ON THE ROLE OF NMDA RECEPTOR SUBUNITS IN THE ACUTE AND CHRONIC EFFECTS OF NICOTINE

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To my dear mother Ludmila and to my love Ann-Charlott
ABSTRACT

Nicotine is considered the main dependence-producing constituent in tobacco products. In analogy with other drugs of abuse, nicotine enhances dopamine (DA) neurotransmission within the mesocorticolimbic DA system. This increase in DA release is thought to be at least partially responsible for the reinforcing and dependence-producing effects of nicotine. However, accumulating evidence suggests that also glutamatergic neurotransmission is involved in the dependence-producing effects of nicotine since nicotine also enhances the release of glutamate release in the mesocorticolimbic DA system, and glutamate receptor antagonists inhibit the nicotine-induced release of DA in the nucleus accumbens (NAcc).

The aim of this study was to investigate how NMDA and AMPA receptor antagonists modulate the acute and chronic effects of nicotine on locomotor activity (LMA) and mesocorticolimbic DA release. In particular, we wanted to examine the possible role of NMDA receptor subunits in the stimulatory actions of nicotine following acute and chronic administration of the drug.

Acute administration of nicotine produced a dose-dependent increase in LMA and DA release in the NAcc. Administration of the novel AMPA receptor antagonist ZK200775 and the competitive NMDA receptor antagonist CGP39551 attenuated the effects of nicotine. In contrast and unexpectedly, the NR2B subunit selective NMDA receptor antagonist, Ro 25-6981 potentiated the acute effects of nicotine on LMA (without producing stereotypies) as well as on DA release in the NAcc.

Chronic administration of nicotine resulted in the development of behavioral sensitization and nicotine-conditioned locomotor stimulation. Behavioral sensitization developed two days prior to the onset of the conditioned response. When Ro 25-6981 was given to naïve rats at a dose, which by itself had no effect on LMA, in rats chronically treated with nicotine, it significantly increased LMA without inducing stereotypies. Moreover, the same dose of Ro 25-6981 had no effect on DA release in the medial prefrontal cortex (mPFC) whereas a trend toward increased DA release was noted in the NAcc. In addition, Ro 25-6981 potentiated nicotine-induced DA release in the mPFC but not in the NAcc.

Chronic administration of nicotine exposure caused no changes in the expression levels of NR2A or NR2B mRNA in the prefrontal cortex (PFC) or ventral striatum (VStr) but Western blot revealed that there was an upregulation of the NR2B subunit protein but not NR2A subunit protein in the PFC with no corresponding changes in the VStr.

Taken together, our data suggest that both AMPA and NMDA receptors are involved in the acute effects of nicotine on LMA and NAcc DA release. However, whereas a non-selective blockade of NMDA receptors inhibits the acute effects of nicotine on LMA and NAcc DA release, a selective blockade of the NR2B subunit enhances nicotine's acute effects. In addition, chronic nicotine treatment upregulates the NR2B subunit in the PFC but not in the VStr suggesting that chronic nicotine exposure induces regionally selective neuroadapative changes in the composition of NMDA receptor subunits. These observations are of potential interest not only for our understanding of the neurochemical mechanisms involved in nicotine dependence but also for some psychiatric and neurodegenerative disorders known to be associated with disturbances in the interplay between dopaminergic and glutamatergic neurotransmission.
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IV. Kosowski AR, Cebere A, El-zaqzouq R, and Liljequist S Chronic nicotine upregulates NMDA NR2B subunits in the prefrontal cortex and produces locomotor stimulation after a subthreshold dose of the NR2B-selective NMDA antagonist Ro 25-6981 without changing dopamine release. Manuscript
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>AP-5</td>
<td>DL-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>CGP39551</td>
<td>(DL-[E]-2-amino-4-methyl-5-phosphono-3-pentanoic acid ethyl ester)</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>GYKI52466</td>
<td>4-(8-methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepine-5-yl)-benzenamine dihydrochloride</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>LMA</td>
<td>locomotor activity</td>
</tr>
<tr>
<td>MK-801</td>
<td>5-methyl-10,11-dihydro-5H-dibeno(a,d)cyclohepten-5,10-imine maleate</td>
</tr>
<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NAcc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7sulfamoyl-benzo[f]quinoxaline</td>
</tr>
<tr>
<td>PCP</td>
<td>phencyclidine</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>[(+/)-(R*,S*)-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidine propanol]</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>VStr</td>
<td>ventral striatum</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>ZK200775</td>
<td>[1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl)quinoxaline-1-yl]methylphosphonate</td>
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1. INTRODUCTION

1.1 General Background

Nicotine is considered to be the major dependence producing constituent in tobacco products (Benowitz 1988; Henningfield and Goldberg 1983). Despite the established knowledge and awareness that smoking may result in severe health problems, people continue to use tobacco products. This behavior and the difficulty to quit smoking (that is 80% of those who try to quit fail to do so on their first attempt) confirm the strong drug dependence liability of nicotine (Stitzer and Gross 1988). It is generally assumed that nicotine, like several other drugs of abuse, induces its reinforcing and dependence producing effects by enhancing dopamine (DA) release in the mesocorticolimbic DA system of the brain (Balfour et al. 2000; Benwell and Balfour 1992; Corrigall et al. 1992; Di Chiara and Imperato 1988; Imperato et al. 1986). For this reason, most attention of nicotine research has, until recently, been focused on dopaminergic neurotransmission. However, there is evidence that nicotine also influences several other neurotransmitters in the brain such as GABA, noradrenaline, acetylcholine, endogenous opioid peptides and glutamate (Grady et al. 1992; Lu et al. 1998; Lu et al. 1999; McGehee et al. 1995; Reid et al. 2000; Toth et al. 1993; Watkins et al. 2000). In fact, an interaction between glutamatergic and dopaminergic systems may play a key role in the processes underlying the development of dependence to nicotine as well as other drugs of abuse (Fu et al. 2000; Schilstrom et al. 1998; Sziraki et al. 1998; Tzschentke and Schmidt 2003). Several studies show that nicotine induces release of glutamate in the mesocorticolimbic DA system and that nicotine’s effect on DA release can be blocked by glutamate receptor blocking agents (Fu et al. 2000; Lambe et al. 2003; Schilstrom et al. 1998; Sziraki et al. 1998; Toth et al. 1993).

Although chronic intake of nicotine, e.g. smoking, causes drug dependence, there are also studies suggesting that nicotine has beneficial effects in some situations (Balfour and Fagerstrom 1996; Singh et al. 2004). For example, since patients with schizophrenia show high prevalence of smoking (80-90%) (Dalack et al. 1998; Diwan et al. 1998; Hughes et al. 1986) it has been suggested that nicotine might alleviate some of the pathophysiological deficits observed in schizophrenic
patients and that smoking therefore may represent a form of self-medication (Adler et al. 1992; Dalack et al. 1998; Dalack and Meador-Woodruff 1999; Svensson et al. 1990). Moreover, a high prevalence of smoking (40-70%) is also reported in patients with anxiety disorders and depression, suggesting that nicotine might ameliorate some of the symptoms observed in these diseases as well (Breslau et al. 1991; Covey et al. 1990; 1998; Glassman 1993; Glassman et al. 1992; Picciotto et al. 2002). In addition, smokers exhibit a lower incidence of Parkinson’s disease (Quik 2004; Wirdefeldt et al. 2005) although it is not yet fully confirmed that the protective effects of smoking can be exclusively attributed to nicotine. In addition, nicotine might improve cognitive functions in Alzheimer’s patients (Jones et al. 1992) and have some beneficial effects in the treatment of Tourette’s syndrome and attention-deficit hyperactivity disorder (Conners et al. 1996; Levin et al. 2001; Levin et al. 1996; Mihailescu and Drucker-Colin 2000).

Overall, the dependence producing effects of nicotine are largely due to enhanced DA transmission in the brain although glutamate may also be critically involved in nicotine’s effects on DA release. Accumulated evidence suggests that nicotine may have several pharmacologically interesting beneficial effects. Therefore, novel information about the effects of nicotine on dopaminergic and glutamatergic neurotransmission may increase the understanding of both the adverse and beneficial effects of nicotine. Such information might pave the way for the development of novel pharmacological strategies for the treatment of drug dependence and perhaps for a variety of psychiatric and neurodegenerative disorders.

1.2 Brain Glutamate Receptors

In the 1940s, some clinical and preclinical observations suggested that glutamate could play an important functional role in the central nervous system (CNS). For instance, glutamate was claimed to improve cognitive acuity in patients with mental impairment and to terminate hypoglycemic coma (Albert et al. 1951; Waelsch 1951; Weil-Malherbe 1950). In 1949, Krebs and co-workers reported high concentrations of glutamic acid in animal brain tissue (Krebs et al. 1949). However, the first who actually suggested that glutamate might act as a brain neurotransmitter
was Takashi Hayashi in 1954. He discovered that intracerebroventricular or intracarotid injection of glutamate caused convulsions in dogs and monkeys (Hayashi 1954; 1956). More evidence to support the idea that glutamate acts as a neurotransmitter in the CNS emerged in the late 1950s by Curtis who found that glutamate depolarized and excited individual neurons in the cat spinal cord (Curtis et al. 1959; 1960; Curtis and Watkins 1960). However, it was not until the late 1970s and early 1980s that glutamate was established to act as an independent neurotransmitter in the CNS (McLennan 1983; Watkins and Evans 1981). Since then, numerous studies have helped to shed light on the function and regulation of glutamate and glutamatergic neurotransmission (Danysz and Parsons 1998; Monaghan and Cotman 1982; 1985; Monaghan and Larsen 1997; Monaghan et al. 1984; Seeburg 1993).

Today, glutamate is considered to be the principal excitatory neurotransmitter in the CNS where it has been shown to modulate much of the synaptic activity in brain (Bleich et al. 2003; Dingledine et al. 1999). Glutamate is involved in regulating sensory, motor, cognitive, and emotional functions (Ikonomidou and Turski 2002) and, importantly, is a critical component in the generation of long-lasting changes in synaptic function (termed synaptic plasticity). Two such forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD), have been extensively characterized and are believed to underlie some forms of learning and memory (Holscher 1997; Massey et al. 2004). Recently, LTP and LTD have become an important focus of addiction research (Wolf 2003b; Wolf et al. 2004). Glutamate has also been demonstrated to play a crucial role during ontogenesis and early development by modulating the proliferation, migration, and differentiation of immature neurons and for synaptogenesis (Ikonomidou et al. 2000; Rzeski et al. 2001). Deficient glutamate signaling has been implicated in several psychiatric and neurological diseases such as stroke, epilepsy, Parkinson’s disease, anxiety disorders and schizophrenia as well as drug dependence (Tzschentke 2002).

Glutamate is a non-essential amino acid that does not cross the blood brain barrier. It is synthesized directly in the brain from α-ketoglutarate in the mitochondrial compartment of glutamatergic nerve terminals either through transamination of aspartate or by conversion from glutamine (Tapiero et al. 2002). Glutamate is subsequently packaged into synaptic vesicles and released from nerve terminals in response to nerve impulses. Glutamatergic neurotransmission is terminated by
glutamate uptake into neurons or glia cells through specific glutamate transporters, termed excitatory amino acid carriers (EEAT’s) (Shigeri et al. 2004).

Glutamate receptors are divided into two large families: the metabotropic and the ionotropic glutamate receptors. The metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors and, so far, eight such receptor subtypes have been cloned and termed mGluR1 through mGluR8. The mGluRs are suggested to modulate numerous ligand- and voltage gated ion channels located both pre-synaptically and post-synaptically on central neurons and to influence neuronal function through regulation of second messenger cascades and protein phosphorylation (Pin and Acher 2002).

The ionotropic glutamate receptors are ligand-gated ion channels permeable to Na$^+$, K$^+$, and Ca$^{2+}$ (Bleakman and Lodge 1998; Dingledine et al. 1999). Based on pharmacological properties, they are divided into three distinct groups of receptor subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors (Hollmann and Heinemann 1994; Nakanishi 1992; Seeburg 1993).

1.2.1 NMDA receptors

NMDA receptors are highly permeable to Ca$^{2+}$ ions. The NMDA receptor is blocked by Mg$^{2+}$ (within the ion channel) and a certain degree of depolarization is needed for the Mg$^{2+}$ block to be removed. Moreover, NMDA receptors require glycine as a co-agonist. Several other allosteric ligands can modulate the NMDA receptor such as D-serine, polyamines, and protons (Danysz and Parsons 1998).

The NMDA receptor is considered to be a heteromer composed of five subunits (although some studies suggest a tetrameric structure); NR1 and NR2A, B, C, and D-subunits (Ferrer-Montiel and Montal 1999; Hollmann and Heinemann 1994; Planells-Cases et al. 1993). The NR1 subunit, which holds a glycine-binding site, is transcribed from one gene that undergoes alternative splicing thereby generating eight unique splice variants (Moriyoshi et al. 1991). The NR2-subunits, which form the glutamate-binding site, are encoded by four different genes termed NR2A, NR2B, NR2C, and NR2D, respectively (Ishii et al. 1993; Kutsuwada et al. 1992). Recently, two additional subunits have been identified, NR3A and NR3B subunits, which
appear to encode a glycine-binding site not associated with the NR1 subunit (Mayer and Armstrong 2004).

By using recombinant receptor systems, it was found that co-expression of NR1 and NR2 subunits is essential for the formation of functional NMDA receptors (Grimwood et al. 1996; Hollmann and Heinemann 1994; Nakanishi 1992; Seeburg 1993). It is suggested that the NMDA receptor comprises two copies of one or several splice variants of the NR1 subunit and at least two different NR2 subunits within a single heteromeric channel (Behe et al. 1995; Blahos and Wenthold 1996; Chazot et al. 1994; Didier et al. 1995; Sheng et al. 1994; Wafford et al. 1993). Thus, the NMDA receptor may form various combinations of NR1 and NR2A-D subunits with different electrophysiological and pharmacological properties.

The subunit composition of NMDA receptors determines their pharmacological and pharmacokinetic properties such as Ca\(^{2+}\) permeability, degree of voltage dependent Mg\(^{2+}\) block and activation/deactivation kinetics. For instance, the NR1/NR2A and NR1/NR2B channels are more sensitive to Mg\(^{2+}\) block than the NR1/NR2C and NR1/NR2D channels (Mori and Mishina 1995). NMDA receptors containing the NR2B subunit appear to have higher affinity for glutamate than those containing the NR2A subunit. Moreover, glycine binds with highest affinity to NR1/NR2C channels and with lowest affinity to NMDA receptors containing the NR2A subunit (Sucher et al. 1996). Diverse NR2 subunit content can also result in different deactivation time constants of glutamate induced currents. Deactivation time has been shown to follow the sequence: NR2A<NR2C=NR2B<<NR2D (Vicini et al. 1998). In conclusion, there is a large regional variation in NMDA receptors with large diversity in subunit composition.

Autoradiographic studies have shown that NMDA receptors are widely distributed in the brain, particularly in the forebrain, the midbrain, the CA1 region of the hippocampus (Buller et al. 1994; Dana et al. 1991; Goebel and Poosch 1999; Monaghan et al. 1989). Additional studies using radioactive ligands, Northern blot and in situ hybridization have revealed that there are multiple pharmacologically distinct NMDA receptor subtypes with specific anatomical distribution in the brain. Thus, the NR1 subunit mRNA is evenly expressed in most of the rat brain whereas the NR2 subunits display distinct regional distributions. The NR2A subunit mRNA shows high density particularly in the cerebral cortex, hippocampal formation, and cerebellar cortex. NR2C is predominately expressed in the granule cell layer of the
cerebellum whereas the NR2D subunit appears to be primarily present in the thalamus, brainstem, and spinal cord (Wenzel et al. 1995). In both non-human primates and rats, the NR2B subunit is highly expressed in the cerebral cortex (particularly in pyramidal like cells in layer II/III and V). Also, NR2B-subunits are highly abundant in the thalamus, in the striatum, in the fields of Ammon’s horn, and in the CA1, CA3, dentate gyrus of the hippocampus (Allgaier et al. 1999; Loftis and Janowsky 2003). Moderate levels of NR2B subunit expression are evident in the midbrain and low expression occurs in the cerebellum and spinal cord.

The relative expression of various NMDA receptor subunits appears to vary with age (Bai et al. 2004; Monyer et al. 1994). In rats, levels of NR1 protein are low at birth and gradually increase in all brain regions to reach adult levels at approximately three weeks after birth (Luo et al. 1996). To add to the complexity, the different NR1 splice variants also appear to have distinct regional and developmental expression patterns (Laurie et al. 1995; Laurie and Seeburg 1994). In analogy with NR1 subunits, the expression of NR2 subunits also displays temporal variation. Thus, the NR2B and NR2D subunits predominate prenatally and at early stages postnatally. In contrast, NR2A and NR2C subunits can first be detected near birth with increased expression postnatally (Laurie et al. 1997; Laurie and Seeburg 1994; Zhong et al. 1995).

Numerous different NMDA receptor antagonists have been used to study the physiological function of NMDA receptors. For instance, the non-competitive NMDA receptor antagonists MK-801, phencyclidine and ketamine act by interfering within the NMDA receptor ion channel (Bresink et al. 1995). Therefore, these are activity-dependent since the Mg$^{2+}$ block must be removed before e.g. MK-801 can bind. Other NMDA receptor antagonists such as CGP39551, CPP, AP-5 and CGS19755 or glycine site antagonists act by competitively binding to the agonist binding sites (Danysz et al. 1994; Danysz and Parsons 1998). However, due to a wide distribution of NMDA receptors with differing subunit composition, non-selective blockade of those receptors induces many adverse side effects (Davis et al. 2000; Parsons et al. 1998). Recently, several novel subunit-selective NMDA receptor antagonists have been developed such as NR2B selective CP101,606 or Ro 25-6981, and the NR2A selective subunit antagonist NVP-AAM077 (Mallon et al. 2004; Menniti et al. 1997; Mutel et al. 1998). In contrast to the non-competitive and competitive NMDA receptor antagonists, these compounds display fewer side effects.
Figure 1. The NMDA receptor showing the various binding sites of endogenous and exogenous agonists, modulators, and antagonists. Modified from Cebere (2003). The white box within the channel is the binding site for non-competitive NMDA receptor antagonists like MK-801 and PCP.
1.2.2 AMPA receptors

AMPA receptors, to a large extent, mediate the fast excitatory transmission in the mammalian CNS (Bleakman and Lodge 1998; Frerking and Nicoll 2000). For instance, AMPA receptors are suggested to be important in synaptic plasticity, maturation of glutamatergic synapses, dendritic growth, processes for memory formation, and cognition (Black 2005; Bleakman and Lodge 1998; Perez-Otano and Ehlers 2004; Zivkovic et al. 1995). In addition, a crucial role for AMPA receptors has been established in excitotoxic neuronal cell death, cognitive deficits, epilepsy, pain and also drug dependence (Bleakman and Lodge 1998; Wolf 2003a).

AMPA receptors are composed of four subunits called GluR1-4 and studies using in situ hybridization, immunohistochemistry and single-cell RT-PCR have revealed a widespread distribution of these subunits in the brain (Bochet et al. 1994; Hollmann and Heinemann 1994; Jonas et al. 1994). However, the expression of different AMPA receptor subunits differs between brain regions and even among cellular layers in the same structure. For example, in the cortex, GluR1 and GluR3-4 are expressed differently while GluR2 shows a uniform distribution. In addition, there is evidence to suggest that AMPA receptors with different subunit composition exist within the same neuron (Lerma et al. 1993; Molnar et al. 1993). Thus, the large variation in the distribution of AMPA receptor subunits suggests regional variations in AMPA receptor pharmacology. Indeed, quantitative receptor autoradiography studies reveal regional variations in the pharmacological specificity of AMPA receptors (Porter and Greenamyre 1994). To add to the complexity of AMPA receptor ion channels, the GluR subunits can be expressed in two forms: the "flip" and "flop" splice variants. These two isoforms differ in regards to their desensitization profiles (Bleakman and Lodge 1998). Furthermore, in addition to Na⁺, AMPA receptors possess a certain degree of Ca²⁺ permeability which is determined by the GluR2 subunit (Jonas et al. 1994). A low proportion of GluR2 subunits in the receptor render them more permeable to Ca²⁺ ions. A subset of Ca²⁺ permeable AMPA receptors have been found in, for instance, hippocampal neurons, medial septal neurons and cerebellar Purkinje neurons (Bleakman and Lodge 1998).

Newly developed specific AMPA receptor antagonists have become useful tools for the study of the functional role of AMPA receptors. The most prominent group of classical AMPA receptor antagonists are a series of quinoxalidione derivatives,
including CNQX, DNQX and NBQX (Honore et al. 1988). However, these compounds show poor solubility, short duration of action, and in the case of CNQX, a lack of selectivity since it also binds the glycine site of the NMDA receptor (Pellegrini-Giampietro et al. 1989). Other more selective AMPA receptor antagonists are the 2,3-benzodiazepine derivatives GYKI52466 and GYKI53655 (Lerma et al. 1993). These compounds act as allosteric modulators. Recently, novel AMPA receptor antagonists have been developed such as LY326325, LY293558 and ZK200775 (Schoepp et al. 1995; Turski et al. 1998). These compounds possess an improved pharmacological profile with better selectivity, solubility, and longer duration of action. ZK200775 for instance, is water soluble at physiologic pH with retained high selectivity and potency at AMPA receptors.

1.3 Brain Nicotinic Acetylcholine Receptors

Nicotine exerts its effects in the brain by stimulating neuronal nicotinic acetylcholine receptors (nAChRs) (Gotti and Clementi 2004; Haass and Kubler 1997; Huh and Fuhrer 2002; Wessler et al. 1998). The neuronal nAChRs are pentameric structures composed of various combinations of α- and β-subunits organized around a central pore and are permeable to Na⁺ and K⁺ and Ca²⁺ (Changeux et al. 1998; Mulle et al. 1992; Rogers et al. 1997). To date, twelve subunits, each encoded by a separate gene (Gotti and Clementi 2004; Paterson and Nordberg 2000) have been identified in the mammalian nervous system and divided into two subfamilies of receptor subunits: the α-subunits (α2-α7, α9, α10) and the β-subunits (β2-β4) (Lindstrom 2000; McGehee and Role 1995; Role and Berg 1996). The α-subunits have been demonstrated to hold two adjacent cysteine residues which are proposed to form the ligand binding site (Kao and Karlin 1986) whereas the β-subunits lack these two cysteines and are suggested to be structural subunits. Studies with Xenopus oocytes have shown that both α- and β-subunits contribute to the pharmacological specificity of nAChR subtypes (Corringer et al. 2000; Luetje and Patrick 1991). Based on radioligand binding studies using the curaremimetic neurotoxin, 125I-α bungarotoxin, and ³H-nicotine, respectively, nAChRs have been divided into two main classes: one that binds α-bungarotoxin with high affinity and
nicotine with low affinity and another that binds nicotine with high affinity and is insensitive to α-bungarotoxin. These two classes of receptors are sometimes also referred to as low and high affinity nAChRs, respectively (Lukas 1984; Lukas and Bencherif 1992; Wonnacott 1986). The low affinity nAChRs can be homomeric (comprised of α7-α9 subunits) or heteromeric (made up of α7, α8 or α9, α10 subunits) while the high affinity nAChRs appear to form heteromers from a combination of α2-α6 and β2-β4 subunits (Gotti and Clementi 2004). The large number of nAChR subunits and the fact that these subunits can form multiple combinations suggest that there is a large variation in nAChR subtypes in the CNS. This notion is also supported by autoradiographic observations and various in situ hybridization studies which have revealed that nAChRs are widely and differentially distributed in the CNS and exist on various cell populations (Clarke and Pert 1985; Clarke et al. 1984; Deneris et al. 1989; Wada et al. 1989; Wilson and Karlin 2001). Also, nAChRs can be located both pre- and postsynaptically (McGehee et al. 1995; McGehee and Role 1995; Wonnacott 1997).

The two most abundant neuronal nAChRs subtypes in the CNS are the high affinity binding α4β2-subtype (Flores et al. 1992; Whiting et al. 1987) and the low affinity binding and highly Ca²⁺-permeable α7-subtype (Couturier et al. 1990; Seguela et al. 1993). It is suggested that nicotine preferentially acts via the highly Ca²⁺ permeable α7-subtype which presynaptically facilitates the release of several neurotransmitters including GABA, acetylcholine, DA, noradrenaline, and glutamate (McGehee et al. 1995; Wonnacott 1997). However, at drug concentrations relevant to smoking, nicotine activates DA neurons in the VTA mainly by stimulating β2 containing nAChRs (Picciotto et al. 1998; Pidoplichko et al. 1997).
Figure 2. Top: A schematic view of the nAChR and its diverse ligand binding sites. Left: Two nAChR subtypes. Filled circles represent agonist binding sites.
1.4 The Mesocorticolimbic DA System

In the late 1950s, to a large extent through the important work of Carlsson and co-workers, DA was demonstrated to act as an independent neurotransmitter in the brain (Carlsson 1959; Carlsson et al. 1957; Carlsson et al. 1958). Today, DA is known to have many different and complex actions in the CNS (Seamans and Yang 2004).

With the introduction of histochemical methods (formaldehyde- or glyoxylic acid-induced fluorescence), it was possible to characterize and map the anatomical connections for DA-containing neurons in the rat brain (Björklund and Lindvall 1984; Dahlström and Fuxe 1964; Falck et al. 1962).

There are three major dopaminergic pathways innervating the forebrain and the basal ganglia. The nigrostriatal DA system originates in the substantia nigra (SN) and projects to the caudate nucleus and putamen i.e. the dorsal part of striatum (Anden et al. 1964). This pathway is considered important for movement control. The mesolimbic DA pathway originates in the ventral tegmental area (VTA) and projects via the medial forebrain bundle to the amygdaloid complex, the nucleus accumbens (NAcc) shell and core, the olfactory tubercle and septum. The shell subregion of the NAcc is associated with limbic structures such as the amygdala whereas the core subregion is connected with motor structures such as the striatum (Pierce and Kalivas 1997). The mesolimbic DA system is implicated in emotions and reward (Wise 2002). The third DA system, the mesocortical DA pathway, also originates in the VTA but projects to cortical structures i.e. the prelimbic, infralimbic, and cortex cinguli (Anden et al. 1966; Ungerstedt 1971). The mesocortical DA pathway regulates higher motor execution of behavior, motivation and cognition (Seamans and Yang 2004). The mesolimbic and mesocortical DA systems are collectively termed the mesocorticolimbic DA system.

DA acts by stimulating DA receptors that are divided into two subgroups: the D1-like receptors (D1 and D5) and the D2-like receptors (D2, D3, D4). The D1-like receptors are coupled to $G_s$ complex and thus stimulate the formation of the second messenger cyclic adenosine 3′5′-monophosphate (cAMP). In contrast, the D2-like receptors interact with the $G_{i/o}$ complex and thus decrease the levels of cAMP (Missale et al. 1998). In rat brain, D1 and D2 receptors exhibit similar patterns of distribution. Their mRNA is expressed in the neostriatum, olfactory tubercle and NAcc
(Cortes et al. 1989; Meador-Woodruff et al. 1989). D1-like receptors are mainly located postsynaptically (Caille et al. 1996) whereas D2-like receptors in addition to postsynaptic location, also are located presynaptically where they act as autoreceptors (Carlsson 1977).

The DA neurons in the midbrain display two different firing modes in vivo: single spike firing and burst firing (Grace and Bunney 1984a; b). Burst firing is expressed as a major phasic response and single spike firing represents tonic activity burst (Gonon 1988; Gonon and Buda 1985). The firing activity of midbrain DA neurons is modulated by autoinhibitory mechanisms, mainly through presynaptic D2 receptors, and by afferent input from several neurotransmitter systems including GABA, excitatory amino acids, acetylcholine, noradrenaline, serotonin and neuropeptides (Adell and Artigas 2004).

The midbrain DA systems are involved in the functional regulation of many important basic functions such as movement control, natural reinforcement, emotions, cognition and stress (Tzschentke 2001). For instance, deficient DA signaling in the nigrostriatal system causes Parkinson’s disease, one of the most common movement disorders (Birkmayer and Hornykiewicz 1961; Carlsson 1959; Hornykiewicz 1973). Another disorder suggested to arise from dysfunctional DA signaling in the mesocorticolimbic DA system is schizophrenia (Weinberger 1987). However, altered DA signaling could not explain all the symptoms observed in schizophrenia and other mediators such as glutamate and serotonin have also been suggested to be involved (Lewis and Lieberman 2000).

Mesocorticolimbic DA signaling is suggested to play an important role in natural reward. For instance, administration of a DA receptor antagonist attenuates lever pressing and running for food (Wise et al. 1978). In conditioning and operant responding paradigms, it seems that the increase in DA release in the NAcc is associated with preparatory rather than consummatory feeding behavior (Blackburn et al. 1989). Moreover, feeding and access to water in rats deprived of water, increases DA levels in the NAcc (Bassareo and Di Chiara 1997; 1999; Westerink et al. 1997; Yoshida et al. 1992). Thus, it seems that DA neurons in the mesocorticolimbic DA system do not only respond to actual consumption of food, water or exposure to reward but also to the anticipation of reward (Schultz 1998; 2004). Hence, mesocorticolimbic DA transmission appears to be activated in association with activities that serve to promote the survival of an individual or
species. Interestingly, the perception of reward and thus the mesocorticolimbic DA system (other structures like ventral pallidum, amygdala, and mediodorsal thalamus however are also involved) coincides remarkably well with behavioral activation (Kalivas and Nakamura 1999). This is probably a consequence of the fact that the perception of or encounter with reward involves the initiation of an appropriate behavioral response to obtain or investigate a rewarding stimulus. Interestingly, the NAcc, VTA, and prefrontal cortex (PFC) appear to have different functional roles in natural reward. Dopaminergic inputs to the VTA appear to provide a prediction error signal that cues novel aspects of reward. Consequently, DA plays an important role when the stimulus is either novel or does not match previous experience. The PFC functions to integrate very recent experiences with the rewarding stimulus to help to shape the appropriate behavioral response. The NAcc, on the other hand, is suggested to function as an integrating site or as a gating site, which, depending on degree of depolarization, regulates the excitatory input thereby amplifying strong excitatory signals and dampening less potent ones (Kalivas and Nakamura 1999).

Substantial evidence suggests that the reinforcing and dependence-producing properties of drugs of abuse result from enhanced DA release in the mesocorticolimbic DA system (Imperato et al. 1986; Koob 2000; Self and Nestler 1998). For instance, drugs of abuse are all readily self-administered by rats (Deneau et al. 1969; Smith and Davis 1974; Wilson et al. 1971; Yokel and Pickens 1973) and lesions of dopaminergic terminals in the NAcc reduce or abolish self-administration (Kelly and Iversen 1976; Lyness et al. 1979; Roberts et al. 1977; Singer et al. 1982) although see (Dworkin et al. 1988). In addition, in vivo microdialysis studies have shown that ethanol, cocaine, amphetamine, morphine, and nicotine increase the levels of DA in the NAcc and PFC (Di Chiara and Imperato 1986; Imperato et al. 1986; Kuhar et al. 1991; Nisell et al. 1996; Yoshimoto et al. 1992). Drugs of abuse have also been shown to stimulate LMA in rats and mice, which has been ascribed to increased DA release in the mesocorticolimbic DA system. Evidence to support such a correlation comes from studies where lesions in the DA terminals in NAcc attenuate or abolish drug-induced LMA (Babbini and Davis 1972; Kelly and Iversen 1976; Morrison and Stephenson 1972; Weissman and Koe 1965). Consequently based on these studies, it has been postulated that the locomotor stimulant and euphoriant properties of drugs of abuse might be associated with enhanced dopaminergic neurotransmission in the mesocorticolimbic DA system (Wise and Bozarth 1987).
Figure 3. Schematic presentation of glutamatergic and dopaminergic projections within the mesocorticolimbic DA system.
1.5 Effects of Nicotine on Dopaminergic and Glutamatergic Transmission in the Mesocorticolimbic DA System

1.5.1 Acute effects

Numerous biochemical, behavioral and electrophysiological findings, suggest that, in analogy with other drugs of abuse, the reinforcing and rewarding properties of nicotine may be associated with an activation of the mesocorticolimbic DA system. Thus, acute nicotine administration enhances DA release in the NAcc (Imperato et al. 1986) and PFC (Nisell et al. 1996). Furthermore, nicotine is readily self-administered in rats (Corrigall and Coen 1989; Donny et al. 1995) while lesions of the mesolimbic DA system (Corrigall et al. 1992) or administration of D2 antagonists (Corrigall and Coen 1991) reduces nicotine self-administration.

The VTA appears to be an important site for mediating and modulating the reinforcing effects of nicotine. Systemically administered nicotine increases burst firing in the VTA DA neurons (Grenhoff et al. 1986). In addition, blocking nAChRs by local infusion of mecamylamine in the VTA but not NAcc inhibits systemic nicotine-induced DA release in the NAcc (Nisell et al. 1994). Interestingly, microdialysis studies show that systemic administration of nicotine results in a more pronounced and longer-lasting effect of DA release in the NAcc (Di Chiara 2000). This effect is suggested to arise from the involvement of two different nAChR subtypes in the VTA. Accordingly, nicotine directly activates the DA neurons via $\alpha_4\beta_2$ containing nAChRs distributed on the cell surface of DA neurons, which results in depolarization and removal of $\text{Mg}^{2+}$ from postsynaptic NMDA receptors also located on DA neurons. Due to the fact that $\alpha_4\beta_2$-containing nAChRs desensitize rapidly, much of the direct nicotine stimulation on the DA neurons ceases. At the same time, nicotine enhances glutamate release mainly via $\alpha_7$ nAChRs on glutamatergic afferents onto DA neurons. The enhanced release of glutamate activates postsynaptic NMDA receptors which continue to drive the DA neurons. Presynaptic $\alpha_7$ nAChRs are much less susceptible than the somal $\alpha_4\beta_2$ to desensitization to low doses of nicotine. Consequently, activation of presynaptic nAChRs via the $\alpha_7$-subtype continues to enhance glutamate release onto DA neurons, which could explain the finding that DA is elevated in the NAcc for hours after nicotine injection. Thus, nicotine is suggested
to mediate its reinforcing effects in the VTA not only through both α7 and α4β2 subtype receptors but also via the NMDA receptors (Dani et al. 2001; Picciotto et al. 1998; Rahman et al. 2003; Schilstrom et al. 2000).

The mesocorticolimbic DA system is connected with glutamatergic afferent projections in a complex manner. Both the VTA and the terminal region in the NAcc receive glutamatergic input from several corticolimibic structures such as the amygdala, PFC and hippocampus (Carr et al. 1999; Carr and Sesack 1999; Christie et al. 1987; Conde et al. 1995; Gorelova and Yang 1997; Kelley and Domesick 1982). At the level of the VTA, glutamatergic input increases the activity of dopaminergic cells and augments DA release in the NAcc (Westerink et al. 1992). Glutamate, although to a lesser extent, also facilitates DA release at the level of NAcc (Youngren et al. 1993). Thus, glutamate neurotransmission appears to perhaps directly influence the mesocorticolimbic DA system.

In addition to enhancing dopaminergic activity, several studies have provided evidence that acute nicotine stimulates the release of glutamate in the striatum (Toth et al. 1993), in the PFC (Lambe et al. 2003), in the NAcc (Fu et al. 2000; Reid et al. 2000), and in the VTA (Fu et al. 2000; Schilstrom et al. 2000). In addition, intrategmental infusion of the competitive NMDA receptor antagonists 2-amino-5-phosphopentanoic acid (AP-5) or cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755) significantly reduces nicotine-induced DA release in the NAcc. However, neither intra-tegmental infusion of CNQX or GYKI52466 nor intra-accumbal infusion of CNQX alters nicotine-induced DA release in the NAcc (Fu et al. 2000; Schilstrom et al. 1998; Sziraki et al. 2002). Consequently, it seems that nicotine-induced DA release is regulated by glutamate and that the NMDA but not AMPA glutamate receptor subtype is involved in the acute effects of nicotine.

Acute nicotine administration stimulates LMA in rats although higher doses can produce an initial transient decrease (Clarke and Kumar 1983b). The stimulatory effect of nicotine can be abolished by lesions of either the NAcc (Clarke et al. 1988) or the VTA (Louis and Clarke 1998). In addition, nicotine-induced locomotor stimulation is blocked by selective D1 and D2 antagonists (O’Neill et al. 1991) which suggests that the locomotor stimulatory effect of nicotine is mediated by the mesocorticolimbic DA system. Several studies indicate that the locomotor stimulating effects of nicotine are preferentially mediated via the α4β2-ubtype in the VTA.
1.5.2 Chronic effects

Acute as well as chronic nicotine administration to rodents, in doses relevant to smoking, causes rapid desensitization of the nAChRs (Marks et al. 1983; Pidoplichko et al. 1997). Chronic nicotine exposure in rats, however, results in upregulation of nAChRs (Buisson and Bertrand 2002). A similar effect is seen in postmortem human brains of tobacco users (Benwell et al. 1988; Fenster et al. 1999; Marks et al. 1983). The upregulation of nAChRs is opposite of what is observed in other receptor systems, which tend to become downregulated (Flugge 2000). It is hypothesized that chronic desensitization (i.e. inactivation of the receptor) results in a compensatory up-regulation of nAChRs. Recent studies also show that the subunit composition of nAChRs changes upon chronic nicotine treatment, which perhaps is a consequence of a compensatory mechanism in response to changed homeostasis (Lai et al. 2005; Nguyen et al. 2003).

Chronic systemic nicotine administration causes sensitization or tolerance. Sensitization is defined as increased effect of a drug upon repeated administration of the same dose whereas tolerance is defined as a gradual decrease in effect of a certain drug dose (Clarke and Kumar 1983a; b; Hakan and Ksir 1988; Marks et al. 1983; Robinson and Berridge 2000). For instance, a sensitized response in DA release (i.e. an increased release of DA) is evident in both the medial PFC and in the NAcc (Benwell and Balfour 1992; Reid et al. 1998; Shoaib et al. 1994) although some studies have failed to observe such an effect (Nisell et al. 1996). The effect of chronic nicotine on glutamate release has, to our knowledge not yet been described. However, MK-801 and the competitive NMDA receptor antagonist CPP prevent the development of sensitization to nicotine-induced DA release in the NAcc (Shoaib et al. 1994).
1.5.2.1 Behavioral sensitization

Behavioral sensitization manifests itself as a progressive and enduring enhancement in the motor stimulant effects of nicotine following intermittent chronic administration of nicotine (Nisell et al. 1996; Reid et al. 1998). Thus, the behavioral effect of the drug will be enhanced in animals that have been exposed to the drug earlier compared to those that have not. By inference, changes in the brain must have taken place that make the animals more sensitive to the drug following repeated exposure. Sensitization is shown to persist for months in rats (Robinson and Berridge 1993; 2001). This suggests that sensitization may represent a behavior that is equivalent or related to the addictive process. Therefore, behavioral sensitization in animals has been extensively used as a model to study changes in the brain which may be responsible for the development and maintenance of drug addiction and for relapse into drug seeking behavior (Robinson and Berridge 2000; 2001).

Behavioral sensitization is divided into two phases: induction and expression (De Vries et al. 1998; Robinson and Becker 1986). These two phases are believed to represent acquisition and maintenance of drug addiction, respectively. The VTA and NAcc are thought to be critically involved in the induction and expression of behavioral sensitization, respectively (Robinson and Berridge 2000). The induction is suggested to be mediated primarily at the level of the VTA whereas expression is thought to be mediated at the level of NAcc (Pierce and Kalivas 1997; Vanderschuren and Kalivas 2000; Vezina et al. 1987). Both D1 receptors as well as AMPA and NMDA receptors in the VTA have been implicated in the induction of behavioral sensitization (Kalivas and Alesdatter 1993; Pierce et al. 1996; Vezina 1996; Vezina and Stewart 1989). In addition, some of the earliest cellular alterations, such as somatodendritic subsensitivity of DA autoreceptors and consequently increased neuronal DA activity, occur in the VTA (Ackerman and White 1990). In the NAcc, sensitization-related cellular changes are expressed as functional supersensitivity of the postsynaptic D1 receptor, which is suggested to correspond to the persistence of behavioral sensitization (Henry and White 1991; 1995). Moreover, D1 receptor sensitivity is changed in the mPFC in sensitized animals and lesions in mPFC prevent induction of sensitization (Li et al. 1999; Sorg et al. 2001).

There are two distinguishable forms of sensitization termed context-dependent and context-independent sensitization. Context-dependent sensitization has been
suggested to result in a more robust response in LMA or NAcc DA release (Benwell and Balfour 1992; Reid et al. 1998). Accordingly, a group of animals that have received drug injection in a specific environment and that are then given a challenge injection in that same environment usually express sensitization. In contrast, animals that have been administered drug injections in environment different from that in which a challenge injection is given tend to show weak or no behavioral sensitization (Anagnostaras et al. 2002). On the other hand, existing data claims that a sensitized response will eventually develop irrespective of context if the pretreatment protocol is extended to at least twelve days of treatment (Nisell et al. 1996; Reid et al. 1998; Vezina et al. 1992). However, the context still induces a stronger and more robust sensitized response (Reid et al. 1998). The mechanisms underlying these two forms of sensitization are still largely unknown. There are indications, however, that the changes in the CNS that occur in the process of context-dependent sensitization develop in different brain regions compared to context-independent sensitization (Badiani et al. 1999; Delamater 2004; Ferguson et al. 2003; Johnson et al. 2000).

Recent studies propose an alternative explanation of behavioral sensitization which states that only one form of sensitization exists and that this form is considered to be a non-associative form of neuroplasticity manifested behaviorally as an increase in an unconditional drug effect (Anagnostaras and Robinson 1996; Anagnostaras et al. 2002; Vanderschuren and Kalivas 2000). Depending on treatment conditions, behavioral sensitization may or may not be modulated by associative learning. Thus, animals tested in an environment different from the one in which they received drug treatments (unpaired animals) were found to develop neural sensitization (Castaneda et al. 1988; Henry and White 1991; Nestby et al. 1997; Robinson and Becker 1982), but not behavioral sensitization (Robinson and Berridge 2001). Specifically, the neural circuits underlying the development of behavioral sensitization appear to have been engaged although these neuroadaptations were not expressed in behavior (Anagnostaras et al. 2002). Consequently, rather than the environmental context markedly potentiating behavioral sensitization in a familiar environment, it may instead block behavioral sensitization in a novel environment. In other words, the absence of a familiar context might suppress the expression of sensitization. Obviously, much more work is needed to unravel the mechanisms underlying the various forms of behavioral sensitization.
As noted, glutamatergic transmission seems to play a significant role in induction and expression of behavioral sensitization (Domino 2001; Kelsey et al. 2002; Shoaib et al. 1994). Accordingly, both AMPA and NMDA receptors antagonists prevent the induction of behavioral sensitization (Karler et al. 1994; Karler et al. 1989; Shoaib et al. 1994; Shoaib et al. 1997). However, most studies elucidating the mechanisms involved in the development of behavioral sensitization have focused on the psychostimulants cocaine and amphetamine and only a few have investigated the role of glutamate receptors in nicotine-induced behavioral sensitization. For instance, pretreatment with MK-801 or CPP attenuates sensitization to the locomotor stimulant effect of nicotine (Shoaib et al. 1997). On the other hand, MK-801 and CPP given prior to an acute dose of nicotine increase LMA (Shoaib et al. 1994).
2. AIMS OF THE STUDY

- To investigate the effect of the novel AMPA receptor antagonist ZK200775 on acute nicotine-induced LMA and DA release in the NAcc
- To examine the effects of the NR2B subunit-selective NMDA receptor antagonist Ro 25-6981 on acute nicotine-induced LMA and DA release in the NAcc
- To investigate the effect of chronic nicotine administration on the development of behavioral sensitization and nicotine-conditioned LMA
- To study the effects of the NR2B subunit-selective NMDA receptor antagonist Ro 25-6981 on nicotine-induced LMA and DA release in the NAcc and mPFC in rats chronically treated with nicotine
- To explore the effects of chronic nicotine exposure on the expression of NR2B and NR2A subunits in the PFC and VStr
3. MATERIALS AND METHODS

3.1 Animals

Male Wistar rats (Scanbur BK AB, Sollentuna, Sweden) weighing between 250 and 350 g were used in all experiments. Upon arrival to the animal facility, rats were housed in groups of four in a temperature- (22°C) and humidity- (50%) controlled environment on a 12-h light/dark cycle (lights on at 7 am) and given free access to standard rat chow and water. Before the start of the experiments the rats were allowed to adapt to the novel environment for at least 7 days.

All experiments were performed in compliance with the animal care guidelines approved by the local Ethical Committee (Norra Stockholms Djurförsöksetiska Nämnd) with the permit numbers N48/98, N57/98, N311/00, N155/03, N314/00, and N103/04.

3.2 Drugs

(-)-Nicotine hydrogen tartrate salt (Sigma-Aldrich St.Louis, MO, USA) was dissolved in saline (0.9%), pH-adjusted to 7.2 ± 0.2 with 1M NaOH and administered subcutaneously (s.c.) in all experiments. MK-801 (hydrogen maleate form) (Sigma-Aldrich, St.Louis, MO, USA) was dissolved in distilled water and administered intraperitoneally (i.p.) ZK200775 (a generous gift from Schering AG Gmbh, Berlin, Germany), NBQX disodium salt, CGP39551, and Ro 25-6981 (Tocris Cookson Ltd, MO, USA) were dissolved in saline and administered i.p. Sodium pentobarbital, ketamine (Ketalan®), xylazine chloride (Rompun®), and bupivacaine chloride (Marcain®) were purchased from Apoteket AB, Stockholm, Sweden.
3.3 Locomotor Activity

3.3.1 Acute nicotine administration

LMA was measured using four AccuScan activity meters (42x42x30 cm) (AccuScan Instruments Inc, Columbus, OH, USA) equipped with three rows of infrared photo sensors. Each row consisted of 16 sensors, 2.5 cm apart where two rows were placed around the bottom of the activity boxes and the third row was placed 10 cm above the floor to measure vertical activity. The lighting was dim with one dim light source above each activity box. All measurements were conducted (according to a between or within subject experimental design) between 8 am and 5 pm. Each time a photo beam was crossed, it was recorded as one activity count. The animals were habituated to the LMA boxes for two days before any drug treatment commenced. On the first day of habituation, the rats were allowed to freely explore the LMA boxes for one hour. The second day of habituation was designed to familiarize the rats to the injection and to simulate the test situation. Thus, the rats were allowed to freely explore the activity boxes for 30 min and were then given an injection of saline (1 ml/kg, s.c.). Following the saline injection, the rats were returned to the activity boxes and allowed an additional period of 60 min for free exploration (Papers I and II). If two compounds were to be administered, two saline injections either thirty or ten min apart were given. On the third day, rats were placed in the activity boxes and given 30 min to habituate to the activity boxes and were then administered either saline, ZK200775, MK-801, CGP39551 or Ro 25-6981. Thirty min (Paper I) or ten min (Paper II) later, either saline or nicotine was administered and LMA was recorded for 60 min. Behavior was recorded with digital video cameras (Panasonic, NV-DS27EG) set up in front of each activity cage. The behavior of the rats was rated once every 5 min for 30 min using a 9-point scale developed by (Ellinwood and Balster 1974) Scores ranging from 1 to 4 define normal activity behavior from asleep, score 1, to running around, sniffing and rearing, score 4. Stereotypy scores ranging from 5 to 9 define increased severity of stereotypic behavior where score 5 represents hyperactive movements with jerky moves and score 9 is characterized by seizures, abnormally maintained postures and dyskinesias.
3.3.2 Chronic nicotine administration

Paper III: As described above, all rats were habituated to the LMA boxes for two days before any drug treatment commenced. After two days of habituation, the rats were placed in the activity boxes and thirty min later administered either saline (1 ml/kg, s.c.) or nicotine (0.6 mg/kg, s.c.) after which LMA was recorded for 60 min (as described in section 3.3.1). To avoid any diurnal variation, the animals from different cages were run on an alternating schedule. Thus, the cages were numbered, cage 1 to 4, and the first day measurements started with cage number 1 followed by number 2, 3, and 4. The second day the measurements started with cage number 2 followed by cages number 3, 4, and so forth throughout the 21-day regimen. Between every run, throughout the 21-day regimen, the Plexiglas boxes were rinsed with water and 10 % v/v ethanol solution and wiped clean with paper towels. Thus, for each rat the effects of repeated administration of nicotine on nicotine-conditioned locomotor stimulation and on nicotine-stimulated LMA could be monitored. Nicotine-conditioned locomotor stimulation is the increase in LMA observed compared to saline treated animals during the 30 min prior to drug administration whereas behavioral sensitization to nicotine is the progressive increase in locomotor activity seen after intermittent administration of nicotine.

Paper IV: All rats were habituated to the LMA boxes for two days before drug treatment (as described in section 3.3.1). On the third day, rats were placed in the activity boxes and given 30 min to habituate to the experimental environment before they were injected with saline followed 10 min later by administration of either saline or nicotine (0.6 mg/kg, s.c.). Using this paradigm, the effects of nicotine on LMA in naïve rats could be measured and the rats were familiarized with the activity boxes under the influence of nicotine. Thereafter, rats were administered nicotine (0.6 mg/kg, s.c.) once a day for 12 consecutive days, in their home cages. On day 13, rats were again placed in the activity boxes for 30 min and injected with saline or Ro 25-6981 (1.0, 3.0, and 10 mg/kg, i.p.) followed 10 min later with either saline or nicotine (0.05, 0.1, and 0.6 mg/kg, s.c.) and LMA was recorded for 60 min. The gross behavior of the animals was also recorded as described above.
3.4 Microdialysis

Rats were anaesthetized with sodium pentobarbital (Paper I) or with a mixture of ketamine and xylazine chloride (Papers II and IV) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Guide cannulas for the probe (CMA 12, CMA Microdialysis, Stockholm, Sweden) were implanted into the NAcc [AP: 1.6, ML: 1.2 and DV: -8.0 (Papers I and II)], alternatively in NAcc [AP: 1.6, ML: 1.3, and DV: -8.0] or mPFC [AP: 3.0, ML: 0.6, and DV: -3.0 (Paper IV)] according to the brain atlas of Paxinos and Watson (Paxinos and Watson 1986) and anchored to the skull with stainless steel screws and dental cement. After surgery, bupivacaine chloride was applied on the wound to induce post surgical analgesia. The animals were housed individually in single housing cages for 48 or 72 days before the start of microdialysis, which was conducted in awake freely moving rats. In the morning of the experimental day the animals were transferred to an separate room and microdialysis probes (CMA12/2 mm, CMA Microdialysis, Stockholm, Sweden) were inserted and connected to a perfusion line via a two-channel liquid swivel (AgnTho’s AB, Sweden) where the perfusion solution (artificial cerebrospinal fluid containing 147 mM NaCl, 3.0 mM KCl, 1.3 mM CaCl$_2$, 1.0 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$, and 0.2 mM NaH$_2$PO$_4$) was perfused via a Univentor 801 syringe pump (AgnTho’s AB, Sweden) and collected in a refrigerated Univentor 820 microsampler (AgnTho’s AB, Sweden) at a flow rate of 1 µl/min. Following the probe insertion a 2-hour wash out period preceded the sampling of a total of 17 samples where the first six samples served as baseline samples. Thirty min following the first injection of saline or either of the glutamate subtype receptor antagonists, the rats were administered nicotine (Paper I). In Papers II and IV, the perfusion solution was collected at a flow rate of 2 µl/min and glutamate receptor antagonist and nicotine were administered 10 min apart. The sample vials were prefilled with 10 µl of 0.3 mM perchloric acid and the temperature of the microsampler was constantly held at 8°C. Samples were collected every 20 min (Paper I) or every 10 min (Papers II and IV) and were immediately frozen at -80 °C.

At the end of each experiment, the animals were given a sub-anaesthetic dose of sodium pentobarbital, decapitated and the brains were removed and immediately frozen in dry-ice chilled acetone. Alternatively, rats were anaesthetized with sodium pentobarbital and intracardially perfused with phosphate buffered saline and 4%
parafomaldehyde, respectively. Brains were removed and stored in 30% PBS/sucrose solution. Probe position was verified histologically with sectioning of the brains performed in a cryostat (Zeiss 500, Oberkochen, Germany) with 20 or 25 µm thick coronal slices mounted on slides followed by staining with thionin. Only data obtained from animals with probes correctly placed within the NAcc or mPFC were used in the studies. A probe was considered correctly located when it transverses the mediodorsal core and ventral shell (Papers I, II) or the NAcc core subregion or the medial part of the PFC (Paper IV).

3.4.1 Analysis of dialysate

The concentration of DA was analyzed with reverse phase HPLC systems (ESA Inc., Chelmsford, MA, USA) with electrochemical detection using a Coulochem II detector (5200A) with a conditioning cell (5021) and an analytical cell (5011) where one of the systems only had the analytical cell. The mobile phase (Na-acetate; 7.465 mg/l, Na$_2$EDTA; 3.7 mg/l, octanesulfonic acid monohydrate; 140.79 mg/l, and HPLC-graded methanol; 110 ml/l and pH adjusted to 4.1 with concentrated acetic acid) was delivered by an HPLC-pump (Model 582, ESA Inc., Chelmsford, MA, USA) through a C18-AQ column (Reprosil-Pur, 150 x 4 mm, 5µ) at a flow rate of 1 ml/min. Samples (25 µl) were automatically injected by an autosampler (Model 830, Midas, Spark Holland, The Netherlands). The potentials were set as follows; HPLC-system 1: conditioning cell: +175 mV, analytical cell R2 +400 mV and HPLC-system 2: analytical cell R1 +75mV, R2 +350 mV. Alternatively, when analyzing samples from the mPFC the potentials were set as follows; HPLC-system 1: conditioning cell: +175 mV, analytical cell R2 +400 mV and HPLC-system 2: analytical cell R1 +75mV, R2 +450 mV. The microdialysis samples were randomly assigned to one of the two HPLC systems; all samples from a given subject were analyzed with the same system. Chromatographic analysis was performed using CSW 1.7 software (DataApex Ltd, Czech Republic).
3.5 Primary Cultures of Cerebellar Granule Cells

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat (Scanbur BK, Sollentuna, Sweden) cerebella as previously described (Cebers et al. 1996). Briefly, after dissection, 8 cerebella were pooled and sliced with a McIlwain tissue chopper in two orthogonal directions (slices were 0.3 mm thick), incubated in a 0.025% trypsin solution, and dispersed by trituration in a DNase and soybean trypsin inhibitor containing solution (0.01% and 0.05%, respectively). Cells were plated (2 x 10⁶ cells/2ml/dish) onto 6-well plates coated with 5 µg/ml of poly–L-lysine (MW=30,000-70,000). Cells were cultured for 8 days at 37°C in an atmosphere of 5% CO₂/95% air in Basal Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 µg/ml gentamicin. Cytosine-β-arabinofuranoside (10 µM) was added 24 h after plating to limit the number of non-neuronal cells. The medium was not changed until the cultures were used in the experiment.

3.5.1 Drug treatment

NMDA and AMPA receptor-mediated neurotoxicity, and its modulation by ZK200775, was examined by applying the relevant drug concentrations dissolved in Mg²⁺-free Locke’s buffer containing 154 mM NaCl, 5.6 mM KCL, 2.3 mM CaCl₂, 3.6 mM NaHCO₃, 5.5 mM D-Glucose, and 5 mM HEPES (pH 7.4). To begin the experiment on DIV 8, the medium was collected from cerebellar granule cells, after which they were washed once with pre-warmed Mg²⁺-free Locke’s buffer to remove traces of the growth medium before the drug-containing Mg²⁺-free Locke’s buffer was added. ZK200775 was added at concentrations of 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 100, and 300 µM). The collected medium was filter-sterilized and stored until needed. After 2 h incubation at 37°C, the buffer was removed; cells were washed with pre-warmed drug-free Locke’s buffer containing 1 mM Mg2+ and returned to the original culture medium collected previously. Cell viability was assessed 24 h later.
3.5.2 Assessment of cell viability

The MTT assay was used to assess the viability of cerebellar granule cells in culture. Earlier it was widely assumed that mitochondrial dehydrogenases in living cells convert soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble blue formazan product that can be dissolved in isopropanol and the colour intensity measured spectrophotometrically (Mossman 1983). In this way, the MTT assay would assess the integrity of mitochondria characteristic of viable cells. However, later findings suggest that MTT is taken into cells through endocytosis and reduced primarily in the endosome/lysosome compartment instead of the mitochondria (Liu et al. 1997). Nevertheless, the MTT assay, as a measure of cell viability, is still valid because it measures endocytosis, a fundamental feature of most living cells. The MTT assay was performed as described previously (Cebers et al. 1996). The assay was initiated by removing the culture medium and adding MTT (0.3 mg/ml) dissolved in serum-free culture medium. Following 1 h incubation at 37°C, the medium was aspirated and 0.5 ml of isopropanol added to lyse the cells and to dissolve the formazan crystals. Aliquots (100 µl) of this solution were pipetted into 96-well microplates and absorbency was recorded at 570 nm using a microplate reader. Cell viability was expressed as percentage of the absorption in control cells (100%).

3.6 Nicotine Receptor Binding Assay

Rats were sacrificed by decapitation, the brains removed and the cerebral cortex was dissected out on an ice-cold glass plate and stored at -80°C until used. A total of 8 (Papers I and IV) or 9 (Paper III) cortices were pooled to acquire enough tissue for the binding assays. The tissue was homogenized in an ice-cold hypotonic buffer solution (0.1 x HEPES buffer: 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 0.02% w/v sodium azide) using a Polytron homogenizer (10 seconds at setting 5) (Kinematica, Switzerland). The crude particulate fraction was obtained by centrifugation at 15 000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was washed twice more by resuspension in ice-cold homogenization buffer.
using a glass-Teflon tissue grinder and centrifugation. The final pellet was stored at -80°C until use.

The pellet was resuspended in HEPES buffer (pH 7.4) and the protein concentration was measured using the Bio-Rad Protein KIT (Bio-RAD Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. Aliquots of tissue homogenates (≈0.2 mg of protein) were incubated in polypropylene test tubes in 50 mM Tris-HEPES buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, pH 7.4) containing 100 pM [³H]Epibatidine [(56.2 Ci/mmol), Perkin-Elmer Life Sciences Inc. Boston, MA, USA] and increasing concentrations of Ro 25-6981 or nicotine (1 nM to 100 µM) in final volume of 5 ml to avoid ligand depletion. The concentration of [³H]Epibatidine was chosen based on a previously determined saturation binding curve which had a Kₐ of 10.4 ± 1.4 pM (data not shown). The binding assay was run in triplicates and the reaction was started by the addition of tissue. The mixture was incubated for two hours in the dark at room temperature. Incubation was terminated by vacuum filtration through Whatman GF/C filter (presoaked in binding buffer containing 0.5% polyethyleneimine) using a Brandel cell harvester (Gaithersburg, MD, USA). The filter was rapidly washed four times with 4 ml aliquots of cold 50 mM Tris-HCl buffer (pH 7.4). Subsequently, filters were placed in scintillation vials with 5 ml Ready Safe liquid scintillation cocktail (Beckman Fullerton, Coulter, CA, USA) and counted in a β-counter (Wallac, Finland) at 45% counting efficiency. The IC₅₀-value for nicotine was calculated from the curve in the graph, which was fitted by nonlinear regression analysis (GraphPad Prism, GraphPad Software, San Diego, CA, USA).

3.7 Real Time Reverse Transcriptase Polymerase Chain Reaction

NMDA receptor subunit NR2A and NR2B mRNA expression in the PFC and ventral part of the striatum of saline and nicotine treated rats was analyzed using the LightCycler Instrument (Roche Biochemicals, Idaho Falls, ID, USA) as described previously (Kovacs et al. 2002).
3.7.1 Isolation of total RNA and first strand cDNA synthesis

The brains from rats chronically treated with either saline or nicotine (0.6 mg/kg) were removed, cut in two halves along the longitudinal fissure, put in RNAlater solution (Ambion Inc., Austin, TX, USA) and stored in -20 °C freezer. Total RNA was extracted from the PFC and VStr using TRIzol® Reagent (Invitrogen™ Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. To remove residual genomic DNA samples the samples were treated with DNase I (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. The concentration of RNA was measured spectrophotometrically at 260 nm.

2 µg of total RNA from each sample were reverse transcribed for 60 min at 37°C using a random hexamer primer (pd (N)6; Pharmacia Biotech, Uppsala, Sweden) and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WT, USA) in a 25 µl reaction volume in the presence of RNase inhibitor (Promega, Madison, WT, USA) and an equimolar (2.5 mM) mixture of nucleotide triphosphates (NTPs). Resulting cDNA samples were brought to 60 µl with DEPC-treated water.

3.7.2 Real time RT-PCR with NR2A and NR2B specific primers

One of the saline-treated rats PFC and one of the saline-treated rat VStr cDNA preparations were serially diluted (1:1, 1:4, 1:16) and used to generate the assay standard curves for the respective areas. For the PCR reaction, 2 µl of the standard cDNA dilutions (1:1, 1:4, 1:16) and the unknown samples were diluted to 1:4 (to adjust the cross-point value approximately in the middle of the corresponding standard curve), mixed with 2 µl of NTPs, Hot-start Taq polymerase, reaction buffer and SYBR Green I dye (LightCycler DNA Master SYBR Green I kit, Roche Biochemicals, Idaho Falls, ID, USA) whereafter they were placed into individual LightCycler glass capillaries. The reactions were supplemented with 3 mM Mg²⁺ and 0.5 µM of each gene specific primer (forward and reverse). Gene-specific primers for NR2A and NR2B were custom made at TAG Copenhagen AS (Copenhagen, Denmark) using previously published oligonucleotide sequences (Sun et al. 2000): NR2A 5´-GAC GGT CTT GGG A TC TTA AC- 3´, 5´-TGA CCA T GA ATT GGT GCA
3.8 Western Blot

3.8.1 Protein isolation

PFC and VStr from 8 saline treated and 8 nicotine treated rats were dissected. Tissues of the respective brain areas from two rats were pooled and samples were homogenized in ice-cold lysis buffer A, [0.01 M TrisHCl, 0.320 M sucrose, and 0.005 M EDTA, 0.1 mM PMSF, 1 μg/ml proteinase inhibitors (aprotinin, leupeptin, pepstatin A) pH = 7.4] using a Polytron™ homogenizer (15 seconds at setting 5). After centrifugation at 3000 rpm for 20 min (4°C) the supernatant was collected and centrifuged at 20000 rpm for 20 min (4°C). The supernatant was discarded and the pellet was washed twice with 10 ml of ice-cold lysis buffer B (0.01 M TrisHCl, 0.005 M EDTA, 0.1 mM PMSF, 1 μg/ml proteinase inhibitors (aprotinin, leupeptin, pepstatin A, pH=7.4) by resuspension and centrifugation. An aliquot (0.5 ml) from each sample was used for protein concentration measurement using the Bio-Rad kit (Bio-Rad laboratories, Hercules, CA, USA). To the final pellet a specific volume of loading
buffer (SDS 4%, Glycerol 20 %, β-mercaptoethanol 10%, BromoPhenol Blue 0.01 %, Tris-HCl 10mM, pH = 6.8) was added so that the protein concentration would amount to 5 µg/µl. The samples were then re-homogenized and stored at –80°C.

3.8.2 Immunoblot analysis

The protein samples were heated at 80 °C for 5 min and loaded (25 µg/lane) together with Precision plus protein standard (Bio-Rad laboratories, Hercules, CA, USA) on Ready Gel Tris-HCl Gel 10 % (Bio-Rad laboratories, Hercules, CA, USA) submerged in running buffer (0.1 M TrisHCl, 0.1 M glycine, 0.02 M SDS) and electrophoresed at 100 V for 2 hours. Proteins were then transferred to PolyScreen polyvinylidene difluoride (PVDF) membranes (DuMedical Scandinavia, Stockholm, Sweden) washed (2 x 10 min) in Tris-buffered saline/Tween 20 (TBS-T; Tris, 20 mM; NaCl, 150 mM; Tween 20, 0.1%) and incubated for 1 hour in Blotto containing 5% dry milk in TBS-T. The membranes were then incubated for 2 hours in a Blotto solution of mouse purified monoclonal antibody against the NR2A or NR2B subunit protein (1:250 and 1:500, respectively, BD Biosciences, Santa Cruz, San Diego, CA, USA) washed (3 x 10 min) in TBS-T and incubated for 1 hour in Blotto solution of anti-mouse horseradish peroxidase-conjugated secondary antibody (1:20000; Santa Cruz Biotechnology, CA, USA). Finally, the membranes were washed (3 x 10 min) in TBS-T, incubated in substrate solution (ECL plus Western Blotting detection system, Amersham Biosciences, NJ, USA) for 5 min, and exposed to Hyperfilm™ ECL (Amersham Biosciences, NJ, USA). The membranes were subsequently incubated in stripping buffer (62.5 mM TrisHCL, ph 6.8, 2% SDS, 100mM β-mercaptoethanol), washed (3 x 10 min) in TBS-T and reprobed with β-actin antibody (1:5000, BD Biosciences, San Diego, CA, USA). NR2A and NR2B subunit proteins as well as β-actin had the expected size (180, 180, and 40 kDa respectively). The levels of immunoreactivity for NR2A, NR2B and β-actin were quantified densitometrically.
3.9 Data Analysis

LMA data are presented as mean (± SEM) total horizontal activity counts over 60 min and analyzed using one-way ANOVA followed by Bonferroni’s or Dunnett’s test for multiple comparisons when appropriate. Alternatively, LMA data are presented as mean (± SEM) total activity counts per 5 min over the hour after the last drug administration and analyzed using two-way ANOVA (treatment x time) with repeated measures followed by Bonferroni’s test for multiple comparison when appropriate. Stereotypic behavior scores are presented as (± SD) and were analyzed using Friedman test for comparisons within groups and the Kruskal-Wallis test for between group comparisons.

DA levels are expressed as percent of baseline, which was defined as the average of the three samples immediately preceding treatment. The mean percent changes were then calculated for each 10- or 20-minute sample for all rats in each group. Data were analyzed statistically with two-way ANOVA (treatment x time) with repeated measures followed by Bonferroni’s test for multiple comparisons when appropriate. The mRNA expression data are presented as the mean (± SEM) and analyzed using unpaired two-tailed t-test. For each brain structure the intensities of NR2A and NR2B bands were normalized to the intensity of β-actin bands on the same membrane and expressed as per cent change compared to saline treated control group (=100%). All statistical calculations were done using the GraphPad Prism, GraphPad Software, San Diego, CA, USA.
4. RESULTS AND DISCUSSION

4.1 Modulation of the Acute Effects of Nicotine by NMDA and AMPA Receptor Antagonists (Paper I & II)

4.1.1 Acute effects of systemically administered nicotine

In agreement with several studies, acute administration of nicotine dose-dependently increased LMA in habituated rats (0.2, 0.3 and 0.6 mg/kg free base, s.c.) (Benwell and Balfour 1992; Clarke and Kumar 1983b). The most pronounced effect in LMA after acute administration of nicotine was observed during the initial 15 min followed by a declining but continuously elevated LMA. Pretreatment with the nicotinic antagonist mecamylamine (1 mg/kg, i.p.) blocked nicotine stimulated LMA (data not shown). Consistent with other studies, we found that nicotine (0.1, 0.3, and 0.6 mg/kg, s.c.) increased the release of DA in the NAcc (Benwell and Balfour 1992; Imperato et al. 1986). The maximum effect was always observed 20 min after nicotine administration. The nicotine-induced (0.6 mg/kg) DA release in the NAcc was effectively blocked by mecamylamine (1 mg/kg, i.p.), as reported previously (data not shown) (Sziraki et al. 1998). Taken together, these data indicate that the locomotor stimulation and the enhanced DA release after acute nicotine administration were specifically mediated via activation of nicotinic receptors.

Interestingly, as seen in Paper II (Fig. 4 and Fig. 10) 0.1 mg/kg nicotine had no effect on LMA but significantly increased DA release in the NAcc, suggesting that there is not an absolute correlation between nicotine-induced LMA and DA release. As pointed out by Deutch and Cameron, the shell and core subregions of the NAcc have different functional properties. The shell subregion is intimately connected with limbic structures whereas the core subregion and the dorsal striatum are more extensively connected with motor circuits (Deutch and Cameron 1992; Kalivas and McFarland 2003). Thus, since acute nicotine administration induces a stronger and more pronounced DA release in the shell compared to the core subregion (Cadoni and Di Chiara 2000) one possibility for our observation could be that DA was predominately measured in the shell subregion of the NAcc. Moreover, acute administration of nicotine only modestly elevated DA in the dorsal striatum in rats at a dose that significantly increases DA release in the VStr (Seppa and Ahtee 2000).
Thus, a plausible explanation for the lack of locomotor stimulation after 0.1 mg/kg nicotine could be that this dose of nicotine is too low to activate circuits involved in LMA but high enough to activate DA release in the limbic part of the NAcc.

### 4.1.2 Effects of the competitive NMDA receptor antagonists CGP39551

When administered alone, CGP39551 (10 mg/kg) had no effect on LMA in naïve rats (Paper I, Fig. 10; Paper II, Fig. 5). Pretreatment with CGP39551 significantly reduced nicotine-induced (0.6 mg/kg s.c.) LMA and DA release in NAcc (Paper I, Fig. 7 and 8). Accordingly, CGP39551 reduced nicotine-stimulated LMA to about 50% (Paper I, Fig. 8). In CGP39551-treated rats, nicotine increased DA to about 40% after 20 min and DA returned to baseline levels 60 min after nicotine administration. Thus, CGP39551 decreased both the magnitude and duration of nicotine-induced DA release in the NAcc (Paper I, Fig. 7).

### 4.1.3 Effects of competitive AMPA receptor antagonists NBQX and ZK200775

Nicotine’s effect on both LMA and DA release in the NAcc was inhibited by various doses of ZK200775 in a U-shaped manner (Paper I, Fig. 5 and 8). Thus, only the medium dose (3.0 mg/kg) of ZK200775 [shown to be neuroprotective against ischemia and head trauma in rodents (Turski et al. 1998)] significantly decreased nicotine-induced LMA and DA release in the NAcc. Neither a lower (1.5 mg/kg) nor a higher (6.0 mg/kg) dose of ZK200775 was effective in attenuating the responses to nicotine. Interestingly, pre-treatment with ZK200775 almost completely blocked (~80%) the initial peak of nicotine-induced DA release at 20 min after injection. However, DA levels slowly increased and 60 min after nicotine administration the DA release reached about 50% above the baseline, which is similar to the DA release observed in animals that were treated with nicotine alone (Paper I, Fig. 5). ZK200775 (1.5, 3.0, 6.0 mg/kg) given alone had no effect on DA release or LMA (Paper I, Fig. 6 and 10). In contrast to the effects observed with ZK200775, the selective AMPA receptor antagonist NBQX (10 mg/kg), [at a dose that is reported to be neuroprotective (Sheardown et al. 1990)], did not influence nicotine-induced DA release in the NAcc (Paper I, Fig. 7). The selectivity and specificity of ZK200775...
using cerebellar granule cell cultures revealed that ZK200775 displayed about 30-fold higher potency for AMPA receptors compared to NMDA receptors (Paper I, Fig. 1). IC$_{50}$ against AMPA receptor- and NMDA receptor-mediated neurotoxicity was found to be 0.34 µM and 11.27 µM, respectively, which is in good agreement with the ZK200775 binding profile reported earlier, where the IC$_{50}$-values of ZK200775 for [$^3$H]AMPA and [$^3$H]TCP were 0.12 µM and 11 µM, respectively (Turski et al. 2000). Furthermore, [$^3$H]Epibatidine binding assay revealed that ZK200775 has no affinity for nAChR (Paper I, Fig. 2).

4.1.4 Relative importance of NMDA vs. AMPA receptors

The inhibitory effect of CGP39551 on nicotine-induced DA release in the NAcc is in agreement with previous studies. For instance, intrategmental infusion of the competitive NMDA receptor antagonists AP-5 or CGS19755 decreases nicotine-induced DA release in the NAcc (Fu et al. 2000; Schilstrom et al. 1998). In addition, systemic or intrategmental administration of the non-competitive NMDA antagonist MK-801 also blocks nicotine's effects on DA release in the NAcc (Sziraki et al. 1998; Sziraki et al. 2002). However, in contrast to our results, previous findings report that systemic administration of MK-801 or the competitive NMDA receptor antagonist D-CPPene enhances the locomotor response to an acute dose of nicotine (Shoaib et al. 1994).

As noted, nicotine's acute effects on LMA and DA release is suggested to predominately be mediated via presynaptic α7 nAChRs as well as postsynaptic α4β2 nAChRs and NMDA receptors in the VTA (Dani et al. 2001; Grottick et al. 2000; Kempsill and Pratt 2000; Nisell et al. 1994). One recent study demonstrates that systemic CGP39551 administration inhibits nicotine's effects on burst firing and also attenuates the nicotine-induced increase in firing rate in VTA DA neurons (Schilstrom et al. 2004). It seems therefore plausible that CGP39551 reduces nicotine-induced DA release and LMA by blocking NMDA receptors in the VTA. Interestingly, CGP39551 only partially blocked LMA and NAcc DA release, which suggests that the direct effect of nicotine on nAChRs on dopaminergic neurons in the VTA was not affected.

One explanation for the enhanced effect in LMA when MK-801 was given prior to acute administration of nicotine might be that MK-801 by itself stimulates LMA.
Thus, MK-801 in combination with nicotine might potentiate each other. In analogy with MK-801, D-CPPene also is reported to stimulate LMA when given alone which therefore could explain the increase in LMA when given prior to nicotine (Svensson et al. 1991).

Based on previous findings, it has been suggested that AMPA receptors are not involved in the acute effects of nicotine. Accordingly, neither intrategmental nor intraaccumbal infusion of the competitive AMPA antagonist CNQX nor intrategmental infusion of the non-competitive AMPA antagonist GYKi52466 altered nicotine-induced DA release in the NAcc (Fu et al. 2000; Schilstrom et al. 1998; Sziraki et al. 2002). Caution should, however, be exercised when interpreting results from studies using AMPA receptor antagonists since some of them, e.g. NBQX and CNQX display relatively low selectivity, poor solubility, and short duration of action (Jackson et al. 2000). For instance, studies using CNQX report very small or no effects at all on basal levels of DA in either the NAcc or PFC with intrategmental infusion of CNQX (Feenstra et al. 1998; Karreman et al. 1996; Westerink et al. 1998; Westerink et al. 1996). On the other hand, the more selective AMPA antagonists LY293558 (Liljequist et al. 1995; Schoepp et al. 1995), when infused intrategmentally, was demonstrated to significantly increase DA release in the NAcc and decrease DA release in the PFC (Takahata and Moghaddam 1998; 2000). In addition, infusion of LY293558 directly into the PFC significantly reduced cortical DA release. Thus, the use of different AMPA receptor antagonists may result in different outcomes although the same parameters are investigated. In our hands, NBQX and ZK200775 influenced nicotine-induced DA release differentially. Moreover, ZK200775 inhibited both nicotine-stimulated LMA and NAcc DA release in a U-shaped fashion. One simple explanation for the difference in effects between NBQX and ZK200775 might be that too low a dose of NBQX was used. However, higher doses decrease basal DA release in the striatum (Karcz-Kubicha and Liljequist 1995; Sakai et al. 1997) and also reduces LMA (Vanover 1998). In addition, ZK200775 displayed about 5-fold higher anticonvulsant effect than NBQX against AMPA receptor-mediated seizures (Turski et al. 1998), suggesting that the observed difference in the action of ZK200775 and NBQX on DA release in the NAcc could be due to the difference in the potency of those compounds.

The substantial regional differences in the pharmacological specificity and distribution of AMPA receptors could perhaps contribute to the diverse effects
observed (Kessler et al. 1998; Martin et al. 1993; Monaghan et al. 1984; Porter and Greenamyre 1994). For example, a recent study reports that the ability of AMPA receptor antagonists to suppress spontaneous LMA in rats is associated with greater affinity for the GluR2 subunit (O’Neill M et al. 2005). Thus, the difference between ZK200775 and NBQX could be that ZK200775 has higher affinity for a certain AMPA receptor population in the mesocorticolimbic DA system.

The effect of ZK200775 on nicotine-induced NAcc DA release and LMA is difficult to explain. We tested the possibility that ZK200775 might have affinity for nAChRs. This was, however, not the case since [3H]Epibatidine binding assay revealed that ZK200775 did not interact with nAChRs. At high doses, ZK200775 has been shown to interact also with NMDA receptors but no other non-glutamatergic interactions were detected in brain synaptosomes (Turski et al. 2000) suggesting that the effect is restricted to inhibition of glutamatergic neurotransmission. The most likely site of action would be the VTA where nicotine is known to increase glutamate release. Alternatively, glutamatergic input to the NAcc, which is reported to facilitate dopaminergic transmission, is predominantly mediated by AMPA receptors (Blaha et al. 1997; Floresco et al. 1998; Youngren et al. 1993) and could be blocked by ZK200775. Moreover, recent studies report the existence of presynaptic AMPA receptors in the striatum which, when activated, produce both glutamate and GABA release (Fujiyama et al. 2004; Patel et al. 2001). Furthermore, AMPA receptor subunits but not NMDA receptor subunits are located on axon terminals of corticostriatal and thalamostriatal afferents suggesting that glutamate released from these axon terminals may control the activity of the terminals through the presynaptic AMPA autoreceptors (Fujiyama et al. 2004). Consequently, the complex inhibitory profile of ZK200775 could be related to interactions with AMPA receptors in several brain regions although blockade of pre- and postsynaptic AMPA receptors in the VTA and/or NAcc appears to be the most likely explanation.

Taken together, our data demonstrates differential effects in the pattern of inhibition of nicotine-induced DA release in NAcc produced by ZK200775 (3.0 mg/kg) and CGP39551, respectively. Therefore, it can be concluded that both NMDA and AMPA receptors are involved in regulating nicotine-induced DA release.
4.2 Modulation of the Acute Effects of Nicotine by the NR2B-Selective NMDA Antagonist Ro 25-6981 (Paper I & II)

The non-selective NMDA receptor antagonist CGP39551 reduced nicotine-induced LMA and DA release, probably by inhibiting NMDA receptors in the VTA (Schilstrom et al. 2004). Interestingly, it is reported that in the VTA there is a proportionally high expression of the NR2B subunits compared to NR2A-, NR2C-, and NR2D-subunits (Allgaier et al. 1999). Therefore, we tested whether the observed effect produced by CGP39551 could be specifically mediated through the NMDA NR2B subunit receptor.

In analogy with CGP39551, Ro 25-6981 given alone had no effect on spontaneous LMA or DA release in the NAcc (Paper II, Fig. 2 and Fig 10). However, in contrast to CGP39551, Ro 25-6981 dose-dependently potentiated the effect of the high dose (0.6 mg/kg) and a subthreshold dose (0.1 mg/kg) of nicotine on LMA producing a 2-fold increase compared to nicotine alone, respectively (Paper II, Fig. 3 and 4). Importantly, the increased locomotor response was well coordinated and non-stereotypic. In contrast, although significantly increasing LMA, MK-801 (0.15 and 0.3 mg/kg) also induced stereotypic behaviors (Paper II, Fig. 7). Furthermore, administration of subthreshold doses of MK-801 (0.02 and 0.05 mg/kg) produced a dose-dependent stimulatory effect on LMA in the presence of a subthreshold dose (0.1 mg/kg) of nicotine and the magnitude of the effect was comparable to that of Ro 25-6981 (Paper II, Fig. 4 and 8). CGP39551, on the other hand, had no effect on LMA when given prior to a subthreshold dose of nicotine (Paper II, Fig. 6). Moreover, in analogy with effects on LMA, Ro 25-6981 potentiated nicotine-induced DA release in the NAcc (Paper II, Fig.10).

4.2.1 Role of subunit specific vs. non-selective NMDA receptor antagonists

In analogy with previous studies, the non-competitive NMDA receptor antagonist MK-801 increased LMA and induced stereotypic behaviors (Liljequist 1991; Shoaib et al. 1994). Also, MK-801 in combination with nicotine enhanced LMA. In our study, however, subthreshold doses were used for both nicotine and MK-801 whereas in previous studies, these drugs were used in doses that per se increase LMA. Thus, a
combination of subthreshold doses of MK-801 and nicotine caused an increase in LMA. Similarly, Ro 25-6981, at a dose with no effect by itself, also caused an increase in LMA when combined with a subthreshold dose of nicotine suggesting synergistic effects between these NMDA receptor antagonists and nicotine. CGP39551, on the other hand, when given alone or together with a subthreshold dose of nicotine, had no effect on LMA. In addition, CGP39551 blocked the stimulatory effects of nicotine on both LMA and NAcc DA release. One explanation for the difference between those NMDA receptor antagonists might be that, in contrast to CGP39551, both Ro 25-6981 and MK-801 are activity-dependent. MK-801 is an open-channel blocker acting in a use-dependent manner, which means that the NMDA receptors have to be activated (the physiological Mg^{2+} block has to be removed) before an inhibitory effect of MK-801 is achieved. In comparison, Ro 25-6981 has high efficiency and faster onset of blocking NMDA receptors at higher agonist concentrations. In other words, Ro 25-6981 has higher affinity for the agonist-bound activated state of the NMDA receptor relative to the resting, agonist-unbound state (Kew et al. 1996, Fischer et al. 1997, Mutel et al. 1998, Zhang et al. 2000). Thus, a common feature in their mode of action is that the magnitude of their inhibitory effect is dependent of the activity state of the NMDA receptor.

In analogy with the effect observed in LMA, Ro 25-6981 potentiated DA release in the NAcc. In contrast, MK-801, when given intrategmentally or systemically, has been shown to block nicotine-induced DA release in the NAcc (Sziraki et al. 1998; Sziraki et al. 2002). Thus, Ro 25-6981 enhances both LMA and DA release in the NAcc in rats treated with nicotine whereas MK-801 only increases LMA but blocks NAcc DA release. A dissociation in NAcc DA release and LMA after blockade of NMDA receptors has been reported earlier. Intrategmental infusion of the competitive NMDA receptor antagonist AP-5 dose-dependently increases LMA without a concomitant increase in DA metabolism in the NAcc, striatum, or PFC (Cornish et al. 2001). Furthermore, perfusion of the NAcc with AP-5 was without effect on DA efflux in the NAcc but still increased arousal and LMA (Feenstra et al. 2002). In contrast, perfusion of AP-5 into the mPFC dose-dependently induced arousal and increased cortical DA efflux (Feenstra et al. 2002). There is some evidence to suggest that stimulation of LMA and, to some extent, DA release induced by NMDA receptor antagonists is mediated via the activation of non-NMDA receptors in the PFC (Takahata and Moghaddam 2003). The difference between Ro 25-6981 and
CGP39551 on nicotine-induced DA release and LMA might be due to that Ro 25-6981 specifically antagonizes NR2B containing NMDA receptors. In this regard, NR2B subunits are primarily expressed in forebrain structures (such as cortex, hippocampus, striatum, thalamus) with moderate expression levels in the midbrain, and the distribution of $[^{3}H]$Ro 25-6981-binding sites correlates well with that of NR2B (Loftis and Janowsky 2003). Also, Ro 25-6981 binds to NR2B-containing receptors with high affinity regardless of the NR2 subunit composition (NR1/NR2B or NR1/NR2A/NR2B) (Chazot et al. 2002). Thus, if Ro 25-6981 acts in the PFC by blocking NR2B-containing NMDA receptors, one would expect an increase in LMA, which however, at the dose administered, did not occur. On the other hand, higher doses of Ro 25-6981 than those used in the present study have been shown to stimulate LMA (Loschmann et al. 2004). Another way that Ro 25-6981 may exert its effects in the PFC could be related to the fact that DA affects glutamatergic neurotransmission in the PFC. Glutamatergic pyramidal neurons in the PFC show membrane potential fluctuations between relatively hyperpolarized ‘down’ states and relatively depolarized ‘up’ states (Yang et al. 1996). DA released from the mesocortical projection promotes the ‘up’ state in these cells, thus increasing the probability that these cells fire action potentials (Lewis and O'Donnell 2000). Therefore, DA and glutamate released in response to a low dose of nicotine would enhance the activity of NMDA receptors making them accessible for e.g. MK-801 as well as increasing the efficiency of Ro 25-6981 and in both cases lead to the inhibition of NMDA receptors. If so, this might lead to enhanced glutamatergic input to the VTA DA neurons through disinhibition of GABAergic interneurons (Conti et al. 1997; Yonezawa et al. 1998) and hence potentiation of the effects of nicotine, which was observed as higher DA release in the NAcc (Paper II, Fig. 10) with concomitant increase in LMA (Paper II, Fig. 4).
4.3 Modulation of the Chronic Effects of Nicotine by the NR2B-Selective NMDA Antagonist Ro 25-6981 (Paper III & IV)

4.3.1 Behavioral sensitization vs. nicotine-conditioned locomotor stimulation

In agreement with previous studies, chronic intermittent nicotine administration (0.6 mg/kg) resulted in behavioral sensitization. After seven days of daily treatment, the progressive increase in LMA leveled out and reached a steady and consistent plateau phase, which persisted throughout the 21-day treatment regimen (Paper III, Fig. 1). We also confirmed that repeated pairing of nicotine with the testing environment elicited nicotine-conditioned locomotor stimulation (Paper III, Fig. 4). When investigating the temporal development of behavioral sensitization, we noted that the expression of behavioral sensitization preceded the onset of nicotine-conditioned locomotor stimulation. Our experiments demonstrated that the conditioned response develops two days after the sensitized LMA response has reached a plateau phase. Thus, behavioral sensitization to nicotine and nicotine-conditioned locomotor stimulation appear to develop in parallel but the temporal difference suggests that a maximal level in the expression of behavioral sensitization to nicotine precedes the onset of conditioned increase of LMA.

Behavioral sensitization has been divided into context-dependent and context-independent sensitization on the basis that context-dependent sensitization develops more rapidly and is expressed more potently, thus suggesting that the environment in which the drug has been administered enhances the expression of behavioral sensitization (Robinson and Berridge 2000). Accordingly, existing data confirm that a sensitized response will eventually develop if the pretreatment protocol is extended to at least twelve days of treatment (Nisell et al. 1996). Nevertheless, in rats treated for 15 days with nicotine, a context-dependent effect on LMA and DA release is still discernible (Reid et al. 1998). Interestingly, one view states that the context functions as an “inhibitory occasion-setter”, i.e. the absence of a familiar context might suppress the expression of behavioral sensitization. Thus, instead of the familiar context enhancing behavioral sensitization, an unfamiliar context inhibits or reduces behavioral sensitization (Anagnostaras et al. 2002). In support of this view, neural but not behavioral sensitization has been demonstrated in animals that have been
chronically treated with amphetamine or cocaine in a different environment from the one where expression of sensitization was measured (Anagnostaras et al. 2002; Robinson and Berridge 2001). In other words, the neural circuitries underlying the development of behavioral sensitization appear to have been engaged although these neuroadaptations were not expressed in behavior. Accordingly, recent publications have shown that the levels of c-fos mRNA vary substantially in different brain areas when amphetamine or cocaine is administered in a novel environment, relative to when they are administered drug in the home cage (Badiani et al. 1999; Uslaner et al. 2001). For example, in the dorsal portion of the caudate putamen, core and shell of the NAcc, and in several cortical regions, both amphetamine and cocaine induced higher levels of c-fos mRNA expression when administered in a novel environment, relative to when they were administered in the home cage (Uslaner et al. 2001). It seems therefore that chronic drug treatment causes changes in gene expression in certain brain areas whereas the absence or presence of a familiar context in combination with chronic drug treatment appears to modulate the degree of gene expression in these brain circuits. Thus, the expression of behavioral sensitization and conditioned locomotor response could involve different adaptational changes but, with the available data, it cannot be deduced whether these two phenomena develop interdependently or in parallel.

4.3.2 Effects of nicotine and Ro 25-6981 on LMA

A challenge dose of nicotine (0.6 mg/kg) to rats chronically treated with nicotine (0.6 mg/kg, 12 days) produced an almost 3-fold increase in LMA compared to saline challenge. The same dose of nicotine in naïve rats increased LMA only 1.5-fold (Paper III, Fig.1; Paper IV, Fig. 1A). A time course graph revealed that a challenge with 0.1 mg/kg nicotine, a dose that had no effect on LMA in naïve rats, significantly increased LMA during the first 15 min of the LMA-measurement (Paper IV, Fig. 1B). Thus, in concordance with other studies, chronic intermittent nicotine administration results in a sensitized response manifested as higher LMA response with the same nicotine dose or as an increased response to a low dose of nicotine (Domino 2001; Nisell et al. 1996; Reid et al. 1998; Shim et al. 2001).

Ro 25-6981 potentiated the effect of a challenge dose of nicotine on LMA 3-fold (Paper IV, Fig. 3A-B) and this effect was stronger compared to the one observed in
naïve rats (2-fold) (Paper II, Fig. 3). In addition, a time course graph demonstrated that in contrast to naïve rats, combined treatment with Ro 25-6981 and nicotine caused an immediate and pronounced increase in LMA which after a rapid decline again rose and remained at significantly elevated levels throughout the 60 min of LMA measurement (Paper IV, Fig 3B). Ro 25-6981, when given alone to rats chronically treated with nicotine at a dose that had no effect in naïve rats, significantly increased LMA without inducing any stereotypic behaviors (Paper IV, Fig 3 and 4). Interestingly, although total LMA was similar in magnitude compared to rats treated with both Ro 25-6981 and nicotine, the time course graph reveals a different pattern in LMA (Paper IV, Fig. 3B). Accordingly, Ro 25-6981 alone increased LMA gradually until reaching a peak at 25 min followed by a constant decline whereas the maximum effect of nicotine was observed during the initial 10 min.

4.3.3 Effect of nicotine and Ro 25-6981 on DA release in the NAcc and mPFC

In line with previous studies, a challenge dose of nicotine (0.6 mg/kg, s.c) to rats chronically treated with nicotine, rapidly increased DA release in the NAcc (Benwell and Balfour 1992; Reid et al. 1998). The initial release of DA in sensitized rats was, however, lower (175%) than in naïve rats (220%) (Paper I, Fig. 4). In addition, the DA levels remained elevated at the same level (175%) for two hours (Paper IV, Fig. 7). Moreover, nicotine (0.1 and 0.6 mg/kg) also enhanced DA release in the mPFC (Paper VI, Fig. 8). However, in the mPFC, nicotine significantly increased DA release 20 and 30 min after nicotine administration, respectively. DA returned to baseline levels within one hour. Ro 25-6981 potentiated nicotine-induced DA release in the mPFC after both the low and the high dose of nicotine, respectively (Paper IV, Fig. 8). Specifically, Ro 25-6981 potentiated the effect of the higher dose of nicotine during the entire microdialysis measurement whereas, in combination with the low dose of nicotine, there was only a clear potentiation for 40 min. In contrast to the mPFC, Ro 25-6981 did not further potentiate DA release in the NAcc (Paper IV, Fig 7).
4.3.4 Effect of nicotine on NR2B and NR2A subunit expression

Chronic nicotine treatment did not alter the mRNA expression of NR2A or NR2B subunits in the PFC and ventral striatum (VStr) (Paper VI, Fig 8). However, there was a pronounced upregulation of the NR2B subunit protein in the PFC but not in the VStr (Paper VI, Fig 9-10). No changes were found in the NR2A subunit protein.

4.3.5. Effect of mecamylamine and CGP39551 on nicotine and Ro 25-6981-induced LMA

The non-selective nAChR antagonist mecamylamine completely blocked LMA to a challenge dose of nicotine. CGP39551, on the other hand, only partially blocked LMA to a challenge dose of nicotine (Fig. A). In contrast, neither mecamylamine nor CGP39551 influenced LMA induced by Ro 25-6981 in rats chronically treated with nicotine (Fig. B).

Figure A. Effect of the central nicotinic receptor antagonist mecamylamine (1 mg/kg, i.p.) and the competitive NMDA receptor antagonist CGP39551 (10 mg/kg, i.p.) on nicotine-stimulated (0.6 mg/kg, s.c.) horizontal LMA in rats chronically treated with nicotine. Shown are the means ± (S.E.M.) analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. +++p<0.001, +++p<0.001, **p<0.01 compared to the Sal + Nic0.6 treatment group.
Figure B. Effect of the central nicotinic receptor antagonist mecamylamine (1 mg/kg, i.p.) and the competitive NMDA receptor antagonist CGP39551 (10 mg/kg, i.p.) on horizontal locomotor stimulation induced by the NR2B selective NMDA receptor antagonist Ro 25-6981 (10 mg/kg, i.p.) in rats chronically treated with nicotine. Shown are the means ± (S.E.M.) analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. **p<0.01 compared to the Sal + Sal treatment group.

4.3.6 Effects of acute vs. chronic nicotine administration on LMA and DA release

In naïve rats, an acute dose of nicotine rapidly increased DA release in the NAcc. Also in sensitized rats nicotine rapidly increased DA levels but in contrast to naïve rats DA remained elevated at 175% for the entire experiment. Thus, chronic nicotine treatment appears to result in adaptive changes that lead to a prolonged effect of nicotine on NAcc DA release. nicotine (0.6 mg/kg) produced a biphasic pattern in DA release in the mPFC of rats chronically treated with nicotine. Accordingly, there was a low initial non-significant enhancement in DA release (~125%) which 20 min after administration increased to 175%. This increase in DA lasted for an additional 30 min and then returned to baseline levels. Since mPFC DA release was not measured in naïve rats, it is not possible to conclude whether DA release in the mPFC is sensitized. Nevertheless, the magnitude and rate of the DA release in the NAcc and mPFC after systemically administered challenge dose of nicotine in sensitized rats correlates well with results from other studies (Balfour et al. 1998; Benwell and Balfour 1992; Nisell et al. 1994; Reid et al. 1998; Shim et al. 2001).

In general, NAcc DA release in sensitized animals correlated with LMA. However, while DA release in the NAcc remained elevated throughout the entire
experiment, there was a transient peak in LMA, which lasted for 15 min. Thereafter, LMA slightly declined to a plateau level of LMA that also lasted throughout the experiment. The enhancement of DA release in the mPFC after 20 min in turn correlated with that small decline, indicating that DA release in the mPFC might exert an inhibitory effect on LMA. This particular assumption is highly speculative but it seems reasonable to conclude that chronic exposure to nicotine results in altered DA neurotransmission and LMA as a consequence of neuroadaptive changes in several parts of the mesocorticolimbic DA system.

4.3.7 Possible site of action of Ro 25-6981 in nicotine-induced LMA and DA release

Additional LMA studies demonstrated that the nAChR antagonist mecamylamine (1 mg/kg) completely blocked LMA to a challenge dose of nicotine whereas administration of NMDA antagonist CGP39551 (10 mg/kg) only partially blocked LMA (Fig. A) which suggests that nicotine’s stimulatory effect on LMA in both naïve and sensitized rats is critically dependent on stimulation of nAChRs but only partially on NMDA-receptors (tentatively in the VTA). As noted, experimental evidence indicates that both mecamylamine and CGP39551 inhibit nicotine-induced responses by acting in the VTA (Schilstrom et al. 2004).

In contrast to CGP39551, Ro 25-6981 enhanced the effect of a high challenge dose of nicotine on LMA (Paper IV, Fig. 3A). The effect of Ro 25-6981 in naïve rats was similar but less pronounced (Paper II, Fig. 3). The time course graph reveals that Ro 25-6981 enhanced nicotine-induced LMA during the whole hour of LMA (Paper IV, Fig. 3B and Fig. 4). In contrast, in naïve rats, Ro 25-6981 potentiated LMA only during the initial 15 min after nicotine administration (data not shown). Thus, in sensitized animals, changes in the brain have occurred that make the animals sensitized not only to nicotine but also to Ro 25-6981 when combined with nicotine. In addition, and in analogy with naïve rats, Ro 25-6981 and CGP39551 produced opposite effects on nicotine-induced LMA in sensitized rats suggesting that they act through different mechanisms and/or in different brain areas.

Interestingly, Ro 25-6981 did not potentiate nicotine-induced DA release in the NAcc in rats chronically treated with nicotine (Paper IV, Fig. 7). In contrast, Ro 25-6981 significantly enhanced nicotine-induced DA release in the mPFC. Thus, similar to the response seen after a nicotine challenge in sensitized animals the transient
peak in LMA cannot be explained by changes in NAcc DA levels but rather by changes in prefrontal DA levels. The potentiation of Ro 25-6981 on nicotine-induced DA release in the mPFC could arise if Ro 25-6981 inhibited glutamatergic input to inhibitory GABAergic interneurons in the VTA (Carr and Sesack 2000) leading to disinhibition. Such an effect would facilitate the stimulatory effect of nicotine on mesocortical DA neurons in the VTA and result in additional increase of DA release in the PFC. In conclusion, the potentiating effect of Ro 25-6981 on nicotine-induced DA release in the mPFC further points to the involvement of the mPFC in the regulation of nicotine-induced behavioral sensitization (Feenstra et al. 2002; Seamans and Yang 2004; Tzschentke 2001).

4.3.8 Role of NMDA receptors on LMA and DA release

In contrast to naïve rats, Ro 25-6981, when administered alone to rats chronically treated with nicotine, significantly increased LMA in a well-coordinated, non-stereotypic manner (Paper IV, Fig. 4). This increase in LMA is similar in magnitude when compared to rats given both Ro 25-6981 and nicotine. However, the time course graph (Paper IV, Fig. 3B) shows that Ro 25-6981 alone induced a completely different temporal development in the locomotor stimulatory effect compared to Ro 25-6981 in the presence of a challenge dose of nicotine. Additional experiments demonstrated that neither mecamylamine nor CGP39551 influenced the Ro 25-6981-induced locomotor stimulation (Fig. B) suggesting that the effect is not due to activation of either nAChRs or NMDA-receptors. However, since all drugs were given systemically, the mechanism(s) that might underlie the increased response in LMA after administration of Ro 25-6981 in sensitized rats cannot be explained on the basis of the LMA or microdialysis data. In addition to the observation that mecamylamine could not affect Ro 25-6981-induced LMA, Ro 25-6981 had no affinity to nAChRs in rats chronically treated with nicotine (Paper IV, Fig. 5) further supporting the notion that Ro 25-6981 does not interact with nAChRs.

There was a clear dissociation between locomotor stimulatory effects and DA release after treatment with Ro 25-6981 in rats chronically treated with nicotine. Accordingly, a challenge dose of Ro 25-6981 did not produce any significant changes neither in NAcc nor mPFC (although a slight but not significant increase in the NAcc
was discernible) (Paper IV, Fig. 7 and 8). Thus, the enhanced effect of Ro 25-6981 on LMA cannot be attributed to increased DA release.

As previously mentioned, a dissociation between NAcc DA release and LMA after blockade of NMDA receptors may be due to mechanisms within the PFC. Intrategmental infusion of the competitive NMDA receptor antagonist AP-5 dose-dependently increases LMA without a concomitant increase in DA metabolism in the NAcc, striatum, or PFC (Cornish et al. 2001). Furthermore, perfusion of the NAcc with AP-5 was without effect on DA efflux in the NAcc but still increased arousal and LMA (Feenstra et al. 2002). In contrast, perfusion of AP-5 into the mPFC dose-dependently induced arousal and increased cortical DA efflux (Feenstra et al. 2002). Thus, it appears that DA release in the PFC, induced by NMDA receptor antagonists, may also increase LMA. Ro 25-6981, on the other hand, had no effect at all on mPFC DA release while still stimulating LMA. This difference could result from Ro 25-6981 being NR2B-specific while AP-5 is non-selective as discussed previously.

There is some evidence to suggest that stimulation of LMA and, to some extent, DA release induced by NMDA receptor antagonists is mediated via the activation of non-NMDA receptors and GABAergic neurotransmission. Application of CNQX or baclofen in the mPFC blocked AP5-induced cortical DA release and behavioral activation (Feenstra et al. 2002). The non-competitive NMDA receptor antagonist MK-801 increases LMA and also DA release in PFC and VStr (Kretschmer 1999; Mathe et al. 1999; Mathe et al. 1998). Moreover, MK-801-induced hyperlocomotion was reduced by co-administration of GYKI 52466. However, in rats treated with MK-801 plus GYKI 52466, DA metabolism was increased only in the PFC, but not in the NAcc (Bubser et al. 1995). The GABAergic involvement regulating LMA and DA release in the mesocorticolimbic DA system is suggested to arise, in part, from GABAergic interneurons that may exert a local inhibitory mechanism or in part from GABAergic neurons in the ventral mesencephalon which project to a number of nuclei in parallel with DA neurons including NAcc and PFC (Steffensen et al. 1998; Van Bockstaele and Pickel 1995). Cortical interneurons have been shown to express NMDA receptors and cortical terminals carrying presynaptic NMDA receptors are reported to be exclusively GABAergic (Conti et al. 1997; DeBiasi et al. 1996). It is therefore suggested that NMDA receptors activate GABA efflux which in turn inhibit DA efflux (Yonezawa et al. 1998). Thus, blocking NMDA receptors inactivates GABA transmission, which ultimately disinhibits DA transmission. However, as noted,
administration of Ro 25-6981 to rats chronically treated with nicotine had no effect on DA release in mPFC and only a very small effect in NAcc.

In contrast to the acute situation, Ro 25-6981 administered to rats chronically treated with nicotine significantly increased LMA suggesting that adaptive changes in NMDA receptor subunit composition might have occurred, which would explain the increased functional sensitivity to Ro 25-6981. Indeed, there was a marked upregulation of the NR2B but not NR2A subunit proteins in the PFC (Paper IV, Fig. 10E) while no changes in either NR2A or NR2B protein were observed in the VStr. Our results therefore suggest that changes in LMA induced by chronic nicotine treatment may not be exclusively be dependent on increased DA neurotransmission in the NAcc or mPFC but may also involve a glutamatergic mechanism.

Whether Ro 25-6981 exerts its effects by binding to NMDA receptors located on GABAergic neurons and/or by inducing hyperactivity on non-NMDA receptors cannot be deduced at this point.

4.3.9 The upregulation of the NR2B subunit and functional consequences

The effects of both acute and chronic nicotine administration on LMA and DA release can be attenuated by non-selective NMDA receptor antagonists. Surprisingly, the NR2B selective NMDA receptor antagonist Ro 25-6981 potentiated nicotine-induced LMA and DA release both in naïve rats and rats chronically treated with nicotine. In addition, in chronically nicotine treated rats, Ro 25-6981 by itself increased LMA. The effects induced by Ro 25-6981 may be mediated by NMDA receptors in the PFC since DA release was potentiated in the PFC and the NR2B subunit was upregulated in the PFC.

The major characteristic of NR2B subunit-selective antagonists, including Ro 25-6981, is that they have higher affinity for the activated state of the NMDA receptor functionally seen as enhanced inhibitory potency (Fischer et al. 1997; Kew et al. 1996; Mutel et al. 1998; Zhang et al. 2000). In naïve rats, the effect of Ro 25-6981 on LMA could only be observed in the presence of nicotine, which most likely was due to nicotine-induced glutamate release. In rats chronically treated with nicotine, Ro 25-6981 alone stimulated LMA. The upregulation of NR2B subunits in the PFC in rats chronically treated with nicotine could therefore be an indication for a changed activity state of the NMDA receptors. Thus, it could be hypothesized that the changed
activity state of the NMDA receptors strongly contribute to both the sensitized LMA response to the challenge dose of nicotine and Ro 25-6981 on LMA in rats chronically treated with nicotine.

The observed upregulation of NR2B subunit is most likely a neuroadaptive response to the nicotine-induced DA and glutamate release in the PFC (Loftis and Janowsky 2003). Glutamate concentrations were not measured, but a growing body of anatomical and physiological evidence supports the notion that DA and glutamate are co-released (Seamans and Yang 2004). It has also been demonstrated that stimulation of nAChRs by nicotine induces a large increase in release of glutamate onto layer V pyramidal neurons of the PFC (Lambe et al. 2003).

Too much activity via NMDA receptors results in increased excitability and ultimately excitotoxicity (Obrenovitch 1999). Thus, one possibility to reduce a possible overactivation via NMDA receptors would be to downregulate NMDA receptors and several findings support such a notion. For instance, results from in vitro studies imply that increased synaptic glutamate concentration results in the down-regulation of NR2B subunits (Cebers et al. 2001). Our results demonstrate a hypersensitive response to Ro 25-6981 following chronic nicotine administration and an upregulation of NR2B subunits in the PFC indicating that nicotine might inhibit NMDA receptors indirectly. One mechanism through which such an inhibition might occur could be through the stimulation of D1 receptors. A recent report provides evidence that D1 receptors interact directly with NMDA receptors (Lee et al. 2002). Functionally, the D1-NR2A interaction inhibits NMDA-mediated currents. Therefore, it is tempting to speculate that nicotine, through the stimulation of D1 receptors, may produce an inhibition of NR1- and NR2A-containing receptors. As a consequence, there is a net reduction in NMDA receptor activity, which therefore might induce a compensatory upregulation of the NR2B-subunit. On the other hand, D1 receptors have also been reported to enhance NMDA receptor mediated currents in the PFC and in the striatum, D1 receptor activation induces an upregulation of NMDA receptors (Chen et al. 2004). Thus, the upregulation of NR2B in the PFC could also be a direct result of nicotine-induced increase in DA release in this brain region.

Taken together, our current findings strongly suggest that a selective blockade of NR2B-containing NMDA receptors produces substantial changes in the interplay between nicotine-activated dopaminergic and glutamatergic neurotransmission resulting in a series of functional consequences, that is altered LMA and release of
DA following acute administration of nicotine. Although the altered interplay between DA and glutamate could be demonstrated already after acutely given nicotine, this change in the interaction was further modulated following chronic nicotine administration which, among other things, was accompanied not only by the development of behavioral sensitization, but also by an altered composition of NMDA receptor subunits in some, but not all brain regions investigated. As discussed above, some earlier observations concerning the hitherto known interactions between DA and glutamate can be used to promote our understanding of the neurochemical mechanisms involved in the concomitant and complex effects of nicotine on the interaction between these two neurotransmitter systems. The results in this thesis points out a novel role for NR2B-containing NMDA receptors in the effects of nicotine on dopaminergic neurotransmission and behavior. Needless to say, more research is required to fully elucidate the involvement of NMDA receptors containing the NR2B subunit in both the acute and chronic effects of nicotine.
5. GENERAL CONCLUSIONS

- Systemic administration of the novel competitive AMPA antagonist ZK200775 inhibited acute nicotine-induced LMA and DA release in the NAcc in an inverted U-shaped manner. Systemic administration of the competitive NMDA antagonist CGP39551 also inhibited acute nicotine-induced responses. However, the observed differences in the inhibitory action of these compounds on nicotine-induced DA release in the NAcc suggest that NMDA and AMPA receptors convey regulation of DA release via different mechanisms.

- In contrast to the effect of CGP39551, systemic administration of Ro 25-6981, a NR2B-subunit selective NMDA antagonist, potentiated acute nicotine-induced LMA and DA release in the NAcc. The enhanced LMA was observed only in the presence of nicotine. Unlike CGP39551, both Ro 25-6981 and the non-competitive NMDA antagonist MK-801, in doses without effect when administered alone, enhanced LMA in the presence of a subthreshold dose of nicotine. High affinity for forebrain NR2B-containing NMDA receptors in combination with the activity-dependent mechanism of action and opposite effect to CGP39551, suggests that the effects of Ro 25-6981 could be mediated via NMDA receptors located in distinct brain regions.

- Chronic intermittent nicotine treatment induced behavioral sensitization and conditioned locomotor stimulation. Full expression of behavioral sensitization to nicotine preceded the onset of nicotine-conditioned locomotor stimulation, suggesting that the development of these two phenomena is temporally dissociated.

- Chronic nicotine treatment resulted in a sensitized response in both LMA and DA release to a challenge dose of nicotine and also upregulated the NR2B subunit in the PFC. In addition, Ro 25-6981 potentiated nicotine-stimulated LMA and DA release in the mPFC without an effect in DA release in the NAcc. These findings suggest that the PFC is an important site for the development of behavioral sensitization to nicotine.

- Ro 25-6981 alone, at a dose with no effect in naïve rats, enhanced LMA in rats chronically treated with nicotine without inducing stereotypies. Ro 25-6981 had no effect on DA release in the mPFC and there was only a small non-significant release in the NAcc. Thus, Ro 25-6981-induced LMA does not seem to depend on increased DA transmission in the mPFC or NAcc but may be mediated by glutamatergic mechanism, which can be modified by repeated nicotine treatment.

- The effects of Ro 25-6981 on LMA in rats chronically treated with nicotine reveal the importance of glutamatergic neurotransmission in behavioral sensitization.
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