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Christin Schifani (M.Sc.)
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Robustness of a neurodevelopmental animal model of schizophrenia: combining immune stimulation with glutamatergic insult

Referees: Prof. Dr. Rainer Spanagel
Prof. Dr. Peter Gass

To my beloved husband Marcello

Summary

Schizophrenia is a neurodevelopmental disorder affecting about 1 % of the population. This thesis focuses on the development of an animal models of schizophrenia-related pathophysiology based on the so called "two-hit" hypothesis. According to this hypothesis, schizophrenia is the product of a combination of at least two factors, acting during the critical phases of development of the central nervous system. One "hit" reproduces hypofunction of the NMDA receptor signaling via early postnatal treatment with the NMDA receptor antagonist phencyclidine (PCP), a treatment that is toxic to inhibitory networks of brain areas such as the cerebral cortex. Second "hit", prenatal treatment with polyinosinic : polycytidylic acid (Poly I:C), mimics maternal infection, one of the most commonly reported environmental risk factors of schizophrenia.

In a sharp contrast to acute models (e.g. amphetamine-induced locomotor hyperactivity), developmental models are inherently suffering from long periods between the sickness-inducing treatments and the test phase, when various compensatory and other confounding environmental factors can interfere with the model performance. As a result, developmental models often lack robustness that is most commonly expressed as high inter-individual variability. There are several approaches to deal with these problems that are proposed and presented in this thesis.

First, current analysis emphasizes the need to develop and validate tasks that can be applied repeatedly to monitor the development of the disease-like state (and potential response to pharmacological and other treatment). One such task is presented in details – a novel reinforcement learning task that is not sensitive to practice effects and robust enough to be administered for many weeks, if not months.

Second, an intermediate test phase was introduced to bridge manipulations during the perinatal period of development and assessments made in adult subjects. Based on the juvenile social play behavior of the rat, correlation and cluster analyses were conducted in order to develop predictors of adult behavioral and non-behavioral abnormalities (i.e. to identify adult animals most affected by the Poly I:C/PCP treatment).

In schizophrenia research, adolescence is seen as a critical time period and it was hypothesized, that pharmacological intervention during this phase could have a preventive effect on brain pathology and behavioral abnormalities in adults. Two drugs, minocycline and pregnenolone, previously reported to have anti-inflammatory, neuroprotective and disease modifying potential, were shown to prevent brain pathology (alterations in microglia density and GAD67 expression) in rats of the two-hit group when applied during adolescence. These results have two important consequences. On the one hand, they call for more discussion on the need to develop disease-modifying therapy of schizophrenia, a subject that is still not well accepted in the medical community at least in part due to stigmatization of the disease. On the other hand, such studies are the best illustration of the potential value of neurodevelopmental models. Present thesis clearly indicates that, given the limited robustness, the value of these models is not as unequivocal as one may want to have. These issues are currently not discussed in the literature that is dominated by positive evaluations of dozens of various neurodevelopmental models.

Despite challenges, the Poly I:C/PCP model can be recommended for the further development and validation. Developmental models not only help to understand the impact of factors implicated in the pathophysiology of schizophrenia but are also the key to study novel therapeutic approaches.

Zusammenfassung

Schizophrenie ist eine Neuroentwicklungskrankheit, die etwa 1 % der Bevölkerung betrifft. In dieser Doktorarbeit steht die Entwicklung eines Tiermodells mit Schizophrenie-ähnlicher Pathophysiologie, welche auf der sogenannten „Two-Hit"-Hypothese basiert, im Vordergrund. Dieser Hypothese nach ist Schizophrenie das Produkt einer Kombination zweier Faktoren, die während kritischen Entwicklungsphasen auf das zentrale Nervensystem einwirken. Ein „Hit" bildet die Unterfunktion der NMDA-Rezeptor-abhängigen Signalvermittlung nach, hervorgerufen durch frühe, postnatale Behandlung mit dem NMDA-Rezeptor-Antagonisten Phencyclidin (PCP). Diese Behandlung wirkt toxisch auf inhibitorische Netzwerke von Hirnarealen wie dem zerebralen Kortex. Als Einzelbehandlung war dieser „Hit" nicht ausreichend um robuste Anomalien hervorzurufen, die eine regelmäßige Verwendung rechtfertigen würde (z.B. um Bemühungen der Medikamentenentwicklung zu befürworten). Um die Robustheit des Modells zu erhöhen wurde ein zweiter „Hit" hinzugefügt: Eine prenatale Behandlung mit dem Immunstimulanz „Polyinosinic : Polycytidylic acid" (Poly I:C) wurde dazu verwendet eine Infektion während der Schwangerschaft nachzuahmen, welche einer der häufigsten Umweltfaktoren mit Risiko für Schizophrenie ist. Im starken Gegensatz zu Akutmodellen (z.B. Amphetamin-induzierte, lokomotorische Überaktivität), sind Entwicklungsmodelle von Natur aus langen Perioden zwischen Krankheits-induzierender Behandlung und Testphase ausgesetzt, in denen unterschiedlichste kompensatorische oder andere umweltbedingte Störfaktoren die Effektivität des Modells beeinträchtigen können. Daraus resultierend mangelt es Entwicklungsmodellen oft an Robustheit, die sich am häufigsten in einer hohen interindividuellen Variabilität wiederspiegelt. Einige Vorgehensweisen zur Vermeidung solcher Probleme werden in dieser Arbeit aufgezeigt.

Erstens: Die durchgeführten Analysen heben die Notwendigkeit hervor, neue Testverfahren zu entwickeln und diese anschließend zu überprüfen. Nur wenn diese Testverfahren wiederholt am Tiermodell angewandt werden können, ist es möglich den Verlauf eines Krankheits-ähnlichen Zustandes zu beobachten. In diesem Kontext wird ein neuartiger „Reinforcement Learning" Test genauer vorgestellt, welcher unempfindlich gegenüber Lerneffekten ist. Auf Grund seiner Robustheit kann er über Wochen oder gar Monate hinweg im Tiermodell angewendet werden.

Zweitens: Um das Zeitfenster während der perinatalen Entwicklungsperiode und der späteren Untersuchung der erwachsenen Tiere zu überbrücken, wurde eine zusätzlich Versuchs-Phase zwischen diesen beiden Zeitpunkten eingeführt. Hierbei wurden auf Grundlage des beobachteten jugendlichen Spielverhaltens der Versuchstiere Korrelations- und Clusteranalysen durchgeführt. Ziel dieser Untersuchungen war Prädiktoren für adulte Verhaltens- und Nicht-Verhaltensauffälligkeiten zu entwickeln (bzw. adulte Tiere zu identifizieren, die am stärksten von der Poly I:C/PCP Behandlung betroffen sind).

In der Schizophrenieforschung wird die Adoleszenz als eine kritische Entwicklungsphase angesehen und es wird angenommen, dass eine pharmakologische Behandlung während dieser Zeit vorbeugende Wirkung auf die adulte Gehirnpathologie wie auch auf Verhaltensanomalien haben kann. Die Arzneimittel Minozyklin und Pregnenolon, welche Berichten zufolge anti-inflammatorisches, neuroprotektives und krankheitsmodifizierendes Potential haben, beugten Gehirnpathologie (Veränderungen in Mikrogliadichte und Expression von GAD67) in Ratten der „Two-Hit"-Gruppe vor, wenn sie während der Jugend verabreicht wurden. Diese Ergebnisse

führen zu zwei wichtigen Konsequenzen. Zunächst einmal ermutigen sie zu einer stärkeren Diskussion über den Nutzen von krankheitsverändernden Therapien für Schizophrenie. Allerdings ist diese Thematik auf Grund der Stigmatisierung der Krankheit in der Ärzteschaft nicht allgemein akzeptiert. Weiterhin zeigen solche Studien welcher potentielle Nutzen aus Neuroentwicklungsmodellen hervorgehen kann. Auf Grund der eingeschränkten Robustheit zeigt die vorliegende Arbeit auch, dass der Nutzen solcher Modelle jedoch nicht immer so eindeutig ist wie man es sich erhofft. Leider wird diese Problematik in der Literatur aktuell nicht beachtet, da sie von zahlreichen, positiven Einschätzungen verschiedenster Neuroentwicklungsmodelle beherrscht wird.

Trotz der Herausforderungen, welche das Poly I:C/PCP Modell mit sich bringt, sollte es weiterentwickelt und validiert werden. Entwicklungsmodelle wie dieses helfen nicht nur die Einflussgrößen, welche mit der Pathophysiologie von Schizophrenie in Verbindung stehen, besser zu verstehen, sondern ermöglichen auch neue therapeutische Ansätze zu evaluieren.

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Abbreviations

(m)PFC	(medial) Prefrontal cortex
5-HT	Serotonin
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Amy	Amygdala
ASR	Acoustic startle response
CA	Cornu ammonis
Cb	Cerebellum
CD	Cluster of Differentiation
CNS	Central nervous system
CNTRICS	Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia
CPu	Caudate putamen
DA	Dopamine
dB	Decibel
ddH_20	Bidestilled water
DG	Dentate gyrus
dHPC	Dorsal hippocampus
DISC1	Disrupted in schizophrenia-1
FI	Fixed interval
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
GAT	GABA transporter
GD	Gestational day
GLU	Glutamate
GP	Globus pallidus
GWAS	Genome wide association study
HPC	Hippocampus
Hypoth	Hypothalamus
i.p.	Intraperitoneal
i.v.	Intravenous
Iba1	Ionized calcium-binding adapter molecule 1
IFN	Interferon
IL	Interleukin
KCC2	Potassium chloride cotransporter 2
Lx	Lux
MATRICS	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MINO	Minocycline
MK-801	Dizocilpine

MPP+	1-Methyl-4-phenylpyridinium
mRNA	Messenger ribonucleic acid
Nacc	Nucleus accumbens core
ND	Not determined
NIH	National institute of health
NMDA	N-methyl-D-aspartate
p.o.	Per os
PAG	Periaqueductal grey
PCP	Phencyclidine
PFC	Prefrontal cortex
PND	Postnatal day
Poly I:C	Polyinosinic : polycytidylic acid
PPI	Prepulse inhibition of the acoustic startle
PREG	Pregnenolone
PRP	Post-reinforcement pause
PV+	parvalbumine positive
R	Receptor
RD	Response discrimination
RNA	Ribonucleic acid
s.c.	Subcutaneous
Sep	Septum
Thal	Thalamus
TNF	Tumor necrosis factor
Two-hit-HIGH	Two-hit animals with normal to high play engagement
Two-hit-LOW	Two-hit animals with low play engagement
VCD	Visual-cue discrimination
VEH	Vehicle
vHPC	Ventral hippocampus
VI	Variable interval
VIFI	Variable-interval fixed-interval
VTA	Ventral tegmental area

1. Introduction

1.1. Schizophrenia

Schizophrenia is one of the most prevalent psychiatric disorders affecting about 1% of the population worldwide (see Figure 1; Miyamoto et al. 2012). It is associated with a high financial burden with estimates ranging from 9.63 to 13.52 billion Euro per year for the German society (Frey 2014). Apart from the financial perspective, it is a major burden for the patients and families (Ganguly et al. 2010;von Kardorff et al. 2015). Patients can suffer from a variety of symptoms, which develop individually in strength and appearance. Content of delusions, for instance, can vary dependent on personal beliefs and socio-cultural settings (Tandon et al. 2009). The heterogeneity of the disorder can be also seen in the pathology.

1.1.1 Symptoms

There are two main clusters of schizophrenia symptoms: positive and negative. Positive symptoms are normally not experienced by healthy people and include hallucinations, which may occur in any sensory modalities (most commonly auditory such as hearing voices), delusions (fixed beliefs persisting despite being in conflict with reality or rational arguments and the content can include persecutory, referential, somatic, religious or grandiose themes) as well as disorganization in speech and thinking. In contrast, negative symptoms are features which are present in healthy subjects but absent or reduced in patients. This can be presented by a reduction of emotional responsiveness, lacking motivation, asociality leading to social withdrawal and poverty in speech and movement (American Psychiatric Association 2013;Tandon et al. 2009).

Schizophrenia is diagnosed based on criteria laid down in manuals such as the Diagnostic and Statistical Manual of Mental Disorders (DSM 5) (American Psychiatric Association 2013), published by the American Psychiatric Association and used in the United States and the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (World Health Organization 1993), published by the World Health Organization and used in rest of the world. Diagnosis is based essentially on the five key features: delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior and negative symptoms.

In addition, patients with schizophrenia suffer from disabilities in various cognitive domains, with estimates of 90 % of patients having deficits in at least one cognitive domain and 75 % in at least two (Green 2007). These deficits are very disabling for the patients translating into difficulties to find a job, form a network of friends, or live independently (Green 2007;Harvey et al. 2012).

Cognitive disabilities are seen as a core feature of schizophrenia (Green 2007), a view that is supported by various studies showing the presence of cognitive deficits before the onset of psychosis, often already during childhood (Woodberry et al. 2008), similar strength of deficits between never-medicated and chronic patients (Barnett et al. 2010;Carbon and Correll 2014) and relatively stable individual magnitude of cognitive impairment across different schizophrenia states, like psychosis or remission (Albus et al. 2002;Barder et al. 2013).

The severity of cognitive symptoms is predictive of the functional outcome and patients suffering from stronger cognitive deficits have poor overall prognosis (Bowie and Harvey 2006;Green 2006). Therefore, it is suggested that amelioration of the cognitive deficits would improve the overall functional performance (Green 2006;Green 2007). Antipsychotics, the common pharmacological treatment of schizophrenia, can have negative effect on cognition (Moritz et al. 2013) and can induce secondary negative symptoms (Kirkpatrick 2014).

An initiative named MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) was sponsored by the National Institute of Mental Health with the goal to formulate standards in clinical research on cognition in schizophrenia to unitize clinical studies (Marder and Fenton 2004;Nuechterlein et al. 2004). The project identified seven cognitive domains affected in patients with schizophrenia: working memory, attention/vigilance, verbal learning and memory, visual learning and memory, speed of processing, reasoning and problem solving, and social cognition (Nuechterlein et al. 2004;Nuechterlein et al. 2008). The MATRICS consensus battery included the best validated tests that addressed these cognitive domains but had a disadvantage of focusing on cognitive processes dependent on multiple neural and neurochemical systems. A follow-up initiative, CNTRICS (Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia), targeted the selection of narrower defined cognitive constructs and development of novel tasks for their analysis (Barnett et al. 2010).

Typically, schizophrenia onset occurs between an age of 15 and 45 years, and diagnoses before 20 years are seen as early onset schizophrenia. Rarely, cases are diagnosed before the onset of puberty, called childhood schizophrenia. Both early forms suffer from stronger

negative and cognitive problems with inferior overall prognosis (Tandon et al. 2009). The typical disease onset varies between men and women. Men are affected earlier with an average onset peak at an age of 25 years. In women, the onset peaks on average at an age of 28 years (Cascio et al. 2012).

First symptoms precede the final diagnosis of schizophrenia already by an average of five years. The so-called prodromal phase starts with negative symptoms and non-specific changes in behavior. The reduction in the level of functioning is one of the most consistent features. Prodromal subjects often experience mood changes like anxiety, depression, irritability and anger, social withdrawal, and school or occupational failure because of a loss of interest in vocational or academic activities and cognitive problems. This may be accompanied by attenuated positive symptoms that are getting more severe by the end of the prodromal phase while still being subpsychotic in terms of frequency, duration and intensity. Attenuated positive symptoms include suspiciousness, preoccupation and development of odd ideas. The prodromal phase ends with reaching the psychotic threshold based on the severity of symptoms and presence of frank psychotic symptoms (Larson et al. 2010;Tandon et al. 2009). There are attempts to recognize individuals within the prodromal phase and at high risk for schizophrenia (Mokhtari and Rajarethinam 2013). A study by Davidson and colleagues suggested that combined data on interpersonal and cognitive functions may predict later breakout of schizophrenia with a high level of accuracy (Davidson et al. 1999). More recently, Yung and colleagues developed “ultra high risk” criteria to predict the onset of psychosis (Yung et al. 2003;Yung et al. 2008).

1.1.2 Etiology

Schizophrenia is a multifactorial disorder caused by genetic and/or environmental factors (Tandon et al. 2008a). The greatest risk factors of schizophrenia seem to be a positive family history, confirmed by family, adoption and twin studies (Picchioni and Murray 2007). There is 6.5 % risk for a first degree relative of a patient (Kendler et al. 1993) which rises to more than 40 % for monozygotic twins (Cardno et al. 1999;Gottesman et al. 1987).

Genetic studies have identified various genes as increasing the risk for schizophrenia (Farrell et al. 2015) and many of these genes are related to the glutamatergic system. To mention some examples, neuregulin 1, disrupted in schizophrenia-1 (DISC1), dystrobrevin-binding protein 1, D-amino acid oxidase activator and regulator of G-protein signaling were all shown to affect N-methyl-D-aspartate (NMDA) type glutamate receptor function (Snyder and Gao

2013;Stahl 2008). For instance, DISC1 does not only influence the glutamate (GLU) release from axonal terminals directly (Maher and LoTurco 2012), but it also regulates cyclic adenosine monophosphate signaling involved in the GLU neurotransmission via metabotropic receptors (Millar et al. 2005). DISC1 is also important for D-serine generation, an important co-agonist at the NMDA receptors. DISC1 mutation leads to a D-serine deficiency accompanied by behavioral abnormalities indicating a hypofunction of NMDA receptors (Ma et al. 2013).

As another example, neuregulin is involved in the regulation of the postsynaptic density, a dense protein network containing NMDARs as well as membrane proteins involved in synaptic signal transduction and cell adhesion (Snyder and Gao 2013). Neuregulin also activates members of the ErbB family that, in their turn, modulate NMDA receptor transmission via interaction with the postsynaptic density. Stimulation of neuregulin suppresses NMDA receptor activation. As the neuregulin function was reported to be more pronounced in patients with schizophrenia compared to healthy controls, this led to the conclusion that an enhancement in neuregulin signaling contributes to the NMDA receptor hypofunction in the disease (Hahn et al. 2006).

In addition to genetic heritability, a variety of environmental factors are known to increase the risk of the development of schizophrenia (reviewed by Brown 2011). Prenatal risk factors include obstetric complications, like premature birth, hypoxia during birth or low birth weight (Cannon et al. 2002), as well as maternal infection (Brown 2006), stress (Khashan et al. 2008) and malnutrition (Brown and Susser 2008). Postnatally, the risk to develop schizophrenia is increased in individuals who grow up in urbanized areas (Krabbendam and van Os 2005), belong to an immigrant ethnical group especially in an area with low ethnic density or migrant background (Cantor-Graae and Selten 2005;Veling et al. 2008), and use cannabis during adolescence (Moore et al. 2007).

The fact that various developmental factors increase the disease risk (Brown 2011) and findings of neuronal abnormalities with fetal origin (Jakob and Beckmann 1986) led to the hypothesis that schizophrenia is a neurodevelopmental disorder, formulated in the late 1980s (reviewed by Fatemi and Folsom 2009;Murray and Lewis 1987;Weinberger 1987).

It has also been long acknowledged that a single insulting event during the brain development or maturation is rather unlikely to cause the neuroanatomical and behavioral changes seen in schizophrenia (Bayer et al. 1999). More than 15 years ago there was a hypothesis put forward postulating an interplay between genes and environment in the mechanisms of the disease. According to this hypothesis, two “hits” are necessary for the development of schizophrenia -

a first hit during early development (prenatal) leads to changes in the brain that make it vulnerable to the second hit (during pre- or postnatal brain development) triggering the disease process (Bayer et al. 1999;Maynard et al. 2001;van den Buuse et al. 2003).

Since the postulation of the two-hit hypothesis, animal models were discussed and developed following this framework (Robertson et al. 2006;van den Buuse et al. 2003). For instance, maternal immune activation, as a model for maternal infection, was combined with repeated unpredictable stress (Giovanoli et al. 2013;Giovanoli et al. 2014), cannabinoid exposure (Dalton et al. 2012;Stenson et al. 2012) or a genetic manipulation (e.g. DISC1 mutation, Abazyan et al. 2010).

1.1.3 Neurobiology

Most research efforts have focused on alterations in dopamine (DA), GLU and γ-aminobutyric acid (GABA) neurotransmission and their link to the pathophysiology and symptoms of schizophrenia, though it is generally acknowledged that other transmitters may play a role as well (Keshavan et al. 2008).

The DA hypothesis is the most enduring and remains to be the key hypothesis explaining generation of the positive symptoms of schizophrenia (Kapur et al. 2005). Ability of dopaminomimetic agents such as amphetamine to induce psychosis (Lieberman et al. 1987) and dopaminolytic agents such as reserpine to reduce psychotic symptoms (ARNOLD and FREEMAN 1956;CARLSSON et al. 1957) led to the idea of dysfunctional DA transmission as a central mechanism in the disease development (Matthysse 1973;Snyder 1976). Positron emission tomography studies supported this hypothesis by revealing increases in striatal DA release during acute psychotic episodes (Laruelle et al. 1996). DA hypothesis of schizophrenia focuses specifically on D2 receptors because all currently used antipsychotic drugs block D2 receptors (Tandon et al. 2010), their clinical effectiveness correlates well with the affinity to these receptors (Creese et al. 1976;Seeman and Lee 1975), and D2 receptor density is elevated in patients with schizophrenia (Laruelle 1998). However, two distinct pathways have to be distinguished. Whereas a hyperfunction in the mesolimbic DA pathway is evident, the mesocortical DA pathway was shown to be hypofunctional. The observed deficits in prefrontal DA D1 transmission are assumed to contribute to cognitive impairments and negative symptoms seen in schizophrenia (Abi-Dargham and Moore 2003).

Besides DA, abnormalities of the GLUergic signaling are also implicated in the disease pathophysiology of schizophrenia. Research in this area has largely followed clinical observations that blockade of NMDA receptors produces transiently schizophrenia-like

symptoms (positive, negative and cognitive) (Javitt et al. 2012;Javitt and Zukin 1991). Identification of various risk genes involved in NMDA receptor signaling (Snyder and Gao 2013; see section "1.1.2 Etiology") as well as reduced GLU levels and those of associated enzymes (Kim et al. 1980;Tsai et al. 1995), and alterations in GLU receptor levels (Humphries et al. 1996;Kornhuber et al. 1989;Sokolov 1998) found in patients, led to a hypothesis of reduced GLUergic signaling in schizophrenia (Tsai et al. 1995). More specifically, a NMDA receptor hypofunction is proposed to underlie the disease (Moghaddam and Javitt 2012). Therefore studies were conducted stimulating the NMDA receptor function in different ways. For instance agents enhancing the NMDA receptor function at the glycine modulatory site are expected to reduce negative symptoms and improve cognitive impairments in patients when used as an add-on treatment to general antipsychotics (Coyle 2012).

The reduced activity of NMDA receptors located on GABAergic interneurons in the cortex is proposed to decrease the activity of these inhibitory neurons. This results in a downregulation of GABA synthesis, release and reuptake in the cortical inhibitory neurons found in schizophrenia patients (Lewis and Gonzalez-Burgos 2006). In turn a reduction in inhibition is proposed to lead to disinhibition of GLUergic neurons increasing the synaptic activity of GLU, especially in the prefrontal cortex. The excessive activity of non-NMDA receptors in particular α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors due to the increase in GLU is proposed to be one source of cognitive deficits upon NMDA receptor blockade and could be involved in the mechanism mediating cognitive problems in schizophrenia (Moghaddam and Javitt 2012). Further, the lack of inhibition by fast-spiking GABAergic neurons is also suggested to lead to deficits in cognitive function, due to an absent synchronization of pyramidal neuron activity (Lewis and Moghaddam 2006).

Apart from the prefrontal cortex, hippocampal dysfunction is linked to cognitive deficits observed in schizophrenia as well (Heckers and Konradi 2015). Hippocampal hyperactivity is proposed to be an important feature of schizophrenia, a view that was first supported by a study reporting positive correlations between cerebral blood flow in the left temporal lobe and reality distortion symptoms (Liddle et al. 1992). Other studies endorsed these initial findings: higher hippocampal activation in schizophrenia patients when viewing passively facial expressions, a decrease in the hippocampal habituation to repeated presentations of faces that correlated with the degree of memory deficit, etc. (reviewed by Heckers and Konradi 2015). This hippocampal hyperactivity is believed to result from GABAergic hypofunction

supported by findings of a decreased number of GABAergic interneurons in patients with schizophrenia (Benes et al. 1998).

Apart from changes in neurotransmission, schizophrenia patients were found to have various pathological abnormalities, like a reduction in whole brain volume, grey matter volume including the frontal and medial temporal lobe, thalamus, and amygdala regions as well as an increase in the lateral and third ventricles (Matheson et al. 2014). Those anatomical alterations are seen even in recently diagnosed patients (Fannon et al. 2000) and unaffected relatives (McDonald et al. 2002), and are therefore unlikely to be a matter of disease chronicity.

1.1.4 Management

The main treatment of schizophrenia is antipsychotic drugs. They are classified into typical and atypical, all of which interact with DA D2 receptors and modulate DAergic transmission (Tandon et al. 2010). Typical antipsychotics like haloperidol or chlorpromazine block strongly the nigrostriatal D2 receptors, leading to the extrapyramidal side effects as one example of side effects. Atypical antipsychotics are less likely to produce extrapyramidal side effects due to their great affinity to serotonin type 2A receptors but some of them may induce metabolic side effects like weight gain as well as glucose and lipid dysregulation (Picchioni and Murray 2007). All antipsychotics, except for clozapine, mainly reduce positive symptoms and have little or even negative impact on negative symptoms or cognitive dysfunctions (Tandon et al. 2010).

The overall poor tolerability and limited efficacy of the existing antipsychotics justify the search for agents with novel mechanisms of action. These novel agents may target DAergic, GLUergic, serotonergic, GABAergic and cholinergic neurotransmission or may go beyond that including neurosteroids or anti-inflammatory treatments, like pregnenolone or minocycline, respectively (Miyamoto et al. 2012;Tandon et al. 2010).

Another important aspect of the treatment of schizophrenia is psychosocial therapy that includes psychoeducation and coping-oriented intervention, cognitive behavior therapy, cognitive remediation, social skills training, assertive community treatment, supported employment as well as others (Tandon et al. 2010). Psychosocial therapy as adjunctive treatment to antipsychotics helps to reduce symptoms and relapse as well as improve drug adherence, social functioning and quality of life (Patterson and Leeuwenkamp 2008).

As mentioned above, first symptoms precede diagnosis of schizophrenia often by years (Tandon et al. 2009) and breakout can be predicted by symptoms (Cannon 2008;Davidson et

al. 1999;Yung et al. 2008). Despite the awareness that earlier intervention can be associated with better outcome (Malla et al. 1999), drug treatment during the prodromal phase is still questionable due to ethical reasons (Bosanac et al. 2010;Buckley and Miller 2015;de Koning et al. 2009). Yet, one needs to acknowledge that studies on early intervention can bring significant benefit as demonstrated in case of cognitive behavioral therapy, omega-3 fatty acids and low-dose antipsychotic treatment (Mokhtari and Rajarethinam 2013).

1.2 Immune system of the brain

The immunological capacity of the central nervous system (CNS) differs from that of peripheral tissues and specialized innate cells, including microglia, macrophages and dendritic cells, help to ensure immune reactions. The most common CNS-resident innate immune cells are microglia regarded as the first and main form of CNS immune defense (Nayak et al. 2014).

1.2.1 Microglia: Appearance and function (during brain development and adulthood)

"Resting" microglia have small cell bodies with many branched (ramified) processes, first described by William Ford Robertson and Pío del Río-Hortega (Rio-Hortega 1917;Robertson 1899). One special feature of ramified microglia is that they make no connections with each other, differentiating them from other brain cells like neurons or astrocytes (Graeber 2010). While their cell bodies remain stationary, their long, motile processes continuously scan the surroundings for damaging or infectious agents as well as other homeostatic changes. As part of this function they communicate with neurons, astrocytes and blood vessels (Franco and Fernandez-Suarez 2015;Nimmerjahn et al. 2005;Prinz et al. 2014).

In response to injury or infectious agents, they can quickly morphologically transform by increasing and rounding their somas and withdrawing their processes (de-ramification) up to the point of an amoeboid state (Walker et al. 2014). They can secrete chemo- and cytokines (or other factors) to communicate with surrounding cells, can translocate their processes or even their whole cell body to the site of interest and can phagocytize debris or agents (Nayak et al. 2014;Nimmerjahn et al. 2005;Walker et al. 2014). If they engulf infectious agents, they can act as antigen-presenting cells activating T-cells.

For many years, it was believed that insults or injury lead to the "reactive" state of microglia, characterized by the amoeboid shape and the secretion of pro-inflammatory factors (M1 or classical activation pathway) contributing to inflammation and being cytotoxic (see Figure 1; Franco and Fernandez-Suarez 2015;Gehrmann et al. 1995). More recently, another

polarization of activation was revealed, the M2 or alternative activation pathway. This pathway is beneficial rather than being harmful for neurons due to the secretion of anti-inflammatory factors. Activated M2 microglia are supposed to contribute to repair and regeneration, and are characterized by an increase in ramification (see Figure 1; Franco and Fernandez-Suarez 2015).

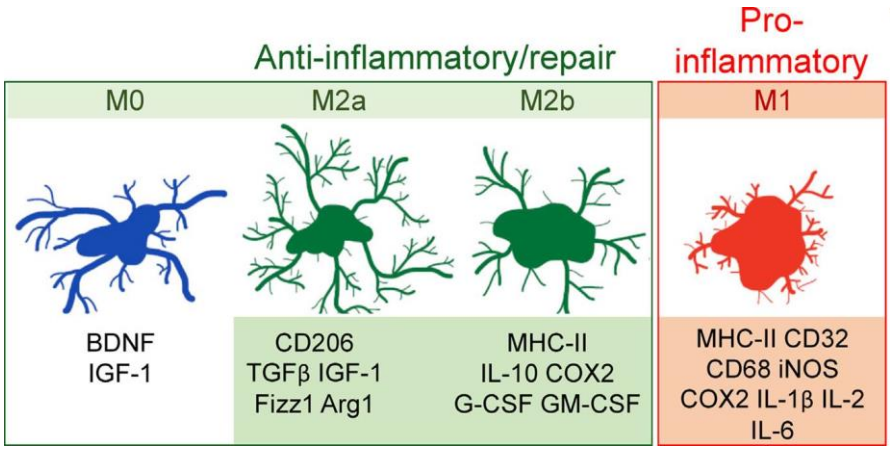

Figure 1: Schematic drawing of the activation state of microglia. Resting microglia (M0) can be activated via two distinct pathways, the classical (M1) pathway, leading to pro-inflammatory factor release and cytotoxicity, or the alternative (M2) pathway, leading to repair and regeneration through anti-inflammatory factor secretion. Molecules expressed by microglia of each polarization are highlighted in the colored boxes. Taken and adjusted from Franco and Fernandez-Suarez, 2015.

More recently, other important non-phlogistic functions were linked to microglia cells. They were found to regulate the CNS activity in the absence of harmful agents in order to ensure CNS homeostasis (Nayak et al. 2014;Salter and Beggs 2014).

During the brain development, microglia contribute to neuronal survival by releasing neurotrophic factors, which support neuronal circuit formation beneficial for the neuron's survival. These factors play a role not only in the development of the brain, but also in maintenance and function later on (Nayak et al. 2014).

However, during the brain development, half of the immature neurons are destined to die because of defective differentiation and migration or when they have only a transient function. This is arranged primarily via programmed cell death due to cell intrinsic factors and accessory cells like microglia. Thus, microglia not only clear the dead cell bodies by engulfing them (phagocytosis) but can actively kill neurons. Phagocytosis by microglia is not only important during development but plays an important role in maintaining a healthy adult CNS too (Nayak et al. 2014;Salter and Beggs 2014).

Accompanying the excess of neurons in the developing CNS, many neuronal connections are built during the development, which need shaping and refinement upon CNS maturation through partial elimination of synapses, the so called synaptic pruning (Chechik et al. 1998).

Inappropriate or superfluous synapses are phagocytized by microglia upon signals from the targeted neurons (Nayak et al. 2014;Salter and Beggs 2014).
Further, microglia seem to be necessary for the proper functional maturation of GLUergic synapses. However, their function at those synapses is not limited to the developing brain. They were found to be involved in the regulation of the activity-triggered, persistent changes in synaptic plasticity via long-term potentiation or depression. This seems to be achieved mainly by brain-derived neurotrophic factor release form the microglia (Salter and Beggs 2014).

1.2.2 Developmental neuroinflammation and schizophrenia

Microglia regulate the induction of inflammation just as they limit it via synthesis of cytokines (pro- and anti-inflammatory) and up-/down-regulation of cell surface receptors (for pathogen recognition, cytokines and antigen presentation) (Boche et al. 2013). Due to their dual function, they play a crucial role in the progressive pathology of brain diseases. If chronically activated, they excessively secrete pro-inflammatory factors linked to neurodegenerative processes for instance within Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and amylotrophic lateral sclerosis (Nayak et al. 2014;Wu et al. 2014).
Cytokines not only play a role in the immune regulation but also fulfill a myriad of important regulatory and modulatory functions during all stages of brain development including neuronal induction, proliferation, migration and survival. These functions are crucially dependent on their concentration and developmental stage (Deverman and Patterson 2009). Therefore, it was hypothesized "[…] that abnormal levels of these molecules during critical periods of early brain development adversely affect neurodevelopmental processes and contribute to a higher susceptibility for complex brain disorders of developmental origin such as schizophrenia" (Meyer 2013).

As mentioned above, infection during pregnancy is an important environmental factor that increases the risk of schizophrenia in offspring. The risk of causing schizophrenia seems to be the highest for infections during early to middle stage of pregnancy (Brown 2012;Khandaker et al. 2013). These are not only infections induced by viral pathogens, like influenza, rubella, measles, polio and herpes simplex, but also bacterial pathogens causing genital and/or reproductive infections and the protozoan parasite *Toxoplasma gondii* (Meyer and Feldon 2010). However, most of the pathogens are unable to penetrate the placenta barrier and,

therefore, do not affect the fetus directly. This led to the hypothesis that the mediators of the high risk linking gestational infection and schizophrenia are not pathogen-specific, but general immunological factors involved in the immune response towards multiple pathogens, like pro-inflammatory cytokines (Meyer et al. 2009;Patterson 2002). For instance, there were reports on a relationship between high maternal levels of tumor necrosis factor (TNF)-α and interleukin (IL)-8, and an increased risk of schizophrenia in offspring (Brown et al. 2004;Buka et al. 2001). The association between elevated levels of maternal IL-8 and schizophrenia risk was strengthened by findings of structural brain changes in offspring with schizophrenia. IL-8 increase during the second/third trimester of pregnancy was associated with greater ventricular cerebrospinal fluid volume as well as lower volume of the entorhinal cortex and posterior cingulate (Ellman et al. 2010). However, causality between cytokine secretion and disease can hardly be proven in humans (Meyer 2013). To better understand the association between prenatal neuroinflammation and mechanisms likely involved in schizophrenia, animal models of prenatal immune activation were developed (See also paragraph: "1.3.5 Developmental animal models of maternal immune activation").

Immune stimulation in pregnant female rodents causes long-term pathological and behavioral abnormalities in the offspring (Boksa 2010;Meyer 2013) that could be related to neuroinflammation during important stages of the fetal and neonatal brain development (Meyer 2013). For instance, maternal immune activation enhances the concentration of pro-inflammatory cytokines in the maternal-fetal compartments, like placenta, amniotic fluid and the fetus/fetal brain (Hsiao and Patterson 2011;Meyer et al. 2006;Urakubo et al. 2001). Further, it leads to microglia activation (Pratt et al. 2013) and the expression of pro-inflammatory transcription factors (Briscoe et al. 2006) as well as can cause white matter injury in fetal and/or neonatal brains (Kumral et al. 2007). Blockade of the pro-inflammatory cytokines IL-1β and IL-6 as well as overexpression of the anti-inflammatory cytokine IL-10 during gestation prevent long-term consequences of maternal immune activation (Girard et al. 2010;Meyer et al. 2008c;Smith et al. 2007) supporting the relationship between developmental neuroinflammation and long-term abnormalities as well.

Inflammation triggered by prenatal immune activation leads to an interference with the course of fetal neurogenesis that may continue to be seen as a reduction of hippocampal neurogenesis during postnatal development and in adulthood (Cui et al. 2009;Graciarena et al. 2010;Meyer et al. 2006;Soumiya et al. 2011). Additionally, alterations in the fetal development of DAergic neurons were observable upon prenatal immune activation, including changes in the expression of genes involved in DA neuron development and an increase in the amount of

midbrain DA neurons (Meyer et al. 2008a;Vuillermot et al. 2010). An increase in mesolimbic DAergic neurotransmission is still present in adult animals (Hadar et al. 2015). Due to these results, postnatal abnormalities in the GABAergic, GLUergic and serotonergic transmitter systems were hypothesized to be induced by disturbing effects of prenatal immune activation during fetal transmitter systems' development as well (Meyer 2013).

There are several immunological abnormalities reported in patients with schizophrenia, including changes in pro-inflammatory and anti-inflammatory cytokine profiles (Miller et al. 2011;Upthegrove et al. 2014) as well as an increase in microglial activation (Bernstein et al. 2015) compared to healthy controls. An increase in both microglia activation and pro-inflammatory cytokines was already reported for recently diagnosed patients (Miller et al. 2011;van Berckel et al. 2008) linking neuroinflammation to the early disease progression. Similarly as in patients, long-lasting inflammatory changes (i.e. increased pro-inflammatory cytokine levels, enhanced microglia activation) are obtained upon preclinical prenatal immune activation (Borrell et al. 2002;Romero et al. 2010;Van den Eynde et al. 2014).
It is hypothesized that prenatal inflammatory processes induce long-lasting neuroinflammatory abnormalities, which can be unmasked upon exposure to distinct environmental stimuli afterwards (i.e. "second hit"), the so called priming effect (Bilbo and Schwarz 2009;Meyer et al. 2011). Supporting this hypothesis, individuals primed by prenatal immune activation exhibited an exaggerated immune response to an immunological challenge (Bilbo and Schwarz 2009). Further, non-immunological challenges such as stress during adolescence in primed animals result in behavioral abnormalities and brain pathological as well as long-lasting signs of neuroinflammation absent or lower in non-primed individuals (e.g. Giovanoli et al. 2013).

1.3 Animal models

To study disease mechanisms, typically animal models are used. For schizophrenia there are about 150 published animal models to date (Koenig 2014).

1.3.1 Validity

Evaluation of a model often follows three criteria: face, construct and predictive validity.
Face validity refers to the apparent similarities between the animals' behavior and the actual disease symptoms (Tordjman et al. 2007). Animal models of schizophrenia are not expected to have desired face validity since most core disease symptoms and signs like delusions and

hallucinations cannot be observed in animals. However, meaningful face validity can be achieved for certain aspects of the disease pathophysiology that may be expressed similarly in humans and animals (e.g. social withdrawal that can be studied using social play behavior; Table 1).

If similar mechanisms (etiology or pathophysiological mechanisms) are thought to underlie the disorder and the animal model, the latter is said to have **construct validity** (Nestler and Hyman 2010;Novak and Meyer 2014). A more specific term "etiological validity" refers exclusively to an identical etiology of a phenomenon in animal and human (Tordjman et al. 2007). Etiology of schizophrenia is not fully understood and this makes it difficult to claim etiological validity, especially for simpler models. However, given the growing knowledge on genetic and environmental risk factors, neurodevelopmental models may target higher levels of construct validity. For instance, prenatal immune activation, an environmental factor that strongly increases the risk to develop schizophrenia, induces in animals a number of pathophysiological changes that appear similar to those in schizophrenia. Those similarities include changes in various neurotransmitter systems like GABA, DA and GLU or morphological abnormalities (Meyer and Feldon 2010).

Table 1: Main factors that drive method selection for assessment of a developmental model of schizophrenia.

Clinical appearance of schizophrenia	Strategies to build animal models	Supporting evidence	Examples of animal models
Positive symptoms	Predictive validity	Reversal by antipsychotic drug treatment	• Locomotor activity
Negative symptoms	Face validity	Species-specific social behavior	• Social play behavior
Cognitive deficits	Construct validity	Cross-species cognitive domains with identified neuronal circuits	• Prepulse inhibition • Set-shifting • Social discrimination

Robust construct validity can be claimed for certain aspects of a model. For instance, there are several functionally equivalent cognitive domains that can be studied in humans and laboratory rats and that share similar neuroanatomy and neurochemistry. To give examples, PPI and set shifting are often used to reveal deficits in pre-attentive processing or executive functioning, respectively, which are dependent on comparable neuronal circuits in humans and animals (Table 1).

If an animal model's response to therapeutic treatment predicts the effect of this treatment in humans, the model is said to have **predictive validity** (Nestler and Hyman 2010). Evaluation of most animal studies aimed to model schizophrenia focused on predictive validity utilizing clinically used antipsychotic drugs. For example, psychostimulants can be used to induce locomotor hyperactivity that is readily reversed by antipsychotic drugs (Table 1).

1.3.2 Pharmacological (acute) vs. neurodevelopmental animal models of schizophrenia

There is a number of animal models of schizophrenia developed over the last 50 years (Jones et al. 2011;Koenig 2014;Ratajczak et al. 2013) with the aims to study the disease pathophysiology (Ratajczak et al. 2013) and to develop effective treatments (Barak and Weiner 2011). For preclinical drug screening purposes, acute pharmacological models like amphetamine-induced hyperactivity are used most commonly (e.g. Smith 1965) and their utilization is based on the dopamine hypothesis of schizophrenia (Howes et al. 2015). Acute pharmacological models are very easy to use and their penetrance is very high. For instance, all animals injected with a stimulating dose of amphetamine will show hyperactivity and all antipsychotic drugs will block this hyperactivity reliably in all animals. Hence, this model is very robust and was claimed to have predictive validity (Young et al. 2010). However, a major disadvantage of acute models is that testing cannot go beyond the mechanistic level and, therefore, they do not show construct or etiological validity (Lipska and Weinberger 2000).

To advance the knowledge about the etiology of schizophrenia and to develop novel drugs (mechanistically different from D2 receptor antagonists), more naturalistic models need to be developed. Genes or environmental factors proposed to be the risk factors for schizophrenia, form the basis of such neurodevelopmental models (Bosia et al. 2015;Lipina and Roder 2014;Meyer and Feldon 2010;Ratajczak et al. 2013). For instance, maternal infection or developmental abnormalities in the GLUergic system can be modelled using maternal immune activation or neonatal phencyclidine (PCP) treatment, respectively.

1.3.3 Temporal aspects of model development

When designing developmental animal models, there are several critical aspects of the model that are related to timing of the manipulations and test procedures.

First, with regard to the brain development, rat pups are delivered much earlier compared to humans (Clancy et al. 2001). The stage of the rat brain development at birth resembles that of human brain at the beginning of the second trimester of pregnancy (Bernal 2000;Clancy et al.

2001). Further, the first two weeks of postnatal life in rats are comparable to the second trimester of gestation in humans in terms of similar neurodevelopmental changes (Clancy et al. 2001), including for instance the development of the granule cells within the dentate gyrus of the hippocampus, the olfactory bulb or the cerebellum (Bayer et al. 1993). This has to be taken into account when selecting the time point for prenatal or postnatal challenges. For further details on prenatal challenge with Poly I:C or neonatal treatment with PCP see section 1.3.4 and 1.3.5.

Second, in contrast to acute models like amphetamine-induced hyperactivity, where treatment (challenge) is followed by testing almost without any delay, developmental models are characterized by long periods separating challenge (e.g. prenatal) from testing (e.g. adulthood). These long delays are among the main factors that contribute to higher variability in the expression of challenge-associated disturbances and make the developmental models less robust compared with the acute models. One strategy to cope with this issue is based on introducing intermediate time points at which the test organisms are assessed with the aim to bridge the time between pre-/postnatal challenges and tests in adulthood. For developmental models of schizophrenia, this approach is especially well justified given that schizophrenia diagnosis is preceded by symptoms seen already in adolescence (Tandon et al. 2009).

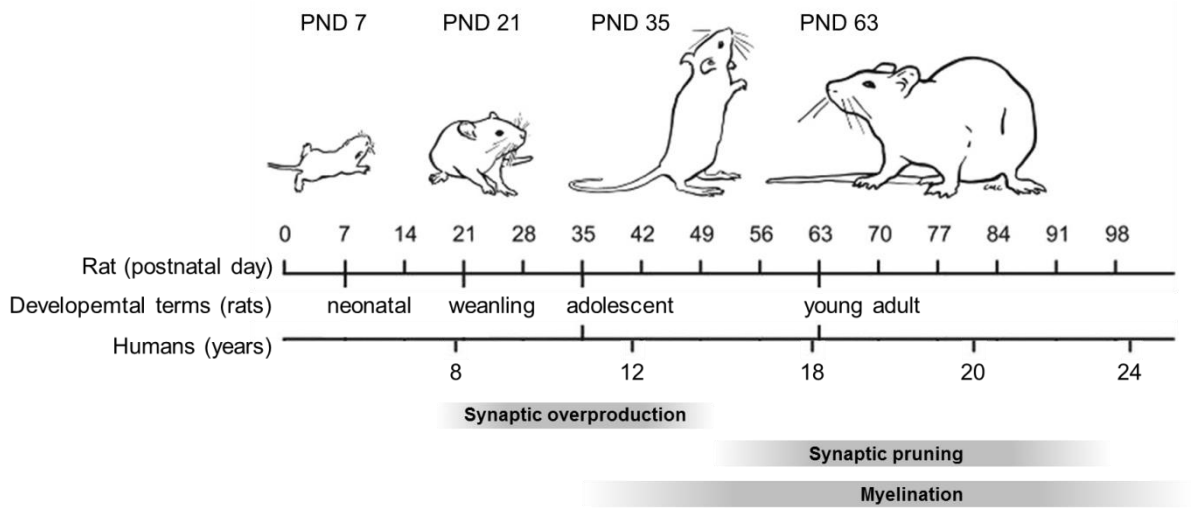

Figure 2: Rats' development compared to humans. Rough timing of developmental stages (*upper part*) and chronological time course of the neuroanatomical developmental processes (*lower part*) in male rats compared to humans. Taken and modified from Sengupta, 2013; Brenhouse and Andersen, 2011. PND: postnatal day.

Adolescence is defined as the time span from childhood (shortly before puberty) to adulthood and characterized by hormonal, neuroanatomical, neurochemical and behavioral changes. Very important for maturation, synaptic overproduction and subsequent pruning, the maturation of neurotransmitter systems, and myelination take place during this period (Figure

2). The exact timing of adolescence is difficult to define, however, puberty as part of adolescence can be defined relatively precisely because it refers to sexual maturation of the individual. In male rats, it usually starts between PND 38 and PND 45 and ends around PND 60 with the achievement of fertility. Based on that, age between 28 and 42 days is often taken as potential starting point for adolescence (Schneider 2013). In addition to adolescence, the term "juvenility" is often used referring to the early phase of adolescence (before puberty starts) (Horovitz et al. 2012). In the framework of the thesis, rats were regarded as adult at PND 70 and later.

Third, whenever pharmacological studies are planned in order to reverse or correct behavioral or non-behavioral disturbances, the time of treatment is an important point to consider. Developmental models are mostly used in the same way acute models are. In both types of models, there is a readout selected for analysis (e.g. hyperactivity in an open field) and drugs are applied acutely in attempt to impact this readout (e.g. Depoortere et al. 2005;Ozawa et al. 2006;Zuckerman et al. 2003). Surprisingly few studies take an advantage of the longitudinal aspect of the developmental models (Piontkewitz et al. 2009;Piontkewitz et al. 2012). In other words, the biggest value of developmental models is in identifying therapeutic interventions that can be applied before the "disease-like state" is fully developed. To that end, adolescence appears to be a well justified time point, both clinically and experimentally, when drug treatment can be administered to prevent development of brain pathology and expression of associated behavioral abnormalities. In rats, preventive drug treatment is applied from PND 34 to 47 (Piontkewitz et al. 2009;Piontkewitz et al. 2012).

1.3.4 Developmental animal models based on the glutamate hypothesis

As mentioned earlier, many genes associated with an increased risk for schizophrenia are linked to NMDA receptor signaling (Kantrowitz and Javitt 2010;Snyder and Gao 2013) and a deficiency in GLUergic signaling is one of the core mechanisms underlying schizophrenia (Howes et al. 2015). Additionally, GLU and especially NMDA signaling play a major role in the brain development by regulating neuronal migration, differentiation and survival as well as controlling the brain's structure and plasticity (Komuro and Rakic 1993;Shatz 1990). Therefore, there were several attempts to model these developmental GLUergic alterations using both genetic and pharmacological tools (Bubenikova-Valesova et al. 2008;Wilson and Terry, Jr. 2010). One of the most commonly reported models is based on administration of PCP to rat pups during the early postnatal period of life (Harich et al. 2007;e.g. Wang et al. 2001).

The late second trimester of pregnancy in humans is a vulnerable period of the fetal CNS development when susceptibility to neurotoxic effects is especially high. During this sensitive period, environmental or genetic factors may disturb developmental programs and maturation of neuronal circuitry and thereby increase the probability of schizophrenia development (Murray et al. 1992;Pilowsky et al. 1993). As mentioned above, neurodevelopment in rats during the first two weeks of postnatal life is comparable to that during the second trimester of human gestation (Clancy et al. 2001). In rats, this window of seven to ten days appears to be a critical period where even a brief exposure to NMDA receptor antagonists may induce deleterious effects on development and function of the CNS (Haberny et al. 2002).

PCP treatment is often administered repeatedly on days 7, 9 and 11 according to a protocol reported to result in a variety of alterations (reviewed by Mouri et al. 2007). Early postnatal (PND 12) biochemical analyses revealed neurodegeneration caused by this repeated PCP treatment determined via upregulation of several apoptotic markers and genes as well as the appearance of neuronal death (Liu et al. 2011;Wang et al. 2001;Wang et al. 2003). Apart from behavioral abnormalities, which are summarized in Table 2, animals treated with PCP on PND 7, 9 and 11 have an overall reduction in brain weight observable from early juvenile period until adult stage (Boctor and Ferguson 2009;Boctor and Ferguson 2010). Additionally, various changes in the neurotransmitter systems were reported, including alterations in the NMDA receptor subunits (du Bois et al. 2012) and loss of GABAergic inhibition due to loss of interneurons (Kaalund et al. 2013;Radonjic et al. 2013;Redrobe et al. 2012) in cortex and hippocampus of adult animals. Due to pathophysiological similarities to schizophrenia, this postnatal PCP treatment protocol was selected to be one of the two hits in the model developed within the framework of this thesis.

Table 2: Behavioral abnormalities caused by repeated postnatal PCP treatment on PND 7, 9 and 11 with 8.7, 10 or 20 mg/kg PCP per injection day.

Dosing protocol	Human / Rats / Age of testing	Impaired cognition									Negative symptoms	Positive symptoms			Reference
		Prepulse inhibition (sensori-motor gating)	Latent inhibition (sensori-motor gating)	Set-shifting (executive functioning)	Reversal learning (executive functioning)	NOR (visual learning and memory)	Spatial alternation (spatial memory)	Water maze task (working memory)	Water maze task (reference memory)	Social novelty discrimination (social discrimination)	Social behavior	Spontaneous exploration	Sensitivity to DA receptor agonists	Sensitivity to NMDA receptor antagonists	
PND 7, 9, 11 with 8.7, 10 or 20 mg/kg PCP	PND 21 – puberty (< PND 35)	↓ →	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	↑ (PCP)	Anastasio & Johnson, 2008a; Boctor & Ferguson, 2010; Rasmussen et al., 2007; Wang et al., 2001, 2003
	Puberty – adulthood	↓	ND	↓ ↓	↓	↓	↓	ND	↓	↓	ND	ND	ND	↑ (PCP)	Andersen & Pouzet, 2004; Boctor & Ferguson, 2010; Broberg et al., 2008; Broberg et al., 2008; Clifton et al., 2013; Kjaerby et al., 2013; Redrobe et al., 2012; Terranova et al., 2005; Wang et al., 2001; Wiley et al., 2003
	Adulthood (≥ PND 65)	ND	ND	↓ ↓	ND	ND	→	↓	ND	↓	→	→ →	↑ (AMPH)	ND	Andersen & Pouzet, 2004; Broberg et al., 2008; Broberg et al., 2008; Clifton et al., 2013; Depoortere et al., 2005; du Bois et al., 2008; Harich et la., 2007; Kaalund et al., 2013; Redrobe et al., 2012; Wang et al., 2001; Wiley et al., 2003

AMPH amphetamine, *ND* not determined, *NOR* novel object recognition, *PCP* phencyclidine, *PND* postnatal day.
↑: upregulation, ↓: downregulation, →: no differences to control animals.

Table 3: Behavioral abnormalities in offspring caused by maternal Poly I:C treatment on GD 15 with 4 mg/kg Poly I:C.

Human		Impaired cognition							Negative symptoms	Positive symptoms			
Rats		Prepulse inhibition (sensori-motor gating)	Latent inhibition (sensori-motor gating)	NOR (visual learning and memory)	Fear conditioning (associative learning)	Water maze task (reference memory)	Reversal learning (executive functioning)	Set-shifting (executive functioning)	Social behavior	Spontaneous exploration	Sensitivity to DA receptor agonists	Sensitivity to NMDA receptor antagonists	Reference
Dosing protocol	Age of testing												
GD 15 with 4 mg/kg Poly I:C i.v.	≥ PND 35) puberty – adulthood	→ ↓	→	ND	ND	ND	ND	ND	ND	→	→ (AMPH) ↑ (AMPH)[a]	ND	Hadar et al., 2015; Piontkewitz et al., 2011a; Wolff & Bilkey, 2008, 2010; Zuckerman et al., 2003a; 2003b
	Adulthood (≥ PND 65)	↓	↓	↓	→	→	↑ (wet t-maze) ↑ → (operant) ↑ (water maze)	↓ →	ND	→	↑ (AMPH)	↑ (MK-801)	Ballendine et al., 2015; Dickerson et al., 2010; Hadar et al., 2015; Klein et al., 2012; Mattei et al., 2014; Piontkewitz et al., 2009; 2011a; 2011b; Vernon et al., 2015; Wolff et al., 2011; Wolff & Bilkey, 2010; Yee et al., 2011; 2012; Zhang et al., 2012; Zuckerman et al., 2003; Zuckerman & Weiner, 2003; 2005

AMPH amphetamine, *GD* gestational day, *i.v.* intravenous, *ND* not determined, *NOR* novel object recognition, *PND* postnatal day, *Poly I:C* polyinosinic : polycytidylic acid.
a: since late adolescence (PND 61)
↑: upregulation, ↓: downregulation, →: no differences to control animals.

Table 4: Morphological and neurotransmission abnormalities in offspring caused by maternal Poly I:C treatment on GD 15 with 4 mg/kg Poly I:C.

Dosing protocol	Age of testing	Abnormalities in Neuro-transmission	Morphological brain abnormalities						Reference
			Neurogenesis	Neuronal morphology	Lateral ventricle size	Hippocampus size	PFC size	Striatum size	
GD 15 with 4 mg/kg Poly I:C, i.v.	≥ PND 35) puberty – adulthood	Depression of excitatory transmission (HPC)	↓ (DG)	ND	→[b,c] ↑[d]	→[b] ↓[c,d]	→	↓	Dalton et al., 2012; Hadar et al., 2015; Partirch et al., 2016; Piontkewitz et al., 2009; 2011a; 2012; Vernon et al., 2015
	Adulthood (≥ PND 65)	Depression of excitatory transmission (HPC)	→ (DG ↓ (DG)	Abundance of pyknotic neurons (HPC)	↑	↓	↓	↓	Dalton et al., 2012; Dickerson et al., 2014; Hadar et al., 2015; Jing et al., 2013; Mattei et al., 2014; Patrich et al., 2016; Piontkewitz et al., 2009; 2011a; 2012; Vernon et al., 2015; Zuckerman et al., 2003; Zuckerman & Weiner, 2003

DG dentate gyrus, *GD* gestational day, *HPC* hippocampus, *i.v.* intravenous, *PFC* prefrontal cortex, *Poly I:C* polyinosinic : polycytidylic acid, *PND* postnatal day, *ND* not determined.

b: early adolescence (PND 35)

c: early adolescence (PND 46)

d: late adolescence (PND 56)

↑: upregulation, ↓: downregulation, →: no differences to control animals.

Table 5: Neurochemical abnormalities in offspring caused by maternal Poly I:C treatment on GD 15 with 4 mg/kg Poly I:C.

Dosing protocol	Age of testing	Abnormalities in brain neurochemistry (content/expression level)											Reference
		PV+ neurons	GAD67	5-HT	DA	GABA	Glu	GABA-R	DA-R	NMDA-R	5-HT1A-R	Glucose metabolism	
GD 15 with 4 mg/kg Poly I:C, i.v.	(≥ PND 35) puberty – adulthood	ND	ND	↓ (CPu, GP) → (PFC, HPC, Thal, Nacc, VTA)	↑ (Nacc) → (CPu, GP, PFC, HPC, Thal, VTA)	→ (CPu, GP, Nacc, PFC, HPC, Thal, VTA)	→ (PFC) → (CPu, GP, Nacc, PFC, HPC, Thal, VTA)	ND	ND	ND	↑ (CA1)	↓ (vHPC, PFC, Cb) ↑ (Thal, GP) → (Amy, Nacc, PAG, Sep, Hypoth)	Dalton et al., 2012; Hadar et al., 2015; Partirch et al., 2016; Piontkewitz et al., 2009; 2011a; 2012; Vernon et al., 2015
	Adulthood (≥ PND 65)	↓ (DG) → (CA1)	↓ (in PV+ cells in dHPC)	↑ (VTA) → (CPu, GP, PFC, HPC, Thal, Nacc)	↑ (Nacc) → (CPu, GP, PFC, HPC, Thal, VTA)	↓ (GP) → (CPu, Nacc, PFC, HPC, Thal, VTA) → (HPC, PFC, Cb)	→ (PFC) → (HPC, PFC, Cb) → (CPu, GP, Nacc, PFC, HPC, Thal, VTA)	ND	ND	ND	↑ (CA1)	↓ (vHPC, PFC) ↑ (Thal, Amy, NAcc) → (Cb, GP, PAG, Sep, Hypoth)	Dalton et al., 2012; Dickerson et al., 2014; Hadar et al., 2015; Jing et al., 2013; Mattei et al., 2014; Patrich et al., 2016; Piontkewitz et al., 2009; 2011a; 2012; Vernon et al., 2015; Zuckerman et al., 2003; Zuckerman & Weiner, 2003

5-HT serotonin, *Amy* amygdala, *CA* cornu ammonis, *Cb* cerebellum, *CPu* caudate putamen, *DA* dopamine, *DG* dentate gyrus, *dHPC* dorsal hippocampus, *GABA* γ-aminobutyric acid, *GAD* Glutamate decarboxylase, *GD* gestational day, *Glu* glutamate, *GP* globus pallidus, *HPC* hippocampus, *Hypoth* hypothalamus, *i.v.* intravenous, *PAG* periaqueductal grey, *PFC* prefrontal cortex, *Poly I:C* polyinosinic : polycytidylic acid, *PND* postnatal day, *Nacc* Nucleus accumbens core, *ND* not determined, *NMDA* N-methyl-D-aspartate, *PV+* parvalbumine positive, *R* receptor, *Sep* septum, *Thal* thalamus, *vHPC* ventral hippocampus, *VTA* ventral tegmental area.
↑: upregulation, ↓: downregulation, →: no differences to control animals.

1.3.5 Developmental animal models of maternal immune activation

As mentioned already, prenatal infection with various pathogens increases the risk to develop schizophrenia. To study the mechanisms mediating the association between prenatal infection and schizophrenia as well as the potential preventive treatments, animal models of maternal immune activation are used (Meyer 2013). Different kinds of models can be distinguished. One model using direct maternal infection is based on the increased risk of prenatal influenza infection to induce schizophrenia. The human influenza virus is infused directly intranasal in mouse dams. Other models are based on the maternal exposure to viral- or bacterial-like immune activating agents, such as polyinosinic : polycytidylic acid (Poly I:C) and lipopolisaccharide (LPS), or pro-inflammatory cytokines like IL-6. They induce maternal immune activation without real infection (Meyer and Feldon 2010).

Immune stimulating agents like Poly I:C or LPS have some advantages over direct viral infection, such as: no biosafety concerns, easy handling and commercial availability. Importantly, the strength of the immune reaction can be controlled more easily using dose-response studies. Furthermore, the agent-induced immunological response is time-limited (24-48 h, dependent on the dose). Therefore, it is possible to control the overlapping time window with a specific period of the fetal development. Finally, those agents do not penetrate the fetal brain directly, allowing the study of indirect effects by maternal immune activation rather than direct "infection" of the fetus (Meyer and Feldon 2012).

The most extensively studied model is maternal immune activation by Poly I:C, which was chosen as the second hit for the model developed within the framework of this thesis. Poly I:C is a synthetic form of double stranded RNA. Upon injection, it is recognized by toll-like receptor 3 (Alexopoulou et al. 2001) stimulating the production and release of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-α and IFN-β (Cunningham et al. 2007;Fortier et al. 2004;Meyer et al. 2006) that is the acute phase response to a viral infection (Traynor et al. 2004). In the literature, different injection protocols are described.

Administration with Poly I:C (4 mg/kg, i.v.) in middle / late gestation to pregnant rats (GD 15) revealed a broad spectrum of cognitive, behavioral, neuroanatomical, neurotransmitter and pharmacological abnormalities in the offspring with relevance to schizophrenia (summarized in Table 3 to 5). The rats' middle / late pregnancy is characterized by similar developmental processes as the humans' early / middle pregnancy (1st and 2nd trimester) (Bernal 2000;Clancy et al. 2001). Therefore, this protocol was used in this work.

1.3.6 Introduction into specific methods

As mentioned already, patients with schizophrenia exhibit various symptoms, summarized in three categories, positive, negative and cognitive, which seem to underlie various neurobiological abnormalities (Tandon et al. 2008b; see section "1.1 Schizophrenia"). Therefore, different methods should be used to characterize an animal model of schizophrenia. Behavioral tests as well as immunohistological and neurochemical methods used in the framework of the thesis are described below in detail.

Locomotor activity

Assessment of locomotor activity is a simple behavioral test commonly used in laboratory rodents. Animals are typically placed into a featureless arena and the distance travelled is recorded for a certain period of time (commonly between 15 min and 2 h). In addition to distance travelled, there may be other recorded and analyzed behaviors such as rearing or stereotypies (Whimbey and Denenberg 1967;Yee and Singer 2013). Rodents have an innate drive to explore novel environments (Schmitt and Hiemke 1998) provoked by their natural foraging behavior. Therefore, when animals are exposed to the open field for the first time, measured activity reflects not only the motor capacity but also the exploratory drive that is diminishing over time due to habituation to the environment (Yee and Singer 2013).

Locomotor activity is influenced by a number of factors, including the shape of the arena and the level of illumination. Rats and mice naturally avoid open, lit spaces and stay more in the border areas (thigmotaxis behavior) or hide, if possible (Prut and Belzung 2003). Therefore, locomotion is studied best using squared arenas under low-level illumination (Walsh and Cummins 1976). The time the animals spend in the central part of the arena is typically used as an index of anxiety (Prut and Belzung 2003).

There are two main reasons to study locomotor activity in animal models of schizophrenia. First, psychostimulants like amphetamine or others, which induce an increase in DA release in striatal regions (Sharp et al. 1987), lead to increased locomotor activity (e.g. Smith 1965). Second, antipsychotic treatment (D2 receptor antagonists) reverses effects of amphetamine on locomotor activity (Young et al. 2010). Given that both hyperactivity and psychosis are associated with increased striatal DA (Reith et al. 1994), locomotor activity is one of the most frequently studied behavior in animal models of schizophrenia.

Social play behavior

Social play behavior is one of the earliest forms of non-mother directed social behavior and the most common form of play behavior in mammals (Poole and Trevor 1975;Vanderschuren et al. 1997). During these social play activities, behavioral patterns similar to social, sexual and aggressive behavior are exercised but in an exaggerated, fragmented and context-independent manner (Vanderschuren et al. 1997).

Rats are a very playful species. That explains why most studies on mammalian social play behavior are done in rats (Pellis and Iwaniuk 2004). This so called *play fighting* starts to appear at PND 18-19, peaks between PND 30 and 40 and declines thereafter to lower levels but does not disappear completely (Pellis et al. 1997). It is characterized by the playful fight of two or more individuals with behavioral patterns of attack and defense but in the absence of the aim of aggression. The goal of the attacker is the nape that differs from serious fights where the rump and lower flanks are targeted (Pellis 1988).

A typical sequence starts with the approach of one animal soliciting the partner to play by attacking its nape (Figure 3). If the attack is successful, the nape of the recipient will be shortly nuzzled with the snout. Normally, an approach will be defended by the recipient expanding the play bout. There are several defense mechanisms used by rats to secure the nape from the attacker including evasion (running, jumping or swerving away) or turning around and facing the attacker (facing defense) mostly combined with the attempt to contact the partner's nape. The facing defense tactics fall into two categories. In the first, the recipient rotates around its longitudinal axis (complete rotation) lying supine on the ground and defending the attacks by using its paws. This situation is called "pinning" meaning that one partner is on his back and the other standing above him. In the second, the partial rotation, the defender rotates only partially by remaining with its hindpaws in contact with the ground (Pellis et al. 1997).

Social play has a high incentive value for rats as shown in place preference tests and maze learning where it serves as a reward. In contrast, play deprivation during the period of highest social play leads to lasting disturbances in the normal patterns of social, aggressive and sexual behavior of rats. This emphasizes the role of play behavior during the adolescent period to facilitate social development by establishing social organization, improving the understanding of communicative signals in a group and facilitating the coping with social conflicts (Vanderschuren et al. 1997).

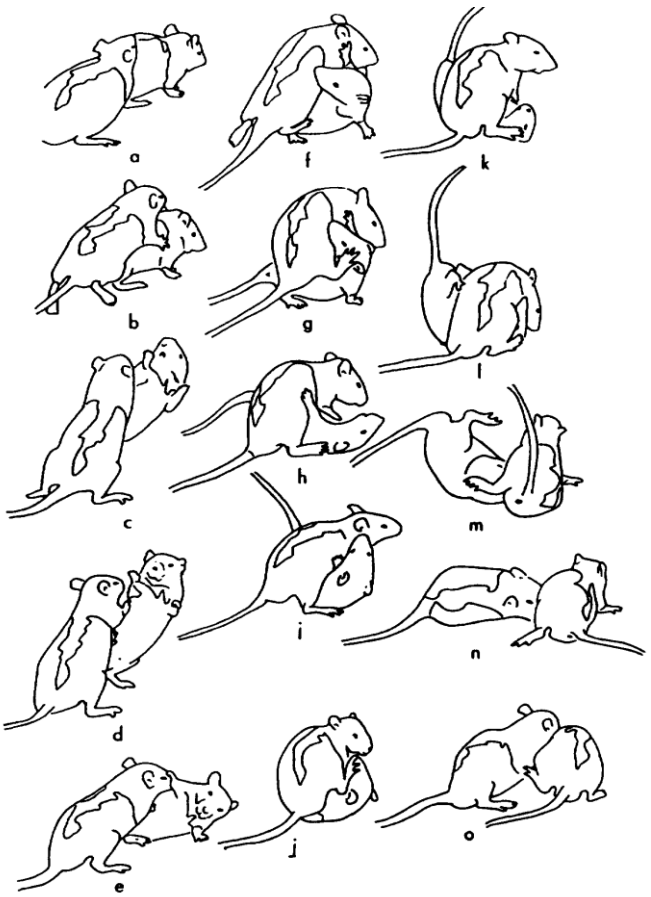

Figure 3: Play fighting sequence of 30 days old male rats. The play bout is initiated by one rat attacking the nape of the other (*a, b*). The recipient defends itself by using the complete rotation (*c-h*) ending up in pinning (*h-i*). This situation is utilized by the animal lying supine which initiates a new attack expanding the play bout (*i-o*). Taken from Pellis et al., 1997.

Social withdrawal and isolation are key features of the negative symptoms of schizophrenia (American Psychiatric Association 2013). Those symptoms are often already visible during the prodromal phase, preceding the diagnosis of schizophrenia (Larson et al. 2010). Social play behavior is a robust and very well studied juvenile behavior. Due to the fact that during these playful fights the whole spectrum of social behavior is modelled and trained, its analysis can reveal inadequate social reactions (Schneider 2004).

There are different methods to analyze social play behavior. One the one hand, it can be assessed under naturalistic conditions in animals that are group-housed where rats are observed undisturbed in their home cage. An advantage of this method is that it does not require any isolation or exposure to a novel environment and therefore eliminates potential stress. However, long observation periods are needed to see an adequate amount of play bouts. Another method is the paired-exposure testing. For that purpose, the animals are isolated before testing to increase their motivation to play with each other. This method makes it possible to assess many play bouts during a rather short time window of testing (Auger and

Olesen 2009). We decided to use the second method but, to minimize stress, observed play behavior in the test arena identical to the home cage under dim light conditions.

Cognitive tasks

The selection of behavioral tasks to assess cognition was based on cognitive domains that are thought to be most affected in schizophrenia. These domains can be clustered into two groups.

One group is represented by seven domains that were identified via the MATRICS discussion and for which there are commonly agreed tools to study them in laboratory animals (Nuechterlein et al. 2004;Nuechterlein et al. 2008). Of this group, I focused on three domains. Pre-attentive processing was assessed by means of prepulse inhibition of the acoustic startle response (PPI), that was chosen for being one of the most robust methods in the preclinical laboratory toolbox. Operant set-shifting task was expected to address executive cognition, one of the key domains affected in schizophrenia and several other neuropsychiatric diseases and that is fairly well characterized in animals. Social novelty discrimination task was added not only because of the role social cognition plays for the functional state of schizophrenia patients but also because of the extensive experience with the task, accumulated during the preceding work with the one-hit neonatal PCP model in the laboratory.

Second group is represented by cognitive domains that are commonly agreed to be strongly impaired in schizophrenia but for which no standard assessment tools exist – reinforcement learning and aberrant salience stimulus learning. Reinforcement learning reflects the modulation of behavior as a function to changing reinforcement contingencies (Ragland et al. 2009) and to track reinforcement learning efficiency, a novel task was developed with the goal to enable longitudinal studies, called variable-interval fixed-interval (VIFI) task. Aberrant learning assessed by the autoshaping task is based on the construct of aberrant salience, discussed as the mechanism contributing to generation of psychotic symptoms in schizophrenia (Kapur 2003).

– *Operant set shifting task*

Executive functioning like attention, working memory, reasoning and problem solving, help to alter established behavior in response to changes in the environment. Reflecting problems in these functions, patients with schizophrenia often exhibit deficits in different forms of behavioral flexibility (Floresco et al. 2009). One of the most commonly used tasks to determine cognitive flexibility in patients is the Wisconsin Card Sorting test (e.g. Berman et

al. 1986;Morice 1990). Additionally, the Intradimensional / Extradimensional shifting (IDS/EDS) task (e.g. Pantelis et al. 1999) and the Stockings of Cambridge test (e.g. Feldmann et al. 2006) are used. During these tasks, patients have to shift between different rules or strategies based on trial-and-error feedback. A great problem in schizophrenia appears to be in the ability to shift attentional set from one stimulus dimension to another (extra-dimensional shift) (Jazbec et al. 2007;Murray et al. 2008;Pantelis et al. 1999). However, also a simpler form of flexibility was shown to be deficient in patients, the reversal learning (intra-dimensional shift) (Murray et al. 2008;Waltz and Gold 2007).

Multiple tasks were developed to investigate the cognitive flexibility in problem solving in rodents with the aim of achieving translatability between species (Barnett et al. 2010). In a rat version of the IDS/EDS task, called attentional set-shifting task, rats have to discriminate between two bowls that can be distinguished by a variety of stimuli (e.g. digging media, odor or the outer texture of bowl) (Birrell and Brown 2000). The task assesses different aspects of learning, including discrimination learning, reversal learning as well as intra- and extra-dimensional shifting (Birrell and Brown 2000;Garner et al. 2006). However, this method is very labor- and time-consuming (Yee and Singer 2013).

Another task used to assess set-shifting abilities require rats to shift between visual-cue and egocentric spatial response-based discrimination strategies and is conducted either in a cross-maze (e.g. Block et al. 2007;Floresco et al. 2006;Ragozzino 2002) or in an operant chamber (Enomoto et al. 2011;e.g. Floresco et al. 2008). During the first discrimination session in operant chambers, rats are trained to learn to press the lever signaled by a visual cue to earn a reward. During the set shift, the rule is changed so that, in order to get a reward, rats have to press the right lever, as an example, and disregard the visual cues (Floresco et al. 2008). In contrast to the IDS/EDS, where novel stimuli are used during the EDS stage, in the operant and maze tasks, the same stimuli are utilized during the entire experiment, resembling more the human Wisconsin Card Sorting test (Floresco et al. 2009). An advantage of maintaining the same set of stimuli is the possibility to distinguish between different set-shifting errors like impaired perseveration or ability to acquire and maintain a novel strategy (Floresco et al. 2008).

– *Variable-interval fixed-interval task:*

The construct of reinforcement learning is defined as “acquire(d) behavior as a function of both positive and negative reinforcers including the ability to (a) associate previously neutral stimuli with value as in Pavlovian conditioning; (b) rapidly modify behavior as a function of

changing reinforcement contingencies; and (c) slowly integrate over multiple reinforcement experiences to determine probabilistically optimal behaviors in the long run" (Ragland et al. 2009) and proposed to be a key finding in schizophrenia (Deserno et al. 2013;Gold et al. 2008).

There are a number of reinforcement learning tasks that were characterized with respect to psychometrics and involved neural systems and were applied in healthy and diseased individuals (also Schizophrenia, see Ragland et al. 2012). One such task is the probabilistic reward task that is "based on a differential reinforcement schedule that provides an objective assessment of participants' propensity to modulate behavior as a function of reward history". Under this task, appropriate answer to one of two stimuli is rewarded more often than to the other and these differences in reward probability generate a response bias that is present in healthy subjects but may be absent in some categories of patients (Pizzagalli et al. 2005). Based on certain construct validity, initial information on underlying neuroanatomical substrates and evidence for pharmacological and behavioral modifiability of task performance, CNTRICS initiative recommended this task for immediate development. There is an ongoing work to adapt this task for the use in animals (Der-Avakian et al. 2013).

Challenges associated with the translation of originally human probabilistic tasks into preclinical laboratory work are well illustrated by studies on reversal learning phenomena. Besides difficulties with making the human and animal tasks truly symmetrical (e.g. deterministic vs probabilistic contingencies, nature of reward and punishment; Ragland et al. 2009), animal reversal learning tasks suffer from a practice effect that prevents longitudinal studies and within-subjects designs (see however, Wallace et al. 2014).

It is not surprising that some human tasks are too difficult or too complex to be implemented in animals while others loose certain qualities when back translated. Therefore, one may also want to consider the development of a task in animals to assess a certain cognitive construct and then taking it forward into humans. Reinforcement learning is the area of cognitive neuroscience that is very well researched in animals and there are a number of phylogenetically well conserved phenomena that may serve as a basis for developing novel translatable tasks.

Reinforcement learning has been studied in animals for more than 100 years and various schedules of reinforcement have been extensively characterized. Even simple reinforcement schedules, ratio or interval, generate responding that differs markedly in terms of the overall response rate and response patterning. For example, variable interval schedule can be used to produce relatively high and steady response rate. In contrast, fixed interval schedule will

produce lower response rates that accelerate towards the interval end and pause after the reinforcer delivery. Response patterning including the post-reinforcement pauses (PRP) directly depends on the interval size (e.g., larger intervals produce larger PRPs; Ferster and Skinner 1957). However, what is most important, these differences between fixed and variable interval schedules exist even if the overall density of reinforcement is equal (i.e. rate of reinforcer delivery averaged across the session).

A novel reinforcement-learning based task was developed for rats in the framework of this thesis. According to the protocol, animals are trained on a variable interval (VI) schedule and, once training is completed, are repeatedly switched to a fixed interval (FI) equivalent. This leads to an adjustment in the PRP during the FI session, a process that is expected to be dependent on cognitive and behavioral flexibility.

– *Prepulse inhibition of the acoustic startle response:*

The acoustic startle reflex is a defensive response to a sudden and intense (>80 dB) auditory stimulus. It is characterized by a pattern of movements including eye-lid closure and contraction of facial muscles at the beginning, followed by neck and skeletal muscle contraction. Meanwhile, all ongoing behaviors are interrupted and heart rate accelerates (Koch 1999;Leumann et al. 2001). This behavior is believed to serve as preparation of a flight-or-fight response against an injury or a predator (Koch 1999). The magnitude of the startle reflex can be measured by using various methods in different species. In humans, usually the contraction of the orbicularis oculi muscle is recorded (e.g. Ebner-Priemer et al. 2005) whereas, in rodents, it is measured as a whole body reaction in a movement sensitive chamber (e.g. Varty et al. 2000). The magnitude of the acoustic startle reflex can be modulated by a variety of conditions. For example, it can be attenuated by prepulse, repeated startle stimulus presentation and positive affect or enhanced by unconditioned and conditioned aversive events (Koch 1999).

The acoustic startle reflex magnitude is reduced if a non-startling sensory event precedes the startle stimulus by 30-500 ms, the so-called prepulse inhibition (Hoffman and Ison 1980). In experimental settings, most commonly acoustic prepulses are used (Klein et al. 2013;e.g. Varty et al. 2000). It is expected that such a prepulse activates a pre-attentional gating mechanism that inhibits the acoustic startle reflex (Swerdlow et al. 2001). This makes it a good operational measure to study sensorimotor gating i.e. pre-attentive processing mechanisms (Braff et al. 2001;Ellenbroek 2004). The inhibition of a response by weak sensory events gives individuals the ability to focus their attention on the most salient aspects

of a stimulus-rich environment (Braff and Geyer 1990). A deficit in this inhibition mechanism is a pronounced problem reported in patients with schizophrenia (Ellenbroek 2004).

– *Social novelty discrimination task:*

Social novelty discrimination addresses the rat's ability to discriminate between a familiar and a novel juvenile rat. During the course of an experiment, the adult test rat is exposed to a juvenile rat for 30 minutes, directly followed by the introduction of a second juvenile (novel) for another 5 minutes (Harich et al. 2007). During this second presentation period, the adult rat prefers to investigate the novel juvenile. This task requires social cognition, a domain where deficit in schizophrenia are prominent (Choi and Kwon 2006;Nuechterlein et al. 2004). With this social novelty discrimination task general social interaction can be assessed as well.

– *Autoshaping task:*

During the autoshaping task, rats receive one food pellet every 60 seconds. Every pellet delivery is preceded by a lever insertion. Pressing the lever results in an immediate pellet delivery. Not pressing the lever has no consequences - the pellet is delivered automatically after the eight seconds interval (Meneses 2003). Because of the close temporal relationships between these two events (lever insertion and pellet delivery), rats start to explore the lever by sniffing it, licking, gnawing or chewing (called pavlovian sign-tracking), and occasionally press it. The resulting food delivery increases the probability of the lever-pressing response being repeated, although, there is still no need for the animal to press the lever to receive food (Tomie and Sharma 2013). An increase in sign-tracking can be interpreted as aberrant learning.

Aberrant learning due to aberrant salience was shown in patients with schizophrenia (Jensen et al. 2008;Roiser et al. 2009). Aberrant salience is based on increased striatal dopamine release and, therefore, is proposed as one of the mechanisms underlying psychotic symptoms of schizophrenia (Jensen et al. 2008;Kapur 2003;Roiser et al. 2009).

Immunohistochemistry

– *Microglia:*

Due to the immune stimulating effect of Poly I:C and studies showing changes in microglia activation in offspring from Poly I:C treated dams (Van den Eynde et al. 2014;Zhu et al. 2014b), the density and state of microglia were evaluated in the framework of the thesis.

Ionized calcium binding adapter molecule (Iba1) is responsible for the cytoskeletal remodeling of macrophages and microglia, uniquely expressed in those cells at all states (Ito et al. 1998;Sasaki et al. 2001). An upregulated expression of Iba1 is seen in activated microglia cells due to inflammation, injury, ischemia or several brain diseases. Therefore, it is often not only used as a marker for microglia, but also as an index for microglia activation (Bland et al. 2010;e.g. Sominsky et al. 2012).

To study microglia activation, there are also other microglia-expressed markers that target, for instance, CD11b (also known as complement receptor 3 or OX-42) and CD68 (also known as ED-1) receptors. Although CD11b is not exclusively expressed in pro-inflammatory macrophages or microglia (Saijo and Glass 2011), an increased expression of this marker is often interpreted as a sign of M1-activation (Perego et al. 2011;Roy et al. 2006). CD68 is expressed in lysosomes, endosomes and on the cell surface of macrophages, neutrophils and reactive microglia (Franco and Fernandez-Suarez 2015;Van den Eynde et al. 2014). For the purpose of studies reported in the thesis, I assessed Iba1 expression as a measure for microglia density, whereas the activation state was determined by staining against CD11b and CD68.

– *Glutamate decarboxylase type II:*

In prefrontal cortex and hippocampus of patients with schizophrenia a reduction in synthesis of GABA was repeatedly reported, measured via expression of glutamate decarboxylase type II (GAD67) mRNA and protein (Akbarian and Huang 2006;Gonzalez-Burgos et al. 2010;Thompson et al. 2011). GAD67 is the enzyme that catalyzes the carboxylation from glutamate into GABA and is commonly used to study GABAergic changes in patients with (Akbarian and Huang 2006;Gonzalez-Burgos et al. 2010) and animal models of schizophrenia (Harvey and Boksa 2012;Labouesse et al. 2015;Nouel et al. 2012).

NMDA receptor antagonism during the neonatal period induces death of GABAergic interneurons (Roux et al. 2015). Therefore, GAD67 was selected as a marker of GABAergic transmission within the experiments of this thesis.

Neurochemistry

– *[^{14}C]-2-deoxyglucose utilization:*

Local cerebral energy metabolism in animals can be determined via the [^{14}C]-2-deoxyglucose utilization paradigm. The amount of glucose taken up by selected brain regions is assessed using a 2-deoxyglucose that is radioactively labelled with the isotope [^{14}C]. The [^{14}C]-labelled glucose is injected in vivo and can be measured ex vivo in the brain slices via

autoradiography. A higher energy demand, measured as increased glucose uptake, can be directly seen by higher amount of radioactivity (Dedeurwaerdere et al. 2011;Sokoloff et al. 1977).

For this technique, normal glucose labelled with [^{14}C] is not suitable as a substrate because it is too rapidly converted to carbon dioxide. Therefore, the analogue [^{14}C]-2-deoxyglucose is commonly used, having special properties that make it more practical for this application. It is metabolized analog to glucose until being phosphorylated by hexokinase. The following product, [^{14}C]-2-deoxyglucose-6-phosphate, cannot be converted into fructose-6-phosphate (like glucose) because of the lack of a hydroxyl group on its second carbon atom (see Figure 4). Its metabolism ceases at this point and it accumulates in the tissue, allowing the application of the quantitative autoradiographic technique (Sokoloff et al. 1977).

In humans local metabolic brain activity is studies by positron emissions tomography using [^{18}F]-fluorodeoxyglucose as radiotracer. Metabolic levels can be determined at resting conditions but also while performing cognitive tasks (during cognitive stimulation). A changed cerebral metabolic rate of glucose in patients with schizophrenia was first reported in 1982 by Buchsbaum and colleagues (Scholl et al. 2014). Commonly, a hypoactivity and hypometabolism in frontal, prefrontal and anterior cingulate cortical regions at rest are seen in patients and are associated with negative symptoms, while the temporolimbic system, the basal ganglia and temporal cortical regions are linked to hypermetabolism during rest and are associated with positive symptoms (Mingoia et al. 2012;Scholl et al. 2014).

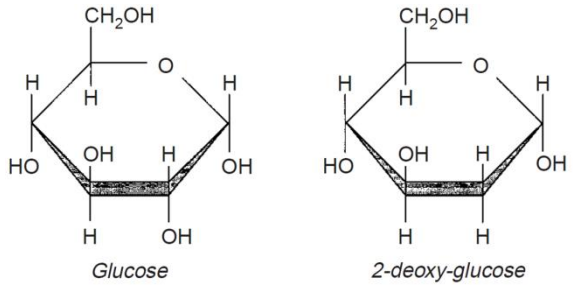

Figure 4: Chemical structure of glucose and 2-deoxyglucose. Only a hydrogen instead of a hydroxyl group at C2 distinguishes 2-deoxyglusose from common glucose. Taken and modified from Aft et al., 2002.

1.4 Study aims

In the framework of the present thesis, three related projects were conducted, which investigated a novel two-hit animal model of schizophrenia combining prenatal immune activation (by Poly I:C) with early postnatal glutamatergic insult (by PCP).

The **first project** was aimed to **establish the two-hit model**. Reasons for the development of a two-hit model were the low robustness of the one-hit models like neonatal PCP treatment, and the fact that schizophrenia likely results from an interplay of multiple developmental factors. **First step** of the model development was to increase the robustness of the one-hit model by adding a second developmental insult. Prenatal maternal immune activation by Poly I:C at GD 15 was chosen, because it mimics viral infection during the first/second trimester of pregnancy. **Second step** was the choice of the rat strain showing the highest effect by combined prenatal Poly I:C and neonatal PCP treatment. Wistar rats were identified as the preferred strain and therefore, all further experiments were conducted in these rats. **Third step** was the selection of methods that could assist analysis of the effects of Poly IC/PCP treatment in further studies.

The **second project** aimed to test the **hypothesis that juvenile behavior could serve as a predictor for adult abnormalities** and to identify analytic tools (based on correlation or cluster analyses) for **identifying responders and non-responders in the population exposed to Poly I:C/PCP treatment**. Reason for this hypothesis was the natural high variability seen in developmental animal models such as Poly I:C/PCP.

The **third project** aimed to assess the **adolescent anti-inflammatory/neuro-protective treatment as a preventive therapeutic approach for schizophrenia**. Used were minocycline and pregnenolone, both discussed in the literature as potential disease modifying treatment.

2 Materials and Methods

2.1 Ethics

All experiments were approved by the AbbVie Animal Welfare Office (Ludwigshafen, Germany) and were performed in accordance with the European and German national guidelines as well as the recommendations and policies of the U.S. National Institutes of Health "Principles of Laboratory Animal Care (1996 edition)". Animal housing and experiments were conducted in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2 Animals

Rats were housed under controlled environmental conditions (ambient temperature 21±1°C, humidity 40–70%, two different enrichment items per cage, 12-h day / night cycle lights on at 6:00 or 6:30 a.m.) in Macrolon cages (Tecniplast, Buguggiate, Italy). During all studies, animals had unlimited access to drinking water. Food was provided ad libitum except for the time of operant testing were rats were fed restrictively (for details see test protocols). Upon delivery from the supplier, animals were allowed to acclimatize to housing conditions for at least one week prior to start of experiments.

Pregnant female rats at gestation day (GD) 7 for the treatment with polyinosinic : polycytidylic acid (Poly I:C) or saline were purchased from Janvier (WistarHan (Wistar) or Lewis; Le Genest-St-Isle, France) or Charles River (Fischer344 (Fischer); Sulzfeld, Germany) and housed individually (before delivery of pups) or together with their litter in type IV cages. In total, male pups from 67 Poly I:C and 38 saline treated dams were used.

For the development of the variable-interval fixed-interval (VIFI) and the operant set-shifting tasks, 36 male Wistar rats (12 for VIFI and 24 for set-shifting) were purchased from Janvier at an age of 9 weeks and were housed individually in type III cages.

As social partners for the test animals in the social novelty discrimination task, 16 male Wistar rats were purchased from Janvier at the age of 21 days and were housed 4 per cage (type IV).

2.3 Prenatal and postnatal treatments

On GD 15, pregnant rats were brought to an experimental room and let acclimatize for at least one hour. They were briefly anesthetized one at a time with Isoflurane (Forane; Abbvie, Ludwigshafen, Germany), and one group of rats was injected intravenously with Poly I:C (4

mg/kg, 1 ml/kg), whereas rats in the other group received an equal volume of saline injection. Body weight was assessed directly before treatment and one day later to monitor weight changes due to the Poly I:C treatment. Delivery date was designated as postnatal day 0 (PND 0). Delivery rate per strain was calculated by dividing the number of females that delivered live pups (male and/or female pups) by the total number pregnant females, expressed as a percentage. One day after delivery, pups were culled to give litters of 8 males, if possible. On PND 7, 9 and 11, pups born from Poly I:C-treated dams were treated intraperitoneally with 10 mg/kg PCP (10 ml/kg) and formed the two-hit group used in subsequent experiments. This was done by short separation of the pups from their mothers (one litter after another) and injection in a separate room. Pups from saline-treated dams received no PCP treatment (control group). Rats of both treatment groups were earmarked on PND 20 or 21 and weaned on PND 21 or 22 (see study designs). About one week later, the offspring were rehoused in groups of 2-3 rats per cage with animals belonging to the same treatment group and sex (Figure 5).

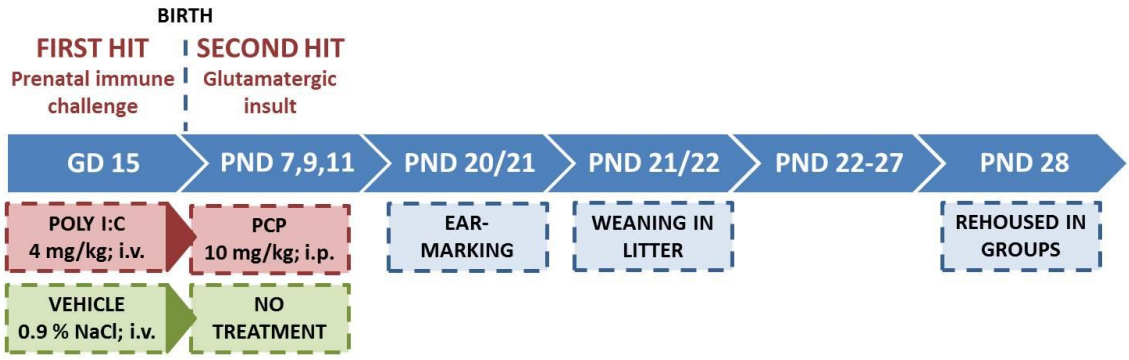

Figure 5: Schema of the pre- and postnatal treatments. See text for details.

2.4 Drugs

Poly I:C was purchased from Calbiochem (Merck, Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany). Minocycline and pregnenolone were purchased from Sequoia Research Products (Pangbourne, UK) whereas all other drugs, including phencyclidine (PCP), D-amphetamine sulfate (amphetamine), diazepam and MK-801 hydrogen maleate (MK-801) were obtained from Sigma-Aldrich.

All solutions were prepared fresh before the use. Poly I:C, PCP and amphetamine were dissolved in 0.9 % NaCl. MK-801 was dissolved in sterile water, whereas diazepam, minocycline and pregnenolone were suspended in 0.5% hydroxyl-propyl-methylcellulose / 0.02% Tween-80® in sterile water.

Poly I:C was injected intravenously (i.v.) in a volume of 1 ml/kg. For neonatal treatment, PCP was injected intraperitoneally (i.p.) in a volume of 10 ml/kg. Minocycline and pregnenolone were administered orally (p.o.) in a volume of 5 ml/kg. In adult rats, amphetamine and PCP were injected subcutaneously (s.c.) in a volume of 1 ml/kg while diazepam and MK-801 were given i.p. at 1 ml/kg.
For all drugs, doses refer to the base forms.

2.5 Behavioral Testing

All experiments were performed during the light phase of the dark / night cycle. Before each experiment, animals were brought from the animal facility to the experimental room and let acclimatize for at least 30 minutes except for VIFI and operant set-shifting where this was not necessary. After finishing the experiments, rats were brought back to the animal facility as soon as possible.

2.5.1 Social play behavior

Prior to the tests, rats were housed individually for 24 hours to increase the likelihood to see play behavior over the short recording time (Panksepp 1981;Pellis and Pellis 1990;Siviy et al. 1997). At least 1 h before testing started, rats were marked using a water resistant pen. Animals were placed pairwise ("unfamiliar" partners – from a different litter that received the same treatment but was housed separately since PND 21) in the test enclosure (home cage-like, macrolon type IV cage without enrichment items, food or water) and interaction was videotaped for 5 minutes under dim light conditions (20 lx, white light). Social play behavior was evaluated offline by a trained experimenter blind to group assignments. Play bouts were analyzed either in slow motion or by frame-by-frame advancement, depending upon the complexity of the sequence. Analysis focused on three measures of social play behavior: attacks initiated, total defense and facing defense.
A **playful attack** (play initiation) was scored when one of the pairmates brought its snout either into contact with, or within 1 cm of, the partner's nape. Withdrawing the nape from an approaching partner was scored as a defense (**total defense**) by that animal. The recipient of the attack could use two defensive tactics including evasion (running or leaping away, Figure 6A) or facing defense (Figure 6B and 6C). In the latter case, the recipient not only withdrew the nape, but also turned to face the attacker while often initiating a counterattack. For a detailed description see Pellis, 1990 and Smith et al., 1997. Total defense was calculated by

dividing the number of defense by the total number of attacks from the pairmate, expressed as a percentage:

$$Total\ defense = \frac{number\ of\ evasions + number\ of facing\ defenses\ of\ rat\ A}{number\ of\ attacks\ by\ rat\ B} * 100$$

Facing defense was calculated in the same way:

$$Facing\ defense = \frac{number\ of facing\ defenses\ of\ rat\ A}{number\ of\ attacks\ by\ rat\ B} * 100$$

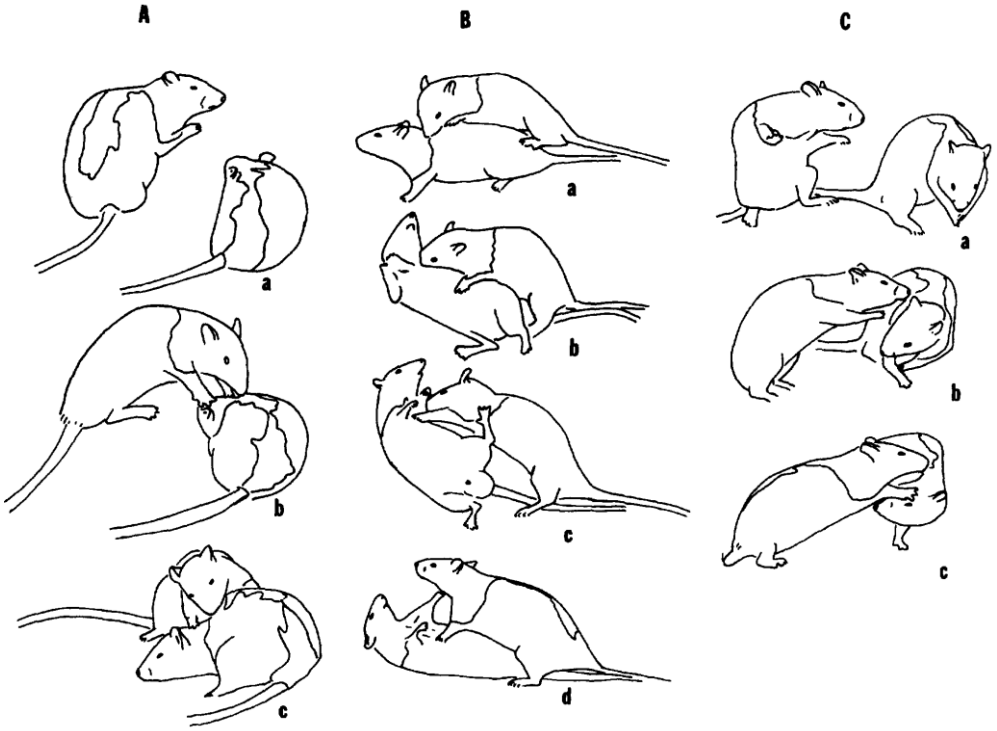

Figure 6: Schematic drawing of possible defense mechanisms in social play behavior. There are three major kinds of defense mechanisms including evasion (*A*), complete rotation (*B*) and partial rotation (*C*). The latter both can be combined as facing defense. Taken from Pellis et al., 1997.

2.5.2 Social novelty discrimination task

The experiment was carried out like described elsewhere (Harich et al. 2007). At least one hour before testing started, juvenile partner rats were marked using a Raidl maxi pen (Raidex GmbH, Dettingen / Erms, Germany). Social discrimination was assessed in large open field boxes (60×60×35 cm, L×W×H) made of black plastic under dim light conditions (30 lx, red light). Each adult rat was placed into the test enclosure together with a juvenile for a period of 30 minutes. After this time, a second juvenile (novel) was introduced for another 5 minutes. Behavior of the test rats during this 5-minute period was videotaped and evaluated offline by a trained experimenter blind to group assignment. The duration of social exploratory behavior, including sniffing, grooming and close following, of the adult rat directed towards

either of the two juveniles was analyzed using the Noldus Observer software (version 5.0, Noldus, Netherlands). The discrimination index was calculated as the time spent investigating the novel juvenile divided by the time spent investigating the familiar juvenile.

2.5.3 Locomotor activity

Motor activity was studied using small (40×40×35 cm, L×W×H) or large (60×60×35 cm, L×W×H) open field boxes made of black plastic under dark (<1 lx, red light), bright light (525-555 lx, white light) or dim light (around 5 lx, white light) conditions. Tests under bright and dim light conditions were conducted in the experimental room other than the tests under dark conditions. Rats were placed into the test arenas and their movements were videotaped. Recorded activity patterns were processed using Ethovision software (version 3.1 or 8.5, Noldus, Netherlands) to obtain parameters such as total distance travelled (cm) as well as distance travelled and duration (s) in the center zone (small: 13×13 cm; large: 20×20 cm).

2.5.4 Spontaneous locomotor activity in adult rats

Testing under dark conditions was conducted in small or large open field boxes, whereas motor activity under bright light conditions was assessed using large open field boxes. Rats were tested for 30 or 60 minutes.

Locomotor activity after acute drug challenge in adult rats

As a part of all experiments, rats were habituated one day before treatment with the first drug dose. For the MK-801 experiment (0.056 or 0.1 mg/kg), only one dose per animal was tested. For amphetamine (0.25, 0.5 and 1 mg/kg) and PCP (1 mg/kg) experiments, drug treatment was repeated once a week until every animal received all doses (*latin square* design).

Animals were tested in small open field boxes under dark conditions. Testing lasted for 60 minutes and started directly after injection of vehicle or compound (for MK-801) or after a 30 minutes latency period in the home cage (for amphetamine and PCP). Vehicle and compound injections were counterbalanced between treatment groups.

Spontaneous locomotor activity in adolescent rats

In experiments where locomotor activity was tested in the same rats during adolescence and in adulthood, adolescent testing was conducted in small open field boxes under dim light conditions in an experimental room other than that used for adult testing. If locomotor activity

was assessed during adolescence only, animals were tested in small open field boxes under dark conditions. Motor activity was monitored for 30 minutes.

2.5.5 Prepulse inhibition

The prepulse inhibition (PPI) experiments were performed at the Central Institute of Mental Health (Mannheim, Germany). PPI of the acoustic startle response (ASR) was measured using a standard startle chamber (SR-Lab; San Diego Instruments, San Diego, USA) (Goepfrich et al. 2013). The system contained a sound-attenuating chamber with fan and loudspeaker, a piezoelectric accelerometer attached underneath a plexiglas cylinder containing the test animal, and was controlled by a computer using SR-Lab software.

The experiments were performed with the following settings: For Wistar rats, white noise with an intensity of 115 decibel (dB) sound pressure level was used as startle pulse with a duration of 40 ms (0 ms rise / fall time). Background noise was white noise with 65 dB. For Lewis rats, I used the same settings except for the startle pulse, which was adjusted to 120 dB. Prepulse intensities were the same for both strains – 72, 76, 80 and 84 dB with a duration of 20 ms. Prepulses preceded the startle pulse always by 100 ms.

The PPI program was the same for both strains and consisted of 5 minutes of acclimatization period when animals were only exposed to the background noise, followed by 5 initial startle stimuli. Afterwards, the test period began comprising 6 different kinds of trials presented in a pseudorandomized order: startle pulse alone trials, startle pulse trials preceded by a prepulse of 4 different intensities (see above) and trials where only background noise was presented. The inter-trial interval varied from 10 to 20 seconds. Every trial type was presented 10 times resulting in a total of 60 test trials.

The PPI was calculated from the 60 trials of the test period according to the following formula:

$$\%\, PPI = 100 - \frac{100 \times mean\ amplitude\ of\ the\ ASR\ \ on\ pre-pulse\ trials}{mean\ amplitude\ of\ the\ ASR\ \ on\ pulse\ alone\ trials}$$

2.5.6 Operant conditioning chambers

Experiments were conducted in 12 identical standard operant conditioning chambers (MED Associates Inc., East Fairfield, Vermont, USA; model ENV-008; 31×27×33 cm). Each chamber was located in a sound- and light-attenuating cubicle (ENV-022V) and was equipped with a grid floor, a house light (ENV-215M), a 2.8-kHz, 78-dB sound generator (ANL-926), two retractable levers (ENV-112CM), a triple LED stimulus light (green, yellow, red; ENV-

222M) located above each lever, a food pellet dispenser (ENV-203) ejecting 45-mg pellets (Bioserve F0165, Flemington, NJ, USA), a food receptacle (ENV-200R2M) with a head-entry detector (ENV-254) and a nose-poke hole (ENV-114M). The food receptacle was located in the middle of one of the walls between the retractable levers. The house light was located on the opposite wall with the nose poke underneath. Chambers were connected to an operating computer through a MED interface.

2.5.7 Variable-interval fixed-interval (VIFI) task

Training and testing procedure

Animals were weighed and food-restricted one day before the experiment started. They got a daily amount of 15 g food (including reward and home cage food) for the first two months followed by 17 g to limit the body weight gain to 5-6 g per week. At the first training day, rats were shaped to press a lever for food under a continuous reinforcement schedule until they reached a criterion of 100 lever presses during a 30-minutes session. Animals were then shifted to a variable interval 15 seconds (VI15) schedule of reinforcement (intervals ranging from 5 to 25 s) for 3 days, followed by a VI30 schedule of reinforcement (5 to 70 s) for 3 (animal group for task establishment) or 7 (animal group to assess the effect of Poly I:C/PCP) weeks. After that, a testing procedure was introduced whereby "training" VI30 schedule was operating during 4 of the 5 weekly sessions and one "test" session was conducted under fixed interval 30 seconds (FI30) (varying between Wednesdays, Thursdays and Fridays). In addition, there were weeks when one or two VI sessions were designated as "test" sessions (in the animal group for task establishment only). There was a maximum of 84 pellets that could be earned during training and test sessions.

Drug testing (in the animal group for task establishment only) did not commence until each rat was exposed to at least 8 FI30 test sessions. All drugs were injected 20 minutes before the test sessions started. Pseudo-random sequence of drug dose testing was based on a *latin square* design. FI test sessions with drug pretreatment were conducted maximum once a week (varying between Wednesdays, Thursdays and Fridays) while VI training sessions with drug pretreatment were conducted twice a week (Tuesdays and Fridays).

Data analysis

During each session, every event (lever press response and food delivery) was recorded with the time stamp. Based on these raw data, the following parameters were calculated

individually for each rat and session for subsequent analysis. First, average duration of the post-reinforcement pauses (PRP) was calculated as the mean of all 84 values for a given session (**average PRP**). Second, to estimate the change of the PRP duration over the FI30 session, mean of the last 28 PRP values was divided by the mean of the first 28 PRP values to obtain the **PRP adjustment ratio**. Third, linear regression analysis was applied to yield the slope of the PRP adjustment over the session (**regression slope**). To calculate that, successive blocks of 14 PRPs were averaged to result in 6 values that gave the regression slope. Fourth, **response rate** (responses / sec) was calculated as the total number of lever presses per session divided by corrected session duration (total session time minus cumulative duration of all PRPs).

2.5.8 Operant set-shifting task

The protocol was adapted from that published by Floresco and colleagues (Floresco et al. 2008).

Training and testing procedure

– *Training procedure*

Rats were restrictedly fed for 3 days and, in addition to the lab chow, were given 50 pellets to habituate to their taste one day before the experiment started. During the set-shifting experiment, rats received a daily amount of 15 g food (including reward and home cage food) to limit the body weight gain to 5-6 g per week. Before the training started, each rat was placed into an operant chamber for a period of 15 minutes and received 45 pellets delivered to the food receptacle. Only if all pellets were eaten, training was started one day later.

During the first days of training, animals were shaped to press lever for food under two different continuous reinforcement schedules. On the first schedule, a lever press on any of two levers was reinforced with one food pellet. Only if the criterion of 100 earned pellets within 30 minutes was reached, the schedule was changed the following day. The second schedule was designed to prevent the development of a side bias. To that end, half of the total pellets could be earned by pressing the left lever and the other half by pressing the right lever. Again, only if the criterion (100 pellets within 30 min) was reached, the schedule changed one day later.

If the rats passed both initial schedules, they were familiarized with the insertion of the levers into the chamber which started one day later. Each session consisted of 90 training trials and

began with the levers retracted and the houselight switched off. Every 20 seconds a trial started with the illumination of the houslight and the insertion of one of the two levers. If the animal pressed the lever within 10 seconds, the lever was retracted, a single food pellet was delivered into the food tray and the houslight stayed illuminated for another 4 seconds. This was counted as a correct response. If the animal did not respond within the given time, the lever was retracted and the houselight was turned off immidiatly without food delivery. This was counted as an omission. Side of the lever presentation was randomly determined. Before proceeding to the side-bias determination session, rats had to achieve a criterion of less than 5 omissions out of 90 training trials, but received a minimum of 4 training sessions.

– *Side-bias determination session*

Within this session, the side bias of every rat was determined. Procedure was similar to that of the lever-insertion training (described above) except that both levers were inserted into the chamber at the beginning of each trial and both were retracted after any response. At every trial, a food pellet was delivered after responding on either lever. Upon next insertion of levers, a lever press was only rewared if the opposite lever was chosen. If the rat chose the same lever as before, the houslight was turned off and no food was delivered. This procedure continued until the rat responded on the lever opposite to one initially chosen. After responding on both levers within a trial, a new trial was initiated. This session was conducted one day before the visual-cue discrimination session and comprised 45 trials (Figure 8).

– *Visual-cue discrimination (VCD) session*

During this procedure, every trial began with the presentation of a cue-light illuminated above one of the levers. Three seconds later, the houselight was illuminated and both levers were inserted. Lever pressing was rewarded only if the animal responded on the lever containing the illuminated cue-light, counted as a correct response (Figure 7, left panel). If the rat responded on the correct lever, both levers were retracted and a single food pellet was delivered. The houselight remained on for another 4 seconds. If the rat responded on the incorrect lever, both levers were retracted and the houselight was turned off immediately. This was counted as an incorrect response. Failure to respond on either lever within 10 seconds resulted in the retraction of both levers and extinguishing of the houselight, and was counted as an omission. Trials started every 20 seconds. Position of the illuminated cue-light was choosen pseudo-randomly. The session continued until a criterion of 10 consecutive correct

responses in minimum 30 trials was achieved or ≥ 150 trials were reached, whichever came first (VCD criterion). Number of trials and errors to criterion were recorded.

SHORT testing protocol: After one day of VCD testing, the response discrimination (RD) session was conducted (Figure 8, upper panel). Only animals that reached the VCD criterion were considered for the analysis of VCD and RD.

LONG testing protocol: VCD testing was conducted for 5 days before continuing with the RD testing (Figure 8, lower panel). If a rat did not reach the VCD criterion in one session, a value of 150 for "number of trials to criterion" was recorded for this session.

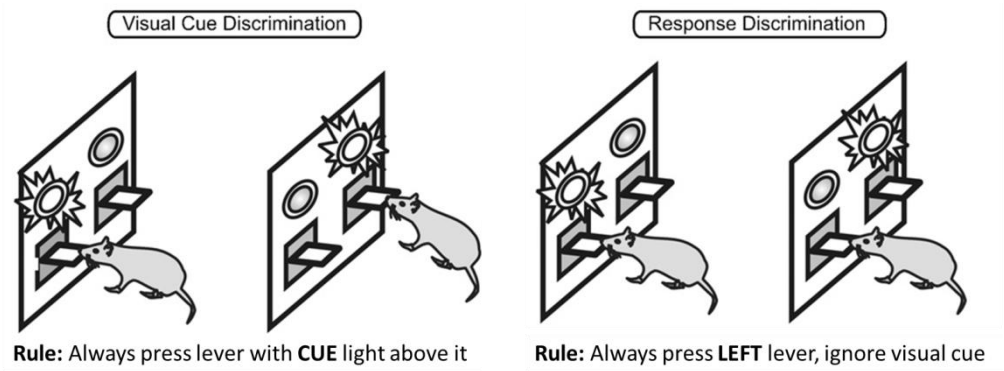

Figure 7: Test protocol. Animals were trained to discriminate the correct lever dependent on the visual cue (*left panel*). This was shifted later to a response-based rule (*right panel*). Taken and modified from Floresco et al., 2009.

– *Response discrimination (RD) session*

While the general procedure was the same as before, the rule for a correct response changed from one stimulus dimention (visual cue) to another (response). This means that a lever press on one of the levers was reinforced independent of the position of the illuminated cue light (Figure 7, right panel). The "correct" lever was set before the session started individually for each animal (see analysis of the side bias below). A RD session lasted until rats achieved a criterion of 10 consecutive correct responses or ≥ 150 trials were reached (RD criterion). Number of trials and errors to criterion were recorded.

SHORT testing protocol: RD testing stopped after one session and the side-bias determination was repeated followed by the RE-LONG testing protocol.

LONG and RE-LONG testing protocol: For animals which underwent LONG and RE-LONG testing RD protocol was continued for 5 sessions before the experiment was finished (Figure 8). If a rat did not reach the RD criterion within one session, a value of 150 for "number of trials to criterion" was recorded for this session.

Figure 8: SHORT and LONG / RE-LONG test protocols. SHORT protocol (*upper panel*) comprised of one day side-bias determination, followed by one day VCD and RD testing. The LONG and RE-LONG protocol (*lower panel*) was a prolonged version, including one day side-bias determination, 5 days VCD and 5 days RD testing. VCD: visual-cue discrimination, RD: response discrimination.

Data analysis

– *Analysis of the side bias*

The initital responding of the animal in every side-bias-determination trial was counted towards its side bias. If the animal made a comparable amount of initial lever presses on the left and right lever over the entire session, the lever that was chosen 4 or more times during the first 7 trials was designated as the animal's side bias. If the initial responses between left and right lever were disproportional (> a 2:1 ratio), the lever chosen more often was designated as the animal's side bias. For the SHORT RD session, correct response entailed responding on the lever opposite of the rat's side bias.

The assessment of the side bias one day before the first VCD session within the RE-LONG test protocol showed that the bias changed towards the lever which was required to press for a correct response during the SHORT RD testing for most of the animals. Therefore, for the RD testing within the RE-LONG test protocol, for all animals the lever opposite to that rewarded during RD within the SHORT test protocol was now required to press for a correct response, even if a bias for that lever was determined in the second side bias determination session.

– *Error analysis*

There were three types of errors analyzed during the first RD session: the perseverative error that reflected the animal's ability of shifting away from the previously learned strategy, as well as the regressive and the never-reinforced error, displaying both the ability to acquire and maintain a new strategy after perseveration had ceased (Floresco et al. 2009).

A **perseverative error** was scored when an animal responded on the lever with the cue-light illuminated above it, but for a correct response the press on the opposite lever was required. In 8 out of 16 consecutive trials, the rat was required to press the lever opposite of that guided by

the cue-light. For the analysis, trials of this type were divided into consecutive blocks of 8 trials each.

An error per trial was scored to be a perseverative error as long as a rat pressed the incorrect lever in 6 or more trials within one block. If an animal made less than 6 perseverative errors within any block for the first time, all errors of this and all following blocks were not counted as perseverative anymore, but as **regressive errors**, because from this point on, the rat used the original strategy less than 75 % of time (Floresco et al. 2008). A **never-reinforced error** was scored when the rat pressed the incorrect lever although the cue light was illuminated above the lever which was required to press for a correct response. This means the animal made a choice that was never reinforced neither during the VCD nor the RD sessions.

2.5.9 Autoshaping

Rats were restrictedly fed for 3 days and, in addition to the lab chow, were given 50 pellets to habituate to their taste one day before the experiment started. During the autoshaping experiment, rats received 11 g of normal lab chow after each training session. Experimental protocol was adapted from Meneses (Meneses and Hong 1994). First, each rat was placed into an operant chamber for a period of 30 minutes and received 50 pellets delivered to the food receptacle. If the animal ate all pellets, training began one day later. The protocol consisted of the presentation of a retractable lever for 8 seconds followed by the delivery of a food pellet, on average every 60 seconds (30-90 s). Each time an animal pressed the retractable lever, the trial was shortened, meaning the lever was retracted, and the food was delivered immediately (Figure 9). This was considered as a "response" and was recorded during the last session only, performed as test session (session 4). The first 3 sessions consisted of 20 trials; the test session consisted of 40 trials.

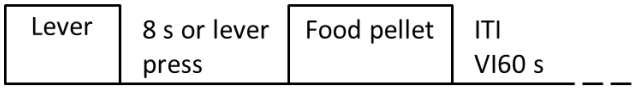

Figure 9: Schema of one trial of an autoshaping test session. Eight seconds upon insertion of the lever, a food pellet was delivered, followed by an ITI of 60 s (on average). If the lever was pressed during the 8 seconds, the food was delivered immidiately. ITI: inter-trial interval.

2.6 Brain metabolic activity

2.6.1 [^{14}C]-2-deoxyglucose utilization

Metabolic brain activity was measured using the fully quantitative [^{14}C]-2-deoxyglucose autoradiographic technique (Dedeurwaerdere et al. 2011). Animals were injected

intraperitoneally with 100 µCi/kg [^{14}C]-2-deoxyglucose (Perkin-Elmer, Waltham, USA) in 0.9 % NaCl. 45 minutes later, the animals were sacrificed by rapid intraperitoneal injection of sodium pentobarbitone (Merial GmbH, Hallbergmoos, Germany), and the brains were immediately removed from the skull, rapidly frozen in pre-cooled 2-methylbutane (-40 °C, on dry ice) and stored at -80 °C. Frozen brains were sectioned (20 µm) in the coronal plane in a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). A series of 3 sections were retained from every 200 µm, thaw mounted onto glass coverslips and dried on a hot plate (37 °C for 15 min). Autoradiograms were prepared by applying these sections to Biomax film (Kodak, Rochester, USA) in light-tight cassettes, together with a series of 16 precalibrated [^{14}C] standards (2-3580 nCi/g tissue equivalents; GE Healthcare GmbH, Frankfurt, Germany), and stored at room temperature for 4 days. Sections were digitized using a light box and digital camera. Local tissue isotope concentration was derived from the optical density of the autoradiographic brain images relative to the [^{14}C] standard using ImagePro (version 5, Media Cybernetics Inc., Rockville, USA) and GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Areas of interest were limited to 3 brain regions: prefrontal cortex (prelimbic + infralimbic cortex) (3.20 – 2.20 mm from bregma), dorsal hippocampus (-2.56 – (-3.80) mm from bregma) and ventral hippocampus (-5.20 – (-6.04) mm from bregma) (Paxinos and Watson, 1998). Optical density was measured in 5 sections for each brain region. To reduce variability, two measurements per section were assessed and averaged. For the prefrontal cortex, bilateral density readings were done, whereas for dorsal and ventral hippocampus, unilateral readings were assessed (because two measurements were necessary to cover the complete hippocampus area of one hemisphere).

2.7 Immunohistochemistry

2.7.1 Tissue preparation for free-floating staining

Animals were deeply anesthetized with a mixture of ketamine : xylazine (100 mg : 5 mg, i.p.; 0.125 ml solution per 100 g body weight) (Ketamin: CP Pharma, Burgdorf, Germany; Xylazine: Bayer HealthCare, Berlin, Germany) and then transcardially perfused with ice-cold 1 % phosphate buffered saline pH 7.4 (PBS; Roche Diagnostics Deutschland GmbH, Mannheim, Germany) for 10 minutes followed by perfusion with 4 % ice-cold paraformaldehyde (PFA; J.T. Baker, Griesheim, Germany) for another 10 minutes with a flow rate of 8-10 ml/min. Brains were removed and stored at 4 °C in 15 ml 4 % PFA. After 24 hours post-fixation, brains were re-bedded in a 30 % sucrose solution (sucrose diluted in a 1 % PBS solution; Sigma-Aldrich, Deisenhofen, Germany) as cryoprotection and stored at 4 °C

until sectioning. Brains were cut in 40 µm slices in the coronal plane using standard microtomes (Leica Microsystems GmbH, Wetzlar, Germany). A series of 6 sections were collected from every 240 µm and stored in 1 % PBS until staining.

2.7.2 Antibody staining

Solutions and antibodies:

0.3 % Hydrogen peroxide (H_2O_2) in 80 % methanol:

- 100 % methanol 800 ml/l
- Bidestilled (dd) H_2O 190 ml/l
- 30 % H_2O_2 10 ml/l

Avidin-biotin-complex solution (Vectastain Elite ABC kit):

- Reagent A 4 drops/10 ml
- Reagent B 4 drops/10 ml
- 1 x TBST
  - ➔ 30 minutes shack

3,3′-Diaminobenzidine solution (DAB substrate kit):

- Buffer 4 drops/10 ml
- DAB stock 8 drops/10 ml
- H_2O_2 4 drops/10 ml
- ddH_2O

Table 6: Antibodies used for staining including concentrations and manufacturers.

1st Antibody	Concentration	Manufacturer
anti-Iba1, rabbit	1:1000	Wako Chemicals GmbH (Neuss, Germany)
anti-CD68, mouse	1:1000	AbD Serotec (Puchheim, Germany)
anti-CD11b/OX-42, mouse	1:300	AbD Serotec (Puchheim, Germany)
anti-GAD67, mouse	1:3000 (free-floating) 1:1000 (on-slide)	Millipore Chemicals GmbH (Darmstadt, Germany)
2nd Antibody	**Concentration**	**Manufacturer**
donkey anti-rabbit IgG	1:500	Jackson ImmunoResearch (Newmarket, UK)
donkey anti-mouse IgG	1:500	Jackson ImmunoResearch (Newmarket, UK)

Staining protocols:

Slices were stained using the 3,3′-diaminobenzidine (DAB) technique for chromogenic reaction.

– *Free-floating*

Before starting, sections were transferred into 6 well plates (Corning Inc., New York, USA) filled with 1 % PBS. Slices were incubated in 0.3 % hydrogen peroxide in 80 % methanol (Millipore Chemicals GmbH, Darmstadt, Germany) for 30 minutes, and washed afterwards with tris-buffered saline containing 0.05 % Tween-20® (TBST; Dako Deutschland GmbH, Hamburg, Germany) 3 x for 5 minutes, followed by the blocking of nonspecific sites with 5 % donkey serum (Bio-Rad Laboratories GmbH, Munich, Germany) in TBST. Over-night (minimum 20 hours), sections were incubated with the primary antibody (see Table 6): Iba1 (1:1000), CD68 (1:1000), CD11b (1:300) or GAD67 (1:3000) at room temperature diluted in 1 % serum / TBST, followed by rinsing (3 x 5 min TBST) and incubation with the matching biotinylated secondary antibody (donkey anti-rabbit or mouse IgG; Table 6) diluted 1:500 in 1 % serum / TBST for 30 minutes at room temperature. After another washing (3 x 5 min TBST), slices were incubated in the avidin-biotin-peroxidase complex solution (Vectastain Elite ABC; Vector Laboratories Ltd., Peterborough, UK) for 30 minutes, rinsed (3 x 5 min TBST), and the reaction product visualized by incubating the sections in the DAB solution (Vector Laboratories Ltd., Peterborough, UK). The reaction was stopped with ddH_2O, followed by washing (3 x 5 min) with 1 % PBS. All steps were done using a shaking device.
After mounted in ddH_2O, slices were dehydrated in graded ethanol (60, 90 and 100 %; 5 min each), cleared in XTRA-Solve for 5 minutes (J.T. Baker, Griesheim, Germany), and coverslipped with UltraKit (J.T. Baker).

– *On-slide*

One series of slides from the [^{14}C]-2-deoxyglucose utilization experiment was used for on-slide staining with GAD67. Slides were let dried at room temperature for about 12 hours, followed by rehydration with 1 % PBS for 5 minutes. Slices were fixed in 4 % PFA for 10 minutes, rinsed (3 x 5 min 1 % PBS) and incubated in 0.3 % hydrogen peroxide in 80 % methanol. All following staining steps were the same as described for free-floating. All steps, except for antibody incubation, were done in stain dishes (Medite GmbH, Burgdorf, Germany) using a shaking device. For antibody incubation steps, slides were removed from the slide holder and arranged in stain trays (Simport, Beloeil, Canada). 750 µl antibody

solutions were pipetted directly on each slide. Primary antibody was diluted 1:1000 (GAD67) and secondary antibody was diluted 1:500 (donkey anti-mouse IgG) (Table 6).

After the last washing with 1 % PBS, slices were rinsed with ddH_2O for 5 minutes and dried, before being dehydrated in graded ethanols (60, 90 and 100 %; 5 min each), cleared in XTRA-Solve for 5 minutes, and coverslipped with UltraKit.

2.7.3 Analysis

For analysis, slides were scanned with a slide scanner (Mirax Scan or Axio Scan.Z1; Zeiss, Oberkochen, Germany) and converted to grey scaling. Analysis was done with ImagePro (version 5, Media Cybernetics Inc., Rockville, USA). If possible, 5 sections per area of interest were used: 1) mPFC (2.70 – 2.20 mm from bregma; Paxinos and Watson 1998), including infralimbic and prelimbic cortex; see Figure 10 for schematic drawing and nissle staining of the mPFC as well as Figure 11 for first and last slice used for analysis); and 2) HPC (-2.56 – (-3.60) mm from bregma; Paxinos and Watson 1998), see Figure 12 for schematic drawing and nissle staining of the HPC as well as Figure 13 for first and last slice used for analysis).

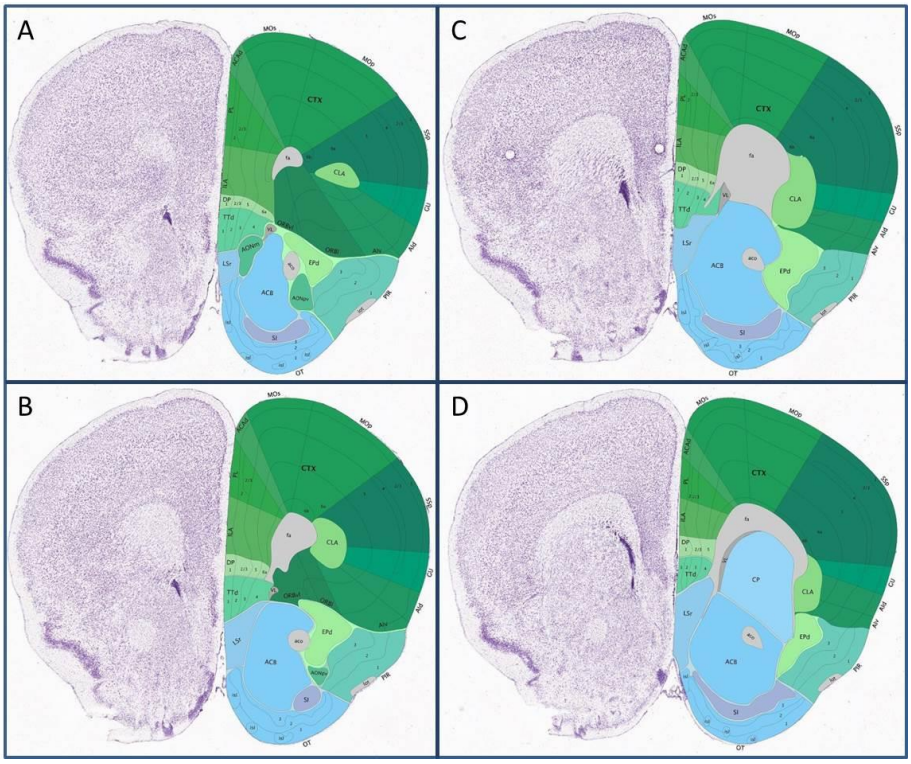

Figure 10: Schematic drawing and nissle staining of the mPFC. Pictures show the course of the mPFC, from anterior to posterior (in the mouse). For analysis, only the infralimbic and prelimbic cortex were used. Taken from Allen Brain Atlas (http://mouse.brain-map.org/).

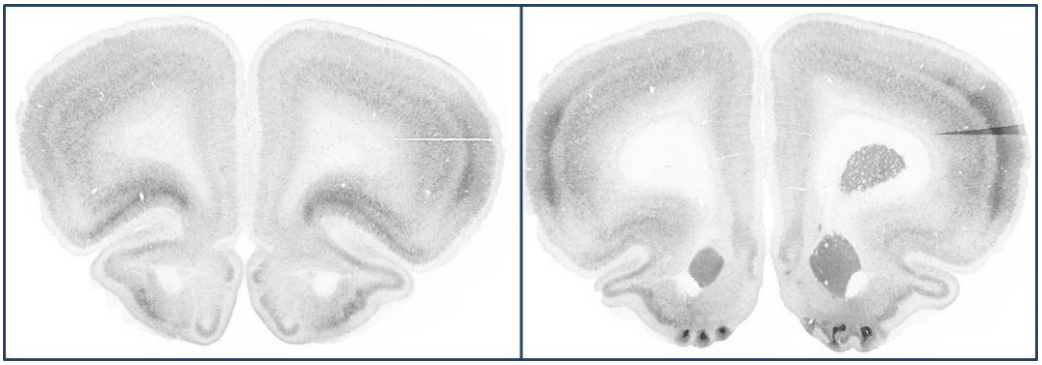

Figure 11: First and last slice of the PFC. Pictures show examples of the first (*left panel*, right hemisphere) and last (*right panel*, right hemisphere) slice used for analysis of the PFC.

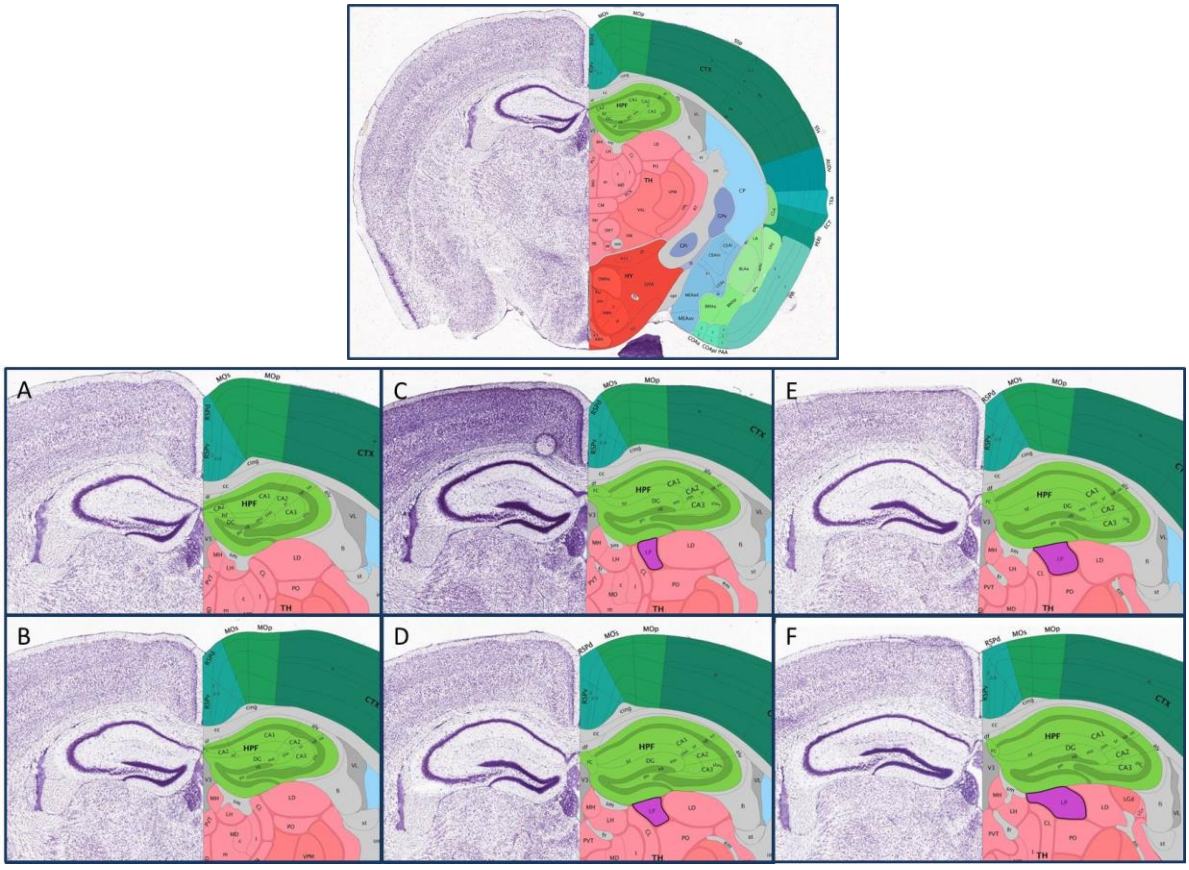

Figure 12: Schematic drawing and nissle staining of the HPC. Pictures show the position (*upper panel*) and the course (*lower panel*) of the HPC, from anterior (*A*) to posterior (*B*) (in the mouse). Taken from Allen Brain Atlas (http://mouse.brain-map.org/).

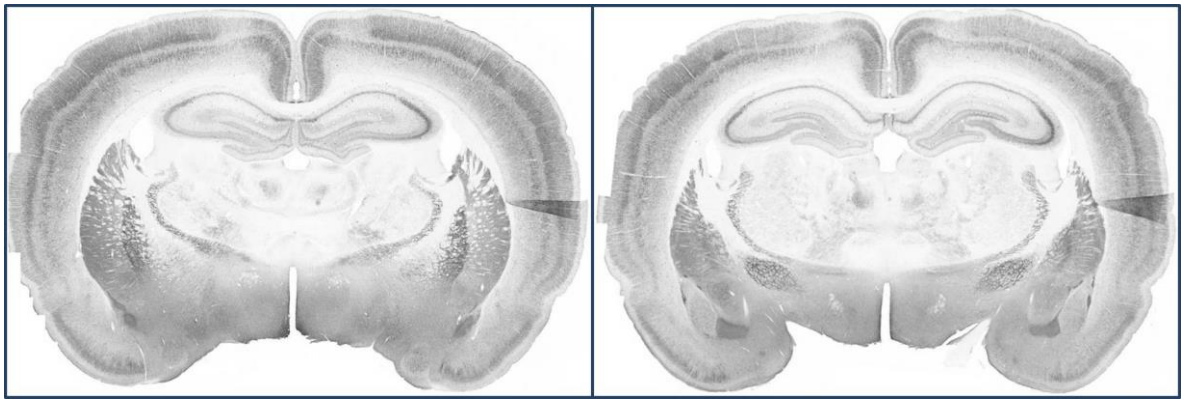

Figure 13: First and last slice of the HPC. Pictures show examples of the first (*left slide*, right hemisphere) and last (*right slide*, right hemisphere) slice used for analysis of the HPC.

Both hemispheres were analyzed separately by measuring the covered area (for Iba1 staining; see Figure 14) or the optical density (for GAD67 staining, see Figure 15 and 16) per area and calculating the mean value per animal and hemisphere. Finally, the mean values of covered area or optical density of both hemispheres were combined and presented per animal.

For the **covered area** analytical approach, the maximal and minimal value of greyscale per pixels was determined, leading to the full detections of the objects in questions (see Figure 14, detected objects in red). These threshold settings were implemented for each study. The region of interest (ROI) was delineated manually (green) following anatomical boarders, and only objects within the threshold as well as within the ROI were counted (red: immunohistochemical positive pixels). The number of positive pixels within the ROI was calculated.

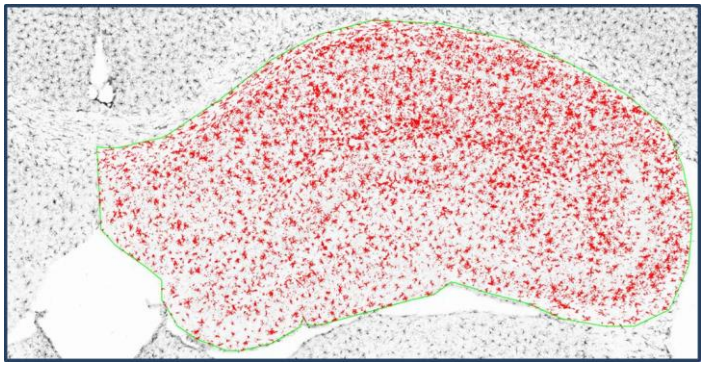

Figure 14: Example of the covered area measurement. Green line shows the region of interest delineated manually and red displays the staining within the threshold.

For **optical density** measurements (Figure 15 and 16), the ROI was delineated manually following anatomical boarders and the greyscale value for each pixel was determined. The optical density of the ROI was obtained by calculating the mean greyscale value for each pixel and normalized by subtracting the slide background (not shown) and tissue background.

Figure 15: Example of the optical density measurement in the mPFC. Green line shows the region of interest delineated manually and red displays the area used to determine the background staining in tissue.

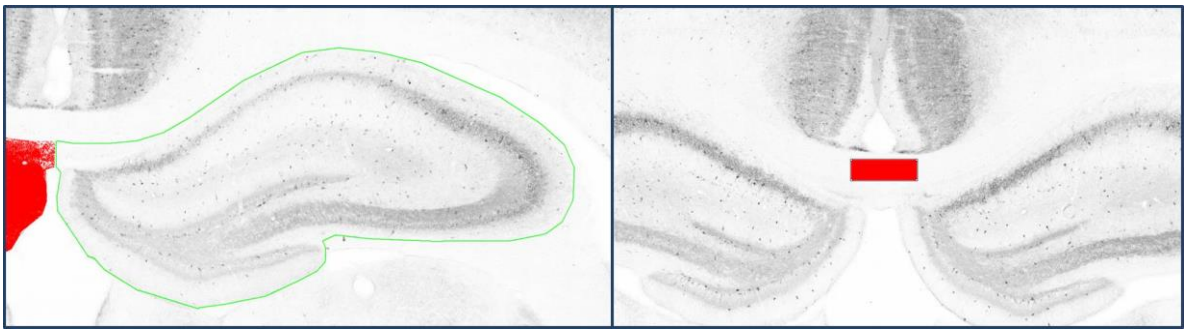

Figure 16: Example of the optical density measurement in the HPC. Green line (*left panel*) shows the region of interest delineated manually and red (*right panel*) displays the area used to determine the background staining in tissue.

2.8 Basic calculations

All basic calculations were performed using Excel (Microsoft Office 2010, Windows, Redmond, USA) and R (version 3.1.3).

Pooled standard deviation (SD_P) was calculated using SD of the treatment group (SD_T) and of the control group (SD_C) as well as the number of animals of both groups (n).

$$SD_p = \sqrt{\frac{SD_T^2(n_T - 1) + SD_C^2(n_C - 1)}{n_T + n_C - 2}}$$

Effect size (ES) was calculated by subtracting the mean of the control group (M_C) from the treatment group (M_T) taking the absolute value and dividing by the pooled standard deviation.

$$ES = \frac{|M_T - Mc|}{SD_P}$$

2.9 Methods of statistical analysis

For statistical analysis, GraphPad Prism 5 or 6 (GraphPad Software Inc., San Diego, CA, USA), SigmaPlot 12.5 (Systat Software GmbH, Erkrath, Germany) and SPSS (version 21.0; IBM, Armonk, USA) were used.

As the d'Agostino & Pearson's omnibus normality test failed at least for some data sets, all statistical analysis was conducted using non-parametric tests. For simple pairwise comparisons between animals of the two-hit and control group the Mann-Whitney's U-test was used. For experiments with two independent variables (e.g. treatment group and time), data were first rank transformed and then subjected to two-way analysis of variance (ANOVA) with repeated measures on dose or time followed by a *post hoc* pairwise comparisons between treatment groups using Bonferroni's test. For the re-analysis of data containing groups separated by correlation analysis or other experiments with 3 groups Kruskal-Wallis-test was used, followed by *post hoc* pairwise comparisons between treatment groups using Dunn's test.

For the VIFI task development, data were analyzed using Wilcoxon's matched-pairs signed rank test (for comparison of two groups) or Friedman's nonparametric repeated measures ANOVA (for comparison of more than two groups). Friedman's ANOVA was followed by *post hoc* analysis (Dunn's multiple comparison test).

A value of $p \leq 0.05$ was considered to represent a significant difference (*) and a value of $p < 0.1$ a trend (#).

2.10 Data exclusion

Data were excluded from analysis or omitted only under pre-specified conditions or in case of a technical failure as described below:

- Spontaneous locomotor activity: When testing locomotor activity under bright light conditions (study 4) the center activity could not be assessed for 8 control and 8 animals of the Poly I:C/PCP group due to technical problems.
- Locomotor activity upon drug challenge: During locomotion testing upon acute challenge with amphetamine and MK-801, center zone was not defined before testing and therefore, this was not analyzed.
- Operant set-shifting task: For both set-shifting protocols, criteria were predefined due to which values were excluded from analysis (see operant set-shifting task). In addition, during the RE-LONG testing, pellets were not delivered continuously in some operant chambers during some sessions. Results of those sessions were excluded

from the analysis. Due to the choice of a two-way ANOVA using reapeated measures for the main factor session over the VCD sessions (Figure 50, upper row panels), data set of one control animal had to be discarded completely from the analysis because of repeatedly missing values. Otherwise analysis would not have been possible. Additonally, data of the last VCD session (session 5) were excluded from the analysis too, due to repeatedly missing values. Analysis of errors with a two-way ANOVA repeated measures was not conducted because of too many missing values.

- VIFI task: All individual PRP values > 500 ms were excluded from analysis. Additionally, FI test data for one diazepam-treated rat were excluded from the analysis because this animal did not finish the session within 120 minutes.
- Social play behavior: This test was conducted in pairs of the same treatment group. Due to an uneven number of animals, one animal per treatment group (two-hit and control) had to be omitted for testing.
- Immunohistochemical analysis: If less than 3 sections per brain region and hemisphere were available for one animal due to tissue disruptions, values for this hemisphere were excluded from analysis.
- [^{14}C]-2-deoxyglucose utilization and on-slide GAD67 staining: In rats of study 4 analysis of one animal from the two-hit and another animal from the control group was not possible because the brain material got lost. In another animal from the control group [^{14}C]-2-deoxyglucose utilization analysis of mPFC slices was not possible because of strong tissue disruptions.

2.11 Overall experimental plan

The following section gives an overview of the design of the 3 related projects that I worked on within the framework of the thesis. The experiments conducted per study are summarized in Table 7. Due to limited space, not all of the collected results are discussed in the result and discussion sections.

Table 7: Experimental plan.

Project	Study number	Strain	Prenatal/ postnatal treatment	Preventive treatment (PND 34-47)	Behavioral tasks	Non-behavioral assessments
Project I (Development of a two-hit model of schizophrenia)	Study 1	Wistar	Neonatal PCP	-	Social novelty discrimination task	-
	Study 2a	Lewis, Fischer, Wistar	Prenatal Poly I:C + neonatal PCP	-	-	Microglia, GABA (PND 48/49)
	Study 2b	Lewis, Wistar	Prenatal Poly I:C + neonatal PCP	-	Spontaneous juvenile OF (dim), spontaneous adult OF (dark), operant set-shifting task, PPI	-
	Study 3a	Wistar	Prenatal Poly I:C + neonatal PCP	-	OF with AMPH or PCP (dark), spontaneous adult OF (bright), social novelty discrimination task, autoshaping task	
	Study 3b	Wistar	Prenatal Poly I:C + neonatal PCP	-	Spontaneous adult OF (dark), VIFI task	Microglia (PND 27)
Project II (Evaluation of methods to enhance robustness of developmental models)	Study 4	Wistar	Prenatal Poly I:C + neonatal PCP	-	Social play behavior, spontaneous adult OF (dark + bright), OF with MK-801 (dark), autoshaping task	[14C]-2-deoxiglucose utilization, GABA (adult)
Project III (Preventive treatment during adolescence)	Study 5	Wistar	Prenatal Poly I:C + neonatal PCP	Minocycline or pregnenolone	-	Microglia, GABA (PND 48/49)
	Study 6a	Wistar	Prenatal Poly I:C + neonatal PCP	Pregnenolone	Spontaneous juvenile OF (dark)	Microglia (PND 48/49)
	Study 6b	Wistar	Prenatal Poly I:C + neonatal PCP	Pregnenolone	Spontaneous adult OF (dark)	

Table contains an overview of strains used per study with detailed information on treatment and methods used. *AMPH* amphetamine, *bright* bright light conditions, *dark* dark conditions, *dim* dim light conditions, *GABA* γ-aminobutyric acid, *PCP* phencyclidine, *PND* postnatal day, *Poly I:C* polyinosinic : polycytidylic acid, *PPI* prepulse inhibition, *OF* open field.

2.11.1 Project I: Development of a two-hit model of schizophrenia

Project I aimed at the development of the two-hit model in rats. Within its framework, a series of studies were conducted to justify the selection of developmental insults, rat strains and methods.

One-hit vs. two-hit model:

Study 1 has addressed robustness of the effects produced by a single hit (neonatal PCP treatment) by comparing effect size, pooled standard deviation, power and p-values of 20 studies conducted under identical conditions. As the effects of one-hit treatment were not robust and given that schizophrenia pathophysiology is likely a product of more than one

developmental insult, a second hit was added – maternal immune activation by Poly I:C. This two-hit model was the main focus of the thesis (studies 2-6).

Selection of rat strains:

The genetic background is a factor that could influence the homogeneity of treatment groups and therefore the robustness of a treatment effect. In study 2, the effect of Poly I:C/PCP treatment was characterized in two inbred (Lewis, Fischer) and one outbred (Wistar) rat strains using behavioral and non-behavioral methods. It was speculated that variability of data collected in inbred rat strains would be lower due to the more uniform genetic background. As inbred strains, Lewis and Fischer rats were selected because they have been repeatedly characterized in direct comparisons and were shown to display a number of differences (Chaouloff et al. 1995;Siviy et al. 2003;Stein et al. 2012). Because the strongest effects of Poly I:C/PCP treatment were seen in Wistar rats, all subsequent studies were performed in this strain (studies 3-6).

Selection of methods:

Different behavioral methods were used to study the effects of Poly I:C/PCP. Locomotor activity assessment was included because it is one of the most frequently studied behaviors in the animal models of schizophrenia and, therefore, allowed comparisons between present results and those reported elsewhere.

Cognitive task selection was based on cognitive domains that are thought to be the most affected in schizophrenia. Prepulse inhibition addresses pre-attentive processing, operant set-shifting task provides estimates of executive cognition and social discrimination task was added due to its role in determining social cognition but also because of the extensive experience with the task in the neonatal PCP model. Further, reinforcement learning was studied using a new developed test called VIFI task, and aberrant learning was addressed by means of the pavlovian autoshaping.

In addition to behavioral methods, immunohistochemistry was used to study the effects of Poly I:C/PCP. Due to the immune stimulating effect of Poly I:C and studies showing changes in microglia activation in offspring from Poly I:C treated dams (Van den Eynde et al. 2014;Zhu et al. 2014b), microglia density was assessed using Iba1 staining. Further, administration of NMDA receptor antagonists to rats shortly after birth was shown to be toxic to GABAergic interneurons (Roux et al. 2015). Therefore, long-term effects on GABAergic signaling were assessed by determining GAD67 expression.

Individual study designs:

– *Study 1:*

I have used access to a database containing a larger number of the neonatal PCP (single hit) studies conducted in rats between 2006 and 2009. In those studies, the social discrimination was used as the primary readout and, based on validation studies, was expected to be a robust and reliable measure. Each study included independent groups of rats treated neonatally with either PCP or saline. During adulthood, rats received acute injections of various test compounds or their vehicles prior to social discrimination tests. For the purposes of the present project, data from vehicle-treated animals from 20 of these studies were taken and compared with regard to effect size, pooled standard deviation, power and P-value (see also Table 7).

– *Study 2:*

Goal of this study was to compare effects of Poly I:C/PCP treatment in Lewis, Fischer and Wistar rats to identify the strain with the smallest data variance. Differences between Poly I:C/PCP treated and control rats were compared in terms of adolescent immunohistochemistry (study 2a) as well as adolescent and adult behavior (study 2b) in all 3 strains.

– *Study 2a:*

As shown in figure 17 (see also Table 7), adolescent rats were perfused at PND 48/49. Microglia density in the hippocampus was assessed using Iba1 staining while GABA levels were determined via optical density of GAD67 in the hippocampus and prefrontal cortex.
I used offspring from two Poly I:C and two saline treated Lewis dams with a total number of n=9 (two-hit) and n=12 (control), offspring from three Poly I:C and two saline treated Fischer dams with a total number of n=8 (two-hit) and n=5 (control), and offspring from eight Poly I:C and seven saline treated Wistar dams with a total number of n=11 (two-hit) and n=11 (control). These Wistar rats are the same as the vehicle treated rats of study 5. Brain preparation and analysis were done in collaboration with Sonja Abele and Ayla Rodriguez Ehrenfried.

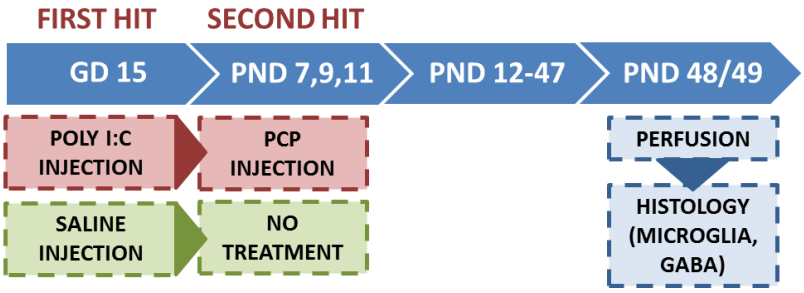

Figure 17: Design of study 2a. Effect of Poly I:C/PCP treatment (red) compared to controls (green) on microglia density and GABA level in Lewis and Fischer rats was assessed in adolescence (PND 48/49). *GABA* γ-aminobutyric acid, *GD* gestation day, *PND* postnatal day.

– *Study 2b:*

This study was conducted in Lewis and Wistar rats only. The rats' spontaneous locomotor activity was assessed in adolescence and adulthood, followed by the determination of their cognitive performance using the operant set-shifting task and prepulse inhibition test (Figure 18; see also Table 7). I used offspring from six Poly I:C and six saline treated Lewis dams with a total number of n=13 (two-hit) and n=16 (control), and offspring from eight Poly I:C and five saline treated Wistar dams with a total number of n=17 (two-hit) and n=19 (control).

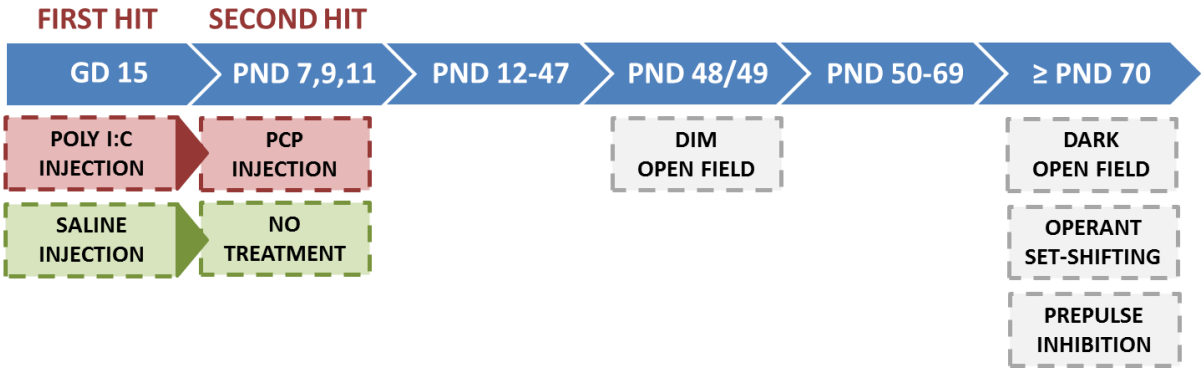

Fig. 18: Design of study 2b. Effect of Poly I:C/PCP treatment (red) compared to controls (green) on spontaneous locomotor activity was assessed at PND 48/49 and PND ≥ 70. Further, animals were tested in the operant set-shifting task and prepulse inhibition. *Dark open field* open field under dark conditions, *dim open field* open field under dim light conditions, *GD* gestation day, *PND* postnatal day.

– *Study 3:*

This study aimed to further characterize the effects of Poly I:C/PCP on behavior in Wistar rats. Two independent batches of animals were used, splitting the study into two parts.

– *Study 3a:*

The study design can be found in figure 19 (see also Table 7). I used offspring from three Poly I:C and one saline treated Wistar dams with a total number of n=11 (two-hit) and n=6

(control). Adult animals underwent testing of spontaneous locomotor activity (under bright light conditions), locomotor activity upon psychostimulant challenge with amphetamine or phencyclidine, social discrimination and autoshaping. Experiments were done in collaboration with Regina Eichhoff-Diefenbach.

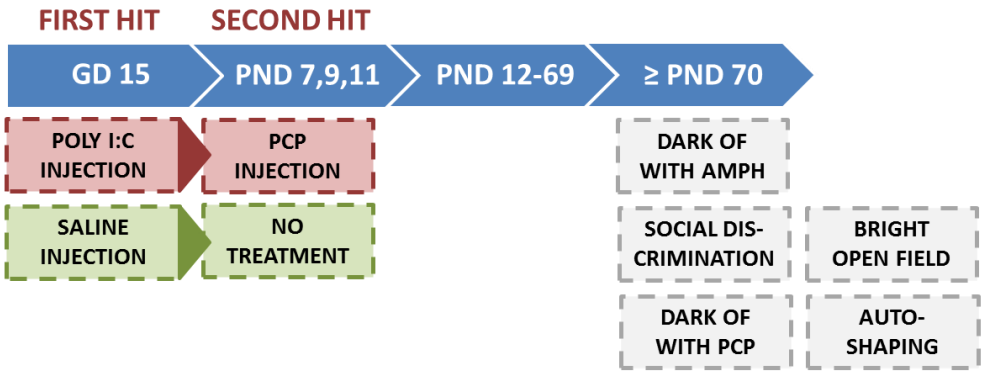

Figure 19: Design of study 3a. Effect of Poly I:C/PCP treatment (red) compared to controls (green) on behavior was assessed at PND ≥ 70, including spontaneous locomotor activity under bright light conditions, locomotor activity upon psychostimulant challenge with amphetamine or phencyclidine, social discrimination and autoshaping. *AMPH* amphetamine, *bright open field* open field under bright light conditions, *GD* gestation day, *dark OF* open field under dark conditions, *PCP* phencyclidine, *PND* postnatal day.

– *Study 3b:*

The study design can be found in Figure 20 (see also Table 7). I used offspring from seven Poly I:C and three saline treated Wistar dams with a total number of n=21 (two-hit) and n=15 (control). Groups were split into two parts. The first half contained 3 animals from the control and 9 from the two-hit group. They were perfused on PND 27 (Figure 20, upper panel) and microglia density was assessed via Iba1 staining. The other half contained 12 control and 12 Poly I:C/PCP treated animals. When they reached adulthood, the rats' spontaneous locomotor activity was assessed and afterwards, they were trained in the VIFI task (Figure 20, lower panel).

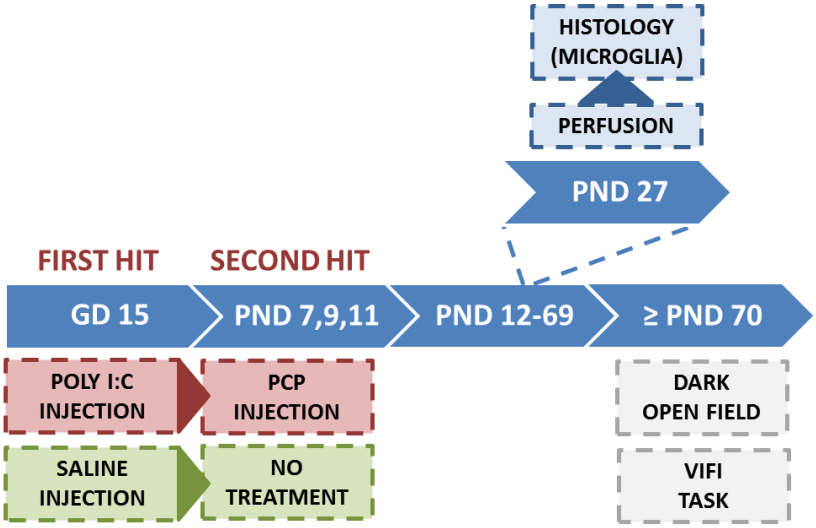

Figure 20: Design of study 3b. Effect of Poly I:C/PCP treatment (red) compared to controls (green) on microglia density as well as spontaneous locomotor activity and cognitive flexibility (VIFI task) was assessed at PND 27 and PND ≥ 70, respectively. *Dark open field* open field under dark conditions, *GD* gestation day, *PND* postnatal day, *VIFI task* variable-interval fixed-interval task.

2.11.2 Project II: Evaluation of methods to enhance robustness of developmental models

Long delays separating challenge (such as Poly I:C or PCP) from the ultimate tests are among the main factors that contribute to higher variability in the expression of challenge-induced disturbances that make developmental models less robust compared with acute models. One strategy to cope with this issue is based on introducing intermediate time points at which the test organisms are assessed with the aim to bridge the time between pre-/postnatal challenges and tests in adulthood. It was hypothesized that juvenile behavior may serve as a predictor for adult abnormalities. This hypothesis was tested within the framework of the project II using correlation and cluster analyses.

In study 4 (Figure 21; see also Table 7), juvenile social play behavior was assessed (PND 35) followed by behavioral measures in adulthood including spontaneous locomotor activity under dark and bright light conditions, locomotor activity upon psychostimulant challenge of MK-801 and autoshaping. Afterwards, brain metabolism was studied by assessing glucose uptake via [^{14}C]-2-deoxyglucose autoradiography in the hippocampus and prefrontal cortex. Additionally, the GABA level was determined via optical density of GAD67 within the hippocampus.

I used offspring from seven Poly I:C and five saline treated Wistar dams with a total number of n=41 (two-hit) and n=31 (control). The autoshaping experiment was done in collaboration with Regina Eichhoff-Diefenbach.

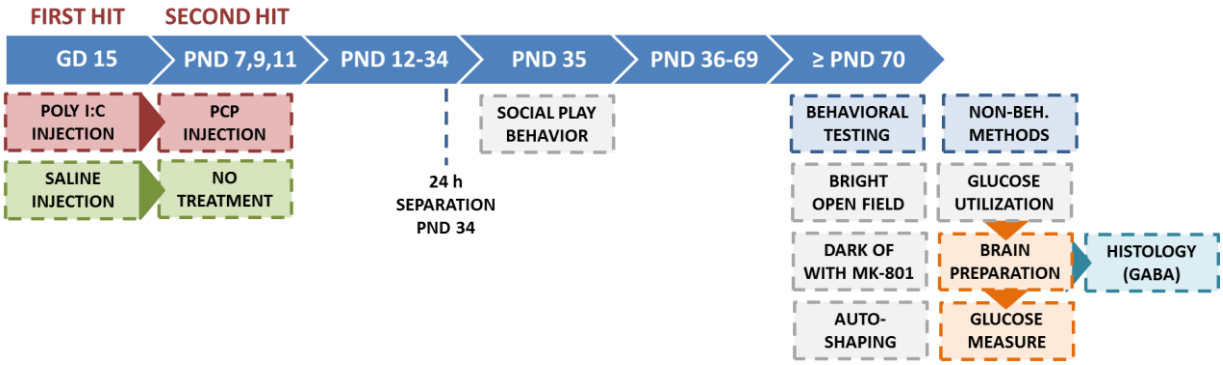

Figure 21: Design of study 4. Effect of Poly I:C/PCP treatment (red) compared to controls (green) on social play behavior was assessed first (PND 35) followed by behavioral, metabolic and immunohistochemical analysis at PND ≥ 70. Behavioral measurements used were spontaneous locomotor activity under dark and bright light conditions, locomotor activity upon psychostimulant challenge of MK-801 and autoshaping. For metabolic analysis glucose levels within hippocampus and prefrontal cortex were measured, followed by GABA level determination in the hippocampus. *Bright open field* open field under bright light conditions, *dark OF* open field under dark conditions, *GABA* γ-aminobutyric acid, *GD* gestation day, *non-beh. Methods* non-behavioral methods, *PND* postnatal day.

Data analysis:

Results generated in juvenile and adult rats were subjected to correlation and cluster analyses.

– *Correlation analysis:*

The strength of bivariate correlations is reported as Spearman's rank correlation coefficient.

– *Cluster analysis:*

A set of % total defense data from social play behavior experiments (see section 2.5.1) was subjected to cluster analysis whereby individual observations were clustered using an objective algorithm (Euclidean distance). Analysis of the two-hit group enabled segregation of two subgroups, two-hit-LOW (low play engagement) and two-hit-HIGH (normal / high play engagement) (Figure 46). I analyzed all data with treated animals divided into these two groups and included the control group, having three groups in total. Statistical analysis of the data was done as described in section "2.9 Methods of statistical analysis".

2.11.3 Project III: Preventive treatment during adolescence

This project aimed to test, if an anti-inflammatory (minocycline) or neuroprotective (pregnenolone) treatment during adolescence could prevent the neurohistochemical and behavioral alterations induced by Poly I:C/PCP treatment. To do so, an exploratory study (study 5) was conducted, assessing the effect of Poly I:C/PCP on microglia and GABA

signaling in adolescence and evaluating the effect of minocycline and pregnenolone on those measures. This was followed by a confirmatory study (study 6), which aimed to reproduce the effect of pregnenolone on microglia density.

Study 5:

I used offspring from eight Poly I:C and seven saline treated Wistar dams with a total number of n=32 (two-hit) and n=31 (control). Animals of both groups were treated with minocycline, pregnenolone or vehicle during adolescence (PND 34-47), as displayed in figure 22 (see also Table 7). To do so, they were randomly assigned to 6 groups: controls treated with vehicle (n=11), minocycline (n=10) or pregnenolone (n=10) and animals of the two-hit group treated with vehicle (n=11), minocycline (n=10) or pregnenolone (n=11). Rats were perfused on PND 48/49, followed by immunohistochemical assessments. Various microglia analyses were conducted, including microglia density via Iba1 staining and activation state via CD11b and CD68 staining in the hippocampus. GABA levels in the hippocampus and prefrontal cortex were analyzed via optical density of GAD67. Experiments were done in collaboration with Sonja Abele and Ayla Rodrigues Ehrenfried.

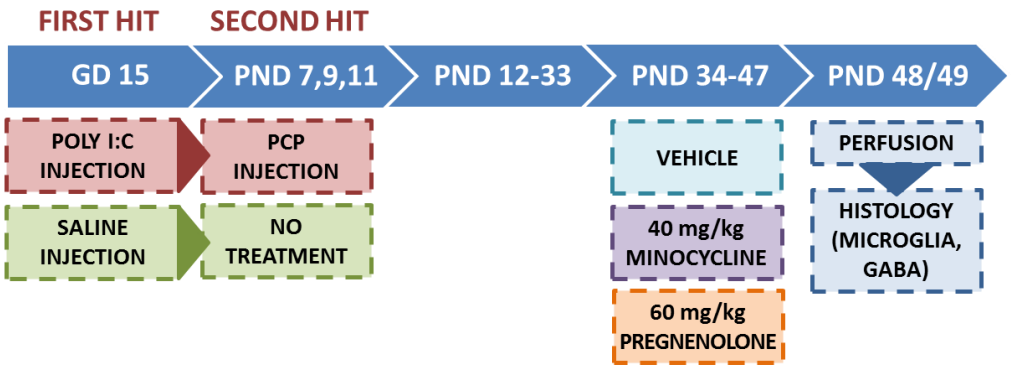

Figure 22: Design of study 5. Poly I:C/PCP treated (red) and control (green) rats were treated with vehicle, minocycline or pregnenolone during adolescence (PND 34-47) and perfused on PND 48/49 followed by immunohistochemical assessments, including microglia and GABA analyses. *GABA* γ-aminobutyric acid, *GD* gestation day, *PND* postnatal day.

Study 6:

I used offspring from twenty-three Poly I:C and seven saline treated Wistar dams with a total number of n=52 (two-hit) and n=38 (control). Animals of both groups were treated with pregnenolone or vehicle during adolescence (PND 34-47; Figure 23 & Figure 24; see also Table 7). They were assigned to 4 groups: control group rats were treated with vehicle (n=24)

or pregnenolone (n=14) and animals of the two-hit group were treated with vehicle (n=26) or pregnenolone (n=26).

– *Study 6a:*

During the period of pregnenolone/vehicle treatment (PND 34-47), animals underwent locomotion test at PND 46. Behavioral testing took place before the daily treatment was applied. At PND 48/49 they were perfused. To replicate study 5, microglia density was determined using Iba1 staining (Figure 23). Histology was done in collaboration with Ayla Rodrigues Ehrenfried.

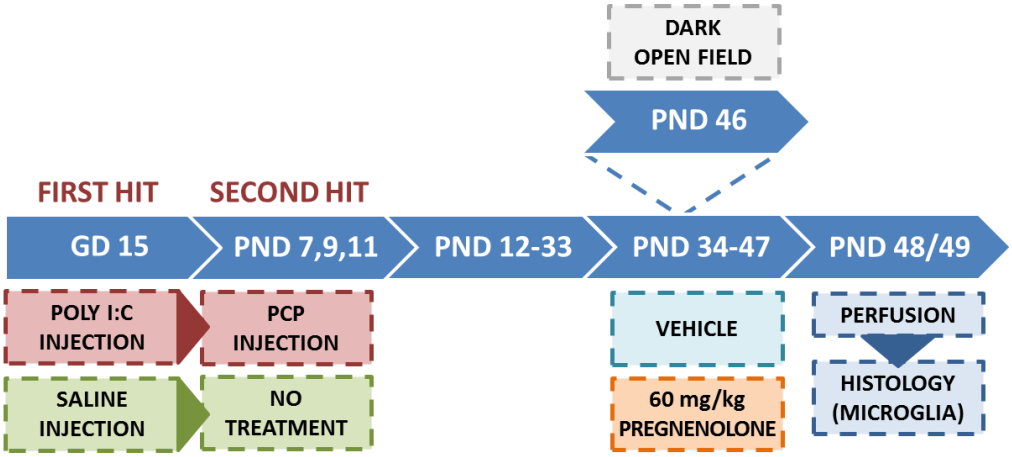

Figure 23: Design of study 6b. Poly I:C/PCP treated (red) and control (green) rats were treated with vehicle or pregnenolone during adolescence (PND 34-47). Animals underwent testing of spontaneous locomotor activity at PND 46, followed by perfusion on PND 48 and the analysis of microglia density. *Dark open field* open field under dark conditions, *GD* gestation day, *PND* postnatal day.

– *Study 6b:*

Animals were treated with pregnenolone or vehicle (PND 34-47) and subjected to tests of spontaneous locomotion during adulthood (Figure 24). Goal of this study was to test whether pregnenolone treatment during adolescence prevent development of behavioral abnormalities expressed in adulthood.

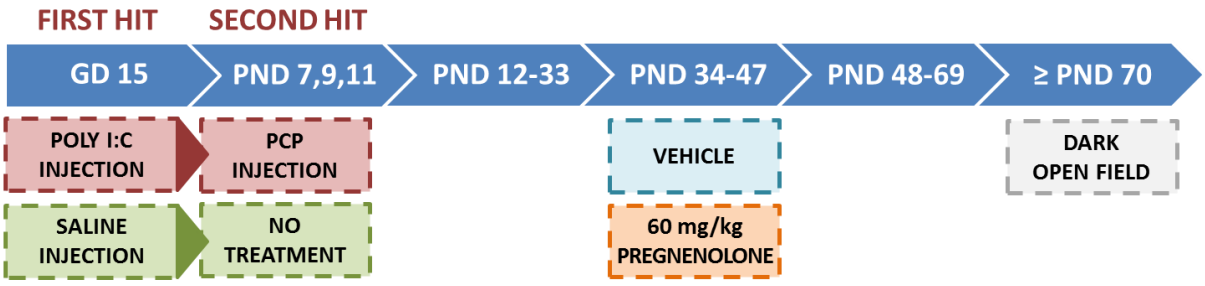

Figure 24: Design of study 6c. Poly I:C/PCP treated (red) and control (green) rats were treated with vehicle or pregnenolone during adolescence (PND 34-47). Animals were tested on spontaneous locomotor activity at PND ≥ 70. *Dark open field* open field under dark conditions, *GD* gestation day, *PND* postnatal day.

3 Results

3.1 Project I: Development of a two-hit model of schizophrenia

3.1.1 Robustness of the neonatal phencyclidine model (one-hit model)

To evaluate the robustness of the effects produced by neonatal PCP treatment, I used the data from 20 studies comparing the social discrimination in rats treated neonatally with PCP and saline. To illustrate the group differences between studies, effect size, pooled standard deviation, statistical power and the P-value were calculated for each study (Figure 26).

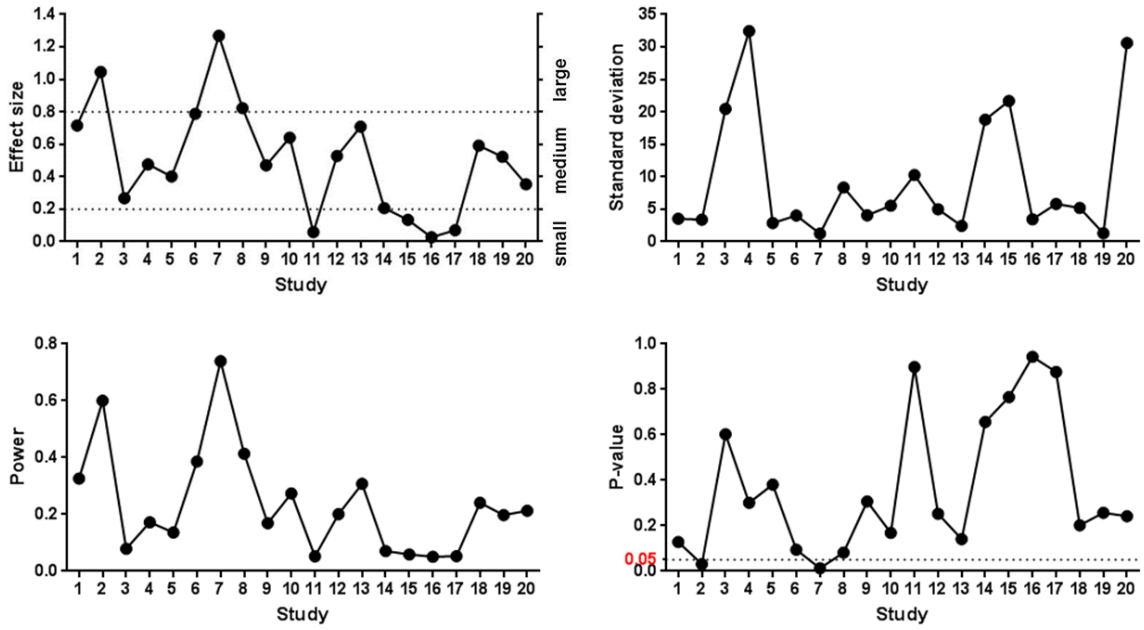

Figure 25: Comparison between 20 neonatal PCP studies. Effect size (*upper row, left panel*), standard deviation (*upper row, right panel*), power (*lower row, left panel*) and P-value (*lower row, right panel*) are plotted for each of the 20 studies. See text for details.

The effect size is conventionally classified into small (<0.2), medium (0.2-0.8), or large (>0.8) (Cohen 1988). A large effect size was observed only in 3 of the 20 studies. In four studies, the effect size was small, whereas for the remaining 13 the effect size was in the medium range. Summarizing these results, one can say that the effect size was highly variable across studies (Figure 25, upper row, left panel). Similar observations were made for statistical power, a measurement dependent on the effect size (Figure 25, lower row, left panel), as well as standard deviation and P-value (Figure 25, right panels), all found to be highly varying between studies.

Therefore, in order to increase the robustness of the model, a second "hit" factor, prenatal treatment with polyinosinic : polycytidylic acid (Poly I:C), was added. This two-hit model,

prenatal Poly I:C treatment combined with neonatal PCP treatment, is characterized in the following sections.

3.1.2 Selection of a rat strain

Two studies were performed in Lewis, Fischer and Wistar rats to identify the strain with the best response to Poly I:C/PCP treatment. The delivery rate was very low in both Lewis and Fischer rats. From 39 pregnant Lewis rats treated with Poly I:C or saline, only 19 delivered that corresponds to a delivery rate of 48.7 % (Figure 26, 1st bar). Similarly, only 5 out of 10 Fischer rats delivered (50 %) (Figure 26, 2nd bar). In contrast, the delivery rate in Wistar rats was 89.7 % (87 out of 97 rats delivered; females from study 2-6) (Figure 26, 3rd bar). These results led to the decision to use Wistar rats in subsequent studies and not to take either of the two strains, Lewis or Fischer, with low delivery rate due to animal welfare reasons.

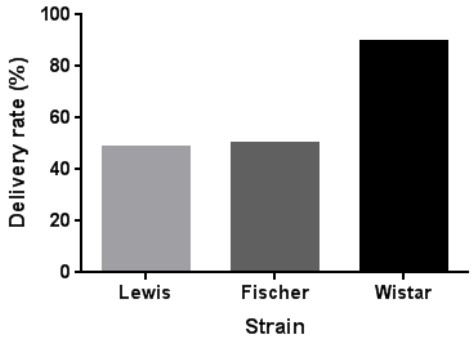

Figure 26: Delivery rate in three rat strains. Data are presented as delivery rate (in %) in Lewis (*1st bar*), Fischer (*2nd bar*) and Wistar (*3rd bar*) females.

In these studies, effects of Poly I:C/PCP treatment in Lewis and Fischer rats were further assessed using different behavioral (open field testing in juvenile and adult rats, operant set-shifting and PPI) and non-behavioral (immunohistochemical analysis of microglia density via Iba1 staining and GABAergic synthesis via GAD67 expression) methods. As the Lewis and Fischer rats were not used in other studies, the results of tests using those strains are not presented in details and are briefly summarized in Table 8.

Table 8: Behavioral and non-behavioral testing performed in Lewis and Fischer rats.

	Iba1	GAD67		Open field adolescence		Open field adulthood		Operant set-shift		PPI
		mPFC	HPC	Arena	Center	Arena	Center	SHORT	LONG	
Lewis	-	-	-	+	-	+	+	-	-	-
Fischer	-	-	-	ND	ND	ND	ND	ND	ND	ND

Plus indicates statistically significant differences ($P<0.05$) between animals of the two-hit and control groups. ND indicates that tests did not take place. Brain material was collected at PND 48/49 for immunohistochemical analysis. Microglia density was assessed via covered area of Iba1 staining and GABA synthesis was determined via optical density of GAD67 staining. Behavioral tests conducted were open field, operant set-shifting and PPI. Open field tests took place in adolescence (PND 48/49) or in adulthood, and the primary analyzed parameters were total distance in the arena (*Arena*) and in the center region (*Center*). The operant set-shifting task was conducted in adulthood using first the *SHORT* and afterwards the *LONG* protocol. Measured parameters were numbers of trials to criterion and errors during the visual-cue and response discrimination sessions. Prepulse inhibition (*PPI*) testing included the assessment of the startle amplitude and the PPI. *mPFC* medial prefrontal cortex, *HPC* hippocampus, *PND* postnatal day.

3.1.3 Selection of methods to characterize the two-hit model

The studies presented here aimed to identify behavioral measures that would be suitable to study the effects of Poly I:C/PCP treatment. In this section, the development of two cognitive tasks is presented first ("Method development") followed by the analysis of effects of Poly I:C/PCP treatment in various behavioral tests ("Effect of Poly I:C/PCP treatment on cognition" & "Effect of Poly I:C/PCP treatment on locomotor activity"). For individual study designs, please refer to the methods' section ("2.11 Overall experimental plan").

Method development

– *Variable-interval fixed-interval (VIFI) task*

Training under VI30 schedule of reinforcement produced a characteristic response patterning with steady lever pressing during the intervals, fairly high response rates (see Figure 29, right panels, VI30-PRE, filled circles; on average 0.8-0.9 responses per second) and short post-reinforcement pauses (PRPs) that averaged 6-7 seconds under baseline conditions.

Introduction of the FI30 schedule during the weekly test sessions was expected to result in longer PRPs compared with those under the training VI30 conditions (see Figure 27 for a comparison of cumulative recordings made for one rat under VI30 and FI30 conditions).

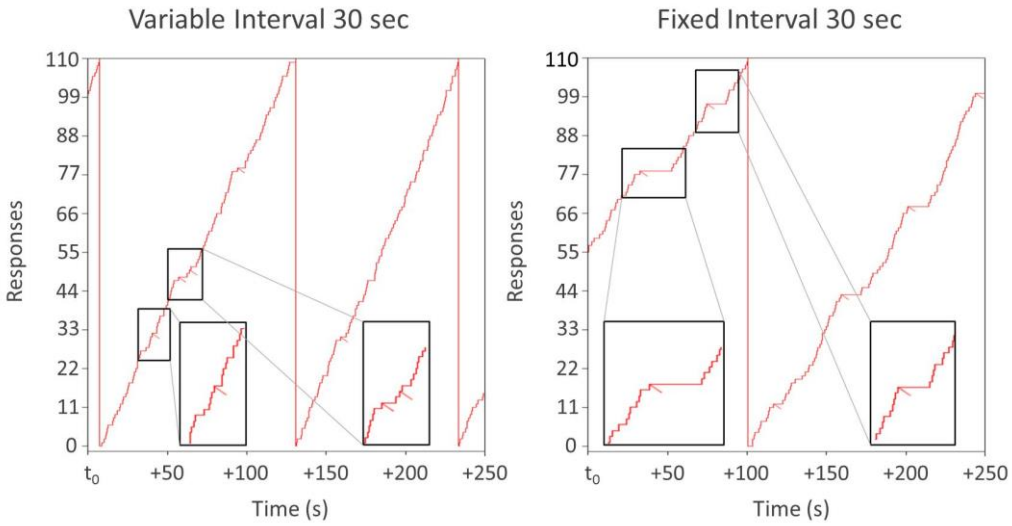

Figure 27: Examples of cumulative recordings from one animal performing under VI30 (*left panel*) and FI30 (*right panel*) schedules of reinforcement. X axis – elapsed time (in seconds). Y axis – recorded responses (each lever press is reflected by the recording pen moving one step up). Switch from VI30 to FI30 schedule resulted in longer pauses after reinforce deliveries (indicated by diagonal pen marks).

Indeed, already during the first FI30 test session, there was a gradual increase in the PRP duration (Figure 28, left panel, FI30-TEST). Two-way ANOVA revealed a significant main effect for bins ($F(5, 110)=7.15$, $p<0.0001$) and interaction of bins × session type ($F(5, 110)=7.86$, $p<0.0001$), as well as no difference for session type ($F(1, 22)=0.34$, $P=0.57$). The schedule change induced adjustment of PRP in all rats (Figure 28, right panel; Wilcoxon's rank test: $W=78$, $p<0.001$). One day after the FI30 test session, animals were switched back to VI30 training conditions and, within one session, the PRP values were back to pre-test levels and remained stable during the session (data not shown).

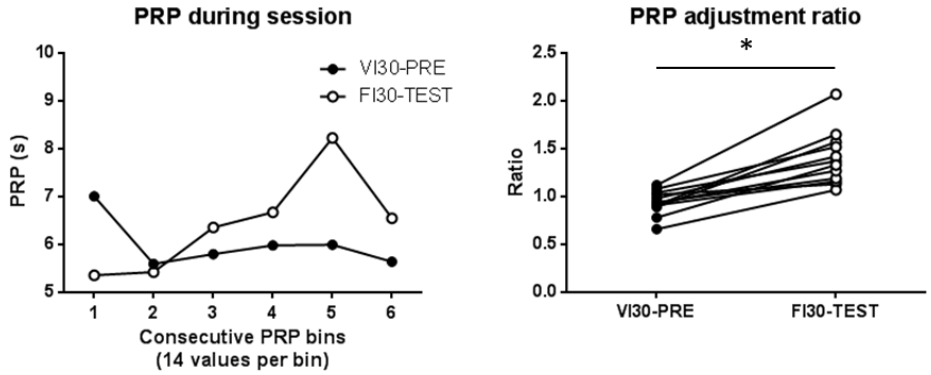

Figure 28: Changes in the post-reinforcement pause (*PRP*) duration during the first FI30 session. Animals (*n*=12) were trained to lever-press under VI30 schedule of reinforcement (*VI30-PRE*, filled circles) and then the reinforcement schedule was switched to FI30 (*FI30-TEST*, open circles) for the first time. VI30 and FI30 sessions were conducted on two consecutive days (Wednesday and Thursday). *Left panel:* Average PRP duration (in seconds) during VI30 and FI30 sessions. As there were a total of 84 pellet deliveries received during the session, PRPs were averaged across 6 consecutive bins of 14 values each. *N*=12. *Right panel:* PRP adjustment ratio values of individual rats under VI30 and FI30 conditions. **P*<0.05 (Wilcoxon's rank test), comparison between VI30 and FI30 test conditions.

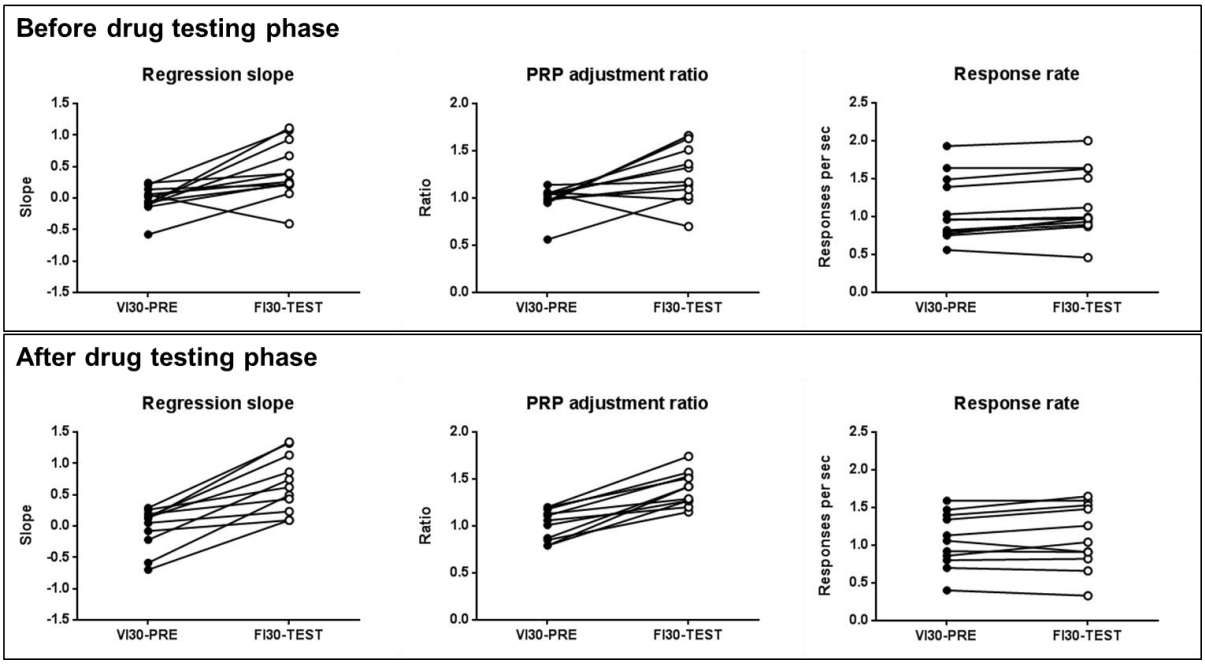

Figure 29: Changes in the post-reinforcement pause (*PRP*) duration and response rate during the VI30 and FI30 sessions at the beginning of the study and at the end. For both time points, before the drug testing phase started (*upper row panels*) and after the drug testing phase (*lower row panels*), VI30 (filled circles) and FI30 (open circles) sessions were conducted on two consecutive days. Regression slope (*left panels*), PRP adjustment ratio (*middle panels*) and response rate (*right panels*; responses per second) are presented for individual rats (n=12).

The present study continued for a period of 9 months. Throughout the study, performance under training VI30 conditions remained fairly stable – for the response rate, PRP adjustment ratio and regression slope (Figure 29, VI30-PRE, filled circles). Switching from the VI30 schedule training conditions to FI30 schedule occurred repeatedly for each rat and, when assessed under drug-free conditions, effects of introducing the FI30 schedule were essentially similar before and after the drug treatment phase (Figure 29, FI30-TEST, open circles). These results were possible to reproduce in another group of animals in house (data not shown) and in another laboratory (personal communication).

The original intention was to develop a task that could be used to characterize a variety of pathological conditions including those induced by drug treatments. Many of such treatments can be expected to have general effects on operant behavior that could complicate analysis of the changes associated with the switch from one reinforcement schedule to another. Therefore, initial validation of the task included assessment of rats' behavior after administration of a prototypic sedative-hypnotic drug, diazepam, as well as a psychostimulant, amphetamine. Drugs were given prior to either FI30 test sessions (Figure 30) or VI30 training conditions (Figure 31).

Pretreatment with vehicle (intraperitoneal or subcutaneous) had no appreciable effects. Under FI30 conditions, most of the vehicle-treated animals demonstrated increases in the PRP duration during the test session as reflected by both PRP adjustment ratio values greater than 1 (Figure 30B,F, data points above '0') and regression slope values greater than 0 (Figure 30A,E). These changes were similar to what was described above for control tests (Figure 29). As expected, no such adjustments were seen in vehicle-treated rats during VI30 sessions (Figure 31A,B,E,F).

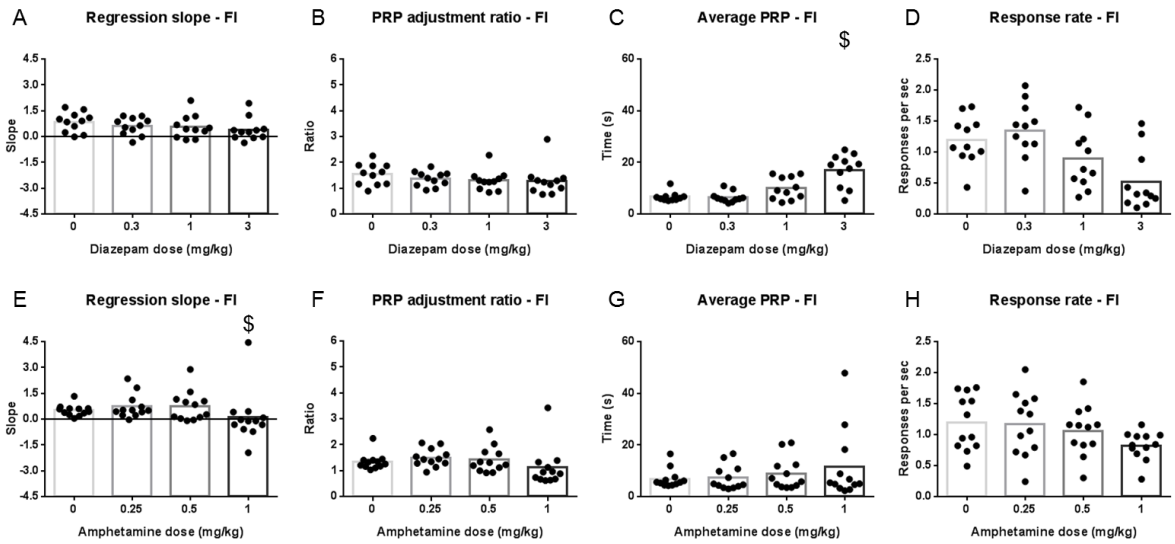

Figure 30: Effects of diazepam and amphetamine on rats' performance during the FI30 test sessions. Data are presented as mean regression slope (A, E), post-reinforcement pause (PRP) adjustment ratio (B,F), average PRP (C, G) and response rate (D, H). Black dots per column indicate individual rat values (*n*=11-12). Symbol (*$*) indicates *P*<0.05 (Dunn's test), compared with corresponding control group treated with vehicle. *FI* fixed interval.

For FI30 sessions, treatment with diazepam had no significant impact on either regression slope (Figure 30A; Friedman's ANOVA: v=4.5, P=0.2) and PRP adjustment ratio (Figure 30B; Friedman's ANOVA: v=4.2, P=0.2). Similarly, diazepam had no effects during the VI30 sessions on either regression slopes (Figure 31A; Friedman's ANOVA: v=4.3, P=0.2) or PRP adjustment ratio (Figure 31B; Friedman's ANOVA: v=3.1, P=0.4). Under both FI30 and VI30 conditions, higher doses of diazepam reduced the overall response rates (Friedman's ANOVA; Figure 30C, FI30; v=11.9, P=0.008; Figure 31C, VI30; v=17.1, P=0.0007). Further, analysis of the VI30 session performance indicated that the average PRP during these sessions seemed to increase as a function of diazepam dose (Figure 31C; Friedman's ANOVA: v=11.5, P=0.009). Similar effects could be seen for FI30 sessions (Figure 30C; Friedman's ANOVA: v=10.3, P=0.02).

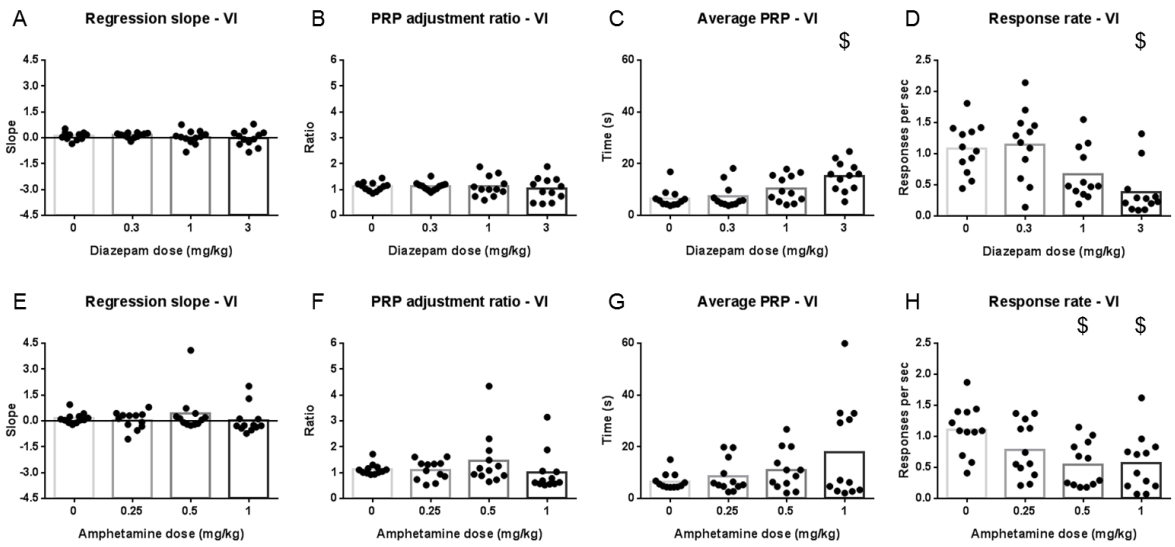

Figure 31: Effects of diazepam and amphetamine on rats' performance during the VI30 test sessions. Data are presented as mean regression slope (A, E), post-reinforcement pause (*PRP*) adjustment ratio (B,F), average PRP (C, G) and response rate (D, H). Black dots per column indicate individual rat values (n=12). Symbol ($) indicates P<0.05 (Dunn's test), compared with corresponding control group treated with vehicle. *VI* variable interval.

Similar to diazepam, higher doses of amphetamine reduced the overall response rates. These effects were statistically significant for the VI30 (Figure 31H; Friedman's ANOVA: v=18.4, P=0.0004) but not for the FI conditions (Figure 30H; Friedman's ANOVA: v=6.1, P=0.1). Treatment with the highest dose of amphetamine (1 mg/kg) resulted in significant decreases in the regression slope (Figure 30E; Friedman's ANOVA: v=13.5, P=0.004) as well as the PRP adjustment ratio during the FI30 test sessions (Figure 30F; Friedman's ANOVA: v=12.4, P=0.006). Interestingly, during the VI30 sessions, these effects of amphetamine were less pronounced and did not reach the level of statistical significance (Friedman's ANOVA; Figure 31E; regression slope, v=7.1, P=0.07; Figure 31F; PRP adjustment ratio, v=7.3, P=0.06). Nevertheless, these results highlight the importance of comparing effects of treatment under training (VI30) and test (FI30) conditions. Such comparisons may especially be important for pharmacological treatment where effects may develop over the time and may differentially apply at the beginning vs at the end of the session.

When given at lower doses (0.25 and 0.5 mg/kg), effects of amphetamine appeared to be milder and rather in the opposite direction. After 0.25 mg/kg treatment, 10 out of 12 animals showed longer PRP duration under FI30 schedule (i.e. higher PRP adjustment ratios compared to corresponding vehicle treatment conditions). However, pairwise group comparisons (Dunn's test) did not confirm statistically significant effects of 0.25 mg/kg of amphetamine on either regression slope or PRP adjustment ratio.

– *Operant set-shifting task*

One group of animals was used to establish the protocol originally described by Floresco et al. (2008). For that, rats were trained to perform a visual-cue discrimination (VCD) task (position of the illuminated cue light indicates the "correct" lever) on one day, followed by response discrimination (RD) testing (one of both levers was defined to be the "correct" lever, independent of the position of the cue light) on the next day. Animals needed on average 67 trials and 18 errors to achieve the criterion of 10 consecutive, correct trials during the VCD session (Figure 32A). During the RD session, animals achieved the criterion after an average of 92 trials and made approximately 30 errors (Figure 32B) that could be split into perseverative, regressive and never-reinforced errors (Figure 32C).

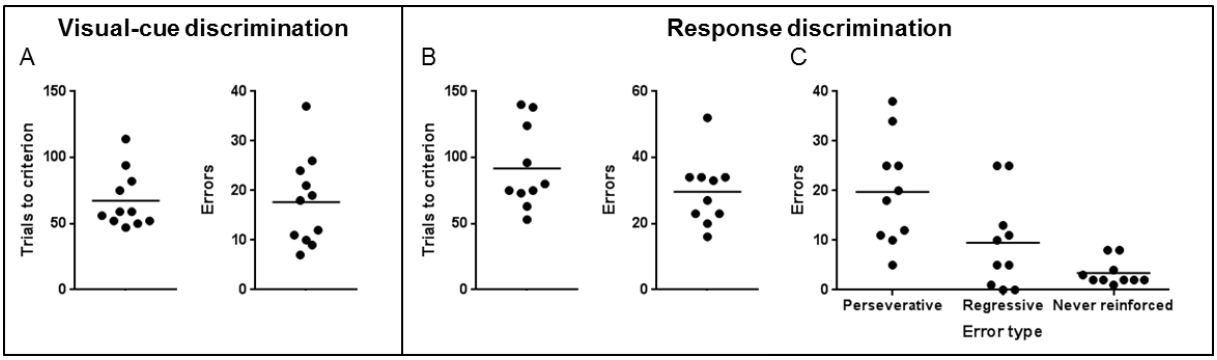

Figure 32: Set-shifting performance with the SHORT test protocol. *Left panel:* Each dot represents an individual data point and lines present the mean numbers of trials to criterion (A, *left*) and errors (A, *right*) during the visual-cue discrimination session (*n*=11). *Right panel:* Each dot represents an individual data point and lines present the mean numbers of trials to criterion (B, *left*), total errors (B, *right*) and errors split into different types (C; perseverative, regressive and never-reinforced) during the response discrimination session (*n*=10).

The originally developed two-day set-shifting protocol is commonly used to assess the effects of acute treatments on executive functioning (Dalton et al. 2011;Enomoto et al. 2011;Floresco et al. 2008). Treatment with Poly I:C/PCP was expected to produce long-lasting changes in the behavior. Therefore, the task was adjusted using a longer training for VCD (five days) and RD (five days) to increase the probability to detect differences between animals of the two-hit and control groups. This longer training protocol was developed using two groups of animals: one group was the same as that tested already under the short protocol (RE-LONG testing) and a second experimentally naïve group (LONG testing).

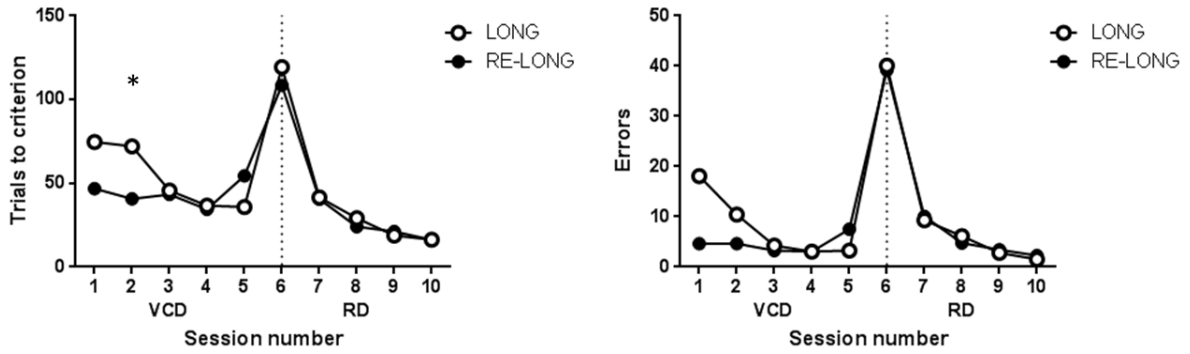

Figure 33: Set-shift performance with the LONG/RE-LONG test protocol. Data are presented as mean numbers of trials to criterion (*left panel*) and errors (*right panel*) per session of rats which underwent the RE-LONG (n=12; filled circles) or the LONG (n=12; open circles) testing. *P<0.05 (Bonferroni's test), comparison between LONG and RE-LONG test conditions. *VCD* visual-cue discrimination; *RD* response discrimination.

During sessions 1-2, animals that were tested for the first time (Figure 33, LONG, open circles) were found to have increased numbers of trials to criterion and errors compared to the re-tested animals (Figure 33, RE-LONG, filled circles). Although when regarding sessions 3-10, rats of both groups made comparable numbers of trials to criterion and errors for the VCD and RD. Importantly, numbers of trials to criterion and total errors during the first RD session (session 6, dotted line) were comparable between both groups. Two-way ANOVA for trials to criterion revealed a main effect of session ($F(9, 198)=30.63$, $P<0.0001$) as well as group × session interaction ($F(9, 198)=2.68$, $P=0.006$), but no main effect of group ($F(1, 22)=2.94$, $P=0.1$). *Post hoc* analysis showed significant differences between groups during session 2 only.

Effects of Poly I:C/PCP treatment on cognition:

– *Operant set-shifting task*

Rats were trained with the SHORT protocol first and afterwards underwent re-testing with the LONG protocol. Using the SHORT protocol (Figure 34), no differences between Poly I:C/PCP treated and control animals were observed in the numbers of trials to criterion (1st panel; Mann-Whitney's $U=112.0$, $P=0.79$) and errors (2nd panel; Mann-Whitney's $U=108.0$, $P=0.67$) for the VCD session. Further, no differences between groups were assessed in the numbers of trials to criterion (3rd panel; Mann-Whitney's $U=110.5$, $P=0.75$) and errors (4th panel; Mann-Whitney's $U=90.0$, $P=0.52$) for the RD session.

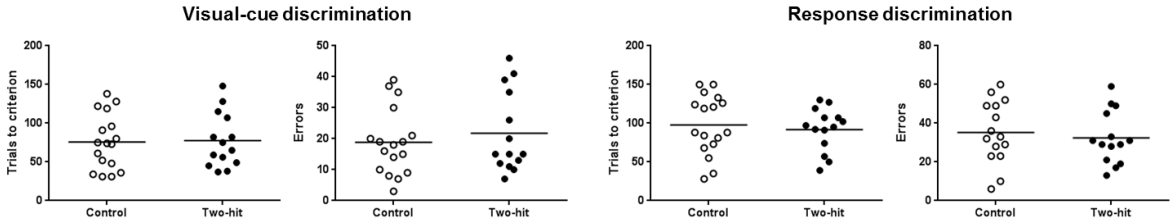

Figure 34: Effects of Poly I:C/PCP treatment on the set-shifting performance (SHORT protocol). Each dot represents an individual data point and lines present the mean numbers of trials to criterion (*1st & 3rd panel*) and errors (*2nd & 4th panel*) of rats of the two-hit (*n*=14; filled circles) or control (*n*=15-17; open circles) groups during the visual-cue discrimination (*1st & 2nd panel*) and response discrimination (*3rd & 4th panel*) session. Data from study 2b (see experimental design in the methods' section).

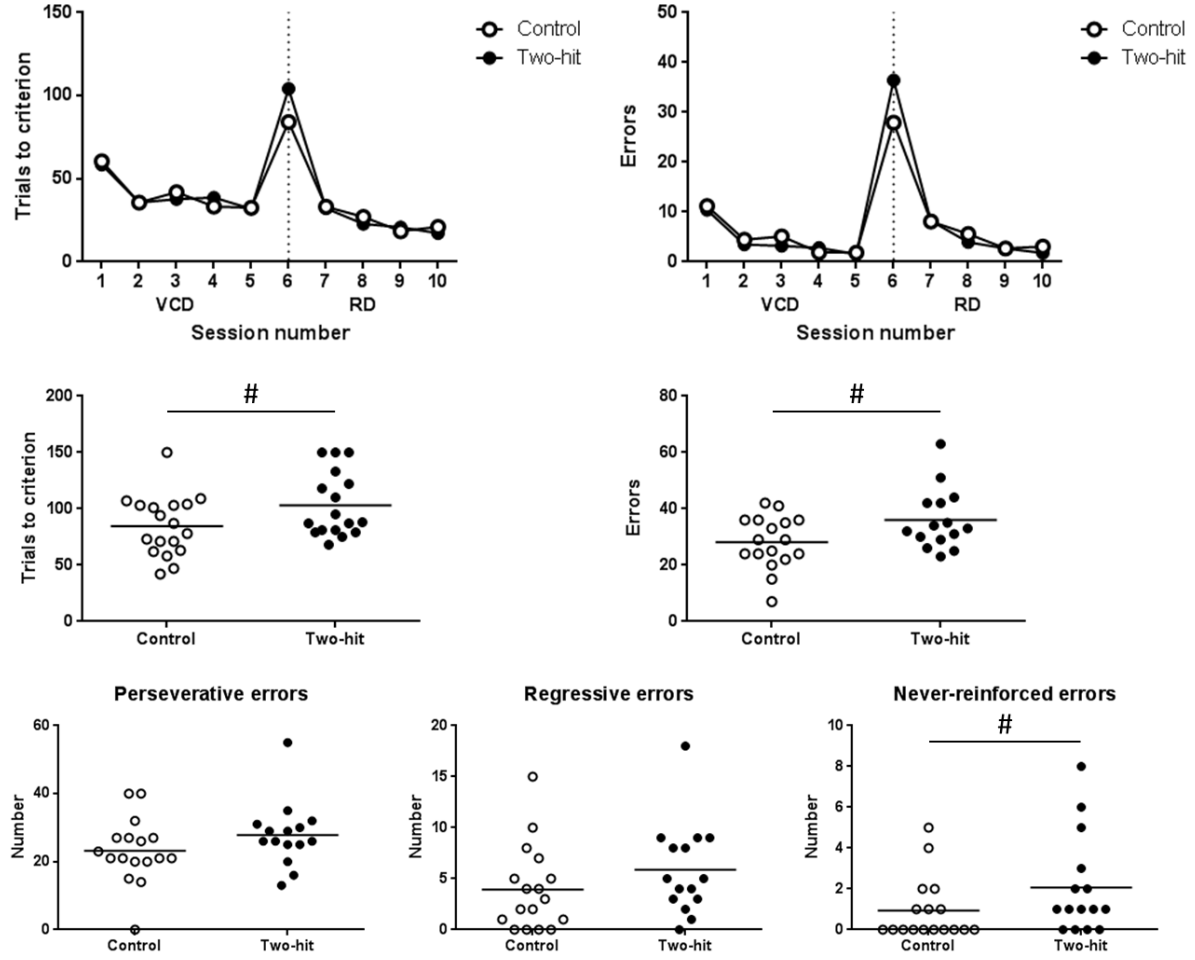

Figure 35: Effects of Poly I:C/PCP treatment on the set-shifting performance (LONG protocol). *Upper row panels:* Data are presented as mean numbers of trials to criterion (*left panel*) and errors (*right panel*) per session of rats of the two-hit (*n*=17; filled circles) or control (*n*=18; open circles) groups. *Middle row panels:* Each dot represents an individual data point and lines present the mean numbers of trials to criterion (*left panel*) and errors (*right panel*) during the first RD session of rats of the two-hit (*n*=17; filled circles) or control (*n*=18; open circles) groups. *Lower row panels:* Each dot represents an individual data point and lines present the mean numbers of perseverative (*left panel*), regressive (*middle panel*) and never-reinforced (*right panel*) errors to criterion during the first RD session of rats of the two-hit (*n*=17; filled circles) or control (*n*=18; open circles) groups. #*P*<0.1 (Mann-Whitney's U test), comparison between control and two-hit groups. *VCD* visual-cue discrimination, *RD* response discrimination. Data from study 2b (see experimental design in the methods' section).

When tested with the LONG protocol, rats displayed no obvious differences between treatment groups during VCD learning, neither in trials to criterion nor in errors (Figure 35, upper row, trial 1-5). Statistical analysis of trials to criterion (trial 1-4; see explanation in section "2.10 Data exclusion") revealed no differences between treatment groups (two-way ANOVA; treatment: $F(1, 32)=1.43$, $P=0.24$; session: $F(3, 96)=2.81$, $P=0.04$; treatment × session ($F(3, 96)=1.33$, $P=0.27$). However, animals treated with Poly I:C/PCP appeared to need more trials to reach the criterion during the first RD session and made more errors than controls (Figure 35, 6th session in upper row panels (dotted line) and middle row panels). Statistical analysis revealed a trend for a difference between treatment groups upon direct comparison on the first RD day (Figure 35, middle row panels; trials to criterion: Mann-Whitney's $U=96.5$, $P=0.06$; errors: Mann-Whitney's $U=79.5$, $P=0.07$).

The analysis of errors during the first RD session indicated that rats of the two-hit group had a numerical increase in all types of errors compared to the control group (Figure 35, lower row panels), with a statistical trend revealed for never-reinforced errors only (perseverative errors: Mann-Whitney's $U=93.0$, $P=0.20$; regressive errors: Mann-Whitney's $U=89.0$, $P=0.15$; never-reinforced errors: Mann-Whitney's $U=84.0$, $P=0.087$).

– *Prepulse inhibition*

During the PPI testing, the startle amplitude was found to be comparable between treatment groups (Figure 37, left panel; Mann-Whitney's $U=117.0$, $P=0.16$). Further, PPI of rats treated with Poly I:C/PCP was not different from that of controls (Figure 37, right panel). Two-way ANOVA confirmed that there was only a main effect of the different prepulse intensities ($F(3, 102)=45.01$, $P<0.0001$) but no main effect of treatment ($F(1, 34)=1.12$, $P=0.30$) or interaction of treatment × prepulse intensity ($F(3, 102)=0.41$, $P=0.70$).

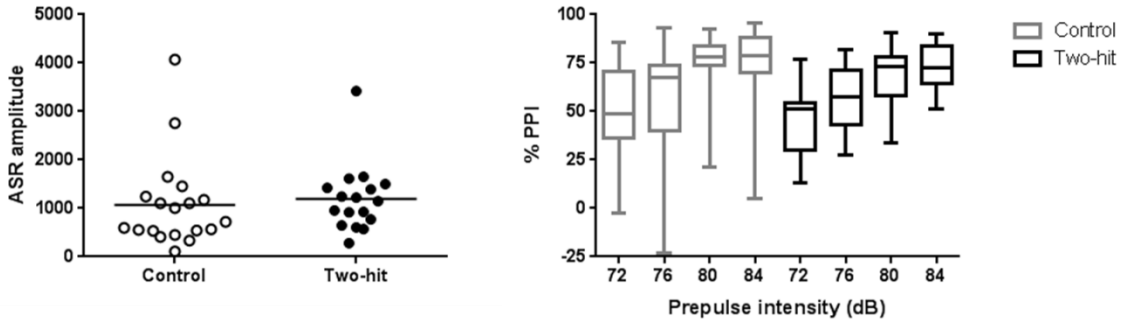

Figure 36: Effects of Poly I:C/PCP treatment on prepulse inhibition. *Left panel:* Each dot represents an individual data point and lines present the mean amplitude of the acoustic startle response (*ASR*) of rats of the two-hit (n=17; filled circles) or control (n=19; open circles) groups. *Right panel:* Boxplots depict % PPI at different prepulse intensities (72, 76, 80, 84 dB) for the two-hit (n=17; black) and control (n=19; grey) groups. Data from study 2b (see experimental design in the methods' section).

– *Social novelty discrimination*

Animals treated with Poly I:C/PCP were not different from controls in terms of social discrimination (Figure 38, left panel; Mann-Whitney's U=17.0, P=0.18). Two-hit treatment did not reduce social interaction towards the juvenile rats (results not shown).

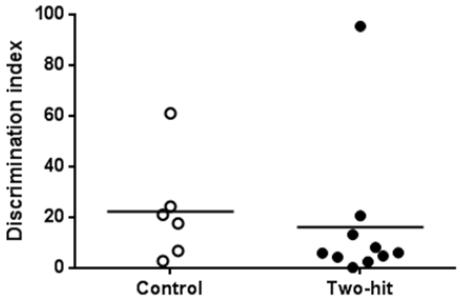

Figure 37. Effects of Poly I:C/PCP treatment on social discrimination. Each dot represents an individual data point and lines present the mean discrimination index (time spent investigating the novel juvenile divided by the time spent investigating the familiar juvenile) of rats of the two-hit (*n*=10; filled circles) or control (*n*=6; open circles) groups. Data from study 3a (see experimental design in the methods' section).

– *Autoshaping task*

During the test session, Poly I:C/PCP treated animals emitted an increased number of responses compared to controls (Figure 39; Mann-Whitney's U=7.0, P=0.006).

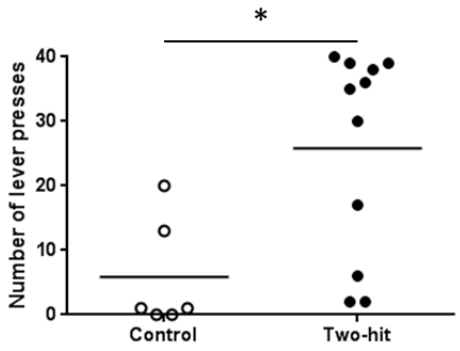

Figure 38: Effects of Poly I:C/PCP treatment on autoshaping performance. Each dot represents an individual data point and lines present the mean number of lever presses (during the test session at day 4) of rats of the two-hit (*n*=11; filled circles) or control (*n*=6; open circles) groups. ***P*<0.05 (Mann-Whitney's U test), comparison between control and two-hit groups. Data from study 3a (see experimental design in the methods' section).

– *Variable-interval fixed-interval task*

During the 3rd FI30 test, animals of the two-hit group displayed no adjustment of the PRP pause over the session, indicated by a regression slope of 0 (Figure 40, test 3). Statistical analysis confirmed a decreased adjustment of the regression slope compared to control rats (Mann-Whitney's U=32.5, P=0.02). No differences between treatment groups were observed

during any other FI30 test session (Figure 40; test 1: Mann-Whitney's U=51.0, P=0.23; test 2: Mann-Whitney's U=68.5, P=0.85; test 4: Mann-Whitney's U=71.0, P=0.96; test 5: Mann-Whitney's U=61.5, P=0.80; test 6: Mann-Whitney's U=60.5, P=0.52).

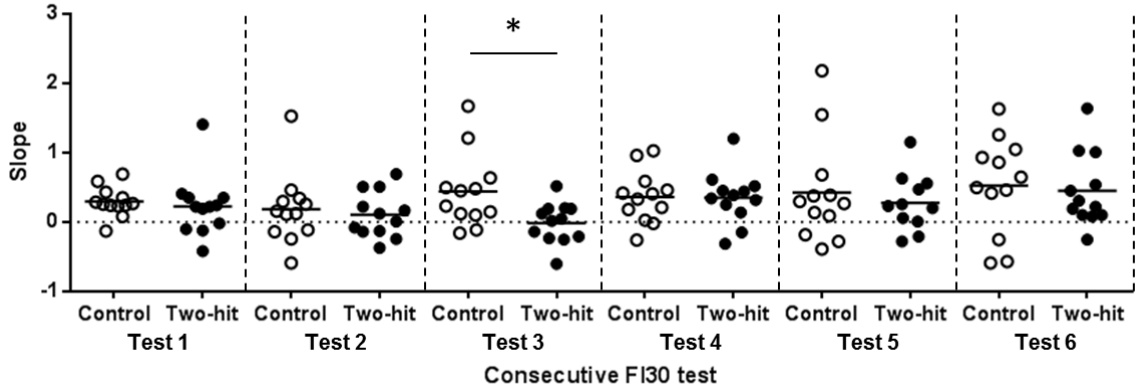

Figure 39: Effects of Poly I:C/PCP treatment on changes in the post-reinforcement pause (*PRP*) duration. Each dot represents an individual data point and lines present the mean regression slope of rats of the two-hit (*n*=12; filled circles) or control (*n*=12; open circles) groups over six consecutive FI30 tests. **P*<0.05 (Mann-Whitney's U test), comparison between control and two-hit groups per test session. Data from study 3b (see experimental design in the methods' section).

Effects of Poly I:C/PCP treatment on locomotor activity:

– *Spontaneous locomotor activity*

Motor activity was assessed using an open field protocol under bright light conditions (~540 lx). Animals treated with Poly I:C/PCP displayed increased spontaneous motor activity compared to controls (distance in arena; Figure 42, left panel; Mann-Whitney's U=9.0, P=0.01). Further, rats of the two-hit group tended to be more active in the center region than controls (Figure 42, right panel; Mann-Whitney's U=16.0, P=0.096).

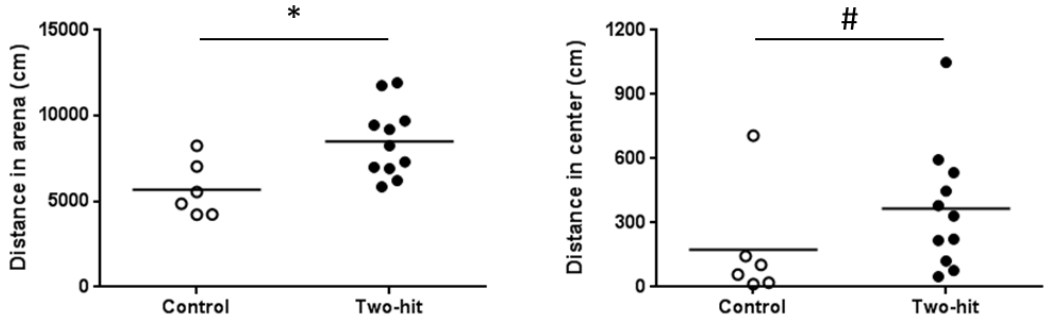

Figure 40: Effects of Poly I:C/PCP treatment on spontaneous locomotor activity. Each dot represents an individual data point and lines present the mean distance moved in the arena (*left panel*) or in the center region (*right panel*) (in cm; during 60 minutes testing, under bright light conditions) of rats of the two-hit (*n*=11; filled circles) or control (*n*=6; open circles) groups. **P*<0.05 or #P<0.1 (Mann-Whitney's U test), comparison between control and two-hit groups. Data from study 3a (see experimental design in the methods' section).

– *Locomotor activity after acute challenge*

To test the rats' sensitivity to psychostimulants, they were challenged with amphetamine or MK-801 and the locomotor activity was measured. In the experiment using amphetamine, rats were treated with one of the doses or vehicle 30 min before being placed into the test apparatus and start of the recording. On the contrary, in the experiment using MK-801, animals were challenged directly before the recording started. For the analysis of these studies, the term "treatment" refers to perinatal administration of Poly I:C and PCP while the term "drug" refers to acute treatment with amphetamine or MK-801.

Animals of the two-hit group displayed an increased motor activity at all tested doses of amphetamine compared to controls (distance in arena; Figures 43, upper row panel; main effect of treatment: $F(1,14)=5.88$, $P=0.03$). *Post hoc* analysis did not show significant differences between treatment groups at any selected dose. Further, there was no treatment × dose interaction ($F(3,42)=0.79$, $P=0.51$), however, analysis revealed a main effect of dose ($F(3,42)=24.96$, $P<0.0001$).

MK-801 challenge led to a dose-dependent increase in total activity in both two-hit and control groups (Figures 43, lower row, left panel; main effect of dose: $F(2, 66)=44.56$, $P<0.0001$). Irrespective of the MK-801 test dose, animals of the two-hit group were more active than controls, an observation that was confirmed by two-way ANOVA (main effect of treatment: $F(1, 66)=5.95$, $P=0.02$; interaction of treatment × dose ($F(2, 66)=0.35$, $P=0.71$). *Post hoc* analysis did not identify significant differences between treatment groups at any selected dose.

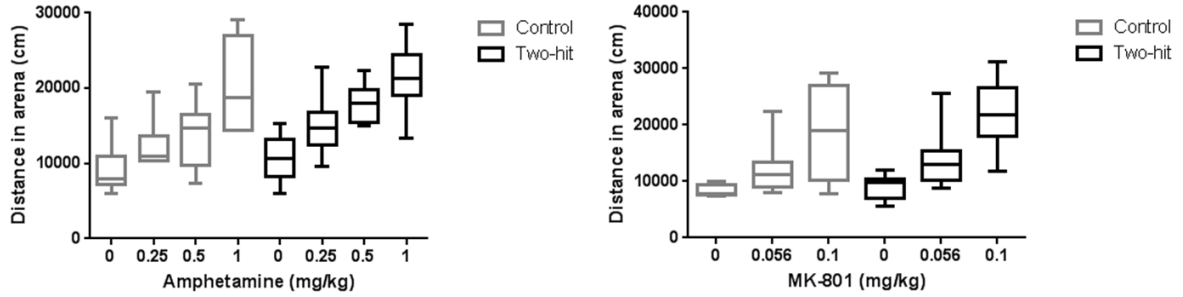

Figure 41: Effects of Poly I:C/PCP treatment on locomotor activity after amphetamine or MK-801 challenge. *Left panel*: Amphetamine (0.25, 0.5 or 1 mg/kg) or vehicle were injected 30 min before the test started. Boxplots depict distance moved in the arena (in cm; during 60 minutes testing) of rats of the two-hit (*n*=11 per dose; black) or control (*n*=6 per dose; grey) groups. *Right panels*: MK-801 (0.056 or 0.1 mg/kg) or vehicle were injected directly before the test started. Boxplots depict distance moved in the arena (in cm; during 60 minutes testing) of rats of the two-hit (*n*=13-14 per dose; black) or control (*n*=10-11 per dose; grey) groups. Data from study 3a and 4 (see experimental design in the methods' section).

3.2 Project II: Evaluation of methods to enhance robustness of developmental models

3.2.1 Body weight

Administration of Poly I:C at the gestation day 15 resulted in a pronounced weight loss that was absent in saline-treated rats (Figure 42, left panel; Mann-Whitney's U=0.0, P=0.003). The body weight change was calculated as % change from the body weight assessed at the day of injection and one day after. This indicated that treatment produced effects that were in line with the expectations (Ballendine et al. 2015;Cunningham et al. 2007;Fortier et al. 2004). Despite treatment with agents like Poly I:C and PCP during important periods of development, no obvious abnormalities in rats' appearance or behavior were observable except for lower body weight at day of weaning and during adolescence compared to controls (data not shown). At PND 70, there were no body weight differences between treatment groups any longer (Figure 42, right panel; Mann-Whitney's U=533.0, P=0.25).

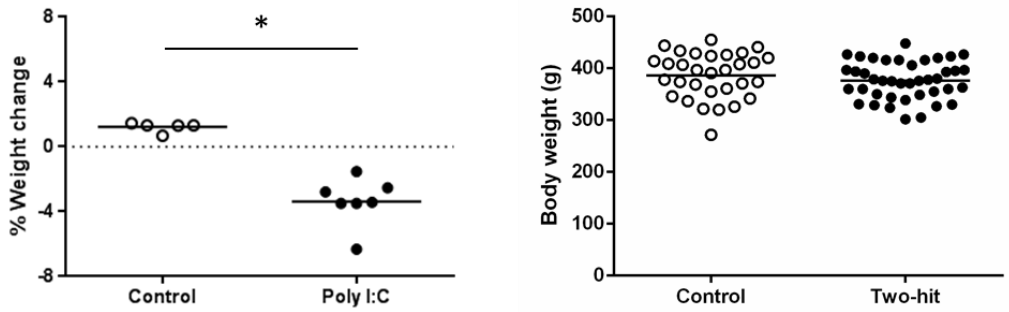

Figure 42: Effects of treatment on body weight. *Left panel*: Body weight changes of pregnant female rats. Each dot represents an individual data point and lines present the mean % weight change 24 hours after injection with Poly I:C (*n*=7; filled circles) or saline (*n*=5; open circles). **P*<0.05 (Mann-Whitney's U test), comparison between Poly I:C and saline treatment. *Right panel*: Effects of Poly I:C/PCP treatment on body weight in the offspring. Each dot represents an individual data point and lines present the mean body weight (in grams) of rats of the two-hit (*n*=41; filled circles) or control (*n*=31; open circles) groups at PND 70. Data from study 4 (see experimental design in the methods' section).

3.2.2 Juvenile social play behavior

Analysis of social play behavior focused on whether or not an offer to play (called "playful attack") was accepted (called "defense") or rejected. As shown in Figure 45, treatment with Poly I:C/PCP led to an overall increase in play engagement as revealed by a higher rate of defense behaviors and, more specifically, by more frequent facing defense responses that translate most often into prolongation of play bouts. Statistical analysis confirmed significant differences between the two-hit and control groups for both total defense and facing defense (% total defense: Mann-Whitney's U=406.5, P=0.02; % facing defense: Mann-Whitney's U=328.0, P=0.001).

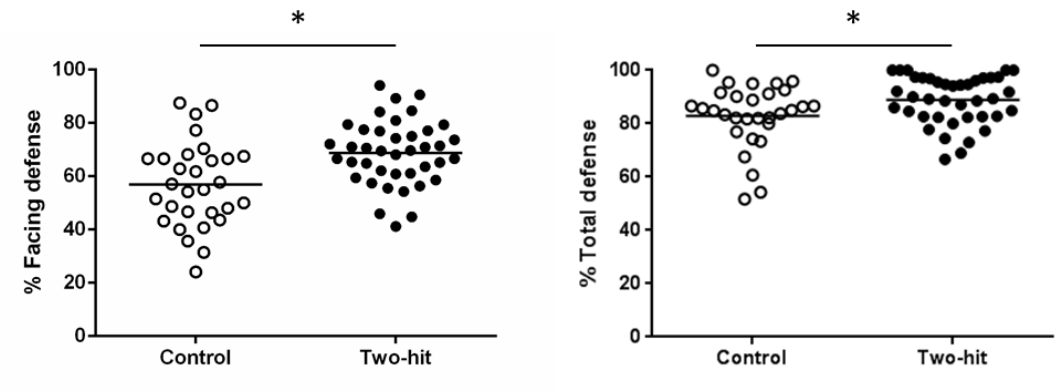

Figure 43: Effects of Poly I:C/PCP treatment on social play behavior. Each dot represents an individual data point and lines present the mean % facing defense (*left panel*) and % total defense (*right panel*) (during 5 minutes testing) of rats of the two-hit (*n*=40; filled circles) or control (*n*=30; open circles) groups. **P*<0.05 (Mann-Whitney's U test), comparison between control and two-hit groups. Data from study 4 (see experimental design in the methods' section).

3.2.3 Correlation analyses

Large group sizes enabled correlation analyses between juvenile social play behavior parameters and different recorded measures in adult rats. Only selected correlations are shown here.

Spontaneous locomotor activity (under dark conditions)

Spontaneous locomotor activity was assessed using an open field protocol in complete darkness (<1 lx). Animals treated with Poly I:C/PCP displayed a higher motor activity than controls (distance in arena; Figures 46, left panel; Mann-Whitney's U=363.0, P=0.002). In addition to higher overall activity, Poly I:C/PCP treated rats were more active in the center region compared to controls (Figures 46, right panel; Mann-Whitney's U=239.0, P<0.0001).

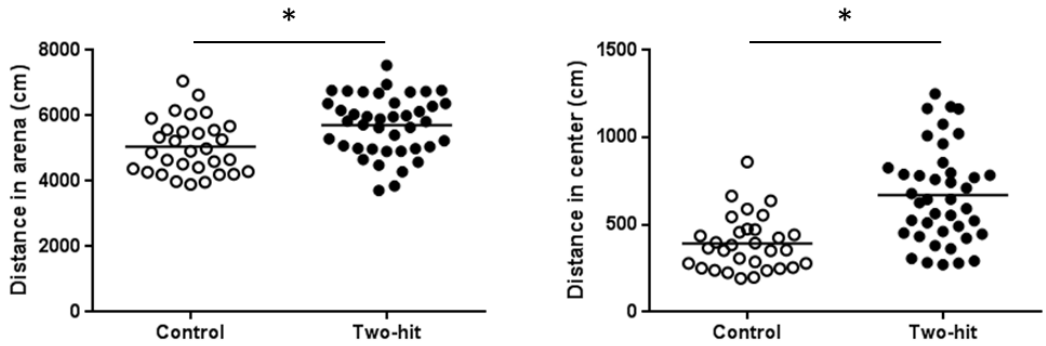

Figure 44: Effects of Poly I:C/PCP treatment on spontaneous locomotor activity. Each dot represents an individual data point and lines present the mean distance moved in the arena (*left panel*) or in the center region (*right panel*) (in cm; during 30 minutes testing, under dark conditions) of rats of the two-hit (*n*=41; filled circles) or control (*n*=31; open circles) groups. **P*<0.05 (Mann-Whitney's U test), comparison between control and two-hit groups. Data from study 4 (see experimental design in the methods' section).

Juvenile social play behavior parameters were indeed found to correlate with the adult locomotor readouts. More specifically, total defense as well as facing defense negatively correlated with different measures of locomotor activity in Poly I:C/PCP treated but not control rats (Table 9). For instance, correlations between % total defense and distance in arena as well as distance in center can be found in the two-hit group (distance arena: Spearman's r=-0.48, P=0.002; distance center: Spearman's r=-0.36, P=0.02) but not in the control group (distance arena: Spearman's r=0.25, P=0.18; distance center: Spearman's r=-0.21, P=0.25). This means that the Poly I:C/PCP treated animals with low amount of play defense (low play engagement) were more active in the open field arena (in general as well as in the center region).

Table 9: Correlations between social play behavior and locomotor activity.

Play			% Facing defense		% Total defense	
			Control	Two-hit	Control	Two-hit
Open field	Distance arena	Spearman's coefficient	0.25	-0.14	0.25	**-0.48**
		P-value	0.19	0.39	0.18	**0.002***
	Distance center	Spearman's coefficient	-0.12	**-0.34**	-0.21	**-0.36**
		P-value	0.54	**0.03***	0.25	**0.02***

Social play behavior variables included in the analysis were % facing defense and % total defense. Open field (under dark conditions) variables included in the analysis were distance moved in arena and center. The analyses were performed using Spearman's rank correlations for the two-hit (n=40) and control (n=30) groups. The coefficients of correlation are given in the table for each comparison. ***P**<0.05, comparison between control and two-hit groups. *Play* social play behavior.

[^{14}C]-2-deoxyglucose utilization

Local metabolic brain activity of the animals was determined by measuring the amount of radioactively labeled glucose in the HPC (Figure 47, left & middle panel) and mPFC (Figure 47, right panel). No differences between treatment groups were assessable, neither in the dorsal HPC (Mann-Whitney's U=470.0, P=0.12), the ventral HPC (Mann-Whitney's U=488.0, P=0.19) nor the mPFC (Mann-Whitney's U=537.0, P=0.61).

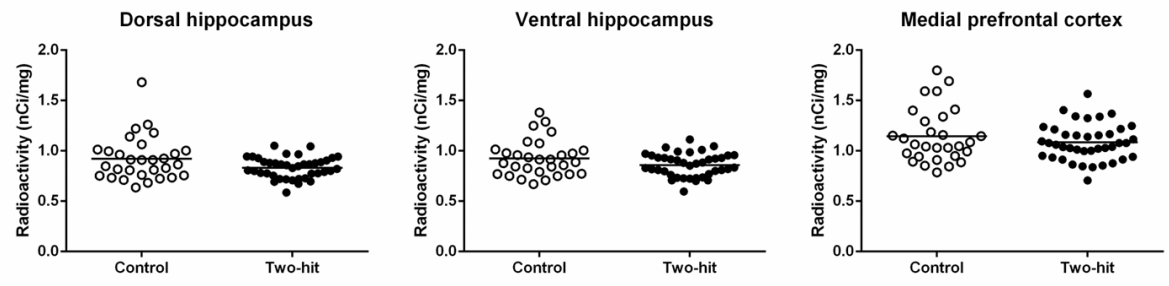

Figure 45: Effects of Poly I:C/PCP treatment on local meatbolic brain activity. Each dot represents an individual data point and lines present the mean measured radioactivity (in nCi/mg) in the dorsal HPC (*left panel*), ventral HPC (*middle panel*) and mPFC (*right panel*) of rats of the two-hit (n=40; filled circles) or control (n=29-30; open circles) groups. Data from study 4 (see experimental design in the methods' section).

Social play behavior parameters were found to correlate with the local brain activity. Negative correlations between defense behaviors and glucose uptake in the three measured brain regions (Table 10) indicated that the Poly I:C/PCP treated animals with lower play engagement had higher brain activity. For instance, correlations between % facing defense and glucose utilization in the dorsal HPC as well as mPFC can be found for animals of the two-hit group (dHPC: Spearman's r=-0.62, P<0.0001; mPFC: Spearman's r=-0.47, P=0.002) but not for controls (dHPC: Spearman's r=-0.18, P=0.34; mPFC: Spearman's r=-0.16, P=0.41).

Table 10: Correlations between social play behavior and local metabolic brain activity.

Play			% Facing defense		% Total defense	
			Control	Two-hit	Control	Two-hit
Glucose utilization	dHPC	Spearman's coefficient	-0.18	**-0.62**	-0.17	**-0.40**
		P-value	0.34	**< 0.0001***	0.38	**0.01***
	vHPC	Spearman's coefficient	-0.20	**-0.58**	-0.23	**-0.32**
		P-value	0.29	**0.0001***	0.22	**0.049***
	mPFC	Spearman's coefficient	-0.16	**-0.47**	-0.23	-0.24
		P-value	0.41	**0.002***	0.24	0.14

Social play behavior variables included in the analysis were % facing defense and % total defense. Brain regions of glucose utilization measurement included in the analysis were dHPC, vHPC and mPFC. The analyses were performed using Spearman's rank correlations for the two-hit (n=39) and control (n=28-29) groups. The coefficients of correlation are given in the table for each comparison. *P<0.05 or #P<0.1, comparison between control and two-hit groups. *dHPC* dorsal hippocampus, *play* social play behavior, *vHPC* ventral hippocampus, *mPFC* medial prefrontal cortex.

3.2.4 Cluster analysis

Cluster analysis for % total defense of the two-hit group revealed the separation of two clusters at the Euclidean distance lower than 15. One cluster was formed by two-hit animals with normal-to-high play engagement (two-hit-HIGH) and another by those animals with low play engagement (two-hit-LOW) (Figure 46). The latter cluster of seven animals was clearly segregated from the cluster with animals that displayed higher play engagement. Therefore, I analyzed the data generated in adult animals divided into three groups: two-hit-HIGH, two-hit-LOW and the control group. Only selected analyses are shown here (Figure 47).

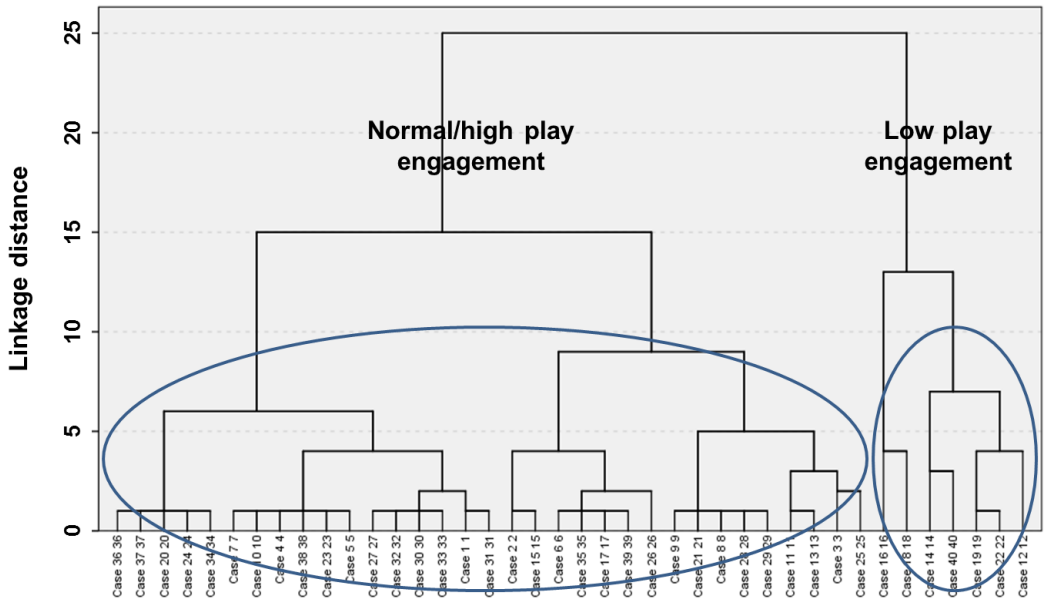

Figure 46: Dendrogram of the cluster analysis. Cluster analysis grouping the Poly I:C/PCP treated animals using the variable % total defense of social play behavior. At the Euclidean distance lower than 15, two groups are clearly separated, as shown in the graph. For details see text.

Autoshaping

As Poly I:C/PCP treated animals differed from controls in their autoshaping performance in study 3a, the autoshaping task was used in this study (study 4) as well. Emitted responses during the test session (session 4) did not differ between animals of the two-hit group and controls (data not shown).

Based on the groups identified by the cluster analysis of the juvenile social play engagement, animals of the two-hit-LOW group were shown to emit a higher number of lever-press responses compared to animals of the control and two-hit-HIGH groups (Figure 47, upper row, left panel; Kruskal-Wallis test; H=6.647, df=2, P=0.04).

Spontaneous locomotor activity (under bright light conditions)

In addition to locomotor activity under dark conditions (Figure 44), the spontaneous locomotor activity was assessed under bright light conditions (~540 lx). Animals treated with Poly I:C/PCP displayed a higher motor activity in arena and more time in the center region than controls (data not shown).

Based on the groups identified by the cluster analysis of the juvenile social play engagement, animals of the two-hit-LOW group were shown to display a higher locomotor activity in general and spent more time in the center region compared to animals of the control or the

two-hit-HIGH groups (Figure 47, upper row, middle and right panels; Kruskal-Wallis test; distance in arena: H=10.22, df=2, P=0.006; duration in center: H=9.966, df=2, P=0.007).

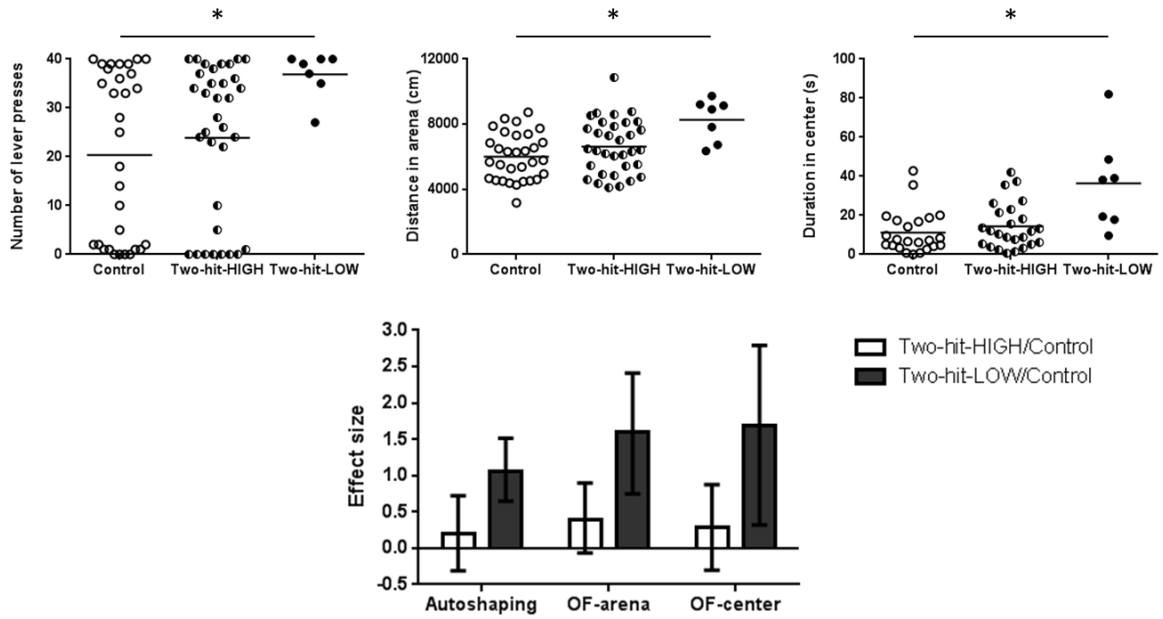

Figure 47: Effects of Poly I:C/PCP treatment on autoshaping performance and locomotor activity after the two-hit group had been split into two separate groups based on the cluster analysis of the juvenile play engagement. *Upper row panels*: Each dot represents an individual data point and lines present the mean number of lever presses in the autoshaping task (*left panel*; during the test session on day 4) as well as distance moved in the arena (in cm; *middle panel*) and duration in the center region (in seconds; *right panel*) (during 30 minutes testing, under bright light conditions) of rats of the two-hit-LOW (*n*=7; filled circles), two-hit-HIGH (*n*=25-33; half-filled circles) or control (*n*=23-31; open circles) groups. **P*<0.05 (Dunn's test), comparison between control and two-hit-LOW groups. *Lower row panel*: Data are presented as mean effect size with 95 % CI calculated for number of emitted responses during session 4 of the autoshaping task (*autoshaping; left bars*), distance in arena in the open field (*OF-arena; middle bars*) and duration in center in the open field (*OF-center; right bars*) from animals of the two-hit-LOW and control group (grey bars) or the two-hit-HIGH and control group (white bars). *OF* open field. Data from study 4 (see experimental design in the methods' section).

Grouping based on the results of the cluster analysis provided clear differentiation of the low play engaged Poly I:C/PCP treated animals (two-hit-LOW) from animals of the Poly I:C/PCP group with normal or high play engagement (two-hit-HIGH). These two subgroups differed in the number of responses emitted during the final test session of the autoshaping task as well as their locomotor activity (total distance traveled in arena and duration spent in center). This difference is further substantiated by comparison of the effect sizes for Poly I:C/PCP treatment in these two subgroups. As shown in Figure 47 (lower row panel), effect size for the two-hit-LOW (vs control group) was considerable higher than the effect size for the two-hit-HIGH (vs control group).

3.3 Project III: Preventive treatment during adolescence

3.3.1 Exploratory studies

Microglia density at postnatal day 48/49

Microglia density was determined by calculating the area covered by Iba1 staining. Animals of the two-hit group displayed an increased microglia density in the HPC compared to controls at PND 48/49 (Figure 50). The effect of Poly I:C/PCP treatment on density was completely reversed by 14 days of treatment with minocycline or pregnenolone (post-treatment) administered between PND 34 and 47 (Figure 50). Two-way ANOVA confirmed main effects of treatment and post-treatment, as well as a treatment × post-treatment interaction (treatment: $F(1, 55)=4.16$, $P=0.046$, post-treatment: $F(2, 55)=3.73$, $P=0.03$, treatment × post-treatment: $F(2, 55)=5.11$, $P=0.009$). *Post hoc* analysis illustrated a difference between animals of the two-hit and control group post-treated with vehicle (control-VEH and two-hit-VEH).

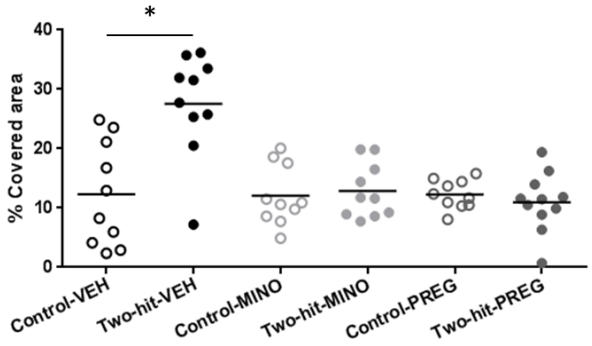

Figure 48: Effects of minocycline and pregnenolone treatment on microglia density at PND 48/49. Each dot represents an individual data point and lines present the mean % area covered by Iba1 staining in the HPC of rats of the two-hit (n=10-11; filled circles) or control (n=10; open circles) groups. Animals were treated with minocycline (*MINO*), pregnenolone (*PREG*) or vehicle (*VEH*) from PND 34 to 47. *P<0.05 (Bonferroni's test), comparison between control and two-hit groups treated with vehicle from PND 34 to 47. Data from study 5 (see experimental design in the methods' section).

Microglia state at postnatal day 48/49

Activation state of microglia was evaluated using two antibodies that mark pro-inflammatory activated microglia, CD11b and CD68. Quantitative analysis revealed no differences between vehicle-treated animals of the two-hit and control groups (control-VEH and two-hit-VEH) neither for number of CD11b- nor CD68-positive cells. Representative pictures of one animal per group show hardly any CD11b- or CD68-positive cells (CD11b, Figure 51; CD68, Figure 52). Control experiments excluded the possibility of technical failure and confirmed the quality of the antibodies and staining protocols.

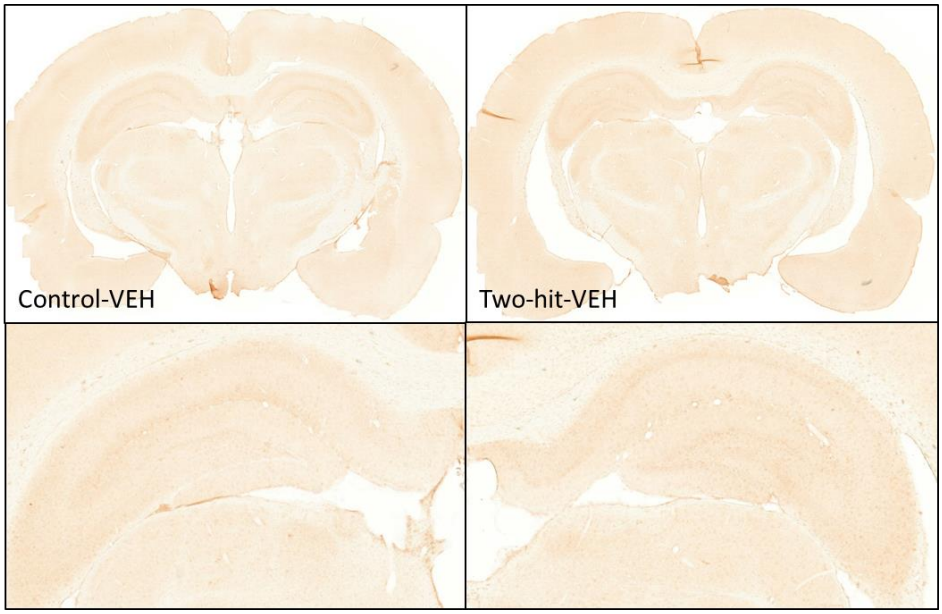

Figure 49: CD11b staining in the HPC at PND 48/49. Pictures illustrate representative slices of one animal of the control (*left panels*) and two-hit (*right panels*) groups, treated with vehicle (*VEH*) from PND 34 to 47. Pictures indicate CD11b-positive cells in the whole slice (*upper row panels*) or in the HPC upon magnification (*lower row panels*). Data from study 5 (see experimental design in the methods' section).

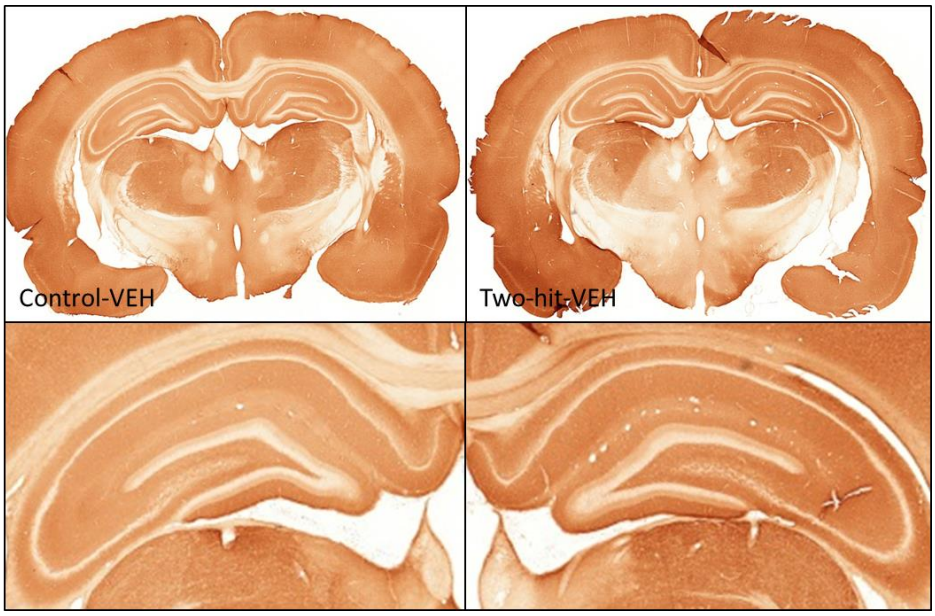

Figure 50: CD68 staining in the HPC at PND 48/49. Pictures illustrate representative slices of one animal of the control (*left panels*) and two-hit (*right panels*) group, treated with vehicle (*VEH*) from PND 34 to 47. Pictures indicate CD68-positive cells in the whole slice (*upper row panels*) or in the HPC upon magnification (*lower row panels*). Data from study 5 (see experimental design in the methods' section).

Quantitative examination of Iba1-stained material from animals of the two-hit group treated with vehicle during adolescence (two-hit-VEH) showed microglia cells with rather smaller cell bodies and long processes not connected to each other (for a representative picture see Figure 53, right panel), but not the typical pro-inflammatory activation state (increased cell bodies with shortened processes, cells clustered together). Only very few microglia cells with

bigger cell bodies containing few processes, probably M1-activated, were observed. This corresponded the lack of CD11b- and CD68-positive cells in Poly I:C/PCP treated animals (Figures 49 & 50).

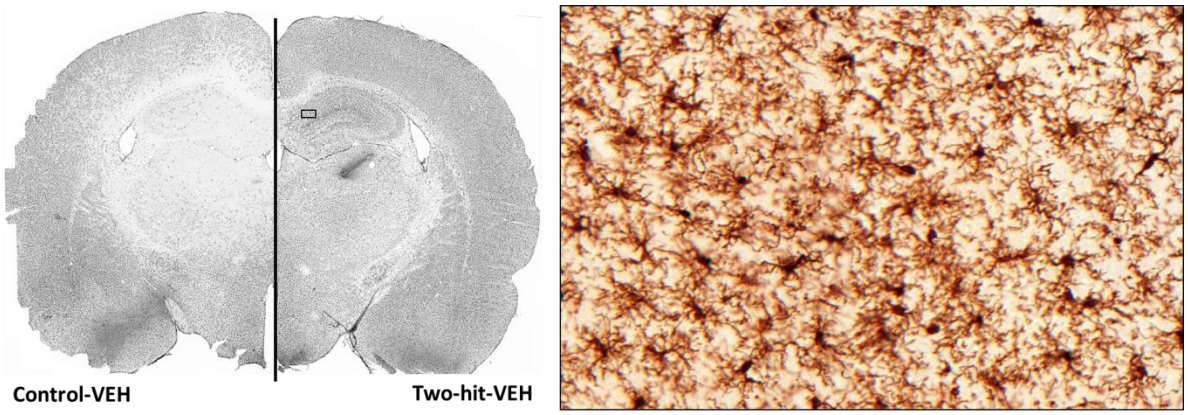

Figure 51: Quantitative examination of the microglia activation state at PND 48/49. *Left panel:* Representative picture (converted to grey scale) of an Iba1-stained slice from one animal of the two-hit and control groups treated with vehicle (*VEH*) during PND 34 and 47. The picture includes a *rectangular box* in the HPC of the Poly I:C/PCP treated animal (*two-hit-VEH*). *Right panel:* This *rectangular box* was magnified (in original color) to provide a representative picture of microglia appearance. Data from study 5 (see experimental design in the methods' section).

Microglia density at postnatal day 27

In addition to PND 48/49, microglia density in HPC slices from 27 days old rats was assessed using Iba1 staining. Both controls and Poly I:C/PCP treated animals displayed very low amounts of microglia that did not differ between groups (Figure 54; Mann-Whitney's U=13.0, P=0.89).

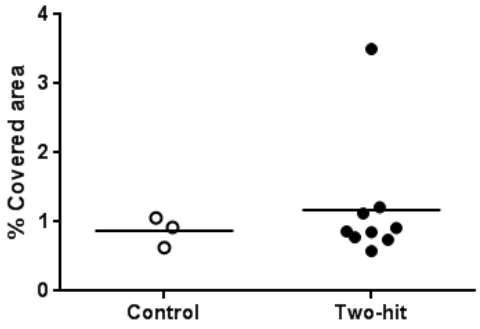

Figure 52: Effects of Poly I:C/PCP treatment on microglia density at PND 27. Each dot represents an individual data point and lines present the mean % area covered by Iba1 staining in the HPC of rats of the two-hit (*n*=9; filled circles) or control (*n*=3; open circles) groups at PND 27. Data from study 3b (see experimental design in the methods' section)

GAD67 expression at postnatal day 48/49

Poly I:C/PCP treatment increased the expression of GAD67 in the HPC and mPFC of 48/49 days old rats when compared to controls (Figure 55, left and right panel, respectively). Further, 14 days of treatment with minocycline or pregnenolone (post-treatment) between PND 34 and 47 completely prevented the increase in GAD67 expression in HPC and mPFC (Figure 55). Two-way ANOVAs confirmed these results. For the HPC a main effect of post-treatment and a treatment × post-treatment interaction as well as no main effect for treatment (treatment: $F(1, 56)=3.75$, $P=0.058$; post-treatment: $F(2, 56)=13.39$, $P<0.0001$; treatment × post-treatment: $F(2, 56)=4.24$, $P=0.019$), and for the mPFC main effects of treatment and post-treatment, but no treatment × post-treatment interaction (treatment: $F(1, 55)=7.00$, $P=0.01$; post-treatment: $F(2, 55)=33.46$, $p<0.0001$; treatment × post-treatment: $F(2, 55)=2.14$, $P=0.13$) were revealed. *Post hoc* analysis for both regions illustrated a difference between vehicle-treated animals of the two-hit and control groups (control-VEH and two-hit-VEH).

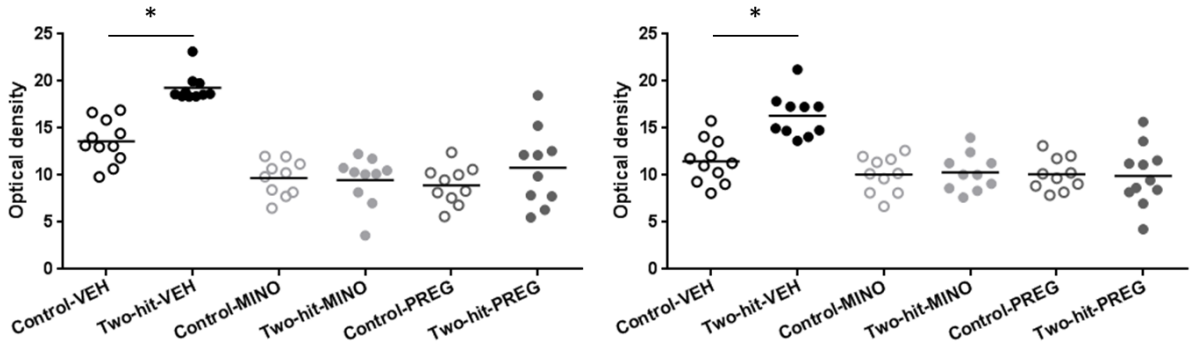

Figure 53: Effects of minocycline and pregnenolone treatment on GAD67 expression at PND 48/49. Each dot represents an individual data point and lines present the mean optical density (of GAD67 staining) in the HPC (*left panel*) and mPFC (*right panel*) of rats of the two-hit (n=10-11; filled circles) or control (n=10-11; open circles) groups. Animals were treated with minocycline (*MINO*), pregnenolone (*PREG*) or vehicle (*VEH*) from PND 34 to 47. **P*<0.05 (Bonferroni's test), comparison between control and two-hit groups treated with vehicle from PND 34 to 47. Data from study 5 (see experimental design in the methods' section). Data produced jointly with Sonja Abele and Ayla Rodrigues Ehrenfried.

GAD67 expression in adulthood

GAD67 expression tended to be reduced in the HPC of adult Poly I:C/PCP treated compared to control rats (Figure 56; Mann-Whitney's U=437.5, P=0.08).

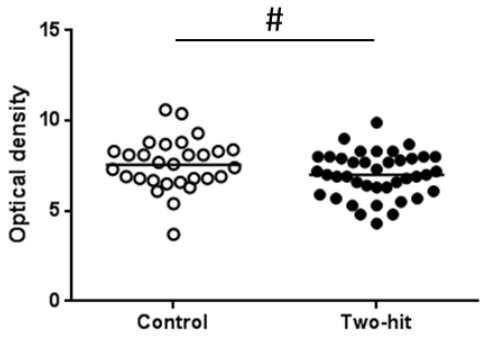

Figure 54: Effects of Poly I:C/PCP treatment on GAD67 expression in adulthood. Each dot represents an individual data point and lines present the mean optical density (of GAD67 staining) in the HPC of rats of the two-hit (*n*=40; filled circles) or control (*n*=29; open circles) groups. #*P*<0.1 (Mann-Whitney's U test), comparison between control and two-hit groups. Data from study 4 (see experimental design in the methods' section).

3.3.2 Confirmatory studies

Two further studies were conducted with the goal to repeat the experiment with the preventive treatment by pregnenolone. The first study aimed to reproduce the effects of pregnenolone on microglia density, whereas the second study was performed to evaluate if pregnenolone treatment during adolescence can prevent locomotor hyperactivity of adult Poly I:C/PCP treated rats.

Microglia density at postnatal day 48/49

Vehicle-treated rats of the two-hit group displayed an increased microglia density in the HPC compared to controls at PND 48/49 (Figure 57). This was not prevented by treatment with pregnenolone for 14 days between PND 34 and 47 (Figure 57). Statistical analysis revealed a main effect of group (Kruskal Wallis, H=7.003, df=2, P=0.03).

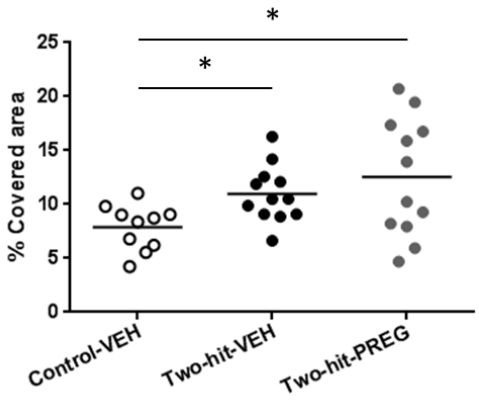

Figure 55: Effects of pregnenolone treatment on microglia density at PND 48/49. Each dot represents an individual data point and lines present the mean % area covered by Iba1 staining in the HPC of rats of the two-hit (*n*=12; filled circles) or control (*n*=10; open circles) groups. Animals were treated with vehicle (*VEH*) or pregnenolone (*PREG*) from PND 34 to 47. **P*<0.05 (Dunn's test), comparison between control and two-hit groups. Data from study 6a (see experimental design in the methods' section). Data produced jointly with Ayla Rodrigues Ehrenfried.

Locomotor activity in adulthood

The effects of pregnenolone treatment on spontaneous locomotor activity were studied in adult rats using open field boxes under dark conditions (<1 lx) (study 6b). Animals of all groups displayed a comparable amount of motor activity (Figure 56, left panel; two-way ANOVA; treatment: F(1, 56)=1.21, P=0.28; post-treatment: F(1, 56)=0.54, P=0.47; treatment × post-treatment: F(1, 56)=0.25, P=0.62).

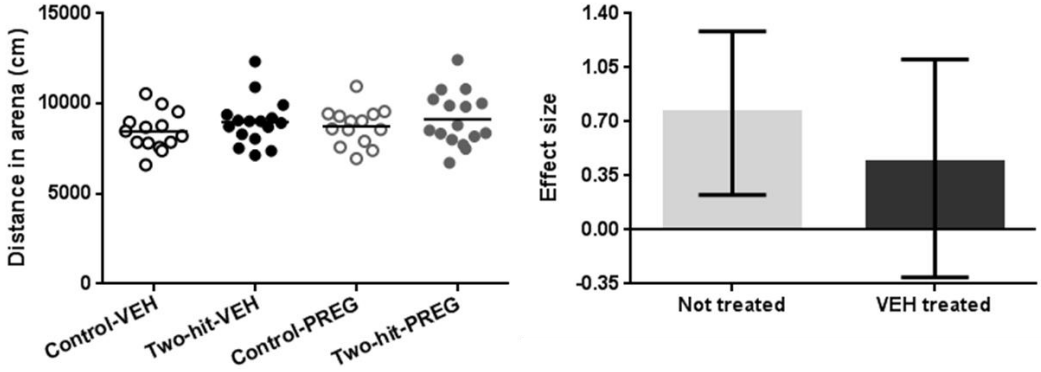

Figure 56: Effects of pregnenolone treatment on spontaneous locomotor activity. *Left panel*: Each dot represents an individual data point and lines present the mean distance moved in the arena (in cm; during 30 minutes testing) of rats of the two-hit (*n*=16; filled circles) or control (*n*=14; open circles) groups. Animals were treated with pregnenolone (*PREG*) or vehicle (*VEH*) from PND 34 to 47. *Right panel*: Data are presented as mean effect size with 95 % CI for distance in arena under comparable conditions (dark conditions, testing in adulthood, 30 minutes) calculated from data of animals of the two-hit and control groups not treated during adolescence from study 4 (*not treated*) and from data of animals of the two-hit and control groups treated with vehicle during adolescence from study 6b (*VEH treated*) (see experimental design in the methods' section).

To illustrate that treatment during adolescence can confound the effects of Poly I:C/PCP treatment, locomotor activity (distance in arena) results were compared between the current study (study 6b) and study 4, where animals were not treated during the adolescent phase but data were collected under comparable conditions (dark conditions, testing in adulthood, 30 minutes). The effect size in study 6b (rats repeatedly injected with vehicle during adolescence; Figure 56, right panel, dark grey bar) was about half of the effect size in study 4 (rats not exposed to injections during adolescence; Figure 56, right panel, light grey bar).

4 Discussion

4.1 Robustness of developmental one-hit models

Prompted by serendipitous discovery of anti-dopaminergic treatments of schizophrenia, preclinical research on pathophysiological mechanisms focused on models that simulated various aspects of behavioral abnormalities as the result of acute or subacute administration of psychotomimetic agents such as D-amphetamine. At the expense of ignoring neurodevelopmental nature of schizophrenia, advantage of such models is in their robustness. However, largely driven by the lack of any truly novel non-dopaminergic treatments of schizophrenia, there have been multiple efforts to develop models that would better reflect the nature of processes that may lead to abnormalities associated with schizophrenia (e.g. DISC1 mutant mice: Johnstone et al. 2011; cannabinoid models: Rubino and Parolaro 2014; ventral hippocampus lesion in neonatal rats: Tseng et al. 2009, etc.). Opposite to (acute) mechanistic models, these neurodevelopmental models may gain in terms of construct validity and be closer to the disease mechanisms but are predictably at risk of being less robust. Indeed, given the long time intervals between the application of the disease-triggering events (pre- or early postnatal) and the time its consequences are assessed (adulthood), there are multiple environmental and other factors that may interfere with the development and expression of the expected abnormalities. Many of these factors are likely to vary between experiments and are difficult to control. For example, different laboratories have shown that neonatal PCP treatment leads to deficits in social novelty discrimination in adult rats (Clifton et al. 2013;Terranova et al. 2005). However, despite early successes in establishing such deficits in our laboratory (Harich et al. 2007), analysis of the subsequent studies conducted over a period of 4 years challenged the robustness of the model in general and social discrimination readout in particular.

When comparing the results of 20 social novelty discrimination studies conducted over this time in neonatal PCP vs. saline treated rats, I saw strong differences in effect size and standard deviations between studies. Similarly, power and p-values were also highly variable (see section 3.1.1 Robustness of the neonatal phencyclidine model (one-hit model)) indicating that the effect produced by neonatal PCP treatment was not stable between studies.

Therefore, the overarching aim of the experiments described in this thesis was to explore options to enhance performance of the model in terms of delivering more robust and reliable results. First, in the neonatal PCP studies, an average of 10 animals per group was used, a sample size suggested by *a priori* power analysis conducted on the basis of the pilot studies as

well as published reports (e.g. Anastasio and Johnson 2008b;Andersen and Pouzet 2004;Wiley et al. 2003). In developmental models, the mean differences between the control and treatment groups are rather small. If a low sample size in models with a rather small true effect is used, this results in a big sample-to-sample variability in the p-value and measured effect size per study. This in turn indicates that such a low number of animals may give rise to many false positive (type I error) or false negative results (type II error) and has to be increased in order to approximate the real effect size (Halsey et al. 2015). Based on all neonatal PCP studies conducted in-house, a sample size of 30-35 animals was calculated to be necessary to see a group effect. Such a high animal number is, however, rather difficult to justify *a priori* and can only be supported by *post hoc* power analysis.

Second, effect size of the neonatal PCP treatment could theoretically be enhanced by increasing the dose of PCP. However, my attempts to increase the dose resulted in strong overall toxicity accompanied by the loss of many animals. Therefore, such an approach would be difficult to justify from an animal welfare perspective.

A third possibility to make the developmental model more robust is to add a second insult, an approach chosen for the studies described in this thesis.

4.2 Maternal immune activation as a second hit factor

4.2.1 Choice of the second hit

Schizophrenia is hypothesized to be a product of a combination of at least two insulting factors that act during the CNS maturation and result in the disease development. The first factor is thought to increase the individual's vulnerability while the second triggers the disease process (Bayer et al. 1999;Maynard et al. 2001;van den Buuse et al. 2003). This hypothesis justifies the decision to increase robustness of the neonatal PCP model by adding a second hit factor.

There are four main types of insults that could be considered as a second hit factor: a) a genetic manipulation (e.g. DISC1, Shen et al. 2008), b) another pharmacological manipulation (e.g. cannabinoid exposure, Leweke and Schneider 2011), c) repeated stress exposure (e.g. post-weaning isolation rearing, Amitai et al. 2014; prenatal repeated stress, Koenig et al. 2005; postnatal repeated restrain stress, Luo et al. 2005), or d) an environmental risk factor (e.g. a prenatal immune activation, Meyer and Feldon 2010).

Selection of a second hit factor followed certain criteria such as expected robustness, compliance with the animal welfare standards, and mechanistic redundancies. For example, I could not identify a suitable genetic manipulation because *a prior* extensive work in the

laboratory failed to reproduce published data on several genetic models (e.g. DISC1, Sakae et al. 2008;Shen et al. 2008; sp4, Zhou et al. 2010). Further, repeated stress exposure was not chosen as a second insult for two reasons. First, from the animal welfare perspective, we rather avoid exposing animals to explicit stress procedures. Second, while drug doses can be easily compared and aligned between laboratories, control over repeated stress protocols, and, hence, their reproducibility is usually limited. Finally, a pharmacological manipulation was also de-prioritized because PCP already represents such an insult and it was deemed most optimal to have it combined with another type of a stimulus. Further, second hit should preferably impact a different stage of CNS development and, therefore, be administered at a distinct time point (such as prenatally as opposed to postnatal PCP injections). Thus, I decided to model prenatal immune activation by Poly I:C treatment that is aimed to mimic prenatal infection, an environmental factor that increases the risk to develop schizophrenia later in life (Brown 2011).

4.2.2 Effectiveness of the perinatal treatment

Maternal immune activation by Poly I:C is known to induce various abnormalities in the offspring's behavior, cognition, neuroanatomy and neurotransmission (Meyer and Feldon 2010). This treatment only mimics maternal infection, what makes it a safer option compared to the maternal influenza infection model. Upon injection of Poly I:C, its double-stranded RNA is recognized by the immune system's toll-like receptor 3 (Alexopoulou et al. 2001) stimulating the production and release of the pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α as well as interferons like IFN-α and IFN-β (Cunningham et al. 2007;Fortier et al. 2004;Meyer et al. 2006). This mimics the acute phase response to a viral infection (Traynor et al. 2004).

Immediate treatment effects

A positive aspect of the Poly I:C treatment is that it leads to various sickness symptoms in the treated animals that are rather immediate, short-lasting and easy to monitor – e.g. reduced activity (locomotion, burrowing) and food intake, hyperthermia as well as weight loss (Ballendine et al. 2015;Bronson et al. 2011;Cunningham et al. 2007;Fortier et al. 2004;Gibney et al. 2013;Zhang et al. 2012).

In my experiments, pregnant females treated with Poly I:C were observed to lose body weight due to the injection when measured 24 h post-injection (see section 3.2.1), a confirmation that the treatment had an expected impact. Apart from that, Poly I:C treatment was accompanied

by a reduction in the litter size per dam that was in line with the published evidence (Ballendine et al. 2015;Yee et al. 2011). Despite these strong acute symptoms induced by Poly I:C as well as impairments induced by PCP treatment, adult Poly I:C/PCP treated rats were indistinguishable from controls in terms of their body weight, general appearance and gross behavior.

Delayed treatment effects

In acute models like amphetamine-induced hyperactivity, the effectiveness of the treatment can be immediately monitored. However, neurodevelopmental models are characterized by long periods between treatment and testing. Therefore, the reliability of the delayed treatment-induced effects is much more difficult to estimate. As a kind of quality control, one may want to refer to long-term brain pathology that is usually expected to characterize the developmental model.

There are two reasons to expect long-term immunological abnormalities in the CNS of rats treated with Poly I:C/PCP. First, immune stimulating effects of Poly I:C in the dams and fetus are thought to induce an inflammatory response in the CNS of the developing rat pups (Meyer et al. 2006). Second, PCP treatment is neurotoxic, especially in developing brains (Wang et al. 2001;Wang and Johnson 2005), and was shown to induce an inflammatory response as well (Nakki et al. 1996). The assessment of the immunological abnormalities can, therefore, help to estimate the effectiveness of the two-hit model. Changes in microglia density and activation have already been reported in the offspring from Poly I:C treated female rats (Van den Eynde et al. 2014;Zhu et al. 2014b) and, therefore, were chosen as a readout in experiments of this work (see section 3.3.1).

Poly I:C/PCP treatment led to an increased microglia density at PND 48/49, assessed by means of Iba1 staining. It is believed that an increase in Iba1 expression is the result of microglia activation (Ito et al. 1998;Sasaki et al. 2001). However, in the present study, microglia showed neither positive staining for pro-inflammatory markers like CD11b and CD68, nor morphological signs of M1 polarization.

To evaluate if the increased microglia density was due to inflammation fading away until PND 48/49, the microglia density was assessed at PND 27. Interestingly, at this age, no increase in microglia density was seen in Poly I:C/PCP treated animals. This led to the conclusion that the increased recruitment of microglia has to be explained by a mechanism that starts after PND 27, e.g. during puberty beginning at around PND 38 in male rats (Schneider 2013).

Puberty is the period characterized by vast changes within brain systems (Brenhouse and Andersen 2011). During this time, many synapses are pruned to build new and more complex circuits of neurons necessary for maturation (Chechik et al. 1998). In doing so, the to-be-eliminated synapses, pre- and postsynaptic elements, are phagocytozed by surrounding microglia (Salter and Beggs 2014). Various pathways and genes involved in synaptic pruning, especially associated with the hippocampus, were linked to schizophrenia pathophysiology (Cocchi et al. 2015;Marco et al. 2015). This supports the hypothesis that exaggerated synaptic pruning during adolescence is involved in the pathogenesis of schizophrenia (Feinberg 1982). An increased density of microglia, seen in animals of the two-hit group, could argue for an exaggerated synaptic pruning during adolescence as well. This is supported by studies showing that prenatal treatment with Poly I:C in combination or alone leads to pre- and postsynaptic deficits, detectable already at adolescent age (Giovanoli et al. 2015;Oh-Nishi et al. 2010) as well as to a deficit in spine density (Abazyan et al. 2010;Li et al. 2014), hypothesized to be the result of exaggerated pruning. Synaptic pruning is part of the microglial homeostatic function and observed to be a nonphlogistic process that is performed by ramified but not reactive microglia (Nayak et al. 2014;Salter and Beggs 2014). This fact could explain the increased amount of microglia in Poly I:C/PCP treated animals without pro-inflammatory activation.

A further explanation of an increased number of microglia without signs of pro-inflammatory activation, is based on the observations that prenatally stressed animals, similar to Poly I:C/PCP treated animals, exhibited an increase in Iba1 immunoreactivity in the hippocampus that was accompanied by microglial hyper-ramification (Diz-Chaves et al. 2012). Also chronic stress leads to an increase in the Iba1 positive cell number in various regions (Hinwood et al. 2012;Hinwood et al. 2013;Tynan et al. 2010) in the absence of pro-inflammatory markers (e.g. IL-1β, MHC-II or CD68) (Hinwood et al. 2013;Tynan et al. 2010), but with microglia showing a hyper-ramified state (Hinwood et al. 2013). Due to the fact that this hyper-ramified state of microglia was not caused by inflammation, Tynan and colleagues argued that changes in neuronal activity may be the trigger of the morphological changes in the microglia (Tynan et al. 2010). Further, neuronal activity was reported to be directly regulated by microglia that interact with the neuronal synapses. Based on these observations, Walker and colleagues suggested that neuronal cues, especially during development, may trigger and modulate a change in the microglia morphology (Walker et al. 2014). Hence, one may speculate that the increase in Iba1 staining, seen in Poly I:C/PCP treated animals, could be also the result of an M2-activation of microglia.

To my knowledge, there are no studies that assessed the effects of maternal immune activation by Poly I:C in adolescent rats using a treatment protocol similar to mine. However, in adult rats, prenatally treated with Poly I:C, rather inconsistent findings were reported. Only one study showed microglial M1-activation (Van den Eynde et al. 2014), whereas two others revealed no changes in the number of microglia or their activation state compared to controls (Mattei et al. 2014;Missault et al. 2014).
To conclude, mechanism whereby microglia density is increased remains unclear. However, based on the results reported here, the increase in density does not seem likely to be the product of an M1-polarization of microglia.

Limitations: Even if this is unlikely, presence of inflammation in juvenile/adolescent rats upon Poly I:C/PCP treatment cannot be fully ruled out and may need to be addressed in further studies. To detect the microglia polarizations, M1- or M2-activation, additional immunohistological markers (Franco and Fernandez-Suarez 2015) as well as morphological methods, measuring the diameter of the microglia cell soma (Watson et al. 2012) or counting different morphological distinct phenotypes (Diz-Chaves et al. 2012), can be used. Further, flow cytometry could be applied based on the principle of separating microglia by their expression of cell surface markers (Dick et al. 1995;Stevens et al. 2002). Independent on microglia, the amount of pro- and anti-inflammatory cytokines and chemokines in the brains or serum could be assessed as well (Meyer et al. 2006).

These results support the assumption that the developmental insults, prenatal Poly I:C and neonatal PCP, produce long-term immunological abnormalities.

4.3 Developmental animal models need novel analysis tools

Developmental models are expected to have construct validity but, their application may be challenging due to the long time separating the triggering challenge (pre- or postnatal) from ultimate testing (most often adulthood). There are multiple environmental and other factors that may interfere with the development and expression of the expected abnormalities. This may lead to a higher variability between subjects (see e.g. Vorhees et al. 2012;Vorhees et al. 2015) that in turn could be one reason why effects produced by developmental insults are often rather subtle and not robust. To handle these problems, there is a need to develop and implement novel analytical tools and methods such as those discussed in this section.

4.3.1 Acute tests to characterize developmental animal models

To identify subtle changes between subjects of the two-hit and control group, I utilized different methods that: a) were robust in previous publications (PPI testing), b) were robust in our laboratory (operant set-shifting task) and have been used in house studies before (social novelty discrimination task), and c) allowed multi-parametric optimization (open field) to identify the best parameters.

Prepulse inhibition

PPI deficits were previously observed in the offspring from Poly I:C treated mice (O'Leary et al. 2014;Shi et al. 2003;Vuillermot et al. 2011;Zhu et al. 2014b) and rats (Dickerson et al. 2010;Hadar et al. 2015;Klein et al. 2012;Song et al. 2011;Wolff and Bilkey 2010;Yee et al. 2011). Due to these repeatedly reported impairments upon Poly I:C treatment, I expected a robust difference between Poly I:C/PCP treated and control rats with regard to sensorimotor gating as well, and therefore, decided to use PPI testing. Despite the expectations, Poly I:C/PCP treatment induced rather very subtle, statistically non-significant reductions in PPI (see section 3.1.3). When taking a closer look at studies using offspring from Poly I:C treated females, impairing effects due to prenatal treatment on PPI were not revealed consistently in all experiments or laboratories. Some reports showed an effect at specific age only (e.g. 16 weeks of age, but not 8 weeks: Lipina et al. 2013; PND 90, but not 56 and 180: Van den Eynde et al. 2014). Other studies suggested that the treatment produced only minor impairments, like deficits at only one or two out of the four prepulse intensities (Ballendine et al. 2015;Missault et al. 2014;Ozawa et al. 2006;Wolff and Bilkey 2008). For instance, in studies by Vorhees and colleagues, offspring were grouped by mothers' body weight loss due to Poly I:C treatment. Only offspring from females with higher weight loss showed PPI deficits (Vorhees et al. 2015). However, even after pre-selecting treated animals upon the body weight changes of the mothers, the differences to controls were not seen robustly in all studies (Vorhees et al. 2012). Deficits in PPI were also shown in neonatal PCP treated animals (PND 7, 9 and 11), but only during the adolescence (Anastasio and Johnson 2008a;Kjaerby et al. 2013;Wang et al. 2001;Wang et al. 2003) and not in all studies (Boctor and Ferguson 2009). In sum, effects of the developmental treatments with prenatal Poly I:C or neonatal PCP on PPI performance were not robustly seen in all studies and were often rather mild.

This is supported by own attempts to establish PPI deficits in different animal models of schizophrenia. Consistent effects could only be shown when using acute pharmacological challenges with apomorphine, amphetamine or PCP but not when using developmental

models including the prenatal methylazoxymethanol acetate injection, the neonatal PCP treatment or the neonatal ventral hippocampal lesion model (unpublished observations).
It should also be mentioned that the rationale for using PPI in preclinical studies is based on deficits in pre-attentive processing in patients suffering from schizophrenia. These deficits have been confirmed using a variety of research tools including PPI, P50 or P100 gating and mismatch negativity (Braff et al. 2007;Kim et al. 2015;Salisbury et al. 2002). However, robust correlations between deficits in PPI and P50 gating have failed to be detected (Braff et al. 2007). A similar lack of robust correlation between PPI and N40 gating (seen as a cross-species analogue to the human P50 gating) was identified in a developmental animal model (Swerdlow et al. 2012). This lack of robust correlations could be based on a different neurobiology underlying those tasks (PPI and P50/N40 gating). Possibly, PPI is not sensitive enough to assess subtle abnormalities produced by Poly I:C/PCP treatment due to a high variability in the treatment groups. But maybe testing N40 gating would have been more appropriate in the Poly I:C/PCP model.

Social novelty discrimination

Social novelty discrimination was repeatedly shown to be impaired in neonatal PCP treated rats (Clifton et al. 2013;Terranova et al. 2005) and this was the reason for including it in the present studies. As discussed already above (see section 4.1), neonatal PCP treatment could not induce robust deficits in social discrimination in our laboratory. Even the addition of a second hit factor (prenatal Poly I:C treatment) was not sufficient to increase the impact of treatment on social discrimination (see section 3.1.3).

Operant set-shifting

Since the tasks described above may not be sensitive enough to detect differences between the Poly I:C/PCP and control group, I tried to identify analysis methods with higher sensitivity. To that end, a set-shifting task, assessing cognitive flexibility (Floresco et al. 2008), was first established in the laboratory and then modified in order to increase its sensitivity (see section 3.1.3).
With the originally reported protocol, my results were very similar to published data (Floresco et al. 2008) in terms of the trials to criterion and errors in both visual-cue discrimination (VCD) and response discrimination (RD). This protocol suits well for testing effects of acute treatment on cognitive flexibility/executive functioning (Dalton et al. 2011;Enomoto et al. 2011;Haluk and Floresco 2009). However, it may not be appropriate to evaluate a

neurodevelopmental model like Poly I:C/PCP. When using a one-day-VCD protocol, it is likely that an animal stays more flexible to learn a new rule than it would be after a longer training.

In order to increase sensitivity of the task, I prolonged the initial VCD learning to five days to ensure stronger learning. For rats that were well trained to perform on one rule, learning a new rule is more difficult and takes longer (Garner et al. 2006). This may increase the chance to see deficits in learning the new RD rule.

Two test this prolonged paradigm, two groups were used: one was the same group as used to establish the original 2-day (SHORT) protocol; the other was a naïve group. Number of trials to criterion and errors in the VCD and RD were compared, in order to assess if this prolonged protocol can be used to study the shift between dimensions. Further, both conditions were compared in order to assess the possibility of re-testing (testing the same animal first in the SHORT protocol and then in the LONG). Apart from faster achievement of the visual-cue learning criterion by re-tested animals, results in both groups, naïve and re-tested, were very similar. They needed a comparable number of trials to criterion during the first day of response discrimination meaning that re-testing did not facilitate shifting to a new rule. Therefore, I decided to test animals of the two-hit and controls group with both protocols, starting with the acute (SHORT) and re-testing the animals afterwards with the LONG protocol.

Poly I:C/PCP treatment did not affect the ability to shift from VCD to RD when using the SHORT protocol. This is in apparent contrast to results of the studies on single-hit prenatal Poly I:C or neonatal PCP treatment. Offspring from Poly I:C treated rats had deficits in RD and showed a higher number of perseverative errors (Zhang et al. 2012) or subtle deficits in VCD learning and RD, with an increase in regressive errors (Ballendine et al. 2015). Similar to these results, adult rats neonatally treated with PCP also needed more trials to criterion when tested in the extradimensional shift using the attentional set-shifting task (Broberg et al. 2008;Redrobe et al. 2012).

However, when animals were tested under the LONG protocol, Poly I:C/PCP treated rats differed from controls. Rats of the two-hit group displayed an increased number of trials to criterion and errors compared to controls in the first RD session after five days of VCD training. This can be interpreted as a deficit in cognitive flexibility due to Poly I:C/PCP treatment.

Errors to achieve RD criterion can be split into 3 different types (Floresco et al. 2009). Animals of the two-hit group were found to make an increased number of never-reinforced

errors compared to controls, whereas no differences between groups were seen regarding other error types. These deficits resemble those seen upon bilateral inactivation of the Nacc core (Floresco et al. 2006) or disconnection inactivation of the mediodorsal nuclei of the thalamus to the Nacc core (Block et al. 2007).

Even if differences in the number of trials and errors to achieve the RD criterion are rather small between animals of the two-hit and control group, Poly I:C/PCP treatment can be evaluated to lead to a decrease in cognitive flexibility. The observation that only a rather challenging protocol can show those deficits, supports the hypothesis, that sensitive tests are needed for neurodevelopmental models.

Locomotor activity

I further evaluated the open field test with regard to its robustness by applying various conditions for testing – under different illumination, with different sizes of open field boxes and testing spontaneous or drug-stimulated motor activity –to identify the most optimal ones.

Overall, rats of the two-hit group were found to be more active than controls, independent of the light conditions or size of the open field box. Due to the generally higher activity under dark conditions, this configuration was used for most of the conducted open field tests.

Neither single-hit Poly I:C (Zuckerman et al. 2003;Zuckerman and Weiner 2005) nor neonatal PCP treatment (Harich et al. 2007;Kaalund et al. 2013;Nakatani-Pawlak et al. 2009) led to significant changes in the spontaneous locomotor activity, in studies using a treatment protocol similar to mine. One exception was a study from van den Eynde and colleagues who reported a mild decrease in activity at one tested time point (at PND 180, but not at PND 56 and 90; Van den Eynde et al. 2014). Locomotion was measured in the Van den Eynde study at a much later time point than in mine or any of the other studies that could explain the differences in results. In contrast to these overall negative results in the single-hit models, Poly I:C/PCP treatment resulted in stronger alterations in spontaneous locomotor activity that were reproduced using different batches of animals under various conditions. A potential stronger effect due to a two-hit treatment was in line with other animal models. Genetic manipulations combined with prenatal Poly I:C treatment led to stronger increases in locomotor activity compared to single-hit Poly I:C treatment (Abazyan et al. 2010;Vuillermot et al. 2012).

Increased exploration and novelty-seeking behavior in rodents is suggested to be rooted in DA dysregulation, i.e. hyperdopaminergic state (Kelly and Iversen 1976). An increased DA content (and its metabolites) was found in striatal regions in offspring of Poly I:C treated

female rats (Deslauriers et al. 2013;Hadar et al. 2015;Ozawa et al. 2006). Based on that, an increase in baseline DA levels is expected to be present in Poly I:C/PCP treated rats as well. Novel stimuli are known to induce DA release in the striatum (Yee and Singer 2013), and a novel environment used for locomotion testing is an example for those stimuli. Upon an already elevated basal DA content in striatal regions, a DAergic stimulation would lead to exaggerated locomotor activity. This may be hypothesized to be the mechanism of the increased locomotor activity seen in Poly I:C/PCP treated compared to control rats.

Instead of an increase in spontaneous locomotion, it was more commonly reported that treatment with neonatal PCP or prenatal Poly I:C sensitized rats to subsequent challenge with PCP, MK-801 or amphetamine (Nakatani-Pawlak et al. 2009;Zuckerman et al. 2003;Zuckerman and Weiner 2005). Upon challenge with either amphetamine or MK-801, Poly I:C/PCP treated animals displayed increased motor activity compared to controls. This was however rather an overall treatment effect (Poly I:C/PCP vs control), not linked to a specific drug and dose (see section 3.1.3).

To summarize, the difference in baseline (spontaneous) activity between Poly I:C/PCP and control groups was repeatedly seen in different experiments using spontaneous motor assessment protocols. Acute drug challenge by MK-801 or amphetamine was not able to increase the difference between the treatment groups further. Due to the robust effects of Poly I:C/PCP treatment on spontaneous motor activity, this task was used in most studies to characterize alterations induced by Poly I:C/PCP treatment.

4.3.2 Longitudinal approaches to characterize developmental animal models

Novel tasks with no practice effects

Due to the progressive nature of developmental models, behavioral abnormalities should be assessed longitudinally rather than just once at an arbitrarily chosen time point. However, there are very few tasks suitable for continuous, repeated monitoring of the behavior (e.g. 5-choice serial reaction time task, Winstanley et al. 2004). This is a particular challenge for cognitive tasks where long-time measurements can be contaminated by practice effects. To address this issue, the variable-interval fixed-interval (VIFI) task, a tool to determine rats' cognitive flexibility capacity, was developed and characterized in the framework of the thesis.

The task was based on the classical observations that response patterning is determined by the reinforcement schedule. For simple interval schedules, it is well known that post-reinforcement pausing (PRP) is almost absent under variable schedule (Ferster and Skinner

1957;Schlinger et al. 2008) and is longer for fixed interval schedules of comparable length (e.g., Schneider 1969). Such differences in the response patterning are consistent with the view that behavior is adjusted to maximize net gain, where net gain is defined as the benefits obtained from responding (e.g. reinforcer rate) minus the costs of responding (e.g. response effort; Baum 1981). Therefore, I expected adjustment in the PRP when changing from VI schedule of reinforcement to a corresponding FI schedule (i.e. FI schedule with the same theoretical density of reinforcement but lower costs of responding).

A study in drug naïve rats showed that, in animals trained to press a lever under VI30 schedule, occasional switching to FI30 schedule during one session resulted in longer PRPs (see section 3.1.3). One day after the session under FI30 schedule, training conditions were returned to VI30 and PRPs were again at the pre-switching level and stable throughout the session. These adjustments were present already during the first session when the schedule was switched and were repeatedly observed during several months of the experiment. In other words, there seemed to be no practice effects that would facilitate adjustment of PRP duration in response to the schedule change.

There are a number of tasks that were developed to study adaptions in the animals' behavior in response to changing reinforcement contingencies (e.g. reversal learning tasks). However, there are several limitations associated with administering such tasks in laboratory animals, too. These limitations may be addressed by the task that is developed specifically for laboratory animals and later translated to higher species.

First, deficits in reinforcement learning are associated with a number of human psychopathologies and there is a need to have learning tasks in animals that are close to those used in humans. For example, patients with schizophrenia are impaired in the ability to rapidly modify behavior as a function of changing reinforcement contingencies and specialized probabilistic reversal learning tasks were developed to study such deficits in humans (Ragland et al. 2012). However, some human tasks are too difficult or too complex to be implemented in animals while others loose certain qualities when back translated. Therefore, one has to consider developing a task in laboratory animals that is based on a very basic learning phenomenon that is likely to be translatable across species (including humans). The PRP adjustment task that was evaluated in the present work is based on basic differences in response patterning under FI and VI schedules of reinforcement that are likely to be observed in various species. Having said this, one needs to acknowledge that further studies will be necessary to identify reinforcement schedule conditions that will enable rapid and reliable PRP adjustments in animals of other (higher) species (e.g. primates).

Second, utility of the tasks largely depends on their susceptibility to carry-over or practice effects. Many of the tasks assessing reinforcement learning in animas cannot be used in longitudinal studies or experiments with repeated drug administration. As mentioned above, there seemed to be no practice effects in the task used in the present study. However, one needs to acknowledge that further studies will be necessary to confirm that the PRP adjustment can be repeatedly induced in rats of different strains and in animals of other (higher) species (e.g. primates).

Furthermore, the present study leaves several other open questions. For example, it has to be established whether longer sessions (more than 84 trials) will enhance the signal. There may be a delicate balance between achieving longer PRPs at the end of the session and other important aspects of the task performance (fast reversal when the schedule is switched back to VI schedule, lack of practice effect, etc.). Another key question concerns the potential impact of the response topography. In other words, it would be necessary to demonstrate that task performance is not affected by using operant responses other than lever pressing (e.g., nose-poking, touch screens).

Whether aimed at reinforcement learning or any other cognitive function, it is expected that a task is amenable to pharmacological manipulation. The first steps in determining pharmacological sensitivity of the task are the analysis of effects of well-established drugs and identification of criteria for deciding on whether or not effects of drugs were behaviorally specific. For example, in the present experiment, diazepam was chosen to represent a class of sedative-hypnotic agents that are known to interfere with operant performance when the doses are high enough. Indeed, higher dose of diazepam was found to reduce response rates under both VI and FI schedules of reinforcement. Under VI schedule, PRP duration was elevated from the beginning of the session in diazepam-treated animals but was stable throughout the session irrespective of diazepam treatment. Under FI schedule, diazepam treatment had a similar effect on PRP duration that was present from the beginning of the session and therefore may have masked the effects of the schedule change (i.e. from VI to FI). Thus, for the present task, adjustments in the PRP duration can only be studied after pharmacological or other manipulations that have minimal or no acute effects on PRP duration.

Amphetamine is another drug that was very often applied to modify operant behavior. Under FI conditions, D-amphetamine (1-2 mg/kg) was shown to shorten PRP (Brown and Seiden 1975) and these effects are sometimes explained by the rate-dependency phenomenon, i.e. dependence of amphetamine's effects on the baseline rate of responding. In the case of operant behavior maintained by the FI schedule, amphetamine is expected to enhance local

response rates during the early to middle segments of a fixed interval while producing no such effects at the end of the interval when the rate of responding is the highest (McMillan 1968).

Interesting enough, in the present experiment, amphetamine did not shorten the PRPs when animals were switched to the FI schedule. Instead, in most animals given low doses of amphetamine, there was an increase in the PRP durations over the course of the session. Thus, unlike the experiments with diazepam, where reductions in response rates and increases in PRPs were in agreement with the expected action of a sedative agent, effects of amphetamine (low doses) were rather unexpected. However, it should be noted that, although effects of low-dose amphetamine were observed in most animals, they were not confirmed with an *a priori* defined statistical analysis. Previous studies have revealed different effects of amphetamine on reversal learning dependent on study protocol, animals' sex and strain, route and schedule of drug administration. Although most studies have found impairing effects of amphetamine (Beckwith et al. 1974;Idris et al. 2005), there were also reports on facilitating effects (Weiner and Feldon 1986). However, none of these studies explored effects of amphetamine on reversal in a reinforcement learning task, similar to the one used in the present study. Therefore, further studies are warranted to confirm and extend the current findings.

All together, these results provide initial evidence for the possibility to use different response patterning under VI and FI schedules with equivalent reinforcement density for studying adaptive guidance of behavior by reward contingencies. Further studies will be necessary to complete methodological validation of this task (e.g. validation of the stable baseline criteria, interval schedules of varying duration, longer training and test sessions, replication in male and female rats of other common strains, etc.).

This task was also successfully established in PolyIC/PCP animals (see section xx) and performance was assessed over 12 weeks. Only in one of the six conducted test sessions, rats of the two-hit group were performing inferior to controls in terms of the PRP adjustment, whereas the values of the regression slope in the other five sessions were similar in both groups. This is a very important observation illustrating the value of longitudinal measurements in developmental models. Indeed, the failure of the two-hit group rats to adjust PRP during session 3 could be falsely interpreted as a deficiency in cognitive flexibility, if this would have been assessed only once. However, due to the possibility to test the rats' behavior repeatedly, observations made during the session 3 could be classified as outliers and the overall conclusion is that the Poly I:C/PCP treatment does not produce robust effects on reinforcement learning in the VIFI task.

Lack of practice effects and long-term stability of the performance argues for the use of tests such as the VIFI task in developmental animal models. The possibility to assess behavior repeatedly not only helps to avoid false positive and false negative results, but may be useful in assessing a "disease-like" progression in models of developmental disorders (e.g. in model of neurodegenerative diseases).

Correlation and cluster analyses

Besides repeated testing approach, there are other methods that may also be applied with the goal to increase the model robustness. One possibility is to introduce an intermediate time point in order to bridge the long phase between early treatment and adult testing. Tools that may help to link treatment-related abnormalities in adolescence and adulthood are cluster and correlation analyses.

Since low sample size is one of the most obvious reasons for research results being not robust (Halsey et al. 2015), I have used preliminary results to design a project with the sufficient power to detect effect sizes estimated from previous studies (see section 3.2). Large sample sizes facilitated the planned analysis of correlations between results of different tests, an approach that is rather uncommon in preclinical schizophrenia model research but, may be instrumental in solving the robustness problems of neurodevelopmental models. Further, cluster analysis was conducted in order to confirm results of correlations and presenting another tool.

In schizophrenia, first disease symptoms already manifest during prodromal phase often starting with negative and cognitive symptoms (Davidson et al. 1999;Hafner et al. 1993) and their severity predicts the later breakout of schizophrenia (Davidson et al. 1999). Several previous studies on neurodevelopmental models of schizophrenia revealed behavioral alterations in adolescent rats treated perinatally with PCP or prenatally with Poly I:C. For example, at PND 56-58, rats that received PCP injections shortly after birth demonstrated impairments in social novelty discrimination (Clifton et al. 2013;Terranova et al. 2005). Further, at the age of PND 35-42, acute challenge with either amphetamine or PCP produced stronger hyperlocomotor response in the rats that received perinatally either Poly I:C or PCP compared to vehicle-treated controls (Boctor and Ferguson 2010;Meyer et al. 2008b;Wang et al. 2001). Present results extend these observations to social play behavior. However, somewhat contrary to what is usually expected (e.g. deficits in social interaction in adult animals from PCP treatment groups in Bitanihirwe et al. 2010;Nakatani-Pawlak et al. 2009), effects of perinatal treatment with Poly I:C and neonatal PCP cannot be interpreted as

impairment. Indeed, animals of the two-hit group showed an increase in social play behaviors compared to controls. Somewhat counter-intuitive at the first glance, these results make more sense when considered in a broader context along with other observations discussed below.

When the rats became adult, their locomotor activity in novel environment has been assessed. It was found that spontaneous, unstimulated motor activity in the open field as well as in the center zone was higher in the two-hit group, confirming the results of initial experiments with the two-hit model (see section 4.3.1). Taking advantage of the study being well-powered, I have evaluated correlations between social play behavior in the juvenile rats and their exploratory activity in the open field when they became adult. In line with the project hypothesis, juvenile play behavior and adult locomotor activity were found to be negatively correlated in the two-hit group. Most importantly, those rats that showed the lowest play engagement were most active in the open field in general as well as in the center.

Furthermore, there were differences between two-hit and control groups detected with regard to several other readouts. When these results were analyzed along with the juvenile play behavior, potentially meaningful correlations emerged. Rats with lower social engagement displayed highest brain activity and these negative correlations were again observed in the two-hit group only. High activity of the hippocampus could be the result of hippocampal disinhibition due to decreased GABA levels. Supporting this, adult Poly I:C/PCP treated rats were shown to have a tendency to decreased GAD67 expression in the hippocampus probably resulting in decreased GABA synthesis. However, it has to be noted that GAD67 expression did not correlate with play behavior. Corroborating my data and the hypothesis of disinhibition due to reduced GABAergic signaling, single-hit prenatal Poly I:C and neonatal PCP treatment were also reported to result in a loss of GABAergic markers as well as parvalbumin- and reelin-positive cells in the hippocampus (Bitanihirwe et al. 2010;Kaalund et al. 2013;Nakatani-Pawlak et al. 2009;Radonjic et al. 2013;Richetto et al. 2014).

Therefore, analyses indicated that group differences in individual behavioral or biochemical parameters may be rather difficult to detect and interpret especially for the neurodevelopmental models. Instead of whole group analysis, correlation analysis may be applied to detect heterogeneity within the experimental groups. This conclusion is further strengthened by the cluster analysis that was applied to the juvenile play behavior and that allowed to cluster Poly I:C/PCP treated rats into two groups – a group with very low social play engagement and a group with high social play engagement (based on Euclidian distance analysis). These two groups turned out to be different with regard to open field behaviors, confirming the outcome of the correlation analyses. Interestingly, animals with the low play

engagement were also those that acquired the operant lever pressing faster than the control group. This may indicate aberrant learning due to highly salient stimuli in this group as discussed in section (see section 4.3.3).

Thus, in this project, despite the sufficiently high statistical power, I could detect only modest differences between two-hit and control groups. Behavioral measurements during the adolescent period correlated with certain behavioral and non-behavioral phenomena in the adult rats. Despite receiving identical treatments and being handled in a very similar, standardized way, the two-hit group appeared highly heterogeneous with some animals showing clear signs of impairments (low social engagement, high locomotor activity and high brain activity) and others being at the level of control subjects. Such heterogeneity may be natural for neurodevelopmental models where the interval between the insult application (pre- or early postnatal) and the test (adulthood) usually exceeds several months (see e.g. Vorhees et al. 2012;Vorhees et al. 2015). During this time, various environmental and other factors may interfere with the development and expression of the expected abnormalities. Given that not every human exposed to maternal infection will develop schizophrenia (Brown and Derkits 2010), the inter-individual variability among Poly I:C/PCP treated rats may seen rather natural. However, among subjects identified to be at risk of schizophrenia, the probability of conversion into psychosis was evaluated to be very high (Yung et al. 2008). This study suggests two approaches to handle this heterogeneity in animals based on the administration of early prediction tests (correlation or cluster analyses) that may help select subjects that are more likely to progress to develop impairments in the adult phase. Although these approaches may appear appealing, one needs to caution the necessity of large sample sizes to enable selection of at-risk subjects in such longitudinal neurodevelopmental models.

4.3.3 Developmental animal models trigger re-interpretation of conventional tasks

Apart from an increased general locomotor activity, rats of the two-hit group were also more active in the center region of the open field box than controls (e.g. see section xx). Increased activity in the center region was repeatedly observed in two-hit models combining a genetic manipulation with prenatal Poly I:C treatment (O'Leary et al. 2014;Vuillermot et al. 2012). To my knowledge, there are no studies that assessed the effects of prenatal Poly I:C or neonatal PCP treatment on locomotor activity in the center region using a treatment protocol similar to mine. However, with different treatment protocols for prenatal Poly I:C application in mice and rats, no differences in center activity could be revealed between offspring of treated or control mothers (mice, 20 mg/kg Poly I:C i.p. at GD 9, Li et al. 2014; rats, 8 mg/kg i.p. at GD

14, Vorhees et al. 2012;Vorhees et al. 2015; mice, 20 mg/kg Poly I:C i.p. at GD 12, Xuan and Hampson 2014). This argues for the need of a second hit factor to be added to the prenatal immune stimulation in order to induce enhanced activity in the center zone.

Traditionally, more time spent in the center of the open field is interpreted as reduced anxiety since rats naturally avoid open space and stay more to border regions (thigmotaxis behavior) or hide if possible (Prut and Belzung 2003). Such interpretation, at least in part, is based on observations that anxiolytic drugs like benzodiazepines increase the time spent in open spaces like in the center of the open field (Prut and Belzung 2003) or the open arms of the elevated plus maze (Pellow et al. 1985). Thus, such readouts are commonly used to assess anxiety-like behavior (Prut and Belzung 2003;Rodgers and Dalvi 1997) and to test novel anxiolytic drugs (e.g. de Mello Schier et al. 2014;de Sousa et al. 2015). If such readouts indeed reflect anxiolytic drug action, then the related tests would be successfully used to identify novel treatment opportunities. If drugs affect time spent in the open space via mechanisms not related to anxiolytic effects (e.g. impulsivity enhanced by benzodiazepine drugs, as argued by Thiebot et al. 1985), then the translational value of these tests is limited and may explain lack of true innovative anxiolytic drugs.

Indeed, spending more time in such "to-be-avoided" areas can be interpreted as a sign of impairment because the animals ignore species-specific and ecologically relevant stimuli. This hypothesis has been first proposed by Thiebot & colleagues (Thiebot et al. 1985) but experimental support is still limited although there are several tools available for such analysis (e.g. ethological analysis of behavioral dimensions in the elevated plus maze, Rodgers and Dalvi 1997).

Thus, enhanced time spent in the center of the open field by animals of the two-hit group may indicate impaired attention or responding to ecologically relevant stimuli, a hypothesis that may be further addressed using other types of such stimuli (e.g. social transmission to food preference task, Drew et al. 2007; enhanced acoustic startle response in presence of a predator odor, Endres et al. 2005).

Further, given that schizophrenia is characterized by cognitive deficits, it is assumed that models of schizophrenia-related aspects of pathophysiology should also deliver evidence of cognitive impairment. There are two fundamental problems with this way of thinking. First, cognitive impairments may not be present in every cognitive domain. Second, schizophrenia is often discussed as an aberrant learning state with exaggerated responding to certain "hyper-salient" stimuli. These arguments have two important consequences. On the one hand, under certain conditions, pathological state may be characterized by improved, rather than impaired,

learning and memory. On the other hand, pathological state may be corrected by drugs that impair cognition in healthy, undisturbed subjects. For example, agonists acting at metabotropic glutamate 2/3 receptors impair various aspects of cognition in healthy rats and mice but nevertheless reverse cognitive deficits induced by phencyclidine (Higgins et al. 2004;Moghaddam and Adams 1998).

An autoshaping task, originally developed by Alfredo Meneses (Meneses and Hong 1994), was used in the present studies and further illustrates this point. In the autoshaping task, lever is presented repeatedly shortly before the food delivery, no matter whether the rat approaches the lever and presses it or not. Because of the close temporal relationships between these two events, rats start to explore the lever (pavlovian sign tracking) and occasionally press it which increases the food delivery. This in turn increases the probability of the lever-pressing response emitted, although there is still no need for the animal to press the lever to receive food. Poly I:C/PCP treated animals were found to acquire operant lever pressing faster than the control group (see section 4.3.3) and, at the first glance, these results invalidate the model as they argue against what is usually expected from a model of a disease state. However, it may also point at the directions how to study aberrant learning in laboratory animals.

Aberrant salience learning is discussed as one of the mechanisms underlying generation of psychotic states (Kapur 2003) and is dependent on high DA neurotransmission in striatal regions hypothesized to be generated by a hippocampal disinhibition (Grace 2010;Lodge and Grace 2011). The hypothesis of an increased aberrant salience learning in Poly I:C/PCP treated rats compared to controls could be supported by two lines of evidence. First, the adult Poly I:C/PCP treated animals were found to have a decrease in GAD67 expression in the hippocampus that is indicative for an attenuated GABAergic transmission in this region. Second, offspring from Poly I:C treated females were shown to have an increased striatal DA content (Deslauriers et al. 2013;Hadar et al. 2015;Ozawa et al. 2006).

Results obtained in the present studies, using activity in the center of the open field and the autoshaping task, clearly do not fit the conventional expectations. It may well be that these results argue against the validity of the two-hit model. Or, they may indicate the need for the fresh look at the interpretation of common behavioral tasks, a process that may be very instrumental in enhancing translational value of currently used methods.

4.3.4 Stress as a confounding factor in developmental animal models

Stress is often found to be an important factor that contributes to disease pathology and is, therefore, used to build preclinical models (e.g. Horovitz et al. 2012). However, as mentioned

above, we have intentionally decided against using stress as one of the hit factors because of the difficulties with controlling the stress exposure (within and between laboratories). This may be especially relevant for neurodevelopmental models.

Apart from the use of stress to build the model, it may also have a confounding influence on the model performance. Indeed, there are multiple sources of environmental stress that are poorly controlled but are unavoidable during the critical developmental periods. These stress factors (e.g. related to housing and handling) may interfere with the effects produced by the primary insult factors. For example, locomotion testing was found to be a very robust method to assess the effect produced by Poly I:C/PCP treatment as animals of the two-hit group repeatedly displayed increased locomotor activity compared to controls. However, during the experiments with preventive pregnenolone treatment when rats were exposed to multiple oral applications during adolescence, no increase in the motor behavior of Poly I:C/PCP treated rats was seen any longer (see section 3.3.2). These results point at injection stress as a potential confounding factor that masks the effects of Poly I:C/PCP treatment possibly due to an increased motor activity of control animals.

Stress during adolescence was repeatedly shown to induce brain and behavioral changes (Novick et al. 2013;Saul et al. 2012;Wright et al. 2008). Cruz and colleagues have even reported that repeated restraint stress during adolescence led to a higher sensitivity to amphetamine-induced locomotion dependent on an increased DA level in the Nacc (Cruz et al. 2012).

These findings lead to the conclusion that it is important to identify and control all potentially impactful environmental stimuli for a developmental model, because they can serve as confounding factors especially during the adolescence. Injection procedure may require a particular attention given that the novel treatments may be applied (sub)chronically and it is not always possible to administer the drug via diet or drinking water.

4.4 Potential impact of compensatory mechanisms in developmental animal models

The brain has a strong compensatory capacity. In rodents, brain may restore its function and partially even structure after major insults (e.g. Kelche et al. 1995). One example of such compensatory capacity is provided by the so called cuprizone model that is used to induce massive demyelination in the brain. Cuprizone provided via the diet over several weeks causes olidgodendroglia to undergo apoptosis followed by recruitment of microglia and phagocytosis of myelin (Matsushima and Morell 2001;Wang et al. 2013). Upon termination

of the cuprizone challenge, an almost complete remyelination takes place within weeks (Matsushima and Morell 2001).

Poly I:C/PCP treatment is administered during critical phases of brain development but, when animals mature, there are no obvious abnormalities in general appearance or gross behavior and rather minor differences to control groups seen only using a limited set of specific behavioral tests. There are also rather mild neuroanatomical abnormalities observed in both adolescent and adult animals.

Adult rats of the two-hit group displayed a trend towards lower GAD67 expression levels in the hippocampus compared to controls, indicating reduced GABA synthesis (see section 3.3.1). Similar to Poly I:C/PCP treatment, adult rats treated neonatally with PCP displayed a reduction in GABA signaling in the frontal cortex and hippocampus, indicated by a reduction in the number of parvalbumin positive cells (Nakatani-Pawlak et al. 2009;Radonjic et al. 2013). Further, the GABA system was shown to be highly sensitive to developmental disruptions by prenatal Poly I:C treatment (Bitanihirwe et al. 2010;Harvey and Boksa 2012;Nyffeler et al. 2006). Adult offspring from Poly I:C treated females displayed lower GAD67 levels both on mRNA and protein levels (Richetto et al. 2013;Richetto et al. 2014), a reduction in GABA transporter (GAT) 1 level (Richetto et al. 2014), and a decrease in the number of parvalbumin positive interneurons (Meyer et al. 2008c;Piontkewitz et al. 2012) in the prefrontal cortex and hippocampus, compared to controls, supporting a reduction in synthesis and reuptake of GABA.

Due to the progressive nature of developmental models, I studied changes in the GABAergic system also during adolescence that is often neglected in the prenatal Poly I:C or neonatal PCP models. In contrast to the reduced GAD67 expression in adulthood, an increase in GAD67 expression was apparent in animals of the two-hit group in the hippocampus as well as in the prefrontal cortex when compared to controls at the age of 48/49 days. One may hypothesize that GABAergic changes are secondary to the alterations in other neurotransmitters with GLU being the most likely candidate. One of the plausible hypotheses is that prenatal Poly I:C combined with neonatal PCP treatment lead to increased GLUergic neurotransmission during development, possibly due to the neurotoxic effect of PCP in the early postnatal brains (Wang et al. 2001;Wang and Johnson 2005). This view is supported by changes in the GLUergic neurotransmission due to neonatal PCP treatment such as an increased sensitivity towards NMDA receptor blockade (PND 28-81; Anastasio and Johnson 2008a;Boctor and Ferguson 2010;Broberg et al. 2013) and changes in NMDA receptor composition (PND 28-35; du Bois et al. 2012). In its own turn, prenatal Poly I:C treatment

caused an increased level in GLU in the prefrontal cortex of adolescent rats (PND 56; Roenker et al. 2011) state mediated by combined action of Poly I:C and PCP can trigger a compensatory upregulation of the GABAergic neurotransmission, producing more GABA to defend against the enhanced excitatory drive. And this defense response would be seen early in the development (i.e. increase in GAD67 expression in adolescent Poly I:C/PCP treated animals compared to controls).

However, acute excessive activation of GLU receptors by GLU (Lipton and Rosenberg 1994;Olney and de 1978) and also chronically increased GLU levels (Rothstein et al. 1993;Rothstein and Kuncl 1995;Urushitani et al. 1998) can trigger neuronal death (excitotoxicity). Loss of GABAergic neurons due to chronically elevated GLU levels may eventually be observable later in life. This may explain why GAD67 levels were markedly elevated in adolescent Poly I:C/PCP treated animals but were below the levels of controls when animals became adult. Supportive for this hypothesis, GLU-induced excitotoxicity was indicated to reduce the GAD67 expression in GABAergic neurons (Monnerie et al. 2010;Monnerie and Le Roux 2007). Changes in GABAergic transmission from adolescence to adulthood were also seen in studies on prenatal Poly I:C treatment, where opposite levels of $GABA_A$ receptor subunits in the prefrontal cortex were reported at PND 35 vs 100 (Richetto et al. 2014).

Overall, an increase in GABA levels in adolescent rats treated with Poly I:C/PCP is thought to result from compensatory responses triggered by increased GLUergic signaling. This increased GLU signaling in turn is expected in the long-term perspective to lead to excitotoxicity that explains a decreased GABA signaling in adult rats. Due to the progressive nature upon treatment with Poly I:C/PCP it can be highly recommended to study CNS abnormalities longitudinally in developmental animal models rather than just assessing them at a single time point.

4.5 Preventive treatment

While the long delays between sickness-inducing insults and the testing are likely to have an overall negative impact on the robustness of developmental models, this time window may also have a positive value as it enables development of truly novel therapeutic interventions. Upon developmental insult, the “disease-like” state in animals is slowly progressing. Adolescence, an important phase in development characterized by vast changes within brain systems (Brenhouse and Andersen 2011), is one of the time periods suitable for administering

drug treatment to stop further development of brain pathology and prevent the expression of associated behavioral abnormalities when subjects become adult.

Although there is still no ethical framework for administering such early treatment in young, not yet diagnosed humans, there appears to be a need to develop interventions for subjects in the prodromal phase and at risk to develop schizophrenia. This is based on the fact that cognitive deficits that are common already during this phase are the reason for occupational failure and school/university drop out (Mokhtari and Rajarethinam 2013;Tandon et al. 2009). Findings that an earlier intervention is associated with a better prognosis for schizophrenia (Malla et al. 1999) support the idea of treatment during the prodromal phase. Drug intervention during this phase showed already some efficacy (Mokhtari and Rajarethinam 2013) with the potential neuroprotective agents omega-3 fatty acids reducing transition to psychosis and improving positive, negative and general symptoms as well as functioning in individuals at ultra-high risk for psychosis (Amminger et al. 2010). Due to the fact that infection and inflammation seem to play an important role in the etiology and pathology of schizophrenia, anti-inflammatory drugs have been proposed as an intervention (Keller et al. 2013).

Minocycline is a second generation tetracycline with neuroprotective and anti-inflammatory activity, independent of its antibacterial profile (Zhang and Zhao 2014). Its anti-inflammatory potential was repeatedly demonstrated via inhibition of microglia activation (Martin et al. 2011;Tikka and Koistinaho 2001;Yrjanheikki et al. 1999). Various animal studies linked its anti-inflammatory action to a positive effect on cognition (Biscaro et al. 2012;Hou et al. 2016;Zhu et al. 2014a) and social behavior (Zhu et al. 2014a;Zhu et al. 2014b). Due to its positive effects, minocycline was tested as an add-on to antipsychotics for the treatment of schizophrenia. Several double-blind randomized clinical trials indicated a strong potential for minocycline to improve cognitive and negative symptoms (Chaudhry et al. 2012;Chaves et al. 2015;Ghanizadeh et al. 2014;Khodaie-Ardakani et al. 2014;Levkovitz et al. 2010). It is well-tolerated by patients (Oya et al. 2014), and hence, may be a good candidate for preventive treatment.

Therefore, ability of minocycline to prevent brain pathology produced by Poly I:C/PCP treatment was tested in the present studies. Treatment with minocycline during the adolescent phase (PND 34-47) completely prevented the increase in hippocampal microglia density in adolescent rats (see section 3.3.1). A similar effect was reported by Zhu and colleagues in early adult (minocycline treatment: PND 42-56; microglia assessment: PND 62) offspring from Poly I:C treated mice. Additionally, minocycline prevented behavioral abnormalities

such as locomotor hyperactivity, deficits in social behavior and sensorimotor gating deficits (Zhu et al. 2014b). My data together with the data of Zhu et al. suggest that minocycline may act via inhibition of microglia up-regulation and, although functional significance of these effects are yet to be established, they may be of therapeutic relevance.

Interestingly, minocycline not only prevented an increase in microglia density in Poly I:C/PCP treated rats, but also an increase in GAD67 expression in the hippocampus and prefrontal cortex. Minocycline may have direct effects on GABAergic neurotransmission since it was shown to affect expression of $GABA_A$ receptor subunits (Ahmadirad et al. 2014) and to reduce expression of potassium chloride co-transporter 2 (KCC2) that is known to shift the GABA action from inhibitory to excitatory (Morgado et al. 2011). However, one may also speculate that effects on microglia and GAD67 are mechanistically related. Given the role of microglia in the pruning processes, minocycline may be capable of inhibiting the exaggerated pruning or developmental inflammatory processes that underlie changes in GABAergic signaling.

Another drug with a potential to be used preventively is **pregnenolone** that is a very important endogenous, neuroactive steroid. It was shown to have pleiotropic actions in rodents, including the enhancement of learning and memory, neuritic outgrowth and myelination (Marx et al. 2011). Unlike other neurosteroids, it does not modulate $GABA_A$ or NMDA receptors by itself but functions as a proneurosteroid. This means it is metabolized into other neurosteroids including allopregnanolone and pregnenolone sulfate (Ritsner 2010). Pregnenolone administration results in the elevation of these neurosteroids in the rodents' brain and plasma (Wang et al. 1997) and humans' serum (Marx et al. 2009;Marx et al. 2014).

Further, the pregnenolone metabolites have various functions by themselves. Allopregnanolone potentiates $GABA_A$ receptor response, has neuroprotective effects, increases neurogenesis, decreases apoptosis and inflammation, and modulates hypothalamic-pituitary-adrenal axis. In contrast, pregnenolone sulfate is a positive modulator on the NMDA receptor but decreases $GABA_A$ current (Marx et al. 2011;Maurice et al. 1999). Due to their functions, neurosteroids and especially pregnenolone are proposed as potential therapeutic targets for schizophrenia (reviewed by Keller et al. 2013;Marx et al. 2011;Vallee 2015).

Several studies showed that neurosteroids are implicated in the schizophrenia pathophysiology (Marx et al. 2011). Altered levels of pregnenolone and other neurosteroids were revealed in postmortem brain tissue and serum of schizophrenia patients (Marx et al. 2006c;Marx et al. 2014;Ritsner et al. 2007). Further, different antipsychotics like clozapine or olanzapine dose-dependently elevate pregnenolone and allopregnanolone levels in the rodent

brain (Barbaccia et al. 2001;Marx et al. 2003;Marx et al. 2006a;Marx et al. 2006b). This led to the idea that neuroactive steroid induction may contribute the therapeutic efficacy of antipsychotic treatment (Marx et al. 2006a). Clinical trials suggested efficacy of pregnenolone as an adjunctive treatment to antipsychotics to improve negative and cognitive symptoms of schizophrenia (Kreinin et al. 2014;Marx et al. 2009;Ritsner et al. 2010;Ritsner et al. 2014) as well as to decrease positive symptoms and extrapyramidal side effects (Ritsner et al. 2010;Ritsner et al. 2014). Further, the elevation of pregnenolone and allopregnanolone levels in serum correlated with cognitive improvements (Marx et al. 2009). Similar to minocycline, pregnenolone is well tolerated by patients (Marx et al. 2014;Ritsner et al. 2014) and may, therefore, be a good candidate for preventive treatment of individuals at risk for schizophrenia and psychosis.

In the present studies, treatment with pregnenolone during adolescence (PND 34-47) prevented increased microglia density and GAD67 expression in the hippocampus and prefrontal cortex of adolescent rats treated with Poly I:C/PCP (see section 3.3.1). As mentioned, the increase in GAD67 expression probably leads to an increased GABA synthesis. This increased GABA level could have been a compensatory result of a hyperexcitability of the brain due to increased GLUergic signaling during adolescence (see section 4.4). Allopregnanolone treatment in rodents during puberty was shown to reduce GABAergic transmission in the hippocampal dentate gyrus due to an increased expression of the δ subunit of the $GABA_A$ receptor at this age (Shen et al. 2007). This presents one possible mechanism which may explain a decrease in GAD67 expression upon pregnenolone treatment. However, due to the myriad functions of pregnenolone and its metabolites, other mechanism may be involved in the reduction of GAD67 expression as well.

Similar to minocycline, neurosteroids were shown to have anti-inflammatory potential (Marx et al. 2011). Allopregnanolone and progesterone, for instance, reduced inflammatory cytokines after traumatic brain injury (He et al. 2004). Further, progesterone, allogregnanolone and dehydroepiandrosterone were shown to attenuate microglia activation in different animal models of injury, neurotoxicity or neurodegeneration including spinal cord injury (3 or 21 days treatment with progesterone startling 3 hours after injury), intrastriatal infusion of 1-methyl-4-phenylpyridium (MPP+; con-injection of MPP+ with dehydroepiandrosterone) or the Ncp 1-/- mouse (a model for the Niemann-Pick type C disease; injection of allopregnanolone at PND 7)(Labombarda et al. 2011;Liao et al. 2009;Tomas-Camardiel et al. 2002). A similar anti-inflammatory effect may be induced in

Poly I:C/PCP treated animals by pregnenolone or its metabolites which could be hypothesized to reduced microglia density.

To summarize this part, treatment with minocycline and pregnenolone during adolescence was shown to counteract Poly I:C/PCP treatment effects on microglia density and GAD67 expression completely. Even if the society is not ready yet to consider preventive treatments for diseases such as schizophrenia (Bosanac et al. 2010;Buckley and Miller 2015;de Koning et al. 2009), potential approaches to develop preventive treatment interventions should be studied. Due to the preclinical preventive treatment potential of minocycline and pregnenolone as well as the positive effects on negative and cognitive symptoms and good tolerability in schizophrenia patients, they may have a value as a therapeutic intervention of subjects with high risk to develop schizophrenia and psychosis.

4.6 Exploratory vs confirmatory studies

There are many concerns about the way preclinical studies are performed and reported for the development of novel drugs. The vast majority of drugs that are safe and efficacious according to preclinical animal studies fail in clinical trials. This growing evidence has prompted analysis of the current approaches to design and perform preclinical studies. There were two conceptually different study designs identified for analysis of translational failures - exploratory vs confirmatory studies. It was found that most of the published studies are performed in an exploratory mode that is believed to be insufficient to support translational medicine efforts.

The basic idea of exploratory studies is the generation of novel hypotheses and collection of preliminary data supporting or refuting these hypotheses. Hypotheses may relate to disease mechanisms, development of a model or a test, drug effects or any other aspect of biomedical research that may later need to be confirmed and translated. Exploratory studies are typically small, may only rely on *a priori* power analysis (if at all, given that the effect size may not be known), have flexible designs and, what is most important, may require creative approaches to data analysis and presentation. The main goal of these studies is enable maximum freedom of scientific endeavor.

On the contrary, confirmatory studies resemble clinical trials in a sense of being well powered as well as using pre-described designs (using conventional study tools), pre-defined hypotheses, and pre-specified endpoints. Whether the confirmatory study is testing drug effects or not, such experiments are built on hypotheses indicated by exploratory studies and their outcome is expected to provide ultimate proof of these hypotheses. In the context of drug

discovery, confirmatory studies are meant to avoid advancing ineffective treatments into clinical testing phase. Unlike exploratory studies that may pursue several hypotheses simultaneously, confirmatory studies should clearly identify the primary outcomes to focus on (Kimmelman et al. 2014).

As a consequence to small sample sizes typical in exploratory research, measured effect size may appear larger than the true effect size is (i.e. false positive results) (Halsey et al. 2015). To estimate the latter, experiments need to be replicated using the original sample size until the positive predictive value reaches the desired level, or use a power analysis to design a confirmatory study with a sufficiently large sample size in order to support the interpretation of the study results (Lenth 2001). Confirmatory studies have an advantage of being able to use the results of exploratory experiments to support calculation of the effect size.

In my studies, Poly I:C/PCP treatment was found to increase density of microglia, and this effect could be completely prevented by a 14-day long treatment with pregnenolone during adolescence (see section 3.3.1). This study was performed in an exploratory mode but, based on its results, the number of rats needed to see an effect was calculated and a confirmatory study was designed with the aim to replicate the preventive effect of pregnenolone on microglia density (see section 3.3.2). To do so, a stricter randomization paradigm for the pups at time of re-housing (about one week after weaning) was chosen. Whereas in the exploratory study the cage mates were selected to be from the same litter (siblings), this was controlled in the confirmatory study, and cage mates were chosen to be from different litters. This aimed to reduce a possible confounding litter effect. Preventive treatment application and ex vivo experiments were blinded for both exploratory and confirmatory studies: group assignment was kept blind for the preventive application and ex vivo analysis was blinded at all steps (slicing, staining and analysis). Confirmatory study reproduced the effects of Poly I:C/PCP treatment on microglia density but pregnenolone treatment was no longer effective in reversing effects of Poly I:C/PCP. Conventional logic suggests that there were two studies, of which one was positive and another negative, and further studies should be conducted in order to support positive or negative evidence. However, the confirmatory study approach assigns a higher value to the second study that had a clear, pre-specified focus on a well-defined outcome that was not supported by an appropriately powered study.

Use of confirmatory studies may be different in other areas of biomedical research but for the field of preclinical research on novel therapeutic interventions, this approach is currently the best recommendation. The general paucity of drug efficacy tests conducted in a confirmatory

mode may be one of the important contributors to preclinical-to-clinical translation failures resulting in high costs and waste of resources (Kimmelman et al. 2014).

4.7 Critical discussion of the Poly I:C/PCP model and future perspectives

Treatment with prenatal Poly I:C and neonatal PCP engages two types of mechanisms that likely contribute to mechanisms underlying pathophysiology of schizophrenia. Therefore, combining these two factors in one model has an advantage of achieving a higher level of construct validity. For the purpose of my work, this combination also served the goal of a two-hit approach producing more robust alterations compared to single-hits. Indeed, while there are a number of single-hit models proposed, there is often not much follow-up research published, even for the genetic models that can be expected to be more robust and reproducible than models based on environmental manipulations. Thus, building a two-hit model requires to take this factor into account and to focus on individual hits that are most commonly researched. While the present results may argue for the value of this two-hit approach, my work also revealed several limitations inherent in such models.

Two closely related factors that have to be seen as critical are the sample size needed to sufficiently power a study and the strength of the sickness effects produced by Poly I:C and PCP treatment. Prenatal treatment with Poly I:C or saline may lead to miscarriage due to injection stress (see section 3.1.2) while immune stimulation itself results in some cases in very strong sickness state promoting the euthanasia decision. Further, Poly I:C treated females commonly deliver a lower amount of pups (Ballendine et al. 2015;Yee et al. 2011). This illustrates the very strong effect due to the prenatal treatment that needs to be seen critically from the animal welfare prospective. Indeed, routine use of developmental models can result in a high number of pregnant females needed in order to design a study with an appropriately high number of offspring. Required sample sizes are expected to be rather high, much larger than in acute studies, due to a relatively low effect size of such models.

In the present studies, effects of Poly I:C/PCP treatment on behavior and CNS anatomy were rather low, even though the studies were well powered. This limited effect size of the treatment is the result of a high inter-individual variability within groups inherently seen in developmental animal models. In order to reduce the inter-individual variability, one can follow several strategies.

For instance, a third hit factor could be added as it was suggested elsewhere (Chen et al. 2011). This approach as well as the attempts to increase the dose of Poly I:C and/or PCP, however, cannot be recommended. Sickness induced by these treatments, acutely and in

combination, may not be well tolerated and will result in the loss of animals that should clearly be avoided.

To decrease inter-individual variability, one could also think of the use of animals with a more homogenous genetic background or with a genetic predisposition that could enhance certain aspects of Poly I:C/PCP treatment. Therefore, I examined two inbred strains, Lewis and Fischer rats, with Fischer rats demonstrating altered responding to DAergic manipulations (Kuribara 1983). However, pregnant females of the Lewis and Fischer strains had a very high abortion rate upon treatment with Poly I:C or saline (see section 3.1.2) and, therefore, these strains could not be used further.

One certainly needs novel tools to overcome the robustness problems that are seen not only in the two-hit animal model but also other developmental models. Several of these novel tools were explored and characterized in this thesis.

In order to increase model robustness even with the rather low overall effect size of the two-hit model, I focused on longitudinal aspects of the developmental models. On the one hand, developmental models require functional and other measurements to be made repeatedly in order to monitor the development of pathology and to support characterization of treatment interventions. This argument highlights the need for functional tests that do not suffer from practice effect. An example of such a test is the novel VIFI task that, as demonstrated in this thesis, enables long-term monitoring of reinforcement learning. On the other hand, I have introduced correlation and cluster analyses. These methods offer unique tools to identify animals that are most affected by the developmental insults. Most importantly, currently presented approaches suggest to select animals with the strongest "phenotype" at a rather early age of the subjects, avoiding any potential biases and enabling truly prospective studies. This approach could help to develop novel therapeutics because only truly affected animals are used but not the whole population, which reduces the inter-individual variability to an acceptably low level.

Despite the challenges mentioned above, the Poly I:C/PCP model can be recommended for the further development and validation. Developmental models not only help to understand the impact of factors implicated in the pathophysiology of schizophrenia but are also the key to study novel therapeutic approaches.

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