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MODULATING CHOLINERGIC SYSTEMS

POSSIBLE RELEVANCE FOR SCHIZOPHRENIA

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To my brother Totte

wherever you are

ABSTRACT

Schizophrenia is a severe, chronic mental disorder. The etiology remains unknown but alterations in cholinergic neurotransmission is one, among other, disturbances in the brain that have been implicated in schizophrenia. The main aim of the thesis projects has been to evaluate if disturbances in central cholinergic function can contribute to schizophrenic symptoms. In all studies an immunotoxin, 192 IgG-saporin (192-sap), was used to selectively kill cholinergic neurons in the basal forebrain. **Paper I** examined the effect of central cholinergic denervation on dopamine (DA)-mediated functions. Because schizophrenia is often considered to be a developmental disorder, we also examined the effect of neonatal cholinergic denervation. Adult and neonatal rats received intracerebroventricular (icv) injections of 192-sap to selectively destroy the cholinergic neurons in the basal forebrain projecting to hippocampus and neocortex. We found that adult lesioned rats showed increased spontaneous horizontal activity and a remarkable increase in locomotor response to amphetamine as evidenced by increased horizontal and vertical activity. The behavioral response to the DA receptor agonist apomorphine was not changed by denervation. There were no marked changes of spontaneous or drug-induced locomotor activity in adult rats that had been treated with 192-sap as neonates. **Paper II** evaluated the relative contribution of the loss of septohippocampal versus basalocortical cholinergic projections for the amphetamine hyper-response seen in icv 192-sap injected rats. Since icv delivery of 192-sap also destroys a population of p75 receptor expressing Purkinje neurons, this cell loss needed to be taken into consideration as well. Cortex cerebri and hippocampus were selectively cholinergically denervated by intraparenchymal injections of 192-sap into nucleus basalis magnocellularis (NBM) and the medial septum/diagonal band of Broca, respectively. Selective loss of Purkinje cells in cerebellum was achieved by icv delivery of OX7 saporin. Selective cholinergic denervation of cortex cerebri, but not denervation of hippocampus or damage to cerebellum elicited DArgic hyper-reactivity similar to that seen in previous icv 192-sap experiments. **Paper III:** Disturbances in glutamatergic functions, and especially hypofunction of the NMDA receptor, is considered as a potential contributing factor in schizophrenia. Since cholinergic afferents from NBM are known to modulate glutamate transmission in neocortex, we hypothesized that cortical cholinergic denervation might result in changes in glutamatergic activity. Therefore, we lesioned the cholinergic corticopetal projections by local infusion of 192-sap into NBM of rats. Possible effects of this lesion on glutamatergic systems were examined by phencyclidine (PCP) (an NMDA receptor antagonist)-induced locomotor activity, and also by NMDA-receptor binding. Cholinergic lesioning of neocortex lead to enhanced sensitivity to PCP in the form of a

dramatic increase in horizontal activity. Further, NMDA-receptor binding was unaffected in denervated rats. **Paper IV** investigated if the increased sensitivity to amphetamine seen in rats with cortical cholinergic denervation was paralleled by an increased amphetamine-induced release of DA in nucleus accumbens (NAC) and/or striatum. The corticopetal cholinergic projections were lesioned by local infusion of 192-sap into NBM in adult rats. Amphetamine-induced DA release in NAC and striatum were monitored by *in vivo* microdialysis two to three weeks after lesioning. Amphetamine caused a greater release of DA in NAC but not in striatum of rats with cortical cholinergic denervation compared to sham lesioned controls. The duration of the amphetamine-effect was also significantly longer in the lesioned group. **Paper V:** Structural changes in schizophrenic brains such as enlarged lateral ventricles and reduced volume of cortical areas have been reported. Therefore we measured possible changes in volume or cortical thickness after cortical cholinergic denervation. Rats received unilateral cholinergic denervation produced by local infusion of the immunotoxin 192-sap into the left NBM while the right side was sham lesioned. MRI scans were made, 3 and 8 weeks postlesion. Age matched naïve rats served as controls. We did not find a significant difference in cortical thickness or hippocampal size between left and right hemispheres in the denervated animals. However, when comparing differences in grayscale values between the left and right side in T2-weighted images 8 weeks after the lesion the left, denervated side was significantly darker in saporin treated rats. This could indicate reduced cerebral blood flow in the cholinergically denervated cortical mantle. **Paper VI:** Schizophrenic patients often have cognitive dysfunctions such as impaired working memory, which might be linked to cholinergic alterations. Thus we investigated if cortical cholinergic deficits affected learning and memory. The corticopetal cholinergic system was lesioned with local infusion of the immunotoxin 192-sap into. Working memory was investigated in a delayed matching-to-place task in the water maze. We found that control animals readily learned the task, while saporin-treated rats clearly had impaired learning and memory abilities. **Conclusions:** Cholinergic denervation of cortex cerebri in adult rats leads to markedly increased behavioral responsiveness to DArgic stimulation by d-amphetamine. This is paralleled by increased release of DA in NAC. The behavioral responses to phencyclidine, a drug used to model aspects of schizophrenia, are also strongly potentiated. Animals with selective cortical cholinergic denervations also display impaired memory functions. A further observation was that effects of cholinergic denervation of cortex cerebri can be detected by standard MR imaging. This suggests that the denervation may alter cortical blood flow and/or overall neuronal activity in cortex cerebri. These observations are compatible with a possible role of cholinergic deficits in schizophrenia, and suggests ways in which cholinergic, glutamatergic and DArgic hypotheses of schizophrenia may be linked.

PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Mattsson A, Ögren SO, Olson L. Facilitation of dopamine-mediated locomotor activity in adult rats following cholinergic denervation. *Exp Neurol 2002 Mar; 174(1):96-108.*

II. Mattsson A, Pernold K, Ögren SO, Olson L. Loss of cortical acetylcholine enhances amphetamine-induced locomotor activity. *Neuroscience 2004; 127(3):579-591*.

III. Mattsson A, Lindqvist E, Ögren SO, Olson L. Increased PCP-induced hyperactivity following cortical cholinergic denervation. *Neuroreport 2005; 16 (16):1815-1819.*

IV. Mattsson A, Schilström B, Svensson TH, Olson L. Cortical cholinergic deficiency enhances amphetamine-induced dopamine release in accumbens but not in striatum. *Manuscript*

V. Mattsson A, Bednar I, Westman E, Olson L, Spenger C. Cortical cholinergic deficiencies revealed by MRI. *Manuscript*

VI. Mattsson A, Olson L, Ögren SO. Impaired spatial working memory in rats with cortical cholinergic denervation. *Manuscript*

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INTRODUCTION

Schizophrenia

The devastating brain disorder today called schizophrenia is believed to have followed mankind through history and can be traced back in written documents all the way to the old Pharaonic Egypt, two millennia before Christ. The disease was first classified as a discrete mental disorder by the German physician Emile Kraeplin in 1887. Dr. Kraeplin believed that it was a particular form of dementia and named the disorder "dementia praecox" (early dementia) in order to distinguish it from other forms of dementia that typically occur late in life. The disease was later named "schizophrenia" (from the Greek roots *schizo*, split, and *phrene*, mind) by the Swiss psychiatrist Eugen Bleuler in 1911.

Schizophrenia is a severe, chronic mental disorder with a lifelong prevalence of about 1% (Jablensky et al., 1992, Carpenter and Buchanan, 1994). The disorder is found throughout the world in all cultures. To date, no single causing factor for the disease has been identified. More likely, schizophrenia results from an interplay of genetic, environmental and other factors. The environmental factors include prenatal and perinatal events, e.g. maternal influenza, rubella, malnutrition, and obstetric complications (Susser and Lin, 1992, Wright et al., 1995, Takei et al., 1996, Thomas et al., 2001), to mention a few. The incidence of schizophrenia is the same across sexes, although women tend to have a later age of onset than men (25-29 years versus 20-24 years in men), with a second smaller peak after age 44, around the age of menopause (Hafner et al., 1993).

Schizophrenia is one of the most common serious human brain disorders, and it is also one of the most difficult to understand. There is a common belief that schizophrenia is the same as having a "split personality" – a Dr. Jekyll-Mr. Hyde switch in character. It is important to note that this is not correct. Schizophrenic symptoms typically emerge during adolescence or adulthood. The symptoms affect the full range of intellectual and emotional functions and fall under three broad categories: Positive/psychotic (i.e. hallucinations, delusions, disorganized speech/thinking), negative (i.e. apathy, lack of emotion, poverty of speech) symptoms, and global cognitive impairments (i.e. poor executive functioning, inability to sustain attention, working memory impairments). The negative and cognitive symptoms are more persistent and chronic, while the psychotic symptoms are typically episodic in nature. Diagnosis of schizophrenia relies on observation-based criteria and includes a

pattern of signs and symptoms in combination with impaired occupational and social functioning (the Diagnostic and Statistical Manual, Fourth Edition, DSM-IV).

The most frequently confirmed neurobiological finding in schizophrenia is enlargement of the lateral and third ventricles, accompanied by overall reductions in brain volume and cortical gray matter (Andreasen et al., 1994, Wright et al., 2000, Cahn et al., 2002). Postmortem studies have reported absence of gliosis (Roberts et al., 1987, Crow et al., 1989, Casanova et al., 1990), suggesting that schizophrenia is a neurodevelopmental rather than a neurodegenerative disorder (Weinberger, 1987). Imaging studies have provided evidence of alterations in cerebral blood flow, especially in the prefrontal cortex in schizophrenic patients (Ingvar and Franzen, 1974, Buchsbaum et al., 1982, Andreasen et al., 1997). Reduced blood flow in prefrontal cortex during cognitive tasks, or hypofrontality (Weinberger et al., 1986), has also been found to be correlated with the negative symptoms of schizophrenia (Andreasen et al., 1992, Knable and Weinberger, 1997).

Although the causes of schizophrenia are unknown, family and adoption studies strongly suggest that there are very significant genetic risk factors (Cardno et al., 1999, Tsuang et al., 2001). Thus, the incidence of schizophrenia is about 50% when both parents are affected (McGuffin et al., 1995) and 60-84% when a monozygotic twin is affected (Cardno et al., 1999). Several putative susceptibility genes have been identified recently, including genes involved in dopamine, glutamate and acetylcholine transmission, dopamine metabolism and synaptic plasticity (for reviews see Owen et al., 2004, Harrison and Weinberger, 2005). However, the mode of transmission is probably polygenetic and quite complex. It seems likely that multiple susceptibility genes acting in concert with environmental factors to cause development of the disorder.

The diversity of clinical signs and variability of possible etiological factors suggest that schizophrenia is not a single clinical entity, but rather a group of diseases with partially overlapping symptomatology. This indicates that there are multiple sources for schizophrenic pathology involving several transmitter interactions.

The Dopamine hypothesis of schizophrenia

The dopamine hypothesis of schizophrenia, formulated almost 40 years ago, postulates that dopamine hyperactivity in the mesolimbic system is the underlying cause for the disease (Carlsson and Lindqvist, 1963, van Rossum,

1966). Several lines of evidence support the concept of abnormal dopaminergic neurotransmission in schizophrenia. Typical antipsychotic drugs, which reduce psychotic symptoms, act by blocking dopamine D2 receptors (Seeman and Lee, 1975, Creese et al., 1976, Nordström et al., 1993). The ability of dopamine-releasing compounds, such as amphetamine, to induce schizophrenia-like psychosis in healthy subjects and worsen the psychotic symptoms in schizophrenics (Angrist and Gershon, 1970, Lieberman et al., 1987) offer further support for the dopamine hypothesis of schizophrenia. More recently, clinical imaging studies have provided additional evidence in favor of abnormal dopamine function in schizophrenia. Thus, an increased amphetamine-induced dopamine release in striatum has been shown in PET-studies of patients with schizophrenia (Laruelle et al., 1996, Breier et al., 1997b, Abi-Dargham et al., 1998). The dopamine hypothesis has been modified to also include dopamine *hypo*function in prefrontal cortex. Diminished dopamine transmission in prefrontal cortex is believed to be associated with the negative symptoms of the disease (Knable and Weinberger, 1997).

However, irregularities in dopamine transmission cannot fully account for all aspects of the disease, particularly not for the negative symptoms and cognitive impairments. For instance, amphetamine-induced psychosis is devoid of negative symptoms. Further, typical antipsychotic drugs, although successful in treating the positive symptoms of psychosis, exert only minor effects on negative symptoms and cognitive dysfunctions (Meltzer et al., 1999). This suggests that other mechanisms than dopamine hyperactivity could be implicated in the pathogenesis of schizophrenia. Mesolimbic dopaminergic hyperactivity may even perhaps be a secondary, although crucial neuropathological event in schizophrenia.

The Glutamate hypothesis of schizophrenia

Recent research on the pathogenesis of schizophrenia has focused on alterations in brain glutamatergic systems, and especially hypofunction of the N-methyl-D-aspartate (NMDA) receptor (Olney and Farber, 1995). The glutamate hypothesis of schizophrenia is mainly based on pharmacological evidence that NMDA receptor antagonists, such as phencyclidine (PCP or "angel dust"), induce clinical symptoms, both positive and negative, indistinguishable from those of schizophrenia (Javitt and Zukin, 1991, Krystal et al., 1994). Genetic linkage and association studies have strongly implicated glutamatergic dysfunction is schizophrenia (Owen et al., 2004, Harrison and Weinberger, 2005). For instance, *neuregulin-1*, a gene involved in the regulation of NMDA receptors, has been identified as a candidate gene for

schizophrenia (Stefansson et al., 2002). While post mortem studies have generally failed to find alterations in NMDA receptor expression or function (Goff and Coyle, 2001, Moghaddam, 2003), altered expression of mRNA coding for glutamate AMPA and kainate receptors have been reported in schizophrenic brains although the results are conflicting (for review see Meador-Woodruff and Healy, 2000).

The Cholinergic hypothesis of schizophrenia

Dysfunction of cholinergic signaling has also been implicated as a putative contributing factor in the pathogenesis of schizophrenia. Altered function and expression of both nicotinic and muscarinic acetylcholine receptors have been reported. Postmortem studies of schizophrenic brains have provided evidence for decreased nicotinic and muscarinic receptor binding in frontal cortex (Guan et al., 1999, Crook et al., 2001) and hippocampus (Freedman et al., 1995, Crook et al., 2000), and also for decreased nicotinic receptor binding in the reticular thalamic nucleus (Court et al., 1999) and decreased muscarinic receptor binding in caudate-putamen (Dean et al., 1996). There is also a significant reduction of muscarinic receptor availability *in vivo* in cortex, basal ganglia and thalamus in unmedicated patients with schizophrenia (Raedler et al., 2003). Further, changed expression and function of the α7 nicotinic acetylcholine receptor has been suggested to be pathogenic in schizophrenia (Leonard et al., 1996, Adler et al., 1998, Freedman et al., 2000). Genetic evidence suggest that the α7 nACh receptor is associated with the auditory sensory gating deficits characteristic of most schizophrenic patients and their relatives (Adler et al., 1985, Freedman et al., 1997). Polymorfisms in the promoter region of the α7 nACh receptor gene are associated with schizophrenia and with diminished inhibition of the P50 auditory evoked responses (Leonard et al., 2002). A further indication of abnormal cholinergic function in the patogenesis of schizophrenia is the exeptionally high rate of cigarette smoking among schizophrenic patients (80-90% compared to about 30% for the general population) (Hughes et al., 1986, de Leon et al., 2002). It has been proposed that smoking is a form of self-medication (Kumari and Postma, 2005). Interestingly, the sensory gating deficits mentioned above can be transiently reversed by nicotine administration (Adler et al., 1993). Finally, there are also indications that compounds which stimulate muscarinic receptors, such as the M1/M4 agonist Xanomeline, have antipsychotic properties (Bymaster et al., 1999, Shannon et al., 2000, Felder et al., 2001).

Cholinergic systems of the brain

Projections

Cholinergic systems in the brain can be classified into six main central pathways (Ch1-Ch6) based on the nuclei of origin of the cholinergic fibers (Figure 1) (Mesulam et al., 1983). Cholinergic fibers from septum (Ch1) and the vertical limb of the diagonal band (Ch2) project to hippocampus, whereas the horizontal limb of the diagonal band (Ch3) projects to the olfactory bulb. The cholinergic innervation of neocortex (Ch4) originates mainly from nucleus basalis magnocellularis (NBM; in humans named nucleus basalis of Meynert). The pedunculopontine nucleus (Ch5) and the laterodorsal tegmental nucleus (Ch6) in the brainstem project mainly to thalamus. The cholinergic projections are often divided into two major groups of cholinergic neurons and are referred to as the basal forebrain cholinergic system (Ch1-Ch4) and the brainstem cholinergic system (Ch5-Ch6). Additionally, striatum has a very dense cholinergic innervation provided by scattered large cholinergic interneurons intrinsic to striatum.

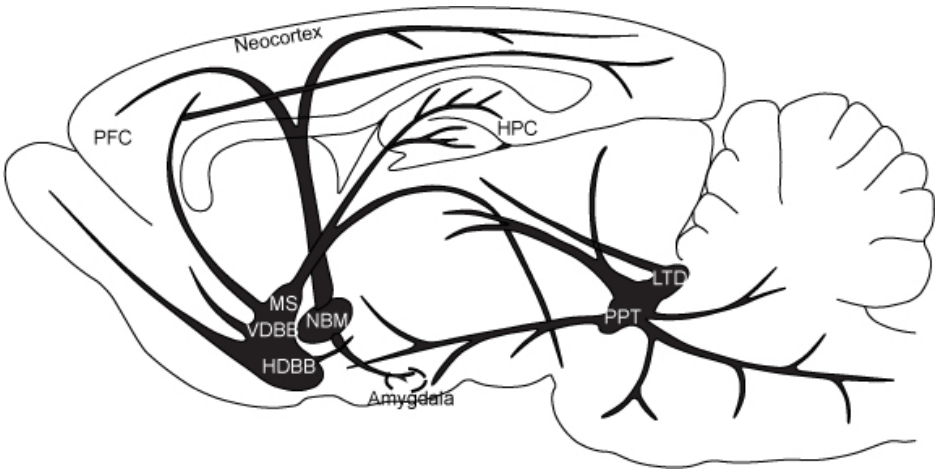

Figure 1. Schematic drawing of the major cholinergic cell groups and their projections in the rat brain. Abbreviations (nomenclature according to Mesulam within brackets): MS, medial septum (Ch1); VDBB, vertical diagonal band of Broca (Ch2); HDBB, horizontal diagonal band of Broca (Ch3); NBM, nucleus basalis magnocellularis (Ch4); PPT, pedunculopontine tegmental nucleus (Ch5); LDT, laterodorsal tegmental nucleus (Ch6); PFC, prefrontal cortex; HPC, hippocampus. Picture modified from Everitt and Robbins (1997).

Brain cholinergic systems are believed to play an essential role in learning and memory (Bartus et al., 1982). Distinct functions have been attributed to different parts of the cholinergic system, mainly based on lesion studies

(Everitt and Robbins, 1997). In short, the NBM-cortical cholinergic system contributes to cortical arousal and attentional function while the septohippocampal pathway has been implicated in spatial learning and memory. Other results, on the other hand, suggests that neocortical acetylcholine (ACh) plays an essential role in spatial memory (Winkler et al., 1995). The brainstem cholinergic projections affect basic arousal processes (i.e. sleep-wake cycle) and behavioral activation. However, in view of the multiple functions performed by ACh (see below), it is difficult to attribute the action of ACh to direct effects in memory processes since ACh also exerts its effects on related cognitive functions such as attention.

Acetylcholine receptors

Acetylcholine targets two major receptor classes: the nicotinic and the muscarinic acetylcholine receptors (nAChR and mAChR). Nicotinic receptors are ligand gated ion-channels with rapid excitatory action. Most nAChRs are pentomers, made up of two alpha and three beta subunits (bind nicotine with high affinity), although homomeric alpha 7 receptors (low affinity for nicotine, sensitive to α-bungarotoxin) are also expressed (Dani, 2001). There are five subtypes of muscarinic receptors (M1-M5) (Bonner et al., 1987) which are all coupled to G-proteins and mediate slow synaptic transmission. Muscarinic receptors are abundantly expressed in the brain and appear to mediate a variety of pre- and post-synaptic actions throughout the central nervous system. In neocortex muscarinic are receptors more plentiful than nAChRs (Parkinson et al., 1988).

Cholinergic innervation of neocortex

The cholinergic neurons of NBM send their axons to the entire cortical mantle (Rye et al., 1984). The terminal fields are restricted and only partially overlapping (van der Zee and Luiten, 1999). The neurons are organized mosaically in such a way that neurons projecting to totally different regions of neocortex can lie next to one another (Bigl et al., 1982). Cholinergic fibers can be found in all cortical layers, with the density of cholinergic fibers differing from one area to another and from one layer to another (Houser et al., 1985, Lysakowski et al., 1989, Mesulam et al., 1992). Cholinergic terminals make synaptic contacts with pyramidal cells as well as both excitatory and inhibitory interneurons in neocortex. In addition to traditional synapses, ACh is thought to exert its action via volume transmission (Umbriaco et al., 1994, Agnati et al., 1995, Mrzljak et al., 1995, Zoli et al., 1998). Possible targets of such extra-synaptic diffusion of ACh might include not only cortical neurons, but vascular structures as well (Chedotal et al., 1994).

Role of ACh in neocortex

ACh is believed to have a predominantly excitatory effect in neocortex. However, this excitation is different from that caused by glutamate, in that it has a slow onset and a prolonged action after removal of ACh (Krnjevic and Phillis, 1963a). This slow depolarizing effect of ACh has been attributed to muscarinic receptors (Krnjevic and Phillis, 1963b, Stone, 1972) since the responses can be blocked by the specific muscarinic antagonists atropine and scopolamine, but are not affected by the nicotinic antagonist mecamylamine. Some cortical neurons are activated via nicotinic receptors (Stone, 1972). These responses are usually of rapid onset and offset. Cholinergic input can also facilitate transmission by thalamic terminals in cortex presynaptically via nicotinic receptors (Prusky et al., 1987). In addition, inhibitory actions of ACh have been shown to occur in a small percentage of cortical neurons (Phillis and York, 1968, Stone, 1972, Sillito and Kemp, 1983, Murphy and Sillito, 1991).

As mentioned previously, cortical ACh is generally believed to play an important part in the regulation of cortical activity and arousal. The release of ACh is much higher during fast cortical activity in wakefulness and REM sleep (Celesia and Jasper, 1966, Jasper and Tessier, 1971) than during non-REM sleep (Jimenez-Capdeville and Dykes, 1993). This effect is likely due to activation of basal forebrain cholinergic neurons by axons from the reticular formation (Rasmusson et al., 1994). Moreover, electrophysiological studies have shown that cholinergic neurons in the basal forebrain are active during the presentation of a novel stimuli or stimuli associated with reward (Richardson and DeLong, 1988, Wilson and Rolls, 1990).

It has long been hypothesized that cortical acetylcholine can improve the "signal-to-noise" ratio for incoming sensory information by modifying the cortical responsiveness to sensory inputs. Thus, following microiontophoretic application of ACh either alone or paired with sensory stimulation, the response to sensory stimulation can be enhanced for several hours (Metherate et al., 1987). Further, ACh has been reported to have a facilatory effect on the responses to visual (Sato et al., 1987, Sillito and Kemp, 1983), auditory (McKenna et al., 1988) or somatic (Tremblay et al., 1990) stimuli in the sensory cortex. This effect has been proposed to be mediated by depolarization via muscarinic receptors (Caulfield, 1993). Activation of muscarinic receptors reduces the membrane K^+ conductance in cortical neurons (McCormick and Prince, 1985), and could thereby enhance depolarization in response to excitatory (glutamatergic) input.

In summary, the role of ACh in neocortex is predominantly as a modulator rather than as a fast synaptic transmitter, such as glutamate.

Lesioning of cholinergic systems with 192 IgG-saporin

The cholinergic neurons of the rat basal forebrain can be selectively lesioned with the immunotoxin 192 IgG-saporin. This toxin is a conjugate of saporin, a ribosome-inactivating protein, and an antibody directed against the low-affinity p75 nerve growth factor (NGF) receptor (Wiley et al., 1991). The selectivity of the toxin results from the abundant expression of p75 NGF receptor by basal forebrain cholinergic neurons (Hefti et al., 1986, Yan and Johnson, 1989). When 192 IgG-saporin is injected into the ventricles or directly into the cholinergic nuclei, the antibody binds to the receptor and the whole immunotoxin-receptor complex is internalized and transported to the soma where the toxin, saporin, inhibits protein synthesis, which results in cell death (Heckers et al., 1994). Virtually all of the cholinergic cells within the basal forebrain are vulnerable to this toxin, with the exception of those projecting to the amygdala (Heckers et al., 1994). The loss of cholinergic nerves reaches its maximum within a week after infusion of the immunotoxin (Berger-Sweeney et al., 1994, Leanza et al., 1995).

The selectivity of 192 IgG-saporin for cholinergic projection systems is supported by the absence of changes in tissue levels of dopamine, serotonin or their metabolites in cortical or subcortical areas following icv-treatment with 192 IgG-saporin (Nilsson et al., 1992, Walsh et al., 1995). However, tissue levels of noradrenaline were increased in hippocampus after such lesions although not significantly altered in other regions.

Immunolesioning differentially change ACh receptors in neocortex. Binding studies have shown an upregulation of binding sites for muscarinic M1 and M2 ACh receptors following icv administration of 192 IgG-saporin (Rossner et al., 1995b). Binding to cortical nicotinic ACh receptors is not significantly changed after icv- or intra-nbm 192 IgG-saporin-treatment (Rossner et al., 1995b, Bednar et al., 1998).

AIMS

The overall goal of the present thesis work was to gain further understanding of the possible interactions between the basal forebrain cholinergic system and the dopaminergic and glutamatergic systems in order to elucidate possible etiological pathways in schizophrenia.

The specific aims were:

- To investigate a possible link between the cholinergic and dopaminergic hypotheses of schizophrenia. More specifically, to examine if cortical cholinergic deficits can lead to dopaminergic hyperactivity.

- To evaluate if cortical cholinergic denervation can result in changes in glutamatergic function of possible relevance for schizophrenia.

- To test if cortical cholinergic denervation will affect working memory functions.

- To measure possible changes in cortical thickness or density after cortical cholinergic denervation.

MATERIALS AND METHODS

The materials and methods used are summarized below. For more detailed descriptions, see appended papers and manuscripts (Papers I-VI).

Animals

Adult male or female Sprague-Dawley rats (B&K, Sollentuna, Sweden) were used in all experiments with the exception for Paper I in which neonatal rats were also used. The animals were housed under standard laboratory conditions with unlimited access to food and water in a temperature- and humidity-controlled room under a 12 h light:dark cycle (lights on at 6 am). All experimental procedures conformed to the Swedish Animal Welfare Act SFS 1988:534, as approved by the local Animal Research Committee of Stockholm. All efforts were made to minimize the number of animals used and their suffering.

Surgery

Intracerebral surgery was performed on anesthetized rats with the head mounted in a stereotaxic frame. The skull was exposed, drill holes were made and toxin was injected at appropriate coordinates relative to Bregma with a 5 or 10 µl syringe (Hamilton) connected to a micropump (Micro 4 Micro Syringe Pump Controller, World Precision Instruments). The infusions were made at a speed of 0.05-1 µl/min depending on the site for injection. The syringe was withdrawn 3-4 min after the injection was completed. In all experiments, sham lesioned controls received equal volume of vehicle.
Anesthesia was maintained by continuous administration of 0.8-1.1% halothane (Fluothane) (Papers I and II) or by intraperitoneal (ip) injections of a mixture of hypnorm (Janssen Pharmaceutica, Belgium) and dormicum (Roche, Switzerland) (Papers II - IV) (one part hypnorm, one part dormicum (5 mg/ml) and 2 parts water; 2.7 ml of the mixture/kg). In the experiment with neonatal rats, the pups were anesthetized by hypothermia (Paper I).

Cholinergic lesioning with 192 IgG-saporin (Papers I-VI)

The immunotoxin 192 IgG-saporin (Advanced Targeting System, San Diego, CA, dissolved in sterile saline or PBS) was injected at different coordinates and doses depending on the desired cholinergic denervation.

In Paper I intracerebroventricular (icv) injections of the toxin were used to remove the cholinergic innervation of the cortical mantle and hippocampus (coordinates relative to Bregma: anterior-posterior (AP) –0.6, lateral-medial (LM) ±1.6, ventral-dorsal (VD) –4.0, with the incisor bar (IB) set at –3.3; total dose/rat: 5 μg; volume: 2.5 μl/site).
In Paper II bilateral intraparenchymal injections into NBM or medial septum/vertical diagonal band (MS/vDB) were used to selectively denervate neocortex or hippocampus, respectively. For lesioning of the NBM: coordinates relative to Bregma: AP +1.0, LM ±3.2, VD –7.5, and IB +5.0; total dose/rat: 0.134 μg; volume: 1 μl/site. For lesioning of the MS/vDB: coordinates relative to Bregma: AP +0.5, LM ±0.6, VD –7.4, -6.2, and IB –3.3; total dose/rat: 0.375 μg; volume: 0.5 μl/side (0.3 μl at –7.4, 0.2 μl at –6.2).
In Papers III-VI bilateral NBM 192 IgG-saporin lesions were performed as described above with the exception of Paper V when only the left side was denervated while the right side was sham lesioned.

Lesioning of Purkinje cells (Paper II)

Icv delivery of 192 IgG-saporin also damages p75 NGF receptor expressing Purkinje cells in cerebellum. In a control experiment (Paper II) we used icv injections of the toxin OX7-saporin (Advanced Targeting System, San Diego, CA, dissolved in sterile saline) which is known to selectively destroy cerebellar Purkinje cells (Davis and Wiley, 1989, Wrenn and Wiley, 2001). The pattern of Purkinje cell loss after icv injection of OX7-saporin is similar to that seen after icv administration of 192 IgG-saporin. Three different doses of the toxin (0.2 μg, 1 μg and 2 μg/rat; volume: 2.5 μl/site) were injected at the following coordinates relative to Bregma: AP –0.6, LM ±1.6, VD –4.0, IB –3.3

Behavioral experiments

Locomotor activity (Papers I, II and III)

Dopamine-stimulating drugs such as amphetamine induce schizophrenia-like symptoms in rodents (increased locomotor activity and oral stereotypies), which are blocked by both typical and atypical antipsychotic drugs (Angrist et al., 1974, Ögren, 1996). The ability of antipsychotic drugs to block the motor stimulatory effect of dopamine-stimulants is probably related to clinical efficacy on positive symptoms. Unlike d-amphetamine, acute administration of NMDA receptor antagonists induces both positive and negative symptoms and also cognitive impairments in healthy individuals (Krystal et al., 1994). In

rodents, acute PCP causes a wider range of schizophrenia-like behavior than amphetamine, including disorganised motor behavior, hyperactivity, cognitive deficits and disruption of prepulse inhibition (Javitt and Zukin, 1991). These motor symptoms caused by PCP seems to be mediated by a combination of effects of the mesolimbic and striatal dopamine systems (Ögren and Goldstein, 1994).

Locomotor activity in rodents is generally associated with limbic-striatal dopaminergic function. Alterations in locomotor activity could thus be considered as one possible functional expression of those limbic abnormalities which in humans may be expressed as psychosis and thought disorders (i.e. schizophrenic symptoms) (Matthysse, 1986).

Before starting the locomotor activity measurements, animals were allowed to habituate to the experimental room for 45 minutes. They were then placed individually in locomotor cages (25 x 40 x 30 cm) and horizontal activity (motility and locomotion) and vertical activity (rearing) were simultaneously recorded in a computerized multicage infrared-sensitive motion detection system (Motor Products, Stockholm, Sweden) (Ögren et al., 1986). Photocells in the cage floor, separated by 4 x 4 cm, detected horizontal motion. Motility was defined as any movement interrupting a photocell beam, i.e. a distance of 4 cm, while movement over eight photocells, or 32 cm, was defined as locomotion. Horizontal infrared beams separated by 4 cm, 10 cm over the cage floor detected rearing. Rearing, motility and locomotion counts were summed every five minutes.

Spontaneous activity was measured for 60 minutes and thereafter drug-induced locomotor activity was measured for another 60 minutes.
Amphetamine-induced (1.5 and 0.5 mg/kg ip) locomotor activity was examined in Papers I and II. Amphetamine acts presynaptically by inhibiting dopamine uptake and reversing the dopamine transporter to release dopamine (Jones et al., 1998). The effect of postsynaptic stimulation of dopamine receptors with the nonselective dopamine receptor agonist apomorphine (1 mg/kg ip) was investigated in Paper I. In Paper III we studied the locomotor response to the N-methyl-D-aspartate (NMDA) receptor antagonist phencyclidine (PCP) (1 and 3 mg/kg sc).

Morris Water Maze (Papers I and VI)

Spatial learning and reference memory

The Morris water maze task is a test for visuospatial learning and memory (Morris, 1981). This test is based on the assumption that animals, over a

number of trials, learn the location of a hidden platform by the use of distal visual cues not directly associated with the platform (Morris, 1981, Ögren et al., 1996). Briefly, a circular tank filled with water, surrounded by several extramaze cues, is equipped with a submerged platform located at a fixed position in the swim tank. Animals are released into the tank and allowed to swim for a set period of time or until they find the platform. Several parameters are recorded for each trial, such as time (escape latency), distance swum to find the platform and swim speed. At the end of the learning period, animals are tested in a spatial probe trial (reference memory test), in which the platform is removed from the pool and number of crossings over the former platform area, latency to first crossing, and swim speed are recorded.

Working memory

The working memory test in the swim maze is a variant of the traditional water maze task described above. The assessment of spatial working memory was based on the protocol described by Steele and Morris (1999) with minor modifications. This test is a form of a one-trial learning task. The rats are given four trials per day to find a hidden platform. The position of the platform varies between the days but stays the same throughout the trials of a given day. After some days of training the behavior of the rats are characterized by long latencies on trial 1 (when the rat does not know the position of the platform) and much shorter latencies during the following trials. Working memory is assessed by the difference in escape latency i.e. the savings in seconds, between trial 1 and 2.

Histology

Tissue preparation

For immunohistochemistry and histochemistry, rats were transcardially perfused under deep sodium pentobarbital anesthesia with 55 ml Tyrode containing heparin, followed by 300 ml 4% paraformaldehyde with 0.14% picric acid. Brains were dissected out, left for postfixation in the same fixative for one hour, and then thoroughly rinsed in 10% sucrose. Brains were frozen and 14 μm cryostat sections were collected at different levels of the brain.
For receptor autoradiography, animals were decapitated, brains quickly removed, frozen on dry ice and stored at –80°C until autoradiography analysis. Frozen sections (14 μm) were prepared in a cryostat at –20°C and stored at –20°C until use.

Immunohistochemistry

Immunohistochemistry is used to visualize molecules in tissue sections using antibodies directed to the compound of interest. We used an antibody against acetylcholine esterase (AChE) (a generous gift from Dr. J. Massoulie) to evaluate the degree of cholinergic denervation following lesion with 192 IgG-saporin (Paper I). Cerebellar Purkinje cells were labeled with a primary antibody against Calbindin 28k (Sigma, St Louis, MO) (Paper II). Secondary biotinylated antibodies were used for amplification and visualization of the signals.

Histochemistry

In this technique the enzyme of interest is provided with a substrate and forms a reaction product that can be visualized. In papers II-VI AChE histochemistry was employed according to the protocol of Karnovsky and Roots (1964). The cholinergic denervation was evaluated in a semi-quantitative manner and expressed as reduced density of AChE-positive reaction products. The mean optical density (OD) was measured in different brain regions from digitized images (Zeiss AxioCam and AxioVision software). Measurements were taken from two to three sections/hemisphere/region/rat. The highest mean OD obtained from denervated cortex (where no cholinergic fibers could be found) was considered as background and all measures were subtracted from this value before analysis.

Receptor autoradiography

This technique is used to localize and characterize neurotransmitter binding sites. In Paper III, glutamate NMDA receptors were detected by the binding of a tritiated ligand, [^{3}H]MK-801 (PerkinElmer Life Sciences, Boston, MA, USA). Radiolabeled slides and calibrated [H^{3}]standards were exposed to Bio Max films (Kodak, Rochester, New York, USA) for 7 weeks before development. Image analysis of cortical and subcortical regions was performed using a computerized image analysis system (ImageJ version 1.32j). MK-801 binding was quantified in two to three sections/hemisphere/region/rat. Non-specific binding was subtracted from total binding before statistical analysis.

Microdialysis

Microdialysis is a method used to monitor the chemistry of the extracellular space in living tissue. This technique was employed to monitor amphetamine-induced release of dopamine in nucleus accumbens and/or striatum of rats with a cortical cholinergic denervation (Paper III). Two to three weeks after cholinergic lesioning, concentric dialysis probes was implanted at the following coordinates relative to Bregma: for nucleus accumbens AP +1.6, LM ±1.4 and VD - 8.2 mm; for striatum AP +0.7, LM ±3.5 and VD –6.2 mm. The incisor bar was set at –3.2. After surgery, rats were housed individually and left to recover for approximately 48 hours. Microdialysis was performed in freely moving rats and the cage was placed on a rotating tray to enable untwisting of dialysis tubings as animals responded to d-amphetamine (0.5 mg/kg ip). Food and water was not available during the experiment. The probe was perfused with a modified Ringer's solution (Apoteksbolaget, Stockholm, Sweden) delivered via a polyethylene tubing from an infusion pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 2.5 μl/min. Dialysate was collected over 15 min intervals (37.5 μl) and automatically injected into a high performance liquid chromatography system. Separation of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was achieved by reversed phase liquid chromatography. Samples were then quantified by sequential oxidation and reduction in a high sensitivity analytical cell (5011 ESA, Chelmsford, MA, USA) controlled by a potentiostat (Coulochem II, model 5200, ESA) with applied potentials of 400 mV and -200 mV for detection of metabolites and dopamine, respectively.

MRI

Magnetic resonance imaging (MRI) is a non-invasive *in vivo* technique that allows analysis of individual, live animals at different time points. This imaging technique is based on the absorption and emission of energy by protons in the radio frequency range. Normally, protons in tissue are directed at random, but when placed in a magnetic field the protons become aligned. If a second magnetic field, formed by a radio frequency pulse, is applied to the tissue peripendicular to the first magnetic field the protons start spinning around their own axis. When the radio frequency pulse is turned off, protons in the tissue relax, generating recordable radio frequency signals. MRI measures the rates of two relaxation processes, T1 and T2. After the radiofrequency pulse has been turned off the spinning protons moves out of phase with one another (T2) and their axes become aligned with the original magnetic field (T1). Protons in tissue have different relaxation times depending on the

composition of the tissue. Images are then created by the fact that proton density, T1 and T2 times differ within and between tissues.

MRI examinations were performed using a 4.7 T magnet with horizontal bore (Bruker Biospec Avance 47/40, Bruker, Karlsruhe, Germany). A commercially available circular resonator (Bruker) with an inner diameter of 35 mm was used for RF pulse application and signal detection. Animals were anaesthetised with isofluran and respiratory activity was monitored continuously. Body temperature was registered and maintained at 37±0.5 ºC with a heated air stream. The sequence employed for 3D-imaging was an inversion recovery (IR) spin echo sequence with rapid acquisition with relaxation enhancement (RARE) imaging (Henning et al., 1986). After acquisition of scout images in all three directions (axial, sagittal and coronal) the 3D volume (1.20x2.20x3.00 cm) was adjusted such that the whole rat brain was included.
Data from the MR imaging were analysed with standard software (Bruker Paravision 3.0.1). MRI signal intensity and cortical thickness was measured from coronal sections.

RESULTS AND DISCUSSION

The results of Papers I-VI are summarized below. For detailed descriptions, see appended publications and manuscripts.

Cholinergic lesion with 192 IgG-saporin (Papers I-VI)

Intraventricular or intraparenchymal injections of 192 IgG-saporin very effectively destroy the cholinergic projections from the basal forebrain without direct effects on other neuronal systems. In Paper I, 192 IgG-saporin was injected into the ventricles lesioning the cholinergic projections to both hippocampus and neocortex. The high dose of toxin used in this study (5 μg /rat) together with the route of administration, causing diffusion in the cerebrospinal fluid, also resulted in destruction of a portion of p75 expressing Purkinje cells in cerebellum. To avoid this 'additional' cell loss, and to make selective lesions of either the hippocampal or the cortical cholinergic projections, 192 IgG-saporin was injected directly into the cholinergic nuclei (Paper II-VI). When 192 saporin was injected into the septal area there was an almost complete loss of cholinergic fibers in hippocampus while cortical areas appeared unaffected. In contrast, when 192 saporin was injected into NBM there were no obvious loss of cholinergic fibers in hippocampus, but cortical regions were almost completely denervated. It is important to note that cholinergic interneurons in striatum appear unaffected by the saporin-injection, demonstrating the selectivity of the toxin. Intraparenchymal injection of the immunotoxin had no effects on Purkinje cells.

Effect of cholinergic denervation on DA systems (Papers I, II & IV)

The dopamine hypothesis of schizophrenia postulates that hyperactivity of mesolimbic dopamine systems is the underlying cause of the disease. However, the possible mechanisms behind this dopaminergic overfunction are not well understood. A more recent theory suggests that disturbances of cholinergic neurotransmission may contribute to the pathogenesis of schizophrenia. In a series of experiments we therefore evaluated the possible effects of cholinergic denervation of the basal forebrain system on central dopaminergic funtions.

In the first study of this thesis (Paper I), the cholinergic innervation of hippocampus and neocortex in rats was selectively destroyed by icv injections of 192 IgG-saporin. Possible effects of such lesions were studied in locomotor cages, both before and after activation of the dopamine system with d-amphetamine and apomorphine. Since schizophrenia is often conceptualized as a neuro developmental disease, we also compared the effect of neonatal cholinergic lesions with those of adult lesions. We found that adult 192 IgG-saporin-treated, cholinergically denervated rats displayed spontaneous hyperactivity and a remarkably increased locomotor response to the dopamine-releasing compound d-amphetamine (1 mg/kg) even at a very low dose (0.5 mg/kg). In contrast, neonatal 192 IgG-saporin-treatement had no such effects on adult locomotor activity. Stimulation of postsynaptic dopamine receptors with apomorphine (1 mg/kg) did not significantly alter locomotor activity in adult or neonatally lesioned rats. This indicates that this wide-spread lesioning of brain cholinergic systems did not result in any apparent dopamine receptor supersensitivity.

The neonatal data are seemingly at variance with a recent report of amphetamine hyperresponsivity in rats treated as neonates by injection of a depo-preparation of 192 IgG-saporin into the prefrontal cortex (Rajakumar et al., 2004). These rats also displayed an impairment of prepulse inhibition of acoustic startle. Interestingly, the behavioral changes became evident only at ten weeks of age but not at five weeks of age. There was no apparent loss of ChAT-immunopositive cells in the basal forebrain in these neonatally lesioned animals. However, as cortical cholinergic fibers were not quantified, the possible contribution of reduced cholinergic activity to the observed effect cannot be evaluated.

The results from Paper I were indicative of an altered state of the dopamine system following cholinergic denervation, mainly involving presynaptic dopaminergic mechanisms. However, since icv injection of 192 IgG-saporin disrupts both the septo-hippocampal and the basalo-cortical cholinergic projections and also destroys a population of Purkinje cells in cerebellum, the question arose: Was it loss of the cholinergic projection to hippocampus, or loss of cholinergic innervation of neocortex that was responsible for the effect? Or, was the effect due to loss of cerebellar Purkinje cells? Or indeed, was it due to some combination of the three effects of icv toxin administration?

The second study (Paper II) was designed to answer these questions. Selective lesions of hippocampal and cortical cholinergic afferents were accomplished by precise injections of 192 IgG-saporin into either MS/vDB or NBM, respectively. Purkinje cells in cerebellum were damaged by icv injections of OX7-saporin. We found that selective loss of cerebellar Purkinje cells did not

significantly alter the locomotor response to amphetamine. We further demonstrated that selective cholinergic denervation of hippocampus also failed to increase the response to amphetamine. If anything, there was a tendency for a reduced response. These results were unexpected since several studies have reported that hippocampal and fimbria-fornix lesions are associated with an enhanced locomotor response to amphetamine (Lipska et al., 1992, Wilkinson et al., 1993, Mittleman et al., 1998, Balse et al., 1999). In our experiments, when such locomotor responses to amphetamine were not seen, there was no or minimal tissue damage and a complete and selective loss of cholinergic afferents to hippocampus. This suggests that additional perturbations associated with the excitotoxic (ibotenic acid) hippocampal lesions and the surgical deafferentiations, e.g. loss of GABAergic neurons, might underlie the observations of increased amphetamine locomotor responses seen by others.

Having ruled out two of the three possibilities for the observed effects of icv administration of the toxin we next tested animals with selective neocortical cholinergic denervations. We found that removal of the cholinergic projection to neocortex was indeed responsible for the marked facilitation of d-amphetamine-induced locomotor activity seen in the previous experiment using icv delivery of 192 IgG-saporin. NBM-lesioned rats displayed a clear hyperreaction even at a very low dose of amphetamine (Figure 2A). This appears to be due to an altered state of the dopamine neurons, since activation of postsynaptic dopamine receptors did not cause the same increase in locomotor activity as dopamine-releasing compounds in rats with cholinergic denervation of the basal forebrain (Paper I). Similarly, as shown in figure 2B, there was no difference in the locomotor response to apomorphine between rats with selective cortical cholinergic denervation and control rats.

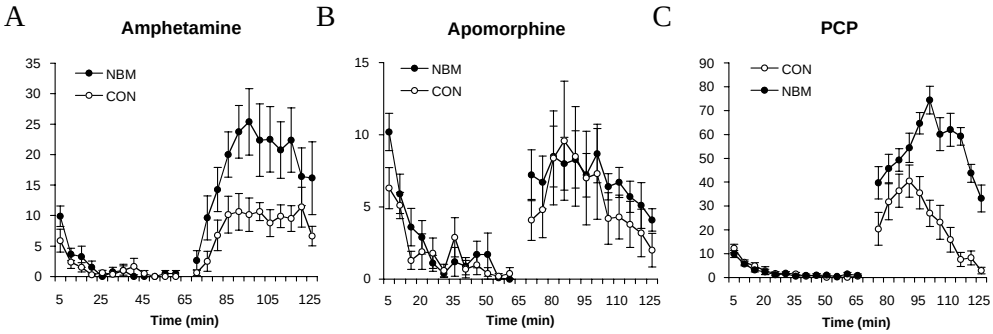

Figure 2. Locomotor activity measurements in NBM 192 IgG-saporin lesioned rats. Sixty min of spontaneous activity was followed by 60 min amphetamine (0.5 mg/kg ip) (A), apomorphine (1.2 mg/kg sc) (B), or PCP (3 mg/kg sc) (C) –induced activity. Loss of cortical cholinergic innervation resulted in a dramatically increased locomotor response to amphetamine and PCP, but not to apomorphine.

The increased locomotor response to amphetamine has been suggested to be mainly mediated by dopamine release in nucleus accumbens, as lesioning of dopamine-terminals in nucleus accumbens by 6-OHDA is known to reduce the motor stimulatory effect of amphetamine (Kelly and Iversen, 1976). However, in addition to causing an increase of dopamine release, amphetamine can also affect other neurotransmitters. It was therefore possible that the hyper-response to amphetamine following cortical cholinergic denervation was not only mediated via the dopamine system.

The main purpose of Paper IV was to investigate if the enhanced locomotor response to d-amphetamine seen after cortical cholinergic denervation was paralleled by an increased amphetamine-induced release of dopamine in nucleus accumbens and/or striatum. The cholinergic projections to neocortex were again selectively destroyed by intraparenchymal injections of 192 IgG-saporin into NBM. D-amphetamine-induced dopamine release in nucleus accumbens or dorsal striatum was monitored by *in vivo* microdialysis two to three weeks after lesioning. The results demonstrated that cholinergic denervation of the rat neocortex leads to a significantly increased d-amphetamine-induced dopamine release in nucleus accumbens. Interestingly, the cholinergic lesion did not affect amphetamine-induced release of dopamine in striatum. The relative levels of the dopamine metabolites DOPAC and HVA after d-amphetamine-challenge were not significantly altered in accumbens or striatum in saporin-treated rats. Basal levels of dopamine, DOPAC and HVA did not differ between groups, consistent with previous findings that striatal and cortical tissue levels of dopamine and its metabolites are not changed after icv 192 IgG-saporin (Nilsson et al., 1992, Walsh et al., 1995). Administration of nicotine before the amphetamine-challenge did not reverse the enhanced dopamine release in the cholinergically denervated rats (Figure 3), suggesting that loss of muscarinic receptor stimulation was likely to have caused the observed effect. Interestingly, mice lacking M1 or M4 muscarinic receptors have also been found to have a hyperresponsive dopamine system evidenced by elevated dopamine transmission in striatum or nucleus accumbens following amphetamine administration (Gerber et al., 2001, Tzavara et al., 2004). The M1 deficient mice had increased spontaneous locomotor activity, combined with an increased response to amphetamine, consistent with our results. There are also additional behavioral evidence of muscarinic/cholinergic inhibition of brain dopaminergic activity. Thus, oxotremorine, a muscarinic receptor agonist, was found to attenuate amphetamine-induced locomotor activity in the rat. Moreover, oxotremorine can reduce amphetamine-induced dopamine release in nucleus accumbens while enhancing dopamine release in the medial prefrontal cortex (Ichikawa et al., 2002a).

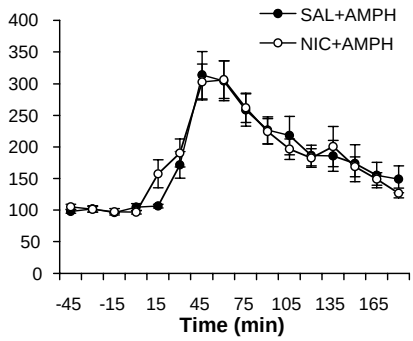

Figure 3. Amphetamine-induced (0.5 mg/kg ip) dopamine release in nucleus accumbens in rats with cortical cholinergic denervation. Pre-treatment with nicotine (1 mg/kg sc) 15 min before amphetamine-challenge did not significantly effect the enhanced dopamine release innucleus accumbens of NBM 192 IgG saporin lesioned rats. Data are expressed as mean percent of baseline release ± sem.

Taken together, these results indicate that cortical acetylcholine exerts a regulatory influence on subcortical dopaminergic activity. Cholinergic regulation of subcortical dopamine release is consistent with previous reports of increased release of striatal dopamine after cholinergic blockade with scopolamine (Dewey et al., 1993). Data from the microdialysis and apomorphine experiments suggest an altered state of the dopamine neurons themselves, allowing amphetamine to cause a larger increase of dopamine in the synaptic cleft of dopaminergic synapses in cholinergically denervated rats.

How then, may loss of cholinergic innervation of cortex cerebri increase dopaminergic activity? One possibility could be that dopamine accumulates in the terminals as a consequence of decreased basal activity of the dopamine neurons. However, the fact that tissue levels of DA in the basal ganglia are not altered after icv 192 IgG-saporin-treatment (Nilsson et al., 1992, Walsh et al., 1995) in combination with the results from the present thesis that basal levels of extracellular dopamine are not significantly changed in animals with cortical cholinergic denervation compared to controls (Paper IV), make this an unlikely explanation.

A more plausible explanation would be that the loss of cortical cholinergic inputs alters the activity of cortical glutamatergic neurons and their regulation of subcortical dopamine neurons in nucleus accumbens and the ventral tegmental area (VTA). The basal forebrain cholinergic system is believed to exert a general activating effect on the cortical mantle. This view is supported by studies showing reduced spontaneous and evoked activity of cortical neurons after 192 IgG-saporin lesions of NBM (Herron et al., 1998, Herron

and Schweitzer, 2000), and also by reports of reduced high frequency EEG activity following lesions of the basal forebrain cholinergic neurons (Berntson et al., 2002). Hence, it might be speculated that loss of cortical acetylcholine alters glutamatergic neurotransmitter function. In fact, we have recently shown that rats with cholinergic denervation of neocortex display an increased sensitivity to the NMDA receptor antagonist PCP, indicated by a drastically enhanced locomotor activity (see next section) (Paper III). This suggests that disruption of cholinergic activity can lead to disturbances of glutamatergic transmission. Anatomical evidence indicates that dopamine neurons are controlled by cortical glutamatergic neurons either directly or indirectly via GABAergic interneurons (Carr and Sesack, 2000), acting as accelerators or brakes, respectively. If dopamine release is increased, e.g. by amphetamine, a negative feedback loop is activated, leading to a much greater effect on the brake, thereby reducing the release of dopamine (Carlsson et al., 2001). Disturbances of cortical glutamatergic function as a consequence of cholinergic denervation could thus lead to a weakened feedback control. This in turn would result in an enhanced amphetamine-induced dopamine release as the breaking effect is diminished. A schematic drawing of the circuits involved is shown in figure 4.

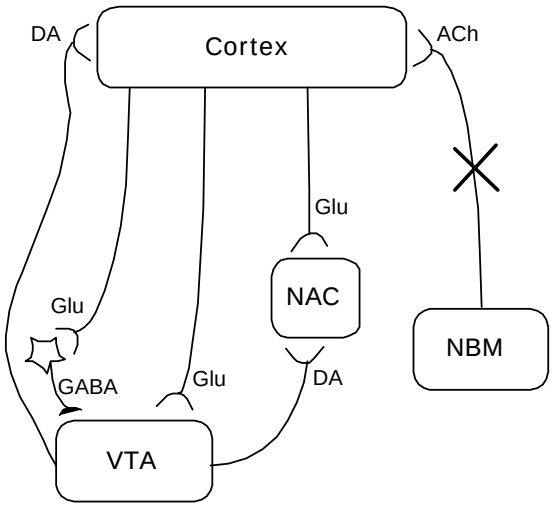

Figure 4. A schematic drawing of the major functional circuits considered in this thesis involving acetylcholine, dopamine and glutamate interactions. For the sake of simplicity, many additional afferent and efferent connections to the illustrated areas have been omitted. In the present work, the cortical cholinergic projections were lesioned with intraparenchymal injections of 192 IgG-saporin into NBM. We hypothesize that the loss of cortical cholinergic innervation results in reduced cortical efferent activity and thereby diminished regulation of mesolimbic dopamine transmission. Abbreviations: ACh, acetylcholine; DA, dopamine; Glu, glutamate, NAC, nucleus acumbens, NBM, nucleus basalis magnocellularis, VTA, ventral tegmental area. The drawing is based on earlier work by Sesack et al (2003).

Effect of cholinergic denervation on glutamate transmission

Acute administration of PCP has been demonstrated to produce schizophrenia-like symptoms in rodents, including increases in locomotor activity which are blocked by all types of antipsychotic drugs (Ögren, 1996). In paper III we investigated the PCP-induced locomotor response in rats with cortical cholinergic denervation. Possible aterations of the NMDA glutamate receptor were studied using receptor autoradiography.

The behavioral results show that cortical cholinergic denervation results in an enhanced sensitivity to PCP (3 mg/kg) evidenced by a dramatic increase in horizontal activity (figure 2C). Both the amplitude and duration of the PCP-effect was strongly and significantly augmented in the saporin-treated animals. In contrast, PCP-induced rearing was significantly reduced compared to controls. The enhanced locomotor response to PCP in rats with cortical cholinergic denervation is consistent with a recent study demonstrating that the AChE inhibitor physostigmine reversed MK-801 induced hyperlocomotion in mice (Csernansky et al., 2005), indicating that cholinergic function might be involved in the regulation of NMDA-receptor mediated glutamatergic function.

Glutamatergic transmission in prefrontal cortex has been shown to play a critical role in the locomotor activating effect of systemically administered NMDA receptor antagonists such as PCP (Takahata and Moghaddam, 2003). NMDA receptor antagonists, in addition to inhibiting NMDA receptors, also increase glutamate neurotransmission via activation of non-NMDA glutamate receptors in cortical and limbic regions (Moghaddam et al., 1997). The increase in prefrontal cortex activity following PCP appears to result in enhanced activity of prefrontal cortex glutamatergic efferents to nucleus accumbens and/or VTA, since blockade of AMPA/kainate receptors in these regions inhibited PCP-induced hyperactivity (Takahata and Moghaddam, 2003). The enhanced locomotor stimulation after PCP in rats with cortical cholinergic denervation is probably due to a marked potentiation of non-NMDA transmission resulting in an enhancement of cortical glutamatergic efferents to subcortical areas such as nucleus accumbens. We suggest that this increase will result in enhancement of dopamine release in nucleus accumbens. This is supported by reports of increased PCP-induced dopamine release in nucleus accumbens in mice lacking the muscarinic M4 receptor (Tzavara et al., 2004). Since we did not observe any changes in NMDA receptors in the 192 IgG-saporin-treated rats, the potentiating effect of PCP in these animals might be due to alterations of non-NMDA receptors. This hypothesis is supported by

findings of upregulated AMPA and kainate binding sites in cortical regions in icv 192 IgG-saporin-treated rats (Rossner et al., 1995a).

Effects of cholinergic denervation on memory functions

The basal forebrain cholinergic system is considered to play an important role in cognitive functions such as learning and memory. In Paper I, spatial learning and reference memory was measured in the Morris water maze task in rats treated with icv 192 IgG-saporin as adults or neonates. The adult lesioned rats, with an extensive cholinergic denervation of both hippocampus and cortex, displayed robust impairments in spatial learning and memory, consistent with previous findings (Nilsson et al., 1992, Berger-Sweeney et al., 1994, Leanza et al., 1995, Walsh et al., 1995). The neonatally lesioned rats also showed deficits in learning or memory when tested as adults, however this deficit could be related to a performance impairment. Thus, unlike the adult lesioned rats there was no significant interaction between days and treatment in the neonatally lesioned rats. Interestingly, the cholinergic denervation in the neonatally lesioned rats was not as substantial as for the adult lesioned rats which could explain the observed difference. As discussed by Wrenn et al. (1999), it seems clear that for learning and memory impairments to occur, an almost complete lesion of the cholinergic basal forebrain neurons is most likely to be required. Thus, the reported lack of impairments in standard watermaze performance following local injection of 192 IgG-saporin into the cholinergic nuclei in the basal forebrain (Torres et al., 1994, Bannon et al., 1996, Baxter et al., 1996, Dornan et al., 1996), may reflect incomplete cholinergic lesions.

Schizophrenic patients as well as Alzheimer patients often display impaired working memory. In paper VI we studied if selective cortical cholinergic denervation would affect working memory functions. The cortical cholinergic projection was destroyed by intra-NBM infusion of 192 IgG-saporin and working memory was subsequently measured in a delayed-matching-to-place task in the water maze. Rats with cholinergic denervation of neocortex demonstrated significant deficits in spatial working memory functions. Thus, in contrast to controls which learned the task without difficulties, lesioned rats were unable to improve the performance in the water maze task over the ten days of testing.

The exact role of cortical acetylcholine in rodent memory functions is still not understood. While some studies have failed to find alterations in spatial working memory in the radial maze or spatial reference memory in the Morris water maze task (Galani et al., 2002, Lehmann et al., 2003), others have

reported mildly impaired performance in spatial working memory tasks (Baxter 1995, Dornan 1997) following 192 IgG-saporin lesions of NBM. Then again, spatial memory deficits in NBM lesioned rats have been reported to be improved by ACh-rich grafts implanted into denervated cortical regions (Winkler et al., 1995), suggesting an important role for cortical ACh in spatial memory functions, consistent with our findings. The differences between our results from the working memory measurements and those from previous studies might be due to several factors such as extent of cortical lesion, design of memory task and rat strain.

Another observation relevant for memory function and cortical acetylcholine is based on results obtained in the locomotor activity measurements in Paper II. Thus, the first time the animals were placed in the locomotor activity boxes, both controls and rats with cortical cholinergic denervation explored the new environment for approximately 20 min before settling down. However, when retested and placed in the boxes the following day, the controls rapidly habituated while the lesioned rats explored the cage with the same intensity as the day before, as if they did not 'remember' that they had previously been there. This might be interpreted as a spatial and contextual memory dysfunction, involving long-term memory.

Several investigators have suggested that impairments in performance following cholinergic NBM lesions are attentional in nature and do not involve learning or memory deficits (Muir et al., 1994, Baxter et al., 1995, Everitt and Robbins, 1997, McGaughy et al., 2002). However, results from a recent study which used a task for simultaneous assessment of attention and memory, indicates that the NBM-derived cortical cholinergic system is involved in both attentional and mnemonic processes (Chudasama et al., 2004). In the aforementioned study, saporin lesions of NBM did not impair attention or memory under baseline conditions. However, increasing the attentional load by decreasing the stimulus duration, significantly impaired memory performance. This suggests a critical role for cortical acetylcholine in rodent memory function, particularly when attentional demands are high.

Cholinergic denervation alters brain MRI signal properties

Structural changes of the brain have been reported in schizophrenia and in Alzheimer's disease (de Leon et al., 1989, DeCarli et al., 1992, Andreasen et al., 1994, Xanthakos et al., 1996, Wright et al., 2000). The study described in paper V was designed to determine a possible causative relationship between

cholinergic denervation and altered brain volume or density as determined by MRI. The main purpose was to investigate if cholinergic denervation of neocortex could lead to alterations in brain size and/or MRI-signal intensity. Rats were infused with 192 IgG-saporin into the left NBM to produce a unilateral cortical cholinergic denervation and were subjected to MRI scans 3 and 8 weeks postlesion.

Three weeks after unilateral infusion of 192 IgG-saporin into NBM there was no significant difference in MRI signal intensity between the two hemispheres. At the time for the second MRI scan, the intensity of the MRI signal was significantly reduced on the denerevated side compared to the sham lesioned side. The denervated side, with reduced MRI signal intensity, appeared as darker. Further, the denervated hemisphere was significantly darker as measured in T2-weighted images two months after the lesion compared to three weeks post lesion, indicating a progressive effect of the denervation that continues after the three week time point. We did not detect any alterations in cortical thickness following cholinergic lesioning of NBM.

The reduced signal intensity in T2 and T2*-weighted MR images observed in the present experiment might be the result of reduced blood flow, blood oxygenation and/or a decreased water proton density in the extracellular space of the tissue. This would lead to faster transverse relaxation of the magnetization and thereby produce a darker image. The findings of reduced MRI signals in the hemisphere with cortical cholinergic denervation compared to the sham lesioned side might therefore be explained by reduced blood flow as a consequence of loss of cortical acetylcholine. This theory is supported by evidence for cholinergic innervation of cortical microvessels which mainly has its origin in the NBM (Vaucher and Hamel, 1995). Stimulation of the cholinergic fibers projecting to neocortex increases regional cerebral blood flow by causing active dilation of cerebral blood vessels since ACh is a potent vasodilatator (Biesold et al., 1989, Lacombe et al., 1989).

While further studies, e.g. of the status of the nitric oxide synthase system and other mechanisms for blood flow control in the cholinergically denervated cortex are needed to elucidate possible reasons for the observed effects on MRI signals of the cholinergic denervation, the most parsimonious explanation is decreased blood flow.

As the function of neurons depend on the adequate supply of oxygen and nutrients provided by blood perfusion, it is also possible that abnormal blood flow might result in insufficient energy consumption. Reduced glucose utilization has been reported in parietal cortex following intra-NBM or icv injection of 192 IgG-saporin (Bassant et al., 2000, Browne et al., 2001). Such

results in combination with those of the present thesis work of possible blood flow alterations following cholinergic denervation, adds to the notion of a functional role for cortical acetylcholine in regulating brain homeostasis.

GENERAL DISCUSSION

Implications for schizophrenia

The findings of the present thesis suggest that cortical cholinergic deficiency can:

a) influence the regulation of subcortical dopamine transmission, leading to a 'sensitized' mesolimbic dopaminergic system,
b) result in glutamatergic dysfunctions,
c) cause impairments in spatial working memory,
d) lead to homeostatic changes in the affected cortex.

All of these findings might be of relevance for the pathophysiology for schizophrenia since they match some of the core features of the illness.

a) Patients with schizophrenia show increased responses to amphetamine (Laruelle et al., 1996, Breier et al., 1997b, Abi-Dargham et al., 1998), hypothesized to be due to dopaminergic sensitization (Laruelle, 2000). Similarly, the present data indicate that cholinergic denervation of cortex cerebri can induce alterations in the mesolimbic dopaminergic function, which become evident when the system is challenged by amphetamine.

b) Several studies have indicated that schizophrenic patients have a decreased metabolic activity ('hypofrontality hypothesis') in prefrontal cortex. This hypothesis has received support by measurements of blood flow (Ingvar and Franzen, 1974), glucose metabolism by PET (Buchsbaum et al., 1982) as well as neuropsychological studies (Andreasen et al., 1992). More recent studies indicate that, besides disturbances in prefrontal cortex, the neuronal basis of schizophrenia involves a more distributed dysfunctional network in cortical and subcortical regions (Andreasen et al., 1997). The changes in metabolic activity may partly be related to the proposed cortical glutamate receptor (NMDA) dysfunction in schizophrenia (Olney and Farber, 1995). Interestingly, NMDA receptor antagonists increase metabolic activity and induce an acute psychotic state in healthy individuals (Breier et al., 1997a). Deficits in cortico-striatal drive have been hypothesized to be involved in the disturbed subcortical dopamine transmission (Grace, 1991). Blocking of

NMDA receptors increases striatal DA transmission (Breier et al., 1998) and increases severity of positive symptoms and cognitive impairments in schizophrenic patients (Malhotra et al., 1997).
In view of the present results, we propose that the depletion of cortical acetylcholine results in a reduced threshold for NMDA antagonist-induced psychosis-like symptoms. This could occur by several different mechanisms. Since acetylcholine is known to modulate glutamate transmission in cortex cerebri, loss of cortical acetylcholine could shift the relationship between the influence of acetylcholine on excitatory and inhibitory transmission (Kimura, 2000). This change could theoretically result into a different modulation of the regulatory action of acetylcholine on afferent sensory inputs versus intracortical connections. The cholinergic depletion will concomitantly shift the balance of dopamine/glutamate interactions in cortex, resulting in a hyper-dopaminergic state of the mesolimbic system. This hyper-dopaminergic state is probably related to changes in the activity of the glutamate afferents to VTA and nucleus accumbens, resulting in increased dopamine release in animals with cortical cholinergic denervation. Taken together, these regulatory changes caused by the loss of cortical acetylcholine will probably manifest itself as symptoms of psychotic disorders as well as cognitive dysfunctions.

c) Spatial working memory has been shown to be impaired in schizophrenic patients (Park and Holzman, 1992, Keefe et al., 1995). This deficit is believed to be partly mediated by dopamine dysfunction in prefrontal cortex (Williams and Goldman-Rakic, 1995, Murphy et al., 1996, Knable and Weinberger, 1997), even though it is likely that other systems, including the cholinergic system are also involved. Thus, spatial working memory deficits in smokers with schizophrenia can be ameliorated by cigarette smoking, presumably due to nicotinic receptor stimulation (Sacco et al., 2005). Anticholinergic drugs, which are commonly used to treat extrapyramidal side effects of neuroleptic treatment, impair spatial memory in schizophrenics (McGurk et al., 2004). Further, atypical antipsychotic drugs such as clozapine, olanzapine and risperidone, have been reported to improve negative symptoms and some cognitive deficits in schizophrenia better than typical antipsychotic drugs (Green et al., 1997, Keefe et al., 1999, Lee et al., 1999, Meltzer and McGurk, 1999), and risperidone appears to be the most effective in improving working memory (Green et al., 1997, Meltzer and McGurk, 1999). The atypical, but not the typical, antipsychotic drugs preferentially enhance efflux of both dopamine and acetylcholine in prefrontal cortex relative to nucleus accumbens (Kuroki et al., 1999, Ichikawa et al., 2002b). However, antidepressant drugs, known to increase prefrontal dopamine release (Tanda et al., 1994) but not ACh release (Bertorelli et al., 1992), are not effective in treating cognitive impairments and negative symptoms in schizophrenics (Plasky, 1991). This indicates that

cortical cholinergic deficiencies might contribute to some of the cognitive dysfunctions such as working memory impairments seen in schizophrenia.

d) Decreases in gray matter density in schizophrenic brains have been reported (Hulshoff Pol et al., 2001). We found reduced MRI signal intensity following cortical cholinergic denervation. It may be speculated that the decreased MRI signal is a consequence of reduced blood flow. Recent imaging studies provide evidence for reductions in resting regional cerebral blood flow in schizophrenic patients (Schultz et al., 2002, Malaspina et al., 2004), confirming the original finding of hypofrontality (Ingvar and Franzen, 1974). Furthermore, mitochondrial dysfunction, compromised brain metabolism and oxidative stress in the prefrontal cortex have recently been implicated in schizophrenia (Prabakaran et al., 2004). Importantly, the authors of the latter report hypothesized that these alterations could be explained by aberrant microvasculature in the prefrontal cortex. Finally, an abnormal blood flow is most likely accompanied by reduced energy supply. Indeed, PET-studies of unmedicated schizophrenics have reported reduced glucose uptake in fronto-thalamic circuits (Siegel et al., 1993, Lehrer et al., 2005).

The present thesis work suggests that cortical cholinergic denervation of the rat brain might be valid as a rodent model for some aspects of schizophrenia. In addition to what has been described in this thesis, 192 IgG-saporin lesions of NBM have been shown to lead to deficits of prepulse inhibition (PPI) of the acoustic startle reflex (Ballmaier et al., 2001). PPI is an operational measure of sensorimotor gating which is usually compromised in schizophrenic patients, i.e. they have a diminished capacity to filter out unimportant features of their environment.

As discussed by Sarter and Bruno (Sarter and Bruno, 1998) there are at least two seemingly contrasting views about the possible involvement of the basal forebrain cholinergic system in schizophrenia, one hypofunction model assuming that hallucinations can be caused by loss of cortical acetylcholine (Perry and Perry, 1995), and one hyperfunction model. According to Sarter and Bruno (Sarter and Bruno, 1998, Sarter et al., 2005), hyperactivity of cortical cholinergic transmission impairs information processing in cortex and hence is responsible for the attentional and cognitive deficits seen in schizophrenia. The increased efflux of cortical ACh is explained by a sensitized dopaminergic system (caused by glu alterations?) which leads to disinhibition of NBM. Instead, in the model put forward in the present thesis, loss of cortical acetylcholine results in a ‘sensitized’ dopamine-system, as evidenced by increased DA release following acute amphetamine challenge. It remains possible that both mechanisms, hypo- and/or hyperfunctioning of cortical ACh, are relevant in schizophrenia (for instance during different

episodes of the disease or in different forms of schizophrenia). This view is supported by the hypothesis put forward by Tandon and Greden (Tandon and Greden, 1989) stating that cholinergic/dopaminergic balance is of central importance in schizophrenic pathophysiology, and that increased cholinergic activity is associated with negative symptoms while decreased cholinergic activity is associated with positive symptoms. Further, treatment with biperiden, a muscarinic antagonist, in unmedicated schizophrenics resulted in worsening of positive symptoms and improvement of negative symptoms (Tandon et al., 1991, Tandon et al., 1992).

CONCLUSIONS

In this thesis it has been shown that cholinergic denervation of cortex cerebri in adult rats leads to markedly increased behavioral responsiveness to dopaminergic stimulation. This is paralleled by increased release of dopamine in nucleus accumbens. The behavioral responses to phencyclidine, a drug used to model aspects of schizophrenia, are also strongly potentiated. Animals with selective cortical cholinergic denervations also display impaired memory functions. A further observation was that effects of cholinergic denervation of cortex cerebri can be detected by standard MR imaging, suggesting that the denervation may alter cortical blood flow and/or overall neuronal activity in cortex cerebri. All of these observations are compatible with a possible role of cholinergic deficits in schizophrenia, and provides a possible link between different hypotheses of the disorder. The MRI findings as well as the memory disturbances also have implications for Alzheimer's disease.

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