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ON THE ROLE OF FOREBRAIN CHOLINERGIC INNERVATION FOR PHENCYCLIDINE-INDUCED BEHAVIORS AND GENE EXPRESSION PATTERNS

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Stockholm 2013

Cover by *Carolina Thörn Pérez*

The cover shows an illustration of the chemical forumla of phencyclidine, with the two behavioral paradigms used within this thesis, the novel object recognition task and social interaction test.

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Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7549-185-1

It is the brain,
the little gray cells on which one must rely.
One must seek the truth within – not without.

– Hercule Poirot

ABSTRACT

The basalo-cortical cholinergic innervation reaches neural targets in cortex cerebri where it modulates the incoming sensory information, regulating arousal, attention, vigilance, memory and cognition. Progressive degeneration of the cholinergic neurons in the basal forebrain is considered a hallmark of Alzheimer's disease related to cognitive impairment in this condition. Dysregulation of cholinergic neurotransmission has also been implicated in schizophrenia, a chronic and debilitating neurodevelopmental disorder characterized by positive and negative symptoms and cognitive impairments, with deficits in thought processes, perceptions and emotional responsiveness. Modeling specific disruption of cholinergic function allows studies of its behavioral and molecular consequences and may contribute to the development of future therapies for Alzheimer's disease and Schizophrenia.

To investigate the role of the cholinergic system, we carried out uni- and bilateral cholinergic denervation of cortex cerebri in male Lister hooded rats using the selective immunotoxin 192 IgG-saporin to specifically target the cholinergic neurons of nucleus basalis of magnocellularis (NBM). IgG-saporin effectively removed cholinergic afferents to the cortical mantle. Intact and denervated rats were challenged with phencyclidine (PCP), a non-competitive NMDA receptor antagonist, which is known to produce schizophrenia-like psychosis in humans and hence used to model aspects of schizophrenia in rats. Experiments were conducted to determine the effects on behavior and to shed light on the underlying molecular mechanisms.

Negative symptoms of schizophrenia include social withdrawal, which is studied in animal models using social interaction tests. We found that cortical cholinergic denervation lead to a significant reduction in the duration of active social interaction in pairs of lesioned animals versus pairs of sham-operated controls. After an acute dose low of PCP (1 mg/kg, s.c.), there was a marked decrease in active social interaction for both groups, such that there was no longer a difference between lesioned and control animals. To evaluate cognitive impairments, particularly those reflecting declarative memory, a novel object recognition test was used. Neither cholinergic denervation alone, nor an acute PCP dose (1 mg/kg, s.c.) alone, blocked the ability of rats to recognize a novel object. However, animals lacking cortical cholinergic innervation and challenged with PCP were no longer able to recognize the novel object (Paper I).

Behavioral paradigms have typically been analyzed manually, though automated hardware and software systems have been introduced to speed up the process and eliminate subjective errors. However, the reliability and accuracy of computerized scoring needs to be verified for each specific paradigm. For novel object recognition, the program scoring reduced analysis time for the task, but needed manual corrections to avoid erroneous data points. Manual for scoring of social interaction revealed that this labor intensive approach was more nuanced and allowed to discern complex behaviors, whereas the automated scoring system registered more global interaction patterns. It is concluded that manual and computerized techniques for scoring social interaction offer complimentary information on various aspects of this complex behavioral task (Paper II).

The next studies were aimed to examine the bases of the behavioral responses to cortical cholinergic denervation and PCP challenge at the molecular level. PCP-induced neuronal activation was mapped using quantitative *in situ* hybridization of the neuronal immediate early gene c-Fos mRNA levels. Transcription of c-Fos responds to many different forms of activation, including stress and toxins. We found that two doses of PCP used (2 and 3 mg/kg, s.c.) caused a marked increase in neuronal c-Fos mRNA expression at 30 and 60 min after PCP administration, though these doses did not alter the levels of BDNF or Nogo receptor mRNA. Importantly, PCP responses were markedly dampened in cholinergically denervated regions of cortex cerebri (Paper III).

RNA-Sequencing was then employed to understand gene regulatory processes at a global level in somatosensory cortex with regard to effects of chronic denervation and/or a PCP challenge (1 and 3 mg/kg, s.c.). A first round of analysis focused on the genes most significantly altered by denervation (Egr1, Dusp6, Ier2, Nr4a1), PCP treatment (Cyr61, Bcl6b, Apold1, Dusp1) and those altered over time (Sox9, Coq10b, Zfp189, Rnf39) (Paper IV). The most common response was for mRNA levels to increase in response to PCP and for cholinergic denervation to dampen these increases.

The findings presented in this thesis support a role for the cholinergic system in assigning significance to incoming stimuli as exemplified by regulating the response of cortical neurons to a PCP challenge. The findings are also compatible with a proposed involvement of cholinergic dysfunction in schizophrenia.

LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts, which will be referred to by the corresponding Roman numerals.

I. **Savage S**, Kehr J, Olson L, Mattsson A. Impaired social interaction and enhanced sensitivity to phencyclidine-induced deficits in novel object recognition in rats with cortical cholinergic denervation. *Neuroscience*, 2011; 195: 60-9.

II. **Savage S**, Holst S. Manual versus automated scoring of novel object recognition and social interaction. *Manuscript.*

III. **Savage S**, Mattsson A, Olson L. Cholinergic denervation attenuates phencyclidine-induced c-fos responses in rat cortical neurons. *Neuroscience*, 2012; 216: 38-45.

IV. **Savage S**, Dillman A, Cookson M, Olson L. Cholinergic denervation and phencyclidine alter patterns of gene expression in rat primary somatosensory cortex. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AChE	acetylcholine esterase
ACh	Acetylcholine
nAChR	nicotinic acetylcholine receptors
mAChR	muscarinic acetylcholine receptor
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA	Cornu ammonis
Ch1	(cholinergic nucleus)
Ci	Curie
cDNA	complementary DNA
Cg	Cingulate cortex
CSPGs	Chondroitin sulphate proteoglycans
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	Dithiothreitol
FDR	false discovery rate
GC-content	guanine-cytosine content
HT-SEQ	python module used statistical analysis of gene expression data
ISH	*in situ* hybridization
NBM	Nucleus basalis magnocellularis
NGF	Nerve growth factor
NgR	Nogo receptor
NMDA	N-methyl-d-aspartate
NOR	Novel Object Recognition Task
M1	primary motorcortex
MATRICS	Measurement and Treatment Research to Improve Cognition in Schizophrenia
PCP	Phencyclidine hydrochloride salt
PPT	partner preference test
PI	preference index
qRT-PCR	quantitative real-time polymerase chain reaction
S1FL	primary somatosensory cortex – forelimb region
SAP	192 IgG-saporin
s.c.	Subcutaneous
RIN	RNA integrity number
MA	manual analysis
PA	program analysis
MCPA	manually corrected program analysis
NMDA	N-methyl-D-aspartate
OD	optical density
RNA-Seq	RNA Sequencing
SEM	standard error of the mean
NGFr	P75 nerve growth factor receptor

1 INTRODUCTION

HISTORICAL BACKGROUND

The cholinergic system has an interesting and varied history, which dates back to ancient times though it was not until the 20th century that acetylcholine (ACh) was discovered and defined. Historically, different toxins that interfere with cholinergic neurotransmission have been used e.g. in hunting with arrows dipped in poison, in rituals, as a plot thickener in literature, and tragically as nerve gas agents during world wars. To determine the guilt or innocence of an accused, the Efik people of Old Calabar, a province of Nigeria, gave a concoction made from the seeds of the calabar bean (*Physostigma venenosum* Balfouri). The survivors were found innocent, while those who died were deemed guilty. It is postulated that if one drank the liquid material without hesitation in one bolus, vomiting was elicited, thus saving the life (Balfour, 1861, as referenced in Giacobini and Pepeu, 2006). Scottish missionaries who became acquainted with this so called "ordeal bean" brought it back from their travels where it was discovered to contain physostigmine, a cholinesterase inhibitor alkaloid whose antidote is atropine. This same bean was used by Agatha Christie to poison a Mrs. Barbara Franklin in the last of the Hercule Poirot mysteries (Christie, 1975).

Acetylcholine (ACh) was discovered by Sir Henry Hallett Dale in 1914 and its existence was later confirmed by Otto Loewi, through a series of elegant experiments utilizing frog hearts. For these findings, Dale and Loewi were awarded the Nobel Prize in Physiology/Medicine in 1936. At the time, there was much debate among scientists whether communication between nerves and muscles was chemical or electrical and ACh was initially only considered a synthetic agent. However, Loewi took a frog heart, perfused with fluid and electrically stimulated the vagus nerve until the heart rate slowed (Loewi, 1924). The fluid was collected, and added to a second frog heart which had been stripped of its vagal and sympathetic nerves. By adding this fluid from the first heart to the second, he caused the heart rate of the second to slow down. This provided evidence that stimulation of the vagus nerved caused the release of a substance which directly acted upon the second heart and slowed it down. He named this substance vagusstoff. This substance was later confirmed to be acetylcholine which had been identified by Sir Henry Hallett Dale (Dale, 1914).

CHOLINERGIC SYSTEMS IN THE BRAIN

The number of cholinergic neurons is not numerous, but virtually every brain region and peripheral target receives a cholinergic input (Woolf and Butcher, 2011). It was investigations of the ascending cholinergic pathways in the rat brain that led to a classification of the cholinergic projecting neurons into six major sectors (Ch1-Ch6) (Figure 1) (Mesulam et al., 1983). According to the nomenclature, cholinergic cells within the medial septal nucleus (Ch1) and those in the vertical nucleus of the diagonal band (Ch2) provide the major cholinergic innervation for the hippocampal complex. The cholinergic cells associated with the horizontal limb of the diagonal band (Ch3)

innervate the olfactory bulb, while those associated with the nucleus basalis magnocellularis (Meynert) (Ch4) project to cerebral cortex and amygdala. The pedunculopontine nucleus of the pontomesencephalic reticular formation (Ch5) and the laterodorsal tegmental gray of the periventricular area (Ch6) provide the major cholinergic innervation of the thalamus. Finally, striatum contains a dense cholinergic innervation provided by large scattered cholinergic interneurons, located within the striatum itself.

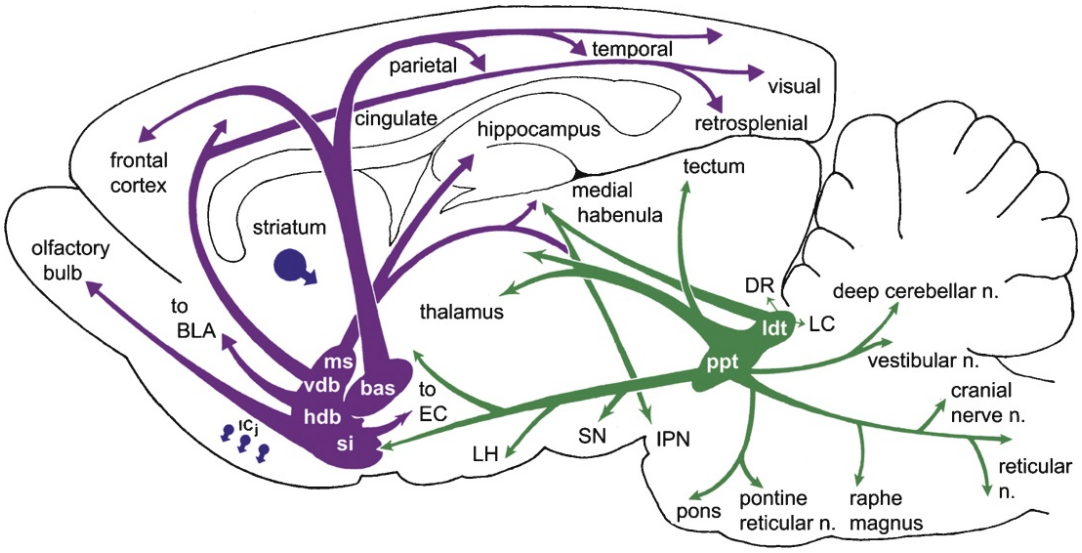

Figure 1. Schematic representation of cholinergic neurons and networks in the central nervous system. Abbreviations: bas, nucleus basalis; BLA, basolateral amygdala; DR, dorsal raphe; EC, entorhinal cortex; hdb, horizontal diagonal band nucleus; Icj, islands of Cajella; IPN, interpeduncular nucleus; LC; locus ceruleus; ldt, laterodorsal tegmental nucleus; LH, lateral hypothalamus; ms, medial septal nucleus; ppt, pedunculopontine nucleus; si, substantia innominata; SN, substantia nigra; vdb, vertical diagonal band nucleus. Reprinted from Woolf and Butcher, Cholinergic systems mediate action from movement to higher consciousness, 2011, with permission from Elsevier.

The cholinergic system is implicated in cognitive functions including attention, arousal, modulation of sensory information, learning and memory processing (Bartus et al., 1982; Everitt and Robbins, 1997). In addition, distinct functions are attributed to different pathways. The NBM-cortical cholinergic pathway is considered to contribute to cortical arousal and attention, whereas the septohippocampal pathway is implicated in spatial learning and memory. The brainstem projections are involved in basic arousal processing.

Acetylcholine receptors

Acetylcholine was the first neurotransmitter identified and has functions in both the peripheral and central nervous system. There are two major classes of acetylcholine receptors (AChR), nicotinic (nAChR) and muscarinic acetylcholine (mAChR) receptors. Nicotinic acetylcholine receptors are ionotrophic receptors with a pentameric structure composed of alpha and beta subunits, and are highly expressed in hippocampus, cortex, striatum, and thalamus (Breese et al., 1997; Freedman et al., 1995). The most prevalent nAChRs in the brain are the α4β2 and α7 subtypes, both of which have been shown to be present in reduced numbers in post-mortem studies of

schizophrenic patients (Breese et al., 2000; Freedman et al., 1995). Muscarinic acetylcholine (mAChR) receptors are metabotropic and found in both the central and peripheral nervous system. These receptors are stimulated by muscarine and acetylcholine, though blocked by atropine. There are five subtypes of muscarinic receptors (M1-M5) (Bonner et al., 1987) which are all G-protein coupled and mediate slow synaptic transmission.

ALZHEIMER'S DISEASE

A deficit found involving the cholinergic projections from nucleus basalis of Meynert to cortex and hippocampus was consistently found in material from Alzheimer's patients. In addition, choline acetyltransferase, which is the enzyme responsible for the synthesis of acetylcholine, was remarkably decreased in pathological samples from cortical and hippocampal areas of Alzheimer's patients. These findings along with others lead to the review article published by Bartus in 1982 which was considered to be the first comprehensive statement for the cholinergic hypothesis of age-related cognitive dysfunction and dementia (Bartus et al., 1982).

As the role of the cholinergic system was tested for Alzheimer's, there was a drive to develop animal models with lesions of the cholinergic projections from the basal forebrain to cortex cerebri and hippocampus. Initial studies used excitotoxins, such as ibotenic or quisqualic acid, stereotaxically injected in NBM of rats (Dunnett et al., 1991; Muir et al., 1993). The results produced consistent depletions of the NBM cholinergic neuron population but contradictory results were in part attributed to the non-specificity of the treatments which resulted in lesioning of non-cholinergic neurons along with the cholinergic neurons. The immunotoxin 192 IgG-saporin was developed to specifically and selectively target the cholinergic neurons, while sparing other cell types (Wiley et al., 1991). Clearly, Alzheimer's disease involves other important pathological processes, notably mutations that lead to the development of amyloid plaques, but treatment with drugs that enhance cholinergic signaling has remained one of very few options to improve cognition.

SCHIZOPHRENIA

Schizophrenia is a chronic, severe and disabling mental disorder. It is listed among the top ten leading causes of disease-related disability in the world due to the pervasiveness of associated deficits, the frequently life-long course and a lifetime prevalence of 1% worldwide (Carpenter and Buchanan, 1994). There is an earlier onset for males, typically during late adolescence and early 20's, while women tend to have a later onset (mid to late 20's) with an additional peak around menopause (Hafner et al., 1993). The disease is associated with a much higher rate of suicide than in the general population; approximately 10% of schizophrenia-patients, especially young adults males, commit suicide (Meltzer, 2002).

Schizophrenia was first classified as a discrete mental disorder by the German physician Emile Kraeplin who separated schizophrenia from bipolar disorder and senile dementia in 1887. He called the illness "dementia praecox", (early dementia) to emphasize that the illness began in early adulthood rather than old age (Kraepelin, 1919). He defined the disorder as involving severe loss of mental function (dementia) as compared to bipolar disorder, which includes periods of recovery for the patient. However, unlike most dementias, some portions of the mind appeared intact while others were severely disturbed. Moreover, there was a marked deterioration in early adulthood which seemed to overcome mental functions which had been intact up to adolescence in the patient's life. As a result, Eugen Bleuler coined the term schizophrenia in 1911, from the Greek *schizo* meaning split, and *phrene*, mind. The split referred to the fracturing of the whole mind, with behavior, emotion, and intellect failing to work together. It was not a reference to multiple personality disorder, clinically termed dissociative identity disorder (Bleuler and Zinkin, 1950).

Causes

A genetic component in schizophrenia is clearly established, though the precise mechanisms of inheritance of risk are not understood. Several environmental factors also show associations with schizophrenia, including winter/spring birth, prenatal infection and famine, obstetric and perinatal complications, cannabis abuse, social stress, older paternal age, and migration (Susser and Lin, 1992; Thomas et al., 2001; Wright et al., 1995).

Symptoms

The symptoms affect the full range of intellectual and emotional functions and fall under three broad categories: positive/psychotic symptoms (i.e. hallucinations, delusions, disorganized speech/thinking), negative symptoms (i.e. apathy, social withdrawal, poverty of speech) and cognitive impairments (i.e. poor executive functioning, inability to sustain attention, working memory impairments). The negative and cognitive symptoms are more persistent and chronic, while the psychotic symptoms are typically episodic in nature. The available treatments (mostly antipsychotic drugs that are dopamine D2 receptor antagonists) only alleviate aspects of the positive symptoms, but do not cure the disease. Because of the fact that negative symptoms and cognitive impairments appear in childhood in individuals prior to the develop psychosis (Woo et al., 2010), it has been suggested that these aspects are the core deficiencies of schizophrenia that drive the progression of the disease (Gandal et al., 2011). When schizophrenia is considered from this point of view it motivates the study of brain areas involved in social interaction and cognitive function.

Diagnosis

Currently, there are no distinct biomarkers for schizophrenia, therefore diagnosis of schizophrenia is based on the presence of a certain combination of symptoms for over a defined period of time. In the United States, the Diagnostic and Statistical Manual of Mental Disorders is in transition to the latest edition (DSM-5). The changes to assessment of schizophrenia focus on changes in the symptoms required to meet criteria. The schizophrenia subtypes, which included paranoid, disorganized, catatonic to name a few, have been removed, and instead the focus is on the symptom types and severity expressed across individuals on a spectrum (APA, 2013). However, there is debate about making a shift between strict categories of distinct mental illnesses, or placing categories across a spectrum which is more clinically relevant (Adam, 2013).

DA hypothesis

The predominant pathophysiological hypothesis of schizophrenia for the past decades, the dopamine hypothesis, postulates that enhanced activity in the mesolimbic dopamine-system is associated with psychotic episodes. The dopamine D2 receptor antagonists can alleviate positive symptoms of the disease (Carlsson, 1988) while dopamine-releasing compounds, such as amphetamine, can induce schizophrenia-like symptoms in healthy volunteers, as well as exacerbate the psychotic symptoms in schizophrenics (Angrist et al., 1974). However, these results do not explain the whole range of symptoms expressed in patients. Therefore, the dopamine hypothesis has been modified to also include dopamine hypofunction in the mesocortical projection which is believed to be associated with the negative symptoms of the disease (Knable and Weinberger, 1997). Even so, irregularities in dopamine transmission cannot fully account for all aspects of the disease, particularly not for the full range of negative symptoms and cognitive impairments. Mechanisms other than dopamine dysfunction must therefore also be involved in the pathogenesis of schizophrenia.

Glutamate hypothesis

The glutamate hypothesis of schizophrenia is mainly based on pharmacological evidence that NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, induce clinical symptoms, both positive and negative symptoms, as well as cognitive deficits, very similar to those of schizophrenia (Javitt and Zukin, 1991). In addition, post-mortem studies have reported reduced expression of glutamate receptors, especially of the NMDA receptor subunit, in several areas of the brain, including prefrontal cortex and hippocampus (Harris et al., 2003).

Cholinergic hypothesis

Another theory suggests that cholinergic disturbances may be involved in the etiology of schizophrenia. This hypothesis is supported by genetic, histological and

pharmacological data. Mutations in the α7 nicotinic acetylcholine receptor gene have been linked to auditory sensory gating deficits characteristic of a considerable number of schizophrenic patients and their relatives (Adler et al., 1985). Altered function and expression of both nicotinic and muscarinic acetylcholine receptors have been reported in cortical and subcortical regions in post-mortem schizophrenic brains (Crook et al., 2001; Freedman et al., 1997). Schizophrenic patients tend to be heavy cigarette smokers, and it has been suggested that this may be an attempt to compensate for a deficit in nicotinic cholinergic receptors. Experimental studies have shown that atypical antipsychotic drugs, which are more effective at treating negative symptoms and cognitive impairments as compared to typical antipsychotic drugs, in addition to blocking the dopamine D2 receptors, also enhance the efflux of acetylcholine in prefrontal cortex (Ichikawa et al., 2002). In addition, muscarinic receptor agonists have been suggested to possess antipsychotic activity in animal models (Shannon et al., 2000) as opposed to muscarinic antagonists, which may exacerbate psychotic symptoms (Yeomans, 1995).

HOW CAN SCHIZOPHRENIA BE MODELED IN ANIMALS?

Schizophrenia is presumably a uniquely human disorder, and it is impossible to fully model its phenotypic spectrum in animals as subjective symptoms such as delusions and hallucinations cannot currently be modeled with rats. Nevertheless, aspects of main symptom clusters can be studied using rodent models, with the help of lesions, different transgenic models as well as challenging the system with various agonist or antagonist drugs (Mouri et al., 2013; Neill et al., 2010; Young et al., 2010).

Positive symptoms can include psychomotor agitation, which can be induced with such drugs as ketamine, phencyclidine and amphetamine, known to produce a psychotic state in healthy subjects (Javitt and Zukin, 1991; Krystal et al., 1994). Rodent models also exhibit drug-induced locomotor hyperactivity which is counteracted by antipsychotic medications (Ögren, 1996).

Currently, there are treatments which address the positive symptoms for patients, however the treatment for particularly the cognitive symptoms is lacking. To meet this need the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative identified seven domains of cognition found to be deficient in schizophrenia and a rodent test battery was compiled to probe each domain to improve drug development for the treatment of these deficits (Young et al., 2009). The novel object recognition task and social interaction chosen in the following studies are two paradigms which are included in this battery of testing.

PCP

Phencyclidine is an N-methyl-D-aspartate (NMDA) receptor antagonist which binds to a specific site located inside this ion channel, blocking the glutamate-induced influx of sodium, potassium and calcium ions (Reynolds and Miller, 1990). This compound was

self-administered by drug addicts as “angel dust”, a substance of abuse producing cognitive disruptions which in sufficient dose lead to psychotic symptoms (Cohen, 1977). The severe but transient psychotic symptoms these individuals developed raised the interest of researchers. In fact, PCP produced psychotic symptoms so closely resembling the psychotic state in schizophrenia that they could not be distinguished by clinicians (Cohen, 1977). For this reason it has become an immensely useful drug in experimental schizophrenia research. Animals challenged with this drug exhibit several behavioral deficits (which will be discussed in results and discussion), recapitulating core deficits found in patients with severe schizophrenia

192 I_GG-SAPORIN

The cholinergic neurons in NBM of the rat basal forebrain project to cortex cerebri (Mesulam, 2004; Mesulam et al., 1983) (See figure 1). Wiley and collaborators (Wiley et al., 1991) developed a potent immunotoxin to specifically target the cholinergic neurons based on their expression of the p75 nerve growth factor (NGF) receptor. An antibody against this receptor is conjugated to the ribosomal inactivating toxin saporin (from *Saponaria officinalis*). After attaching to the target, this conjugate becomes internalized and the saporin will inactivate ribosomes resulting in cell death. This toxin has been used to specifically lesion different cholinergic pathways in the brain (Berger-Sweeney et al., 1994; Torres et al., 1994; Wenk et al., 1994; Wiley et al., 1991). The lesion is very specific with no or minimal damage to other structures at the site of injection. As has been reported the toxin does not significantly decrease the number of non-cholinergic basal forebrain neurons containing: galanin, or somatostatin, (Burk and Sarter, 2001; Kaur et al., 2008; Roβner et al., 1995), and no affect was reported on cortical monoamine levels (Pizzo et al., 1999). In addition to the cholinergic neurons which project to neocortex, a minority of the neurons of Ch4 project to the amygdala. However these cholinergic neurons do not express p75 NGF receptors and are therefore not effected by the toxin (Heckers et al., 1994; Henderson and Evans, 1991).

2 AIMS

The focus of this thesis is to increase understanding of the role of the basalo-cortical cholinergic projections by studies of behavior and gene expression patterns in the presence or absence of cholinergic innervation of cortex cerebri. This role of the cholinergic innervation is evaluated under otherwise normal circumstances, as well as with respect to how this innervation modulates a strong drug-induced experience such as an acute phencyclidine injection. The aim with these two treatments and two levels of analysis is to provide novel information of relevance for memory and memory impairment, as well as for the possible involvement of cholinergic dysfunction in schizophrenia.

To accomplish these overall goals, the aims addressed in the four papers were to:

- evaluate the role of neocortical cholinergic innervation and the NMDA receptor antagonist phencyclidine on behavioral tasks, namely social interaction and novel object recognition, in order to investigate schizophrenia-related negative symptoms and cognitive deficits

- consider methodological advantages and disadvantages including validity and reliability issues of analyzing behavioral paradigms manually versus using automatic program scoring

- localize and quantify c-Fos mRNA expression in rat brain tissue as a marker of neuronal activation after cortical cholinergic denervation and/or PCP challenge

- investigate possible alterations anywhere in the rat transcriptome due to cholinergic denervation and/or PCP challenge by the use of hypothesis-free deep sequencing (RNA-Seq)

3 MATERIALS & METHODS

ANIMALS

Adult male Lister hooded rats (Charles River Laboratories, Sulzfeld, Germany), weighing 250-340g at the time of surgery, were used in all experiments. The animals were housed 4 per cage under standard laboratory conditions with a 12-h light/dark cycle (lights on at 06:00 h), room temperature 21 ± 2 °C, humidity 50%, in transparent cages (1354G Eurostar Type IV, Techniplast, Italy) lined with bedding material (Aspen wood, Tapvei, Harjumaa, Estonia), and had access to food (R34 chow, Labfor, Lantmännen, Stockholm, Sweden) and water *ad libitum*. Experiments were carried out during the light phase. All experimental procedures conformed to the Swedish Animal Welfare Act SFS 1988:534, as approved by the local Animal Research Committee of Stockholm.

SURGERY

The animals were allowed to acclimatize for at least 1 week before surgery commenced to lesion the cholinergic neurons in nucleus basalis magnocellularis (NBM). Anesthesia was induced with 4-5% isoflurane (Baxter medical AB, Kista, Sweden) inhalation. The rats were then mounted in a stereotaxic frame (David Kopf, Tujunga, CA, USA) and anesthesia was maintained via a face mask at 1.5 – 2.5% isoflurane mixed with air set at a flow rate of 500 ml/min. The head was shaved and swabbed with iodine. The skull was exposed and 2 drill holes made. Ophthalmic ointment (Viscotears, Novartis, Sweden) was applied to keep the eyes moist during surgery. The cholinergic immunotoxin 192 IgG-saporin (Advanced Targeting System, San Diego, CA, USA) was diluted in sterile Ringer solution (Fresenius Kabi AG, Bad Homburg, Germany) and infused either bilaterally (Paper I, II, IV) or unilaterally (Paper III) at the following coordinates relative to bregma (in mm): anterior-posterior: +1.2; lateral: ±3.2; ventral: -7.2 (from dura); incisor bar set to +5.0 (Ferencz et al., 2000; Paxinos and Watson, 2007). For the unilaterally lesioned rats in Paper III, the side receiving the saporin injection was alternated between rats. The immunotoxin (volume: 1.5 µl/side; concentration: 0.054 µg/µl; total dose/side: 0.081 µg; lot number: 64-124) was injected with a 10-µl Hamilton injection syringe connected to a micro-infusion pump (KD Scientific Inc., Holliston, MA, USA) set at a flow rate of 0.2 ml/min. After the injection, the syringe remained in place for 3 min before being withdrawn. Sham-operated control rats received equal volumes of sterile saline or Ringer solution. All rats received a bolus injection of buprenorphine (0.3 mg/ml) (Schering-Plough Europe, Brussels, Belgium), saline (1 ml) to prevent dehydration, and lidocaine (AstraZeneca, Södertälje, Sweden) was applied topically to the sutures at the completion of surgery. Each rat was then placed in a holding cage before being returned to the home cage and then allowed to recover for at least 2 weeks before experiments began. Naïve control rats did not undergo any surgical procedure though were housed in home cages with animals which had undergone surgical procedures.

VERIFICATION OF CHOLINERGIC DENERVATION

The potency of the toxin was confirmed using two methods. Acetylcholine esterase (AChE) staining detects the cholinergic fibers in the cortical mantle and cholinergic neurons in NBM. The absence AChE staining can be observed by microscopy and semi-quantified by comparing optical density (OD) values of lesioned and sham-operated control rats. Radioactive *in situ* hybridization with probes for p75 mRNA detects the immunotoxin-sensitive cholinergic neurons in NBM. The absence of p75 mRNA labeling in immunotoxin-injected areas, paired with the presence of p75 mRNA signals in the same areas of sham-operated brains, characterizes successful immune-lesioning. Lack of p75 mRNA hybridization corresponds well with a loss of AChE labeling of the cortical nerve fibers (Singh and Schweitzer, 1995).

HISTOCHEMISTRY AND IN SITU HYBRIDIZATION

Tissue preparation for histology

For perfused tissue, used primarily for AChE staining, rats were anesthetized with an overdose of sodium pentobarbital and underwent cardiac perfusion with 100 ml Tyrode containing heparin, followed by 350 ml 4% paraformaldehyde with 0.14% picric acid. The brains were removed and post-fixed in the same fixative for 1 h and then transferred to a 10% sucrose phosphate buffer solution.

For fresh frozen tissue, used primarily for *in situ* hybridization, rats were anesthetized with isoflurane (5%) and sacrificed by decapitation. Brains were removed, frozen on dry ice, and stored at -80°C. Frozen brains were sectioned (14µm) with a cryostat (Microm HM 500M or Microm HM 560; Thermo Fisher Scientific Inc., Walldorf, Germany) and the slides stored at -20°C until use.

Acetylcholine esterase (AChE) histochemistry

To verify the effect of the 192 IgG-saporin immunotoxin, selected rats from each experiment were perfused and brains processed for AChE histochemistry (Karnovsky and Roots, 1964). Slides were incubated at room temperature for 2 h in a solution containing: 1.7 mM acetylcholine iodide, 0.64 M sodium acetate, 5 mM sodium citrate, 3 mM $CuSO_4$ and 0.5 mM $K_3Fe(CN)_6$. Slides were thereafter rinsed in phosphate-buffered saline, dehydrated and finally cover slipped.

This technique uses a “direct-coloring” method developed by Karnovsky and Roots, where acetylthiocholine iodide is used as a substrate for the detection of cholinesterase activity. The product of the cholinesterase activity, thio-choline, is believed to reduce ferricyanide to ferrocyanide, which precipitates as copper ferrocyanide at the specific site of enzymatic activity, and can be visualized as a yellowish-brown color.

Quantification of AChE products

The cholinergic denervation was evaluated in a semi-quantitative manner using the mean OD of AChE-labeled sections and expressed as percent of control. Measurements were taken from two to three sections/hemisphere/region/rat in defined brain regions from digitized images (AxioCam HRc (Zeiss, German) and using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Different levels of the brain were assessed to determine the extent of lesion. The OD from corpus callosum was considered as background and used to normalize each slide.

Quantitative in situ hybridization

Oligonucleotide probes

The synthetic oligonucleotide probes used for *in situ* hybridization (ISH) were approximately 50 base pairs long and had a guanine-cytosine (GC) content of >45%. The publicly available online software Mfold (version 3.2) was used to calculate the folding energy (Mathews et al., 1999; Zuker, 2003). Probe specificity was controlled by aligning the probe against all sequences available in a public sequence data base (www.ncbi.nlm.nih.gov/BLAST) and only sequences with high specificity were selected. Several probes were designed for each gene and only probes that generated coherent results were used.

The probe (Thermo Scientific, Waltham, MA, USA) sequences were as follows: c-Fos: 5´-TCA GGA GAT AGC TGC TCT ACT TTG CCC CTT CTG CCG ATG CTC TGC GCT CT-3´; p75: 5´-GGC CAC AAG GCC CAC GAC CAC AGC AGC CAG GAT GGA GCA ATA GAC AGG-3´; NgR-1: 5´-AGT GCA GCC ACA GGA TGG TGA GAT TCC GGC ATG ACT GGA AGC TGG C-3´; BDNF: 5´-CTC CAG AGT CCC ATG GGT CCG CAC AGC TGG GTA GGC CAA GTT GCC TTG-3´.

In situ hybridization

High-stringency quantitative radioactive *in situ* hybridization (paper III) is a method for localization and quantification of mRNA transcripts in cells and tissues without the need of any PCR step, by complementary hybridization of radioactively labeled oligonucleotide probes to transcripts of interest. The probe size (~50 bases) allows for easy penetration into the tissue. The method used in the present thesis follows previously published protocols (Dagerlind et al., 1992; Galter et al., 2003a; Galter et al., 2003b).

Oligonucleotide probes were labeled with α-^{33}P-deoxyadenosine 5´-triphosphate (dATP) at the 3´ end (Perkin-Elmer, Boston, MA, USA) using terminal deoxynucleotidyl transferase (TdT) (Amersham Biosciences, Little Chalfont, UK) and were purified (ProbeQuant G50-Micro Columns, Amersham Biosciences). Cryosections were air dried and hybridized overnight at 42°C with a mixture containing 4x SSC solution (3.0 M trisodium citrate and 3.0 M sodium chloride; pH 7.0), 50%

formamide ($HCONH_2$), 1x Denhardt's solution (Ficoll, Polyvinylpyrrolidone, and BSA), 1% sarcosyl, 0.02 M sodium phosphate (Na_3PO_4), 10% dextran sulfate (wt/vol), 0.2 M dithiothreitol (DTT) ($C_4H_{10}O_2S_2$), 0.5 µg/µl sheared salmon sperm DNA, and the radio-labeled oligonucleotide. The next day, slides were washed in 60°C 1x SSC buffer for 1 h and cooled to room temperature, followed by rapid dehydration in increasing concentrations of ethanol (70%, 95%, and 99.5%) and finally, air dried.

Image analysis

For qualitative assurance and to determine the appropriate time for film exposure, slides were first exposed over night to phosphoimaging plates (Fujix BAS-3000; Fuji Photo Film Co., Ltd. Tokyo, Japan) followed by exposure to autoradiographic films (Biomax, Eastman Kodak, Rochester, NY, USA) together with a ^{14}C standard (Amersham) for 13–23 days depending upon the probe. After development, to determine mRNA content, films were digitized using a high resolution scanner (Epson Perfection V750 Pro, Dual lens system, High pass optics; Digital ICE Technologies, Long Beach, CA, USA) and mRNA signal intensities were quantified (ImageJ, version 1.43u, http://rsb.info.nih.gov/ij/) in specific anatomically defined regions. Optical density readings from scanned autoradiographs were converted to nCi/g as determined from the ^{14}C-step standard curve. For each rat, two to three sections were analyzed for mRNA expression and the results averaged. Mean optical density values ± SEM were then calculated for each area for each treatment group. Data were analyzed using a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test.

DRUG TREATMENTS

Phencyclidine hydrochloride salt (PCP, Sigma-Aldrich, St. Louis, MO, USA) solution was prepared fresh daily, dissolving the salt in sterile saline or Ringer, and used for subcutaneous (s.c.) injections in doses of 1.0, 2.0, or 3.0 mg/kg. Control animals received either sterile saline or Ringer injections.

BEHAVIORAL EXPERIMENTS

To study social interaction, a social interaction test was used, in which interactions of pairs of rats were studied to determine effects of lack of cortical cholinergic denervation and PCP challenges. To study non-spatial working memory, the novel object recognition (NOR) task was used. The method was first developed for rodents by Ennaceur and Delacour (Ennaceur and Delacour, 1988).

Social Interaction (Papers I and II)

Experimental Setup

The social interaction experiment paired two unfamiliar rats in an open-field arena (80x74x40 cm). There was no bedding on the dull gray laminate floor, which was evenly illuminated with indirect lighting. Any fecal matter was removed and the arena walls and floor were cleaned with 70% ethanol and allowed to dry thoroughly to remove any odor between each interaction. One rat of each pair was marked with red ink on the rear portion of the body to facilitate manual and automatic tracking. To improve detection of the two-toned coat color (white body, black hood) of the Lister hooded rats, the default settings were adjusted. The behavior was recorded from above by a digital video camera (Canon HF10).

Figure 2. The experimental design for social interaction.

Experimental Design

The social interaction was comprised of a habituation and a test day. On the habituation day, the rats from the same home cage were placed together in the arena and allowed to explore for 45 min. On the test day, two unfamiliar rats from different cages were both injected subcutaneously with either saline or PCP and then individually placed in separate empty cages for 30 min (Figure 2). At the initiation of the social interaction trial, the pair of unfamiliar rats was placed in adjacent corners of the arena, and the behavior was recorded with the camera placed above for the 10-min trial.

In Paper I, pairs of unfamiliar sham rats and pairs of unfamiliar lesioned (sap) rats, were tested following administration of PCP or saline. In Paper II, one sham rat was paired with one lesioned rat after saline administration.

Analysis of Social Interaction

The social interaction films were analyzed with two different methods: manual analysis (MA) (Table 1), and program analysis (PA) (Table 2). In Paper I, the program analysis coded for active and passive social contact, the number of approaches, and the total distance traveled. In Paper II, the MA analyzed 26 behaviors, while the PA analyzed the number of “social approaches”, “social sniffs”, and “social contacts”. The three behavioral parameters used to compare the manual and program analyses were

therefore the individual number of approaches, duration of social sniffs and social contacts.

Manual Analysis (MA)

The manual analysis (MA) was performed by trained observer blind to surgical and drug treatment of the animals. The films were scored with the assistance of the EthoLog® program (Ottoni, 2000) with designated key strokes for 26 distinct behaviors, 13 of which were used (Table 1). The latency (time to the onset of the first bout of each behavior), frequency (total number of bouts of each behavior per session) and duration (total duration in seconds of each behavior per session) of the behaviors were calculated. The observer coded each rat individually for the entire 10-min trial; therefore each video was coded twice, once for each individual rat.

Table 1. Ethogram of manually scored behaviors in the open field arena.

Behavior	**Manual Analysis Description and Definition**
Approach	The rat is walking or running more than three steps without hesitation, with the nose pointed in direction towards the other rat and fulfils the approach within a minimum of 5 cm from the other rat.
Social Sniff	
head-head	The head of the rat touches the head of the other rat.
head-tail	The head of the rat touches the tail of the other rat.
nose-nose	The rat sniffs the other rat's nose in an equal sniff.
nose-side	The rat sniffs between the ventral region and the back of the other rat.
nose-genitals	The nose of the rat touches the genitals of the other rat.
Social Contact	
Passing	The rat passes the other rat either in a direct meeting or from behind.
Following	The rat follows the other rat more than two steps.
Mount	The rat rears and leans its front legs on the other rats' back, from the back.
Chase	The rat runs after the other rat more than two steps.
Nuzzling	The rat sniffs/bites/grooms the other rat in the area between the tip of the nose and the ventral region.
Avoiding	The rat moves or faces in a direction away from the other rat when the other rat is approaching.
Crawling under	The rat is crawling under the other rat.

Program Analysis (PA)

The program (PA) analyzed the films using dedicated software (TopScan, SocialScan 2.0 programs, Clever Sys Inc., Reston, VA, USA) (Table 2). For Paper I, the program

analyzed the following parameters: number of approaches, social contact, passive and active social contact. In Paper II, the program analyzed the number of approaches, social sniff, and social contact. The default settings were also adjusted to improve detection of an approach. However, the default settings were used for the coding of Social Sniff and Social Contact behaviors.

Table 2. Ethogram of behaviors scored using the automated behavioral software program.

Behavior	Program Analysis Description and Definition
Social Approach	One animal approaches the other. It is determined by the initial distance between two animals, the moving direction and the body distance when approaching ends.
Social Sniff	One animal moves to the other one and sniffs. It can be sniffing head, genital or body. It cannot detect the sniffing when two animals are in contact.
Social Contact	Animals stay together when any parts of the two animals are less than 20mm, by default.

Number of Approaches (Paper I and Paper II)

In Paper II, manual analysis scored an approach as one animal moving towards the other without hesitation. The body posture and direction of movement of each animal were considered when scoring (Table 1).

In Paper I, program analysis scored an approach when there was a starting distance ≥ 10 cm between the rats and at least one moved towards the other at a defined angle, and the final distance was within 2 cm of the other rat. In Paper II, the program analysis parameters for an approach were refined and now defined as the two animals starting at a distance greater than 20 cm, traveled a distance of at least 10 cm towards the other, at a defined angle, and ended in close proximity (≤ 10 cm).

Social Sniff (Paper II)

The manual analysis scored the behavior sniffing was defined as one rat sniffing the other rat on the head, tail, nose, side or genitals (Table 1). Each sniffing interaction was coded individually for each rat.

The automated program labels the behavior sniffing as “Social Sniff “ and defines it as when one rat moves to the other rat and sniffs the head, genitals or body with their nose. Scores are determined individually for each rat; however, when the two rats are in close physical contact, the program can lose the nose point detection and therefore not accurately code for sniffing (Table2). It is stated in the definitions for the parameters that the program cannot detect sniffing when the two animals are in physical contact.

Social Contact (Paper I and Paper II)

In Paper I, the duration of passive social contact (TopScan term "immobile contact") was defined as when two animals are sitting or lying within 2 cm of each other and have less than a combined 4% total body movement. The duration of active contact was calculated by subtracting the passive contact (TopScan term "immobile contact") from the duration of total social contact (TopScan term "Social Contact") which included all close (within 2 cm) behavioral interaction. Therefore the duration of active contact included active behavior such as sniffing, grooming, following, fighting, boxing, and pinning.

In Paper II, the manual analysis coded a number of specifically defined behaviors for each rat, and therefore the duration of social contact was calculated as the sum of all behaviors which would involve direct interaction between the pairs of rats. To compare methods of analysis of social contact, all manually scored social sniff and social contact behaviors were summed together (see Table 1).

In Paper II, the program analysis defines social contact as contact in close proximity (within 2 cm) of any body parts (nose, body, or tail point) of the rats. Since the program uses the distance between body points of the two animals, which are either in close proximity or not, the program scores a social contact as the same value for each rat in a paring (Table 2). However, the social contact scoring can be further subdivided into an "active" or "passive" (TopScan terms) contact depending upon who is initiating the contact, and how much joint motion there is.

Distance Traveled (Paper I)

The program analysis tracked the movement of each individual rat following the body point of each rat, during the trial to give the distance traveled and a trace of the pathway. The total distance traveled for the entire trial was assessed for each group. Manual analysis did not code for this parameter.

Thigmotaxis (Paper I)

Thigmotaxis is the tendency for animals to run along the walls of a containment. This behavior signifies increased avoidance of the central open field area, searching for escape points, and increased non-purposeful locomotion due e.g. to anxiety. The program analysis tracked the center body point in the peripheral zone of the arena to record thigmotaxis.

Statistical Analysis

Data for the program analysis are expressed as mean ± SEM. For Paper I, statistical significances were calculated using a two-way ANOVA followed by a Bonferroni post hoc test where appropriate. The assumptions were not met for a two-way ANOVA for the social contact and distance traveled, and the group comparisons were instead

performed with the Mann-Whitney test, while within group comparisons were performed with the Wilcoxon matched-pairs signed rank test (GraphPad Software, Sand Diego, CA, USA). Differences were considered significant when $p < 0.05$.

For Paper II, all data were first analyzed for normality by assessing the sample distribution or skewness (-1.0 to +1.0 considered normally distributed). The results which passed the tests for normality were analyzed with a parametric test, the two-tailed t-test to measure differences in-between groups. The results analyzed parametrically were presented as means ±SEM.

Data which failed normality tests were analyzed with non-parametric tests. The repeated measurement Friedmans's ANOVA was used for individual comparisons of the novel object recognition task exploration time for the three scoring methods used. The Mann-Whitney U-test was used for the preference index of the T1 and T2 results within the three methods of analysis. The results analyzed non-parametrically are presented as scatter plots with medians. The correlations for data not normally distributed were analyzed with the Spearman Rank Order test. The results were analyzed using appropriate software (Statistica® 10.0, Statsoft, Uppsala, Sweden).

Novel object recognition (NOR) (Papers I and II)

Experimental Setup

Novel object recognition was carried out in a Plexiglas box (41x41x41 cm) without bedding as it could interfere with the ability of the program to analyze the images. Blue tinted plastic contact paper on the sides of the boxes obscured the testing room from the subjects. Pilot experiments were carried out to ensure that the there was no preference for either object used and that the objects therefore had no natural significance for the rats. Toy brick towers (Lego Group, Billund, Denmark) were constructed and used together with 50-ml Falcon tubes (Beckton Dickinson, NJ, USA) as test objects, both made of plastic and similar in size (Figure 3). Unscented adhesive putty (Casco, Stockholm, Sweden) was used to affix the objects to the floor to prevent displacement during testing. Each object was wiped with 30% ethanol and then placed 10 cm from opposing corners in the arena. The rat was consistently placed in a third corner equidistant to each object.

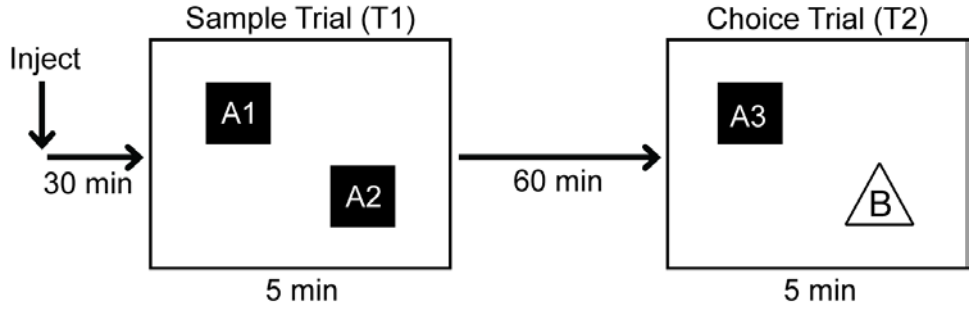

Figure 3. Schematic representation of the procedure for the novel object recognition task. The sample trial (T1) had two identical objects (A1 and A2) and the choice trial (T2) had one familiar (A3) and one novel object (B) *(Paper I and II)*.

Experimental Design

To familiarize the animals to the experimental set up, the day prior to testing, animals were habituated to the arena without objects for 30 min, removed to the home cage for 15 min, and then returned for an additional 20 min. On the test day, each rat was habituated to the empty arena for 20 min, removed and injected with either saline or PCP and then returned to the home cage for 30 min. The rat was then placed in the arena for the sample trial (T1) with two identical objects (A1, A2; half were presented with two toy brick towers, the other half with two Falcon tubes) and allowed to explore the objects for the 5-min trial (Figure 3). After an inter-trial interval of 60 min in the home cage, the rat was returned to the same arena for the choice trial (T2), and presented with two objects, one familiar object (but a new specimen, A3, to prevent a possible scent bias), and one novel object (B); hence always one toy brick tower and one Falcon tube for the 5-min trial. The placement of the novel object was alternated between the A1 and A2 position to prevent a location bias. A digital video camera (Canon HF10) was placed above the box to record each trial for analysis.

There were two criteria for a successful trial, a minimum exploration time, and that the objects remained in an upright position. The 10-s minimum cutoff criterion for total exploration time during each trial was met for all rats. In a few trials, objects were knocked down, and the trial was excluded from analysis.

Analysis of NOR

The object and an area of 1.5 cm around it were defied as the zone of interaction. Exploration of an object was defined as directing the nose at an object within the interaction zone or touching the object with the nose or tongue. The films were analyzed with three different methods: manual analysis (MA), program analysis (PA), and manually corrected program analysis (MCPA).

1. **Manual analysis (MA)** (Paper II) was carried out by a trained observer blind to treatment. Each film was coded at a decreased rate (Medial Player Classic v.6.4.9.0). The space bar key stroke was used to start and stop the clock while coding for the frequency and duration of interaction with each object (including sniffing, licking, and nibbling).
2. **Program analysis (PA)** (Paper II) was conducted using a behavioral software program (TopScan 2.0, Clever Sys Inc., Reston, VA, USA). The user defined zones for the wall, floor, objects, and a 1.5 cm zone around each object. The distance was calibrated from a defined marker of known length. The program identifies three points located at the nose, middle of the body, and at the base of the tail for each rat. The exploration of an object was defined as directing the nose towards an object within a distance ≤ 1.5 cm. The observer initiated the program when the task started with a key stroke, which would then run for 5 minutes. The program tracked the movement of the rat, coding for nose point interaction within the zone of the object, distance and path traveled, speed, and time spent in user defined zones.

3. **Manually corrected program analysis (MCPA)** (Paper I and Paper II) was conducted using parameters within the behavioral software program (TopScan 2.0, Clever Sys Inc., Reston, VA, USA). The program allowed the user to correct for erroneous interactions, resulting in either over-scoring or a missed interaction. The observer used settings within the program to manually enter the program and correct the time settings. An over-scoring occurred if the rat stood or hovered over the zone of the object, though not interacting directly with the object. The program missed an interaction if the nose point was miss-identified. When the rat turns the head at an acute angle from the body, the program can fail to correctly follow, and instead mark the nose as pointing in the direction aligned with the tail and body points, missing an interaction an observer would score. Such missed interactions were identified and corrected for within the program.

Comparison and Statistical Analysis of Scoring Methods

Paper I: Data of the exploration time of each object, total exploration, and total distance traveled were expressed as means ± SEM. Group comparisons were performed with the Mann–Whitney test, and within group comparisons were performed with the Wilcoxon signed rank test (GraphPad Software).

Paper II: The individual exploration time of the familiar and novel object was used to compare the three different methods of analysis. The obtained data from the three methods were correlated for validation. A preference index was calculated using the formula PI = (n) / (n + f), where n is the time actively touching or sniffing the novel object and f is the time actively interacting with the familiar object. The index ranges from 0 to 1.0, where 0 signifies complete preference for the familiar object, 0.5 no preference for either object, and 1.0 complete preference for the novel object.

RNA-SEQ (PAPER IV)

RNA-Seq is a recently developed approach to transcriptome profiling. Complementary DNA (cDNA) is generated from RNA and sequenced using next-generation “short read” technologies. The reads are aligned to a reference genome and a transcriptome map is constructed. It provides a more precise measurement of levels of transcripts and their isoforms than other methods. Analyzing RNA sequences and then quantifying gene expression levels can give insight into the regulatory mechanisms of gene expression with a genome-wide perspective.

Experimental Design

Two weeks post-surgery, intact and chronically cholinergically denervated rats were handled, weighed, and injected with sterile saline solution for two days prior to the test day. On the experimental day, the rats were acclimatized to the procedure room for 60 min prior to administration of 1 or 3 mg PCP/kg or saline subcutaneously in the scruff

of the neck. The rats were returned to the home cage with cage mates comprised of both saline and PCP treated animals. After 30 or 90 minutes, rats were anesthetized with isoflurane (5%) and sacrificed by decapitation. Brains were rapidly removed, a 2 mm thick slice located between 2.00 and 0.00 mm relative to Bregma was removed. Two mm diameter punches of the somatosensory cortex were dissected out (primary somatosensory cortex, upper lip region, including areas of the secondary somatosensory cortex closer to 0.00 relative to bregma). The punches from the right and left side of the same rat were pooled in tubes which were flash frozen in liquid nitrogen. The remaining brain tissue was frozen on dry ice and all tissue was stored at -80°C.

RNA extraction and RNA-Sequencing

Total RNA was extracted from the cortical punches of 3 adult Lister hooded rats from each treatment group, using TRIzol® (Live Technologies™) and a tissue homogenizer. RNA quality was measured (Agilent 2100 Bioanalyzer RNA Nano Chip) and showed that the samples had an RNA integrity number (Kane et al., 2012) of 7.7. RNA-Seq of the RNA samples was carried out at Science for Life Laboratory, a commercial facility operated by the Karolinska Institute together with the Royal Institute of Technology in Stockholm, Stockholm University, and Uppsala University. Poly(A)+ RNA was purified from 250 ng total RNA and cDNA libraries synthesized using the mRNA-Seq prep kit with oligo(dT) priming (Illumina cat. n. RS-100-0801) as per the manufacturer's protocol (http://mmjggl.caltech.edu/sequencing/mRNA-Seq_SamplePrep_1004898_D.pdf). Libraries were multiplexed in groups of six and ran on a single flow cell lane, and 100-bp paired end sequences were generated using an Illumina Hi-Seq 2000 sequencer.

The flow cell surface is coated with single stranded oligonucleotides which correspond to sequences of adapters ligated to the samples during preparation. The single-stranded, adapter-ligated fragments are bound to the surface of the flow cell and exposed to reagents for polyermase-based extension. Priming occurs as the free end of a ligated fragment "bridges" to a complementary oligo on the surface.

Statistical analysis of gene expression

The Illumina pipeline was used to analyze the images and extract base calls for FASTQ file generation. Overall quality and total read counts are reported. One sample did not pass quality control and was removed leaving n = 2 for the one sham (saline, 30 min) group. The FASTQ files were aligned to the rat reference genome (rn4) using Tophat (v2.0.3) and Bowtie (2.0.0.7). Reads were annotated and quantified to a given gene using the Python module HT-SEQ with the RGSC3.4.69 gtf to provide reference boundaries.

The R/Bioconductor package DESeq was used to normalize for library size and to perform a variance-stabilizing transformation. The normalized counts for each

transcript compared all groups using ANOVA and then corrected for multiple testing using the Benjamini-Hochberg procedure.

4 RESULTS & DISCUSSION

This thesis has focused on the role of the cholinergic system and how a challenge with PCP affects sensory experiences in the presence or absence of cholinergic innervation of cortex cerebri as reflected in animal behaviors and gene expression patterns. A lesion model to remove the cholinergic projections from NBM was utilized, and the resulting consequences with and without PCP challenge were analyzed.

CHOLINERGIC DENERVATION AFTER 192 IGG-SAPORIN INJECTION (PAPERS I – IV)

To manipulate the cholinergic system, we utilized an immunotoxin to specifically target the cholinergic neurons in the NBM. The infusion of 192 IgG-saporin into the basal forebrain effectively destroys the cholinergic neurons and their projections to cortical areas without direct effects on other neuronal systems. The immunotoxin was injected bilaterally (Paper I, II, IV) or unilaterally (Paper III) into NBM, and resulted in an almost complete bi- or unilateral loss of AChE-positive nerve fibers in cortical areas, most prominently seen in the primary somatosensory cortex. At the injection site and surrounding NBM, almost no cholinergic cell bodies were identified in lesioned rats by either AChE staining or *in situ* hybridization to locate p75 mRNA.

PAPER I

In a series of experiments (Paper I and II) we evaluated the effects of cholinergic denervation of NBM alone, and/or after an acute dose of PCP, on two behavioral paradigms, social interaction and novel object recognition (NOR). The rationale for the use of these two tests was that social withdrawal is a key deficit of schizophrenia, which is why we chose to include quantitative assessments of social interaction. Symptoms of schizophrenia also encompass cognitive deficits. In order to obtain measurements specific for this aspect of the disorder we assessed performance of the animals in novel object recognition tasks.

In the first study of this thesis (Paper I), pairs of unfamiliar lesioned and pairs of unfamiliar sham-operated rats interacted in an open field arena for a 10-min trial and their interaction was analyzed after injections of saline or PCP (1 mg/kg, s.c.). The animals were placed in the arena 30 min post injection. This time point was chosen based on previous studies from our laboratory which revealed an increase in locomotor activity for both lesion and control rats caused by PCP (1 mg/kg, s.c.), and which returned to habituation levels after 30 min (Mattsson et al., 2005). In addition, there was no statistical difference in locomotor or rearing activity between the lesioned and control groups at this dose after 30 min. By taking this effect into account, we avoid convolving effects on social interaction with effects pertaining to increased locomotor activity.

Social Interaction

Cholinergic denervation decreases active social contact

Pairs of cholinergically denervated rats spent significantly less time (20% decrease) actively interacting as compared to pairs of sham-operated controls after saline administration (Figure 4A). However, there was no difference between groups regarding the number of times a rat approached the conspecific (Figure 4C). These results indicate that the lesioned rats did not avoid an initial contact, but rather discontinued actively interacting once initial contact had been made. Although the total distance traveled was significantly lower for lesioned rats (Figure 4D), this does not seem to be an effect of the lack of cholinergic innervation, as there was no difference between groups after PCP administration at this dose.

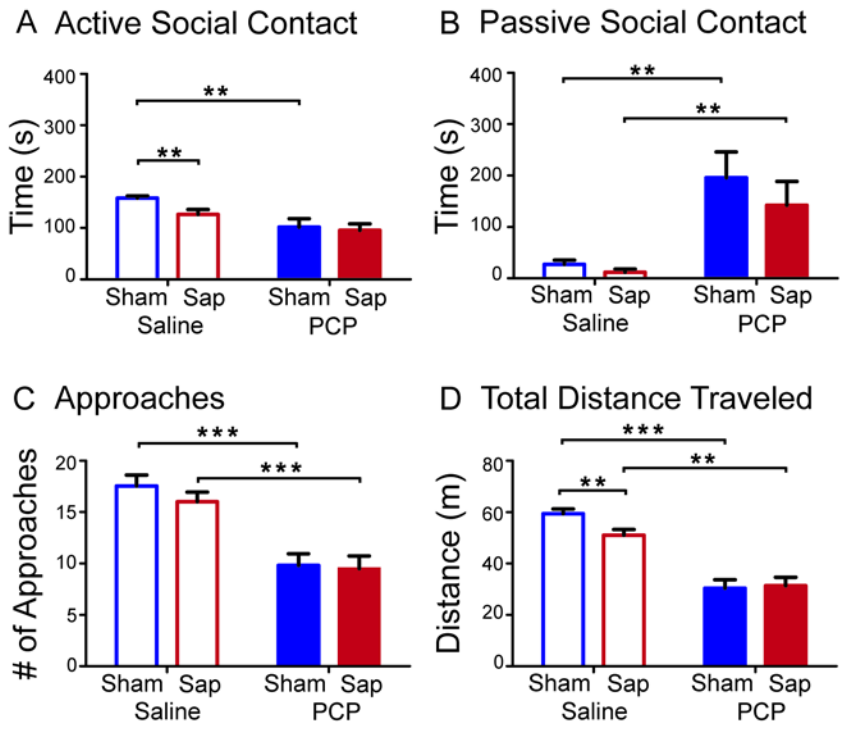

Figure 4. Social interaction after administration of saline (open bars) or PCP (filled bars; 1.0 mg/kg, s.c.) to pairs of cholinergically denervated (sap, red) and control (sham, blue) rats. Duration of active social contact (A), duration of passive social contact (B), the number of approaches by individual rats (C), and total distance traveled by individual rats (D). Mean ± SEM; ** p < 0.01; *** p < 0.001 *(Paper I)*.

PCP decreases active and increases passive contacts, irrespective of cholinergic denervation

We next challenged the pairs of rats with an acute dose of PCP (1 mg/kg, s.c.) administered 30 min prior to the trial. At this dose, the duration of active contacts decreased, while the duration of passive contacts increased for both groups (Figure 4B). As a result, the number of approaches was decreased for both groups, as the pairs spent more time sitting next to each other, in direct or close contact, rarely moving. Overall,

there was little exploration of the arena or one another. These results are in contrast to behavior under saline conditions, where the rats had been moving throughout the arena, interacting, then moving away to explore, before interacting again. The decrease in overall activity level is reflected in the decreased number of approaches (Figure 4C) and decreased total distance traveled (Figure 4D).

Social withdrawal is a prominent feature of the negative symptoms of schizophrenia. In patients, this withdrawal often precedes the onset of the first psychotic episode and is strongly associated with poor psychosocial function (Cramer et al., 1992; Mueser and McGurk, 2004). The social interaction test has been used in animals to model negative symptoms of schizophrenia and PCP challenge (Bruins Slot et al., 2005; Mintz et al., 2005; Sams-Dodd, 1996), anxiety (File and Seth, 2003), and autism (Kane et al., 2012).

Though few studies to date have investigated the role of the cortical cholinergic innervation in social interaction, a number of studies have used pharmacological means to investigate the possibility that cholinergic blockade may impact social interaction. Studies have demonstrated that systemic administration of the muscarinic antagonist scopolamine has been shown to impair social interaction in mice (Wang et al., 2007), and social recognition memory in rats (Van Kampen et al., 2004).

Administration of PCP has been shown to disrupt social behavior (Sams-Dodd, 1995). Bruins Slot and colleagues reported that rats administered PCP (2.5 mg/kg) moved along the periphery of the arena and avoided contact, resulting in a significant decrease in the total number of social behaviors including sniffing, following, climbing over/going under, and aggressive behaviors (Bruins Slot et al., 2005). As expected, active social interaction in Paper I, which includes sniffing, following, climbing and aggressive behaviors, was significantly decreased after PCP administration. However, we were surprised that there was a marked increase in passive behaviors, which were defined as the pairs sitting in close contact, but with very little body movement (4% total for the pair). In open field arenas, pairs of rats typically, though not exclusively, establish a common home base (Barnett, 1963; Haimovici et al., 2001). The home base is both the preferred location within the area which the animal occupies for longer periods of crouching, grooming, turning or rearing, as well as the location to start and stop explorations (Golani et al., 1993; Tchernichovski et al., 1998; Tchernichovski and Golani, 1995). Mintz and colleagues report reduced social home base occupancy after sub-chronic injections of PCP for pairs of rats (Mintz et al., 2005). In contrast, in Paper I we find a significant increase in passive social contact, which was due to shared occupancy of an area in the arena. The discrepancy could be due to the method of administration of PCP, as we gave an acute dose, whereas Mintz et al., administered sub-chronic doses over 3 days. We interpret the increased passive interaction as a tendency for rats to sit near one another when experiencing drug-induced discomfort.

Novel Object Recognition Task

The NOR task assesses declarative memory and is based on an animal's natural tendency to preferentially explore a novel, as opposed to familiar, object (Ennaceur and

Delacour, 1988). This task has been widely used to assess memory function, by varying the interval trial time, and by drug challenge to either enhance or decrease recognition. Notably, the NOR task set up used in this thesis is not dependent upon spatial memory, which is typically assessed in the Morris swim maze. Therefore, the Morris swim maze and the novel object recognition tests are complementary in revealing different aspects of memory function. Previous data have suggested that the cholinergic system is involved in normal object exploration and discrimination. Scopolamine, a mAChR antagonist, has been shown to impair NOR (Ennaceur and Meliani, 1992), and the effect can be reversed by nicotine and acetylcholinesterase inhibitors (Lieben et al., 2005; Sambeth et al., 2007). Pharmacological and lesion studies have implicated the perirhinal cortex in particular as having a crucial role in this task. Infusion of scopolamine directly into the perirhinal cortex impairs NOR in rats (Abe et al., 2004), and injection of the 192 IgG-saporin immunotoxin into the perirhinal cortex disrupts NOR in rats (Winters and Bussey, 2005).

Cholinergic denervation does not impair the ability to recognize novel objects

Following saline administration, there was no difference between the sham and lesioned groups with regard to time spent exploring the two identical objects (A1 and A2) during the sample trial (Figure 5). During the choice trial, both the sham and lesioned rats spent significantly more time with the novel object than the familiar object. Results indicate that lesion of NBM and the cortical cholinergic afferents did not impair the ability to recognize the novel object in the choice trial carried out 1 hour after the sample trial. In contrast, Winters and Bussey report impairment of NOR after 192 IgG-saporin injections directly into perirhinal cortex (Winters and Bussey, 2005). In our study, the NBM-saporin lesion resulted in a near complete cholinergic denervation of cortex cerebri, as well as a modest cholinergic denervation of the rostral portion of perirhinal cortex. The discrepancy between our results and those of Winters and Bussey may be due to the degree and exact location of the perirhinal lesion. We note that for both trials, the total exploration time for both objects, as well as the total distance traveled, were decreased in the denervated animals, showing that the loss of the cholinergic innervation decreased the intensity of exploration, though not the ability to detect novelty a short time after the sample trial. Taken together, these data suggest that it is the degree of cholinergic deficit and in particular, the location of the denervation, which are important to elicit an impairment of novel object recognition.

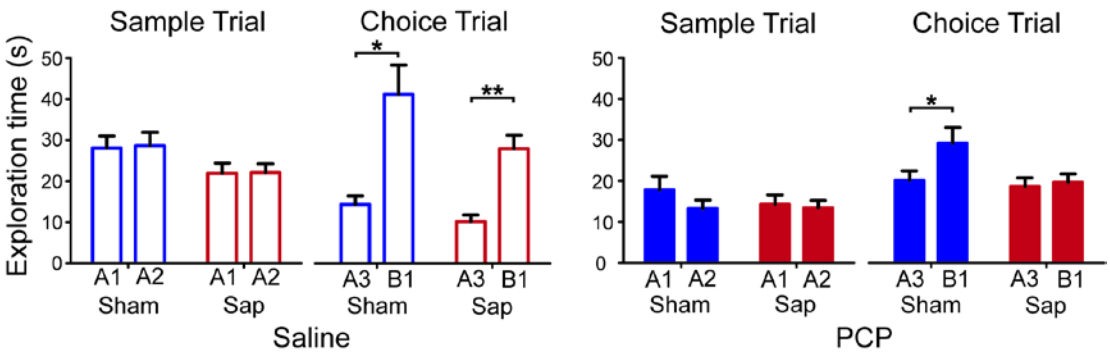

Figure 5. Novel object recognition. Duration of the exploration time for each object after administration of saline (open bars) or an acute dose of PCP (filled bars; 1.0 mg/kg, s.c.) by control (sham, blue) and cholinergically denervated (sap, red) rats. Saline or PCP was injected 30 min prior to the 5-min sample trial with two identical objects (A1 and A2), followed by a 60 min inter trial time, and then the 5-min choice trial with one familiar (A3) and one novel (B1) object. Mean ± SEM; * p < 0.05, ** p < 0.01 *(Paper I)*.

Ability to recognize novel objects is lost in denervated animals given PCP

When administered a low dose of PCP (1.0 mg/kg) 30 min prior to sample trial, there was no difference in exploring the two objects for sham and lesioned groups, and no preference for the identical objects (Figure 5). During the choice trial, 95 min post PCP, the sham group showed a preference for the novel object, but the lesioned group did not. Thus, the low dose of PCP eradicated the preference for the novel object during the choice trial for denervated rats. Studies have shown that when PCP is given at higher doses, or sub-chronically, it impairs NOR (Redrobe et al., 2010). However, the dose of PCP (1 mg/kg) used in this study was sub-threshold, and thus does not impair NOR in sham-operated control rats, though the total exploration time was decreased, as distance traveled was increased. In contrast, the cholinergically denervated rats failed to discriminate between the novel and familiar objects when under the influence of the acute low dose of PCP. Our results therefore suggest that there is an additive interaction between the glutamatergic and cholinergic systems in serving memory functions. These results are in line with reports which show that NOR deficits caused by sub-chronic PCP treatment can be counteracted by activation of α7 nicotinic receptors (McLean et al., 2011) and by the choline uptake enhancer MKC-231 (Shirayama et al., 2007).

Lack of cholinergic innervation dampens intensity of exploration and locomotion in NOR test

Total exploration time and total distance traveled were significantly lower in lesioned as compared to sham-operated rats during both sample and choice trials after saline injection. Chronic lack of cholinergic innervation of cortex dampened the total amount of activity without causing impairment in discrimination of the novel and familiar objects. However, total exploration time after PCP was significantly decreased for both

groups during the sample trial, further reflected in an increase in the total distance traveled for both groups.

In Paper I, we provide experimental evidence for behavioral consequences of cholinergic denervation of cortex cerebri, a significant impairment of active social interaction, and a loss of non-spatial memory when lesioned rats are challenged with the NMDA receptor antagonist PCP at a dose that alone does not block such a memory task. These results add weight to the importance of cholinergic afferents to the cerebral cortex for the maintenance of normal social interaction and for memory function when challenged by pharmacological means.

PAPER II

The behavioral paradigms analyzed in Paper I used a dedicated software program for analysis of NOR and social interaction. As the tasks were being assessed, questions arose as to the most efficient, effective, and valid methods to analyze these behaviors. What considerations should be kept in mind when using a program or manually scoring behavior? What method is best used for the tasks? Do you code the same behavior or miss information with either method? The social interaction analyzed pairs of rats which had undergone the same treatment. How is it best to analyze interaction when the two individuals of a pair have undergone different treatments? The analysis in Paper II was conducted to address these questions.

Animal behavioral studies have traditionally been coded by trained observers. This process is time consuming, requires extensive training, and is susceptible to observer fatigue. A number of programs have been developed to speed up the process and improve reliability. We worked with Lister hooded rats, which have pigmented fur on the head and pigmented markings on the body which is predominately white. The two-toned fur proved difficult for a number of software programs to analyze accurately. Programs would lose part of the body, distorting the analysis of the behavior. One supplier, Clever System Inc., was able to reliably identify the entire body of the rat, which is require when coding two rats engaged in social interaction.

The commercially available program calculates the movement and behavior of each animal by automatically detecting and coding three body points, the nose, body and tail. The program analysis is based on the location and movement of these body points in user defined zones, which may contain objects of another animal.

We hypothesized that the program would be able to effectively analyze “either or” tasks, such as with NOR or the partner preference test, whereas manual scoring would provide better analysis of complex behaviors, such as social interaction.

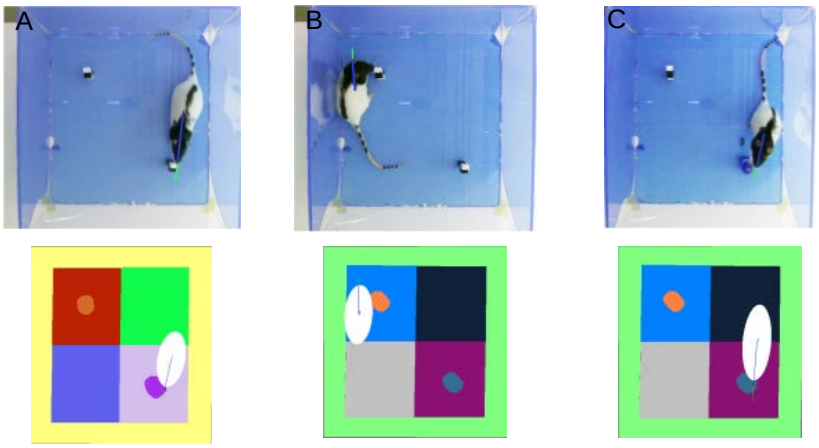

Figure 6. Video images and the corresponding program generated image of NOR from the sample trial (A and B) and the choice trial (C). The program analysis correctly scored an interaction (A) when the nose point was within the zone of the object. The program analysis missed the interaction due to the acute turn of the head which the program failed to detect (B) or if the animal hovered over the object, within the zone, though not interacting (C).

Novel Object Recognition

Video recordings of NOR behavior were assessed by three methods: manual analysis, program analysis, and manually corrected program analysis using parameters within the software set-up. The choice trial after saline administration of sham-operated rats was assessed with the three methods. A difference in exploration of each object was observed between the manual analysis and program analysis. A manual correction of the program showed that two types of errors could occur during the task. If the rat hovered over the object, though not interacting with it, the program could incorrectly score that as a positive interaction, resulting in over-scoring. Alternatively, the program could miss-identify the nose point, which resulted in a missed interaction (Figure 6).

Activity boxes which utilize infrared light to track activity by beam breaks have been used to automate NOR. Silvers and colleagues (Silvers et al., 2007) compared two different sized zones around the objects with manual scoring of NOR. They found that analysis of the zones coded the same preference as the manual analysis. However, it did not actually focus on interaction with the object, but the proximity to the object. As a result, the exploration time as scored manually for each object was markedly different compared the times scored in close proximity by the zones. As a result, small variations between groups may be missed. Rutten and colleagues (Rutten et al., 2008) developed a software to track the nose point of rats. The program analysis was, similar to our analysis, was prone to the false positive exploration scores when a rat hovered over the object, though within the object zone. They found that using small objects, when compared to larger objects, reduced the rearing and leaning behavior, which increased the reliability and accuracy of the program scoring.

The partner preference test (PPT) is a social interaction paradigm often used to examine sexual and social preferences in rodents. The set-up is similar to NOR except the

subjects are offered a choice of two tethered stimulus animals differing in social or sexual valence as opposed to two objects. Ahern and colleagues (Ahern et al., 2009) compared two different automated systems (one of which was Clever System Inc) to manual analysis of the PPT to study pair bonding in prairie voles. Using the program we utilized, they found that automated systems capable of scoring passive social contact ("immobile social contact") yielded results indistinguishable from manual scoring.

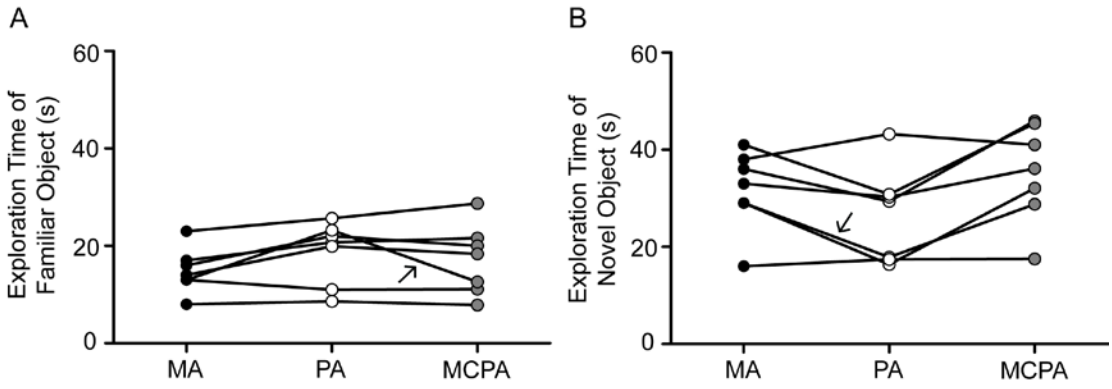

Figure 7. Novel object recognition task scatter plots of the duration of exploration time (s) of the familiar (A) and novel object (B) for each of the three methods of analysis: manual analysis (MA) (●), program analysis (PA) (○), and manually corrected program analysis (MCPA) (◍). Lines connect the different analyses of the same individual. The program analysis was susceptible to two types of errors. The program over-scored if the rat hovered over the object within the detection zone, though not interacting with the object (arrow in 2A). Secondly, the program could miss an interaction due to an acute turn of the head if the nose point was miss-identified as pointing straight ahead away from the object (arrow in 2B). Using settings within the program, the errors could be manually corrected, and placement and choice of objects relative to arena and subject could decrease the instance of these types of errors (Paper II).

The individual scores of the same rat analyzed with the three different methods highlights how scores were either over-scored (arrow, Figure 6A) or missed (arrow, Figure 6B). Analysis of the preference index for NOR resulted in no significant preference for the novel object when program analysis alone was assessed. Both the manual and corrected program had a significant preference index for the novel object. As administration of PCP increases locomotor activity, both objects were placed 10 cm away from opposing corner, to allow the animal to easily pass if engaged e.g. in thigmotaxic behavior. This could result in interaction with objects simply because the animal walked into them. However this placement resulted in missed-interactions if the nose point was miss-identified. A different set up and placement of objects may reduce some of the miss-identified scores, or smaller objects as Rutten et al. concludes.

Social Interaction

To analyze social interaction, cholinergically denervated rats were paired with unfamiliar sham-operated control rats in a familiar open field arena. The video recorded behavior was analyzed using both manual and program analysis and the number of approaches, duration of social sniffing, and duration of social contact were used to compare the two methods of analysis.

Social interaction was developed to use a natural form of behavior while manipulating the environment and then measuring the time spent by pairs in social interaction (e.g. sniffing, following, grooming). File and Hyde proposed this test to study anxiety and found that the behavior of one rat influences that of the other. They argue that the pair of rats should be treated as a unit, and score the pair with one value (File 2003). In Paper I, pairs of lesioned or pairs of sham rats were analyzed using the behavioral program. One value was given for each pair for duration of social interaction, both active and passive. However, we find that both manual and program scoring can reliably score individual values for the number of approaches. An interaction between a pair of rats can be coded with a program as active interaction, but the distinct type of active interaction can be coded by a manual scorer. Therefore, the degree of depth in scoring is determined by the method used.

In Paper II, we coded behavior of a mixed pair of rats, which require different analysis than in Paper I. The number of approaches was assessed individually by both the program and manual analysis, and there was no statistical difference between the two methods (Figure 8A). In line with Paper I, there was no difference between lesioned and sham-operated rats in the number of approaches toward the conspecific.

When two rats are in close proximity, the program can lose track of the nose point, resulting in difficulty determining the specific behavior during the social contact. The program states this point clearly when defining social sniff behavior. The manual analysis coded for sniffing particular areas on the conspecific, and therefore individual values for each rat were obtained. There was no effect of denervation for social sniff, however there was a significant difference between program and manual analysis (Figure 8B). We find that limitations within the program could not accurately determine this parameter.

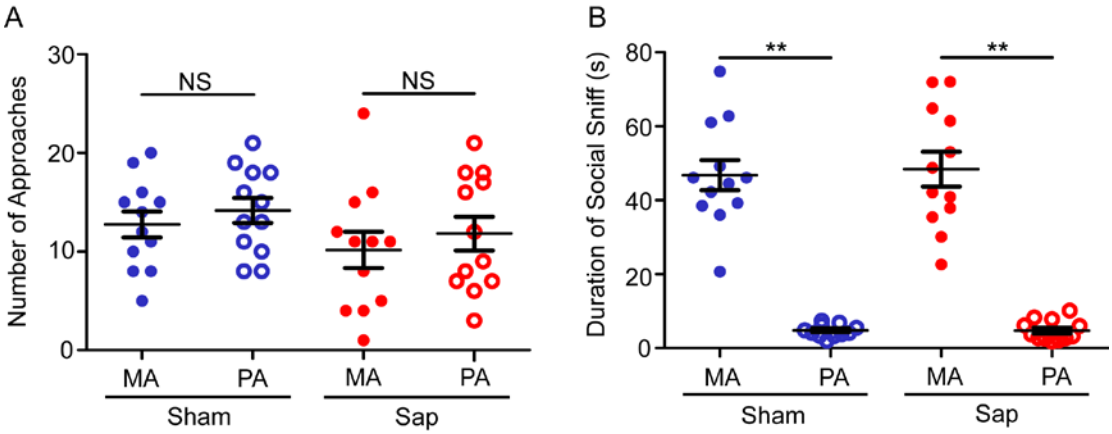

Figure 8. Social interaction scatter plots of the number of individual approaches (A; average ±SEM) and the duration of individual social sniffs (B; average ±SEM) between sham-operated (sham) and saporin-lesioned (sap) animals analyzed either manually (MA) or with the program (PA). There was no difference between the methods of analysis for the number of approaches, however there was a significant difference between methods in how social sniffing was scored. Sham MA (●), sham PA (○), sap MA (●), sap PA (○). **p < 0.01 *(Paper II)*.

Social contact was scored by the program based on the distance between the animals and the direction and amount of movement of each rat. When animals were in close proximity and moving, the program coded for social contact. This could be further

divided into an active or passive interaction. The program defined passive contact as "immobile contact" which was dependent on the close proximity of rats and little body movement. The manual analysis scored each rat individually for 26 distinct behaviors (see Table 1 in Paper II), and 11 of those behaviors which were associated with direct contact between pairs, were summed and compared to the program analysis.

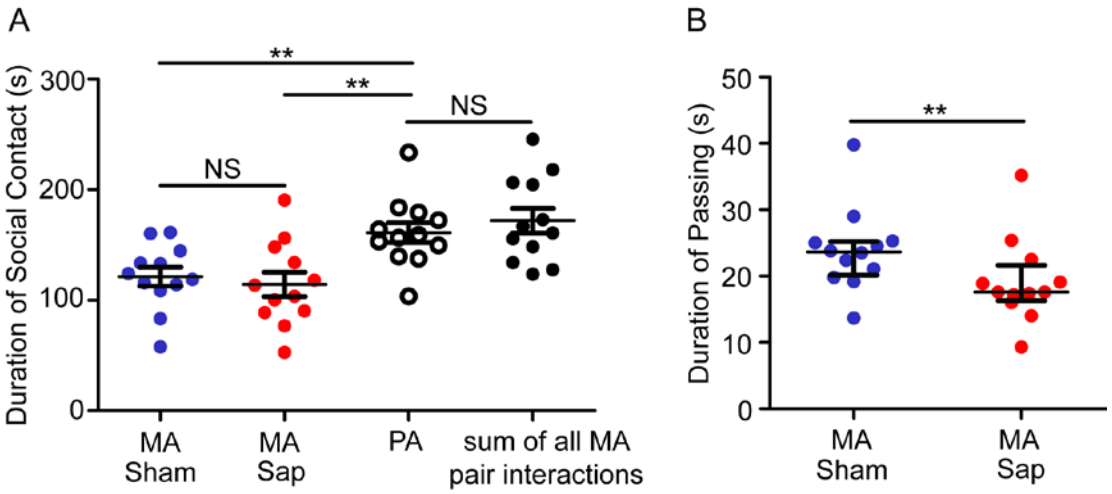

Figure 9 Social interaction scatter plots of the duration of social contact (A). The social contacts (the summation of Social Sniff and Social Contact, Table 1) were manually analyzed in individuals for each pair of sham-operated (sham) and lesioned (sap) animals. The program analysis (PA) of social contacts (Table 2) gives one value for each pairing of sham and sap rats and is based on the distance between the pairs. The sum of all manually analyzed interactions for each animal in a pair results in similar analysis as the program analysis. The total duration of passing (B) of each rat analyzed with MA showed a significant difference between the sham and sap groups, a result missed when analysis is based on the distance between rats. (±SEM, **p < 0.01) *(Paper II)*.

The program analysis gave one value for each pair for social contact. In Paper II, each pair of rats consisted of one lesion and one sham rat, therefore this parameter could not determine if there was an effect of cholinergic denervation on social contact. The manual analysis coded each rat separately, and therefore could determine that after saline administration, there was no difference between intact and denervated animals with respect to social contact behaviors. However, if the behaviors of both rats were combined, the sum of all manually analyzed pairs of interaction were not significantly different from the program analysis. This shows that the program can analyze these behaviors similarly to manual analysis. Further analysis of the manual scoring detected that the duration of passing which occurred after an approach had been made, was significantly decreased for lesioned compared to sham-operated rats. Similarly to Paper I, there was no difference with respect to the number of approaches made, based on cholinergic denervation, but there was a difference in how the interaction progressed after initial contact.

Overall, the program analysis allows screening of large numbers of trials, but must be manually checked for potential errors. It is well suited for tasks where the scoring is "either or" such as NOR or the partner preference test. When analyzing more complex behavioral patterns, such as social interaction between two freely moving animals, a combination of program and manual analysis is recommended to obtain an optimal analysis of the interaction behaviors.

PAPER III

The previous papers looked at the behavioral outcome of cholinergic denervation and/or response to PCP. We then wanted to obtain information about the underlying neuronal mechanisms that produced these behavioral effects. In Paper III, we used the immediate early gene c-Fos to map PCP-induced neuronal activation. We chose this gene because c-Fos is known to increase within minutes in neurons in response to a number of factors, including stress, toxins, trauma, and neural depolarization, (Hughes and Dragunow, 1995; Kovacs, 2008; Sheng and Greenberg, 1990). Due to this fast response, c-Fos mRNA expression can be used as an index of very recent or ongoing neuronal activity, and therefore to map activation of neural pathways by a stimulus (Sharp, 1997).

We unilaterally lesioned one side of the brain and sham-operated the opposite side. Because the basalo-cortical cholinergic system is uncrossed, this procedure results in one fully denervated side and one intact side. To monitor alterations of c-Fos transcriptional activity, we used in situ hybridization to quantify c-Fos mRNA alterations in response to Ringer or PCP (2 and 3 mg/kg, s.c.) at different time points and in defined brain areas.

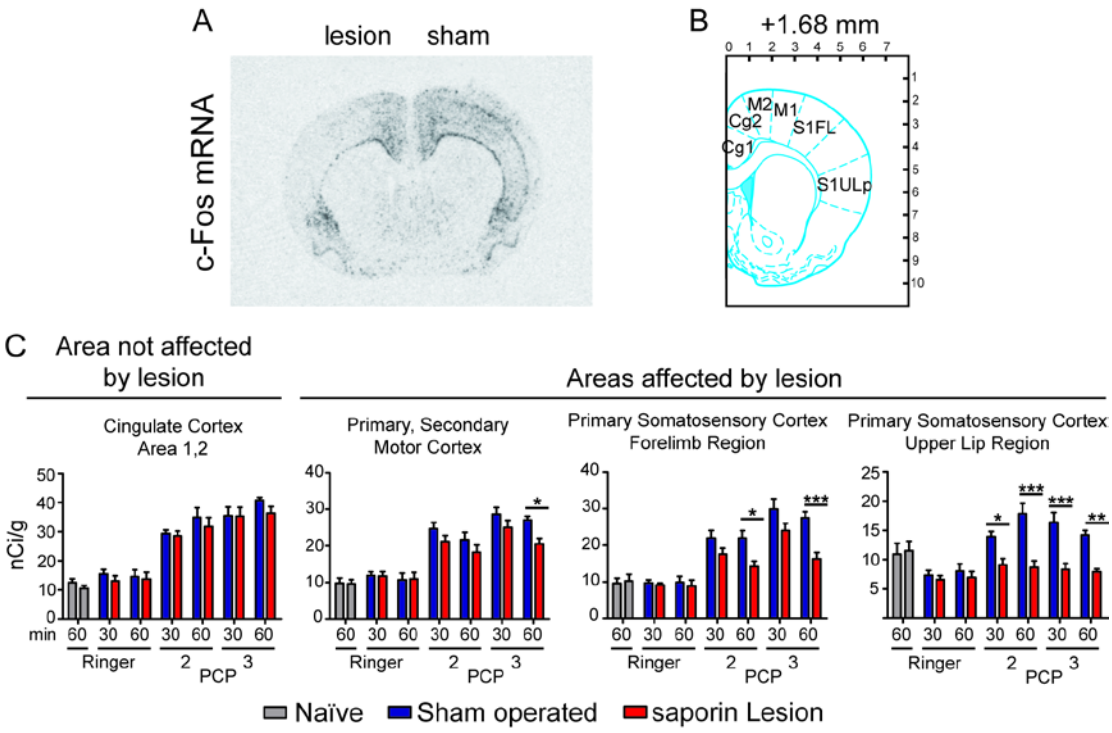

Figure 10. Levels of c-Fos mRNA in different brain areas were measured by quantitative *in situ* hybridization. Representative example (A) of *in situ* hybridization of c-Fos mRNA of unilaterally denervated rat brain (-1.32 mm relative to bregma) which shows the effect of denervation in the form of dampened c-Fos mRNA increases in response to phencyclidine (3.0 mg/kg, s.c.). Areas of analysis (C) are indicated by the atlas image (Paxinos and Watson, 2007) (B). Levels at both side of the brain of naïve rats, and from the sham-operated and denervated side of immunotoxin-treated rats are shown following injections of either Ringer, 2.0 or 3.0 mg/kg PCP. The rats were sacrificed 30 or 60 min after the respective s.c. injections. Values are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences when sham sides are compared to lesioned sides. *Abbreviations*: Cg, cingulate cortex (area 1,2); M1/M2, primary and secondary motor cortex; S1FL: primary somatosensory cortex (forelimb region): S1ULp, primary somatosensory cortex (upper lip region).

No effect of Ringer injections on c-Fos mRNA

We found that 30 and 60 min after an injection of Ringer solution, there was no difference in c-Fos mRNA levels between naïve, sham-operated or lesioned sides in the areas analyzed, regardless if the area was directly affected by the lesion or not. This finding shows that the stress of an injection does not cause neuronal activation at the investigated time points, thus enabling a straight forward interpretation of the effects of the PCP treatment.

PCP induces strong c-Fos mRNA response in intact cortex

As expected, administration of either 2 or 3 mg/kg PCP resulted in robust increases of c-Fos mRNA levels on the sham-operated side when compared to Ringer injected controls, in most areas analyzed. The strongest expression was found in the prelimbic and retrosplenial granular cortex. A comparison of the doses of PCP at different time points after injection indicated that c-Fos mRNA upregulation was typically stronger after the higher dose.

Chonlinergic denervation attenuates PCP-induced c-Fos mRNA response

In areas to which the cholinergic cell bodies in NBM do not project, such as prelimbic, cingulate and retrosplenial granular cortex, and the hippocampal formation (CA1), no differences were noted for the c-Fos mRNA levels between the lesioned and sham-operated sides of the brain after PCP. In contrast, in areas to which the cholinergic cell bodies of NBM do supply a cholinergic innervation, there was an overall tendency for the denervated side to express less c-Fos mRNA than the intact side of the brain after PCP. Chronic lack of a cholinergic innervation dramatically dampened the c-Fos response to PCP, particularly in primary and secondary somatosensory cortex. These findings further underline the potential interaction between the glutamatergic and cholinergic systems, and shows that the normal cholinergic input titrates the cortical response to a novel drug-induced sensory experience.

Low doses of PCP dose not activate transcriptional activity of BDNF or NgR-1

BDNF mRNA levels generally increase and Nogo receptor-1 (NgR-1) mRNA levels typically decrease in response to neuronal activation (see Josephson 2003, Karlén 2009). We therefore examined the transcriptional activity of BDNF and NgR-1 mRNA after PCP challenge. We found that there were no alterations of mRNA levels for either BDNF or Ngr-1 in cortical or hippocampal areas after Ringer or after the relatively low levels of PCP administration at the investigated time points. Furthermore, chronic absence of a cholinergic innervation did not seem to influence the levels of BDNF or

NgR-1 transcripts in the investigated areas. To rule out delayed effects of BDNF and NgR-1 mRNA expression levels, a control experiment was carried out on eight naïve rats given 3 mg/kg PCP and analyzed 4 hours post injection. At this time point the c-Fos mRNA levels had returned to baseline, but there still were no alterations in BDNF or NgR-1 mRNA levels. We assume that the doses of PCP used were too low to alter BDNF and NgR-1 transcript levels. However, it remains to be assessed whether higher doses of PCP might have effects on these two genes.

In summary, using quantitative *in situ* hybridization showed that both doses of PCP used (2 and 3 mg/kg, s.c.) caused a marked increase of neuronal c-Fos mRNA expression 30 and 60 min after drug administration, though no effect was noted for BDNF and NgR-1. This PCP c-Fos mRNA response was ablated or markedly dampened specifically in cholinergically denervated regions of cortex cerebi.

PAPER IV

Papers I and II focused on the effects of cholinergic denervation and PCP challenge on behavior, whereas Paper III focused on the effects on expression patterns of three specific genes. In Paper IV, we wanted to examine the potential global changes of gene expression. To this end, we used RNA-Sequencing (RNA-Seq). We examined bilaterally lesioned and sham-operated groups and administered either saline or PCP (1 and 3 mg/kg, s.c.) followed by sacrifice 30 or 90 minutes post injection. This approach sheds light on how cholinergic denervation alone or in concert with PCP, affects genome-wide patterns of gene expressions, and how expression may change over time. A tissue punch from the primary somatosensory cortex, incorporating a portion of the secondary somatosensory cortex, was analyzed.

Given the results from our previous study (Paper III), we first examined the c-Fos gene expression to determine if the results were in line with our previous study. Overall, the c-Fos gene expression was found by RNA-Seq to be significant for cholinergic denervation, as well as for PCP treatment. Transcript levels in intact cortex did not differ 30 compared to 90 min after saline injection. As expected, the c-Fos expression increased after PCP administration, and in a dose-dependent manner. In addition, these effects were clearly attenuated in the absence of cholinergic innervation (Figure 11). We thus note that alterations of c-Fos mRNA levels by denervation, PCP, and the combination thereof, are strikingly similar when measured by either quantitative *in situ* hybridization or RNA-Seq. This validation procedure suggests that observations of other transcripts by RNA-Seq would also be trustworthy, although validation of key observations with another method, such as quantitative *in situ* hybridization or RT-qPCR should also be carried out.

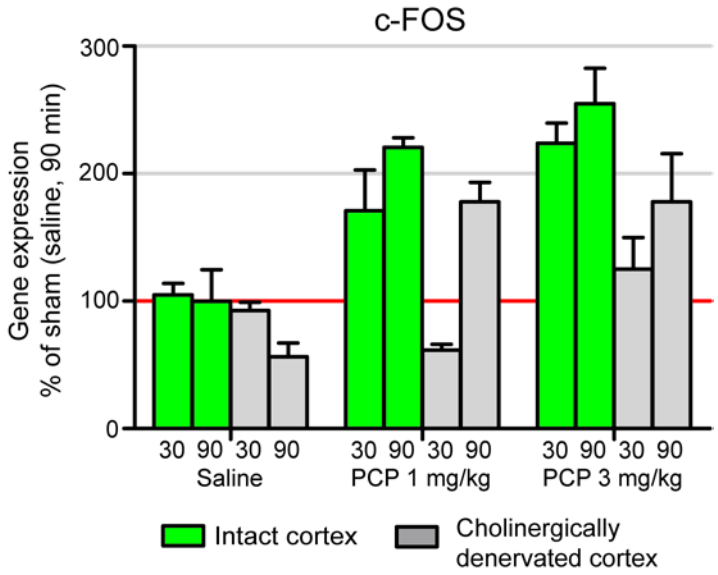

Figure 11. c-Fos gene expression in primary somatosensory cortex as percent of sham (saline, 90 min) measured by RNA-Seq. (Values are mean ± SEM; c-Fos, p(denervation) = 0.027; c-Fos, p(treatment) = 0.0015).

Overall effects of PCP, time, and denervation on the cortical transcriptome

A three factor ANOVA analysis of Lesion x Treatments x Time was conducted, correcting for multiple testing using a false discovery rate (FDR) approach. There was an overall effect of PCP treatment, which typically, though not universally, increased levels of RNA species in a dose dependent manner. An effect of time was seen after PCP treatment, typically, but not always in the form of more pronounced effects 90 min after drug challenge, as compared to 30 min. Certain genes with a rapid response, such as Cyr61 and Apold 1, showed increased levels of mRNA that were higher at 30 than 90 minutes after injection. Together, these observations demonstrate the importance of studying more than one time point to monitor PCP-induced alterations in different genes. Overall analysis of our data showed that time was associated with the largest number of significantly altered gene activities with the strongest significant alterations, followed by PCP treatment. Denervation caused fewer transcripts to be significantly affected in comparison. The most significant differences between denervated and intact cortices was observed after PCP treatment, though there were tendencies for reduced expression compared to controls after saline administration, but not to the same extent.

We looked in greater detail at the most significant transcript alterations for each main term, denervation, dose of PCP, and time after injection. For denervation they were Egr1, Dusp6, Ier2, Nr4a1, for PCP treatment Cyr61, Bcl6b, Apold1, Dusp1, and for alterations over time Sox9, Coq10b, Afp189, Rnf39. Here we will focus on a few of these genes in relation to the larger picture of this thesis. The RNA-Seq analysis contains information on over 20,000 gene transcripts, and the current approach, to look at a few of the most significant ones for each term should be viewed as an initial analysis.

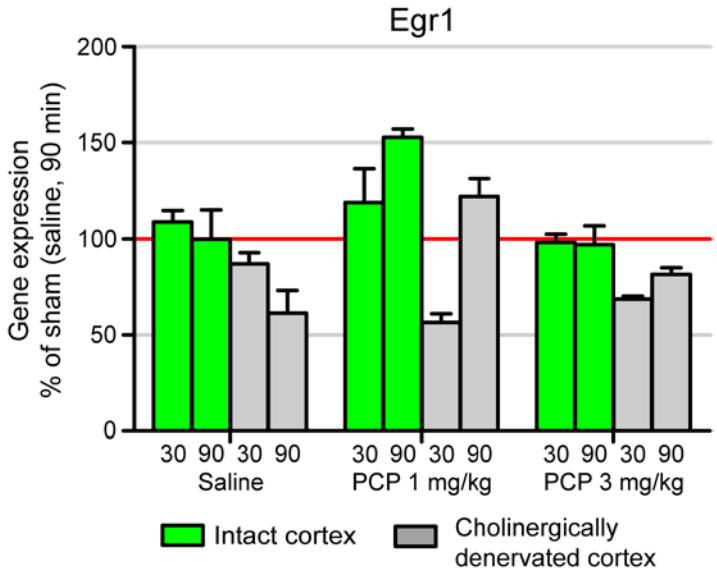

Figure 12. Egr1 gene expression in primary somatosensory cortex as percent of sham (saline, 90 min) measured by RNA-Seq. (Values are mean ± SEM; Egr1 p(lesion) = 0.015).

Egr1 (early growth response 1) is distinctly expressed in neurons in cortex, striatum and stratum pyramidale of hippocampus (Allen Institutet for Brain Science, 2012; Lein et al., 2007) and was the gene most significantly altered by denervation. Overall, Egr1 expression was decreased in cholinergically denervated animals (Figure 12). Egr1 is a member of a family of growth response mediators. These mediators have been implicated in neuronal plasticity, and short and long-term memory (Gomez Ravetti et al., 2010). These aspects of neuronal function are believed to be impaired in schizophrenia (Albus et al., 2006; Albus et al., 2002; Hoff et al., 2005), which made this gene interesting for the present study. Gene expression of human brain tissue from the dorsolateral prefrontal cortex using microarray found Egr1 was downregulated (fold change = -1.31) in schizophrenic patients, and correlated with RT-qPCR analysis (Perez-Santiago et al., 2012). These results were in agreement with previous research which found that Egr1, 2, and 3 were significantly downregulated in schizophrenia patients by RT-qPCR (Yamada et al., 2007). In line with our results, it has been reported that Egr1 expression is stimulated by activation of muscarinic receptors (von der Kammer et al., 1999).

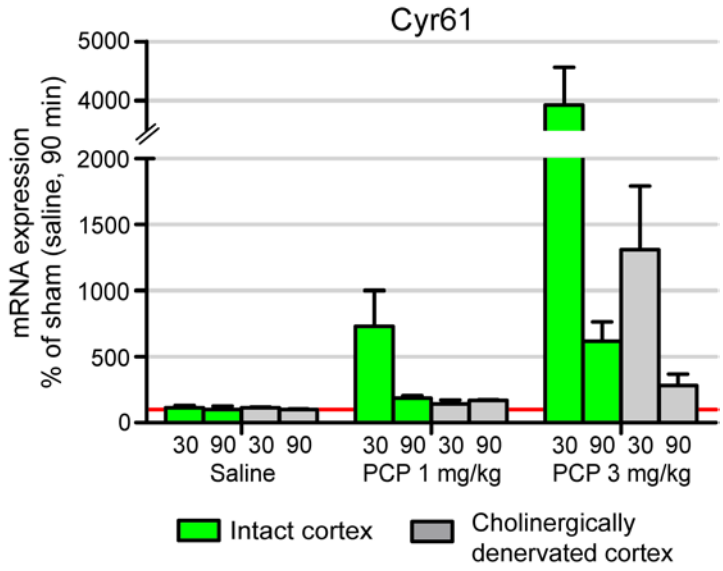

Figure 13. Cyr61 gene expression in primary somatosensory cortex as percent of sham (saline, 90 min) measured by RNA-Seq. (Values are mean ± SEM; Cyr61 p(treatment) = 5.7 x 10e-6).

Cyr61 (cysteine-rich, angiogenic inducer 61) was one of three most significantly altered transcripts for treatment. Limited *in situ* hybridization information reports Cyr61 in deeper cortical layers in mice, mostly primary visual cortex, claustrum and piriform cortex (Allen Institutet for Brain Science, 2012; Lein et al., 2007). Similar to available mouse data, we find very little Cyr61 transcript in our samples after saline injection. In stark contrast, after PCP administration, there is a fast and dose-dependent increase of Cyr61 gene expression, with greatest expression after 30 min, decreased at 90 min. This considerable increase in Cyr61 transcript levels after PCP administration is attenuated in cholinergically denervated rats, with almost no response at the lower dose, and less than half the expression at the higher dose at 30 min. In agreement, a previous study reported strongly increased Cyr61 transcript levels after systemic administration of higher doses of PCP (5, 10, or 20 mg/kg) measured at 60 min after injection (Ito et al., 2007). The cholinergic denervation causes lack of activation of nicotinic and muscarinic AChRs. A previous study treated rats with the muscarinic AChR agonist pilocarpine, resulting in a strong increase of Cyr61 mRNA in neurons in deep cortical layers and thalamic nuclei (Albrecht et al., 2000).

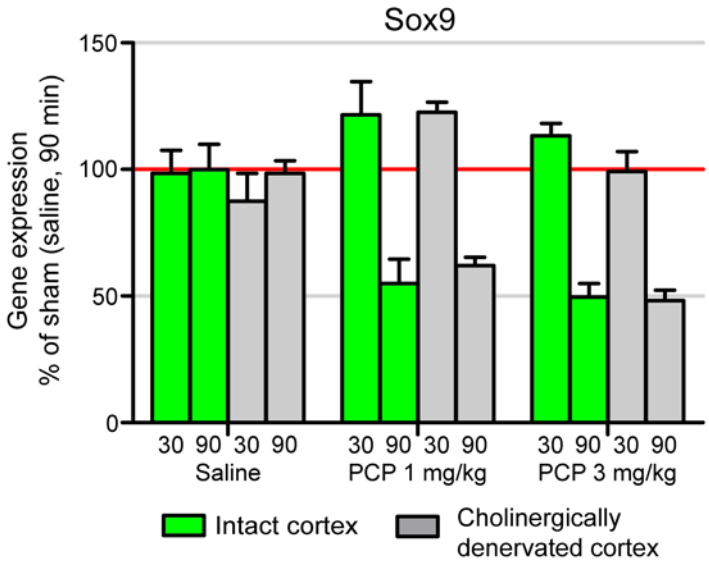

Figure 14. Sox9 gene expression in primary somatosensory cortex as percent of sham (saline, 90 min) measured by RNA-Seq. (Values are mean ± SEM; p(time) = 5.5 x 10e-5).

Sox9 (SRY-box containing gene 9) was the most significantly altered transcript for time. The Sox9 transcription factor is expressed by astrocytes and cerebellar Bergman glia (Gris et al., 2007; Pompolo and Harley, 2001). Unlike neuronally expressed genes, this glial transcription factor was strongly down-regulated, rather than up-regulated by both doses of PCP at 90 min. Also, in contrast to neuronally expressed genes, Sox9 transcript levels were not influenced by the presence or absence of cholinergic innervation. The most direct explanation to the insensitivity of this gene to cholinergic denervation is that astrocytes are not innervated by cholinergic nerves. Sox9 induces expression of chondroitin 4-sulfotransferase and zylosyltransferase-I and –II, which add chondroitin sulfate side chains to the core protein in the formation of growth inhibitory chondroitin sulphase proteoglycans (CSPGs) (Gris et al., 2007). Sox9 knockouts and heterozygous knockouts die before birth (Bi et al., 2001), but conditional Sox9 knockout mice lacking this gene in astrocytes have demonstrated enhanced recovery from spinal cord injury, due to a reduction in deposition of CSPGs (McKillop et al., 2013). We speculate that a strong sensory experience is coupled to down-regulation of Sox9 to facilitate structural synaptic plasticity. Shao and Vawter reported an increase in Sox9 expression in the dorsolateral prefrontal cortex of schizophrenic patients (Shao and Vawter, 2008).

The results from the RNA-Seq analysis of the rat transcriptome have robustly shown that PCP will increase the amount of transcript of many genes, and that this increase is attenuated in rats lacking the basalo-cortical cholinergic input. Our results support the hypothesis that the cholinergic innervation has a role in regulating the intensity by which sensory experiences affect cortex cerebri. Further analysis of the many genes affected by denervation and drug treatment should allow identification of pathways of particular relevance to PCP-induced effects in the intact and cholinergically denervated cortex cerebri.

CONCLUSIONS

Paper I: Impaired social interaction and enhanced sensitivity to PCP-induced deficits in NOR in rats with cortical cholinergic denervation

We examined the cortical cholinergic system with respect to social behavior and declarative memory and found:

- Cortical cholinergic deficits lead to impaired social interaction.
- Cholinergic denervation of neocortex significantly reduces duration of active social interaction.
- A loss of declarative memory when denervated rats are challenged with PCP at a dose that alone does not block the task.
- Impaired novel object recognition in rats lacking cortical cholinergic innervation after PCP.
- These findings are relevant to conditions that involve cholinergic dysfunction, including Alzheimer's disease and schizophrenia.

Paper II: Comparison of manual versus program scoring of two behavioral tasks: NOR and Social interaction

- Program scoring reduced analysis time but needed manual corrections to avoid certain forms of erroneous data points
- Manual scoring was more nuanced but labor intensive.
- Manual and program scoring can offer complimentary data sets.

Paper III: Cholinergic denervation attenuates PCP-induced c-Fos responses in rat cortical neurons

- Low doses of phencyclidine (2 and 3 mg/kg, s.c.) induce c-Fos mRNA in rat cortical neurons within 30 min.
- Cholinergic denervation counteracts PCP-induced cortical c-Fos mRNA expression.
- Low doses of PCP did not influence BDNF or Nogo receptor mRNA levels.
- PCP effects in cortex cerebri are linked to basalo-cortical cholinergic input.
- Results are compatible with a role for basalo-cortical cholinergic pathways in schizophrenia.

Paper IV: cholinergic denervation and phencyclidine alter patterns of gene expression in rat primary somatosensory cortex

- In agreement with Paper III results, c-Fos was increased after PCP, and this response was dampened in the absence of cholinergic innervation, thus providing validation of RNA-Seq methodology.
- Many gene transcripts were increased by PCP treatment.

- In the absence of a cholinergic input to cortex, many genes showed an attenuated response to PCP administration.
- A higher dose of PCP (3 mg/kg) increased the amount of transcript of many genes more than a lower dose (1 mg/kg).
- These findings provide further support for the hypothesis that cholinergic innervation has a key role in regulating the intensity by which sensory experiences affect cortex cerebri

5 ACKNOWLEDGEMENTS

Most any journey in science is a collaborative effort. This is particularly the case in the journey of the PhD student. Whether it be the guiding questions of the supervisors, the stimulating discussions with colleagues, or the supportive words of friends and hugs of family. In the journey I have taken, I have now come to the point where I have the opportunity to thank some of those many people who have helped me and challenged me along the way.

My supervisors

Professor Lars Olson

For accepting me as a graduate student, your encouragement throughout the process, especially on that day when I found that the freezer, with all that work, was no longer frozen. Lars, not only did you help me to pick up the pieces and move forward, but your enthusiasm for science is contagious and kept me going. Not to mention your adeptness at catching those tiny typos that can mean the difference between a shelf above the fireplace and a structure of the brain (cortical mantel vs. cortical mantle).

Associate Professor Jan Kehr

For being a part of this journey from the very beginning when I sparred with HPLC machines. Our discussions have taught me so much about how the different scientific pieces fit together and reminded me of the pharmacology behind it all. I am truly, truly thankful for your calm, steadfast, and warm support.

My external mentor, **Professor Urban Ungerstedt**. Uncles are supposed to be wise and supportive, and you have proven both during this journey. Our long talks have helped me to see the larger context of science and the journey of the scientist, especially the importance to lean back every so often and reflect on what it all means and why it is so.

Collaborators

I began this project with **Anna Mattsson** who guided me as a co-supervisor at the start of this journey. It was her thesis which prompted our first questions. She patiently let me discover the skill of ear bar placement and our work with preparing experiments at the MR centrum illustrated the joys of teamwork through the development of complementary skillsets. Sarah Holst, for all the discussions on the intricacies of coding and analyzing behavior, wonderful discussions on life and statistics, and your never ending support. Allissa Dillman and Mark Cookson, for introducing me to the new world of RNA-Seq.

Eva Lindqvist, Karin Lundströmer, and Karin Pernold, our fantastic technicians. Thank you for your excellent skills, patient teaching, for keeping the ship running smoothly, and for those wonderful outfits with the matching (and very impressive) dance moves. A special thank you and congratulations to Eva as you retire after more than a few

years at KI. And a warm welcome to our new members of the lab, Margareta Widing and Katrin Wellfelt.

For all the hours at the MR centrum, I would like to thank Sahar Nikkhou Aski, Peter Damberg, and Stefan Brené for the time I spent with you, as well as Adam Sierakowiak for long hours of problem solving together and fun conversations.

Professor Yasmin Hurd and her lab, especially Maria Ellgren, for introducing me to surgical techniques and behavioral paradigms.

Selim Sengul for constant help to fix machines so samples could be run.

Members of the Department of Neuroscience
The past and present heads of the department Staffan Cullheim and Sandra Ceccatelli. Ida Engqvist for IT help, and impressing me with knowing the size of the letter "a" when type setting. Sven Ove Ögren for introducing me to the Morris water maze. Rochellys Diaz-Heijtz for discussions on neurodevelopmental disorders and behavioral paradigms, and for all the help with equipment and the development of experimental protocols. Professor Gunnar Grant for a lovely defence dinner conversation and continued encouragement with both my Swedish and my work over the years. The staff at the animal department, especially Niklas Lilja, Tua Finnman, Helena Ericsson, Anna Eriksson.

The Olson group past and present members and affiliates
Dagmar Galter, for introducing me to the world of histology of the human body, which I then applied to the rat, for the wealth of knowledge, fun dinners, long discussions late into the night fueled with chocolate and your mother's cookies.

Current and former doctoral students and colleagues, for creating a fun atmosphere filled with Thursday seminar cake, paper cake, birthday cake, Anna Jo candy encouragements, Monday mornings breakfasts, conferences, and dancing departmental Christmas dinners.
Sandra Gellhaar, Caroline Ran, Lisette Graae, Anna Anvret, Giuseppe Coppotelli, Jaime Ross, Andrea Carmine Belin, Anna Josephsson, Esri Hazael Juárez, Jakob Kjell, Tobias Karlsson, Matthias Erschbamer, Jeff Blackinton, Mathew Abrams, Linus Olson, Simone Codeluppi, Seungmin Lee, Fredrik Sterky Hansson, Mimi Westerlund, Alexandra Karlén, Astrid Björnebekk, Elin Åberg, Toshiki Endo, Silvia Paddock, Robert Karlsson, Magnus Lekman, and the masters students who have made their way through our halls.

Framtidsrådet (The KI Development Council) members including Pamela Mazzocato, Maria Sjören, Patriq Fagerstedt, Maha Yakob, Adina Feldman, Johanna Diel, Italo Masiello and Mia Lindskog. Framtidsrådet was an oasis in which we could discuss the creativity that makes science possible and how this love of questions can be spread through events like TEDx.

Friends from KI and beyond
My neighbor Kim, Jessica, Ann-Charlotte, Maya, Karin, Susanne, Nasren, Rebecca, Simret, Tara, Kristoffer, Adolfo, Lotta, Peter two meter, Amilcar, Michalina, Jorrit, Kalle, Giovanni, April, Michael, Tanaz, Christopher, Marcus, Henrike, Line, Evanthia, Kylie, Shrin, Marta, Andreas, André, Gilli, Johanna, Erik, Anastasia, Danny, Ericka, Parisa, Frances, Rose-Marie, Sam, Tim.

Annie and Robert, my soon to be new neighbors. Lindsey and Nura, Adnan and Missy, my Pomona family. And to my west coast family, Dr. and Mrs. Sasaki, Mat and Lindsey, I have truly enjoyed visiting with you all so many times, I appreciate all that you have done for me.

A special thank you to Carolina for help with the cover! It was a lot of fun designing and working with you.

This journey started with work in Boston, where I was able to hone my lab skills, thanks to my lab mates- Jinhong, Beth, Jane, Nick, and Ben. And to Dr. Suzanne Roffler-Tarlov, I carry with me the wonderful parties in your beautiful home celebrating artistic endeavors through music and poetry, science and dumplings.

Family
Part of every summer I have spent in Sweden, but to move, not just hop, over the pond was a challenge. But with the enthusiasm of my aunt Ulla, uncle Urban, cousins and their spouses, Martin and Cecilia, Carolina and Mikael, and Johan and Johanna, you all have helped me to feel at home and enlarged my understanding of Sweden. I have also enjoyed spending time with the next generation of budding enthusiasm, and want to thank Hedda and Axel, my godchildren, for reminding me about the importance of imagination and play. It has also been a joy to import Thanksgiving, though now we need to make many more pecan pies each year.

To Paul, Doris, Jerry, Rasmus, and the rest of the gang for welcoming me as part of your and Richard's life.

My dear neighbors and adoptive Mormor and Morfar, Gunnar and Lillmor, thank you for your unending encouragement, and for our wonderful lake side tea parties and taxi service.

My parents, Mamma and Dad, thank you for your love and steadfast support throughout this process. Your words of wisdom, not only from your own doctoral experiences, but on the process of learning, have taught Carl and I to see difficulties as challenges, which are really opportunities to learn and grow.

Thanks also to Elizabeth for your support down in Germany and thank you Kevin for your support both in Boston and now in Sweden, and our scientific conversations.

Recent studies have shown that it is not only the parents, grandparents and friends who influence the life choices and outcomes of a child, but that siblings have a much bigger influence than expected. This information is old news to me, as I have had a lifetime to learn from, be inspired by, and encouraged by my big brother, Carl.

And to my Richard, for all your support and especially all the laughs we have shared during this adventure we have been on together (your jokes never fail to make me smile) and to the new adventures we are about to begin.

Funding

This research was supported by the Swedish Research Council, ERC grant no. 322744, Swedish Brain Power, the Karolinska Distinguished Professor Award program, the Alzheimer foundation, Karolinska Institutet (KID; KI fonder), the NARSAD Young Investigator award, and an AstraZeneca postdoc award. In addition, this research was supported in part by the Intramural Research Program of the NIH, National Institutet on Aging.

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