrupt the prepulse inhibition and thus mimic major deficits in schizophrenics (Geyer & Braff 1987, Braff et al. 1992). A recent study also indicates that competitive NMDA receptor antagonists, if they gain rapid and sufficient access to the brain, are capable of causing profound behavioral deficits (Bakshi et al. 1999). All these findings make it tempting to speculate whether the actions of non-competitive or competitive NMDA receptor antagonists give a clue to deciphering the cause of schizophrenia. In this thesis, efforts are made to characterize the electrophysiological effects of endogenous kynurenic acid on brain catecholaminergic neurons and, furthermore, to directly investigate a putative role of the compound in the pathophysiology of schizophrenia by analyzing the levels of kynurenic acid in the cerebrospinal fluid (CSF) of schizophrenic patients.

Specific aims of the study

1. To analyze a putative interaction between centrally active drugs, e.g. muscimol and nicotine, and endogenous kynurenic acid on brain catecholaminergic neurons.

2. To characterize the electrophysiological effects of increased concentrations of endogenous kynurenic acid on midbrain dopamine neurons.

3. To investigate the role of kynurenic acid in the pathophysiology of schizophrenia.

Materials and Methods

Animals

The experiments were performed on male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden) weighing between 180-250 g. The animals were housed in groups of five and free access to food (R34 rat chow) and water was provided. Environmental conditions were checked daily and maintained at constant temperature (25° C) and 40% - 60% humidity in a room with a regulated 12 h light/dark cycle (lights on at 06.00). 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.

Administration of general anesthetic

Animals were weighed before use. Efforts were made to calm them down before an intraperitoneal (i.p.) injection of 8% chloral hydrate (0.5 ml/100 g; 400 mg/kg) to induce general anesthesia. The animal was then left in a quiet environment for 10 minutes in an empty cage before proceeding. If the animal was not fully anaesthetized by the end of this period, a further 0.5 ml of the chloral hydrate was administered.

Pretreatment with PNU 156561A or saline

The animal was placed onto a heating pad to maintain its body temperature at 37° C. An elastic 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 0.5 X 1.6 mm needle was inserted into a lateral tail vein and the elastic band was removed. Blood escaping through the needle indicated that it had been successfully inserted into the tail vein; if this did not occur, the needle was removed from the tail for another attempt. When correct insertion into the tail vein had been achieved, the tail and needle were secured onto the surface using strips of plaster, a syringe containing 0.9% NaCl was inserted onto the needle, and approximately 1 ml was injected into the animal. At this point, no resistance to the injection was felt. Saline (1 ml) or PNU 156561A, dissolved in 1 ml of 10% β-cyclodextrin, was then administered. Thereafter, the needle was removed and the rats were placed in a Plexiglas cage (under a heating lamp in order to maintain body

temperature). About 5 hours later, rats were again anesthetized (chloral hydrate; 400 mg/kg, i.p.) using the same procedure.

Mounting the animal onto the stereotaxic frame

The animal was placed onto the heating pad to maintain its body temperature at 37° C. It was then mounted onto the ear bars of a stereotaxic frame (David Kopf Instruments, Tuajanga, 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.

Maintenance of general anesthesia

The level of anesthesia was maintained via tail-vein administration of 8% chloral hydrate. The necessity for administration of anesthetic was determined by:

I. Visual means, by observing the breathing pattern. A fully anesthetized animal took steady deep breaths, with the majority of breathing movements occurring down the lower part of the body just in front of the hind limbs. Further administration of chloral hydrate was required when breathing movements became shallow and/or the majority of respiratory movements occurred just behind the forelimbs.

II. Response to pinching the hind foot, and testing for resistance in the limb. Further administration of chloral hydrate was required when resistance occurred to pulling the leg.

Surgery

An incision was made from the nose bridge along the center of the head to its base. Remaining flesh was removed by rubbing cotton sticks over it to reveal the skull surface. For recordings from the VTA and SN a hole of approximately 3 mm was drilled immediately anterior to lambda, and lateral to the midline, on the right side of the skull. For recordings from the LC, the 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. If any vessels were damaged, bleeding was stopped by placing a cotton swab over the hole.

Electrodes

A vertical electrode puller (Narishige, Japan) set at 14.5 amperes pulled single barrel microelectrodes. The electrodes were filled with 2 M sodium acetate saturated with Pontamine Sky Blue and microscopically inspected under a X2.5 objective lens. The tip was then broken back on a microscope stage under X10 objective lens against a fire-polished glass rod. The rod was initially brought as close to the electrode tip as possible so that its reflection could be seen in the polished face of the rod. The base of the electrode was then pushed down gently to bring the tip and the glass rod in contact with each other. After breaking back, the tip measured approximately 1-2 μm in diameter. The *in vitro* impedance of the electrode was tested using a microelectrode tester. Impedances were generally 5-8 MΩ (optimal 7.0 MΩ), measured at 135 Hz in 0.9% saline.

Single unit recording

After this procedure, the electrode was put onto a hydraulic microdrive (David Kopf Instruments, Tujunga, CA, USA), which was mounted on the stereotaxic instrument. The electrode was manually lowered as close to the brain surface as possible, without touching it, before setting the electrode tip to the coordinates: 3 mm anterior to lambda, 0.7 mm lateral to the midline or 2 mm anterior to lambda, 2 mm lateral to the midline for recordings in the VTA or SN, respectively. For recordings in the LC, the coordinates were 1.1 mm posterior to lambda and 1.1 mm lateral to midline. Sterotaxic coordinates were according to the atlas of Paxinos and Watson (1998). Initially, the electrode was lowered vertically, using the microdrive until the tip touched the fluid above the brain surface, and the microdrive depth was reset to zero. Contact with the brain was evident from a change in noise level on the oscilloscope, and a change in tone from the audio monitor. The electrode was lowered relatively slowly through the brain to a depth of 7 mm (VTA), 6 mm (SN), or 4.5 (LC), and then slowly (25 μm/sec) to a depth of 7.5 mm (VTA), 6.5 mm (SN), or 5.0 (LC). It is at those depths that VTA dopamine neurons, SN dopamine neurons, or LC noradrenaline neurons initially could be found. Searching for dopamine or noradrenaline neurons was performed very slowly to a depth of 8.5 mm (VTA), 8.0 mm (SN) and 6.5 mm (LC). Upon reaching these depths without finding a dopamine or noradrenaline neuron, respectively, the electrode was raised back to a depth of 7 mm (VTA), 6 mm (SN), or 4.5 mm (LC). The electrode was then moved in any direction, preferably

medially or laterally, by 0.1 mm, before lowering it back into the vicinity of the VTA, SN-ZC, or LC.

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).

In this thesis, only a few histological verifications of recording sites were performed since brains were used for the analysis of kynurenic acid. Thus, identification of noradrenergic or dopaminergic neurons was mainly based upon their typical neurophysiological characteristics as well as their response to pharmacological treatment (see below).

Electrophysiological characteristics of noradrenergic neurons

The electrophysiological characteristics of the neurons were identical to those previously described for noradrenergic neurons of the rat LC (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. To further confirm that recordings had been made only from noradrenaline neurons, the inhibitory action of a single dose of the α_2-receptor agonist clonidine (5 μg/kg, i.v.) was verified at the end of the experiments.

Electrophysiological characteristics of dopaminergic neurons

Using the electrophysiological criteria of VTA dopamine neurons (Wang 1981), and of SN dopamine neurons (Grace and Bunney 1983, 1984a, b), dopaminergic neurons were identified as possessing (Fig. 4):

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

To further confirm that recordings had been made on dopamine neurons only, the inhibitory action of a single dose of the dopamine agonist apomorphine (100 μg/kg, i.v) was verified at the end of the experiments.

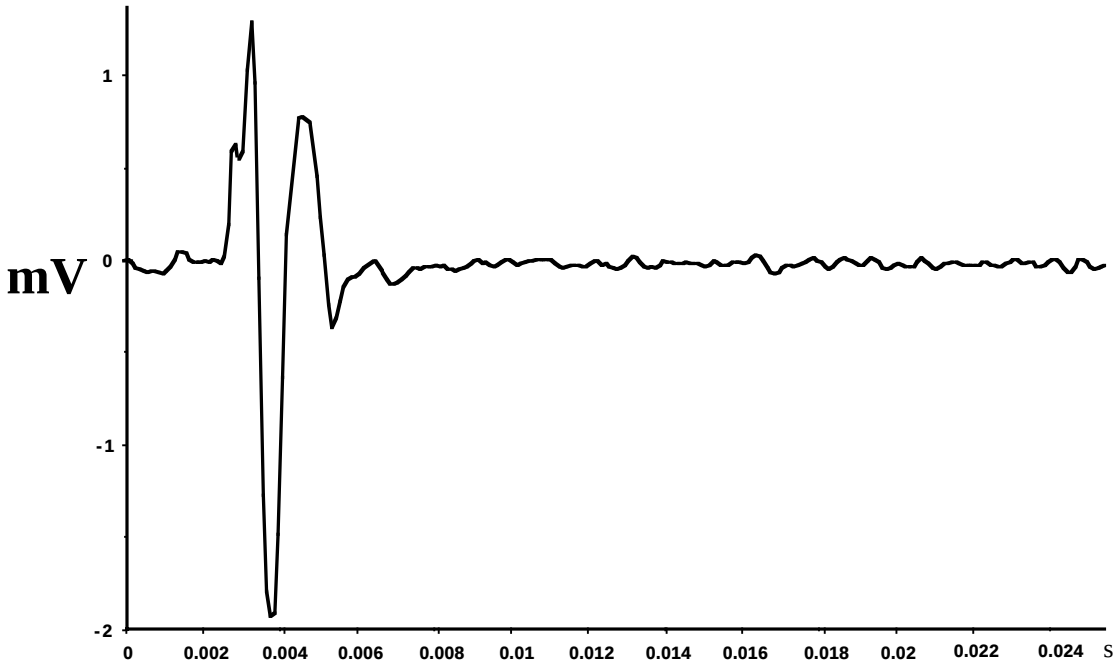

Fig. 4. A representative example of a spontaneous spike discharge, recorded extracellularly from a dopaminergic neuron, exhibiting the characteristic triphasic waveform.

Drug administration

Initially, approximately 3 min of basal activity was recorded prior to i.v. drug injections via the tail vein. Injections were separated from their predecessors by approximately 1.5 min. For analytical purposes, activity recorded during the 90 sec period immediately preceding the first injection of nicotine constituted baseline. Saline injections were performed frequently to maintain fluid balance and to control for injection artefacts.

For intracerebroventricular (i.c.v.) injection of kynurenic acid, a hole was drilled in the skull 2 mm posterior to the bregma and 2 mm lateral from the sagittal suture. A microinjection tubing (HT X-32, Stainless Steel tubing Hypodermic -Type 304

O.D. 0.009", wall 0.0025") filled with kynurenic acid (50 mM solution, 20 µl, pH 7.2) was guided, using a stereotaxic instrument, through this hole so that it reached the lateral ventricle 4 mm below the surface of the skull and to the intended site of local injection.

Influence of anesthesia

Recent investigations have shown that the primary metabolite of chloral hydrate, trichloroethanol, can impair the function of NMDA receptors (Peoples & Weight 1998, Scheibler et al. 1999). Given the role of EAA in the control of firing pattern of midbrain dopamine neurons, such an action of the anesthetic could, theoretically, contribute to the present actions of kynurenic acid. Existing results from chloral hydrate-anesthetized rats, paralyzed rats and freely moving rats show a somewhat conflicting picture of the role of anesthesia on the neuronal activity of midbrain dopamine neurons (Kelland et al. 1990, see Overton & Clark 1997). At any rate, since chloral hydrate was used throughout the present thesis as well as identical routines for maintenance of anesthesia, the results derived are unlikely to be influenced by an NMDA-antagonistic action of the anesthetic.

Data analysis

The distribution of spikes was analyzed on line utilizing a Macintosh computer. The software used for the analysis of firing was written in-house using a high level object oriented programming language called "G" (Lab VIEW; National Instruments, Austin, TX, USA). The software was designed to sample and analyze the intervals of an arbitrary number of TTL pulses (corresponding to spikes passing through the discriminating filter) using a time resolution of one ms. An interspike interval was designated as the time (in ms) elapsed between the rising edges of two sequential TTL pulses. In order to avoid artefacts in the sampling procedure, the spike analyzer ignored time intervals below 20 ms. The onset of a burst was determined as an inter-spike interval shorter than 80 ms and the termination of a burst by the next interval longer than 160 ms (Grace & Bunney 1984a, b). Cells were considered to be bursting if at least one interspike time interval of 100 recorded spikes was below 80 ms. The software program also sorted the intervals of recorded spikes and divided them into 3- ms bins and displayed the results as an interspike time interval histogram (ISH) with regard to the number of intervals corresponding to each bin. The intervals were analyzed with regard to the number of bursts that occurred during e.g. 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 coefficient (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 & Mountcastle 1963)) were expressed as the median of at least three consecutive ISHs. Since the action of nicotine was found to produce both inhibitory and excitatory responses of VTA dopamine neurons (paper IV), firing rate, regularity of firing, and burst firing activity were estimated at a time level when the inhibition or excitation of firing was most pronounced. In addition, since pretreatment with PNU 156561A was associated with an increased activity of VTA dopamine neurons (paper III), VTA dopamine neurons displaying a high percentage of basal burst firing activity (≥ 50 % of spikes fired in bursts) were excluded from this study (paper IV).

To quantify the short-lasting reduction in firing rate produced by nicotine in paper IV the sum of percent inhibition for each 10 s interval during the inhibitory period was calculated. Inhibition of firing was considered to start or terminate when the firing rate exceeded or was below 15 per cent of basal frequency, respectively.

Sampling of rat cerebrospinal fluid and blood

CSF was collected after the electrophysiological experiment, with the rat still positioned in the stereotaxic instrument. As previously described (Consiglio & Lucion 2000) the rat was placed under the superior incisive plane of fixation in such a way that the occipital bone would be almost horizontal and the rest of the body was lying vertically. A needle connected to a syringe was inserted vertically between the occipital protuberance and the spine of the atlas. A gentle aspiration made the CSF flow through it and approximately 70 µl was collected each time. CSF containing blood, according to a visual examination, was not used due to the very high concentrations of kynurenic acid found in blood of controls. Approximately 2 ml blood was collected from each rat before decapitation.

Collection of human cerebrospinal fluid

CSF was obtained by lumbar puncture (L4 - L5). 12 - 18 ml of CSF was collected with a 0.9 mm needle and the samples were immediately frozen (-70°C), coded and sent blindly to the Karolinska Institute.

Analysis of kynurenic acid

The brain was sonicated with sonication solution (perchloric acid 0.4 M, $Na_2S_2O_5$ 0.1%, and ethylenediaminetetra-acetate 0.05% (EDTA)), which was added in the same amount as the weight of the brain before sonication. The samples were centrifuged at 4000 *g* for 10 minutes and 40 µl perchloric acid (70%) was added to the supernatant. Thereafter, the supernatant was centrifuged twice, the last time through a micropore filter (pore size 0.10 µm) under the same conditions as previously. Blood samples were treated the same way. CSF samples were centrifuged at 4000 *g* for 10 minutes. For analysis of kynurenic acid an isocratic reversed-phase HPLC system was used, including a dual piston, high liquid delivery pump (CMA/250 LC Pump; CMA, Stockholm, Sweden), an analytic guard column (Eclipse XDB-C18, 4.6 x 12.5 mm, Hewlett-Packard, USA), an Eclipse XDB-C18 column (4.6 x 150 mm, Rockland Tech, USA) and a fluorescence detector CMA/280 (CMA/Microdialysis; Stockholm, Sweden) with a fixed wavelength (excitation: 315-370 nm emission: 395-545 nm). 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 0.25-0.5 ml/min. Precise acetonitrile concentration and pH of the mobile phase was essential to separate the

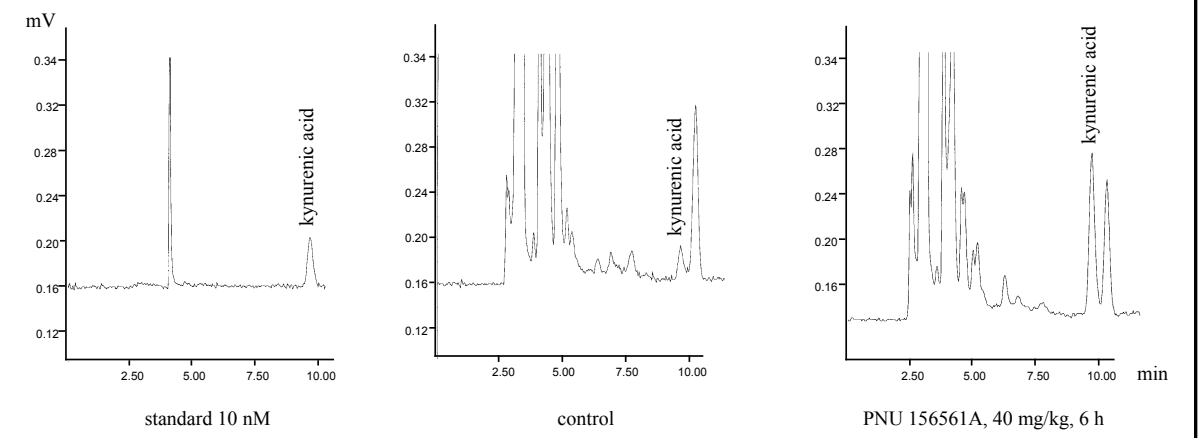

Fig. 5. Chromatograms illustrating the measurement of kynurenic acid in rat whole brain.

kynurenic acid peak from an unknown peak with nearly the same retention time (Fig. 5). Samples were injected into a Rheodyne injector with a single sample loop of 50 µl (Cotati, CA, USA). Then 0.5 M zinc acetate (not pH adjusted) was delivered post column by a peristaltic pump, Pump P-500 (Pharmacia, Sweden) at a flow rate of 0.10 ml/min. The signals from the fluorescence detector were passed through a MacLab analog to a digital converter and transferred to a Macintosh computer. The software used for the analysis was MacLab Chart Software. The

retention time of kynurenic acid differed slightly between each study (due to the column), 9 - 19 min, and the sensitivity of the method was 0.125 pmol (signal: noise ratio 5:1). 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.

Drugs and chemicals

The following drugs and chemicals were used: chloral hydrate (Merck, Darmstadt, Germany), (-)nicotine tartrate (ICN Pharmaceuticals, Plainview, NY, USA). Doses of nicotine are expressed as the salt. 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, zinc acetate, kynurenic acid, and D-cycloserine (Sigma, St. Louis, MO, USA), CGP 35348 (generous gift from Ciba-Geigy), sodium acetate (Riedel-de Haen, Germany), perchloric acid (Kebo Lab, Stockholm, Sweden), muscimol (Bachem AG, Switzerland), MK-801 (Merck Sharp & Dohme, NJ, USA), and acetonitrile (Labasco, Partille, Sweden).

Statistics

The median value from at least three consecutive ISHs recorded from a neuron immediately before drug administration was compared with the median value from at least three consecutive ISHs recorded from the same neuron after each injection of the drug. Since the number of observations in each group is rather limited, no evidence of a normal distribution of measurements was obtained. Therefore, statistically significant differences regarding firing rate, variation coefficient and estimation of kynurenic acid levels were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney U-Test. Burst firing activity was generally analyzed with Kruskal-Wallis analysis of variance followed by the non-parametric Wilcoxon signed rank test. Firing rates, variation coefficient, burst firing activity, and estimation of kynurenic acid levels are presented as means ± SEM. Significance was assumed for all values where $p<0.05$.

Results and Discussion

Pharmacological significance of endogenous kynurenic acid

In the initial studies (paper I and II), a pharmacological role of elevated endogenous kynurenic acid was examined. For this purpose, interactions between endogenous kynurenic acid and nicotine, which has been suggested to release glutamate at the LC level (Engberg 1989), and interactions between endogenous kynurenic acid and the $GABA_A$-receptor agonist, muscimol, were analyzed.

Elevated levels of kynurenic acid prevent the nicotine-induced activation of locus coeruleus noradrenergic neurons. (Paper I)

Previous electrophysiological studies have shown that i.v. administered nicotine in doses relevant to nicotine consumption in man causes two distinctly separated effects on the neuronal activity of noradrenergic neurons in the LC (Engberg & Hajos 1994). Our results confirm that nicotine administration leads to an instantaneous but short-lasting excitation at low doses, and a moderate but long-lasting activation of rat LC neurons following administration of higher doses (Fig. 6a). Previous studies have also shown that the short-lasting excitation of LC neurons by nicotine is blocked by i.c.v. administration of kynurenic acid, suggesting that nicotine produces its effects by release of glutamate at the LC level (Engberg 1989). The activation of LC neurons by nicotine was clearly antagonized by pretreatment with PNU 156561A (40 mg/kg, i.v.; 3-6 h; Fig. 6b; causing an increase in endogenous concentrations of kynurenic acid approximately five times) but not by acute administration of the drug (40 mg/kg, i.v.; 2-12 min), which did not alter concentration of brain kynurenic acid. Since increased levels of kynurenic acid blocked both the short-lasting excitation and the long-lasting activation of LC neurons by nicotine, we suggest that both effects, although being originally executed via two different mechanisms (cf. Introduction), are mediated by release of glutamate within the LC.

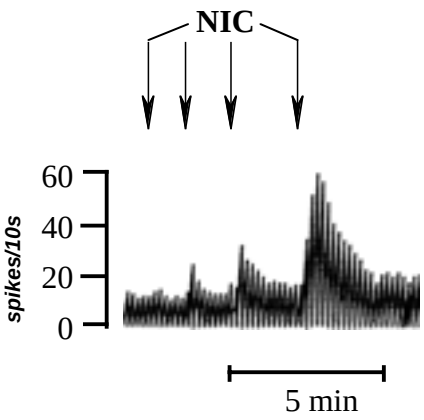

Fig. 6a. Excitation of an LC noradrenergic neuron following i.v. administration of nicotine, depicting both the instantanous short-lasting excitation and the elevated baseline firing rate (NIC, 25 + 50 +100 + 200 μg/kg, at arrows).

The specificity of kynurenic acid as an EAA receptor antagonist has been under investigation in the past few years. Some studies reveal that kynurenic acid does not interfere with nicotinic receptors (Bijak et al. 1991, Bertolino et al. 1997) but a recent communication shows that kynurenic acid blocks the α7* nicotinic receptor with the same IC_{50} as for NMDA receptors (Hilmas et al. 2000). Therefore, the prevention of the stimulatory effect of nicotine on LC noradrenaline neurons by elevated concentrations of kynurenic acid could theoretically be mediated via blockade of nAChR. However, the short-lasting excitatory action of nicotine is originally mediated via activation of nicotine-sensitive receptors located at peripheral primary C-fiber afferents (Hajos & Engberg 1988). The blood concentrations of kynurenic acid was not elevated in rats following pretreatment with PNU 156561A (see Table 2) at the time when recordings were performed. Thus, the blockade of the nicotine-induced excitation of LC noradrenaline neurons by kynurenic acid is unlikely to be mediated via nAChR.

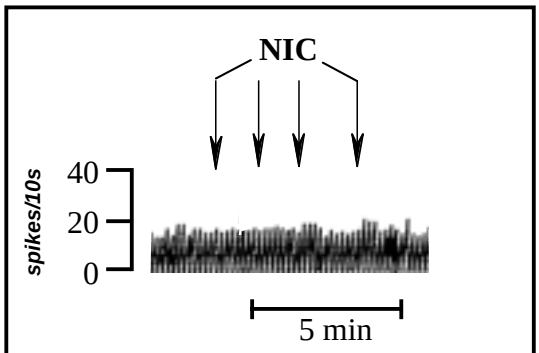

Fig. 6b. Prevention of the nicotine-induced excitatory effects on an LC noradrenergic neuron (NIC, 25 + 50 +100 + 200 μg/kg, at arrows) by PNU 156561A pretreatment (40 mg/kg, i.v., 4 h).

Elevated levels of kynurenic acid block muscimol-induced excitation of nigral dopamine neurons. (Paper II)

Systemic administration of the $GABA_A$-receptor agonist muscimol has previously been shown to increase the firing activity of rat nigral dopamine neurons (Waszczak et al. 1980, Engberg et al. 1993). This action of muscimol is paradoxical since local microiontophoretic application of the drug is associated with a clearcut inhibition of these neurons (Waszczak et al. 1980, Erhardt et al. 1999). In the present study, an attempt was made to analyze the mechanism behind the excitatory action on nigral dopamine neurons following systemic administration of the drug. We found, in line with previous studies (Waszczak et al. 1980, Engberg et al. 1993, Lokwan et al. 2000), that systemic administration of muscimol (0.25-4.0 mg/kg, i.v.) was associated with a dose-dependent increase in firing rate as well as an increased bursting activity of the nigral dopamine neurons (Fig. 7a, b). Both these effects of muscimol were, however, clearly antagonized by pretreatment with the NMDA receptor antagonist MK-801 (1 mg/kg, i.v.) or by i.c.v. administration

of kynurenic acid. Furthermore, pretreatment with PNU 156561A (40 mg/kg, i.v., 5-8 h), causing a rise in endogenous kynurenic acid levels by about 9 times, not only antagonized the actions of muscimol, but also reversed the excitatory action of the compound into an inhibitory effect on the nigral dopamine neurons.

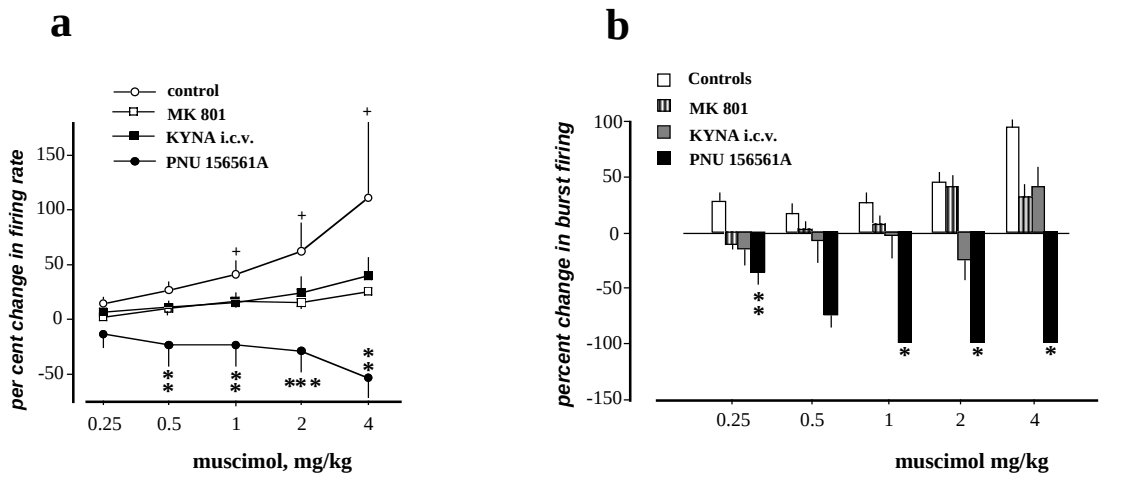

Fig. 7. Dose-response curves illustrating the excitatory action of muscimol on a) the firing rate and b) the burst firing activity of nigral dopamine neurons, its prevention by pretreatment with i.c.v. administered kynurenic acid, i.v. administered MK-801 and the reversed action of muscimol following pretreatment with PNU 156561A. Each value represents mean ±SEM from 5-11 SN dopamine neurons. Statistics: *P<0.05 vs. muscimol control, $^{+}$P<0.05 vs. muscimol 0.25 (Mann-Whitney U-test).

The action of muscimol on SN-ZC neurons is not likely to be mediated via activation of somatodendritic $GABA_A$-receptors located on the dopamine neurons themselves in view of the inhibitory nature of this receptor (Johnson et al. 1992). It has previously been suggested that muscimol via activation of $GABA_A$ -receptors may inhibit GABAergic fibers, e.g. those emanating from substantia nigra pars reticulata, that serve to dampen nigral dopamine neurons (Grace & Bunney 1979, Waszczak et al. 1980, Erhardt et al. 1999). Such a mechanism would result in a disinhibition of nigral dopamine neurons as observed in the present study. However, this idea might be less attractive in view of the fact that the muscimol-induced excitation is not prevented by pretreatment with the $GABA_A$-receptor antagonist picrotoxin (Waszczak et al. 1980). Rather, an indirect action of muscimol unrelated to inhibition of GABAergic neurons may execute the activation of nigral dopamine neurons.

In this study i.v. administration of the NMDA-receptor antagonist MK-801 or i.c.v. administration of kynurenic acid was able to antagonize the muscimol-induced increase in firing rate or burst activity of nigral dopamine neurons. Thus, the action of muscimol appears to be indirect and mediated via activation of afferents containing EAA and projecting to the SN, e.g., from the

medial prefrontal cortex and the pedunculopontine tegmental nucleus (Carter 1982, Jackson & Crossman 1983, Kornhuber et al. 1984, Scarnati et al. 1986, Naito & Kita 1994, Charara et al. 1996, Lokwan et al. 2000). Indeed, activation of EAA projections to the SN are associated with burst firing activity (Zhang et al. 1992, Murase et al. 1993a), suggesting that these afferents are the main target for muscimol in releasing EAA. The view that EAA mediate the paradoxical increase in firing rate and burst firing activity of nigral dopamine neurons by the $GABA_A$ receptor agonist muscimol is strengthened by our results with PNU 156561A. The pure inhibitory action of muscimol in a situation of elevated brain concentrations of kynurenic acid may be attributed to activation of somatodendritic $GABA_A$-receptors on nigral dopamine neurons.

All together, the results of these studies (paper I and II) suggest that a moderate elevation of endogenous kynurenic acid is of a pharmacological importance since it markedly affects the activation of LC neurons by nicotine and reverses the excitatory action of muscimol on the nigral dopamine neurons into an inhibitory effect. Given the relatively moderate increase in the concentrations of kynurenic acid needed to obtain these effects, the present studies support the view that endogenous kynurenic acid acts as a biologically important modulator of brain glutamatergic neurotransmission by counterbalancing the effects of glutamate.

Physiological importance of endogenous kynurenic acid

Activation of midbrain dopamine neurons by pharmacological elevation of kynurenic acid – comparison with MK-801. (Paper III)

In the present study, we found that pharmacologically induced elevation of endogenous brain kynurenic acid levels (3-5 times), caused by PNU 156561A (40 mg/kg, i.v., 5-9 h), increased the firing rate and burst firing activity of VTA dopamine neurons (Table 1). In addition, a decreased regularity of firing, expressed as an increased variation coefficient of an ISH, was found after pretreatment with PNU 156561A. Approximately 25 % of VTA dopamine neurons recorded from were found to display periods of depolarization inactivation following pretreatment with PNU 156561A and these neurons were therefore excluded from the study. Acute treatment with PNU 156561A (40 mg/kg, i.v.), 2-8 minutes before electrophysiological assessment, which was not associated with any changes in brain kynurenic acid concentrations, has previously been shown to be without effect on firing activity of nigral dopamine neurons (Erhardt et al. 2001b).

To further verify that increased endogenous levels of kynurenic acid are responsible for the observed increase in firing rate and burst firing activity of VTA dopamine neurons, a few rats were treated with probenecid (200 mg/kg, i.p., 1 h). Pretreatment with probenecid - thereby inhibiting the efflux of kynurenic acid from the brain - was found to increase the burst firing activity of VTA dopamine neurons (Table 1). In addition, pretreatment with probenecid decreased the regularity of firing, expressed as an increased variation coefficient of an ISH.

Similarly, i.v. administration of MK-801 produced a dose-dependent increase in the firing rate and burst firing activity of VTA dopamine neurons with a maximal effect at 0.8 mg/kg (Fig. 8a, b). All neurons recorded from (n = 10) increased their bursting activity and two of two non-bursting DA neurons were converted to bursting. Two neurons were excluded from further analysis due to the induction of depolarization block after 0.2 and 0.8 mg/kg, respectively.

Table 1. Effects of PNU 156561A on the firing rate and spike distribution of dopamine neurons in the VTA. *P< 0.05 (Mann Whitney U-test).

VTA	**Controls**		**PNU 156561A**		**Probenecid**	
	All neurons (n=93)	Bursting neurons (n=67)	All neurons (n=106)	Bursting neurons (n=104)	All neurons (n=23)	Bursting neurons (n=20)
Firing rate, Hz	3.8 ±0.2	4.5 ±0.2	5.4 ±0.3***	5.5 ±0.3*	4.7±0.6	5.1±0.7
Variation coefficient, %	63.5 ±3.1	62.3 ±3.9	74.6 ±3.2*	75.4 ±3.2**	84.8±6.9**	89.0±7***
% spikes in bursts	12.9 ±1.8	17.9 ±2.3	41.5 ±3.1***	42.3 ±3.1***	47.5±6.6***	54.7±6***
Mean number of bursts^{+}	3.75 ±0.5	5.23 ±0.6	8.42 ±0.6***	8.58 ±0.6***		
Mean spikes per burst	2.34 ±0.2	5.23 ±0.6	6.41 ±1.0***	6.53 ±1.0**		

Values represent means ± SEM,
$^{+}$ during a 100-spike sampling period.

The activation of VTA dopamine neurons observed following increased concentrations of endogenous kynurenic acid appears paradoxical in several aspects. In view of the prominent role of EAA in the control of firing rate and burst firing activity of these neurons (Overton & Clark 1992, Wu et al. 1999, Charléty et al. 1991) one would expect increased concentrations of kynurenic acid to produce a decreased firing rate and burst firing activity. Indeed, local pneumatic ejections of kynurenic acid have previously been found to cause a cessation of burst firing and to induce a pacemaker-like pattern of firing of nigral as well as of VTA dopamine neurons (Charléty et al. 1991). I.c.v. administration of kynurenic

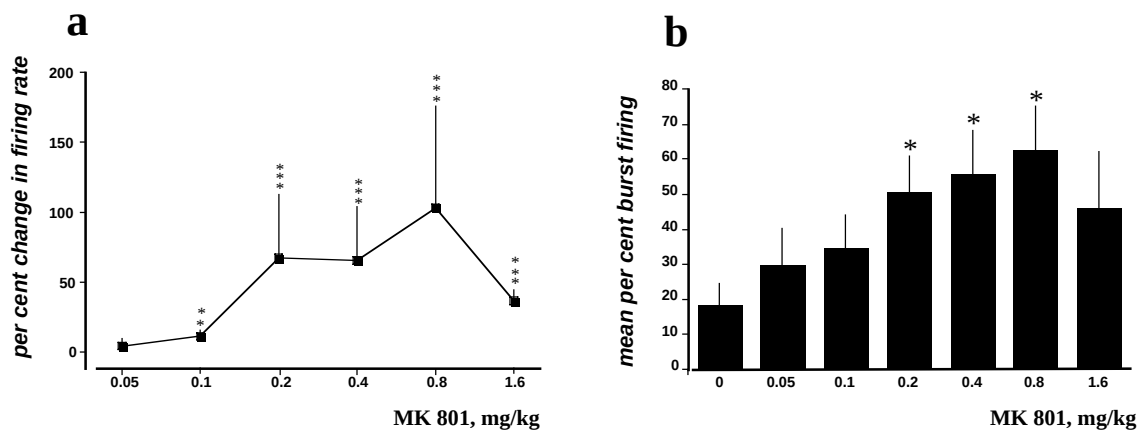

Fig. 8. Dose-response curves depicting the effects of incremental doses of i.v. administered MK-801 (0.05-1.6 mg/kg) on a) the firing rate (statistics: *P<0.05 vs. predrug value, Mann-Whitney U-test) and b) the percentage of burst firing activity (statistics: *P<0.05 vs. predrug value, Wilcoxon signed rank test). Each value represents mean ±SEM from 4-10 VTA dopamine neurons.

acid is also associated with an inhibition of burst firing activity and regularization of the firing pattern of VTA dopamine neurons (Grenhoff et al. 1988), whereas the same route of kynurenic acid administration did not affect the firing rate, bursting activity or firing pattern of nigral dopamine neurons (Tung et al. 1991). However, the effects of increased endogenous levels of kynurenic acid on burst firing activity and firing rate of VTA dopamine neurons bear a striking similarity to the stimulatory actions exerted on these neurons by systemic administration of the non-competitive NMDA-receptor antagonists MK-801 (see also Murase et al. 1993b) and phencyclidine (French et al. 1993). Activation of midbrain dopamine neurons by these drugs has been suggested to involve GABAergic receptor mechanisms (Ceci & French 1989), since i.v. administration of phencyclidine and MK-801 inhibits the activity of putative GABA-containing midbrain interneurons (Zhang et al. 1993). For comparison, micropressure application of kynurenic acid onto the GABA-containing neurons of the SN-zona reticulata decreases the spontaneous firing of these neurons via antagonism of NMDA-receptors (Schmitt

et al. 1999). Thus, the elevated levels of endogenous kynurenic acid may primarily reduce the activity of GABAergic projections to the VTA, thereby decreasing a GABAergic tone onto the midbrain dopamine neurons.

Since somatodendritic $GABA_B$-receptors have been shown to tonically dampen firing rate and burst firing of midbrain dopamine neurons (Erhardt et al. 1999, 2001c), a decreased release of GABA within the VTA would promote increased firing rate and bursting activity of these neurons. In such a situation, where somatodendritic NMDA-receptors are blocked and the GABAergic influence of these neurons is attenuated, EAA activation of AMPA and/or kainate receptors may play a more prominent role to regulate firing activity of midbrain dopamine neurons (Zhang et al. 1994, Mathé et al. 1998).

The discrepancy between pneumatic ejections or i.c.v. administration of kynurenic acid and pharmacologically elevated levels of kynurenic acid appears somewhat puzzling. However, this should be related to different concentrations of kynurenic acid at critical sites of action: local concentration of endogenous kynurenic acid might reach much higher levels than those of externally administered kynurenic acid. The production of kynurenic acid occurs in astrocytes (Schwarcz et al. 1992b, Curatolo et al. 1996) adjoining glutamatergic boutons and therefore the compound could readily be liberated into the extracellular space in relatively high concentrations (Swartz et al. 1990, Gramsbergen et al. 1997). In addition, since KAT, the rate-limiting biosynthetic enzyme of kynurenic acid, has been localized within astrocytes which occasionally adjoin and in some instances partially encircle dopamine cell dendrites in SN-ZC (Schwarcz et al. 1992b), local kynurenic acid concentrations could therefore be high enough to block NMDA-receptors.

Specificity of kynurenic acid and the effects of intravenous administration of D-cycloserine on the firing rate and burst activity of ventral tegmental area dopamine neurons. (Paper III)

In this study, the mechanisms underlying the effects of increased levels of endogenous kynurenic acid on the neuronal activity of midbrain dopamine neurons were more thoroughly investigated. D-cycloserine is a partial agonist, producing approximately 60% activity compared to the full agonist glycine, and acts like an agonist in the presence of low concentrations of glycine and as an antagonist in the presence of high concentrations (Emmett et al. 1991). In rats pretreated with PNU 156561A (40 mg/kg, i.v., 5-9 h, n=10, mean basal firing rate and per cent bursting were 5.8 ±0.8 Hz and 55 ±10%, respectively) i.v. administration of D-cycloserine

was found to significantly decrease the firing rate and per cent burst firing (Fig. 9a, b; mean firing rate and per cent bursting after 128 mg/kg D-cycloserine were 4.9 ±0.9 Hz and 33 ±10%, respectively). Systemic administration of D-cycloserine to saline treated rats (n=8, mean basal firing rate and per cent bursting were 3.4 ±0.9 Hz and 23 ±12%, respectively) was found to significantly increase the firing rate of VTA dopamine neurons (Fig. 9a). The mean percent burst firing was slightly increased, although without reaching statistical significance (Fig. 9b). Since D-cycloserine was found to reverse the excitatory effects on VTA dopamine neurons induced by PNU 156561A, the effects on VTA dopamine neurons of PNU 156561A pretreatment are in all probability mediated via an increased concentration of endogenous kynurenic acid, high enough to block central NMDA-receptors.

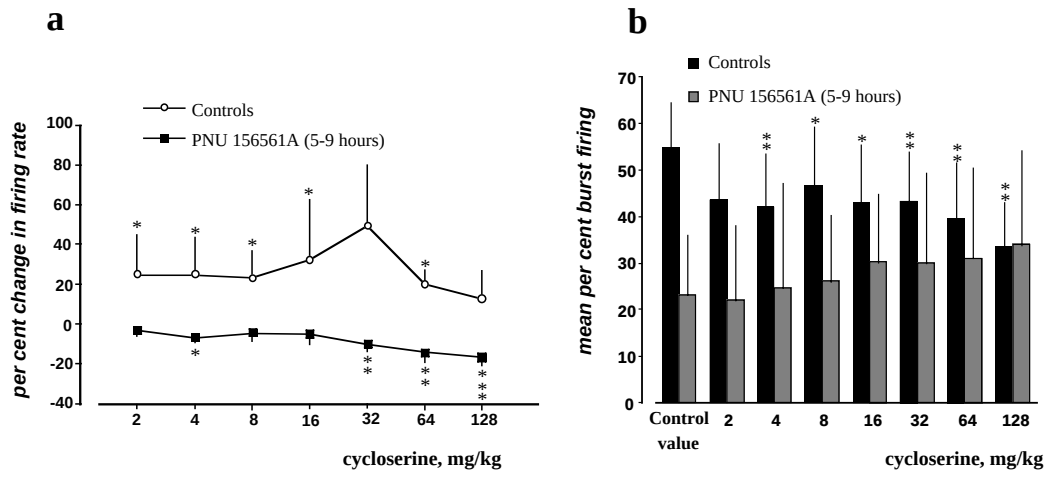

Fig. 9. Effects of incremental doses of intravenously administered D-cycloserine (2-128 mg/kg) on a) the firing rate (statistics: *P<0.05 vs. corresponding predrug value, Mann-Whitney U-test) and b) the percentage of burst firing activity (statistics: *P<0.05 vs. corresponding predrug value, Wilcoxon signed rank test) in control rats and in rats pretreated with PNU 156561A (40 mg/kg, i.v.; 5-9 hours). Each value represents mean + SEM from 5-10 VTA dopamine neurons.

Nicotine-induced excitatory and inhibitory responses of ventral tegmental area dopamine neurons. (Paper IV)

Systemic administration of nicotine has previously been shown to activate midbrain dopamine neurons (i.e. increased firing rate and bursting activity; Grenhoff et al. 1986, Mereu et al. 1987, Erhardt et al. 2001a). In consonance, several studies in the rat indicate that acutely administered nicotine promotes dopamine release in the nigrostriatal and mesolimbocortical systems (Imperato et al. 1986, Mifsud et al. 1989, Nisell et al. 1994). However, the mechanism by which nicotine increases dopaminergic neurotransmission is somewhat disputed. According to *in vitro* studies

on midbrain dopamine neurons, the effect of nicotine is mediated via activation of postsynaptic nAChR located on the soma of the dopamine neurons (Calabresi et al. 1989, Pidoplichko et al. 1997, Piciotto et al. 1998). In this situation, though, only an increase in firing rate is observed since dopamine neurons in the slice preparations do not show burst firing, probably due to the absence of afferent input to the dopamine neurons in the slice preparation. However, other studies show that the nicotine-induced effects on midbrain dopamine are indirect and mediated via activation of afferent terminals impinging upon these systems. During the last decade, presynaptically located nAChR (see Wonnacott et al. 1990, Bertolino et al. 1997, Wonnacott 1997) have been found to promote glutamate release (Bijak et al. 1991, Vidal & Changeux 1993, McGehee et al. 1995, Gray et al. 1996) and interestingly also to facilitate release of GABA in several brain regions (Léna & Changeux 1997, Guo et al. 1998, Alkondon et al. 2000). Thus, given that glutamatergic and GABAergic afferent systems impinge upon VTA dopamine neurons (c.f. Introduction) one would expect nicotine to produce both excitatory and inhibitory responses of these neurons. Indeed, a recent *in vitro* study reveals that the effects of nicotine can be blocked by glutamate receptor antagonists (Grillner & Svensson 2000) and blockade of VTA NMDA receptors *in vivo* diminishes the nicotine-induced dopamine release in the nucleus accumbens (Schilström et al. 1998). Furthermore, systemic administration of nicotine has been shown to increase the extracellular levels of both glutamate and aspartate within the VTA (Toth et al. 1992, Schilström et al. 2000) and the excitation of nigral dopamine neurons by nicotine is abolished by pharmacologically elevated levels of endogenous kynurenic acid (Erhardt et al. 2001a).

The aim of this study was to evaluate the importance of glutamate or GABA release for the actions of nicotine on the firing rate and firing pattern of dopamine neurons in the VTA. For this purpose, the potent and selective $GABA_B$ receptor antagonist CGP 35348 was used. In addition, to antagonize a proposed effect of nicotine on glutamatergic pathways, the levels of kynurenic acid were pharmacologically elevated with PNU 156561A.

A. Effects of systemic nicotine administration on firing rate, burst firing activity and regularity of firing of dopamine neurons in the ventral tegmental area.

In consonance with previous studies (Grenhoff et al. 1986, Mereu et al. 1987), i.v. administered nicotine (1.5-400 μg/kg) was found to dose-dependently increase the firing rate as well as the percentage of spikes fired in bursts of VTA dopamine

neurons (Fig. 10, 11a, b). A significant increase was observed in both firing rate and burst activity when nicotine was administered in doses ≥ 50 μg/kg. A novel finding in this study is that the activation of VTA dopamine neurons by nicotine was preceded by an instantaneous (within seconds after injection) and rather short-lasting reduction of the firing rate, particularly pronounced when higher doses were administered (Fig. 10, 12). This decrease in firing rate lasted less than 120 sec and during this period, no burst firing activity seemed to occur, although it was not possible to measure this parameter due to the short inhibitory period.

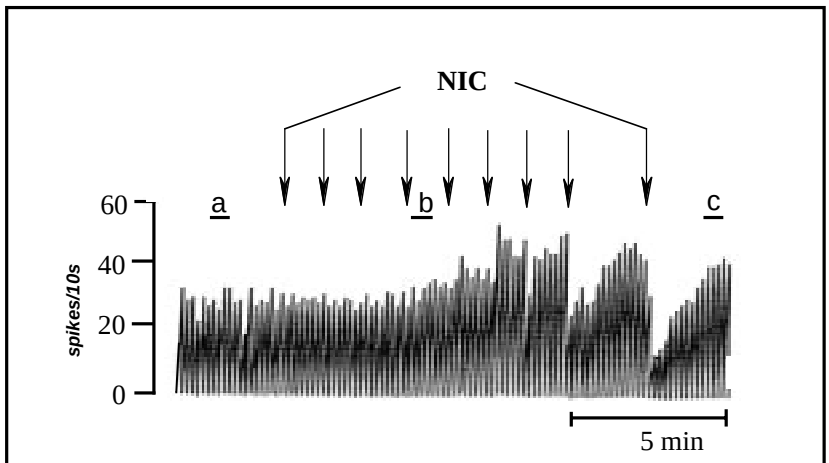

Fig. 10. Cumulative rate histogram from a dopamine neuron in the VTA, depicting the excitatory and inhibitory effects of nicotine (1.5+1.5+3+6+12.5+25+50+100+200 μg/kg, at arrows).

B. $GABA_B$-receptor antagonism of the nicotine-induced effects of dopamine neurons in the ventral tegmental area.

Pretreatment with the selective $GABA_B$-receptor antagonist CGP 35348 (200 mg/kg, i.v., 3 min) promoted the increase in burst firing activity produced by nicotine (Fig. 11b). Here, a considerably lower dose of nicotine (3 μg/kg) was required to produce a significant action on burst firing activity when compared to controls (50 μg/kg). Furthermore, 2 out of 4 non-bursting dopamine neurons begun to burst after nicotine 3 μg/kg and all of them were converted to bursting neurons at doses ≥ 12.5 μg/kg. Some neurons were excluded from further analysis due to induction of depolarization block already after 12.5 μg/kg nicotine. The increase in firing rate induced by nicotine was not affected by pretreatment with CGP 35348. (Fig. 11a). The regularity of firing was significantly decreased (expressed as an increase in the variation coefficient) in this group following nicotine administration. In addition, administration of CGP 35348 (200 mg/kg i.v.) clearly antagonized the initial, short-lasting decrease in firing rate induced by nicotine (Fig. 12).

These actions of CGP 35348 are likely to be related to a specific $GABA_B$-receptor blockade of the drug (Olpe et al. 1990). Thus, the dose used in the present study (200 mg/kg, i.v.) was recently shown to antagonize actions of the $GABA_B$-receptor agonist baclofen on the neuronal activity of midbrain dopamine

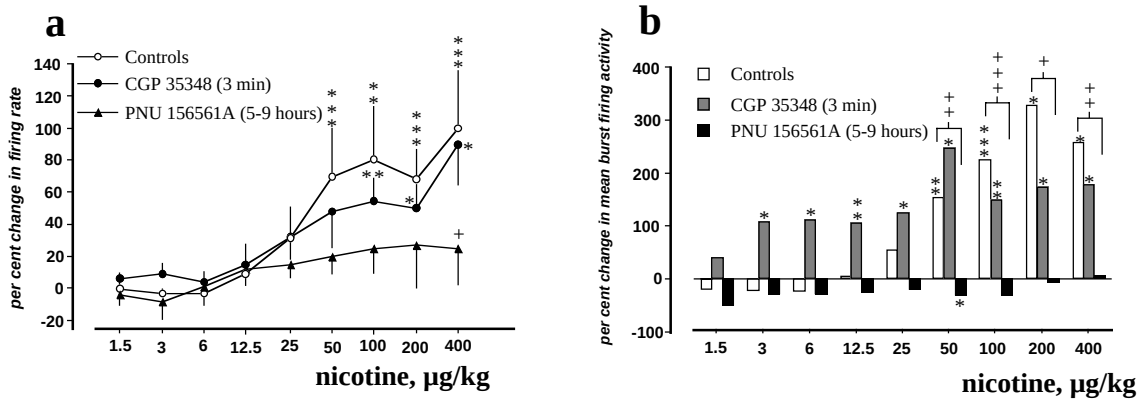

Fig. 11. Effects of incremental doses of i.v. administered nicotine (1.5-400 µg/kg) on a) the firing rate (statistics: *P<0.05 vs. corresponding predrug value , Mann-Whitney U-test and $^{+}$P<0.05 vs. corresponding control value, Mann-Whitney U-test)and b) the percentage of burst firing activity (statistics: $^{+}$P<0.05, Mann-Whitney U-test, *P<0.05 vs. corresponding predrug value, Wilcoxon signed rank test) in control rats, rats pretreated with CGP 35348 (200 mg/kg, i.v.), and in rats pretreated with PNU 156561A (40 mg/kg, i.v.; 5-9 hours). Each value represents mean ± SEM from 6-13 VTA dopamine neurons.

neurons (Engberg et al. 1993, Erhardt et al. 2001c). Previous studies have provided considerable evidence that inhibitory GABAergic afferents, e.g. from neurons within the VTA, modulate the activity of dopamine VTA neurons (Fisher et al. 1998, Steffensen et al. 1998). Activation of such afferents is likely to mediate the short-lasting inhibition of VTA dopamine neurons by nicotine. Recent studies have shown that these GABAergic afferents possess at least four different subtypes of nAChR (Klink et al. 2001) and, indeed, VTA non-dopamine - presumably GABAergic - neurons are excited upon exposure to nicotine (Yin & French 2000). Thus, the presently observed inhibition of VTA dopamine neurons by nicotine may be mediated via stimulation of nAChRs located on GABAergic neurons within the VTA and/or projecting to the VTA, hereby promoting the release of GABA. In addition, the very rapid onset of this nicotine-induced inhibition may indicate a peripheral origin of this effect. If this inhibitory action of nicotine is peripherally mediated then the action of nicotine on VTA DA neurons resembles the nicotine-induced excitation on noradrenergic neurons in the LC, which is originally mediated via activation of peripheral primary C-fiber afferents (Hajos & Engberg, 1988).

C. Elevated levels of kynurenic acid convert the nicotine-induced activation of ventral tegmental area dopamine neurons into an inhibitory response.

Pretreatment with PNU 156561A (40 mg/kg, i.v., 5-9 hours) was found not only to antagonize the increase in burst firing activity of VTA dopaminergic neurons induced by nicotine (1.5-400 μg/kg, i.v) but also to reverse the action of nicotine, leading to a decrease in percentage of spikes fired in bursts (Fig. 11b). Thus, six out of 12 spontaneously bursting neurons were converted to non-bursting neurons by nicotine. This pretreatment also prevented a significant increase in firing rate by nicotine (Fig. 11a). In contrast to its action in controls, nicotine produced an increase in the regularity of firing (expressed as a decreased variation coefficient) in rats pretreated with PNU 156561A. In addition, the antagonistic actions of PNU 156561A on the nicotine-induced increase in firing rate and burst firing activity were prevented by administration of D-cycloserine, a partial agonist at the glycine site of the NMDA receptor. Thus, after treatment with both PNU 156561A (40 mg/kg, i.v., 5-9 h) and D-cycloserine (128 mg/kg, i.v., 10 min) nicotine (1.5-400 μg/kg) had similar effects on firing activity of VTA DA neurons as those observed in control rats (Fig. 13a, b).

The specificity of kynurenic acid as an EAA receptor antagonist has been under investigation in the past few years. Some studies reveal that kynurenic acid does not interfere with nicotinic receptors (Bijak et al. 1991, Bertolino et al. 1997) but a recent communication shows that kynurenic acid blocks the α7* nicotinic receptor with the same IC_{50} as for NMDA receptors (Hilmas et al. 2000). α7* nicotinic receptors have been shown to be preferentially presynaptically located on glutamatergic afferents in the CNS, thereby modulating the release of glutamate (see McGehee et al. 1995, Wonnacott et al. 2000). Therefore, the present blockade of nicotine-induced activation of VTA dopamine

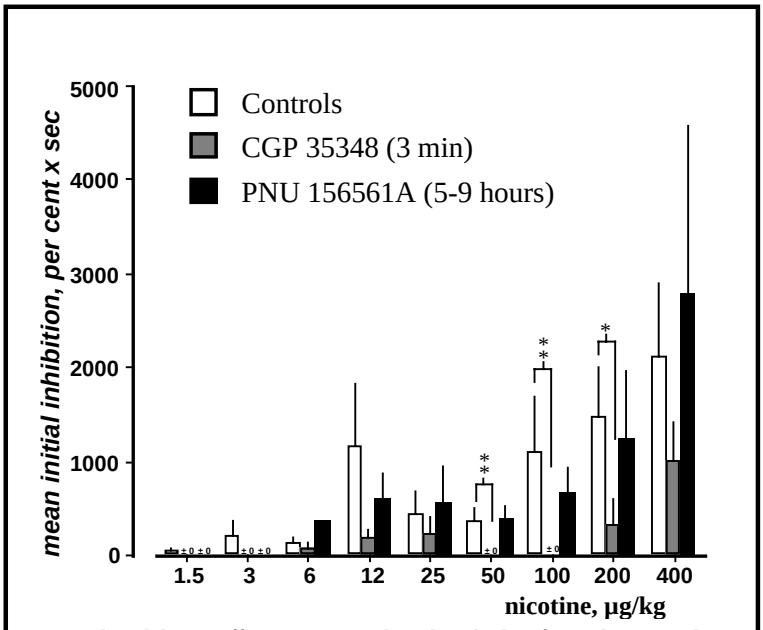

Fig. 12. The inhibitory effect (see Materials and methods) of i.v. administered nicotine in control rats, rats pretreated with CGP 35348 (200 mg/kg, i.v.), and in rats pretreated with PNU 156561A (40 mg/kg, i.v.; 5-9 hours). Each value represents mean ± SEM from 6-13 VTA dopamine neurons. Statistics: *P<0.05 (Mann-Whitney U-test).

neurons by elevated levels of brain kynurenic acid can hypothetically be mediated in at least three different ways: I) blockade of postsynaptic NMDA-receptors, located on cell soma or dendrites of VTA dopamine neurons; II) blockade of presynaptic α7* receptors, located on glutamatergic afferents (leading to a potentially decreased glutamate release by nicotine) or III) blockade of postsynaptic α7* receptors, located on cell soma or dendrites of VTA dopamine neurons. Our findings clearly show that in rats with elevated endogenous levels of kynurenic acid, the excitatory action of nicotine on firing rate and burst activity is restored after administration of D-cycloserine. Thus, stimulation of somatodendritic NMDA receptors of VTA dopamine neurons appears critically involved in the effects of nicotine. This view is also in agreement with a previous microdialysis study in the awake rat showing a specific involvement of NMDA-receptor activation in the VTA in the increased release of dopamine in the nucleus accumbens by systemically administered nicotine (Schilström et al. 1998). Since local application of NMDA-receptor antagonists in the VTA was found to reduce the nicotine-induced nucleus accumbens dopamine release (Schilström et al. 1998, Fu et al. 2000), and local administration of NMDA-receptors antagonists into the nucleus accumbens did not affect the nicotine-induced dopamine release (Fu et al. 2000), the specific site where nicotine acts to affect firing of VTA dopamine neurons seems to be within the VTA itself.

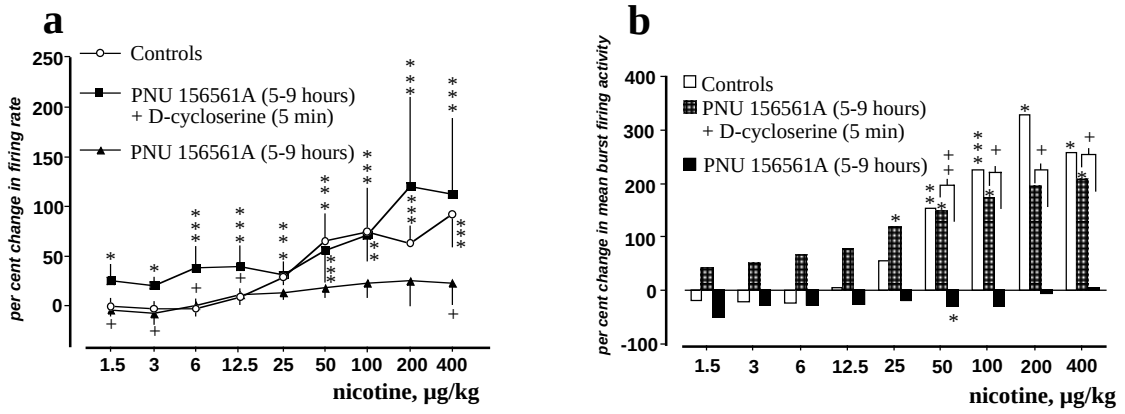

Fig. 13. Effects of incremental doses of intravenously administered nicotine (1.5-400 µg/kg) on a) the firing rate (statistics: *P<0.05 vs. corresponding predrug value, Mann-Whitney U-test and $^{+}$P<0.05 vs. corresponding value from the group treated with both PNU156561A and D-cycloserine, Mann-Whitney U-test) and b) the percentage of burst firing activity (statistics: $^{+}$P<0.05, Mann-Whitney U-test, *P<0.05 vs. corresponding predrug value, Wilcoxon signed rank test) in control rats, rats pretreated with PNU 156561A (40 mg/kg, i.v.; 5-9 hours), and in rats pretreated with both PNU 156561A (40 mg/kg, i.v.; 5-9 hours and D-cycloserine (128 mg/kg, i.v., 10 min). Each value represents mean ± SEM from 6-13 VTA dopamine neurons.

Distribution and significance of endogenous kynurenic acid. (Paper I-IV)

For long time, the physiological significance of kynurenic acid has been a matter of controversy, since the concentrations of kynurenic acid found in brain (Moroni et al. 1988, Turski et al. 1988, see Stone 1993) are below those concentrations reported to be necessary for the antagonism of EAA receptor functions. So far, studies of the physiological role of kynurenic acid in the CNS have been impeded by the lack of specific pharmacological tools affecting the synthesis of the compound. However, the introduction of kynurenine 3-hydroxylase inhibitors that increase endogenous levels of kynurenic acid (Speciale et al. 1996, Cozzi et al. 1999) has greatly improved the studies on the importance of endogenous kynurenic acid in brain. In our studies, pretreatment with PNU 156561A (40 mg/kg, i.v.) caused an increase in endogenous whole brain kynurenic acid concentrations by 3-9 times compared to controls (for summary, see Table 2). In addition, pretreatment with probenecid (200 mg/kg, i.v., 1 h) was found to increase the brain concentrations of kynurenic acid approximately 8-fold. Pretreatment with 10% β-cyclodextrin, in paper I (3-6 h, i.v.; the agent in which PNU 156561A was dissolved) or acute administration of PNU 156561A (5-15 min before decapitation) did not affect brain kynurenic acid levels. Additional treatment with D-cycloserine (128 mg/kg, i.v., 10-15 min, paper III and IV) did not significantly affect the elevated brain levels of kynurenic acid as induced by PNU 156561A (40 mg/kg, i.v.). Furthermore, pretreatment with PNU 156561A (40 mg/kg, i.v., 5-9 h; n = 9) did not significantly alter the levels of kynurenic acid in rat CSF (Table 2). The absence of a rise in kynurenic acid levels in CSF is somewhat obscure but might reflect the relatively moderate increase in whole brain kynurenic acid concentrations.

In all our studies, only a moderate increase in endogenous concentration of brain kynurenic acid was required to affect glutamatergic neurotransmission. Brain concentrations of kynurenic acid in controls (ranging from 22 - 35 nM) or in rats pretreated with PNU 156561A (ranging from 75 - 230 nM) are far below those required to affect NMDA-receptors as revealed from in vitro studies (K_D approximately 8 μM). However, we have measured the concentrations of kynurenic acid in the whole rat brain. Such analyses, like the microdialysis techniques, only allow the measurement of kynurenic acid after diffusion and dilution from the site of release, and therefore they may underestimate the real concentration at the site of action (i.e. the synapse). Moreover, since kynurenic acid is synthesized in brain astrocytes (Schwarcz et al. 1992b, Curatolo et al. 1996) that adjoin glutamatergic boutons, synaptic

concentrations appear high enough to physiologically antagonize NMDA receptors, although average whole brain levels of kynurenic acid are not in the micromolar range.

The results of the present series of experiments, showing that a moderate increase in endogenous brain kynurenic acid concentrations is related to an activation of midbrain dopamine neurons and effectively prevents the activation of centrally acting drugs on brain catecholaminergic neurons, are in agreement with the concept that endogenous kynurenic acid is of physiological significance for modulation of glutamatergic neurotransmission in brain.

Table 2 Concentrations of kynurenic acid (nM)

	Controls	**Controls** (β-cyclodextrin, 3-6 h)	**PNU 156561A** (40 mg/kg, i.v., 5-15 min)	**PNU 156561A** (40 mg/kg, i.v., 3-9 h)	**PNU 156561A + D-cycloserine**	**Probenecid (200 mg/kg, i.p.)**
whole brain (I)	35.3 ±8.1 n=4	30.12±6.7 n=7	27.3 ±5.0 n=4	149.3 ±38.9** n=7		
whole brain (II)	26.3 ±6.0 n=5			230.0 ±170*n=5		
whole brain (III)	22.0 ±2.7 n=30			74.5 ±13.1*** n=17	55.1 ±15.4** n=10	182.5±21** n=4
CSF (III)	3.74 ±0.7 n=5			2.87 ±0.4 n=9	2.94 ±0.4 n=8	
whole brain (IV)	19.1 ±2.5 n=13			66.8 ±19.9*** n=11	44.8 ±15.4* n=8	
Blood	490 ±107 n=6			598 ±143 n=7	568 ±135 n=5	

Values are obtained from saline control rats, β-cyclodextrin (3-6 h) control rats, rats pretreated with PNU 156561A (40 mg/kg, i.v., 5-15 min), rats pretreated with PNU 156561A (40 mg/kg, i.v., 3-9 h), rats pretreated with PNU 156561A (40 mg/kg, i.v., 5-9 h) + D-cycloserine (128 mg/kg, i.v., 10-15 min) and rats pretreated with probenecid (200 mg/kg, i.p., 1 h). *P<0.05 Mann-Whitney U-test. Roman numerals within brackets refer to the study in which analysis were performed.

Pathophysiological significance of kynurenic acid

Increased levels of kynurenic acid in the cerebrospinal fluid of schizophrenic patients. (Paper V)

Based upon the finding that increased endogenous concentrations of brain kynurenic acid were associated with an activation of midbrain dopamine neurons, similar to the effects observed after systemic administration of the psychotomimetic drugs PCP and MK-801, we hypothesized an involvement of kynurenic acid in the pathogenesis of schizophrenia. Therefore, endogenous kynurenic acid concentrations in the CSF of schizophrenics as well as in healthy, age-matched controls were measured.

Kynurenic acid was detected in all controls (n=17), with little inter-individual variation (0.97 ±0.07 nM). In patients with schizophrenia (n=28, 25 of them drug-naive), CSF levels of kynurenic acid were higher (1.67 ±0.27 nM;

p=0.038, Mann-Whitney U-test) than in controls and the variation between individuals was larger, with a maximum value of 6.8 nM (Fig. 14). A correlation between age and CSF kynurenic acid was observed in schizophrenics (r=0.51, p= 0.0054, Fig. 15a) in contrast to healthy volunteers (r=0.08, Fig. 15b).

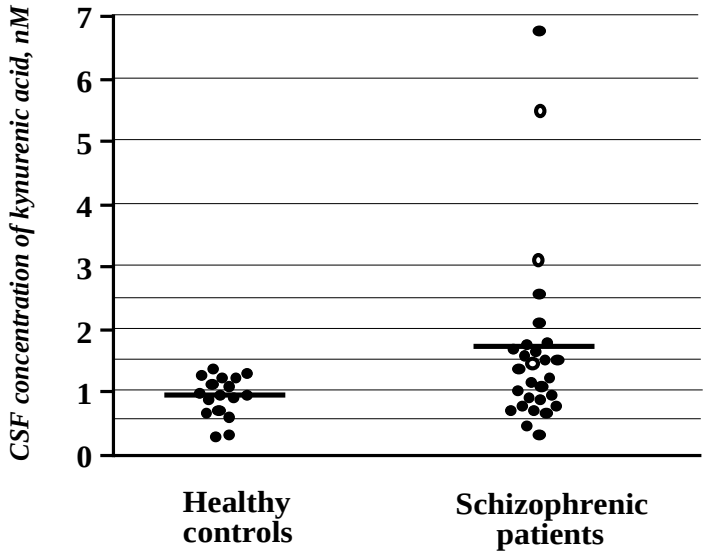

Fig. 14. Kynurenic acid in human CSF. Each point represents the concentration of kynurenic acid in a single CSF sample in units of nM. Open circles represents values from schizophrenic patients under neuroleptic treatment (n=3).

Our results, showing that the levels of endogenous kynurenic acid are elevated in CSF from schizophrenic patients, suggest that kynurenic acid is involved in the pathogenesis of schizophrenia. This is in consonance with the hypothesis of a deficiency of glutamate function in schizophrenia (see Carlsson et al. 2000, Kim et al. 1980). Furthermore, our data are in line with a post-mortem study demonstrating an increase in endogenous cortical kynurenic acid in schizophrenic patients (Rassoulpour et al. 1998b). In the present study, the majority (25/28) of patients were drug naive, first episode patients. Thus, the increased levels of kynurenic acid are not caused by treatment with neuroleptics. At any rate, chronic treatment of rats with antipsychotics does not result in increased levels of kynurenic acid, but rather in a decrease (Ceresoli-Boroni et al. 1999b). Further, since the increase was found in first-episode schizophrenic patients, it is not likely to be a secondary consequence of the disease process, found only at the end stage of the disease. Previous studies have shown an age-related increase of endogenous kynurenic acid in rats (Gramsbergen et al. 1997). In the present study, no such relation was observed in healthy volunteers. However, in schizophrenic patients a positive correlation between CSF kynurenic acid levels and age was found. This finding may suggest different pathogenic mechanisms in older first episode schizophrenics resulting in the late onset of the disease.

Other factors, however, such as heavy smoking, could possibly be responsible for the difference between the schizophrenics and the controls. It was recently

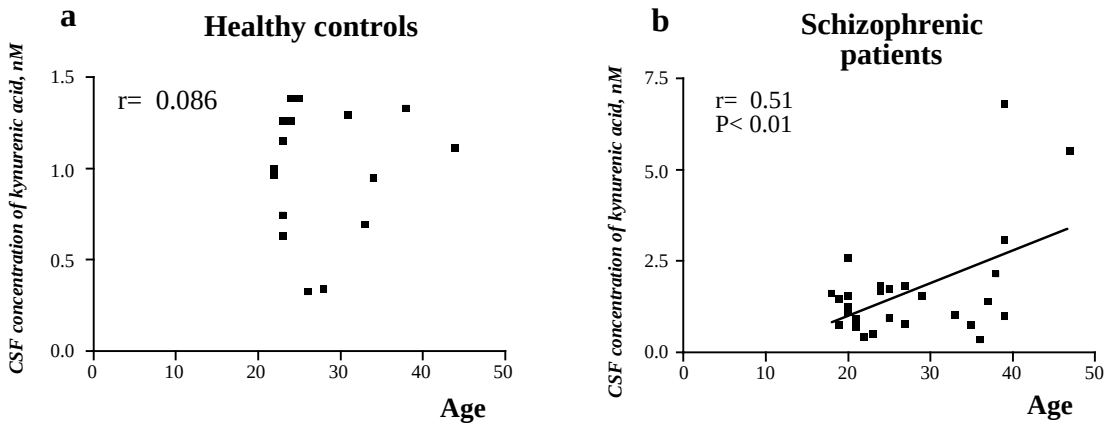

Fig. 15. Correlation between CSF kynurenic acid and age in healthy controls and in schizophrenics.

reported that chronic nicotine administration in rats induces biphasic changes in endogenous kynurenic acid levels (Rassoulpour et al. 2000). However, among the healthy volunteers, at least three were snuff users or smokers and all of them had kynurenic acid levels below the mean value of the control group (0.63, 0.63 and 0.67 nM, respectively), making it unlikely that smoking accounts for the elevated levels of kynurenic acid among schizophrenics. Further studies to correlate the CSF levels of kynurenic acid to symptoms, smoking rates, or antipsychotic treatment are, however, necessary to specifically ascertain the role of kynurenic acid in schizophrenia.

General Discussion

The results of the present thesis suggest that endogenous kynurenic acid is a biologically important modulator of glutamatergic neurotransmission within the brain. We have described the pharmacological and physiological importance of endogenous kynurenic acid and, in addition, revealed a putative contribution of the compound in the pathophysiology of schizophrenia. Furthermore, an attempt to shed some light over the mechanisms behind the high prevalence of smoking among schizophrenic patients was also made.

The question of a physiological role of brain kynurenic acid has been a matter of controversy. In our present series of experiments brain concentrations of kynurenic acid in controls (ranging from 22 - 35 nM) or in rats pretreated with PNU 156561A (ranging from 75 - 230 nM) are far below those required to affect NMDA-receptors as revealed from in vitro studies (K_D approximately 8 μM; see Stone 1993). However, in our studies we have measured the concentrations of kynurenic acid in the whole rat brain. Such analyses, like the microdialysis techniques, only allow the measurement of kynurenic acid after diffusion and dilution from the site of release, and therefore they may underestimate the real concentration at the site of action (i.e., the synapse). Previous findings, showing that kynurenic acid is produced in the astrocytes also indicate that newly produced kynurenic acid, upon release into the extracellular milieu, is likely to be in excellent position to influence NMDA receptor function. This may explain why changes in brain kynurenic acid, though detected at low whole brain concentrations, may produce significant actions on brain glutamatergic neurotransmission, although such concentrations are below those necessary to antagonize glutamate receptors. A possible explanation for the discrepancy between the previously observed effects of intracerebroventricular (i.c.v.) administration of kynurenic acid and the present data,might be that the pharmacological elevation of endogenous kynurenic acid by PNU 156561A is limited to micro-compartments where endogenous kynurenic acid naturally occurs and where the turnover is rapid. This may contrast with the elevation of kynurenic acid in brain following i.c.v. administration of the compound which may result in a more homogeneous distribution in brain. In addition, a pronounced heterogeneity in difference in distribution of kynurenic acid may account for the reversal of the excitatory action of muscimol into an inhibitory response following treatment with PNU 156561A, in contrast to i.c.v. administration of kynurenic acid that only antagonized the excitatory effect of muscimol.

Pharmacologically elevated levels of kynurenic acid are associated with a paradoxical increase in firing of midbrain dopamine neurons, similar to that produced by systemic administration of psychotomimetic agents, e.g. PCP and ketamine (French 1994). These effects are thought to be induced by an inadequate balance of afferent regulation by GABAergic and glutamatergic projections, e.g. from prefrontal cortex and/or subcortical areas. In particular, it is suggested that systemic administration of NMDA-receptor antagonists primarily reduce the activity of GABAergic projections to the VTA, thereby activating dopamine neurons by a decreased GABAergic tone. The presently revealed hyperactivity of midbrain dopamine neurons induced by elevated levels of kynurenic acid should be mediated via similar mechanisms. Schizophrenia is thought to be mediated, at least in part, by dysregulation of the midbrain dopamine pathways and interestingly, both PCP and ketamine induce schizophrenia-like positive and negative symptoms as well as cognitive dysfunction in healthy volunteers, and furthermore, aggravate psychotic symptoms in schizophrenic patients. Recently, strong support for the dopamine hypothesis of schizophrenia was provided by direct evidence of increased occupancy of striatal D_2-receptors by dopamine in schizophrenic patients (Abi-Dargham et al. 2000). Given the fundamental role of burst firing for terminal dopamine release, bursting activity of midbrain dopamine neurons, e.g. induced by the elevated levels of endogenous kynurenic acid found in schizophrenic patients, may play an important role in generating the positive symptoms of schizophrenia by stimulating D_2-receptors. Hereby, a dysfunction of the neuronal circuitries of the basal ganglia and frontal cortex, induced by elevated levels of endogenous kynurenic acid and reflected by a hyperactivity of midbrain dopamine neurons, might contribute to the pathophysiology of schizophrenia. Indeed, the finding of elevated levels of kynurenic acid in the CSF of schizophrenics is in line with a post-mortem study from schizophrenic patients, showing a significant increase in kynurenic acid levels in the frontal cortex (Rassoulpour et al. 1998b). Present studies also provide evidence that, in the rat, the actions of endogenous kynurenic acid on VTA dopamine neurons are antagonized by D-cycloserine. Interestingly, several studies suggest that agonists at the glycine-site of the NMDA-receptor, e.g. glycine and D-cycloserine, ameliorate negative symptoms in schizophrenia (Javitt et al. 1994, Tsai et al. 1998, Goff et al. 1999, Heresco-Levy et al. 1998, 1999). Another interesting finding, also indirectly supporting a role of endogenous kynurenic acid in the pathogenesis of schizophrenia, is that administration of kynurenic acid into the cisterna magna of the rat has been found to block peripheral pain responses

(Hajos & Engberg 1990). Thus, increased endogenous concentrations of kynurenic acid in the brain of schizophrenics may explain the relative insensitivity to pain often observed among these patients (Fishbein 1982, Dworkin 1994, Hooley & Delgado 2001).

As mentioned above, the positive symptoms of schizophrenia are often attributed to hyperactivity in dopaminergic projections to the limbic regions. However, data from schizophrenics indicate that negative symptoms as well as cognitive deficits are associated with a reduction of terminal dopamine release in the prefrontal cortex (Weinberger et al. 1988, Dolan et al. 1995, Karoum et al. 1987). Interestingly, both hypo- and hyperactivity in brain dopamine projections have been proposed to occur simultaneously (Svensson et al. 1995, Svensson 2000). Indeed, recent studies demonstrate that subchronic administration of PCP is associated with a reduction in dopamine utilization in the prefrontal cortex, and an impaired cognition as well (Jentsch et al. 1997). Further, subchronic administration of PCP induces a hyper-responsivity of mesolimbic dopamine neurons to stress or amphetamine administration (Jentsch et al. 1998a). Consequently, cortical dopaminergic hypoactivity and mesolimbic dopaminergic hyperactivity may subserve negative and positive symptoms in schizophrenia, respectively (Jentsch et al. 1998b), and could accordingly be mediated via blockade of NMDA receptors at glutamatergic neurons projecting to the midbrain dopamine neurons or at GABAergic interneurons. In a situation of chronically elevated endogenous levels of kynurenic acid, an increase or decrease in dopaminergic transmission may occur depending on which afferents the blockade prevail. This "brake-and-accelerator hypothesis", based upon the cortical glutamate/GABA-mediated steering of subcortical systems, was introduced by Carlsson and is now becoming widely accepted as a model for how negative and positive symptoms of schizophrenia might occur simultaneously. Furthermore, the hypothesis has during the past few years received strong support from anatomical studies, demonstrating that dopamine neurons projecting to nucleus accumbens are densely innervated by GABAergic neurons, whereas those projecting to prefrontal cortex are not (Carr & Sesack 2000, see Fig. 2). Future studies will reveal if chronically elevated levels of endogenous kynurenic acid are able to induce both types of dopaminergic dysfunction simultaneously.

The reason for the high prevalence of smoking in schizophrenic patients remains elusive; many ideas are speculative, as there has been no study to determine exactly what effects smoking has on psychopathological symptoms in schizophrenic patients. However, two cross-sectional studies have shown that

schizophrenic patients who smoke have higher rates of positive symptoms (Goff et al. 1992, Ziedonis et al. 1994), but there are no longitudinal data to distinguish causality. In addition, the two cross-sectional studies were split in terms of the correlation between negative symptoms and smoking, one suggesting lower rates among smokers (Ziedonis et al. 1994) and the other higher rates among smokers (Goff et al. 1992). The results of the present thesis may shed some light upon the neurobiological mechanism(s) behind the high prevalence of smoking in schizophrenic patients. A novel finding in this thesis is the nicotine-induced inhibition, probably of GABAergic origin, of VTA dopamine neurons especially pronounced in rats with pharmacologically elevated levels of kynurenic acid. 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. In schizophrenia, a dysfunction within the cortico-cortical circuitry is suggested to result in impaired functions of affected cortical regions. As glutamate and GABA constitute the currency of these systems, nicotine, by releasing glutamate and GABA may normalize/stabilize the activity of VTA dopamine neurons. Thus, in a situation of dopaminergic hyperactivity, as induced e.g. by increased endogenous concentrations of kynurenic acid in the brain, the action of GABA, released by nicotine, may prevail. Correspondingly, when dopaminergic hypoactivity dominates, nicotine-induced glutamate release would serve to increase the activity. Hence, nicotine may modulate human behavior by either facilitating or inhibiting dopaminergic neurotransmission, depending on the pre-existent dopaminergic tone.

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 increase in D_2-receptor stimulation. Thus, the increased firing rate and burst firing activity of midbrain dopamine neurons following pharmacologically elevated levels of kynurenic acid, might represent a pathophysiological condition similar to that seen in schizophrenic patients. Hereby, the presently revealed inhibitory action of nicotine on VTA dopamine neurons may serve to mediate a self-controlled relief of psychotic symptoms in smoking schizophrenic patients. This action, in addition to the cognition enhancing effect of nicotine, as well as the drug's ability to also attenuate negative symptoms of schizophrenia could, accordingly, underlie the high prevalence of smoking in schizophrenic patients.

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