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Influenza A Virus Infection and NMDA receptor function:

A behavioral and molecular study of relevance for schizophrenia

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ABSTRACT

Schizophrenia is a neuropsychiatric disorder characterized by positive and negative symptoms, as well as cognitive dysfunctions. There is no single cause of schizophrenia, instead complex combinations of genetic and environmental factors may contribute to the disorder, including exposure to viral infections or other environmental insults during early life. Discoveries during the last 30 years have provided increasing evidence for a glutamatergic hypofunction in schizophrenia. This hypothesis arose from the finding that non-competitive N-methyl-d-aspartate (NMDA) receptor antagonists, such as ketamine and phencyclidine (PCP) can reproduce schizophrenia-like symptoms including cognitive dysfunctions in man. This observation, has led to a search for schizophrenia-related animal models based on NMDA-receptor blockade.

The main aim of these studies was to investigate if exposure to influenza A virus during early life can result in cognitive dysfunctions during adult life. In addition, to allow studies on the potential role of NMDA receptor systems for cognition, a novel NMDA receptor hypofunction model was developed. Both acute and repeated treatment with NMDA receptor antagonists is known to produce a dose-related spectrum of motor dysfunctions that interfere with cognitive performance in rodents. For this reason, PCP was examined in a dose-dependent study to establish the dose-range of cognitive impairments versus motor side effects. Repeated administration of the 0.5 mg/kg dose of PCP impaired spatial learning and long-term memory without affecting non-spatial learning. The PCP-induced impairments in learning and memory were prevented by concomitant treatment with the "atypical" antipsychotic drug clozapine (0.5 mg/kg), but not with the "typical" antipsychotic haloperidol (0.05 mg/kg).

To avoid testing with the drug "on board", the effects of repeated administration of PCP on cognitive and social behavior were examined 24 h after the final dose of PCP. In addition, this study examined the effect of such treatment on the expression of two genes involved in neuronal plasticity and learning. Repeated doses of PCP (1 and 2 mg/kg) produced long-term impairments in spatial learning and working memory performance in the water maze task, without any apparent sensorimotor deficits. Furthermore, mice treated with 2 mg/kg of PCP spent less time in active social interaction, and showed increased non-social and aggressive behaviors. These behavioral effects were associated with altered expression of the genes encoding Arc and spinophilin. The alterations in *Arc* transcripts were observed in areas associated with spatial learning such as the CA1 region of the hippocampus and in the retrosplenial cortex.

To examine if exposure to a maternal infection affects gene expression in the offspring, pregnant C57BL/6 mice were instilled intranasally with influenza A/WSN/33 virus on day 14 of gestation. Differential gene expression in the brains of the offspring could be detected at postnatal days 90 and 280, but not earlier. Thus, a maternal influenza A virus infection can give rise to alterations in gene expression that become apparent only after a considerable period of latency. A subsequent study examined the effects of an influenza A virus infection in 3-4-day old C57BL/6 or immunodeficient *(Tap1$^{-/-}$)* mice. This infection led to transient changes in expression of several genes associated with schizophrenia in C57BL/6 mice while *Tap1$^{-/-}$* mice exhibited more enduring changes. Whereas infected C57BL/6 mice were deficient only in the long-term memory task, infected *Tap1$^{-/-}$* mice showed deficits in working memory performance.

The olfactory tract provides an interesting route of invasion for studies of viruses to the brain. Therefore, mice were injected with influenza A virus into the olfactory bulbs at 5-6 weeks of age. Sixteen to eighteen weeks after the infection these mice showed impaired spatial learning and reduced anxiety-like behavior. Moreover, genes encoding synaptic regulatory proteins were differentially expressed in the amygdala, the hypothalamus and the cerebellum in these mice.

Finally, to link early life virus infection to possible changes in NMDA receptor function, a subchronic dose of PCP (2 mg/kg) was given to control mice and mice infected on day 3. The marked impairment in cognition by subchronic PCP was confirmed in control mice. Virus infected mice tended to be more susceptible to these PCP-induced deficits in cognition and showed a clear difference in the pattern of motor activity compared to control mice.

In conclusion this thesis shows that (i) repeated low dose of PCP impairs cognitive functions and is reversed by clozapine; (ii) repeated administration of PCP can produce long-term effects on cognition, social behavior and gene expression after termination of the treatment; (iii) influenza A virus infections can cause alterations in the expression of (schizophrenia-associated) genes and behavior. The effect of the infection appears to be influenced by the timing and route of infection; and (iv) mice infected with a virus during early life appear more susceptible to repeated PCP treatment in adulthood.

Keywords: Schizophrenia, Influenza A virus, NMDA receptor, glutamate, cognition, learning, memory, hippocampus, water maze, mice, emotional, social interaction, gene expression, brain, behavior, phencyclidine, elevated plus-maze

LIST OF PUBLICATIONS

This thesis is based on the following papers and manuscripts, which will be referred to in the text by their Roman numerals:

I. **Beraki S*,** Kuzmin A*, Tai F, Ögren SO. Repeated low dose of phencyclidine administration impairs spatial learning in mice: Blockade by clozapine but not haloperidol. European Neuropsychopharmacology, 2008, In press

II. **Beraki S**, Diaz-Heijtz R, Tai F and Ögren SO. Effects of repeated treatment of PCP in cognition and gene expression in C57BL/6 mice. Submitted manuscript

III. Tai F, **Beraki S**, Wadenberg S and Ögren SO. Long-term effects of repeated treatment of phencyclidine on social interaction and emotional behaviour in mice. Submitted manuscript

IV. Asp L*, **Beraki S***, Aronsson F, Rosvall L, Ögren SO, Kristensson K, Karlsson H. Gene expression changes in brains of mice exposed to a maternal virus infection. NeuroReport, 2005, 10: 1111-1115

V. Asp L*, **Beraki S***, Kristensson K, Ögren SO and Karlsson H. The effects of early postnatal influenza A virus infection on schizophrenia-related genes and behaviors in mice. Submitted manuscript

VI. **Beraki S**, Aronsson F, Karlsson H, Ögren SO, Kristensson K. Influenza A virus infection causes alterations in expression of synaptic regulatory genes combined with changes in cognitive and emotional behaviors in mice. Molecular Psychiatry, 2005, 10: 299-308

*These authors contributed equally to the study

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LIST OF ABBREVIATIONS

5-HT	Serotonin
AMPA	Amino-3-hydroxy-5-methyl-4-isooxazole propionic acid
Am	Amygdaloid nucleus
Arc	Activity-regulated cytoskeleton-associated gene
ANOVA	Analysis of variance
APV	2-amino-5-phosphonovaleric acid
CA1	Areas of the hippocampus formation (field of Ammon´s 1)
Ca^{2+}	Calcium ion
CaMK2A	calcium/calmodulin-dependent protein kinase II alpha
Cxcl1	chemokine CXC motif ligand 1
COMT	Catechol-O-metyltransferase
CREB	cAMP response element-binding
CSF	Cerebrospinal fluid
DAT	Dopamine transporter
D_{1-5}	Dopamine 1-5
DSM IV	Diagnostic and Statistical Manual of Mental Disorders
DTNBP1	Dystrobrevin binding protein 1
DISC	Disrupted in schizophrenia
DLPFC	Dorsolateral prefrontal cortex
EPSPS	Excitatory postsynaptic potentials
Fisher´s PLSD	Fisher's protected least significant difference
GABA	Gamma-aminobutyric acid
Gcm1	Glial cells missing
Glu	Glutamate
Gly	Glycine
H	Hemaggulutin
Hip	Hippocampus
Hyp	Hypothalamus
KO	Knockout
LI	Latent inhibition
LTD	Long-term depression
LTP	Long-term potentiation
N	Neuraminidase
NMDA	N-methyl D-aspartate
NA	Noradrenaline
NR1-3	NMDA receptor subunits
NRG-1	Neuregulin 1
NAC	Nucleus accumbence
Nurr1	The nuclear receptor related 1
MK-801	Dizocilpine (NMDA receptor antagonist)
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
i.p.	Intraperitoneal
i.v.	Intravenous
Olig2	Oligodendrocyte lineage transcription factor 2
PCP	Phencyclidine
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PET	Positron emission tomography
PolyI:C	Polyriboinosinic-polyribocytidilic acid
PPI	Pre pulse inhibition
PSD	Postsynaptic density
RGS4	Regulator of G-protein signaling 4
s.c.	Subcutaneous
SN	Substantia nigra
Str	Striatum
Th	Thalamus
TAP	Transporter associated with antigen processing
VH	Ventral hippocampal
VMAT	Vesicular monoamine transporters
VTA	Ventral tegmental area
WSN/33	InfluenzaA/WSN/33 virus
WSN	Wilson Smith neurotropic
WT	Wild type

1. INTRODUCTION

1.1. Schizophrenia

1.1.1. Symptomatology

Schizophrenia is a severe, often chronic disorder with a lifetime prevalence of 1% in the world and thereby constitutes a major public health concern (Jablensky et al., 1992; Carpenter and Buchanan, 1994). The onset of psychiatric symptoms usually appears between the age of 15-25 years for males and 15-30 years for females (Hafner et al., 1993). People with schizophrenia have a higher rate of suicide than the general population and approximately 10% of schizophrenia-patients, especially younger adult males, commit suicide (Black and Fisher, 1992; Osby et al., 2000; Meltzer, 2002).

The symptoms of schizophrenia can be classified into three broad categories positive, negative and cognitive symptoms ((Andreasen et al., 1995), Diagnostic and Statistical Manual of Mental Disorders, DSM IV). The positive symptoms, which include delusions and hallucinations, refer to bizarre or abnormal experiences such as seeing or believing things that are not real. Negative symptoms refer to avoidance, emotional withdrawal and flattening and they also involve a lack of feeling as well as a reduced ability to take pleasure in life. This deficit-state is also characterized by a lack of motivation and goal-oriented behavior. Cognitive dysfunctions involve a broad range of symptom involving both perception and cognition such as disorganized speech, thought disorder, disorganized behavior, poor attention, low ability for abstract thinking and problem solving as well as complex disturbances in learning and memory. Individuals suffering from schizophrenia exhibit heterogeneity in the severity and combinations of the symptoms.

1.1.2. Cognitive dysfunctions

Since cognitive dysfunctions in schizophrenia are regarded by some researchers as core symptoms (Goldman-Rakic, 1994; Meltzer et al., 1999) and appear to determine clinical outcome (Kasper and Resinger, 2003), different aspects of cognition will be briefly discussed. Cognitive impairments were recognized as early as 1896 by Kraepelin, who regarded schizophrenia as a dementia disorder and termed it “dementia praecox” (Kreapelin, 1896). Later studies have identified multiple domains of cognitive dysfunctions in schizophrenia patients (Green, 1996). The most characteristic cognitive dysfunctions in schizophrenia involve visual and verbal learning and memory, executive functions, attention, working memory, long-term memory, social learning and intelligence (Goldman et al., 1991; Park and Holzman, 1992; Elvevag and Goldberg, 2000; Sharma and Antonova, 2003; Tamminga, 2006). The impairments in working memory and executive functions have been related to prefrontal cortical dysfunctions and are believed to represent a core feature of the disorder (Goldman et al., 1991; Andreasen et al., 1997; Meltzer et al., 1999). Since the impairments in schizophrenia also involve declarative memory (Clare et al., 1993; Goldberg et al., 1993) and episodic memory (Saykin et al., 1991), it seems likely that there is a

deficiency in hippocampal functions potentially related to disturbances in the adult hippocampal neurogenesis (Bruel-Jungerman et al., 2007).

1.1.3. Etiology

Schizophrenia is considered a multifactoral neurodevelopmental (Murray and Lewis, 1987; Weinberger, 1995) disorder involving complex interactions between genetic and environmental factors. There is strong evidence for a genetic component in the etiology of the disorder (Tienari et al., 1987; Cardno et al., 1999; Tsuang et al., 2001). Monozygotic twins show higher concordance for the disorder than dizygotic twins (Kringlen, 2000; Tsuang et al., 2001). Moreover, siblings of schizophrenia patients and children of parents suffering from schizophrenia show a clearly higher incidence of schizophrenia than the general population (Gottesman and Irving, 1991). A number of environmental factors have also been implicated in schizophrenia. These include exposure to famine during pregnancy (Susser et al., 1996), birth complications (Dalman et al., 1999) as well as urban birth and upbringing (Mortensen et al., 1999). In light of the now well-established seasonal variation in birth patterns of individuals with schizophrenia, the potential role of infections during early life has also been addressed in several studies. During recent years, serological studies of archived maternal blood drawn during pregnancy suggest that increased titers of antibodies directed at *Toxoplasma gondii* (Mortensen et al., 1999; Brown et al., 2005), herpes simplex virus 2 (Buka et al., 2001) or influenza A virus (Brown et al., 2004) increase the risk for the development of schizophrenia in the offspring. Not only fetal, but also exposure to viral CNS-infections during childhood has been reported to confer an increased risk for developing schizophrenia (Rantakallio et al., 1997; Dalman et al., 2008). Furthermore, in a study of samples obtained from US military personnel, Niebuhr and coworkers recently reported increased levels of antibodies to *Toxoplasma gondii* in samples drawn within six months of the onset of schizophrenia (Niebuhr et al., 2008). Such antibodies were not elevated in samples obtained earlier from these same individuals (Niebuhr et al., 2008). It is, however, evident that most individuals do not develop schizophrenia following exposure to infections, which indicates that additional susceptibility factors are involved, such as those relating to the immune status of the individual (Eaton et al., 2006; Shirts et al., 2007).

Moreover, abuse of cannabis has been shown to be an independent factor in the development of schizophrenia (Andreasson et al., 1987). This study following army recruits found that those who admitted taking cannabis on more than 50 occasions had a six-fold risk of schizophrenia compared to non-users (Andreasson et al., 1987).

The heterogeneity of clinical symptoms and the wide range of potential etiological factors suggest that schizophrenia may consist of multiple disorders with partially overlapping underlying mechanisms. This complex symptomatology suggests alterations in several physiological systems involving multiple transmitters such as dopamine, glutamate, gamma-aminobutyric acid (GABA), noradrenaline (NA), and serotonin (5-HT) as well as their receptors (Carlsson et al., 2001). Among the different neurotransmitter dysfunctions, research on dopamine dominated the field during the

past 30 years. Therefore, in the next two chapters, dopamine and the "dopamine hypothesis" will be briefly discussed to give a historical background current research on schizophrenia. In addition, a more recent hypothesis based on dysregulation of brain glutamatergic systems as a pathological factor will be presented.

1.2. Brain dopaminergic system

1.2.1. Pathways and receptors

Evidence for the view that dopamine is a neurotransmitter in the central nervous system was first provided by Carlsson and his coworkers in the late 1950s (Carlsson et al., 1958). Subsequent studies using reserpine showed that dopamine plays an important role in motor control exerted by the basal ganglia (Carlsson, 1959). In the 1960s and 1970s, a number of studies showed that the dopaminergic system regulates a number of functions in the central nervous system including reward, attention as well as various aspects of cognition (Schetz and Sibley, 2007).

The brain dopamine system is organized into four major pathways (see Figure 1), which were originally characterized by the use of the Falk-Hillarp fluorescence-method (Urbanska et al., 1997; Bjorklund and Dunnett, 2007). The nigrostriatal projection, which originates from substantia nigra in the midbrain and terminates in the striatum, has a major role in motor control. The mesocortical projection originates in the ventral tegmental area in the midbrain and projects to cortical areas innervating the prefrontal cortex in which dopamine plays a role in executive functions and working memory. The mesolimbic tract, derived from the ventral tegmental area, projects to the limbic system, including the nucleus accumbens, the cingulate cortex and parts of the amygdala and hippocampus. This pathway seems to be involved in emotional control, reward, and addictive behavior as well as aspects of motivation. The tuberoinfundibular tract, which projects from the arcuate nucleus and paraventricular area in the hypothalamus to the infundibulum and anterior pituitary, is involved in endocrine control, regulating e.g. release of prolactin.

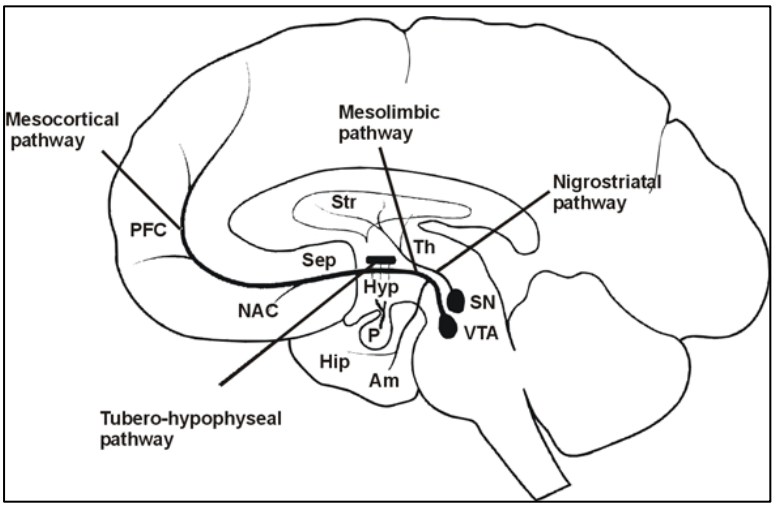

Figure 1: Dopaminergic pathways in the human brain. Abbreviations: PFC = prefrontal cortex; NAC = nucleus accumbens; Sep = septum; Str = striatum; Hyp = hypothalamus; Th = thalamus; P = pituitary gland; Hip = hippocampus; Am = amygdaloid nucleus; VTA = ventral tegmental area and SN = substantia nigra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

Dopamine mediates its physiological actions via five dopamine receptor subtypes (D_1-D_5), classified into two major families (D_1-like- and D_2-like receptors)

based on sequence-homology and transduction mechanisms (Kebabian and Calne, 1979; Civelli et al., 1993; Schetz and Sibley, 2007). The dopamine receptors are differentially localized in the rat brain, which has important functional consequences. For instance, D_1-receptors, which are coupled to G_S G-proteins and increase cAMP-formation, play a major role in prefrontal cognitive functions (Goldman-Rakic, 1994). In contrast, dopamine D_2-receptors, which are coupled to G_i-proteins and thereby decrease cAMP-formation, are localized in the mesolimbic and mesostriatal dopaminergic systems and their projection areas (Civelli et al., 1993).

Despite large efforts to develop novel antipsychotic drugs affecting other transmitter systems, all classical and second generation (atypical) antipsychotic drugs appear to act clinically partially by blocking dopamine D_2-like receptors (Ögren, 1996; Seeman, 2002; Stone and Pilowsky, 2007). The second generation antipsychotic drugs, which have a lower tendency to produce extrapyramidal side effects, appear to preferentially target limbic dopamine D_2-receptors and function rather than striatal dopamine D_2-receptors (Ögren et al., 1993; Pilowsky et al., 1997).

1.2.2. The dopamine hypothesis of schizophrenia

The original dopamine hypothesis of schizophrenia postulates that there exists hyperactivity in the cerebral dopamine function and is based on pharmacological evidences. The discovery by Carlsson and Lindqvist (Carlsson and Lindqvist, 1963) that neuroleptic drugs can increase brain dopamine turnover was suggested to be due to blockade of dopamine receptors. The dopamine hypothesis of schizophrenia was first proposed by van Rossum, who stated explicitly that the mechanism of action of neuroleptic drugs is related to dopamine receptor blockade (van Rossum, 1966; van Rossum, 1967). Direct support for this hypothesis was first provided by the discovery that the therapeutic efficacy of antipsychotic drugs correlates with their affinity with dopamine D_2- but not dopamine D_1-receptors (Seeman and Lee, 1975; Burt et al., 1976). Another line of evidence was based on the observation that indirect dopamine agonists such as amphetamine can induce psychosis in healthy subjects or exacerbate psychotic symptoms in schizophrenia patients (Randrup and Munkvad, 1967; Angrist et al., 1974; Lieberman et al., 1987). Moreover, the behavioral effects of amphetamine, which are mediated by dopamine release, were blocked by antipsychotic drugs (Randrup and Munkvad, 1967; Lieberman et al., 1987). PET-imaging study in schizophrenia patients using the selective dopamine D_2-antagonist C_{11}-raclopride has shown that dopamine D_2-receptors are occupied by antipsychotic drugs at therapeutic doses (Farde et al., 1992). Moreover, studies with C_{11}-raclopride in individuals with schizophrenia have provided evidence for increased or altered dopamine release in the striatum and forebrain respectively (Laruelle, 1998; Lindstrom et al., 1999).

Nevertheless, schizophrenia cannot be explained by the original "dopamine hypothesis" solely, since increases in dopamine transmission are unlikely to give rise to cognitive dysfunctions and negative symptoms. Moreover, classical antipsychotic drugs such as haloperidol, a D_2-receptor antagonist, cannot restore the cognitive dysfunctions in schizophrenia and has marginal effects on negative symptoms (Meltzer et al., 1999).

In contrast, the "atypical" antipsychotic drug clozapine, is the only antipsychotic drug that improves both positive and negative symptoms in treatment-resistant patients (Kane et al., 1988). In addition, clozapine is reported to restore some of the cognitive impairments involving prefrontal executive functions in schizophrenia patients (Meltzer and Gudelsky, 1992; Ichikawa and Meltzer, 1999; Meltzer et al., 1999).

Working memory deficits and negative symptoms in schizophrenia have recently been conceptualized as a prefrontal dysfunction, related to a hypodopaminergic state in the prefrontal cortex (Goldman-Rakic and Selemon, 1997; Abi-Dargham and Moore, 2003). Based on studies in monkeys, dopamine D_1-receptors in the prefrontal cortex have been linked to both negative symptoms and cognitive decline by modulating NMDA receptors on pyramidal neurons (Goldman-Rakic, 1994; Abi-Dargham and Moore, 2003). Also, PET-imaging studies in schizophrenia patients have shown disturbances in prefrontal dopamine D_1-receptors, evidenced by an increased number of dopamine D_1-receptors (Abi-Dargham and Moore, 2003). Further support for a disturbed dopamine transmission in the prefrontal cortex has been provided by genetic studies on the polymorphism of the catechol-O-metyltransferase (COMT)-gene (see chapter 1.4.) (Egan et al., 2001). The evidence for a prefrontal hypofunction has resulted in a "modified dopamine hypothesis", which postulates that a dopaminergic hypofunction in the prefrontal cortex is associated with negative symptoms and cognitive dysfunctions (Andreasen et al., 1997; Abi-Dargham and Moore, 2003). Indeed, the "modified dopamine hypothesis" postulates that the hypofunction of dopaminergic neurotransmission in the PFC results in an increased dopamine release, and stimulation of subcortical dopamine D_2-receptors in the basal ganglia which is secondary to the deficit in the prefrontal cortex (Abi-Dargham et al., 1998; Abi-Dargham and Moore, 2003). Thus, this hypothesis assumes that there is a concomitant hypo- and hyper-function of dopaminergic transmission in schizophrenia patients. In this context, it is important to note that "atypical" antipsychotic drugs such as clozapine, unlike haloperidol, increase dopamine efflux in the medial prefrontal cortex of the rat (Moghaddam and Bunney, 1990; Nomikos et al., 1994; Ichikawa et al., 2001). This property is believed to contribute to the ability of "atypical" antipsychotic drugs to improve cognitive impairments as well as negative symptoms in patients with schizophrenia (Moghaddam and Bunney, 1990; Meltzer et al., 1999).

1.3. Brain glutamatergic systems

1.3.1. Glutamate and its receptors

Glutamate is the primary excitatory neurotransmitter, and is contained in roughly 60% of neurons in the brain. The majority of glutamatergic pathways originates in cortical areas and innervates a number of subcortical regions, including the spinal cord. The major glutamatergic pathways involved in cognition and locomotor control are the cortico-cortical connections including all cortical pyramidal neurons, the perforant pathway from the entorhinal cortex to the hippocampus, the trisynaptic pathway in the hippocampus and the cortico-striatal pathway (Nakanishi et al., 1998). Glutamate mediates fast synaptic transmission via three types of ionotropic receptor, and slow

synaptic transmission via G-protein-coupled metabotropic receptors (Dingledine et al., 1999; Coutinho and Knopfel, 2002) (see Figure 2). Metabotropic receptors (termed mGluR1 through mGluR8) are divided into three groups based on several criteria, including G-protein-coupled transduction mechanisms and modulation of glutamate-transmission.

Three major types of ionotropic glutamate receptors have been identified based on their sensitivity to the synthetic glutamate derivatives: α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)-receptors, kainate-receptors and N-methyl-D-aspartate (NMDA)-receptors.

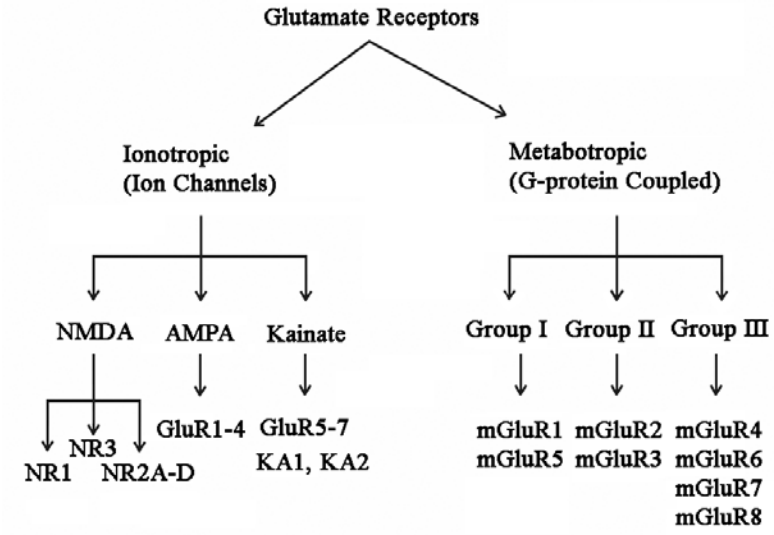

Figure 2. Schematic representation the different families and subtypes of glutamate receptors.

The NMDA receptors play an important role not only glutamatergic neurotransmission but also in synaptic plasticity (see below), synapse development and neurotoxicity. The NMDA receptor consist of different subunits NR1, NR2 (A, B, C, D) and NR3 (A, B). All functional NMDA receptors are heteromeric protein-assemblies containing NR1-subunits in combination with at least one type of NR2 subunit. The NR1 subunits convey basic channel properties, and NR2A-D and/or NR3 subunits modify receptor affinity, channel open time, and ion permeability. NMDA receptors contain a primary binding site for glutamate, as well as an allosteric modulatory site that is sensitive to the amino acid co-agonist glycine (see Figure 3). Extracellular Mg^{2+} blocks the NMDA channel in a voltage-dependent manner, by binding to a site within the ion channel. Activation of the NMDA receptor requires both binding of its agonists (glutamate and glycine) and a depolarization of the membrane-potential (e.g. by AMPA receptor activation) that remove the Mg^{2+} blockade (see Figure 3). The non-competitive antagonists of the NMDA receptor phencyclidine (PCP) and the "dissociative" anaesthetic ketamine, bind to intra-channel sites of the NMDA receptor and, thereby, reduce calcium ion-influx into brain neurons (Anis et al.,

1983). There are also other sites, within or out of the channel, that are sensitive to polyamines and zinc.

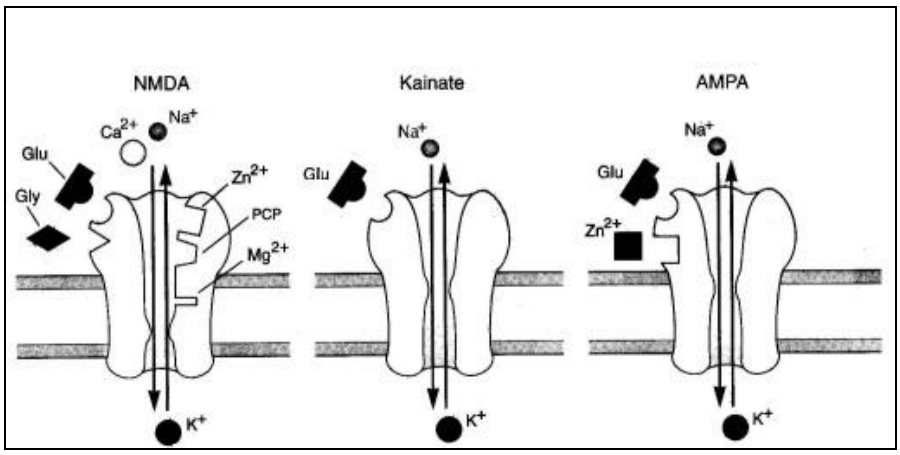

Figure 3. Schematic drawing of the ionotropic glutamate receptors (NMDA, kainate and AMPA). Abbreviations: Glu = glutamate, Gly = glycine, PCP = phencyclidine. (Modified from (Kandel et al., 1991))

1.3.2. NMDA receptors and memory

Analysis of the neural mechanisms underlying learning and memory has focused on how neural activity generated by sensory experiences can produce long-term alterations in neural circuits and their functions. It is generally believed that different forms of synaptic plasticity involving changes in synaptic strength are critical for translating short-term experiences into long-term memory. Synaptic plasticity refers to all processes that participate or play a role in the activity-dependent changes of the synaptic transmission at pre-existing synapses. In this process, the NMDA receptors appear to be critical for some forms of long-term changes in synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD) in various brain regions (Citri and Malenka, 2008). Hippocampal LTP was first discovered in the dentate gyrus. A brief high-frequency stimulation of the perforant path resulted in an increased synaptic transmission in the granule cells of the dentate gyrus (Bliss and Lomo, 1973), which was blocked by NMDA receptor antagonists (Collingridge et al., 1983), leading to the hypothesis that LTP is NMDA receptor-dependent. LTP in the CA1 region of the hippocampus, which is responsible for associative LTP, has been shown to be triggered by activation of NMDA receptors, leading to an increase in postsynaptic Ca^{2+}-concentration (Asztely et al., 1991; Malenka and Nicoll, 1993). The increase in Ca^{2+} serves as a second messenger and activates a number of signal transduction cascades that are important for the maintenance of LTP. The early LTP phase depends on phosphorylation of various proteins by protein kinases such as calcium/calmodulin-dependent protein kinase II alpha (CaMKII) (Soderling and Derkach, 2000). The maintenance of LTP for more extended periods (late phase LTP) is dependent on gene transcription and protein synthesis (Nguyen et al., 1994). Several transcription factors, such as cAMP response element-binding (CREB), have been linked to late-phase LTP, and mice with a mutation of CREB have impaired hippocampal LTP and are deficient in spatial learning (Bourtchuladze et al., 1994; Silva, 2003).

A number of studies have implicated the hippocampus and hippocampal NMDA receptors in declarative memory functions. It has been well-established that lesions of the hippocampus in rodents as well as in humans can produce severe memory impairments (Scoville and Milner, 1957; Morris et al., 1982). For instance, ibotenic lesions of the hippocampus severely retard spatial learning and memory in the rat (Morris et al., 1982). Moreover, systemic administration of NMDA receptor antagonists such as PCP or MK-801 (dizocilpine) severely impairs spatial learning in rodents (Cain et al., 1996; Ahlander et al., 1999). Blockade of NMDA receptors in the hippocampus that disrupts hippocampal synaptic plasticity also impairs spatial memory functions. Thus, intrahippocampal infusion of 2-amino-5-phosphonovaleric acid (APV), an NMDA receptor antagonist, blocked both spatial memory and hippocampal LTP in rodents, giving evidence that spatial memory function is dependent on hippocampal NMDA receptors (Morris et al., 1986). In addition, infusion of APV into the dorsal hippocampus blocked Pavlovian contextual fear conditioning (Stiedl et al., 2000). Further evidence for a critical role of the NMDA receptor in memory is provided by knock-out mice lacking the NR1-subunit only in the CA1 region of the hippocampus. These mice were deficient in spatial memory performance and displayed impaired LTP induction (Tsien et al., 1996).

1.3.3. The glutamate hypothesis of schizophrenia

Since the dopamine hypothesis cannot explain all the symptomatology associated with schizophrenia, several other explanatory theories were developed. In particular, there is accumulating evidence that glutamatergic transmission is abnormal in schizophrenia. Kim and colleagues were the first to suggest disturbances in glutamate transmission, based on the finding that patients suffering from schizophrenia had low cerebrospinal fluid (CSF) glutamate levels (Kim et al., 1980). More recent studies indicate that the disturbance in glutamate involves a dysregulation in NMDA receptor function (Olney et al., 1999; Konradi and Heckers, 2003).

The "NMDA receptor hypofunction hypothesis of schizophrenia" is partly based on clinical observations obtained with NMDA receptor antagonists such as phencyclidine (PCP, "angel dust") and ketamine (Olney and Farber, 1995; Olney et al., 1999). These two compounds produce schizophrenia-like symptoms in healthy individuals and exacerbate symptoms in patients with schizophrenia (Luby et al., 1959; Javitt and Zukin, 1991; Krystal et al., 1994; Lahti et al., 1995; Adler et al., 1999). Unlike amphetamine, ketamine can in volunteers mimic both positive and negative symptoms seen in schizophrenia, including hallucinations, delusions, thought disorders and also cognitive disorders (Krystal et al., 1994). This finding suggests that NMDA receptor dysfunction may be critical for negative and cognitive symptoms in schizophrenia.

Ketamine-induced psychosis is blunted by clozapine but not by haloperidol (Malhotra et al., 1997), indicating that dopamine receptor blockade by itself is not sufficient to reverse the action of NMDA receptor blockade in humans. Furthermore, a direct comparison of healthy volunteers receiving sub-anesthetic doses of ketamine and

individuals with schizophrenia shows disruptions in working memory and thought disorder in both groups (Adler et al., 1999; Honey et al., 2004). These findings suggest that a deficient activation of NMDA receptors may be a critical component of the cognitive deficits observed in schizophrenia.

Moreover, postmortem studies have shown possible changes in glutamate receptor binding, transcription, and subunit protein expression in the prefrontal cortex, thalamus, and hippocampus of subjects with schizophrenia (Clinton and Meador-Woodruff, 2004). Furthermore, imaging studies have indicated reduced NMDA receptor binding in the hippocampus in non-medicated schizophrenia patients (Pilowsky et al., 2006). Also, genetic linkage and association studies have given evidence for a glutamatergic dysfunction in individuals suffering from schizophrenia (Owen et al., 2004). Moreover, Neuregulin 1 (NRG-1), which regulates NMDA receptors, has been identified as a susceptibility gene in an Icelandic cohort of schizophrenia patients (see chapter 1.4.) (Stefansson et al., 2002).

1.4. Schizophrenia and susceptibility genes

Although linkage and association studies during recent years have implicated a number of genes in schizophrenia, no major risk allele has yet been identified. Alleles conferring minor risk have however been suggested, and while defects in these genes are believed to increase the susceptibility for the disorder, the mechanisms remain to be established. COMT catalyzes the transfer of a methyl group from S-adenosylmethionin to orto-position in catecholamine transmitters such as dopamine. An aminoacid polymorphism in the COMT gene (Val158Met) has been found to change the activity of this enzyme and, thereby, the metabolism of dopamine resulting in a possible hypofunction of dopaminergic transmission in the prefrontal cortex (Egan et al., 2001). Impaired performance in prefrontal tasks involving dopamine function has been related to this polymorphism in schizophrenia patients (Egan et al., 2001).

Regulator of G-protein signaling 4 (RGS4) regulates G-protein coupled receptor signaling by accelerating the inactivation of active G-proteins, and it may regulate dopamine D_2 receptor signaling (Koelle, 1997; Neer, 1997). Microarray studies of postmortem brain samples have shown a reduced RGS4 expression in the dorsolateral prefrontal cortex (DLPFC), as well as in the motor and visual cortices, in schizophrenia patients versus control subjects (Mirnics et al., 2000; Mirnics et al., 2001).

The NMDA hypofunction theory of schizophrenia has also received support by recent genetic findings. Several schizophrenia susceptibility genes may function by interacting with glutamatergic transmission through various effects on the NMDA receptors (Moghaddam, 2003; Harrison and Weinberger, 2005). NRG-1 is a neurotrophic factor with a role both in the developing and adult brain. Molecular genetic studies have shown that NRG-1 is a schizophrenia susceptibility gene (Stefansson et al., 2002; Li et al., 2006) but the allelic variants conferring susceptibility have not been identified. Recent studies have implicated NRG-1 in glutamate-transmission, since it is an agonist at the receptor-tyrosine kinase ErbB4, which is enriched in the postsynaptic density (PSD) of glutamate synapses forming a multi-

protein complex with PSD-95 and NMDA receptors (Huang et al., 2000). NRG-1 has been shown to reduce NMDA receptor current by enhancing NMDA receptor internalization (Gu et al., 2005). A recent study has shown that NMDA receptors in the prefrontal cortex are sensitive to the inhibitory effect of NRG-1, possibly by a change in the ErbB4 protein complex (Hahn et al., 2006). Disruptions in this receptor, which is expressed at the postsynaptic protein PSD-95 together with the NMDA receptor, has been reported in schizophrenia patients (Li et al., 2007).

Additional possible susceptibility genes for schizophrenia are the Disrupted in Schizophrenia genes (DISC1 and DISC2) (Millar et al., 2000), which are also involved in a variety of psychiatric disorders (Kilpinen et al., 2008). Genetic evidence suggests that dysbindin (DTNBP1) is involved in psychotic liability not only for schizophrenia, but also for bipolar psychotic disorders and substance induced psychosis (Kishimoto et al., 2008). However, a recent study of 14 schizophrenia candidate genes, including RGS4, NRG-1, COMT and DISC1, showed no statistical significant genetic association with schizophrenia and schizoaffective disorders (Sanders et al., 2008).

1.5. The influenza virus

1.5.1. General background

The influenza virus was responsible for a most devastating epidemic, the Spanish flu of 1918 that killed more than 20 million people worldwide (Patterson and Pyle, 1991). Other notable influenza epidemics are the Asian flu of 1957 and the Hong Kong flu pandemic of 1986 that have swept the world since the "Spanish" flu.

The influenza viruses are enveloped single-stranded RNA, about 120 nm in diameter. The main antigenic determinates of the two integral membrane proteins are hemaggulutin (H) and neuraminidase (N) (see Figure 4). Influenza A virus genomes consist of 8 separate segments (see Figure 4). These segments are covered by the nucleocapsid proteins which together build the ribonucleoprotein (RNP).

The first human influenza A virus to be isolated in 1933 was an H1N1 subtype (Smith et al., 1933) and later this virus propagated to the mouse brain and two different neurotropic strains were achieved, the NWS/33 (Stuart-Harris, 1939) and the WSN/33 strains (Francis and Moore, 1940).

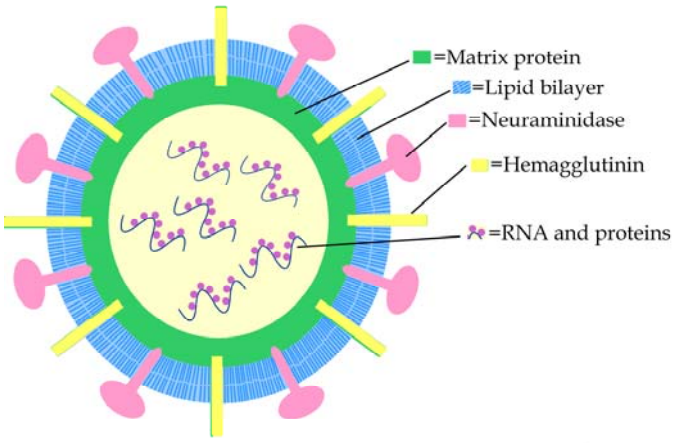

Figure 4. Representation of an influenza virion, features include the surface glycoprotein: hemagglutinin, neuraminidase and the genome segments (Wadenberg S, 2008).

The mouse neuroadapted influenza A/WSN/33 virus strain was chosen for this study because specific areas in the brain such as substantia nigra, ventral tegmental area and hippocampus are targeted after intracerebral injection of the virus (Takahashi et al., 1995). Following olfactory bulb inoculation, the virus was targeted to the ventral tegmental area, as well as thalamic relay and hypothalamic nuclei and following this mode of inoculation the infection was non-lethal (Mori et al., 1999) in contrast to the fatal disease in the study by Takahashi and coworkers (Takahashi et al., 1995).

1.5.2. Schizophrenia and the influenza virus

Individuals suffering from schizophrenia exhibit a birth excess of 10-15% in the winter-spring months (Torrey et al., 1977). Investigations into the potential cause for this seasonal variation, have suggested that individuals exposed to a maternal influenza A virus infection during certain stages of fetal life are at increased risk of developing schizophrenia later in life (Mednick et al., 1988). This study was conducted in Finland and several other studies have demonstrated a similar effect following different influenza epidemics in Australia (McGrath et al., 1994), United Kingdom (O'Callaghan et al., 1991; Takei et al., 1994), Denmark (Barr et al., 1990), and Ireland (Cannon et al., 1993). However, studies by Crow and Dane in the United Kingdom, Torrey and colleagues in ten states in the United States, and others found no association between the rate of schizophrenia and infection in the offspring of women who were pregnant in the second trimester during the 1957 pandemic (Crow and Done, 1992; Susser et al., 1994; Torrey and Rawlings, 1996). However, most of these studies have an ecological design which means that although the women included in these studies were pregnant and living in a region where the infection was prevalent, it was not known if they were actually infected with the influenza virus. Thus, more studies of case records are needed to enable more definitive conclusions (Munk-Jorgensen and Ewald, 2001) whether schizophrenia might be related exposures to an influenza virus infection during early life.

1.6. Animal models of schizophrenia

To develop a relevant animal model that can mimic a human psychiatric disorder as complex as schizophrenia is a great challenge in view of the heterogeneous symptomatology and the lack of any clear neuropathological changes or neurobiological cause of the disorder. The validity of animal models of human disorders should theoretically be reflected in their ability to mimic etiology, pathology, symptomatology and responsiveness to treatment. It is apparent that some of the key symptoms of schizophrenia can most likely not be modeled in rodents, such as thought disorders, delusions, paranoia and also hallucinations. This fact has resulted in an approach in which the animal modeling aims at recapitulating some features of the symptomatology or pathophysiology of the disorder. This means that rodent models of schizophrenia lack construct validity and they may often have doubtful face validity while they may have high predictive validity (McKinney and Moran, 1981; Ögren,

1996; Lipska and Weinberger, 2000). This situation is illustrated by classical pharmacological models such as amphetamine (see below).

Schizophrenia models can be divided in three different categories; pharmacological models, experimentally induced brain lesion models and genetic mouse models. Most pharmacological animal models of schizophrenia are based on the "dopamine hypothesis" and on the use of dopamimetic drugs to elicit a behavioral response, mostly with increases in locomotor activity (see Table 1). Spontaneous or elicited increases in locomotor activity are believed to reproduce positive psychotic symptoms of stereotypic behavior (McKinney and Moran, 1981; Ögren, 1996; Lipska and Weinberger, 2000). These models have been effective in drug development and most current antipsychotic drugs are developed on the basis of these models (Ögren, 1996). However, the main limitation of this dopamine approach is the failure to have an adequate measure of negative symptoms and cognitive dysfunctions (Ögren, 1996). Also animal genetic models have recently focused on the role of dopamine in schizophrenia (Gainetdinov et al., 2001). To study the role of the intrasynaptic dopamine transporter, knockout mice (DAT-KO) were generated by Caron and colleagues (Giros et al., 1996). These mice showed locomotor hyperactivity, which was reversed by haloperidol and clozapine, as well as cognitive impairment and pre pulse inhibition (PPI) disruption (Gainetdinov et al., 2001). Thus, locomotor stimulation caused by dopamine agonists or by increased intrasynaptic dopamine is blocked by most, if not all, current antipsychotic drugs, which indicates that this test has predictive validity. However, there are several problems with this type of circular and analogous reasoning. First, a number of schizophrenia patients do not display agitation or stereotypic behavior but are rather withdrawn (see introduction). Moreover, agitation and even psychotic symptoms are not unique for schizophrenia but can also be seen in several other neuropsychiatric disorders. Thus, it is important in the behavioral analysis consider other ways of interpreting behavioral results, e.g. using an endophenotypic approach.

A major problem in the behavioral analysis is to model negative and cognitive deficits which do not respond to antipsychotic drugs. Since learning and memory impairments involve many domains of cognition, including attention and information processing deficits, it is clear that not one test is able to give an accurate description of the multiple dimensions of cognition. It is therefore preferable to use several behavioral tests in which it may be possible to characterize multiple functional domains including changes in information processing, attention or cognition (see Table 1). By this approach it may be possible to define a subset of symptoms that are possible to assess across species such as PPI and latent inhibition (LI). PPI refers to the reduction in startle response toward a startle-eliciting "pulse" stimulus when it is preceded by a sub-threshold "prepulse" stimulus (Hoffman and Searle, 1965). Schizophrenia patients also often show deficits in attention which is monitored by studies on LI in rodents. LI refers to a process in which exposure to a stimulus, which is not connected to reward, retards conditioned associations with that particular stimulus. LI is part of a process by which overload of information is reduced by inhibiting formation of unnecessary

memories. LI is generally believed to be defective in schizophrenia patients (Baruch et al., 1988) and has been related to increased dopamine transmission (Swerdlow et al., 2003). Importantly, systemic treatment with antipsychotic agent in rats has been reported to facilitate LI (Weiner and Feldon, 1987). Sensorimotor gating deficits refer to a reduced capacity to 'gate out' (filter) sensory and cognitive information and it has been demonstrated in many schizophrenia patients. This deficit is manifested in disrupted PPI and LI. Such deficits have also been found in rodents after administration of dopaminergic agonists and NMDA receptor antagonists (Solomon and Staton, 1982; Weiner and Feldon, 1987; Weiner et al., 1988; Geyer et al., 2001; Gaisler-Salomon and Weiner, 2003). Disruption in PPI is often used as an animal model of sensorimotor gating deficits in schizophrenia as it is impaired in schizophrenia patients (Braff et al., 2001).

The pathogenesis of schizophrenia may also involve subtle disturbances in neurodevelopment resulting into aberrant afferent-efferent functional circuitry (Weinberger and Manreco, 2003). The transcription factor Nurr1, which is a member of the orphan nuclear receptor steroid/thyroid hormone receptor superfamily, has been identified as a critical genetic factor for the development of midbrain dopamine neurons (Zetterstrom et al., 1997). Recently, it has been demonstrated that adult mice with reduced Nurr1 expression (heterozygous male and female mice) showed an increased locomotor response to novelty, amphetamine- and PCP-treatment (Rojas et al., 2007). Furthermore, these animals exhibited decreased emotional memory and had reduced dopamine turnover in striatum and increased turnover in the prefrontal cortex (Rojas et al., 2007).

Pharmacological models based on the NMDA receptor hypofunction model of schizophrenia, have received much attention during the past decade (Olney and Farber, 1995; Olney et al., 1999). The NMDA hypofunction models aim to explore the negative and positive symptoms as well as the cognitive deficits and the neurodegenerative processes observed in schizophrenia patients. The rationale behind the animal model is based on the ability of PCP and ketamine to induce positive, negative and cognitive symptoms in humans (see glutamate hypothesis, chapter 1.3.3.). Phencyclidine (see Figure 5), commonly known as PCP or “angel dust”, is a synthetic arylcyclohexylamine with a complex and unusual pharmacology. In rodents and in monkeys, NMDA receptor antagonists such as PCP, ketamine and MK-801 reproduce a number of behaviors resembling schizophrenic symptoms including locomotor hyperactivitity, behavioral sterotypies, sensorimotor gating deficits, reduced social interactions and various cognitive deficits (Javitt and Zukin, 1991; Jentsch and Roth, 1999; Morris et al., 2005).

It is generally believed that the spectrum of behavioral effects of PCP is primarily due to NMDA receptor blockade mediated by occupancy of the PCP binding site located within the channel (Olney et al., 1999). However, PCP produces a number of behavioral and neurochemical changes, after both acute and repeated administration, which may mediate at least some of its actions. For instance, after acute administration, it increases extracellular levels of glutamate, dopamine, noradrenaline and

acetylcholine in the prefrontal cortex (Verma and Moghaddam, 1996). Moreover, acute PCP also increases the firing rate of dopaminergic nucleus accumbens neurons (O'Donnell and Grace, 1998). Repeated administration of PCP also produces long-term behavioral and neurochemical changes, which can last for weeks after withdrawal of the psychostimulant (Jentsch et al., 1997; Jentsch and Roth, 1999). Therefore, in the behavioral and neurochemical analysis it is critical to distinguish between the acute and long-term effects of PCP, i.e. whether the animals are tested when the drug is on board or whether the effects are tested after withdrawal of the compound. Thus, repeated relatively high doses administration of NMDA channel blockers such as PCP has been shown to cause neurodegenerative changes in the rat cortex, in particular in the posterior cingulate/retrosplenial cortex, anterior cingulate as well as in the hippocampus and amygdala (Olney and Farber, 1995). Since most available studies are based on high doses of PCP, it is difficult to know whether the behavioral effects are due to non-specific effects on behavior or due to a neurotoxic action affecting large areas of the hippocampal-cortical circuitry (Olney et al., 1999). Thus, analysis of the molecular and behavioral mechanisms underlying the effects of low/high doses of acute and repeated PCP in animal models may shed new light on the role of the NMDA receptor in the etiology of schizophrenia. In this analysis, the role of dopamine must also be considered since PCP also binds to the dopamine receptor in its high affinity state (Kapur and Seeman, 2002). Consistent with this observation, the locomotion induced by PCP in rats was found to be blocked by three dopamine D_2-receptor antagonists (Ögren and Goldstein, 1994). These findings suggest that some of the psychomimetic actions of PCP could be due to an increase in dopamine transmission and mediated via high affinity dopamine D_2-receptors.

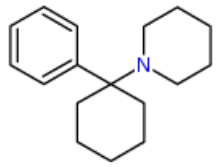

Figure 5. Phencyclidine

Although there is no direct evidence for an abnormal NMDA receptor in schizophrenia, the potential role of the NR1 subunits of the NMDA receptor are evaluated in a genetic mouse model (Mohn et al., 1999). Mutant mice, which express only 5% of the endogenous levels of NR1-subunits, showed hyperlocomotion and stereotypical behavior that were antagonized by haloperidol and clozapine (Mohn et al., 1999). These mice also show deficit in social interaction, which could be improved by clozapine (Mohn et al., 1999). However, the NR1-mutant mice showed a decreased locomotor response to PCP, which is contrary to the behavioral phenotype anticipated from a schizophrenia model. Another animal model related to the NMDA receptor function is the NRG-1 mutant mouse, which showed hyperactivity in the open field test that was reversed by clozapine. Moreover, these mice exhibited disrupted PPI and diminished social recognition memory (Stefansson et al., 2002; O'Tuathaigh et al., 2007).

There is evidence for a role of neurodevelopmental disturbances in schizophrenia, which has led to experiments employing manipulations of brain functions and genes that may play a role in the early development of the central nervous system (Lipska and Weinberger, 2000). The rat neonatal ventral hippocampal (VH) ibotenic lesion model has been proposed as a developmental model of

schizophrenia, since the lesion involves the ventral hippocampus which projects to the prefrontal cortex. This brain region, which corresponds to the anterior hippocampus in humans, is characterized by anatomical abnormalities in schizophrenia patients (Suddath et al., 1990). When tested as adults, lesioned animals display a behavioral phenotype including psychomotor agitation (hyperactivity), deficits in PPI, and in social interaction, measures presumed to reflect positive symptoms and sensory gating deficits and negative symptoms, respectively (Lipska and Weinberger, 2000; Daenen et al., 2003; Lipska, 2004). Moreover, the locomotor responses were blocked by antipsychotic drugs, and the social impairments were reversed by clozapine but not haloperidol.

There is increasing evidence that associates schizophrenia with exposure to toxoplasma parasites and viruses during early life. In experimental animals, infections with *Toxoplasma gondii* (Webster, 2007) and a number of other viruses have been reported to cause a variety of behavior disturbances when the animals reach adulthood (Hornig and Lipkin, 2001; Pearce, 2001; Patterson, 2002; Shi et al., 2003). For instance, Borna virus disease is associated with behavior analogues to autism (Briese et al., 1999; Hornig et al., 2001) and Herpes simplex virus infections with altered PPI (Engel et al., 2000). In light of the many different infections conferring schizophrenia risk, it has also been proposed that an elevation of maternal cytokines caused by the infection is responsible for the behavior changes. This hypothesis has been investigated in another neurodevelopmental animal model of schizophrenia that employs a maternal inflammation. Pregnant mouse dams are administrated the cytokine-releasing agent polyriboinosinic-polyribocytidilic acid (polyI:C) on gestation day 9. This treatment resulted in an offspring which in adulthood displayed multiple schizophrenia-related behaviors, including changes in exploratory behavior, disturbances in PPI and LI as well as spatial working memory deficits. In addition, the pre-exposed animals showed an enhanced locomotor response to systemic administration of amphetamine (Shi et al., 2003; Meyer et al., 2005; Meyer et al., 2006).

Behavioral response in animal model	Predicted clinical symptom
Hyperlocomotion induced by dopamine agonists (amphetamine/apomorphine)	Psychotic (positive symptoms)
Hyperlocomotion induced by NMDA receptor blockade (PCP)	Psychotic (positive symptoms)
Stereotypic behavior induced by dopamine agonists	Stereotypic behavior (mannerism)
Stress-induced hyperlocomotion	Vulnerability to stress
Reduced social interaction with unfamiliar individual(s).	Social withdrawal (negative symptoms)
Sensorimotor-gating deficits (pre pulse inhibition)	Information-processing deficits
Deficits in latent inhibition	Attention deficits
Impairment in delayed alternation and water maze tasks (spatial memory)	Deficits in working- and long-term-memory (cognitive dysfunctions)

Table 1. Behavioral changes in animals and predicted clinical symptoms of schizophrenia. Modified from (McKinney and Moran, 1981; Ögren, 1996; Lipska and Weinberger, 2000)

2. AIMS OF THE THESIS

The overall aim of this thesis is to develop mouse models for analysis of schizophrenia-related pathogenesis with special emphasis on glutamatergic cognitive functions and gene expression.

2.1. Specific aims of the thesis

- To develop a NMDA receptor blockade model by a low doses of phencyclidine without any sensorimotor disturbances, in order to investigate schizophrenia-related cognitive and negative symptoms.

- To examine the validity of the low dose phencyclidine model by studying the ability of "atypical" and "typical" antipsychotic drugs to normalize PCP-induced effects on cognition.

- To investigate the effects of early postnatal exposure to influenza A virus on cognitive and emotional behavior of adult mice, and to study whether the exposure to virus infection will affect gene expression.

- To study whether an early life virus infection exacerbate the behavioral response to NMDA receptor blockade in adult mice.

3. MATERIALS AND METHODS

3.1. Animals

The following animals were used throughout the different studies:

- C57BL/6 mice from Scanbur/B&K (Stockholm, Sweden), Charles River (Germany), MTC (Karolinska institutet, Stockholm, Sweden), and Taconic (Denmark).

- Transporter associated with antigene processing 1 (*Tap 1*) knockout mice generated on a C57BL/6 background were bred at the Microbiology and Tumor Biology Center and at the department of Neuroscience (from Jackson Lab mice homozygous for the $Tap1^{tm1Arp}$ targeted mutation, lack $CD8^{+}$ cells), Karolinska Institutet, Stockholm.

The animals were habituated to the animal facilities for at least five days before the start of the experiments. In all the behavioral experiments, the animals were handled daily 3-5 days during the pre-experimental period by the operator performing the actual experiment. In addition, the animals were habituated to the test rooms at least 45 minutes before the initiation of the experiment. These procedures were used to reduce variations in the experimental results.

All experimental procedures were approved by the local Animal Ethics Committee.

3.2. Compounds

The test compounds used were obtained from commercial sources. The compounds were injected intraperitoneally (i.p.) or subcutaneously (s.c.) in a volume of 4 ml/kg. All doses refer to the salt and/or base of the respective compound.

PCP (1-(1-Phenylcyclohexyl) piperidine hydrochloride; Sigma, USA) in the dose range 0.25-4.0 mg/kg was dissolved in saline and administered s.c.

D-Amphetamine hydrochloride (Apoteket, Sweden), in doses of 1.5 or 3.0 mg/kg was dissolved in saline and administered i.p.

Antipsychotic drug treatment: Clozapine (8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo(b,e) (1,4)-diazepine; Sigma, USA), tested at doses of 0.5 and 1.0 mg/kg, and haloperidol (4-(4-[p-Chlorophenyl]-4- hydroxypiperidino)-4'-fluorobutyrophenone; Sigma, USA), tested at the doses of 0.05, 0.1 and 1.0 mg/kg, were dissolved in 40% propylene glycol solution (v/v) in water. These drugs were administered i.p.
Control animals always received the corresponding vehicle treatment.

The doses of clozapine and haloperidol used in the thesis were selected based on relevant doses for dopamine receptor blockade in the rat (Ögren, 1996). It is difficult

to translate clinical doses of this compound to the rat due to the high protein binding of clozapine in man and the large variation of doses used in the clinic. Clinical doses of clozapine treatment are given in a dose range of 300-600 mg/kg/day (Kinon et al., 2004), while haloperidol is administered in a dose range of 2-40 mg/kg/day (Sacristan et al., 2000).

3.3. Behavioral experiments

Since a detailed materials and methods section is present in each paper, the methodology will be only briefly presented here. In addition, some critical issues related to the tests used are briefly discussed.

In most experiments, the animals were experimentally naïve in order to avoid possible carry-over effects between tests. Moreover, a limited number of behavioral tests were used with a focus on assessment of cognitive dysfunctions in schizophrenia. The range of behavioral domains relevant for the clinical symptoms of schizophrenia is presented in table 2. A particular aim was to examine the contribution of sensorimotor and neurological disturbances produced by NMDA receptor blockade.

The following behavioral methods have been used to examine various domains of schizophrenia-related behaviors in mice (see Table 2). Moreover, the assessment of the anxiety-related behavior was performed using the elevated plus-maze and open field test. The sensorimotor and neurological disturbances were evaluated in the rotarod, a test for motor and balance control.

Behavioral response in animal model	**Predicted clinical symptom**
Spontaneous hyperactivity	Psychosis (positive symptoms)
Enhanced locomotor response to d-amphetamine or PCP	Psychosis (positive symptoms)
Impairments in spatial memory	Cognitive dysfunctions
Impairments in working memory	Cognitive dysfunctions
Reduction in social interaction	Social withdrawal
Changes in anxiety-related behavior	Emotional flattening
Changes in aggression	Problems in interpersonal relationship

Table 2. The behavioral responses in mice and their hypothesized relationships to clinical symptoms of schizophrenia.

3.3.1. Locomotor activity test

The examination of spontaneous or drug-induced locomotor activity is one of the most commonly used tests in the fields of neuropsychopharmacology and behavioral neuroscience. Depending on the design of the test, it is used to study everything from possible disturbances in motor function to spontaneous exploration and basal output of motor mechanisms. Since most types of behaviors of rodents are expressed via motor-responses, it is difficult to interpret the specific functional role of changes in locomotor activity. However, the initial recording when the animal is placed in the locomotor box

allows for examination of the initial exploratory phase, which last for about 20-30 minutes and it can also give information of the rate of habituation to a novel environment.

In this thesis, locomotor activity was in general examined in a novel test environment. Spontaneous, phencyclidine- or amphetamine-induced motor activity was performed using a computerized locomotor activity system. Rearing was measured by counting the number of times an animal stands on its hind legs. Motility was defined as all movements of a distance of 4 cm and represents measurement of general activity. Locomotion was measured by counting the number of times an animal moved from one side of the test cage to the other side (a distance of at least 32 cm).

3.3.2. Motor coordination in the rotarod

Poor performance in behavioral tests may be due to sensorimotor disturbances, rather than impairments in cognitive functions. Therefore, motor performance was assessed using the rotarod (Cat. No. 7650, UGO BASILE, Biological Research Apparatus, Varese, Italy) test, which is sensitive to sensorimotor disturbances involving reduction in dopamine and glutamate receptor function.

In the rotarod, the mice were individually placed on the rotating cylinder and the speed was gradually accelerated from 4 to 40 revolutions per minute (rpm) over 300 sec. The animals were trained three times with a 15 min inter-trial time. Latency and speed to fall from the rotating rod were recorded. The animals were tested prior to and after treatment corresponding with the water maze experiments. The height above the soft bedding material was approximately 20 cm, a height from which mice, when falling, will land on their feet.

3.3.3. Elevated plus-maze test

The elevated plus-maze is an etiologically-based animal model of anxiety-related behavior (Rodgers et al., 1997) that does not require any previous training. Both rats and mice have an innate tendency to avoid open areas or brightly lit arenas and this behavior is related to a safety assessment strategy. The elevated plus-maze has been validated by anxiolytic drugs. However, a problem with the test is that it is sensitive to changes in locomotor activity and it also lacks sensitivity to discover subtle changes in anxiety-related behavior. The test is performed in a plus-shaped maze (see Figure 6), which consists of two enclosed arms, two open arms and a central area, elevated 1 m above the floor. The parameters recorded are time spent in the open arms, closed arms and central region, total distance traveled during the experiment and the number of crossing between closed and opened arms. Open to total ratio (OTR) for time and entries were calculated as index of open arm exploration. Total arm entries and closed arm entries were both analyzed as measures of non specific changes in locomotor activity independent of OTR.

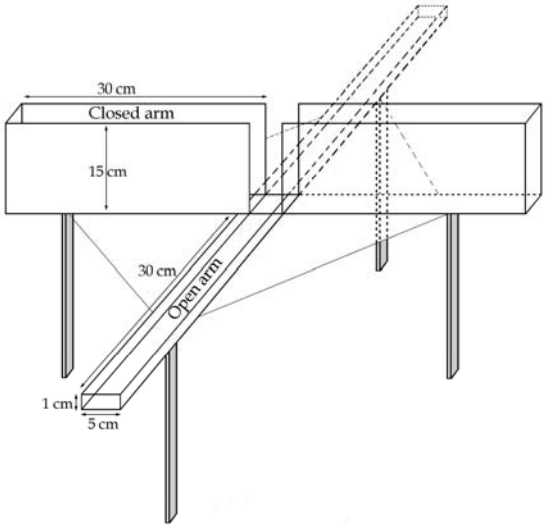

Figure 6. The elevated plus-maze (Wadenberg S, 2008)

3.3.4. The water maze task

Spatial learning and memory were examined using the Morris water maze task (see Figure 7), which has become one of the most frequently used laboratory tools to study learning and memory in rodents. In this thesis, two variants of the water maze task have been used. The "hidden" platform variant, which involves hippocampal functions (Morris et al., 1982) but it also, is dependent on various cortical inputs (Tsien et al., 1996). The "cued"/"visible" platform variant is used to examine visual function and swim motivation.

There are advantages and disadvantages that are associated with the water maze task. The major advantage is that the mice do not need to be food- or water-deprived, since learning is motivated by an aversive stimulus i.e. water exposure. The animal has to acquire the task by using extra-maze cues and it can not depend on olfactory-cues, which is an advantage of this task compared with dry mazes (e.g. radial and Barnes maze).

There are also several disadvantages with the water maze, including the stressful reaction when putting the animal into the water. This means that anxiety states or stress may change or interfere with the performance in the task. Some of these responses, e.g. thigmotactic behavior, must be suppressed in order to have an adequate performance. The water maze is also sensitive to the age of the animal (Brandeis et al., 1991). In our study, age of the testing subject was limited to 3-6 months corresponding to adult age. During the acquisition period where the animals are learning the task, it is assumed that they are processing extramaze cues to form a spatial map of the test arena, which makes it possible to navigate to the hidden platform position. The time it takes a mouse to find a hidden platform in a water pool after the previous trial, using available external cues, is used as a measure of spatial memory. However, it has become increasingly clear that acquisition of this task requires a general knowledge of the entire test procedure. Pre-testing in the water maze has important consequences for subsequent performance in the training sessions. By changing the design of the test

procedure, it is possible to examine both spatial working memory as well as long-term reference memory. Working memory is defined as spatial information that is only useful during a specific training session, whereas reference memory is information that is required to learn the task across all training sessions.

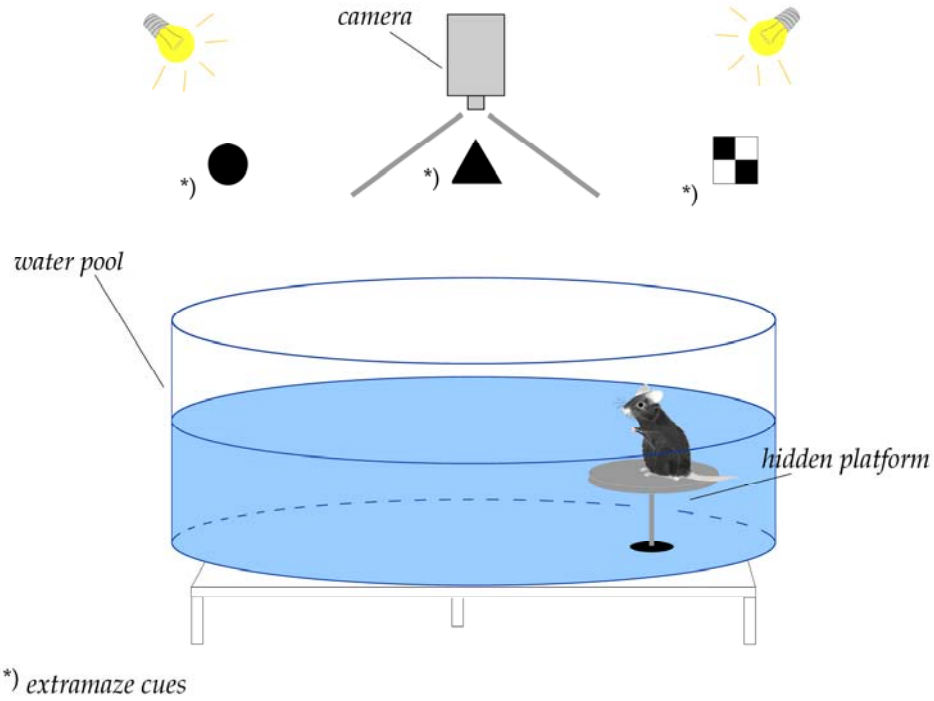

Figure 7. The water maze in the hidden platform task (Wadenberg S, 2008)

Pre-training or cued platform training: The procedure was conducted using a visible platform and different starting points in order to familiarize the animals with the water and the general procedure. No external cues were present during pre-training. Both platform position and starting position were randomized.

Acquisition phase: During training, the hidden escape platform was located in one of the four quadrants (northwest, southwest, northeast or southeast). In each trial, the mouse was placed in the water facing the pool wall at one of four starting points (north, south, west or east pole) to search for a hidden platform. If the mouse did not find the platform within 90 s, it was gently guided to the platform by the experimenter and allowed to remain on the platform for 10 s. During each trial, the mouse's escape latency, swim speed, swim distance and time spent near walls (thigmotaxic swimming) were measured by software (HVS Image Ltd, Hampton UK/WaterMaze Edinburgh, UK) connected to a video camera (JVC, Japan).

Retention test (probe trial): During the retention test, the platform was removed from the pool. The mouse was released into the water tank from a position opposite to the

quadrant where the platform had been during the training sessions. The animals were allowed to swim freely for a 60 s period. The spatial probe trial was used to test the animals' knowledge of the precise location of the platform relative to the available external cues. In the retention test, the time to first cross the area of former platform location, the total number of crossings of this area, time spent in the different quadrants, time spent in target zone (defined as the circular area with a radius of 20 cm from the center of the former platform position) were calculated to evaluate spatial memory performance. All other parameters remained the same as during the acquisition phase.

Working memory: Water maze performance in the spatial working paradigm is dependent upon the temporary storage of the remembered location of the hidden platform while the animal navigates through the maze. The position of the invisible platform changed on each day of testing, but remained in the same position across the two trials on a given day. A different starting position was used for each trial, and different sequences of starting positions were used over days, although the distance between starting position and platform position in these two trials were equal. The assessment of working memory took place over 4-6 days. Mice were allowed a maximum of 60 s to locate the invisible platform. If they failed, they were then guided to the platform. The animals were allowed to spend 10 s on the platform before initiation of the test trial (trial 2). For the working memory test, the interval between trial 1 and trial 2 was maximum 15 s. The critical measure of working memory was the improvement of escape latency from trial 1 (when the location of the platform was unknown) to trial 2. All other parameters remained the same as during the acquisition phase.

3.3.5. Social interaction test

Social interaction has been defined either as active or passive (File and Hyde, 1978) and in this thesis only active social interaction was studied. Testing was conducted in a homecage-like environment instead of an unfamiliar open field (Sams-Dodd, 1995; Sams-Dodd, 1998; Sams-Dodd, 1998). Moreover, only one of the pair was treated with PCP, i.e. the resident mice, which were kept individually in the test cage 24 h before the experiment to habituate themselves to the novel environment. Testing was initiated by placing another mouse (the target mouse) on the other side of the cage. The interaction between the resident mouse and the target mouse was evaluated for 5 min. The total duration and frequency of each behavior expressed by the resident mice were analyzed. The following behaviors, expressed by the resident mice, were recorded by the operator and subsequently analyzed using the software (JWatcher 1.0 http://www.jwatcher.ucla.edu).

Walking: The resident mouse moves around in the cage
Immobility: The resident mouse has no apparent locomotor activity
Rearing: The resident mouse stands on its hind legs and sniffs the air
Sniffing: The resident mouse sniffs the target mouse
Following: the resident mouse follows the target mouse

Body contact: contacts between the two mice, including staying together or sniffing each other
Attacking: The resident mouse attacks, punches or bites the target mouse
Sniffing substrate: The resident mouse has its nose very near to bedding and sniffs
Digging: The resident mouse digs the bedding
Grooming: The resident mouse cleans its fur

3.3.6. Novel cage observation test

The novel cage test has recently been used to characterize multiple behavioral functions in mice and was shown to effectively detect strain differences in pre-weaning mice with regard to risk behavior (Marques et al., 2008). The test procedure used in this study was adapted from Marques and colleagues (Marques et al., 2008). Mice were tested individually in a transparent cage (containing shavings), which was identical with their home cage. The following categories and elements of behavior were recorded for 5 minutes by the operator:
Walking: The mouse moves around in the cage
Immobility: The mouse has no apparent locomotor activity
Rearing: The mouse stands on its hind legs and sniffs into the air
Sniffing substrate: The mouse has its nose very near to the bedding and sniffs
Digging: The mouse digs under the bedding
Grooming: The mouse cleans its fur

3.3.7. Open-field test

Spontaneous motor activity and anxiety-like behavior were assessed in an open field chamber, which is a brightly and evenly illuminated square arena. The arena was illuminated by 4X60 W lamps mounted 1.5 m above the box, resulting into a bright light condition. The area was divided into 16 quadrants (4 central and 12 peripheral, 10 cm from the walls). Mice were placed individually into the centre of the open field and left to explore for 5 min. The time spent in the central and peripheral zones, total distance travelled during the experiment and numbers of crossings were recorded and analyzed. To assess anxiety-like behavior, the percentage of the time spent in the centre of the open field was used.

3.4. Studies on genes expression

3.4.1. Reverse transcription, polymerase chain reaction and real time-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was used in papers IV, V, VI. RT-PCR involves two main consecutive steps, the reverse transcription and PCR amplification. RNAs were first reverse transcribed into cDNAs using a total extracted RNAs and reverse transcriptase, random hexamer primers and dNTPs. The obtained cDNAs were used as template for subsequent PCR amplification using gene specific primers. Quantitative real-time PCR was used for analyzing the levels of specific gene transcripts.

3.4.2. cDNA array

In paper IV, a gene array was used to detect possible effects of the fetal exposure to a maternal virus infection on gene expression. For this purpose, we used the Atlas Mouse 1.2 cDNA Expression Array (CLONTECH) containing cDNA segments representing a total of 1176 mouse genes spotted on a nylon membrane. Briefly, probing of the cDNA arrays was performed as follows: RNAs were extracted from virus-exposed and control animal brains, and used to produce reverse-transcribed probes. A pooled set of primers complementary to the genes represented on the array (CLONTECH) was used for the reverse transcription probe synthesis, which was radiolabeled with P^{32} α-dATP. After hybridization, the array membrane was washed and visualized using a phosphorimaging screen.

Analysis of cDNA arrays: Phosphorimages were scanned and analyzed. Each dot on the membrane represented a gene and its level of expression is represented by the intensity of the dot. Background was subtracted using global image regions, and the intensities are normalized by dividing the intensity of each dot by the total intensity of all dots on each respective array. Ratios of expression, obtained from the virus-exposed animals relative to the control-animals were generated. Genes were considered differentially expressed if this ratio exceeded 1.6. Levels of transcripts from such genes were further analyzed by real time-PCR.

3.4.3. In situ hybridization

In paper IV, we have used *in situ* hybridization using riboprobe, to detect specific mRNA sequences in tissues section. Briefly, the brain tissues of sections cut at thickness of 20 μm were fixed (4% paraformaldehyde). Sections were exposed to a prehybridization treatment before a riboprobe was added to form the hybridization solution. Riboprobes were prepared from cDNAs via an *in vitro* transcription reaction and thereafter labeled. The sections were subsequently processed to remove non specific binding and the labels were detected using photographic film.

3.4.4. Methodological considerations concerning gene expression studies

Gene expression changes have major effects on brain functions; small shifts in the expression level can be related to behavioral differences between individuals. An advantage of methods measuring mRNA-expression is that they are highly sensitive and have a high anatomic resolution since gene-expression can be investigated *in situ*. However, the main disadvantage of measuring mRNA-expression is the fact that it is often not clear to what extent the amount of the transcript is correlated to protein levels and their function. In the present thesis we have used real time RT-PCR and *in situ* hybridization for the analysis of a small number of genes, which are known to play a role in neuronal function. The microarray was used to investigate the expression levels of a larger number of genes in a non-hypothesis driven fashion. Nevertheless, real time RT-PCR is crucial to validate the microarray results obtained from individual animals. A problem with RT-PCR is that the brain-tissue has to be

sonicated and homogenized before the measurement procedure, which means that this method lacks anatomical resolution.

RESULTS AND DISCUSSIONS

4.1. Effects of repeated phencyclidine treatment on spatial learning

4.1.1. Effects of low dose phencyclidine on spatial learning

The NMDA receptor hypofunction hypothesis (Olney and Farber, 1995; Olney et al., 1999) is based on the observation that noncompetitive antagonists at the NMDA receptor such as PCP and ketamine, can reproduce schizophrenia-like psychosis in humans, including positive symptoms, negative symptoms as well as cognitive impairments (Javitt and Zukin, 1991). A number of studies in rodents have also demonstrated schizophrenia-like symptoms in the PCP-model (Jentsch and Roth, 1999; Mouri et al., 2007). It is also well established that acute treatment with relatively high doses of PCP produces cognitive impairments in rodents (Mouri et al., 2007). It has been proposed that chronic or repeated PCP treatment rather than single-dosing is a more optimal model for induction of psychosis-like behavior in rodents and in monkeys (Jentsch and Roth, 1999). Repeated PCP-treatment with high doses (10 mg/kg) has been found to produce behavioral effects which can endure several weeks after withdrawal (Jentsch and Roth, 1999; Qiao et al., 2001; Mouri et al., 2007). However, it is not clear at present whether the long-term effects seen after withdrawal of PCP are due to neurotoxic effects producing behavioral disturbances. The major problem in studies of cognition is that acute or repeated NMDA receptor blockade can produce a number of behavioral effects (Koek et al., 1988) including changes in sensorimotor functions that may confound measures of learning and memory. In fact, studies in the rat have failed to dissociate the effects of doses of NMDA receptor blockers that impair learning from doses that cause dysfunctions in sensorimotor functions (Cain et al., 1996; Ahlander et al., 1999; Creeley et al., 2006).

In view of these considerations, the primary aim of the studies in **Paper I** was to develop and characterize a subchronic, low dose PCP procedure, by which it is possible to study the effects of the antagonist on cognition without any influence on sensorimotor functions. The study in **Paper I** explored the possibility to combine repeated administration with testing of the drug on board while **Paper II** used an approach in which repeated administration was followed by testing without the drug on board i.e. a withdrawal procedure. It was predicted that this design would allow the possibility to dissociate the acute effects of NMDA receptor blockade from long-term effects, where modulation of neuronal plasticity may occur. In addition, by using low doses of PCP it seems likely that the behavioral effects observed would not be related to the neurotoxic action of the compound (Olney et al., 1999; Stone et al., 2007). For this purpose, PCP was given to the mice for seven days, after which spatial training is initiated. The drug treatment continues during training, which means that PCP was present in the brain and thus blocking NMDA receptors, during acquisition and memory retention. This procedure must be clearly distinguished from the drug withdrawal procedure (see below), in which training is performed in a drug-free state at various times after the final dose of the drug (see methods section).

A major concern in studies with mice is that they show marked strain differences in their ability to acquire the water maze task as well as other cognitive tests (Brown and Wong, 2007). In this study, saline-treated C57BL/6J mice performed well in the task with a gradual decrease in latency to find the hidden platform over the training trials, and high spatial selectivity in the retention trial. The rapid acquisition of this spatial task is probably due to the test procedure used, which is somewhat different from the standard water maze procedure (see materials and methods).

To establish a basis for analyzing the effects of PCP, the range of doses producing locomotor stimulation in the mouse was determined. The range of doses tested included doses which either caused strong or no locomotor stimulation. Mice treated repeatedly with 2.0 and 4.0 mg/kg of PCP (these doses produced strong motor stimulation) were severely impaired in the spatial task due to neurological and sensorimotor side effects, which excluded further testing. In fact, swimming ability was severely compromised at these two doses, most likely related to NMDA receptor blockade in motor systems and in the spinal cord (Grillner et al., 1997). PCP produced a dose-dependent impairment in spatial learning and memory in the water maze task at doses of 0.5 and 1.0 mg/kg while the 0.25 mg/kg dose was without effect. A detailed analysis showed however that the 1.0 mg/kg dose of PCP produced neurological side effects which could interfere with the acquisition performance. Moreover, somewhat unexpectedly, PCP at the 1.0 mg/kg dose failed to produce any evidence for a tolerance development to the neurological side effects unlike previous report in the mice (Podhorna and Didriksen, 2005). Subchronic PCP at the 1.0 mg/kg dose also caused deficits in non-spatial training using the visible platform test in the water maze. However, this effect appeared to be more pronounced over days of treatment since acute treatment of PCP failed to impair escape latency to the visible platform. It may be that subchronic PCP either changes swim motivation or that the neurological effects of PCP are more pronounced after repeated versus single-dose administration. It can be concluded from the present studies that repeated treatment with low of PCP (0.5 mg/kg) can produce cognitive dysfunctions in the water maze task without interfering with sensorimotor or motivational functions. It is notable that this dose corresponds to the threshold dose for motor stimulation suggesting a relationship between sensorimotor dysfunctions and motor control.

The behavioral syndrome induced by PCP and MK-801 in rodents has been suggested to represent an animal model of schizophrenia and as a model of the cognitive deficits in schizophrenia (Andine et al., 1999; Bardgett et al., 2003, Mouri et al., 2007). However, the behavioral and neurochemical mechanisms behind the effects of acute PCP on cognition have never been clearly established. It is important to note that the 0.5 mg/kg dose of PCP is low compared to most studies on cognition using acute PCP (Mouri et al., 2007). It is possible that the present results may be due to an inhibition of NMDA receptors in hippocampal glutamatergic systems involved in spatial learning. However, there exists no information on the effects on hippocampal or cortical glutamatergic systems at this low dose of PCP in rodents. At a ten times higher dose, acute PCP (5 mg/kg i.p.) has been shown to increase glutamate release in the

prefrontal cortex (Takahata and Moghaddam, 2003). The increase in glutamate release in the prefrontal cortex is implicated in the regulation of motor behavior since blockade of non-NMDA receptors in this area reduced PCP-induced hyperlocomotion and stereotypies (Takahata and Moghaddam, 2003). It is, therefore, critical to establish whether low doses of PCP can change biological markers relevant for glutamatergic transmission in cortical and hippocampal circuitries.

4.1.2. Effects of haloperidol and clozapine on the spatial learning and memory impairments induced by PCP

To further examine the potential mechanisms underlying PCP effects on cognition, subsequent studies examined the ability of typical and atypical antipsychotic drugs to attenuate or block these PCP-induced impairments. Haloperidol, which acts primarily as a dopamine D_2-receptor antagonist, is regarded as a "typical" antipsychotic drug. The compound is an efficient antipsychotic with a high liability to produce a number of extrapyramidal side-effects due to dopamine D_2-receptor blockade in the basal ganglia. Clozapine is regarded as the prototypical "atypical" drug with a unique clinical profile (Kane et al., 1988) including potential efficacy on cognitive impairments, particularly executive functions (Meltzer and McGurk, 1999). Moreover, the compound has a complex receptor binding profile with relevant affinities for multiple dopamine receptors and also on 5-HT_{2A}- and muscarinic receptors (Zorn et al., 1994; Chung et al., 2004).

The design of this study was the same as for PCP, which means that the antipsychotic drugs were given for seven days prior to training to familiarize the animals to the drugs. Despite pre-exposure to antipsychotic drugs, it is clear that mice are very sensitive to even very low doses of these compounds. These results showed that only very low doses of the compounds (clozapine 0.5 mg/kg and haloperidol 0.05 mg/kg) do not induce any apparent side effects in the water maze task. There was no evidence for a learning and memory facilitation by clozapine, which is consistent with previous animal studies (Hou et al., 2006) but contrary to some human data (Meltzer and McGurk, 1999). The failure to produce facilitation with clozapine, although it has been shown to increase hippocampal acetylcholine release in the rat (Shirazi-Southall et al., 2002; Chung et al., 2004), is probably related to its potent muscarinic receptor blocking properties. However, it is also difficult to demonstrate facilitation of spatial learning in this task, which is easily acquired by control animals.

The spatial learning impairment produced by PCP was blocked by concomitant repeated treatment with the "atypical" antipsychotic drug clozapine (0.5 mg/kg) but not with the "typical" antipsychotic drug haloperidol. The difference between clozapine and haloperidol can not be simply due to motor side effects, as both drugs were tested at doses that do not impair swim speed. This observation indicates that clozapine has a unique action by providing protection from the cognitive deficits induced by NMDA receptor dysfunction. Also, previous data in the rat have shown that clozapine, but not haloperidol, can block the spatial impairment caused by PCP (1.3 mg/kg) (Didriksen et al., 2007). Moreover, several behavioral and neurochemical effects of PCP and other

non-competitive NMDA antagonists are attenuated by clozapine but not by haloperidol in rodents. These include the immobilization in the forced swim test (Corbett et al., 1995), disruption of pre-pulse inhibition (Bakshi et al., 1994; Varty et al., 1999) and social withdrawal (Corbett et al., 1993). In contrast, studies in the rat suggest that the locomotor activating effects of NMDA receptor antagonists are attenuated both by haloperidol and other selective dopamine D_2-receptor antagonists as well as by clozapine (Castellani and Adams, 1981; Kitaichi et al., 1994; Ögren and Goldstein, 1994).

The difference between haloperidol and clozapine may provide a basis for the analysis of the mechanisms underlying "atypical" antipsychotic drugs as well as for studies on mechanisms underlying cognitive deficits. One important feature of animal models of schizophrenia is their ability to distinguish between "typical" and "atypical" antipsychotic drugs, which is not always the case (Ögren, 1996). In this context, the failure of haloperidol to prevent the cognitive dysfunctions of PCP is notable. This finding suggests that dopamine D_2-receptor blockade can not prevent the cognitive dysfunctions caused by NMDA receptor blockade. The ability of clozapine to attenuate the action of PCP is intriguing and may involve several neurochemical and molecular mechanisms of importance for cortical- and hippocampal-mediated cognition including 5-HT_{2A} receptor blockade or increases in acetylcholine release (Tamminga, 1998; Jentsch and Roth, 1999; Meltzer and McGurk, 1999; Ichikawa et al., 2002). It is possible that clozapine affects several different processes which may converge at the glutamate NMDA receptor systems, and thereby preventing a PCP-induced dysfunction of NMDA receptor transmission. Consistent with this notion is the recent observation that subchronic clozapine-treatment in the rat medial prefrontal cortex can prevent PCP-induced hyperactivity of NMDA receptors (Ninan et al., 2003). Moreover, it has been demonstrated that atypical antipsychotic drugs such as clozapine can facilitate glutamatergic neurotransmission, at least *in vitro* (Ninan et al., 2003).

In conclusion, the results from Paper I demonstrate that a low dose subchronic PCP-treatment can impair spatial learning and memory without any apparent motor side effects. The small interval between learning impairments and motor side effects after PCP treatment can be utilized to analyze the contribution of the NMDA receptor system in cognition. Moreover, by this procedure it is possible to analyze the differential mechanisms involved in the action of typical and atypical antipsychotic drugs.

4.2. Cognitive and gene expression changes after withdrawal from repeated PCP treatment

4.2.1. Effects on working and spatial long-term memory

To further assess the validity of the model of the subchronic PCP model for schizophrenia, the experiment in **Paper I** was extended by using a withdrawal design. An important aim was to investigate whether a low dose of repeated PCP treatment can result in long-term changes in schizophrenia-like behaviors, which have been observed after high doses of PCP (Javitt and Zukin, 1991; Mouri et al., 2007). The cognitive tests

included both hippocampal (e.g. long-term memory) and memory functions associated with the prefrontal cortex, e.g. working memory, to model the multiple cognitive impairments seen in schizophrenia patients. In **Paper II**, spatial acquisition, spatial long-term memory and spatial working memory were studied in the water maze. In addition, the study also included investigation of plastic changes in glutamatergic and dopaminergic neurons that may be related to cognitive functions (see below).

Subchronic PCP treatment produced a dose dependent impairment of spatial acquisition in the water maze with a significant effect at 1.0 and 2.0 mg/kg, while the 0.5 mg/kg dose failed to significantly affect spatial acquisition. This finding is notable for two reasons: 1) The animals are examined 24 h after the last dose of PCP and under this condition the 0.5 mg/kg dose is ineffective in contrast to the results obtained after acute treatment (**Paper I**). This indicates that the effects of PCP are much more powerful when the drug is on board compared to the withdrawal state. This finding suggests that the half-life of PCP is short, as shown previously (Proksch et al., 2000). 2) When tested under withdrawal state, the 1.0 and 2.0 mg/kg dose completely failed to produce any evidence for sensorimotor or neurological side effects, suggesting that the neurological side effects of PCP are mainly related to the acute action of the compound. This indicates that the 2.0 mg/kg dose can be used to study the long-term effects of PCP on cognition.

In **Paper II**, memory retention was examined 3 h after the last training trials. Unlike the results in **Paper I**, the performance in the probe test was poor, indicated by the fact that measures for spatial selectivity i.e. quadrant information was not significant. However, measures for latency and platform crossing could be used, showing that animals receiving the 2.0 mg/kg dose of PCP were significantly impaired compared to the control group.

In the working memory task, only the 2.0 mg/kg dose of PCP was examined. This dose of PCP caused a significant impairment in spatial working memory. Unlike the control animals, which improved from trial 1 to trial 2 over the days of training, the subchronic PCP-treated animals failed to show any significant improvement from trial 1 to 2. This impairment in working memory is most likely not due to any motor side effects since swim speed did not differ between the control mice and the PCP-treated mice. Also the dose used in the working memory study is very low compared to previous studies. Repeated administration of PCP to rats and mice at higher doses than used in this study has shown variable effects in the T-maze/radial arm maze test for working memory. PCP in a dose of 5 mg/kg twice daily to rats produced a working memory deficit in the T-maze test when examined seven days after withdrawal (Jentsch et al., 1997). On the other hand, in a similar task a dose of 5 mg/kg twice daily for five days with a withdrawal period of 9 days did not cause any impairment (Stefani and Moghaddam, 2002). Moreover, a dose of 10 mg/kg for 14 days did not cause any deficit in the radial maze when the animals were examined 2 weeks after withdrawal (Li et al., 2003). These results indicate that the time of withdrawal and the dose used are critical in the analysis of the long-term effects of PCP on cognitive functions. However, at present it is not known whether PCP in the cited studies using relatively

high doses produces any neurotoxic changes or whether the results obtained mainly reflect changes in neurotransmitter function (Jentsch and Roth, 1999).

Cognitive deficits are strong predictors of functional outcome in patients with schizophrenia (Green, 1996). Interestingly, a study in humans using a virtual Morris water maze was performed in patients with schizophrenia and controls in two versions of the virtual maze. A hippocampal-dependent hidden-platform version, which depend on navigational abilities, and a non-hippocampal-dependent visible-platform version with cued-navigational abilities were used. Schizophrenia patients had longer distance values and spent more time trying to find the hidden platform over training trials. Moreover, the schizophrenia patients spent less time in the correct quadrant during the probe trial. The patients had no problem in performing in the visible-platform condition (Hanlon et al., 2006). These findings resemble the present findings using subchronic PCP treatment which did not influence the animal's ability to navigate to visible platform. An intriguing question is whether working memory is only dependent on the prefrontal cortex. In animal studies, it is clear that the hippocampus also plays an important role for spatial working memory (Friedman and Goldman-Rakic, 1988) but it can therefore not be excluded that hippocampal/cortical circuitries are involved in the working memory deficits seen after subchronic PCP. Working memory deficits are a common feature in schizophrenia patients and they are believed to mainly reflect impairments in prefrontal cortex functions (Park and Holzman, 1992; Goldman-Rakic, 1994). However, as discussed earlier, schizophrenia patients also show both structural (Tamminga et al., 1992; Arnold et al., 1995) and memory deficits related to hippocampal abnormalities (Boyer et al., 2007), a brain region implicated in spatial learning in both animals and human (Squire and Zola-Morgan, 1991; Morris and Frey, 1997; Scoville and Milner, 2000).

4.2.2. Effects on genes expression

As pointed out earlier, the mechanisms behind the effects of systemic PCP or NMDA receptor blockade on cognitive functions have never been clarified. This may seem surprising in view of the important role for hippocampal NMDA transmission in LTP and spatial learning (see chapter 1.3.2.). Repeated PCP treatment has been shown to produce multiple changes in several neurotransmitter functions (Jentsch and Roth, 1999). Previous results have indicated that subchronic PCP (5 mg/kg twice daily for 7 days) can reduce prefrontal dopamine utilization and also impair spatial working memory (Jentsch et al., 1998). The ability of clozapine but not haloperidol to attenuate the prefrontal cognitive deficits (Moghaddam and Jackson, 2003) has been related to the preferential increase of dopamine transmission in the prefrontal cortex after clozapine treatment (Kuroki et al., 1999). In this context it is notable that the cognitive deficits induced by subchronic PCP treatment were prevented by clozapine but not by haloperidol (see **Paper I**). However, in view of the important role played by the glutamate NMDA receptor system in cognition, it is critical to investigate whether the subchronic low dose PCP can affect biological markers possibly relevant for glutamatergic regulated neuronal plasticity or structural changes in the brain.

It is generally believed that protein synthesis is required for many forms of synaptic plasticity as well as long-term memory (Frey and Morris, 1997). The initiation of protein synthesis involves the rapid expression of various genes (transcription factors and immediate early genes, IEGs) that regulate the expression of other genes. A number of IEGs have been identified in recent years to play a critical role in RNA and protein synthesis dependent synaptic plasticity and memory consolidation (Clayton, 2000).

The main aim of this study was to investigate if the cognitive changes caused by NMDA receptor blockade also involved alterations in the transcriptional activities of two genes involved in neuronal plasticity and learning and memory. The IEG *Arc* (activity-regulated cytoskeleton-associated gene) was selected since it is preferentially expressed in glutamatergic neurons in the hippocampus and cortex, and because it has been found to be critical for the maintenance of LTP and memory consolidation (Guzowski et al., 2000). *Arc* is also rapidly induced by various forms of neuronal activity such as LTP-inducing stimuli, cocaine and behavioral stimulation (Lyford et al., 1995; Rial Verde et al., 2006). Spinophilin, a protein that is highly enriched in dendritic spines, has been reported to modulate dendritic morphology and glutamatergic synaptic transmission (Feng et al., 2000). Moreover, spinophilin mRNA has been shown to be altered in the hippocampus and thalamus of schizophrenia patients (Law et al., 2004; Clinton et al., 2005).

In situ hybridization was performed to detect the expression of transcripts encoding Arc and spinophilin. Twenty four hours after withdrawal from subchronic PCP, *Arc* mRNA expression was found to be up-regulated in the CA1 region of the hippocampus and retrosplenial cortex, but in no other of the cortical or hippocampal regions examined. Importantly, PCP treatment failed to induce *Arc* mRNA expression in basal ganglia or prefrontal cortex. These regional differences are consistent with the important role that the CA1 region of the hippocampus and the retrosplenial cortex plays in spatial learning. For instance, mice with a deletion of the NMDA receptor gene restricted to the CA1 pyramidal cells are deficient in spatial memory but not impaired in non-spatial learning (Tsien et al., 1996). Moreover, the retrosplenial cortex has been shown to play a role in spatial information processing and also in the cognitive processes underlying non-spatial behavioural strategies (Jentsch et al., 1998).

The increase in the expression of *Arc* mRNA may seem paradoxical since previous studies have shown that NMDA receptor activation in the hippocampus induces *Arc* mRNA expression (Steward and Worley, 2001). In contrast, the present results suggest that blockade of NMDA receptors by PCP may facilitate *Arc* expression in hippocampus and retrosplenial cortex. Also, an acute high dose (10 mg/ kg) of PCP increased *Arc* mRNA levels in the prefrontal cortex, nucleus accumbens and posterior cingulate cortex (Nakahara et al., 2000). However, it is possible that the net effect of NMDA receptor blockade is an increase in glutamatergic release, at least in the cortex. Blockade of NMDA receptors expressed on GABAergic interneurons in the thalamus and basal forebrain could result in a disinhibition of glutamatergic projection-neurons (Olney and Farber, 1995). This increase in glutamate release together with the

simultaneous blockade of postsynaptic NMDA receptors in the hippocampus and cortex may result in an imbalance between NMDA and AMPA receptor function. In fact, repeated 2 mg/kg dose of PCP treatment in a withdrawal design resulted in a functional hyperactivity of NMDA receptors in the medial prefrontal cortex (Ninan et al., 2003). This hyperactivity was blocked by clozapine but not by haloperidol. Moreover, both atypical antipsychotic drugs and haloperidol modulated glutamatergic transmission after prolonged treatment, resulting in a hyposensitive response of the rat cortical cells to NMDA (Jardemark et al., 2000). *Arc* mRNA is targeted to dendrites and accumulates in the region of the dendritic tree where the inducing stimuli arrive. Recent studies have also shown that *Arc* is involved in AMPA receptor trafficking in the CA1 region of hippocampal slices (Rial Verde et al., 2006). Increased expression of *Arc* could, at least theoretically, result in a decrease of AMPA-mediated synaptic current (Rial Verde et al., 2006). It therefore, seems possible that the increased expression of *Arc* following PCP treatment may result in disturbances in glutamatergic receptor functions due to an imbalance between NMDA and AMPA receptor function.

In contrast to *Arc*, Spinophilin, a protein that is highly enriched in dendritic spines, was significantly decreased in the striatum in the PCP-treated mice. This finding is important since spinophilin plays distinct roles in dopamine-mediated plasticity in the striatum (Allen et al., 2006). Subchronic PCP was shown to produce increases in dopamine-mediated behavior such as locomotor activity after withdrawal. However, the effect of subchronic PCP after withdrawal is small compared to the acute stimulatory effect of PCP on locomotion. It is possible that the long-term effect of PCP on locomotor activity is related to subtle changes in dopamine dendrites resulting in a sensitization to novel stimuli (Jentsch et al., 1998).

*In conclusion, the results from **Paper II** demonstrate that a low subchronic dose of PCP can impair both spatial working memory as well as reference memory in mice in a withdrawal design. Moreover, this treatment procedure results in changes in gene expression in hippocampal/cortical circuitries involved in synaptic plasticity and learning, as well as in striatal systems implicated in dopamine-mediated plasticity. The changes in gene expression are restricted to certain types of glutamatergic neurons in the hippocampus/cortex and in the striatum.*

4.2.3. Effects on social interaction and emotional behavior

Several studies have shown that both acute and repeated PCP treatment can produce social and behavioral deficits in social interaction tests as well as enhanced immobility in the forced swim test (Sams-Dodd, 1999; Abdel-Naby Sayed et al., 2001). These behavioral changes have been taken as indices of negative symptoms in schizophrenia. However, it is presently unclear whether the indices for positive, negative, sensorimotor gating deficits or cognitive dysfunctions involve similar or different behavioral or neurochemical mechanisms. Moreover, as pointed out earlier, the doses used to study negative symptomatology in rodents have been high and most findings are based on acute PCP. The aim of **Paper III** was to examine whether the subchronic low dose of

PCP (2 mg/kg) which produced learning and memory impairments also may have long-term effects on emotional activity and social interaction in mice. In addition, the behavioral pattern of mice was examined in a new test model, the novel cage test, to perform a more detailed analysis of the effects of repeated PCP administration. Mice were given 2.0 mg/kg PCP daily for one week; emotional activities and social interaction were tested by the novel cage test, social interaction, elevated plus-maze and open field test. In the social interaction test, PCP was given to the resident mice but not to the target mice. The mice were housed individually for 24 h to habituate to the test cage. Testing was performed 24 h, 7 days and 14 days after the last treatment with PCP or vehicle 7 days.

PCP given at a dose of 2 mg/kg decreased time spent in active social interaction but increased non-social exploration such as walking in the social interaction test. This finding suggests that impairment of social behavior occurs at the same dose of PCP which also produces long-term impairment of working- and long-term spatial memory (see **Paper II**). This indicates that the action of PCP on these two domains of behavior might be related to changes in glutamatergic and/or dopaminergic function. However, the effects of PCP on social interaction have also been related to the neuropeptide vasopressin. Thus, in the rat subchronic treatment with PCP which resulted in a reduction in social interaction has been found to reduce the density of one of the vasopressin receptor subtypes i.e. V1aR in several brain regions (Tanaka et al., 2003). Moreover, repeated PCP treatment also affects hypothalamic oxytocin mRNA expression, another peptide with a role in social behavior (Lee et al., 2005). This indicates that there are several mechanisms by which long-term PCP-treatment can affect social behavior in rodents. In this context, it is notable that autistic behavior has been associated with the VlaR gene (Yirmiya et al., 2006).

The changes in social behavior was also indicated by the observation that subchronic PCP treatment elicited aggressive behavior, shown by the increased punching or biting on the target mouse by the resident mouse. This is a novel finding since studies in the rat failed to report increases in aggressive behavior after repeated PCP administration (Sams-Dodd, 1995; Wang et al., 2007). However, clinical studies indicate that use of PCP can increase aggressive and deviant behaviors (McCardle and Fishbein, 1989)

To further validate the PCP-model for schizophrenia, the effects of subchronic PCP treatment were also examined in tests for emotional reactivity and anxiety-related behavior. An important question relates to the interaction between various domains of behavior in the spectrum of schizophrenia-related behavior. For instance, is the decrease in social behavior related to changes in anxiety-states or does it reflect deficits in perception or cognition? Studies of anxiety-related behavior in the elevated plus-maze and in the open field did not give any consistent answer as to the possible states of anxiety after subchronic PCP treatment. Indeed, in this test, total number of transitions, total distance traveled and percent time spent in the open-arms were not markedly affected by the treatment with 2.0 mg/kg PCP. In contrast, subchronic PCP treatment significantly increased the number of transitions between the central area and

peripheral areas in the open field-test 24 h after cessation of PCP treatment. Furthermore, PCP treatment also increased time spent in the central area. In the open field, the native tendency to explore the open unfamiliar arena conflicts with the urge to stay along the walls (thigmotactic behavior), which reflects risk-assessment or approach-avoidance behaviors. The behavior of rodents in the elevated plus-maze is often believed to reflect similar mechanisms as those underlying the behavior in the open field test. Thus, the avoidance of the open-arm would correspond to the avoidance of the central area in the open field-test. It is possible that PCP at this low dose decreases some aspects of anxiety-related behavior but this conclusion must be further substanciated. Thus, a study with repeated administration of 5 mg/kg of PCP for 7 days has shown enhanced anxiety following a seven day withdrawal period in rat (Audet et al., 2007).

*In conclusion, the results from **Paper III** demonstrate that a repeated low dose of PCP induces an impairment of social behavior in mice concomitant with an increase in aggressive behavior, without any clear evidence for changes in anxiety-related behavior. In addition, subchronic PCP caused an increase in locomotor activity after withdrawal but a decrease in investigatory behavior. Moreover, the effects of PCP on behavior lasted for about seven days which indicates that they may be related to functional disturbances but most likely not to neurotoxic actions. Based on these findings it is concluded that similar mechanisms are involved in both the effects of PCP on cognition and on social interaction. However, several neuropeptides may also contribute to the effects of PCP on social behavior.*

4.4. Effects of influenza A virus infection on gene expression combined with alterations in cognitive and emotional behaviors

Disturbances in brain development may predispose individuals to later developing schizophrenia, perhaps triggered by a second hit, e.g. aversive events, drug abuse or infections. As discussed earlier, it is not unlikely that virus also may have a primary etiological role during certain stages of brain development. The potential role of virus infection in schizophrenia is controversial, partly due to the limited knowledge on the possible time-interval during which infection can affect brain development and functions. To shed more light on this complicated issue, models of early life exposure to the neurotropic strain of influenza A virus were studied with regard to the long- and short-term effects on gene expression in the brain as well as the long-term effects on behavior.

4.4.1. Influenza virus infections during early life and alterations in the offspring brain.

It is well known that infections caused by such as *Toxoplasma gondii,* rubella, cytomegalovirus and *Herpes simplex virus* during pregnancy can cause severe malformations and lesions of the nervous system of the fetus both in humans and

domestic animals (Johnson, 1998). However, as described in the Introduction there is only limited knowledge on the effects of virus infections on the differentiation of the nervous system, i.e. synapse and myelin formation (Johnson, 1998), and on cognitive functions, glutamatergic neurotransmission and gene expression.

In this experiment, we hypothesized that maternal exposure to the influenza A virus may lead to different patterns of brain gene expression in the developing offspring. Therefore, pregnant mice were subjected to intranasal infection with influenza A/WSN/33 virus on day 14 of pregnancy (G14). Brains of virus-exposed and control offspring were further analyzed at the following time points: day 17 of gestation (G17) and postnatal days (P) 35, 60, 90 and 280. These animals showed no overt signs of disease or weight loss during their development. According to a previous report from our group, viral RNA could be detected in the brains of fetuses of mothers infected intranasally with the same virus. Viral mRNAs were detected in the brains of these mice three days after birth and, depending on the viral dose administered, the offspring died early or survived without any overt signs of sickness (Aronsson et al., 2002).

Differential gene expression in the brains of the offspring became evident at 90 days of age (P90), but not at earlier time-points. At this time point, expression of the genes encoding Ring 1B, neuroleukin and fibroblast growth factor 5 (FGF5) were significantly elevated. In this study, we found that the gene encoding NLK was detected at significantly elevated levels in the virus-exposed offspring as compared to control offspring at 90 days of age, and even remained significantly elevated by 1.4-fold in the virus-exposed offspring at 280 days of age (**Paper IV**). This shows that a maternal infection can cause gene expression changes in the brain that appear only when the offspring reaches early adulthood. The mice were also examined in a learning and memory tasks in which the hippocampus plays an important role, but no differences were detected.

Previous work by Fatemi and coworkers has shown that influenza virus infection using NWSN/33 strain at G9 of pregnancy can produce behavioral disturbances and neurochemical dysregulation at puberty in the exposed offspring (Fatemi et al., 1999; Fatemi et al., 2002; Shi et al., 2003). However, the virus strain used by Fatemi and coworkers did not invade the brain, but produced a severe sickness behavior in the pregnant mice, while in our study the virus-infected mice did not show any overt signs of disease such as weight loss, and the infection did not adversely affect the size of the offspring. Similar behavioral disturbances as in the studies by Fatemi and coworkers and Shi and coworkers have been observed by injection synthetic cytokine releaser, polyI:C, thus the behavioral effects on the offspring thought to be caused by the maternal inflammatory response rather that the virus itself (Shi et al., 2003).

To address the question if a virus infection during the peak of synaptogenesis in several areas of the brain can cause alterations in behavior and in expression of genes associated with schizophrenia, mice infected intraperitoneally on postnatal day 3-4 were studied. To model the potential effect of a genetic immune dysfunction, we include wild type (WT) C57BL/6 mice and *Tap 1*$^{-/-}$ mice lacking functional cytotoxic T-lymphocytes. Following the virus infection (see Tabel 3), neurons immunopositive

for viral antigens were observed in several different brain areas of WT and *Tap1*$^{-/-}$ mice. These brain areas included the motor, somatosensory, piriform, entorhinal and prelimbic cortices, the visual and the retrosplenial cortices, the hippocampus, the lateral and third ventricles and the thalamus.

Table 3. Study design

Age of mice, postnatal day (P)	Treatment, sampling and behavior tests
P3-4	infection with influenza A/WSN/33 virus
P7, P13, P24, P50 and P90	Sampling of brains for gene expression analysis and immunohistochemistry.
P90-	Behavior tests performed: Spontaneous locomotor activity Elevated plus-maze Working memory Long term learning and memory

At P7 the levels of transcripts encoding the oligodendrocyte lineage transcription factor 2 (Olig2) were found to be down-regulated and the transcripts encoding chemokine CXC motif ligand 1 (Cxcl1) were up-regulated in the brains of WT infected mice. Similar observations were made in the virus infected *Tap1*$^{-/-}$ mice at P7. The levels of Olig2 transcripts were down-regulated whereas the levels of Cxcl1 and glial cells missing 1 (Gcm1) transcripts were elevated at this time-point in the virus infected *Tap1*$^{-/-}$ mice. At P24, all transcripts analyzed were detected at similar levels in brains of infected and control WT mice. Elevated levels of Gcm1 and NRG-1 transcripts were detected in the brains of infected *Tap1*$^{-/-}$ mice compared to control animals at P24.

The next question to address was whether this early life influenza virus infection can cause behavioral changes when the mice reach adulthood. Thus, three months post-infection behavioral changes of the WT and *Tap1*$^{-/-}$ animals were evaluated (see Table 3). The virus infected *Tap1*$^{-/-}$ mice had increased rearing activity compared to control *Tap1*$^{-/-}$ mice. Infected *Tap1*$^{-/-}$ mice also displayed an increase in anxiety-like behavior compared to control *Tap1*$^{-/-}$ mice. The viral infection caused no changes in anxiety-like behavior in the WT animals. This finding suggests that genetic variation influences the effects of an early virus infection on adult mice behavior. Similar observations were previously made in different strains of inbred rats following neonatal Borna disease virus infection (Pletnikov et al., 2002). Anxiety-like behavior was also previously observed in mice infected neonatally with lymphocytic choriomeningitis virus (Hotchin and Seegal, 1977). Maternal infection with influenza virus using NWSN/33 strain in rodents has also been associated with elevated anxiety in offspring (Shi et al., 2003).

4.4.2 Behavioral and gene expression changes after olfactory bulb virus infection.

In experimental animals, it has been shown that a number of viruses can spread along olfactory pathways to target different structures in the brain (Mohammed et al., 1993; Urbanska et al., 1997). Influenza A virus has the capacity to invade the olfactory bulbs

after intranasal inoculation (Park et al., 2002; Aronsson et al., 2003). We aimed to investigate the potential effects of the influenza A/WSN/33 virus infection following olfactory bulb injections in adult mice (Mori et al., 1999) on gene expression and behavioral changes. The WSN/33 strain of influenza A virus was interesting in this respect since it localizes to not only the dopaminergic VTA but also the medial habenula and midline paraventricular thalamic areas (Mori et al., 1999) that serve as relay nuclei. The former nuclei links limbic and striatal forebrain with lower diencephalic and mesencephalic centers (Ellison, 1994), and the midline paraventricular thalamic area projects to the hippocampus, amygdala and nucleus accumbens (Su and Bentivoglio, 1990). In **Paper VI** we used injection of the WSN/33 strain into the olfactory bulb in young adolescent male mice (see Table 4).

Table 4. Testing schedule and treatment

Age (weeks)	Test/treatment
5-6	Infection with the Influenza A/WSN/33 virus
19-20	Spontaneous locomotor activity measurements followed by d-amphetamine i.p. (1.5 mg/kg) induced locomotor activity
21-22	Morris water maze task
23-24	Elevated plus-maze task
24-25	Passive avoidance test
25-26	Spontaneous locomotor activity measurements followed by d-amphetamine i.p. (3 mg/kg) induced locomotor activity

The influenza virus infection caused impairments in spatial learning of mice (see Figure 8A). Memory retention may have also been impaired (see Figure 8D) although that could not be definitely concluded due to the increase in thygmotaxis and floating behavior. However, the virus infected mice showed no impairments in the passive avoidance paradigm. These animals had decreased anxiety-like behavior while showing no changes in spontaneous locomotor activity versus controls. This indicates that the virus affects different brain functions. Moreover, virus-infected mice responded to the low dose of d-amphetamine (1.5 mg/kg) challenge in the same manner as the control groups. However, a challenge dose of d-amphetamine (3 mg/kg) did produce a delayed increase in locomotor activity. This indicates that the virus-infected mice may have a minor disturbance related to dopamine transmission, since the d-amphetamine mediated locomotor stimulation in mice is mainly mediated via dopamine release (Ögren and Ross, 1977).

The reduced level of anxiety-related behavior observed may indicate impaired expression of emotion, which is related to amygdala function (Gallagher and Chiba, 1996). Impairment of emotional response is one of the most common and severe symptoms in schizophrenic psychoses. Therefore, the amygdala and its connections

have been implicated in the pathophysiology of schizophrenia (Benes and Berretta, 2000).

The transcriptional activity of genes involved in the synaptic transmission was then examined. Elevated transcriptional activities of two genes encoding synaptic regulatory proteins, RGS4 and the calcium/calmodulin-dependent protein kinase II alpha (CaMK2A), were detected in the amygdala and the hypothalamus of the infected animals as compared to control animals. RGS4 is one of the regulatory of RGS (regulatory of G-protein signaling) family protein which are a heterogeneous collection of proteins that mediate postsynaptic signal transduction in signaling pathway throughout the brain. RGS4 is highly expressed in neocortex, striatum and in hippocampus (Gold et al., 1997). RGS4 has been implicated as a candidate risk gene for schizophrenia (Mirnics et al., 2000; Mirnics et al., 2001; Chowdari et al., 2002). Furthermore, RGS4 modulates G-protein signaling in a number of neurotransmitter systems, several of which have been linked to schizophrenia, including metabotropic glutamate, dopamine and serotonin receptors (Yan et al., 1997; Saugstad et al., 1998; De Blasi et al., 2001; Beyer et al., 2004; Ghavami et al., 2004). Interestingly, RGS4 also interacts with the ErbB3 receptor, which binds neuregulin, and neuregulin-Erb signaling has been implicated in schizophrenia (Harrison and Weinberger, 2005). The second gene that was altered by virus infection, CAMKII, has been implicated in hippocampal synaptic plasticity and memory in mice (Bejar et al., 2002). This enzyme is activated by calcium influx through NMDA receptors (Bayer et al., 2001) and it appears to be required for permanent memory functions (Frankland et al., 2001).

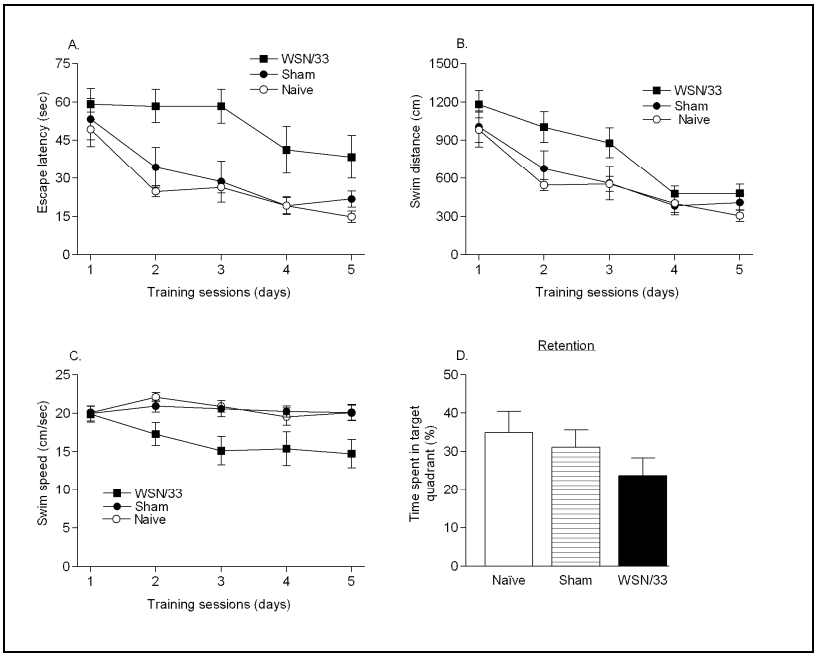

Figure 8. Effects of the influenza virus infection on spatial learning and memory in the water maze. Latency to platform (A), swim distance (B), swim speed (C) and time spent in the target quadrant time (%) (D).

Taken together, these results show that influenza A virus infection may have long-term effects on cognitive and emotional behavior in mice. In addition, the virus infections caused alterations in genes related to neurodevelopment. Moreover, this study showed that an individual response to infection is determined by immune status, timing and route of infection and the genetic composition of the host and organism. These experimental animal studies have provided evidence that early life influenza virus infection can influence the behavior when the mice reach adulthood.

4.5. The effects of an early life virus infection and second-hit treatment by PCP on behavior responses in adult mice.

A critical issue is whether early life virus infections will result in changes in the function of the glutamatergic NMDA receptor systems. At present, there is no direct evidence for alterations in NMDA transmission in virus-infected mice. However, mice inoculated with influenza A virus in the olfactory bulb at 5 weeks of age displayed changes in RGS4-expression (**Paper VI**). RGS4 is known to interact with the ErbB3 receptor which can modulate NMDA receptor function (see above).

In the last study of this thesis, which is in progress, we investigate if an early postnatal infection of influenza A virus could involve changes in NMDA receptor transmission related to cognitive functions. C57Bl/6 male mice were infected with influenza A virus at postnatal day 3, as described in **Paper V**, and examined at 4-5 months of age. The subchronic low dose PCP treatment procedure was used as described in **Paper II**, which means that the animals were first treated for seven days before the behavioral tests were performed. With this procedure the animals are examined under withdrawal condition i.e. behavioral tests are initiated 24 h after the last dose of PCP or saline.

The development of schizophrenia-like symptoms was monitored using the following behavioral measures: motor activity in response to a novel environment, long-term learning and memory in water maze. The virus-infected animals exhibited increased activity as measured by rearing, motility and locomotor activity (see Figure 9). Previous data have shown that an early postnatal influenza A virus infection increases rearing activity but not locomotor activity when examined 3-4 months after infection. The virus-infected animals also had impaired performance in the spatial acquisition task versus control animals with a reduction in swim speed (see Figure 10). These findings recapitulate the observations in a previous study using the same type of infection procedure (**Paper VI**). Memory performance as measured in retention by time spent in the target quadrant, number of crossing of the former platform position and time spent in the target zone differed significantly from the control group ($p<0.05$).

In the previous studies as also shown here, subchronic PCP treatment increased rearing activity when measured 24 h after the final dose of PCP (**Paper III**). Since acute PCP is known to decrease rearing activity, these results suggest differential mechanisms behind acute versus repeated PCP treatment as tested in the withdrawal procedure (Ögren and Goldstein, 1994). Although subchronic NMDA receptor

blockade by PCP markedly increased the rearing response, it did not significantly increase motility or locomotor activity in the infected mice compared to the control mice. In the water maze task, the PCP treatment caused a clear deficit in spatial acquisition in the infected mice but this impairment did not differ from control animals receiving PCP. In the memory retention test, there was a tendency for a somewhat larger impairment in the infected mice compared to the control mice receiving PCP. This difference is illustrated by the time these two groups spent in the target quadrant (see Figure 11). It is possible that the inability to detect a clear difference between control and infected mice is due to the pronounced effect of subchronic PCP treatment on acquisition i.e. a ceiling effect.

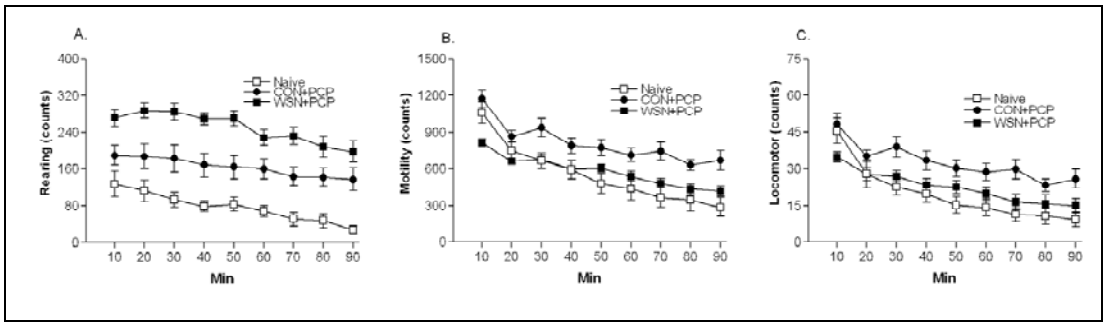

Figure 9. Effects of the combination of early postnatal influenza A virus infection and PCP treatment on spontaneous vertical activity (rearing), motility and locomotor activity in mice. All animals were placed in the locomotor compartment (one mouse per cage) and their activity was recorded for 90 min. The results are presented as mean values ± SEM (n=8-9/group).

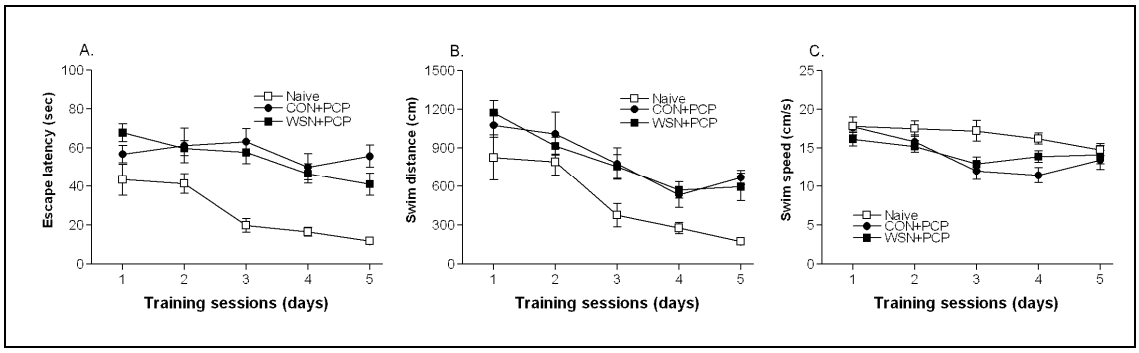

Figure 10. The effect of the combination of early postnatal influenza A virus infection and PCP treatment on spatial learning and memory performance in the water maze. The escape latencies, swim length and swim speed are shown. The results are presented as mean values ± SEM (average over four trials per a day, n=8-9 per group).

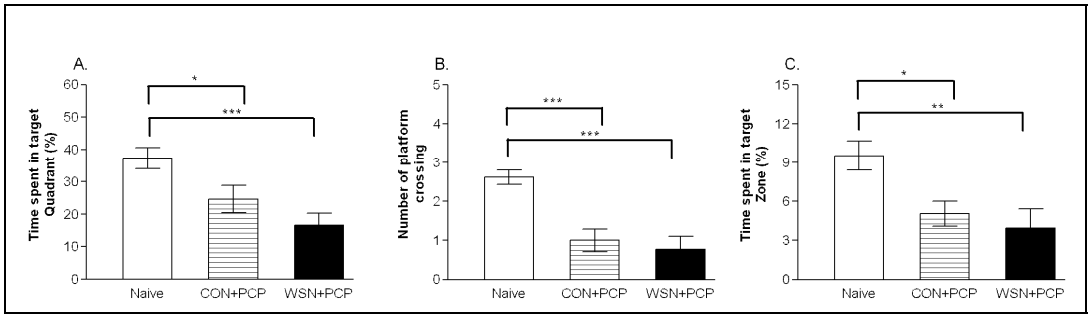

Figure 11. Results obtained from retention trial, the time spent in the target quadrant, number of platform cross and time spent in the target zone. Values represent mean ± SEM, *indicates significant differences between groups, * represents $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

Acute PCP adminstration elicits hyperlocomotion as an index of positive symptoms, as discussed above. Interestingly, virus infected animals showed a differential locomotor response to PCP. Thus, virus infected animals treated with PCP exhibited a lower locomotor response but a higher rearing response than the control animals. Since rearing is related to exploratory/investigatory activity and differs in its neuroanatomical and neurochemical basis from locomotor activity, these results are intriguing. It is possible that the virus infection has caused a dysfunction in NMDA receptor-associated neurotransmission and dopamine transmission in the striatum and in the prefrontal cortex, as discussed in **Paper II**. It has been shown that NMDA receptor antagonist induced hyperactivity can act, at least partially, via enhancing glutamate transmission in the prefrontal cortex (Takahata and Moghaddam, 2003) but also via changes in dopamine neurotransmission in the basal ganglia (Miller and Abercrombie, 1996). It is possible that the reduced response to locomotion and the increased rearing response after PCP in the infected mice reflect a disturbed relationship between striatal and cortical dopaminergic and glutamatergic transmission. From the present results, it is not possible to conclude that the virus infected mice showed a defect in NMDA receptor function as related to cognitive performance. It is possible that such a defect could be detected if a lower dose of subchronic PCP was used. However, this study confirms again the profound effects of subchronic PCP on spatial learning.

Taken together, these results show that the locomotor behavior of the early life virus infected WT animals is differentially regulated by the second-hit treatment of low doses of NMDA receptor antagonist. The virus-infected mice displayed differential sensitivity to PCP by showing a reduced locomotor response and a higher rearing response. Moreover, subchronic PCP tended to enhance the spatial memory deficit seen in infected mice.

4.6. Concluding remarks

The present studies raise a number of intriguing questions, particularly regarding the potential role of early life infections in causing cognitive deficits later in life, or even schizophrenia symptoms? One of the critical questions relates to the "hit and run" hypothesis e.g. how can a transient infection during early life produce long term effects on brain function. Moreover, is a later or second "hit" required for eliciting the actual schizophrenic symptoms? In this context it will be important to further investigate the neuronal systems, both in human and animals that are affected by the virus infection. The present experimental data give evidence that systems in the hippocampal-cortical circuitries are particularly affected by the virus infection investigated here. In light of the important role of glutamate NMDA receptor system in cognitive functions, it will be important to further analyze its contribution to the present findings. Therefore, a further analysis of the subchronic PCP model is required in order to investigate the relative contribution of NMDA receptor hypofunction and dopamine for the observed changes in cognition. Such an analysis should focus on a possible imbalance between dopamine and glutamate activities in the prefrontal cortex and in the hippocampus.

The present findings also suggest that the subchronic PCP model would be useful for evaluating novel therapeutic candidates. One important aspect is to investigate the mechanisms by which atypical antipsychotic drugs may be able to prevent and reverse the cognitive impairments induced by subchronic PCP in the withdrawal model. Such studies may identify novel pathogenetic mechanisms of schizophrenia.

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