Behavioral characterization of two hit mouse models of schizophrenia: Tcf4-4-fl × Camk2a-Cre/social defeat, Tcf4-4-fl × Emx1-Cre/poly(I:C), and Bmal1-ko

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To my family

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ABBREVIATIONS

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analyses of variance
APA	American Psychiatric Association
AUD	Alcohol use disorder
CDA	Canonical Discriminant Analysis
cko	Conditional knockout
CNVs	Copy number variants
C_T	Cycle threshold
DSM-5	5th version of the Diagnostic and Statistical Manual of Mental Disorders(DSM-5)
E-box	Ephrussi-box(DNA-response element, protein binding site)
ED	Embryonal day
ELC	Early live complication
FDR	False discovery rate
fl	Floxed
GD	Gestational day
GWAS	Genome Wide Association Study
ICD-11	11th version of the International Statistical Classification of Diseases and Related Health Problems
IQR	Interquartile range
MANOVA	Multivariate analyses of variance
NIMH	National Institute of Mental Health
NMDA	*N*-methyl-*D*-aspartate
PD	Postnatal day
PRS	Polygenic risk score
PsyCoP	Platform for systematic behavioral and cognitive profiling
PTHS	Pitt-Hopkins-Syndrome
RDoC	Research Domain Criteria
rec	Recombined
RGE	Relative gene expression
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
SUD	Substance use disorder

SVD	Singular Value Decomposition
tg	Transgenic
TLR3	Toll-like receptor 3
VTA	Ventral tegmental area
WHO	Word Health Organization
wt	Wildtype

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I. INTRODUCTION

Schizophrenia is a psychiatric disorder, affecting around 1% of the world wide population (OWEN *et al.*, 2016). This psychiatric disorder leads from mild to severe limitations in the daily live. The classification of symptoms and the diagnosis of schizophrenia follows two different manuals: The 11th version of the International Statistical Classification of Diseases and Related Health Problems (ICD-11), published by the Word Health Organization (WHO), is widely used all over the world. The 5th version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) published by the American Psychiatric Association (APA) is mainly used in the US (AMERICAN PSYCHIATRIC ASSOCIATION, 2022; WORLD HEALTH ORGANIZATION, 2023a). Common symptoms of schizophrenia are delusions, disordered movements, hallucinations, disturbed motivation, reduced sociability, and cognitive impairments (NATIONAL INSTITUTE OF MENTAL HEALTH (NIMH), 2022). For the symptomatic treatment of schizophrenia, evidence-based non pharmacological treatments like psychosocial interventions are used, as well as pharmacological treatments, like suitable neuroleptics (AMERICAN PSYCHIATRIC ASSOCIATION, 2020). The currently available drugs often have side-effects such as extrapyramidal syndrome, agranulocytosis, or weight gain (JM DAVIS & CASPER, 1977; SEDHAI *et al.*, 2022; LEUCHT *et al.*, 2023). Additionally, not all symptoms can be rescued by the commonly used neuroleptics and around 30% of schizophrenic patients do not respond to pharmacological treatment (MELTZER, 1997; LEUCHT *et al.*, 2009). The pathophysiology of schizophrenia is mostly unknown. Different hypotheses include changes in specific pathways of dopamine, glutamate, and serotonin receptors (STAHL, 2018). A working group of the National Institute of Mental Health (NIMH) developed since 2010 Research Domain Criteria (RDoC) to categorize the underlying factors of mental disorders instead of strictly symptomatic categories (CUTHBERT, 2009, 2022; INSEL *et al.*, 2010). These RDoC domains were included in the analysis of behavioral tests in mice by our department (VOLKMANN *et al.*, 2020). To improve the research on pathophysiology of schizophrenia in animal models, the analysis of behavioral patterns should be addressed in order to evaluate the impact of risk factors on sensorimotor gating, cognition, or exploration in mice (KAS, KAHN, *et al.*, 2011).

In our laboratory under the supervision of Moritz Rossner, mouse models for schizophrenia and other psychiatric disorders are used to improve and speed up drug development.

In this study the main goal was to analyze the behavior of specific mouse lines carrying risk genes for schizophrenia in combination with a stress paradigm compared to the corresponding control groups. The behavioral analysis uses an already published behavioral test battery, called platform for systematic behavioral and cognitive profiling (PsyCoP). This platform consists of different behavioral tests, that cover a broad spectrum of behavioral phenotypes in mice (VOLKMANN *et al.*, 2020). The project included the following animal models:

1. Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat
2. Tcf4-4-fl × Emx1-Cre mice subjected to maternal immune activation through poly(I:C) injections
3. Bmal1-ko mice

II. LITERATURE

1 History of schizophrenia

Schizophrenia was first described as "dementia praecox" in 1896 by Emil Kraepelin (KRAEPELIN, 1896). Together with „manic depression“, these two diseases were subsumed under the term "psychosis". The descriptions of Kraepelin were mainly focused on negative symptoms like limited emotions and impaired mental abilities (KRAEPELIN *et al.*, 1919). Later, the term "schizophrenia" was established in 1911 by Eugen Bleuler. Bleuler categorized the symptoms into two groups: typically symptoms, which appeared in each case of schizophrenia and a second group that included symptoms, which are also described for other diseases (BLEULER & ZINKIN, 1950). A classification of symptoms can also be found in early hypotheses of schizophrenia. A loss of function was defined as negative symptom. Positive symptoms were described as excessive function of neuronal processes (HUGHLINGS-JACKSON, 1931). The first "International Pilot Study of Schizophrenia" took place from 1985 to 1998 in nine different countries. The findings of this initial study, as well as the results of the follow up studies, led to a more detailed clinical picture of schizophrenia (SARTORIUS *et al.*, 1972).

2 Causes and consequences of schizophrenia

2.1 Epidemiology of schizophrenia

Schizophrenia is a psychiatric disorder, that affects around 1% of the world`s population (OWEN *et al.*, 2016). Many factors influence the onset, course, and outcome of the disease. Studies identified associations between specific behaviors and schizophrenia, like smoking or addiction (DIXON, 1999; LASSER *et al.*, 2000). Epidemiology was, and is, a very important aspect of schizophrenia research. One important long term study, called "ABC study", took place from 1985 to 1998 in order to analyze the age (A), beginning (B), and course (C) of schizophrenia (HÄFNER *et al.*, 1998).

2.1.1 Incidence

The term "incidence" refers to the total number of individuals, who develop a specific disease during a particular time period, usually during a month or a year (AVENUE *et al.*, 2012). The Global Burden of Disease study is quantifying health

loss by diseases, injuries, and risk factors. According to them 1.29 million new cases of schizophrenia were reported worldwide in 2019. These cases were divided into 590,000 male cases and 700,000 female cases (INSTITUTE FOR HEALTH METRICS AND EVALUATION, 2020).

2.1.2 Prevalence

Prevalence is defined as the proportion of a population that shows a specific characteristic in a given time period (PORTA, 2008). Applied to diseases, it describes the total number of individuals in a population that suffers from an illness during a specific period of time, usually expressed as a percentage of the population (AVENUE *et al.*, 2012).

The prevalence of schizophrenia is the rate of diagnosed schizophrenic patients in a population at a specific time point and can be used as a measure for the frequency of the illness. The lifetime prevalence of schizophrenia is 4.0 per 1000 individuals. The prevalence can be influenced by different factors like sex, living conditions, or migration. Immigrants have a higher risk than native-born people (SAHA *et al.*, 2005). Gender differences are an often analyzed topic in schizophrenia research, especially comparing the disease onset, course of episodes, and symptoms of schizophrenia between male and female patients. The main difference between man and women is found for the age of onset: In men, the disease begins 3 – 5 years earlier than in women (ANGERMEYER & KÜHNZ, 1988). An incidence peak can be found between 15 and 25 years of age for men and 15 and 30 years of age for women. The incidence curve in men shows a higher peak than in women. After the male incidence peak, the curve decreases constantly, while for females a second peak occurs between 45 and 49 years (HÄFNER *et al.*, 1998). Male patients are suffering more often from negative symptoms, while females showed more positive symptoms (LIEBERMAN *et al.*, 1993). Substance abuse, especially alcohol abuse, is commonly associated with the diagnosis schizophrenia. The cumulative prevalence for substance abuse is higher in male patients compared to females (HÄFNER *et al.*, 1998). In particular, smoking is more common in schizophrenic patients than in healthy people. The probability of becoming a current smoker is higher if a mental illness has been diagnosed (LASSER *et al.*, 2000). Sociability and social life can also be influenced by schizophrenia: the rate of unemployment, living alone and being single is increased compared to healthy controls (STILO *et al.*, 2013).

2.2 Symptoms and diagnosis

According to the WHO, a mental disorder can be diagnosed as schizophrenia when at least two of the following symptoms are present for one month or longer. Schizophrenic symptoms are persistent hallucinations, delusions, disorganized behavior or thinking, experiences of influence, passivity or control, avolition, asociality, anhedonia, and disturbed psychomotor activity. It must be excluded, that the symptoms occur because of other reasons, such as a brain tumor, or medication (WORLD HEALTH ORGANIZATION, 2023b). These different symptoms are usually categorized as positive, negative, and cognitive symptoms (EDWARDS *et al.*, 2016). Hallucinations, delusions, disordered thoughts, and uncoordinated movements are summarized as positive symptoms. Hallucinations are defined as seeing, hearing, smelling, tasting, or feeling things, which are not real. Especially hearing voices is very common. Delusions are disturbed perceptions: Patients may be thinking, that they are concerned by objectively neutral situations, e.g. messages from television are aimed at them. Disorganized thoughts are also common: conversations with patients may include interruptions and switching topics. Coordination and motor skills can also be affected by schizophrenia: unusual gestures occur as well as repeated movements. The negative symptom complex contains any problems related to motivation, enjoyment, and sociability. Cognitive limitations affect memory, attention, and concentration. Paying and maintaining attention can be very hard for patients. The ability to apply new information is reduced and the processing of information is affected, including decision making (NATIONAL INSTITUTE OF MENTAL HEALTH (NIMH), 2022). For specific diagnostics and to classify the stage or course of schizophrenia the ICD-11 can be used. The ICD-11 distinguishes between the first episode of schizophrenia, multiple episodes of the disease, and a continuous variant is listed. The different disease variants can also be categorized according to the presence or absence of current symptoms: there can be current symptoms, a partial remission, a full remission, or an unspecified phase (WORLD HEALTH ORGANIZATION, 2023b).

2.3 Co-morbidity and mortality

Schizophrenic patients often also suffer from other diseases and symptoms simultaneously. As described above, the risk of having additional disorders like smoking or alcohol use disorder (AUD) is higher in schizophrenic patients compared to healthy people (HÄFNER *et al.*, 1998; LASSER *et al.*, 2000). Nearly

50% of schizophrenic patients are suffering from a substance use disorder (SUD). Out of patients diagnosed with schizophrenia or schizophreniform disorder, 33.7% suffer from an alcohol use disorder, and 27.5% suffer from another drug abuse disorder. (REGIER, 1990). The period of drug abuse was longer in male than in female patients. There are also gender specific differences in sociability; women maintained more social contacts than men and had an increased number of children. Gender differences in dual-diagnosed-patients exist also for the crime rate, which is higher in men suffering from schizophrenia and substance use disorder (BRUNETTE & DRAKE, 1997). Recovery in case of a dual diagnosis can take a long time. A long-term study investigated the course of treated dual-diagnosed patients over 10 years. Recovery of clinical symptoms and improved living conditions were observed after three years. The recovery rate increased in the following seven years (DRAKE *et al.*, 2006).

Other psychiatric comorbidities often occur with schizophrenia. The prevalence of depression in addition to a diagnosed schizophrenic disorder is about 28.6% (W LI *et al.*, 2020). Depression in schizophrenic patients is difficult to diagnose and can occur at all stages of the illness. Several differential diagnoses for depression have to be excluded. Depressive symptoms, like anhedonia, asociality, and attentional impairment can be mistaken as symptoms of the negative symptom complex of schizophrenia. The depressive subtype of a schizoaffective disorder is also similar to a depression. Misinterpretation of schizophrenic symptoms as demoralization or as a substance use disorder is also common. Symptoms can also overlap with an antipsychotic-induced pseudo-parkinsonism or with dysphoria (NAGUY, 2018). The frequency of depressive symptoms does not correlate with the duration of the illness. An Increase of depressive symptoms seem to be associated with psychotic episodes of schizophrenia (KOREEN *et al.*, 1993; AN DER HEIDEN *et al.*, 2005). Because of increased burden of disease for those double diagnosed patients, a clear diagnosis and an adjusted treatment based on antipsychotics and antidepressant medication is necessary. In addition to that, a psychological therapy including cognitive and behavioral aspects as well as psychological interventions like psychoeducation or family therapy should be applied (UPTHEGROVE, 2009).

The consequences of the additional illness, like depression or substance use disorder, and schizophrenic symptoms shorten the lifetime of patients. The main reasons for death in schizophrenic patients are suicide, cancer, and cardiovascular

disease. The suicide rate is higher in younger patients and at the beginning of schizophrenia (ÖSBY *et al.*, 2000). Cardiovascular diseases such as arterial hypertension are very common in schizophrenic patients. Arterial hypertension can also be an unwanted side-effect of the treatment with antipsychotics (DE CARLO *et al.*, 2023; SUDARSHAN & CHEUNG, 2023).

The potential life time in schizophrenic patients is reduced by 13 to 15 years compared to the average age of healthy people. Men showed a shorter potential life time, up to 8 years shorter than female patients (HJORTHØJ *et al.*, 2017).

2.4 Prediction of disease progression

Usually, there is a prodromal phase before symptoms manifest and before the mental disorder is diagnosed. According to the ABC study, in 75% of the cases a prodromal phase occurred with single symptoms that started up to five years before the first schizophrenic episode was diagnosed (HÄFNER *et al.*, 1998). The clinical phases of schizophrenia can manifest in different ways and at different time points. The course and recovery of schizophrenia showed a high variability between studies, because a general definition for recovery and consistent criteria for convalescence are missing (WING, 1988). The recovery rate after the first episode of illness is 83%. Recovery was defined as a lack of symptoms for 16 weeks. There were no negative symptoms and no or only mild positive symptoms left (LIEBERMAN *et al.*, 1993). In addition to the clinical recovery, the functional recovery can also be included to assess recovery. The median functional recovery rate is 13.5%, independent of sex (JÄÄSKELÄINEN *et al.*, 2013).

Because of the inhomogeneity of recovery definitions, suggestions for a better scale were developed (LIBERMAN & KOPELOWICZ, 2005). An early example is the Social Functioning Scale, which includes questions about the individual and social behavior of the patients (BIRCHWOOD *et al.*, 1990).

3 Treatment

According to the American Psychological Association, treatment of schizophrenic patients should include a treatment plan with evidence-based non-pharmacological and pharmacological treatments. During pharmacological treatment, monitoring for side effects and effectiveness should be established. Also proceeding with the same medication after success is recommended. The use of the antipsychotic clozapine is

indicated when there is a treatment resistance or an existing risk for suicide attempts or aggressive behavior. For non-pharmacological treatment, several psychosocial interventions are suggested including permanent contact to the family and intervention sessions (AMERICAN PSYCHIATRIC ASSOCIATION, 2020). Over the last decades, neuroleptics were the preferred group of pharmaceuticals for treatment of schizophrenia (MÖLLER, 2005). Neuroleptics, also known as antipsychotics, are suitable for the treatment of a wide range of psychiatric disorders. The term "antipsychotic" is more common, because the effect of these drugs is better described by this phrase. Antipsychotics are used to treat acute psychotic, manic, and psychotic-depressive disorders, as well as agitation states associated with delirium and dementia. They are also used as a long-term medication for chronic psychotic disorders, including schizophrenia, schizoaffective disorder, and delusional disorders. Antipsychotics antagonize the dopamine receptor D_2 in the central nervous system. Typical antipsychotics also antagonize serotonine, histamine, muscarinic, and adrenergic receptors (VIDAL MMI GERMANY GMBH, 2023). Other dopaminergic pathways are also blocked by typical antipsychotics, such as the nigrostriatal pathway. Blocking receptors of this pathway lead to disturbances in movements and coordination (MELTZER & STAHL, 1976; STAHL, 2000). Typical antipsychotics, or first-generation antipsychotics, were developed in the 1950s based on the effects of chlorpromazine and haloperidol (CARLSSON & LINDQVIST, 2009; WEINBERGER & HARRISON, 2011). Consequently, several groups of antipsychotics were used to rescue the positive symptoms of schizophrenia such as delusions and hallucinations. In particular phenothiazines (e.g. chlorpromazine), thioxanthene derivatives and butyrophenones (e.g. haloperidol), as well as the dibenzoxazepines such as loxapine and the indoles such as molindone. A general advice for the dosage of first-generation-antipsychotics is missing, because there are differences in drug levels in blood and therapeutic outcomes between patients. This group of drugs leads to mild to severe side effects. The most common side effects are sedation and the extrapyramidal syndrome (JM DAVIS & CASPER, 1977). The extrapyramidal syndrome affects speech and spontaneous motor activity. The symptoms are categorized into hypokinetic and hyperkinetic symptoms. Hypokinetic symptoms are a consequence of short term exposure of dopamine blockers while hyperkinetic symptoms only occur after chronic administration of dopamine blocking drugs (SANDERS & GILLIG, 2012).

To avoid these severe side effects, the use of second generation neuroleptics was preferred (LEUCHT *et al.*, 2009). Antipsychotics of this group are structurally very heterogeneous. Examples of atypical antipsychotics include aripiprazole, clozapine and risperidone (VIDAL MMI GERMANY GMBH, 2023). Despite the advantage of a reduced extrapyramidal syndrome, the second generation antipsychotics cannot ameliorate the negative or cognitive symptoms of schizophrenia (LEUCHT *et al.*, 2009). Studies took place to validate efficiency of different drugs. In 2023 a meta-analysis compared the success of treatment over at least six months between all second-generation and 18 first-generation antipsychotics. Overall changes in symptoms, positive and negative symptoms and depressive symptoms were included. In rescuing symptoms, olanzapine, a second-generation neuroleptic, showed a higher efficiency compared to the other drugs. But patients treated with olanzapine showed a higher weight gain than patients treated with other antipsychotics (LEUCHT *et al.*, 2023). In this study resistant patients were excluded, but 30% of schizophrenic patients are treatment resistant. Treatment resistance is defined as no successful treatment for three or more periods of treatment with at least two different classes of neuroleptics including treatment with at least 60 mg/day haloperidol, no restoration of good functioning within five years, and very high levels of psychopathology according to Brief Psychiatric Rating Scale and Clinical Global Impressions criteria (MELTZER, 1997). According to the APA, clozapine should be used if the patient show resistance against current treatment (AMERICAN PSYCHIATRIC ASSOCIATION, 2020). Clozapine is a serotonin and a D_2 antagonist (MELTZER, 1989; FARDE *et al.*, 1994). The effect on D_1 receptors is not clearly verified. Experiments in rats showed an agonistic effect of clozapine (AHLENIUS, 1999). The affinity for D_1 and D_2 receptors is similar. But in comparison to risperidone, olanzapine and quetiapine, clozapine showed a higher affinity to D_1 receptors and a lower affinity to D_2 receptors than risperidone and olanzapine. These differences between other second generation neuroleptics and clozapine can cause the ability of improving the outcome in treatment resistant schizophrenia (TAUSCHER *et al.*, 2004). Affinity for dopamine D_4 receptors is also described as well as the ability to influence noradrenergic pathways (SEEMAN, 1992; A. BREIER, 1994; Alan BREIER *et al.*, 1994). Blood levels of norepinephrine and dopa were increased and the heart rate was higher in patients treated with clozapine after five weeks of treatment (Alan BREIER *et al.*, 1994). During treatment with clozapine mild to severe side effects may occur.

Weight gain during treatment with clozapine or olanzapine is higher than during treatment with amisulpride, asenapine, iloperidone, paliperidone, quetiapine, risperidone, sertindole, aripiprazole, lurasidone or ziprasidone (MUSIL *et al.*, 2015). The most severe, but also rare side effect is agranulocytosis. The comparison of 260,948 clozapine-treated patients between 1984 and 2018 showed a prevalence for agranulocytosis of 0.4% and a prevalence of death caused by agranulocytosis of 0.05% (X-H LI *et al.*, 2020). In agranulocytosis the blood level of neutrophils is reduced, which increases the risk for severe infections. Monitoring and adjusting the therapy of affected patients protect the patients of severe courses (SEDHAI *et al.*, 2022).

4 Research Domain Criteria (RDoC)

Commonly used classification systems for mental disorders are provided by the WHO with ICD-11 and by the APA with the DSM-5 (AMERICAN PSYCHIATRIC ASSOCIATION, 2022; WORLD HEALTH ORGANIZATION, 2023a) The categories and the structure of DSM-5 are similar to the early version of 1980. The focus was more on symptoms and the number of symptoms than on the pathomechanisms. From a current point of view, this approach is not useful anymore, in particular for the research of the pathophysiology underlying schizophrenia (CUTHBERT, 2022). Using symptoms to categorize the different severities and types of schizophrenia can be complicated. Patients, which show just a few common symptoms can have the same diagnosis, despite the underlying pathomechanisms being completely different. A strict separation of mental disorders limits the possibility of finding similar causes for different psychiatric disorders. Commonly, more than one mental disorder can occur in one patient. But with an exact assignment of symptoms to a diagnosis, co-morbidity is underrepresented. Standardized conditions in patient studies lead to an exclusion of patients suffering from various symptoms. In order to focus on the underlying causes, RDoC domains were introduced by the NIMH in 2013. Six categories were listed, based on human neurobehavioral function. These categories, called major functional domains, include domains for arousal/regulatory, sensorimotor, cognitive parts, social processes, negative, and positive valence (CUTHBERT, 2009).

5 Underlying mechanisms of schizophrenia

The underlying genes and biologic mechanisms leading to schizophrenia are still unknown. Different hypotheses exist in order to explain the causes and consequences of this mental disorder. Schizophrenia has been explained by a dysfunction of the glutamatergic, serotoninergic, or dopaminergic system (STAHL, 2018).

5.1 Dopamine hypothesis of schizophrenia

A very early hypothesis was that the dopaminergic mesolimbic pathway is more activated in schizophrenic patients. This pathway extends from the ventral tegmental area (VTA) of the mesencephalon to the ventral striatum and is assumed to result in auditory hallucinations and delusions. This assumption is supported by pharmacologic evidence: On the one hand drugs that decrease dopamine activity have antipsychotic properties. On the other hand, amphetamine, a drug increasing dopamine activity, can cause psychotic episodes (MELTZER & STAHL, 1976; SIMMLER & LIECHTI, 2018). According to this hypothesis, the pathways of the dopaminergic system play a key role. The mesolimbic dopaminergic pathway, as well as the nigrostriatal, mesocortical, and tuberoinfundibular pathway are formed by the axons of dopaminergic neurons. Dopaminergic neurons built the mesolimbic pathway from VTA to the nucleus accumbens in the ventral striatum, as well as to the amygdala and the hippocampus. The nucleus accumbens is important for the reward system. Amygdala and hippocampus are parts of the limbic system and influence emotion and memory formation (BLAESS *et al.*, 2020; KATOLIKOVA & GAINETDINOV, 2021). The connection between the dorsal striatum (caudate nucleus and putamen) and the substantia nigra pars compacta is known as the nigrostriatal pathway. This pathway is important for regulation of movements. The VTA and the prefrontal cortex are also connected via dopaminergic axons. This mesocortical pathway is involved in cognition and emotion. Prolactin is released via the tuberoinfundibular pathway. Dopaminergic axons combine the hypothalamus and the median eminence (KATOLIKOVA & GAINETDINOV, 2021). Dopamine blocking substances influence all these pathways and can therefore cause unwanted side effects (MELTZER & STAHL, 1976; STAHL, 2000).

5.2 Glutamate hypothesis of schizophrenia

Glutamate is part of important signaling pathways. In addition to its role in signaling, it also affects the metabolism and oncogenic mechanisms. Glutamate is able to activate ionotropic and metabotropic glutamate receptors. The ionotropic receptor subgroups include the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptor subfamilies (WILLARD & KOOCHEKPOUR, 2013). It is assumed that an hypofunction of NMDA receptors leads to schizophrenia (STAHL, 2018). Substances like phencyclidine and ketamine are interacting with the NMDA-type excitatory amino acid receptor. They are able to inhibit that receptor and affect neurotransmission. Subjects treated with phencyclidine and ketamine show effects similar to a psychosis (JAVITT & ZUKIN, 1991). Ketamine, a NMDA antagonist, worsened the function of working memory in the prefrontal cortex of rats after intraperitoneal injection (VERMA & MOGHADDAM, 1996). Patients suffering from schizophrenia show reduced levels of glutamate in the medial prefrontal cortex (SMUCNY *et al.*, 2021). This supports the assumption that glutamate plays a key role in the pathogenesis of schizophrenia (STAHL, 2018).

5.3 Serotonin hypothesis of schizophrenia

Based on these two hypotheses, treatment of schizophrenic patients was adjusted. Dopaminergic receptors can be blocked by suitable drugs in order to treat psychosis (STAHL, 2000). Drug development focused on the mechanisms of second-generation antipsychotics, such as clozapine, quetiapine, olanzapine, risperidone, and ziprasidone. These novel antipsychotics were able to reduce psychosis without showing serious side effects like the extrapyramidal syndrome (MELTZER & MCGURK, 1999). Their mechanism seemed to be based on the antagonism of serotonin receptors, so they have a weaker D_2 receptor antagonism, but a stronger 5-HT_{2a} antagonism (MELTZER *et al.*, 1989). For example the serotonin antagonist lumateperone has an high affinity to 5-HT_{2a} receptors and a lower affinity for D_2 and D_1 receptors (RE DAVIS & CORRELL, 2016). Administration of lumateperone leads to a significant improvement of symptoms compared to the placebo control group (CORRELL *et al.*, 2020). Different serotonin receptor families can be found in the whole body. Either they are ligand-gated ion channels (5-HT_3 receptor) or they are G protein-coupled receptors (5-HT_1; 5-HT_2; 5-HT_4; 5-HT_6; 5-HT_7) (BRADY *et al.*, 1999). The 5-$HT_{2\alpha}$ receptor is mainly located in the

cortex and the hippocampus (HOYER *et al.*, 1986). These receptors are expressed in cortical and hippocampal pyramidal glutamatergic neurons (HIROSE *et al.*, 1990) and in GABAergic interneurons (WILLINS *et al.*, 1997). The serotonin receptors in schizophrenic patients differ from the receptors in the healthy control groups. The density of 5-$HT_{1\alpha}$ receptors was increased in the dorsolateral prefrontal cortex. The number of binding sites in 5-$HT_{2\alpha}$ receptors was reduced in the parahippocampal gyrus. Receptor densities in the dorsolateral prefrontal cortex were also reduced (ARORA & MELTZER, 1991; BURNET *et al.*, 1996).

6 Risk factors

6.1 Genetic risk factors

The heritability of schizophrenia amount to 70 – 95%, depending on the particular study (SULLIVAN *et al.*, 2003). But there is still a lack of information concerning distinct causal genes and pathologic pathways. In order to identify potential risk genes for schizophrenia, genome wide association studies (GWAS) were performed. In 2022, the results of the largest genome-wide association with 76,755 schizophrenic patients and 243,649 controls were published. A higher risk for schizophrenia was associated with 342 single nucleotide polymorphisms in 287 genomic loci (TRUBETSKOY *et al.*, 2022). A single nucleotide polymorphism describes a gene variant, which occurs on a specific gene locus and differs from other gene variants in one base (TOMIUK & LOESCHKE, 2016). Further mutations associated with schizophrenia were identified. In 11 out of 15 different risk loci a higher rate of copy number variants (CNVs) was observed (REES *et al.*, 2014). CNVs are differences in the amount of copies of a specific genomic locus between two genomes. Reasons of the phenomenon can be duplications or deletions for example (BICKHART, 2018).

6.1.1 Transcription factor TCF4

TCF4 encodes the protein "transcription factor 4", a basic helix-loop-helix transcription factor. The encoded protein recognizes an Ephrussi-box (E-box) binding site (CANNTG). This gene is expressed in the whole body, but mainly in cortical and subcortical structures in the developing and adult brain of mice, rhesus monkeys, and humans (JUNG *et al.*, 2018). TCF4 associates with other basic helix-loop-helix transcription factors to form a heterodimer, such as the proneural protein Math1. The lack of Math1/TCF4 heterodimers results in a reduction of neuronal

density in the hindbrain of mice (FLORA *et al.*, 2007). *TCF4* is also involved in the development of the nervous system. During brain development, the expression of *TCF4* increases at the end of prenatal life and decreases after birth and stays at a constant level through adulthood (M LI *et al.*, 2018). Multiple alternatively spliced transcript variants encode 18 protein isoforms, named TCF4A to TCF4R(TCF4 transcription factor 4, 2023). These isoforms, differing in their N-terminal sequence, have alternative transcription start sites in exons 1, 3, 4, 5, 7, 8, and 10 (SEPP *et al.*, 2011). Some of these protein variants are pathogenic and can cause intellectual disabilities to varying degrees (MARY *et al.*, 2018). The Pitt-Hopkins syndrome (PTHS) is one of these diseases. A heterozygous pathogenic variant in *TCF4* or a deletion of the 18q21.2 chromosome causes a haploinsufficiency of *TCF4*. This insufficiency leads to PTHS. Patients suffering from PTHS show developmental delays, including severe intellectual disability and behavioral abnormalities. Speech acquisition is delayed and nonverbal communication is often preferred over using language. Breathing problems and characteristic facial features are also common symptoms (SWEETSER *et al.*, 1993). Various genome-wide association studies showed that variants in the *TCF4* gene locus are associated with schizophrenia, e.g. a variant located in intron 3 (STEFANSSON *et al.*, 2009; STEINBERG *et al.*, 2011). Pluripotent stem cells derived from schizophrenic patients showed a higher *TCF4* expression compared to unaffected individuals (BRENNAND *et al.*, 2011). In animal experiments, a full knockout of Tcf4 in the whole body was not viable (FLORA *et al.*, 2007). In comparison to that loss of function model, a gain of function mouse model showed behavioral phenotypes in transgenic mice. It affects sensorimotor gating and leads to less freezing behavior in the fear conditioning test. The memory in these mice was reduced in contrast to littermates with a normal level of Tcf4 expression (BRZÓZKA *et al.*, 2010).

6.1.2 *BMAL1* gene locus

The *BMAL1* gene encodes a basic helix-loop-helix protein that forms a heterodimer with CLOCK. This heterodimer binds E-box enhancer elements upstream of period (*PER1, PER2, PER3*) and cryptochrome (*CRY1, CRY2*) genes and increases their transcription. *BMAL1*, also known as *MOP3*, is important for the circadian clock of mammals. Defects in this gene can disturb sleep patterns, fertility, gluconeogenesis, and lipogenesis. The highest expression levels can be found in the adrenal glands and the skin (NATIONAL INSTITUTE FOR HEALTH, 2023). Homozygous

Bmal1 knockout mice are devoid of the circadian rhythmicity. The animals showed a locomotor activity independent from light and dark phases and a reduced activity in general. The gene expression of CLOCK-regulated genes in the central pacemaker is disturbed in Bmal1 knockout mice (BUNGER *et al.*, 2000). Knockout of *Bmal1* leads to psychiatric related symptoms by affecting the circadian clock. Macaque monkeys missing *BMAL1* showed more stationary behavior. The animals spent more time off-ground then the control monkeys and did not explore their cages as much as wildtype animals. These findings can be interpreted as anxiety-like behavior (QIU *et al.*, 2019). Association studies in humans identified psychiatric risk genes that are relevant for the circadian clock. SNPs in the *BMAL1* gene are associated with bipolar 1 disorder (MANSOUR *et al.*, 2006). On a molecular level it is possible to influence the circadian clock by blocking the D_2 receptor (YUJNOVSKY *et al.*, 2006). The D_2 receptor is a major target of all current neuroleptics used for treating schizophrenia (MÖLLER, 2005; VIDAL MMI GERMANY GMBH, 2023).

6.2 Environmental risk factors

As described previously, the prevalence of schizophrenia differs enormously between patients. There is an association between other diseases and psychiatric disorders, like SUD or depression. Even behavioral patterns, like smoking occur more often in schizophrenic patients. The environment or living situation of patients seems to have a strong impact on the onset and progress of the disease. In the following sections examples for environmental risk factors are given.

6.2.1 Pregnancy and birth complications

Complication during pregnancy and birth can be separated into three subgroups. Bleeding, diabetes, rhesus incompatibility, and preeclampsia are common pregnancy complications than can affect the newborn. The fetal development can be impaired, manifesting in low birthweight, congenital malformations, and a reduced head circumference. Obstetric complications like uterine atony, asphyxia or emergency cesarean section can increase the schizophrenia risk of the offspring (DALMAN *et al.*, 2001; CANNON *et al.*, 2002).

Prenatal infections and the following response of the maternal immune system can also lead to schizophrenia in the offspring. Immunoglobulin levels in maternal blood samples immediately before birth have been analyzed. The levels of IgG and

IgM antibodies were elevated in mothers of schizophrenic patients, while IgA antibodies and albumin were not (BUKA *et al.*, 2001). The time point of an infection during pregnancy is critical. The risk of developing schizophrenia spectrum disorders was increased by exposure to maternal genital/reproductive infections periconceptionally, but not in the first, second, or third trimester (BABULAS *et al.*, 2006).

6.2.2 Trauma during childhood and adolescence

WHO studies on the relation between childhood adversities and mental disorders in 21 countries showed that 29.8% of all tested mental disorders were associated with childhood adversities including interparental loss, like parental death or divorce, parental maladjustment like criminal behavior, substance abuse, mental illness, family violence, and physical or sexual abuse or neglect. Further adverse experiences were dependent on low economic status and physical diseases. The most common childhood adversity was parental loss, followed by physical abuse, family violence and parental mental illness All the conditions for childhood adversity were associated with an increased risk for mental disorders, with maladaptive family functioning being a robust predictor. This term comprises parental mental illness, substance misuse, criminality as well as family violence, neglect, and physical and sexual abuse (KESSLER *et al.*, 2010). Parental death in the early childhood is associated with an increased risk of developing schizophrenic symptoms up to two- to three-fold compared to healthy controls (AGID *et al.*, 1999; MORGAN *et al.*, 2007; STILO *et al.*, 2013). In order to associate specific events in childhood with symptoms or symptom complexes in the adulthood, more studies were conducted. The way how mothers interacted with their six month old children influenced the development and performance of language and thoughts of their children when they were 45 to 48 month old (MEINS *et al.*, 2002).

6.2.3 Social isolation

Disadvantages in sociability and social life are associated with schizophrenia risk. People which are living alone and do not have a partner, have more contact to psychiatric services than people living in a functional social situation. Living in overcrowded conditions, income below the official poverty, or unemployment are also more common in schizophrenic patients (STILO *et al.*, 2013).

6.2.4 Migration

Studies in different countries confirmed a higher risk of schizophrenia in migrants compared to the origin population (HARRISON *et al.*, 1997; HAASEN *et al.*, 2001; SELTEN *et al.*, 2002; SAHA *et al.*, 2005). The risk is also elevated in the following generations (CANTOR-GRAAE & PEDERSEN, 2007).

6.2.5 Substance abuse

The prevalence of alcohol or substance abuse is increased in schizophrenic patients (HÄFNER *et al.*, 1998). In several studies, 70 – 77% of the subjects suffering from schizophrenia showed a SUD including daily tobacco consumption. Both diseases can start at the same time, but there are also studies observing an earlier onset of substance use disorders (WADE *et al.*, 2005). Especially daily tobacco is associated with an increased schizophrenia risk and earlier outbreak of the disease (GURILLO *et al.*, 2015). The use of cannabis correlates with the schizophrenia risk as well. Particularly, if cannabis was consumed in adolescence. People using cannabis between the age of 15 and 18 showed more schizophrenic symptoms in the adulthood than abstinent people (ARSENEAULT *et al.*, 2002). The abuse in early years and the frequency, as well as using high potency tetrahydrocannabinol (THC) is associated with an increased schizophrenia risk (DI FORTI *et al.*, 2009). Other drugs are known to cause symptoms of a psychosis, such as hallucinations and delusions, especially the long term use of methamphetamine (CHEN *et al.*, 2003).

6.2.6 Cognitive impairments and brain structural abnormalities

Developmental delay was also associated with schizophrenia. Important milestones during early childhood were reached later compared to healthy controls. Milestones included the ability to smile, to lift the head, to sit without support, to crawl longer distances, and to walk without support (SØRENSEN *et al.*, 2010). Deficits and delay in speech, attention, and working memory were observed (REICHENBERG *et al.*, 2010).

6.3 Gene–environment interactions

The risk factors for schizophrenia include genetic risk factors as well as environmental risk factors. The onset, progress, and outcome of the illness is highly individual. Cannabis use in adolescence is associated with an increased risk for schizophrenia. The effect of cannabis use can be increased by genetic factors, e.g. a functional polymorphism of the catechol-O-methyltransferase (COMT) (CASPI

et al., 2005). Other correlations between genetic and environmental factors are also observed. The combination of childhood trauma and genetic liability is associated with an increased disease risk (GULOKSUZ *et al.*, 2019). A similar correlation was measured by investigation of polygenic risk score (PRS) based on GWAS significant alleles and complications in the early live (ELC). The term early live includes the birth and early neonatal days. The risk for a schizophrenic disorder was increased by ELC (URSINI *et al.*, 2018).

7 Animal models of schizophrenia

GWAS studies are used to identify risk genes or risk gene variants, but the study of one particular gene locus and its functional consequences is nearly impossible in patients. Therefore, standardized and genetically identical rodent models are used. For most genes, mouse models with altered gene expression level are available.

Three different levels are possible in animal models. These levels are face validity, construct validity and predictive validity (SOTIROPOULOS *et al.*, 2021). Face validity describes the quality of replicating symptoms in a model, for example a drug-induced behavior, which is comparable to psychosis in humans (LIPSKA & WEINBERGER, 2000). Positive symptoms, such as hallucinations, are hard to observe in animals. Hyperlocomotion after administrating a drug or novel stimuli is used as an equivalent for hallucinations (WINSHIP *et al.*, 2019). Because rescuing symptoms is highly important for patients, these models were mainly used in the beginning of schizophrenia research (KAS, KRISHNAN, *et al.*, 2011). Various doses and administration schemes of ketamine or phencyclidine were used to create a suitable mouse model for psychosis (CHATTERJEE *et al.*, 2011; MOURI *et al.*, 2012). The ketamine or phencyclidine-induced models were used to find compounds that rescue the symptoms of a psychosis, such as hallucinations and delusions (KAS, KRISHNAN, *et al.*, 2011). Based on that first-generation antipsychotics were established (STAHL, 2000).

Drug development has recently shifted from symptom-based analysis to more mechanism-based analysis. The etiology and pathophysiology of schizophrenia are focused (KAS, KAHN, *et al.*, 2011). For research on underlying mechanisms the construct validity of a mouse model is important. The construct validity evaluates the similarity of mechanisms in humans and mechanisms in the corresponding animal model. Because of missing information about the pathomechanisms in

schizophrenia, the validation of the construct validity is limited. The predictive validity evaluates the possibility of rescuing the created phenotype by established antipsychotics (SOTIROPOULOS *et al.*, 2021).

In order to get more detailed information about potentially risk factors and the etiology of schizophrenia, animals are tested in sensorimotor gating, cognition, sociability, and exploratory behavior (KAS, KAHN, *et al.*, 2011).

III. MATERIAL & METHODS

1 Material

Materials for *in vivo* and *in vitro* experiments are summarized in the following tables (Table 1-10).

1.1 *In vivo* experiments

1.1.1 Mouse lines

Table 1: Mouse lines

Name	Specifications
Bmal1-ko	Constitutive knockout, generated by *Bmal1* loxP-flanked exon 8 removed by crossing Bmal1-fl and Cre lines (STORCH *et al.*, 2007)
Camk2a-Cre	Mice expressing Cre recombinase under the control of the CamKIIα (CAMK2A) promoter in excitatory neurons (MINICHIELLO *et al.*, 1999)
Emx1-Cre	Expression of Cre recombinase in neurons of neocortex and hippocampus, in glial cells of the pallium (GORSKI *et al.*, 2002).
Tcf4-4-fl	Inducible conditional knockout of Tcf4, inducible via floxed exon 4, Tcf4tm1c(EUCOMM)Wtsi/WtsiBiat (SKARNES *et al.*, 2011)
Tcf4-4-fl × Camk2a-Cre	Conditional knockout of Tcf4 in the offspring around PD 20 in pyramidal neurons of hippocampus, neocortex, striatum and amygdala (MINICHIELLO *et al.*, 1999), crossed for behavioral experiments
Tcf4-4-fl × Emx1-Cre	Conditional knockout of Tcf4 in 91% of the neurons in cerebral cortex and hippocampus at ED 12.5 (GUO *et al.*, 2000), crossed for behavioral experiments

1.1.2 Pharmaceutical preparation for poly(I:C) injection

Table 2: Pharmaceutical substance

Name	Source
Polyinosinic-polycytidylic acid potassium salt (poly(I:C))	Sigma-Aldrich, CAS 42424-50-0, Darmstadt, Germany

1.1.3 Materials and setup for behavioral testing

Table 3: Materials and setup for behavioral testing

Name	Source/Details
ANY-maze software	v4.98, Stoelting, Wood Dale, IL, USA
Open field box	50 × 50 × 50 cm
Y-maze	Three arms (A, B, C): each arm: 50 × 5cm
Intellicage system for mice	TSE systems, Bad Homburg, Germany (http://www.tse-systems.com/product-details/intellicage)
Chip reader with USB connection	Felixcan F1 Mini white, Animal ID, Albacete, Spain, obtained from chiphandel.de, Tangerhütte, Germany
SR-LAB Startle response system	San Diego instruments, San Diego, USA (sandiegoinstruments.com/product/sr-lab-startle-response)
Tail suspension test box	60 × 10 × 30 cm
Plastic tubes	4 cm × 0.1 cm, Ø = 0.5 cm
Labeled bar	62 × 1 × 3 cm
Adhesive tape	0.71 mm x 180 m , # PAT-18, Labtag, Quebec, Canada
Fear conditioning system	36 cm × 20 cm × 20 cm, Stoelting, Wood Dale, IL, USA (any-maze.com/mazes/fear-conditioning)
80% ethanol	A0565,5000, AppliChem, Darmstadt, Germany
Soap concentrate, 5% SDS solution	Sodium dodecyl sulfate, 436143, Sigma-Aldrich Chemie, Darmstadt, Germany
Laptop	Fujitsu Lifebook A532, #1041U13, Fujitsu Technology Solutions, Munic, Germany
Grey table	104 × 104 × 51 cm
Paper towels	Kimtech Science 200, lab wipes white, Art. No. 7558, Kimberly-clark professional, Coblenz, Germany
Luxmeter	LM 1302, ELV Elektronik AG, Leer, Germany
Handling tube	50/47 × 130 × 1.5 mm, from transparent colorless polycarbonate raw material, Kahmann & Ellerbrock, Bielefeld, Germany
FlowR	XBehavior, Dägerlen, Switzerland
RStudio	RStudio, Boston, MA, USA

1.1.4 Materials for transponder implantation

Table 4: Materials for transponder implantation

Name	Source/Details
Eye and nose ointment	Bepanthen, Bayer, Berlin, Germany
Tissue adhesive	B.Braun Histoacryl, Melsungen, Germany
Metamizole	Novaminsulfon-ratiopharm 500 mg/mL drops, Ratiopharm, Ulm, Germany
Carprofen	Rimadyl 50 mg/mL Injectable for dogs and cats, Zoetis, Parsipanny, USA
Suture material	Serapid, PGA, USP 4.0, DSS-13, 45 cm, 3015131L, Serag Wiessner, Naila, Germany
Transponder	1.4 × 9 mm, RFID Tierchip international – Herstellercode 941, 2005, chiphandel.de, Tangerhütte, Germany
FMI Föhrs Medical Instruments ZUA-82-GME gas mixing unit	14285, FMI Föhr Medical Instruments, Germany
Warming plate	FMI Föhr Medical Instruments, Darmstadt, Germany
Anesthesia unit	FMI Föhr Medical Instruments, Darmstadt, Germany

1.2 *In vitro* experiments

1.2.1 Materials for genotyping

1.2.1.1 Materials for DNA isolation

Table 5: Materials for DNA isolation

Name	Source/Components
Lysis buffer	EDTA Na_2 $2H_2O$ (ethylenediaminetetraacetic acid disodium salt dehydrate), CAS 6381-92-6, Sigma-Aldrich Chemie, Darmstadt, Germany NaOH (sodium hydroxide), CAS 1310-73-2, Sigma-Aldrich Chemie, Darmstadt, Germany
Neutralization buffer	TrisHCl (tris(hydroxymethyl)aminomethane hydrochloride), CAS 1185-53-1, Sigma-Aldrich Chemie, Darmstadt, Germany
NucleoSpin Tissue Kit	Macherey-Nagel, Düren, Germany

1.2.1.2 Materials for PCR

Table 6: Materials for PCR

Name	Source/Components
TBE buffer	Tris base (Tris(hydroxymethyl)aminomethan), 77-86-1, Sigma-Aldrich Chemie, St. Louis, MO, USA Boric acid, CAS 10043-35-3, Sigma-Aldrich Chemie, Darmstadt, Germany EDTA Na_2 $2H_2O$ (ethylenediaminetetraacetic acid disodium salt dehydrate), CAS 6381-92-6, Sigma-Aldrich Chemie, Darmstadt, Germany
GoTaq G2 Flexi DNA Polymerase	M7801, Promega, Fitchburg, USA
Agarose gel	1% agarose in 150 mL TBE buffer heated in microwave until fully dissolved, 0.1 µl/mL ethidium bromide 1% (stock in H_2O: 10 mg/ml)
Ethidiumbromide 1%	3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide, CAS 1239-45-8 , Carl Roth, Karlsruhe, Germany
Gel Doc XR+ Gel Documentation System	Biorad, Feldkirchen, Germany
GeneRuler 100 bp DNA ladder, 50 µg, 0.5 µg/µL	SM0241, Thermo Fisher Scientific, Waltham, USA

1.2.1.3 Primers for genotyping

Table 7: Primers for genotyping

Mouse line	Forward primer	Reverse primer	Product size in base pairs (bp)
Camk2a -Cre tg	TCCAATTTACTGACCGTACAC	CATCAGCTACACCAGAGACGG	694
Emx1-Cre tg	ATTTTCCACCATATTGCCGTCT	AGCCATTTGACTCTTTCCACAAC	321
Emx1-Cre wt	AAGGTGTGGTTCCAGAATCG	CTCTCCACCAGAAGGCTGAG	378
Tcf4-4-fl wt	CCGATGACAGTGATGATGGT	GAACCAGGCACAGGGCTAC	554
Tcf4-4-fl fl	CCGATGACAGTGATGATGGT	GAACCAGGCACAGGGCTAC	637
Tcf4-4-fl rec	CCGATGACAGTGATGATGGT	AAATGACTTCCCGCCAGAC	483
Bmal1-fl	ACTGGAAGTAACTTTATCAAACTG	CTGACCAACTTGCTAACAATTA	420
Bmal1-ko	TTTACTGTGCTGCCTGTAG	CTGACCAACTTGCTAACAATTA	621
Bmal1 wt	ACTGGAAGTAACTTTATCAAACTG	CTGACCAACTTGCTAACAATTA	320

1.2.2 Materials for RNA isolation

Table 8: Materials for RNA isolation

Name	Source
RNeasy Mini Kit	74106, Qiagen, Venlo, Netherlands
RNase-Free DNase Set	79254, Qiagen, Venlo, Netherlands
RNA later (tissue permeable RNase inhibitor)	AM7020, Thermo Fisher Scientific, Waltham, USA
Eppendorf BioPhotometer	6131025057, Eppendorf AG, 22331 Hamburg, Germany

1.2.3 Materials for qPCR

Table 9: Materials for qPCR

Name	Source
2x qPCR SYBR Green Master Mix, high ROX	SL-9902HR, Steinbrenner Laborsysteme, Wiesenbach, Germany
Applied Biosystems High-Capacity cDNA Reverse Transcription Kit	4368813, Thermo Fisher Scientific, Waltham, USA
Applied Biosystems StepOnePlus thermocycler	Thermo Fisher Scientific, Waltham, USA

1.2.4 Primers for qPCR

Table 10: Primers for qPCR

Name	Forward primer	Reverse primer	Target
Tcf4 Exon1-2	CATATTTGTGGCCATTGAAGG	GTCCCTAAGGCAGCCATTC	Exon 1 – 2 (Tcf4)
Tcf4 Exon 3-4	TCTTTGGCGAGTGGACATTT	CTTGGATGGCCTCCAGTTC	Exon 3 – 4 (Tcf4)
ATP5B	GGCACAATGCAGGAAAGG	TCAGCAGGCACATAGATAGCC	Housekeeping gene 1
RPL13a	ATCCCTCCACCCTATGACAA	GCCCCAGGTAAGCAAACTT	Housekeeping gene 2

2 Methods

2.1 *In vivo* experiments

2.1.1 Mice

2.1.1.1 Animals and husbandry

C57BL/6N mice were bred in-house. All mouse lines were kept on a C57BL/6N background.

Mice were kept under a non-inverted 12:12 light/dark cycle, matching the natural

day/night cycle. Lights were switched on at 7 a.m. (daylight saving time). Food was provided ad libitum; water was conditionally restricted in the Intellicage system only. The mice were maintained under specific-pathogen-free condition and the health monitoring was performed according to the FELASA recommendations (MÄHLER *et al.*, 2014). Transgenic mice and littermate controls were weaned at 21 days and group housed in open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2.065 cm²) and individually ventilated type II cages (Tecniplast 1284L, 365 × 207 × 140 mm, 530 cm²). The cages were enriched with red polycarbonate houses (150 × 111 × 55 mm), handling tubes (Ø = 50/47 × 130 mm), wood chips (4 × 1 × 1.5 cm), and nesting material.

The behavioral experiments are summarized in one standardized test battery and all animals go through every test, so the calculation refers to a multivariate analysis of variance (MANOVA) with a significance of $\alpha = 0.05 / 6 = 0.0083$ adjusted to six behavioral domains. In previous studies effect sizes of $d = 0.5 – d = 3$ were measured (D BADOWSKA, 2015). Because effect sizes of $d < 0.8$ led to no significant differences in behavioral tests in previous studies, calculation of group sizes was based on $d < 0.8$, $f > 0.4$, $f > 0.6$, $f \geq 0.4$ (ANOVA), $f^2(V) \geq 0.16$ (MANOVA) (DM BADOWSKA *et al.*, 2015; VOLKMANN *et al.*, 2020). This calculation leads to a minimum group size of 15 animals per group.

The grouping of the experimental animals was not randomized. Littermates were grouped together in order to avoid aggressiveness especially between male mice. The experimenter is blinded to the different genotypes.

2.1.1.2 Tcf4-4-fl mice

The Tcf4-4-fl line was used for creating conditional knockout mice by crossing with different Cre lines. The line itself was created earlier as follows: The Tcf4tm1a strain is a commercially available Tcf4 knockout strain. The expression of Tcf4 in these mice is knocked down by a promoterless lacZ-neo cassette introduced before exon 4. The cassette is flanked by two FRT sites and the exon 4 is flanked by two loxP sites (Figure 1: Breeding strategy Tcf4). The LacZ-neo cassette can be deleted by crossing Tcf4tm1a mice with mice expressing Flp1 recombinase. The offspring, called Tcf4tm1c or Tcf4-4-fl, have a restored Tcf4 gene function. The exon 4 can be conditionally knocked out by crossing the Tcf4tm1c line with a Cre line, for the current experiments either Camk2a-Cre or Emx1-Cre (SKARNES *et al.*, 2011; D

BADOWSKA, 2015). The region of the gene between the two floxP sites will be excised, in this case exon 4. That leads to a premature stop codon and activates the nonsense-mediated mRNA decay. After knockout of exon 4, just the smaller isoforms of TCF4 will be expressed (SEPP *et al.*, 2011).

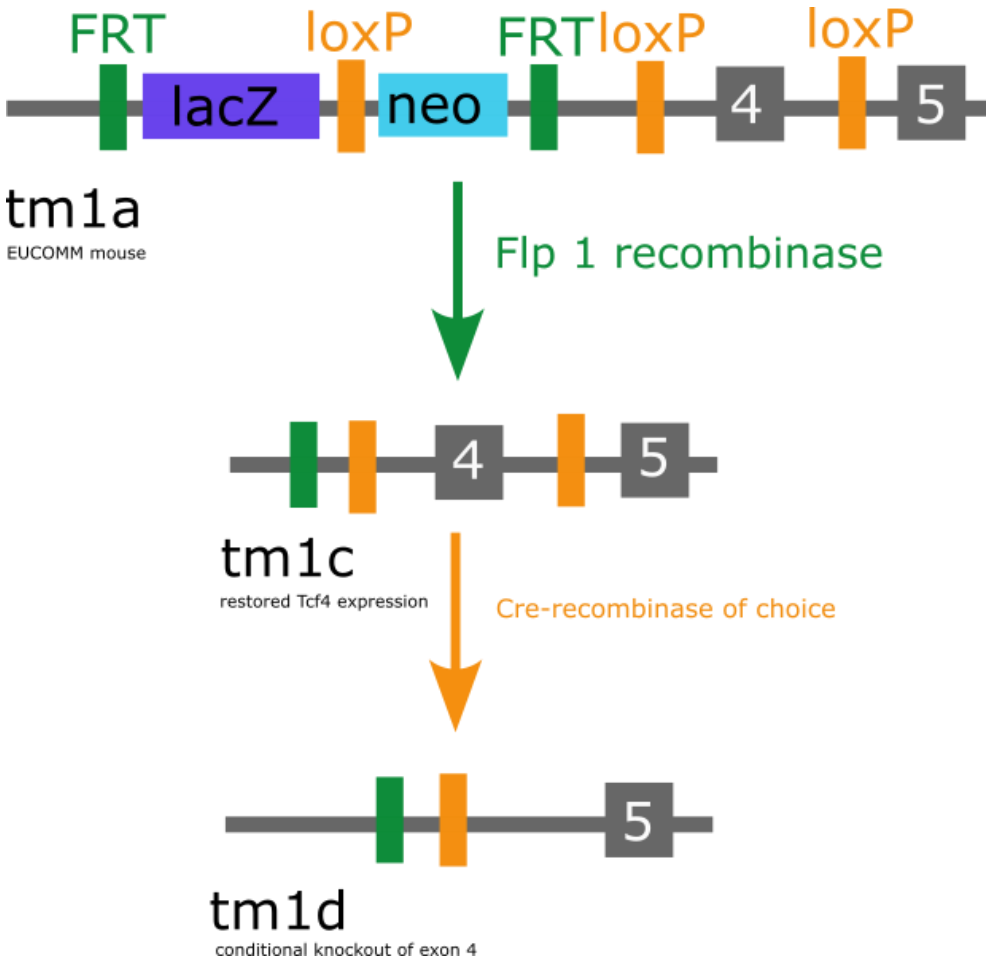

Figure based on Skarnes et al., 2011.

Figure 1: Breeding strategy Tcf4.
Tcf4tm1a: An FRT-flanked lacZ-neo cassette was introduced before floxed exon 4. Crossing with mice carrying flip1 recombinase deleted the lacZ-neo cassette and restored gene function. When Tcf4tm1c mice are bred with mice carrying Cre (Camk2a-Cre or Emx1-Cre) a conditional knockout line Tcf4tm1d was created. (SKARNES *et al.*, 2011; D BADOWSKA, 2015).

2.1.1.3 Camk2a-Cre mice

This line has been used for a tissue specific knockout. From postnatal day 20 Cre recombination can be found in the CA1 region of the hippocampus (TSIEN *et al.*, 1996; MINICHIELLO *et al.*, 1999). Crossing this line with different floxed-lines, for example Tcf4-4-fl, creates knockouts of the targeted gene in the forebrain. Camk2a-Cre is expressed in the male germline, so just female Cre carriers should

be used for breeding (CHOI *et al.*, 2014).

2.1.1.4 Emx1-Cre mice

Similar to Camk2a-Cre, the Emx1-Cre line can be used for a neuron specific knockout. Compared to Camk2a-Cre, the knockout occurs earlier, around embryonal day 12.5 and affects neurons in the cerebral cortex and hippocampus (GUO *et al.*, 2000).

2.1.1.5 Tcf4-4-fl × Camk2a-Cre mice

In order to produce a loss of function-model of Tcf4, Tcf4-4-fl and Camk2a-Cre mice were crossed to create the Tcf4-4-fl × Camk2a-Cre mouse line. Only female Cre carriers were used, because Camk2a-Cre is expressed in the male germline. The experimental animals were conditional knockout mice with a heterozygous expression of Cre (genotype: Tcf4: fl/fl; Camk2a-Cre: tg/+) and wildtype mice without Cre expression (genotype: Tcf4: fl/fl; Camk2a-Cre: +/+). 17 male mice with a conditional knockout of Tcf4 (cko) and 25 wildtype (wt) littermates as control mice were weaned at day 21 and group housed in four open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²) in two groups of ten male animals of mixed genotypes and two groups of 11 male animals of mixed genotypes. Test mice were 7 – 12 weeks old when used for tests. Figure 2.

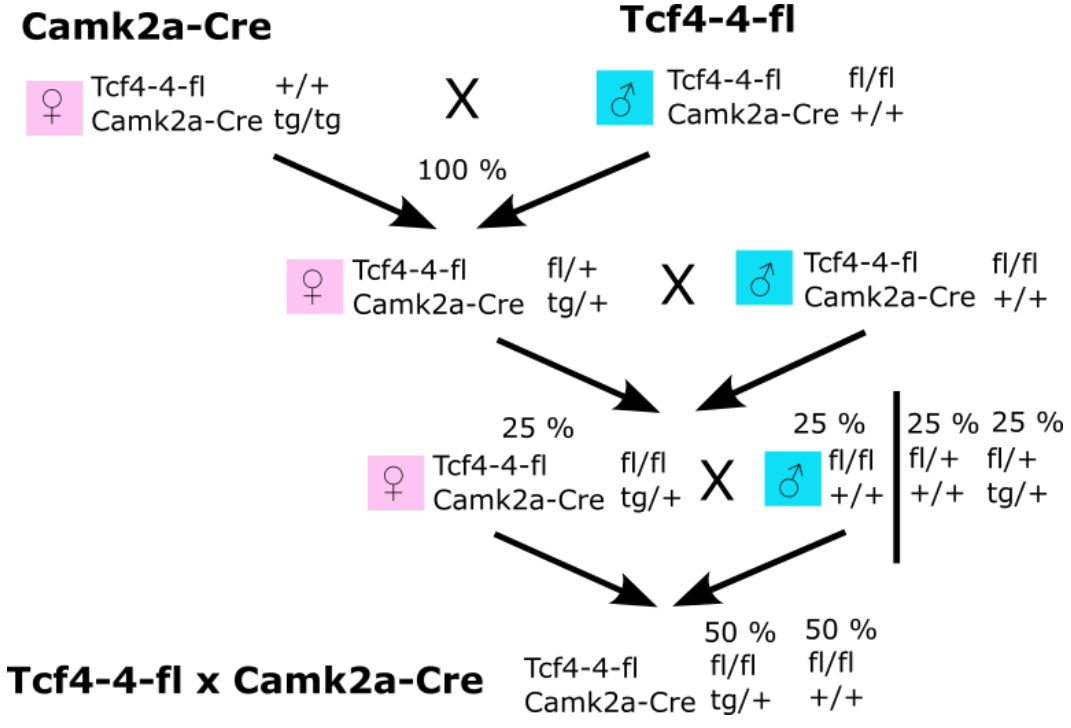

Figure 2: Breeding strategy of Tcf4-4-fl × Camk2a-Cre.
fl/fl, homozygous floxed exon 4; fl/+, heterozygous floxed exon 4; tg/tg,

homozygous expression of Camk2a-Cre; tg/+ heterozygous expression of Camk2a-Cre; +/+, wildtype

2.1.1.6 Tcf4-4-fl × Emx1-Cre mice

Similar to the Tcf4-4-fl × Camk2a-Cre mouse line, Tcf4-4-fl and Emx1-Cre mice were crossed. The knockout of Tcf4 in the offspring starts at embryonal day 12.5 (GUO *et al.*, 2000). Offspring with heterozygous Cre expression (genotype: Tcf4: fl/fl; Emx1-Cre: tg/+) built the group of conditional knockout mice and mice without Cre expression (genotype: Tcf4: fl/fl; Emx1-Cre: +/+) built the wildtype control group. Conditional knockout mice (18 male and 16 female) and wildtype control mice (14 male and 16 female) were weaned at 21 days and group housed in eight groups of mixed genotypes. The 32 male mice were group housed in two groups of 16 animals in open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²). The 32 female mice were group housed in six groups of three to six animals in individually ventilated type II cages (Tecniplast 1284L, 365 × 207 × 140 mm, 530 cm²). During the behavioral tests in the intellicages female mice were grouped housed in two groups of 16 animals in open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²). Test mice were 8 – 14 weeks old at the start of the experiments. Figure 3.

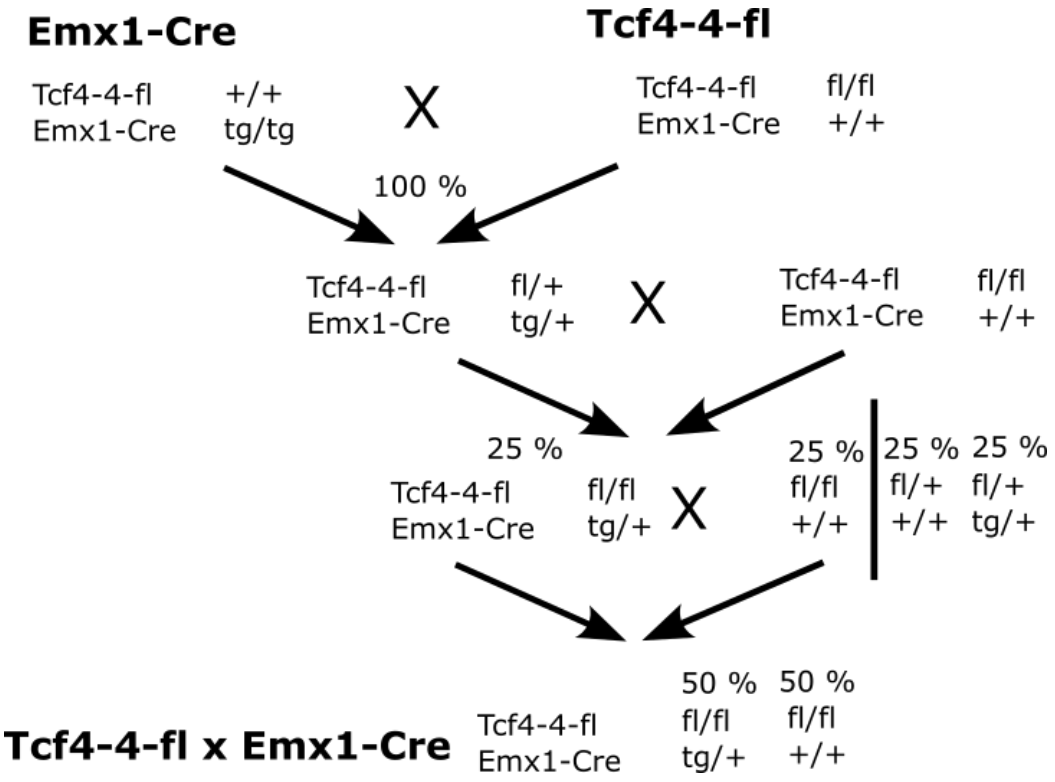

Figure 3: Breeding strategy of Tcf4-4-fl × Emx1-Cre.
fl/fl, homozygous floxed exon 4; fl/+, heterozygous floxed exon 4; tg/tg,

homozygous expression of Emx1-Cre; tg/+ heterozygous expression of Emx1-Cre; +/+, wildtype

2.1.1.7 Bmal1-ko mice

Heterozygous Bmal1 knockout mice (11 male and six female) and wildtype littermate control mice (ten male and nine female) were weaned at 21 days and group housed with animals of mixed genotypes. The 21 male mice were group housed in one group of eleven animals in open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²) and two groups of five animals in individually ventilated type II cages (Tecniplast 1284L, 365 × 207 × 140 mm, 530 cm²). The 15 female mice were group housed in three groups of four to six animals in individually ventilated type II cages (Tecniplast 1284L, 365 × 207 × 140 mm, 530 cm²). During the behavioral tests in the intellicages female mice were grouped housed in one group of 15 animals in open type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²). Experimental mice were 13 – 26 weeks old during the testing period.

2.1.2 Environmental factors

As the environmental factor for a two-hit mouse model, the following already published stress paradigms were used: The social defeat paradigm was used for other cohorts before (VOLKMANN *et al.*, 2020; STEPHAN *et al.*, 2022). The protocol for poly(I:C) injections was established in our lab as a part of my thesis.

2.1.2.1 Social defeat

The social defeat paradigm, also known as resident-intruder paradigm, was essentially conducted as described previously (BRZÓZKA *et al.*, 2011). The mice were subjected to the paradigm from postnatal day 22 to 34. FVB/N male mice, 29 – 66 weeks old, were used as resident stressor mice. Residents are individually housed males that exhibit aggressive behavior toward unfamiliar other male mice when they enter their cage. Male residents were primed by pairing with a female mouse of nearly the same age or younger for 3 days. Their attack latency was observed during the experiment and only residents with attack latencies under 60 s were used. For 21 consecutive days, experimental mice, also called intruders, were once-daily transferred individually into a resident's home cage. After the first physical attack, animals were protected by a perforated metal cage (75 mm × 115 mm × 60 mm) and stayed in the resident cage for another 30 min. None of the animals sustained bite marks or showed other signs of injury. The

intruders were then identified by their ear tags and put back into their home cage. Metal cages were cleaned with water and ethanol between sessions. The test time was randomized daily between 7 a.m. and 7 p.m. The pairing of residents to intruders was rotated in order to minimize repeated contacts. If the attack latency increased during the 21 days of the paradigm, other residents were used or the residents were paired again for one night with a female mouse in order to restore aggressiveness.

2.1.2.2 Poly(I:C) injection

Eleven pregnant dams were injected with poly(I:C) (polyinosinic-polycytidylic acid potassium salt; Sigma-Aldrich, Saint Louis, USA) and nine pregnant dams were injected with saline (sterile pyrogen free 0.9% NaCl) intravenously (i.v.) as a vehicle control at gestational day (GD) 17 at a final volume of 4 ml/kg (5 mg/kg). The pregnant dams were added to the groups randomly. This time point is common to mimic a viral infection during late pregnancy and has been reported to lead to an anxiety-like behavior in the offspring (NYFFELER *et al.*, 2006). The double stranded RNA of poly(I:C) activates the maternal immune system (DA SILVEIRA *et al.*, 2017). The foreign double stranded RNA of poly(I:C) is detected by the toll-like receptor 3 (TLR3) of the mammalian innate immune system (ALEXOPOULOU *et al.*, 2001; HUR, 2019). The signaling pathway of TLR3 leads to an activation of NF-kB and interferon (IFN)-regulatory factor 3 that are transcription factors of the innate immune system (SATO *et al.*, 2003). The levels of pro-inflammatory cytokines in blood of dams and in tissue of fetal brains are altered after three and six hours after poly(I:C) injections in comparison to saline controls. Poly(I:C) injections on GD 17 lead to an increased level of IL-1β, IL-6, IL-10, and TNFα in maternal blood. In the tissue of fetal brain the levels of IL-1β and IL-10 were increased, whereas the level of IL-6 was reduced after three hours. After six hours the levels of IL-1β and IL-10 decreased and the level of IL-6 increased (MEYER *et al.*, 2006). In order to determine GD17, matings were time limited and the plug was checked twice a day. After separating the breeding pairs, the females were group housed and the weight was checked daily. Poly(I:C) was dissolved in sterile saline solution on the day of administration and injected in the lateral tail veins of the pregnant dams. The dose of poly(I:C) (5 mg/kg) was chosen based on previous studies (BITANIHIRWE *et al.*, 2010; DA SILVEIRA *et al.*, 2017). For the tail vein injections, females were restrained and the tails were gently

warmed in water (40 °C) to induce vasodilation for a better visualization of the veins. The injection volume was separated equally to both lateral tail veins, in order to minimize the applied volume. After this procedure, two females were housed together in an enriched environment until the birth of the offspring.

2.1.3 Behavioral experiments

2.1.3.1 Test Battery

To reduce the stress caused by classical tail handling, all experimental mice were removed from their cages and transferred using clear polycarbonate tunnels (HURST & WEST, 2010; SENSINI *et al.*, 2020). Mouse studies were conducted in accordance with the German animal welfare act (TierSchG) and regulations (TierSchVersV) as well as the European parliament directive 2010/63/EU. All animal experiments were authorized by the supervisory authority (Regierung von Oberbayern, Vet_02-16-179). The data collection is fully automatic. The intellicage system measures visit, nosepokes and licks for each mouse using the RFID number of the transponder. In the prepulse inhibition setup, movements of the mice are detected. All other parameters are based on the tracking in the software ANY-maze, v4.98 (Stoelting, IL, USA). Before each experiment, the animals were habituated to the new environment for a minimum of 10 min. All experiments were run during the light phase, except Intellicage experiments, which ran continuously. The order of the animals was selected by chance, but all animals were tested at a similar time point. Test equipment was cleaned before and after tests using SDS solution (5%), followed by ethanol, unless stated otherwise. All animals went through the same behavioral tests in the same order using a standardized test battery. The standardized test battery and the individual test setups were published before (HÜHNE *et al.*, 2020; VOLKMANN *et al.*, 2020). The tests are explained in the following.

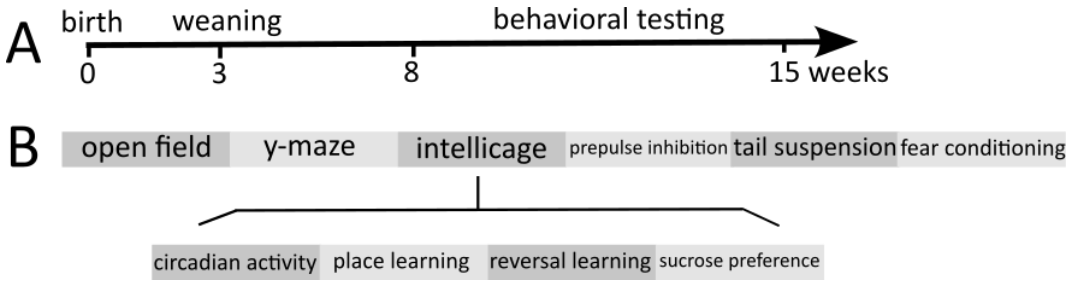

Figure 4: Behavioral testing.
A: experimental timeline; B: behavioral test battery.

2.1.3.2 Open field test

Novelty-induced and spontaneous exploratory behavior was monitored in an open field arena (50 cm× 50 cm × 50 cm). Mice were filmed for 10 min and distance, speed, time spent in predefined areas, number of immobile episodes, and time immobile were assessed with the behavioral tracking software ANY-maze, v4.98 (Stoelting, IL, USA). Illumination during the test was set to 1600 lx (HÜHNE *et al.*, 2020).

2.1.3.3 Y-maze

Working memory capacity was assessed by quantification of spontaneous alternations in the Y-maze. The Y-maze consists of three identical arms (A, B, C; 50 × 5 cm) in the shape of a "Y" and the test was done at 30 lx for 10 min. Spontaneous alternations are the number of full sequences of visits to each arm without repetition (e.g. A-B-C, but not A-B-A). Mice were tracked and the data was analyzed using ANY-maze, v4.98 (Stoelting, IL, USA).

2.1.3.4 Intellicage

Test animals were identified by implanted RFID transponders using a handheld scanner and within the Intellicage. The transponders were implanted one week before starting Intellicage experiments to ensure recovery from surgery. Mice were anesthetized using isoflurane and then shaved in a small area in the dorsocervical region. Eyes were covered with eye ointment (Bepanthen eye and nose ointment, Bayer, Berlin, Germany) during anesthesia. The skin was disinfected with 80% ethanol and the transponder (1.4 × 9 mm) was placed subcutaneously in the neck region. The wound was closed with single stiches and the stiches were fixed with tissue adhesive (Histoacryl, B.Braun, Melsungen, Germany). As pain killer metamizole (200 mg/kg) was applied per os before the transponder implantation and carprofen (5 mg/kg) was injected subcutaneously afterwards.

The Intellicage system consists of four corners that are connected by a frame. The Intellicage system is suitable for type IV cages (Tecniplast 2000, 612 × 435 × 216 mm, 2,065 cm²). Each corner has two doors, one to the left and one to the right side. When a door is open, mice have access to a water bottle. Depending on the current paradigm, mice can open the doors by visiting the corner and disrupting a light

barrier ("poking doors"). Visits to a corner, nosepokes, and licks at water bottle nipples were measured and recorded continuously. The Intellicages contained standardized enrichment from the original cage. Each test paradigm was run for a minimum of 24 h. Experiments were run in the following order:

Table 11: Intellicage Programs

Day	Test	Details
1	Free adaptation	Doors are open, free access to water bottles in all corners
2	Free adaptation	Doors open on visit: doors open when a mouse enters a corner, free access to water bottles in all corners
3 – 4	Nosepoke adaptation	Doors stayed closed until a mouse entered the corner and executed one or more nosepokes on a door; only the corresponding door opened and granted access to water for 7 s or until the mouse withdrew from the corner
5 – 6	Place learning	Each mouse was assigned to one of the four corners in a balanced fashion, ensuring equal distribution; mice could only get access to water after executing a nosepoke in their assigned corner
7 – 13	Serial reversal learning	Place learning with a new assignment of the drinking corner for each mouse every 24 hours, following a randomized order to make the new corner unpredictable for the mouse
14	Sucrose preference	Doors were open with free access to water bottles in all corners; one of the two bottles in each corner (each on the same side) was filled with a 4% sucrose solution, the other bottle containing normal drinking water

For place learning, sucrose preference, and nocturnality, the preference score (A-B)/(A+B) was calculated. Weighted for random expectation, where A equals the number of correct trials (visits in the assigned corner with at least one nosepoke), the number of licks at a sucrose solution bottle, or visits during nighttime and B equals incorrect trials (visits with nosepoke in non-assigned corner), licks at a bottle containing plain water, or daytime visits.

Sequential probability ratio testing (SPRT) was used to analyze the learning performance in the serial reversal learning test. A learning criterion was calculated for the assessment of learning performance (WALD, 1945). A learning criterion is the number of trials - defined as visits with at least one nosepoke - needed to pass the upper bound, i.e. a predefined learning criterion. The upper bound was defined as the random expectation plus 10% (35% for four corners), the lower bound was equal to the random expectation (25% for four corners). Significance levels were set to 5% for both bounds. In case a mouse did not reach the learning criterion

within the duration of one phase (24 h), the total number of trials was used for downstream analysis and plotting instead. Serial reversal learning performance was measured as the area under the curve across all reversal phases (VOLKMANN *et al.*, 2020).

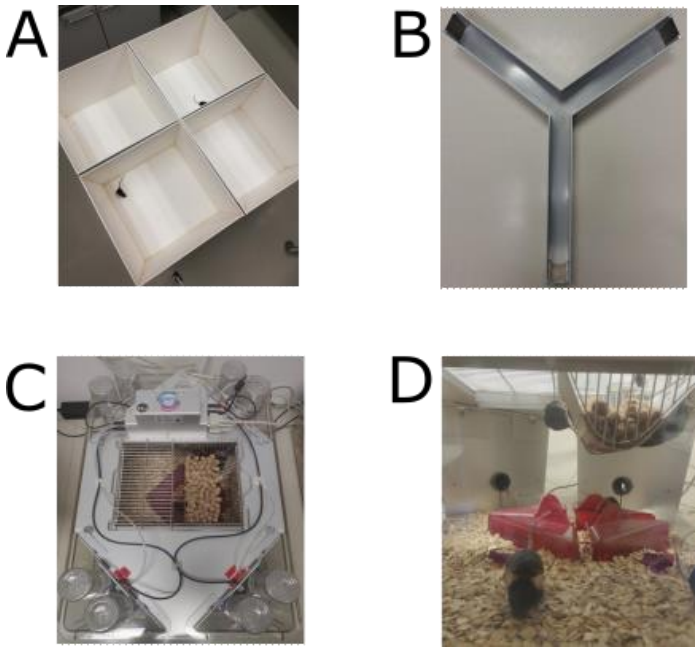

Figure 5: Open field test, y-maze, intellicage, experimental setup.
A: Open field test boxes; B: Y-maze; C, D: Intellicage.

2.1.3.5 Prepulse Inhibition

Three Days before testing, every mouse was habituated daily for 10 min to the enclosure, light, and background noise in order to reduce stress caused by the new environment. On the test day, the startle response was measured via movement transducer at the base plate of startle-response-enclosures (SR-LAB, San Diego Instruments, San Diego, USA). The background noise in the boxes was set to a constant level of 65 dBA. For short term habituation the main 40 ms 115 dBA pulse was presented 10 times before the actual test sequence (baseline startle response). To test the baseline startle response, a 40 ms 115 dBA pulse was presented 10 times before the actual test sequence. Then, a non-startling 20 ms prepulse at an intensity of 70, 75, or 80 dBA was presented, which was followed by a pulse of 115 dBA 100 ms after the start of the prepulse. Each condition was repeated in 10 trials. All trials were presented in pseudorandomized order with inter-trial intervals ranging from 8 to 22 s (HÜHNE *et al.*, 2020).

2.1.3.6 Tail suspension test

For analyzing passive and active behavior in mice, the tail suspension test was used. The tail suspension test box (60 × 10 × 30 cm) includes four identical chambers (15 × 10 × 30 cm). The test animal was suspended freely by its tail using adhesive tape and attaching the mice to a bar located 30 cm above a flat surface for 6 min. Plastic tubes (4 cm × 0.1 cm, Ø = 0.5 cm) were slipped over the tail to prevent mice from climbing up their tail. Periods of no movement of the whole body were measured as time immobile. Whole-body movement was defined as movement of the center of the body. Flailing with the front limbs was not measured as time immobile. Mice were filmed by a camera in front of the test setup and whole-body movements were quantified automatically using ANY-maze, v4.98 (Stoelting, IL, USA) (HÜHNE *et al.*, 2020).

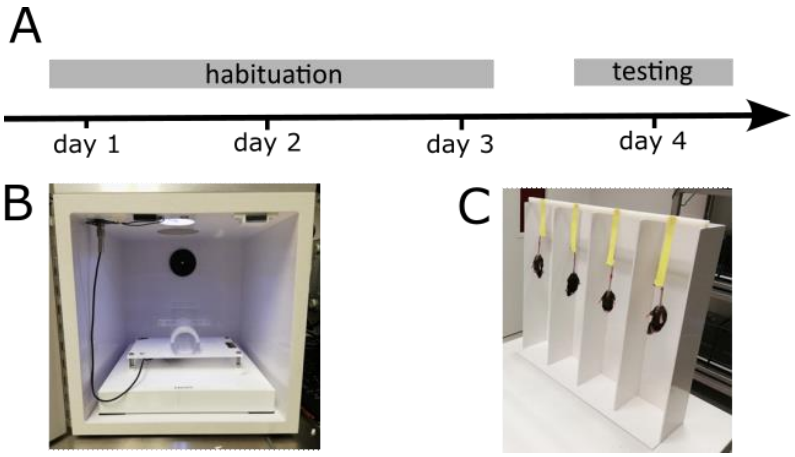

Figure 6: Prepulse inhibition, tail suspension test, experimental setup.
A: Prepulse inhibition timeline; B: Prepulse inhibition box; C: Tail suspension test box.

2.1.3.7 Fear conditioning test

In this test memory is analyzed using classical conditioning. As a read-out for conditioning, freezing behavior was used, defined as a lack of movement (excluding respiratory movements of the chest). The experiments were performed with equipment of TSE Systems (Bad Homburg, Germany). The conditioning and the contextual testing were conducted in an acrylic conditioning chamber (36 cm × 20 cm × 20 cm) with a loudspeaker, a lamp (12 V), and a removable shock grid floor made of stainless rods (4 mm diameter, spaced 6 mm apart) and wall decoration with vertical black and white stripes. The box is connected to the shock-scrambler unit providing an electrical shock. For the cue testing a flat grey plate and a transparent plexiglas tube was used. On the day of conditioning, each

mouse was placed in the conditioning box and freezing was filmed every 3 s for 120 s (context baseline). The baseline scoring was followed by a 30 s tone (tone 1; conditioned stimulus; 10 kHz, 75 dB) and a foot shock (0.4 mA, 2 s duration) simultaneously. After termination of the tone, a pause of 30 s followed with only background sound (white noise) presented (trace 1). Then the same tone as the first one (tone 2; 10 kHz, 75 dB) and a shock of the same intensity and duration as the previous one was applied followed by another pause of 30 s (trace 2). Mice were placed back to the home cage. During the entire training procedure on day 1, the freezing responses of mice were filmed by camera and recorded with ANY-maze, v4.98 (Stoelting, IL, USA). The conditioning box was cleaned with 80% ethanol after each mouse.

On day two the contextual memory was measured by observing freezing behavior in 180 s. The total freezing time was measured. The conditioning box was cleaned with 80% ethanol between the sessions of each mouse.

Day three was about testing the cue memory. A grey flat plate (40 cm × 26 cm × 0.8 cm) and a transparent plastic tube (25 cm × 20 cm × 0.3 cm) were placed in the box. Furthermore for cleaning between the sessions only water was used in order to create a completely different environment compared to the last two days. First, the baseline freezing in the new context was scored every 3 s for 120 s. Then, the same tone as the conditioning one was applied for 30 s (tone 1) and the freezing response as a measure of cued memory was scored. Subsequently, a pause of 90 s followed (post-tone) when anticipation of a possible subsequent foot shock was assessed. After the presentation of the second tone (tone 2, 30 s duration) the freezing behavior during the second post-tone was scored for 90 s (BRZÓZKA & ROSSNER, 2013).

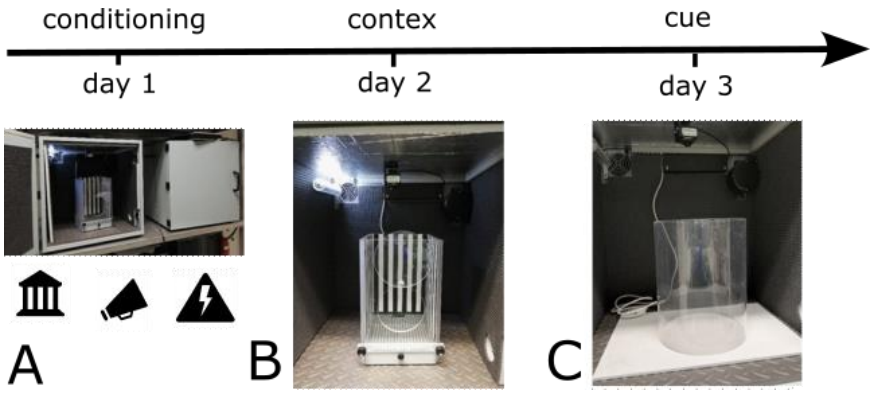

Figure 7: Fear conditioning test, experimental setup.
A: Day 1, conditioning; B: Day 2, contex; C: Day 3, cue.

2.1.3.8 Statistical analysis

The statistical analysis was done as described earlier (VOLKMANN *et al.*, 2020). During open field test, y-maze test, tail suspension test and fear conditioning test the experimental animals were filmed and recorded and analyzed with the tracking software ANY-maze, v48 (Stoelting, Wood Dale, IL, USA). Raw data from the intellicage system and the prepulse inhibition test were analyzed using FlowR (XBehavior, Dägerlen, Switzerland). RStudio (RStudio, Boston, MA, USA) was used for calculating statistical tests and plotting graphs. Boxplots were created using the R function ggplot from the package ggplot2 (WICKHAM, 2016). Whiskers extend no more than 1.5 times of the interquartile range (IQR) from their hinges. Datapoints with a distance of <3% of the total range were shifted horizontally by a random offset. *P*-values refer to scheirer ray hare test or two-way analyses of variance (ANOVA) with Type 2 sum of squares. The normality was tested with a QQ plot and additionally with a density plot. This model was tested in a multivariate analysis of variance (MANOVA) with F-values from a Wilk's lambda approximation. The resulting *p*-values of both analysis were adjusted for multiple comparisons by false discovery rate (FDR) adjustment. In case of a significant interaction term, the corresponding one-way ANOVA was calculated and indicated for each genotype level. For specific analyses that go beyond the standard analysis, the welch`s t-test was used.

Heatmaps were plotted with the R function pheatmap from the pheatmap package. Z-transformation was used for clustering. The method of clustering was the hierarchical clustering of Manhattan distances (STRAUSS & VON MALTITZ, 2017). The distance of two clusters is defined as the maximum distance between their individual components (WARD, 1963). The color depends on the differences

in group mean z-scores between transgenic mice subjected to stress paradigm and the corresponding control group. The rows and the arrangement of them are adjusted to RDoC domains (CUTHBERT, 2009; ANDERZHANOVA *et al.*, 2017). For adjustment in dimension deflation and missing values the R function nipals provided in the ade4 package was used. Nipals executes a non-linear iterative partial least squares (NIPALS) algorithm.

R function candisc from the candisc R package was used for Canonical Discriminant Analysis (CDA) and results were plotted with ggplot2. As input the data of the NIPALS analysis was used. In order to create a maximal separation between the groups, linear combinations (canonical components) of variables were calculated. In CDA there are four steps: The pooled within-group covariance matrix is converted into an identity matrix; the group means of transformed variables are calculated; a Singular Value Decomposition (SVD) of the means is calculated, weighting for the sample size of each group; finally, the resulting values are back-transformed to the original variable space, yielding canonical components. These components summarize variation between groups. The corresponding plots display the first two canonical components with individual data points and data ellipses.

2.2 *In vitro* experiments

2.2.1 Genotyping

Every experimental animal was genotyped by weaning and after death to verify the previous results. In both cases tail biopsies were used.

The DNA was isolated using the Hotshot isolation protocol. 75 µL Lysis buffer (containing $EDTANa_2 2H_2O$ and NaOH) was added and then incubated for 20 min at 95 °C. After cooling down 75 µL neutralization buffer were added and the samples were vortexed and stored at -80 °C.

1 µL DNA solution was used in a volume of 15 µL PCR. Master mixes and programs are presented in Table 12 and

Table 13. PCR products were loaded on 1.5-2% agarose gels in TBE buffer. The PCR products were separated by electrophoresis and visualized with ethidiumbromide 1% (1 g/mL). Pictures of the gel were taken with Gel Doc XR+ Gel Documentation System (Biorad, USA). As examples, images are shown in Figure 8.

Table 12: Genotyping reaction

	Tcf4-4-fl	Camk2a-Cre	Emx1-Cre	Bmal1-ko
	µL	µL	µL	µL
H2O	7.4	7.775	7.025	7,4
Flexi 5X	3	3	3	3
MgCl2	0.9	0.9	0.9	0.9
dNTP	1.5	1.5	1.5	1.5
Primer 1	0.375	0.375	0.375	0.375
Primer 2	0.375	0.375	0.375	0.375
Primer 3	0.375	-	0.375	0.375
Primer 4	-	-	0.375	-
GoTaq	0.075	0.075	0.075	0.075
DNA	1	1	1	1
total	15	15	15	15

Table 13: Genotyping program

Step	Tcf4-4-fl			Camk2a-Cre			Emx1-Cre			Bmal1-ko		
	Temp (°C)	Time (min)	Cycles	Temp (°C)	Time (min)	Cycles	Temp (°C)	Time (min)	Cycles	Temp (°C)	Time (min)	Cycles
0	95	preheating		95	preheating		95	preheating		95	preheating	
1	95	1:00	-	95	1:00	-	95	1:00	-	95	1:00	-
2	95	0:10	36	95	0:10	36	95	0:10	36	95	0:10	38
3	59	0:20		56	0:20		55	0:20		50	0:20	
4	72	0:30		72	0:45		72	0:30		72	0:40	
5	72	2:00	-	72	3:00	-	72	3:00	-	72	2:00	-
6	4	∞	-	4	∞	-	4	∞	-	4	∞	-

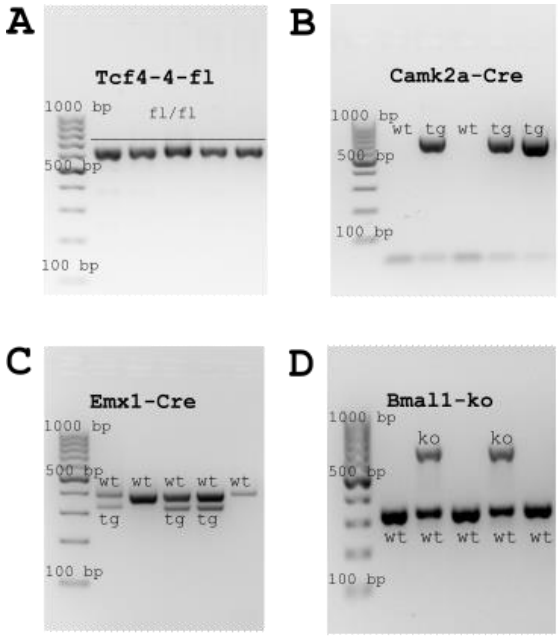

Figure 8: Genotyping PCR electrophoresis.
Products of genotyping PCRs were separated by electrophoresis in agarose gels and visualized using ethidium bromide under UV light. Representative genotyping gels for: A) Tcf4-4-fl allele (637 bp), B) Camk2a-Cre tg (694 bp), wt (not tested), C) Emx1-Cre tg (321 bp) and wt allele (378 bp), D) Bmal1-ko heterozygous knockout, ko (621 bp) and wt (320 bp); 100 bp, 100 base pair DNA ladder; ko, knockout; tg, transgen; wt, wildtype.

2.2.2 Knockout validation

The expression of Tcf4 is expected to be reduced in tissues, which are targeted by the two different Cre lines. In order to verify the conditional knockout in Tcf4-4-fl × Camk2a-Cre and Tcf4-4-fl × Emx1-Cre mouse lines, the relative gene expression of exon 2 and exon 4 of Tcf4 was analyzed with a qPCR. RNA was isolated from two specific brain regions (medial prefrontal cortex, hippocampus) affected by both conditional knockouts and a control region (cerebellum), which is not affected by the Cre-mediated knockout. Relative gene expression (RGE) levels of exon 2 and exon 4 in conditional knockout mice and wildtype mice were measured using one primer pair targeting exons 1 and 2 (1/2) of Tcf4 and one primer pair targeting exons 3 and 4 (3/4) of Tcf4 by referencing to the housekeeping genes RPL13a and ATP5B. The cycle threshold (C_T) is defined as the required cycle number to reach a previously determined fluorescent signal. The difference of the C_T value between the targeted genes (exons 1/2 or exons 3/4) and the mean value of the reference genes (RPL13a and ATP5B as housekeeping genes) was defined as ΔC_T. The relative gene expression was defined as $2^{-\Delta C_T}$.

2.2.2.1 RNA isolation

At the end of the experiment, mice were anesthetized with isoflurane and decapitated. Immediately after death, the whole brain was removed and the medial prefrontal cortex, the hippocampus, and the cerebellum of each hemisphere were isolated and stored in RNAlater at -80 °C. Samples of the hippocampus and the medial prefrontal cortex were taken, because the promoters of the genes Camk2a and Emx1 are active in the forebrain. As a control region, the cerebellum was chosen because it should not be affected by the Cre-mediated conditional knockout (TSIEN *et al.*, 1996; GORSKI *et al.*, 2002).

For isolating RNA, the RNeasy mini Kit (Qiagen, Venlo, Netherlands) was used. The 30 µg brain sample was removed from the RNA later solution and was homogenized with 600 µL RLT buffer. Then one volume 70% EtOH (600 µL) was added, the solution was mixed by pipetting three times up and down and was loaded immediately onto a RNeasy column before proceding with the next sample. The column was centrifuged at 10000 rpm for 30 s. In order to remove any residual DNA, the RNase-Free DNase Set (Qiagen) was used. The DNase solution was diluted in RDD buffer (10 µL Dnase + 70 µL RDD per sample). Then the diluted DNase was added onto the colum followed by a 15 min incubation at room

temperature. The samples were then washed with 350 µL RW1 and 2 × with 500 µL buffer RPE, each followed by centrifugation for 30 s at 10000 rpm. Then the columns were dried by centrifugation for 2 min at 10000 rpm. To eluate the RNA, 30 µL RNA-free water was added.

2.2.2.2 Measuring RNA concentration

2 µL RNA were diluted in 68 µL H_2O. RNA concentration was measured with an UV spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany).

2.2.2.3 cDNA synthesis

The concentration of the reagents for the reverse transcription were adjusted to the cDNA concentration. The high-capacity cDNA reverse transcription kit was used. The reagents and program can be found in the tables below (Table 14, Table 15).

Table 14: qPCR program

Step	Temp (°C)	Time (min)
Annealing	25	10
Elongation	37	120
Heat Inactivation	85	5
Store	4	∞

Table 15: qPCR reaction

Component	1X (µL)
10× RT Buffer	2
25× dNTP Mix (100 mM)	0,8
10× RT Random Primers	2
RT Enzyme	1
H_2O	4.2

2.2.2.4 qPCR analysis

The synthezised cDNA was diluted to 0.25 ng/µL for qPCR. 2x qPCR SYBR Green Master Mix, high (Steinbrenner Laborsysteme, Wiesenbach, Germany) was used as mastermix and the Applied Biosystems StepOnePlus cycler (Thermo Fisher Scientific, Waltham, USA) for qPCR. 10 µL mastermix and 5 µL of the corresponding cDNA were added to each well. In the first row H_2O as negative control was applied with different primers (A1, B1: RPL13a; C1, D1: ATP5B; E1, F1: primer Tcf4 exon 1/2; G1, H1: primer Tcf4 exon 3/4). For each sample four technical replicates were used per primer pair and four samples were quantified per run (Rep1, **Rep1**, Rep2, Rep2). Figure 9.

	1	2	3	4	5	6	7	8	9
A	NTC	Rep1	Rep1	Rep1	Rep1	**Rep1**	**Rep1**	**Rep1**	**Rep1**
B	NTC	Rep1	Rep1	Rep1	Rep1	**Rep1**	**Rep1**	**Rep1**	**Rep1**
C	NTC	Rep1	Rep1	Rep1	Rep1	**Rep1**	**Rep1**	**Rep1**	**Rep1**
D	NTC	Rep1	Rep1	Rep1	Rep1	**Rep1**	**Rep1**	**Rep1**	**Rep1**
E	NTC	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2
F	NTC	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2
G	NTC	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2
H	NTC	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2	Rep2

Figure 9: Template for qPCR analysis.
NTC: Negative control; Rep1, Rep1, Rep2, Rep2: different samples; A1, B1, A2 – H2, A6 – H6: primer RPL13a; C1, D1, A3 – H3. A7 – H7: primer ATP5B; E1, F1, A4 – H4, A8 – H8: primer Tcf4 exon 1/2, G1, H1, A5 – H5, A9 – H9: primer Tcf4 exon 3/4.

IV. RESULTS

1 *In vivo* experiments

1.1 Behavioral experiments

Three mouse models were characterized using a standardized behavioral test battery. The tests and results were categorized into the following RDoC domains: sensorimotor gating system, arousal, cognition, positive valence, and negative valence. For measurement of sensorimotor gating, the prepulse inhibition paradigm was used. Arousal was analyzed by measuring two different locomotor activities: The general locomotor activity includes night and day activity, while the novelty-induced locomotor activity includes the choices in the y-maze and the mean speed in the open field test. The cognitive performance was analyzed in y-maze test, fear conditioning test, and in reversal learning. For the analysis of memory fear conditioning test was used based on classical conditioning. The working memory was evaluated with the analysis of alternations in y-maze. For learning flexibility, the first and serial reversal learning paradigms were used. The positive valence domain was represented by the sucrose preference test and the place learning paradigm. Analysis of negative valence included the center time and the amount of rotations in the open field test, the time immobile in the fear conditioning test, and the tail suspension test.

CDA was used for the analysis of the groups of the Tcf4-4-fl × Emx1-Cre/poly(I:C) model. The groups cko/vehicle, cko/poly(I:C), wt/vehicle, wt/poly(I:C) were separated and dimension plots were plotted.

1.1.1 Tcf4-4-fl × Camk2a-Cre

The TCF4 level in the forebrain of Tcf4-4-fl × Camk2a-Cre mice is decreased from postnatal day 20, induced by crossing Tcf4-4-fl mice with the Camk2a-Cre line (TSIEN *et al.*, 1996; MINICHIELLO *et al.*, 1999). As an environmental factor, the resident-intruder paradigm was used. The experiment was designed as a four arm experiment consisting of the groups wt/social defeat, wt/no stress, cko/social defeat, and cko/no stress. Due to the limitations of manual testing, the four arm experiment had to be split into two runs. The social defeat arm includes 25 wildtype mice subjected to social defeat and 17 conditional knockout mice subjected to social defeat was done first, assuming the strongest behavioral effects. Figure 10.

Groups	Animals
Conditional knockout of *Tcf4*/social defeat	**17 males**
Wildtype/social defeat	**25 males**
Total	**42 males**

Figure 10: Study design Tcf4-4-fl × Cmak2a-Cre/social defeat.
42 Male mice subjected to social defeat were tested in total; 17 conditional knockout mice with reduced expression of Tcf4; 25 wildtype mice with normal Tcf4 expression.

There were no significant differences between conditional knockout mice and wildtype mice observed in alternations in y-maze, reversal learning, serial reversal learning, fear conditioning test, and prepulse inhibition (

Figure 11). Testing sucrose preference, place preference, rotations and center time in open field test, freezing baseline in fear conditioning test and time immobile in tail suspension test showed no significant differences between conditional knockout and wildtype mice (Figure 12). Differences in locomotor activity including general activity, nocturnality, choices in y-maze, and mean speed in open field test were not significant (Figure 13). Because of the absence of strong behavioral effects the second run including the groups wt/no stress and cko/no stress was not done.

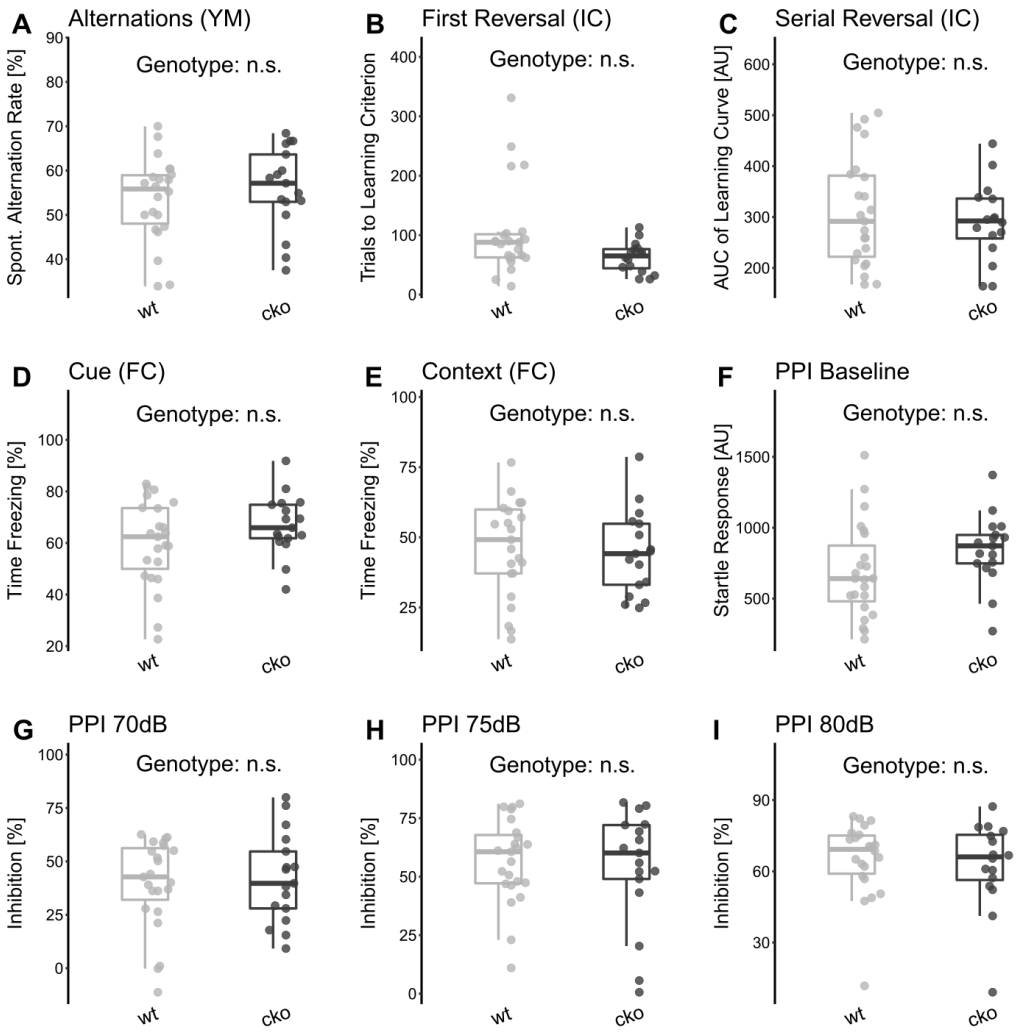

Figure 11: Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat: cognition and sensorimotor gating.

(A-I) No differences between conditional knockout and wildtype mice in cognition and sensorimotor gating. (A) $F_{(1,33)} = 0.36$, $p = 0.83$. (B) $F_{(1,33)} = 4.09$, $p = 0.59$. (C) $F_{(1,33)} = 0.51$, $p = 0.84$. (D) $F_{(1,33)} = 1.63$, $p = 0.8$. (E) $F_{(1,33)} = 0.25$, $p = 0.84$. (F) $F_{(1,33)} = 3.05$, $p = 0.59$. (G) $F_{(1,33)} = 0.10$, $p = 0.84$. (H) $F_{(1,33)} = 0.00$, $p = 0.98$. (I) $F_{(1,33)} = 0.17$, $p = 0.84$. Genotype genotype term; wt, wildtype; cko, conditional knockout; YM, y-maze; IC, intellicage; FC, fear conditioning; PPI, pre-pulse inhibition. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s., not significant; *p*-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 25$ wt males/17 cko males.

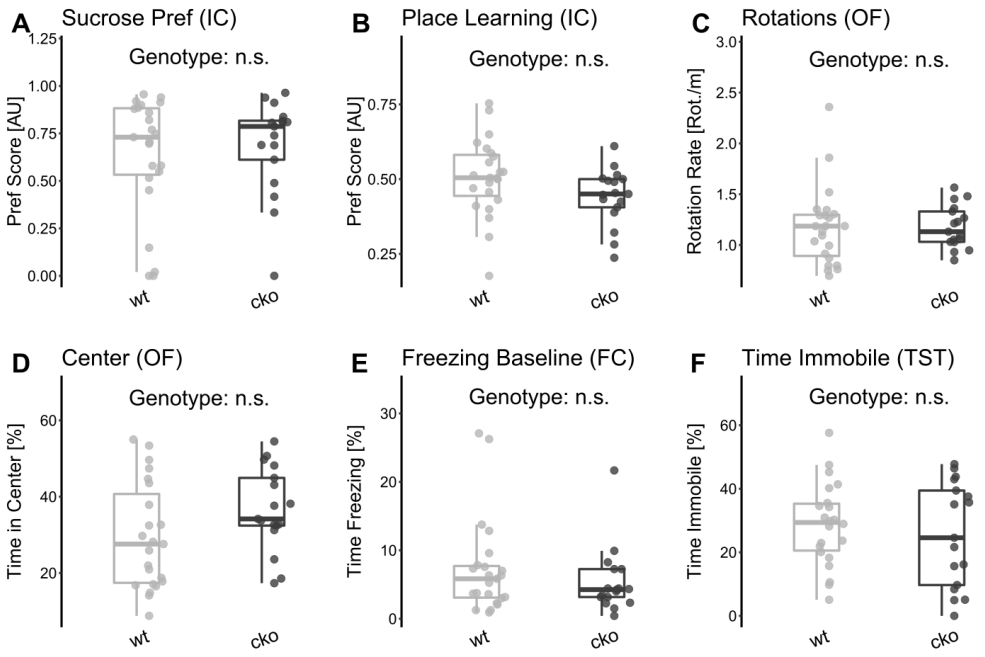

Figure 12: Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat: positive and negative valence.

(A-F) No differences between conditional knockout and wildtype mice in positive and negative valence systems. (A) $F_{(1,33)} = 0.54$, $p = 0.84$. (B) $F_{(1,33)} = 2.22$, $p = 0.69$. (C) $F_{(1,33)} = 0.11$, $p = 0.84$. (D) $F_{(1,33)} = 2.99$, $p = 0.84$. (E) $F_{(1,33)} = 1.07$, $p = 0.84$. (F) $F_{(1,33)} = 0.44$, $p = 0.84$.

Genotype genotype term; wt, wildtype; cko, conditional knockout; IC, intellicage; OF, open field; FC, fear conditioning; TST, tail suspension test. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; p-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 25$ wt males/17 cko males.

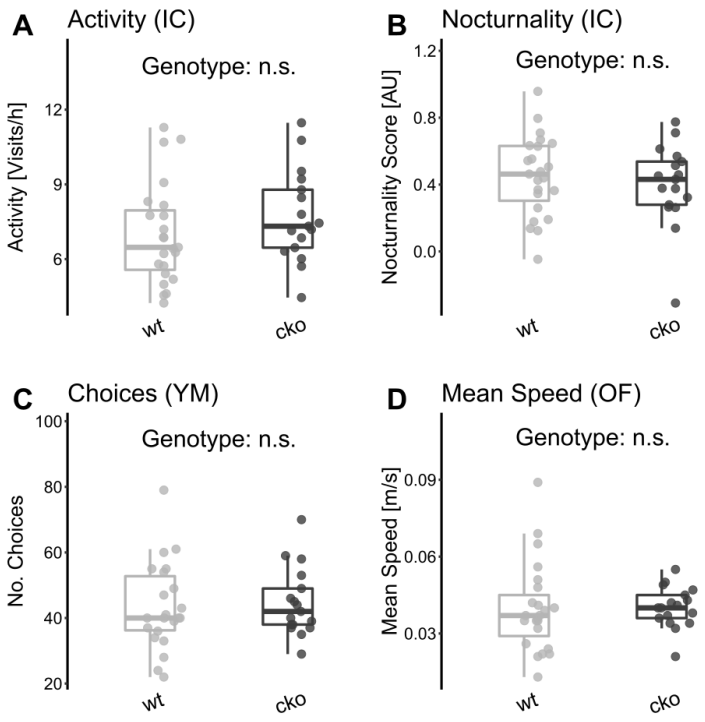

Figure 13: Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat: locomotor activity.

(A-D) No differences between conditional knockout and wildtype mice in locomotor activity. (A) $F_{(1,33)} = 0.93$, $p = 0.84$. (B) $F_{(1,33)} = 0.15$, $p = 0.84$. (C) $F_{(1,33)} = 0.1$, $p = 0.84$. (D) $F_{(1,33)} = 0.00$, $p = 0.99$.

Genotype genotype term; wt, wildtype; cko, conditional knockout; IC, intellicage; YM, y-maze; OF, open field. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; *p*-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 25$ wt males/17 cko males.

1.1.2 Tcf4-4-fl × Emx1-Cre

Crossing Tcf4-4-fl mice with Emx1-Cre mice leads to a reduced level of TCF4 in the forebrain of the offspring. The TCF4 level is reduced in the embryonal phase from ED 12.5 on (GUO *et al.*, 2000). As a second hit, dams were injected with poly(I:C) in late pregnancy. The experiment was designed as a four arm experiment with 34 conditional knockout mice compared to 30 wildtype mice and 32 mice subjected to poly(I:C) compared to 32 mice subjected to vehicle only. The dams were injected with poly(I:C) in late pregnancy as a second hit. Figure 14.

Groups		**Vehicle control**	**Poly(I:C)**	**Total**
Conditional knockout of *Tcf4*	male	11	7	**18**
	female	7	9	**16**
	total	**18**	**16**	**34**
Wildtype	male	7	7	**14**
	female	7	9	**16**
	total	**14**	**16**	**30**
Total		**32**	**32**	**64**

Figure 14: Study design Tcf4-4-fl × Emx1-Cre/poly(I:C).
64 male and female mice were tested in total; 18 conditional knockout mice, offspring of dams injected with vehicle only, 11 male mice, 7 female mice; 16 conditional knockout mice, offspring of dams injected with poly(I:C), 7 male mice, 9 female mice; 14 wildtype mice, offspring of dams injected with vehicle only, 7 male mice, 7 female mice; 16 wildtype mice, offspring of dams injected with poly(I:C), 7 male mice, 9 female mice.

There were no significant differences observed between conditional knockout mice of Tcf4 and wildtype mice and between offspring of dams injected with poly(I:C) or dams injected with vehicle only in alternations in y-maze, fear conditioning test, prepulse inhibition, and first reversal learning. The conditional knockout mice performed better in flexibility learning compared to the wildtype control group ($p = 0.001$). Flexibility learning is analyzed in the serial reversal learning test (Figure 15). In positive valence, tested with sucrose and place preference test, and negative valence, represented by rotations and center time in open field test, freezing baseline in fear conditioning test, and time immobile in tail suspension test, no significant differences between the groups were measured (Figure 16). Significant differences were found in the novel-induced locomotor activity. The mean speed in open field test was reduced in conditional knockout mice of Tcf4

compared to wildtype mice (p = 0.047). The general activity in night and day and the choices in y-maze was not affected (Figure 17). In order to determine the cause of the reduced mean speed, the time immobile in the open field test was analyzed. The conditional knockout mice showed an increased time of immobility compared to the wildtype control mice. Figure 18.

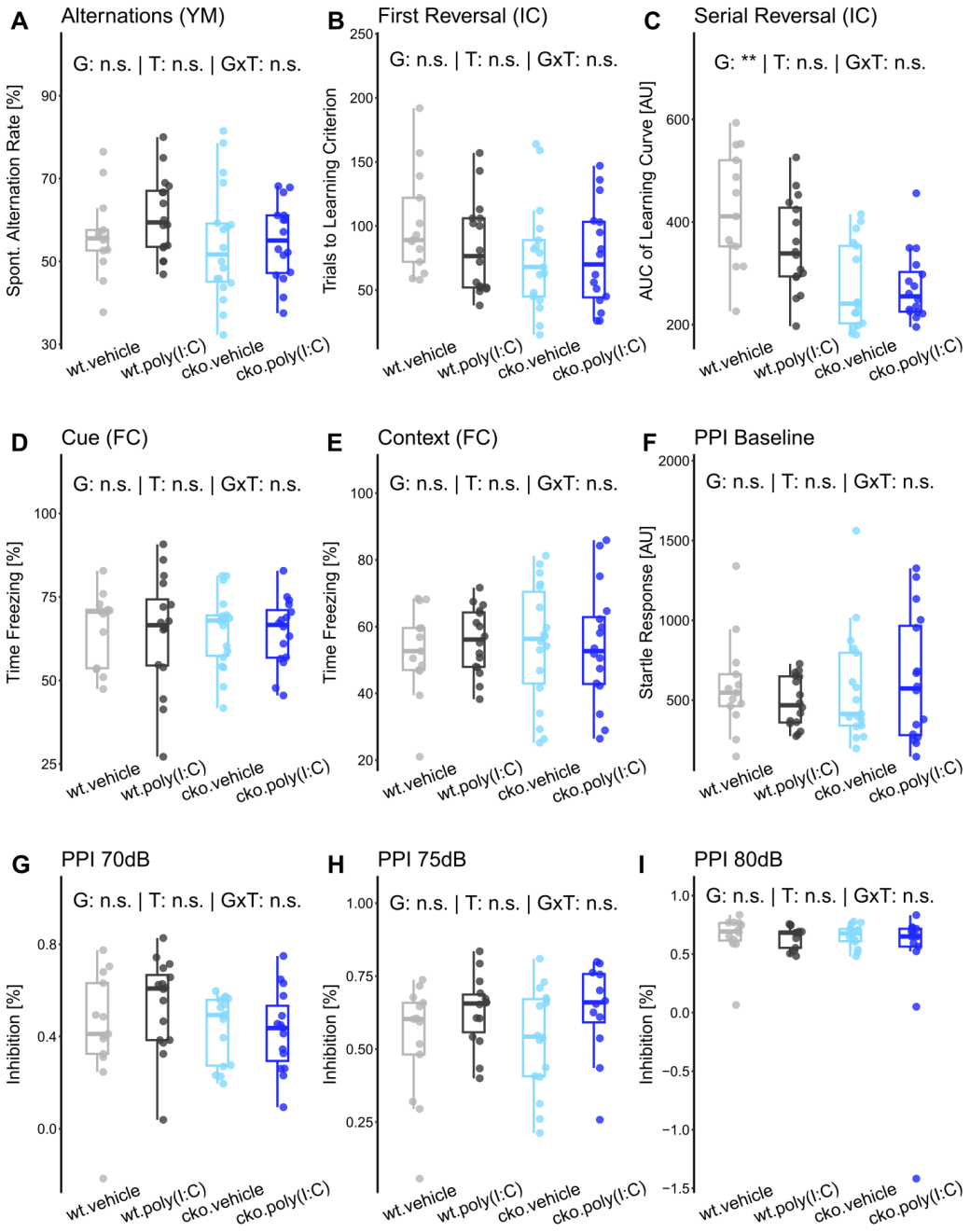
A
Alternations (YM)
G: n.s. | T: n.s. | GxT: n.s.
Spont. Alternation Rate [%]
B
First Reversal (IC)
G: n.s. | T: n.s. | GxT: n.s.
Trials to Learning Criterion
C
Serial Reversal (IC)
G: ** | T: n.s. | GxT: n.s.
AUC of Learning Curve [AU]
D
Cue (FC)
G: n.s. | T: n.s. | GxT: n.s.
Time Freezing [%]
E
Context (FC)
G: n.s. | T: n.s. | GxT: n.s.
Time Freezing [%]
F
PPI Baseline
G: n.s. | T: n.s. | GxT: n.s.
Startle Response [AU]
G
PPI 70dB
G: n.s. | T: n.s. | GxT: n.s.
Inhibition [%]
H
PPI 75dB
G: n.s. | T: n.s. | GxT: n.s.
Inhibition [%]
I
PPI 80dB
G: n.s. | T: n.s. | GxT: n.s.
Inhibition [%]
wt.vehicle
wt.poly(I:C)
cko.vehicle
cko.poly(I:C)

Figure 15: Tcf4-4-fl × Emx1-Cre, offspring of mice subjected to poly(I:C) injections: cognition, and sensorimotor gating.

(A) The Y-maze test showed no significant differences in spontaneous alternation between groups. (B) No significant differences in first reversal between groups. (C) Conditional knockout mice perform better in the serial reversal learning paradigm, $H_{(1,58)} = 17{,}77$, $p = 0{,}001$ (D, E) Fear conditioning test showed no significant differences between groups. (F-I) No significant differences in sensorimotor gating between groups. (A) G: $H_{(1,59)} = 2$, $p = 0.89$; T: $H_{(1,59)} = 1.28$, $p = 0.95$; GxT: $H_{(1,59)} = 0.4$, $p = 0.95$. (B) G: $H_{(1,58)} = 3.04$, $p = 0.76$; T: $H_{(1,58)} = 0.5$, $p = 0.95$; GxT: $H_{(1,58)} = 0.8$, $p = 0.95$. (C) G: $H_{(1,58)} = 17.77$, $p = 0.001$; T: $H_{(1,58)} = 0.71$, $p = 0.95$; GxT: $H_{(1,58)} = 1.17$, $p = 0.95$. (D) G: $H_{(1,59)} = 0.13$, $p = 0.95$; T: $H_{(1,59)} = 0.08$, $p = 0.95$; GxT: $H_{(1,59)} = 0.04$, $p = 0.95$. (E) G: $H_{(1,58)} = 0.004$, $p = 0.99$; T: $H_{(1,58)} = 0.009$, $p = 0.95$; GxT: $H_{(1,58)} = 0.33$, $p = 0.99$. (F) G: $H_{(1,58)} = 5$, $p = 0.99$; T: $H_{(1,58)} = 0.04$, $p = 0.95$; GxT: $H_{(1,58)} = 0.43$, $p = 0.95$. (G) G: $H_{(1,56)} = 2.35$, $p = 0.83$; T: $H_{(1,56)} = 0.6$, $p = 0.95$; GxT: $H_{(1,56)} = 0.93$, $p = 0.95$. (H) G: $H_{(1,51)} = 0.08$, $p = 0.001$; T: $H_{(1,51)} = 3.96$, $p = 0.75$; GxT: $H_{(1,51)} = 0.04$, $p = 0.95$. (I) G: $H_{(1,48)} = 0.28$, $p = 0.001$; T: $H_{(1,48)} = 1.17$, $p = 0.95$; GxT: $H_{(1,48)} = 0.19$, $p = 0.95$.

Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s., not significant; *p*-values are FDR-corrected and refer to scheirer ray hare test; $n = 14$ wt vehicle/16 wt poly(I:C)/18 cko vehicle/16 cko poly(I:C); G, genotype term; T, treatment term; G×T, interaction term; wt, wildtype; cko, conditional knockout; YM, y-maze; IC, intellicage; FC, fear conditioning; PPI, pre-pulse inhibition

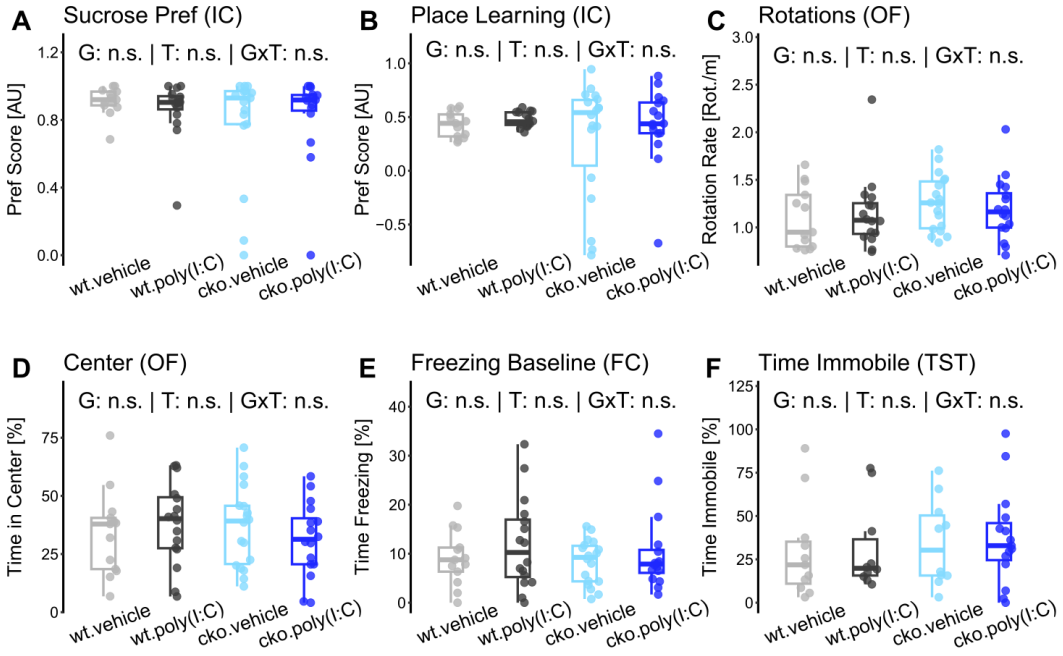

Figure 16: Tcf4-4-fl × Emx1-Cre, offspring of mice subjected to poly(I:C) injections: positive and negative valence.
(A-F) No significant differences between groups in positive and negative valence. (A) G: $H_{(1,57)} = 0.06$, $p = 0.95$; T: $H_{(1,57)} = 0.27$, $p = 0.95$; GxT: $H_{(1,57)} = 0.16$, $p = 0.95$. (B) G: $H_{(1,59)} = 0.37$, $p = 0.95$; T: $H_{(1,59)} = 0.07$, $p = 0.95$; GxT: $H_{(1,59)} = 0.53$, $p = 0.95$. (C) G: $H_{(1,59)} = 2.27$, $p = 0.83$; T: $H_{(1,59)} = 0.17$, $p = 0.95$; GxT: $H_{(1,59)} = 0.46$, $p = 0.95$. (D) G: $H_{(1,59)} = 0.13$, $p = 0.95$; T: $H_{(1,59)} = 0.003$, $p = 0.99$; GxT: $H_{(1,59)} = 1.66$, $p = 0.95$. (E) G: $H_{(1,58)} = 0.39$, $p = 0.99$; T: $H_{(1,58)} = 0.23$, $p = 0.95$; GxT: $H_{(1,58)} = 0.06$, $p = 0.99$. (F) G: $H_{(1,44)} = 0.97$, $p = 0.99$; T: $H_{(1,44)} = 0.14$, $p = 0.95$; GxT: $H_{(1,44)} = 0.0003$, $p = 0.99$.
Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s., not significant; p-values are FDR-corrected and refer to scheirer ray hare test; $n = 14$ wt vehicle/16 wt poly(I:C)/18 cko vehicle/16 cko poly(I:C); G, genotype term; T, treatment term; G×T, interaction term; wt, wildtype; cko, conditional knockout; IC, intellicage; OF, open field; FC, fear conditioning; TST, tail suspension test.

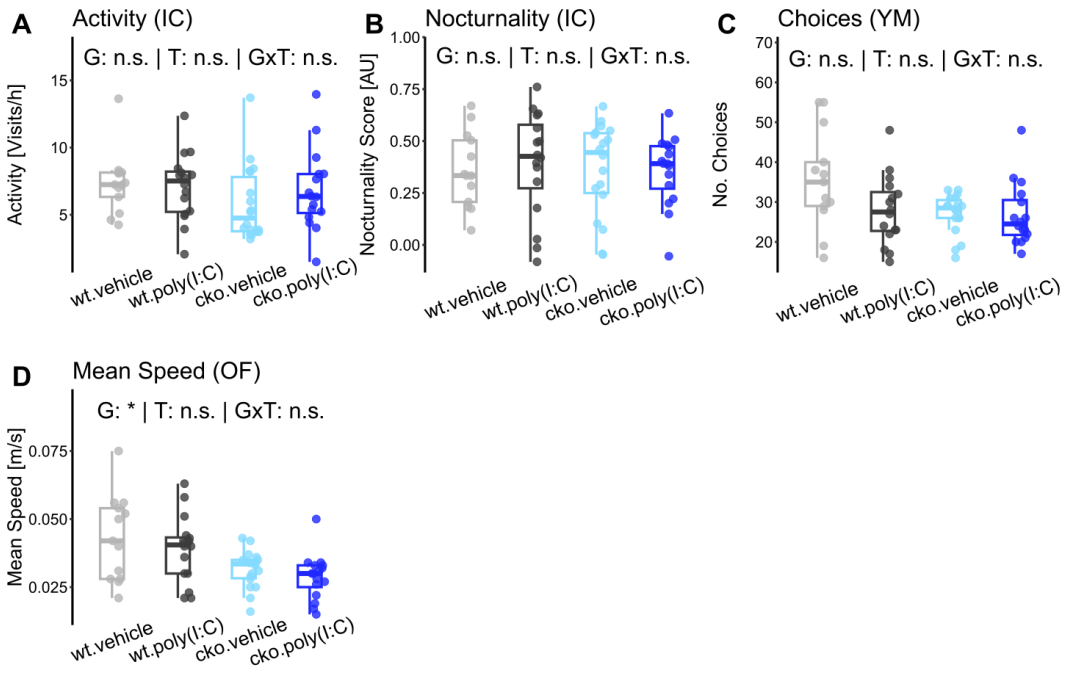

Figure 17: Tcf4-4-fl × Emx1-Cre, offspring of mice subjected to poly(I:C) injections: locomotor activity.
(A-C) No significant difference between groups. (D) Decreased novelty-induced locomotor activity in conditional knockout mice, $H_{(1,59)} = 9.88$, $p = 0.047$.
(A) G: $H_{(1,59)} = 2.82$, $p = 0.76$; T: $H_{(1,59)} = 0.66$, $p = 0.95$; GxT: $H_{(1,59)} = 0.94$, $p = 0.95$. (B) G: $H_{(1,59)} = 0.04$, $p = 0.95$; T: $H_{(1,59)} = 0.02$, $p = 0.98$; GxT: $H_{(1,59)} = 0.8$, $p = 0.95$. (C) G: $H_{(1,59)} = 3.32$, $p = 0.76$; T: $H_{(1,59)} = 2.81$, $p = 0.76$; GxT: $H_{(1,59)} = 1.13$, $p = 0.95$. (D) G: $H_{(1,59)} = 9.88$, $p = 0.047$; T: $H_{(1,59)} = 0.87$, $p = 0.95$; GxT: $H_{(1,59)} = 0.31$, $p = 0.95$.
Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; p-values are FDR-corrected and refer to scheirer ray hare test; $n = 14$ wt vehicle/16 wt poly(I:C)/18 cko vehicle/16 cko poly(I:C); G, genotype term; T, treatment term; G×T, interaction term; wt, wildtype; cko, conditional knockout; IC, intellicage; YM, y-maze; OF, open field.

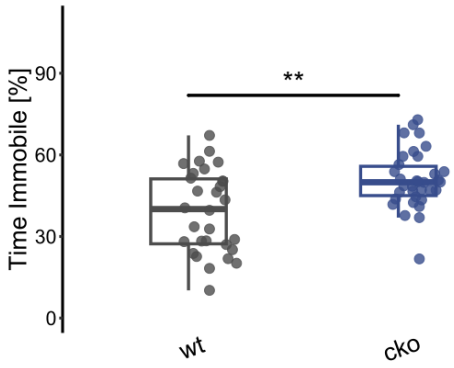

Figure 18: Tcf4-4-fl × Emx1-Cre: Time immobile in open field test.
Increased time immobile in conditional knockout mice. T = - 3.5342, p = 0.00558. Wt, wildtype; cko, conditional knockout; Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant; p-values refer to Welsh t-test.

The CDA is shown in a dimension plot. The separation of the datapoints that are summarized in ellipsoids depends on the measured differences between the tested groups. The separation of the ellipsoids along the genetic factor show a few overlaps. The separation along the environmental factor is lower compared to the genetic factor and more parts of the ellipsoids are overlapping. Figure 19.

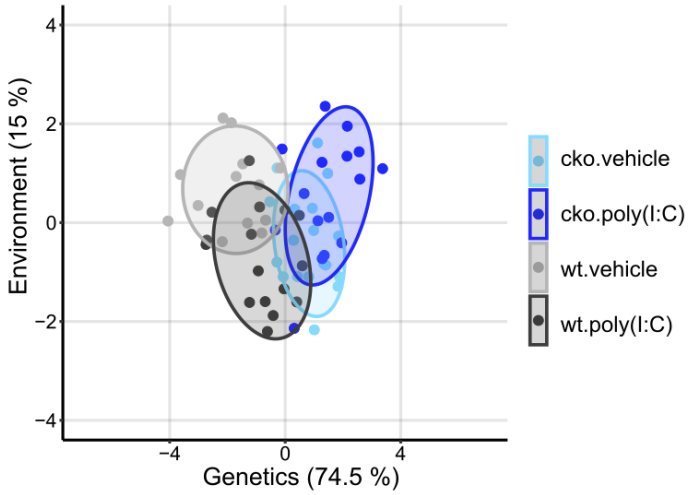

Figure 19: cko/wt, poly(I:C)/vehicle: dimension plot.
The stronger canonical component Genetics, which explains 74.5% of the total canonical correlation, separates the datapoints by Tcf4 gene dosage, while the second component, amounting to 15% of canonical correlation, separates the datapoints by the environmental factor. Ellipsoids visualize 75% coverage of each group and each animal is represented as a correspondingly colored dot; n = 14 wt vehicle/16 wt poly(I:C)/18 cko vehicle/16 cko poly(I:C).

In the heatmap the differences between the genetic factor, environmental factor, and gene × environment interaction are illustrated with intensity of the colors based on the z-score. The tested parameters are arranged according to the RDoC domains cognition including alternations in y-maze, reversal learning performance, serial reversal learning performance, cue and contextual memory, sensorimotor gating including prepulse inhibition, positive valence including performance in sucrose and place preference test, negative valence including rotations and center time in open field, freezing baseline, tail suspension test, and arousal including activity, nocturnality, choices in y-maze, and mean speed in open field. The conditional knockout mice showed a better performance in flexibility learning, measured in the serial reversal learning paradigm, compared to the wildtype control (p = 0.001). The novel-induced locomotor activity of conditional knockout mice of *Tcf4* was significantly reduced compared to the wildtype control mice (p = 0.047). Figure 20.

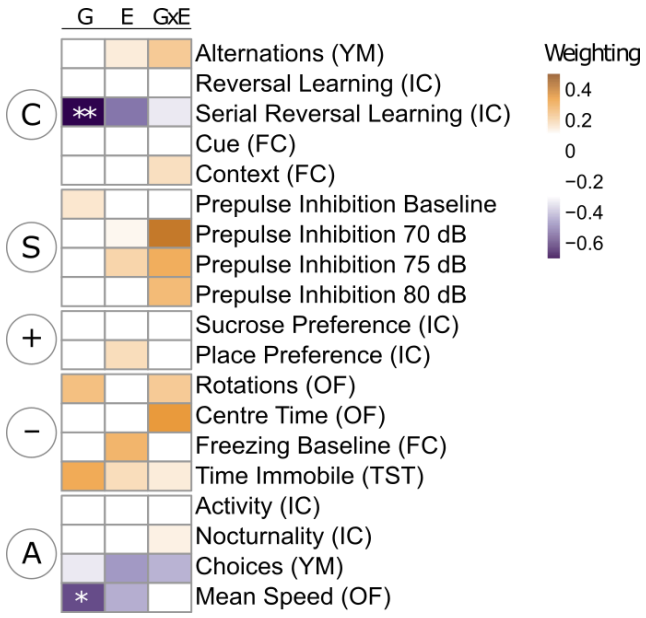

Figure 20: Tcf4-4-fl × Emx1-Cre, offspring of mice subjected to poly(I:C) injections: heatmap: genetic factor, environmental factor, gene × environment interaction.
RDoC-grouping of z-scored data reveals domain-specific differences in the contribution of Tcf4 gene dosage and psychosocial stress to behavioral and cognitive dysfunction. The heatmap highlights changes relative to the healthy control group. Serial reversal learning is improved in conditional knockouts of Tcf4 ($H_{(1,58)}$ = 17,77, p = 0,001). The mean speed is reduced in conditional knockouts of Tcf4 ($H_{(1,59)}$ = 9.88, p = 0.047). Column blocks are RDoC domains; n = 14 wt vehicle/16 wt poly(I:C)/18 cko vehicle/16 cko poly(I:C); G, genotype term; E, environment term; GXE, interaction term; C, cognition; S, sensorimotor gating; +, positive valence; -, negative valence; A, arousal; YM, y-maze; IC, intellicage; FC, fear conditioning; OF, open field; TST, tail suspension test.

1.1.3 Bmal1-ko

To analyze the impact of a reduced expression of the *Bmal1* gene, a genetic mouse model was designed with 17 mice carrying a heterozygous knockout of *Bmal1* compared to 19 wildtype control mice Figure 21.

Groups	**Animals**		
	males	females	**total**
Bmal1 heterozygous knockout	11	6	**17**
Wildtype mice	10	9	**19**
Total	**21**	**15**	**36**

Figure 21: Study design Bmal1-ko.
36 Male and female mice were tested in total; 17 heterozygous Bmal1 knockout mice, 11 males; 6 females; 19 wildtype mice, 10 males, 9 females.

There were no significant differences between heterozygous knockout mice of *Bmal1* and wildtype mice in cognition, measured with alternations in y-maze, reversal learning, serial reversal learning, and fear conditioning test, and sensorimotor gating, measured with prepulse inhibition test (Figure 22). The heterozygous knockout mice showed a reduced preference for sucrose compared to their wildtype littermates. The heterozygous knockout mice were separated into two subgroups. One subgroup showed similar values to the wt control mice (n = 10) and the other subgroup showed values next to 0.0 (n = 7). No significant differences between the groups were observed in place preference, rotations and center time in open field test, freezing baseline in fear conditioning test and time immobile in tail suspension test (Figure 23). The locomotor activity including activity, nocturnality, choices in y-maze and mean speed in open field were not significantly different between the tested groups (Figure 24).

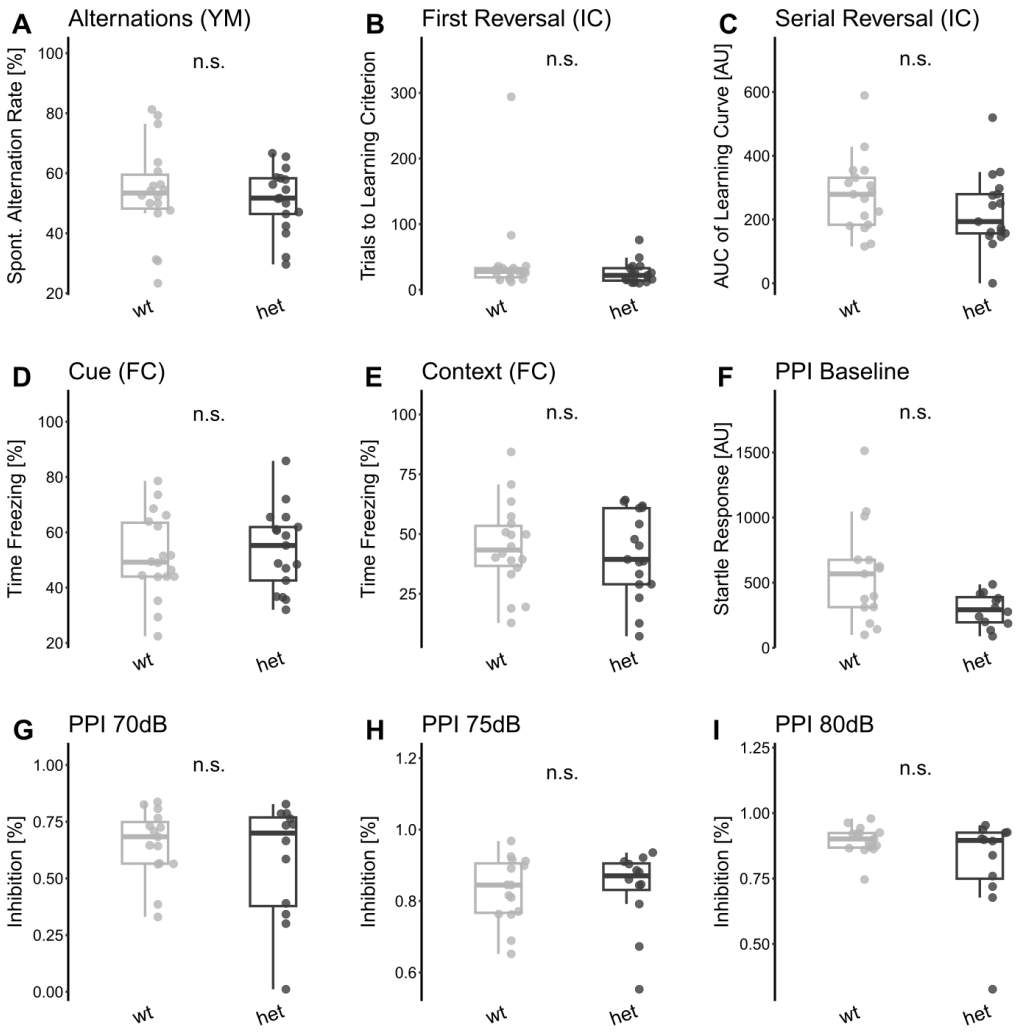

Figure 22: Bmal1-ko mice: cognition, sensorimotor gating.
(A -I) No differences between conditional knockout and wildtype mice in cognition, and sensorimotor gating. (A) $F_{(1,24)} = 0.25$, $p = 0.79$. (B) $F_{(1,24)} = 1.1$, $p = 0.58$. (C) $F_{(1,24)} = 0.42$, $p = 0.71$. (D) $F_{(1,24)} = 1.45$, $p = 0.57$. (E) $F_{(1,24)} = 1.27$, $p = 0.57$. (F) $F_{(1,24)} = 6.88$, $p = 0.14$. (G) $F_{(1,24)} = 0.61$, $p = 0.71$. (H) $F_{(1,24)} = 0.001$, $p = 0.97$. (I) $F_{(1,24)} = 2.9$, $p = 0.48$.
Wt, wildtype; het, heterozygous knockout; YM, y-maze; IC, intellicage; FC, fear conditioning; PPI, pre-pulse inhibition. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; *p*-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 19$ wt/17 het.

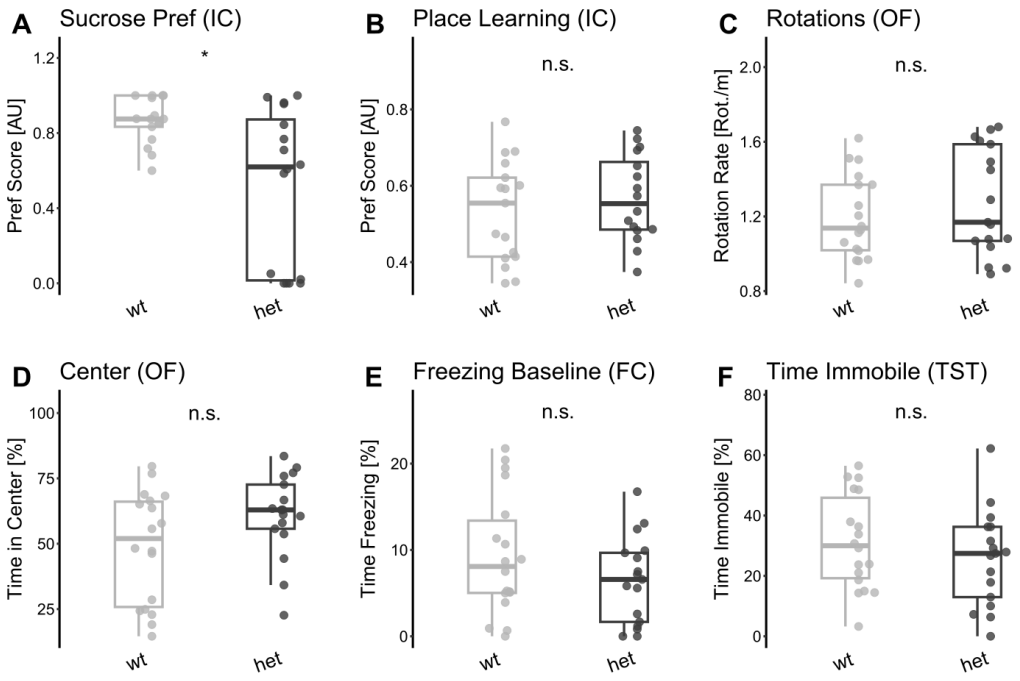

Figure 23: Bmal1-ko mice: positive and negative valence.
(A) Reduced sucrose preference in conditional knockout mice, $F_{(1,24)} = 13.7$, $p = 0.02$. (B-F) No significant difference between heterozygous knockout and wildtype mice for the positive and negative valence domains. (A) $F_{(1,24)} = 13.7$, $p = 0.02$. (B) $F_{(1,24)} = 0.19$, $p = 0.79$. (C) $F_{(1,24)} = 0.5$, $p = 0.71$. (D) $F_{(1,24)} = 3.08$, $p = 0.48$. (E) $F_{(1,24)} = 2.41$, $p = 0.51$. (F) $F_{(1,24)} = 0.02$, $p = 0.94$.
Wt, wildtype; het, heterozygous knockout; IC, intellicage; OF, open field; FC, fear conditioning; TST, tail suspension test. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; *p*-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 19$ wt/17 het.

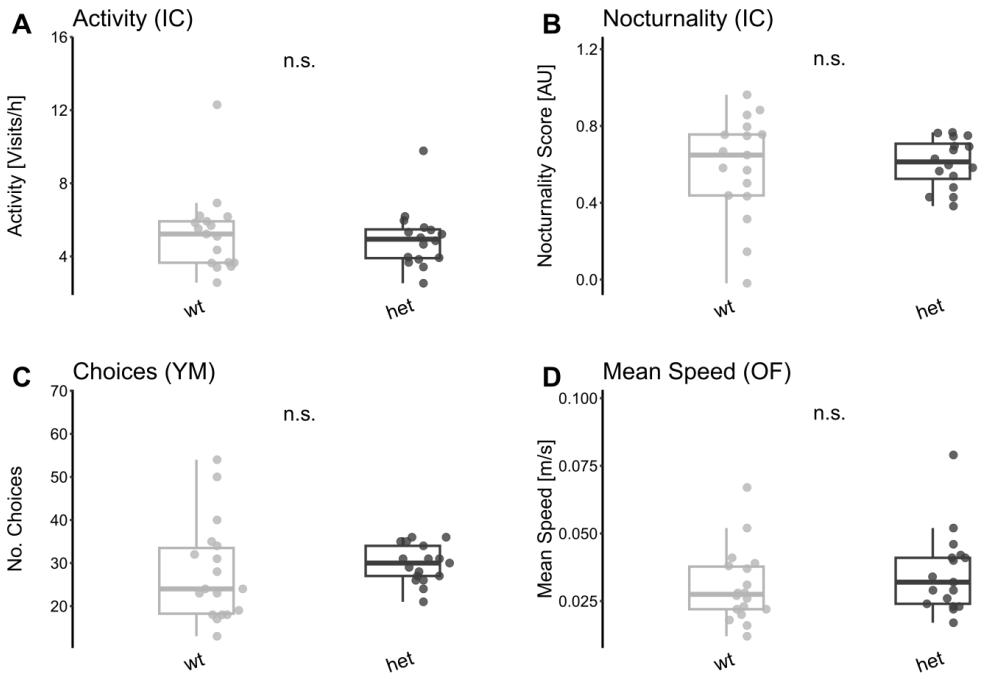

Figure 24: Bmal1-ko mice: locomotor activity.
(A-D) No significant difference between conditional knockout and wildtype mice in general locomotor activity and novelty induced locomotor activity. A) $F_{(1,24)} = 0.06$, $p = 0.91$. (B) $F_{(1,24)} = 0.59$, $p = 0.71$. (C) $F_{(1,24)} = 1.45$, $p = 0.57$. (D) $F_{(1,24)} = 1.49$, $p = 0{,}57$.
Wt, wildtype; het, heterozygous knockout; IC, intellicage; YM, y-maze; OF, open field. Data are shown as box plots with whiskers extending to no more than 1.5-fold IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant; p-values are FDR-corrected and refer to Wilk's lambda testing two-way ANOVA; $n = 19$ wt/17 het.

In the heatmap the differences caused by the gene dosage are illustrated with the intensity of the colors based on the z-score. The tested parameters are arranged according to the RDoC domains cognition including alternations in y-maze, reversal learning performance, serial reversal learning performance, cue and contextual memory, sensorimotor gating including prepulse inhibition, positive valence including performance in sucrose and place preference test, negative valence including rotations and center time in open field, freezing baseline, tail suspension test, and arousal including activity, nocturnality, choices in y-maze, and mean speed in open field. The heterozygous knockout mice of *Bmal1* showed a reduced sucrose preference compared to the wildtype control mice ($F_{(1,24)} = 13.7$, $p = 0.02$). Figure 25.

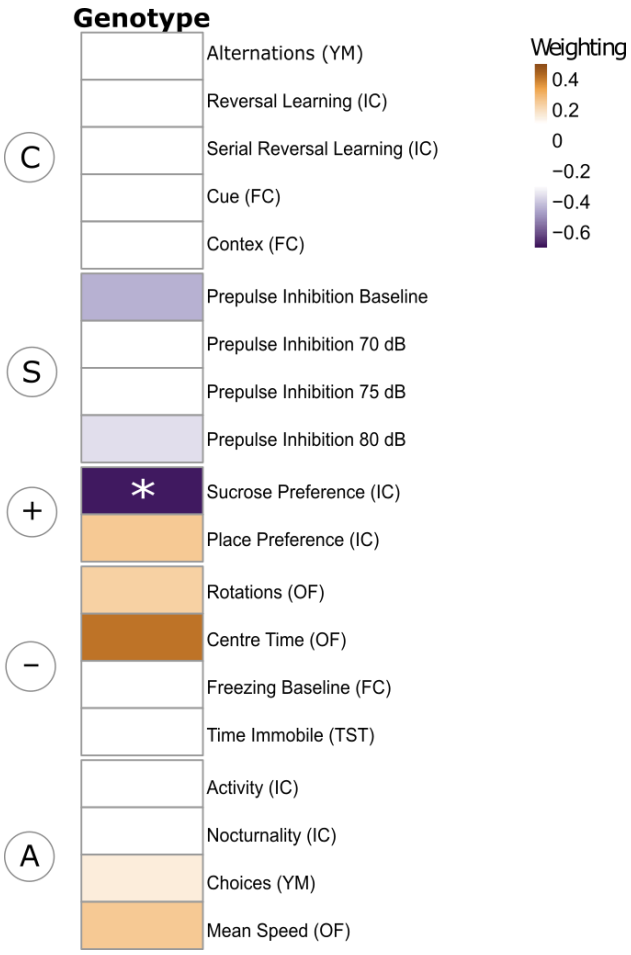

Figure 25: Bmal1-ko mice: heatmap.
RDoC-grouping of z-scored data reveals domain-specific differences in the contribution of *Bmal1* gene dosage. The heatmap highlights changes relative to the healthy control group. Sucrose preference is reduced in heterozygous Bmal1 knockout mice. Column blocks are RDoC domains; *n* = 9 wt/17 het; C, cognition; S, sensorimotor gating; +, positive valence; -, negative valence; A, arousal; YM, y-maze; IC, intellicage; FC, fear conditioning; OF, open field; TST, tail suspension test.

2 *In vitro* experiments

2.1 Genotyping

In order to identify the genotype of the mice tail biopsies were taken after weaning and after death. The results were compared and the PCR were repeated for both tail biopsies when the results were not identical. (Figure 26).

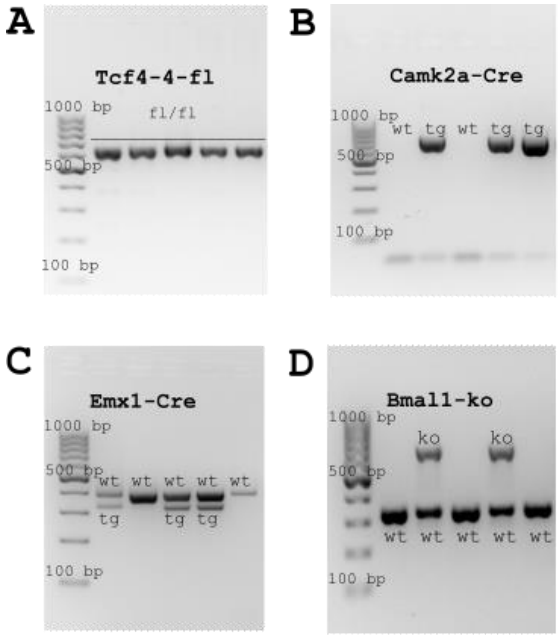

Figure 26: Genotyping PCR electrophoresis.
Products of genotyping PCRs were separated by electrophoresis in agarose gels and visualized using ethidium bromide under UV light. Representative genotyping gels for: A) Tcf4-4-fl allele (637 bp), B) Camk2a-Cre tg (694 bp), wt (not tested), C) Emx1-Cre tg (321 bp) and wt allele (378 bp), D) Bmal1-ko heterozygous knockout, ko (621 bp) and wt (320 bp); 100 bp, 100 base pair DNA ladder; ko, knockout; tg, transgen; wt, wildtype;

2.1.1 Tcf4-4-fl × Camk2a-Cre

After comparing the genotyping results based on biopsies taken by weaning and after death, four mice were initially falsely identified as wildtype and four mice were falsely detected as Cre carriers. Repetition of PCR of both samples led to the same results, so analysis was adjusted afterwards.

2.1.2 Tcf4-4-fl × Emx1-Cre

After comparing the genotyping results based on biopsies taken by weaning and after death, two mice were initially falsely detected as wildtype. Repetition of PCR of both samples led to the same results, so analysis was adjusted afterwards.

2.1.3 Bmal1-ko

After comparing the genotyping results based on biopsies taken by weaning and after death, one mice was falsely detected as heterozygous knockout. Repetition of PCR of both samples led to the same results, so analysis was adjusted afterwards.

2.2 Knockout validation

Two conditional knockout models were used, Tcf4-4-fl × Camk2a-Cre and Tcf4-4-fl × Emx1-Cre. The conditional knockout of Tcf4 is tissue specific in the forebrain and in Camk2a-Cre mice also in the male germline (MINICHIELLO *et al.*, 1999; CHOI *et al.*, 2014). The conditional knockout is mediated trough a Cre-mediated deletion of exon 4, which leads to a premature stop codon and the processing mRNA decays. In order to validate the knockout of Tcf4 in excitatory neurons in the forebrain (Tcf4-4-fl × Camk2a-Cre) and the knockout of Tcf4 in neurons of the neocortex and hippocampus, brain samples were taken after the behavioral experiments. The brains of five mice per genotype were dissected. Samples of the medial prefrontal cortex and hippocampus that are affected by the conditional knockout and samples of the cerebellum, which is not affected by the conditional knockout, were isolated and relative gene expression of exon 4 and exon 2 was measured. Three to five samples per tissue and genotype were used. The primers for exon 2 (1/2) are located in exon 1 and 2 and the primers for exon 4 (3/4) in exons 3 and 4. One sample corresponds to one data point.

2.2.1 Tcf4-4-fl × Camk2a-Cre

The relative gene expression of three samples of medial prefrontal cortex, five samples of hippocampus, four samples of cerebellum from conditional knockout mice and the relative gene expression of three samples of medial prefrontal cortex, five samples of hippocampus, three samples of cerebellum from wildtype mice was measured. Figure 27.

Groups	**Tissue samples**		
	Medial prefrontal cortex	**Hippocampus**	**Cerebellum**
Conditional knockout mice	3	5	4
Wildtype mice	3	5	3
Total	6	10	7

Figure 27: Experiment design: knockout validation Tcf4-4-fl × Camk2a-Cre. Samples of the medial prefrontal cortex (cko: three, wt: three), hippocampus (cko: five, wt: five), cerebellum (cko: four, wt: three).

The relative gene expression of exon 2 in the medial prefrontal cortex consisted of one group of clustered datapoints and three outliers. Two samples of wildtype mice and one sample of conditional knockout mice were at a similar level ~ 0.25 RGE. The relative expression levels of exon 2 in the other samples were higher: the relative gene expression of exon 1/2 of the third wildtype sample was ~3.5 RGE and the relative gene expression of the two cortex samples of the conditional knockout were ~2.5 RGE and ~6 RGE. The relative gene expression of exon 4 in conditional knockout samples was reduced (~0.1 – 0.5 RGE) compared to the relative gene expression of exon 4 in samples of wildtype mice (~0.5 – 1.5 RGE).

Figure 28.

The relative gene expression level of exon 2 in the hippocampus was similar in samples of wildtype and conditional knockout mice (~0.5 RGE), except three data points (~2.75 RGE, ~4 RGE, ~1.5 RGE). In conditional knockout samples the relative gene expression of exon 4 was at a constant low level (~0.1 – ~0.2 RGE) and reduced compared to the wildtype samples (~0.5 RGE). Two samples of wildtype mice show a higher expression level compared to the other wildtype samples (~2 RGE). Figure 29.

The relative gene expression of exon 2 and exon 4 in the cerebellum of both genotypes is spread widely: exon 2 (~0.1 – ~3 RGE), exon 4 (~0.1 – ~1.75 RGE). The relative expression of exon 4 in conditional knockout mice is reduced (~0.1 – ~0.5 RGE) compared to the wildtype mice (~0.75 – ~1.75 RGE). Figure 30.

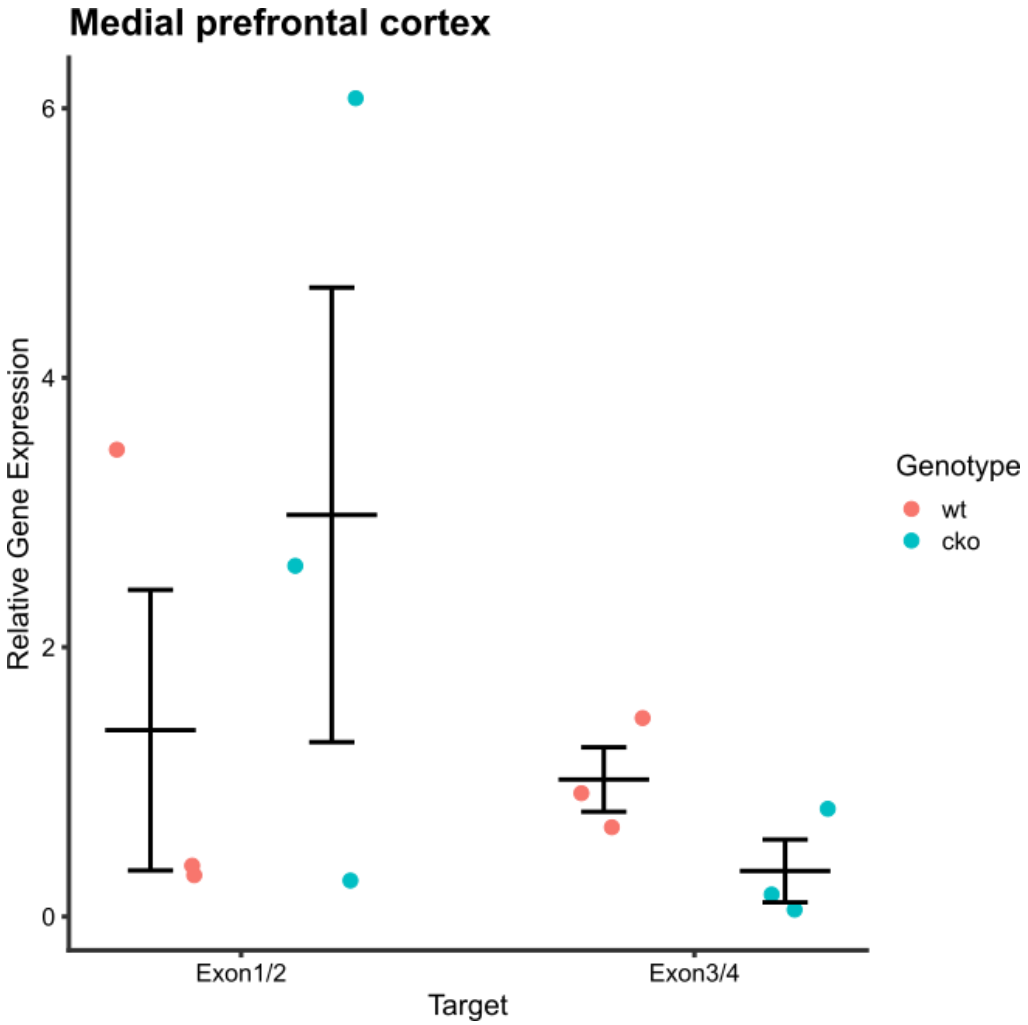

Figure 28: Relative gene expression of exon 1/2 and exon 3/4 in medial prefrontal cortex of Tcf4 - 4 - fl × Camk2a - Cre mice subjected to social defeat.
Relative gene expression wt, wildtype; cko, conditional knockout.

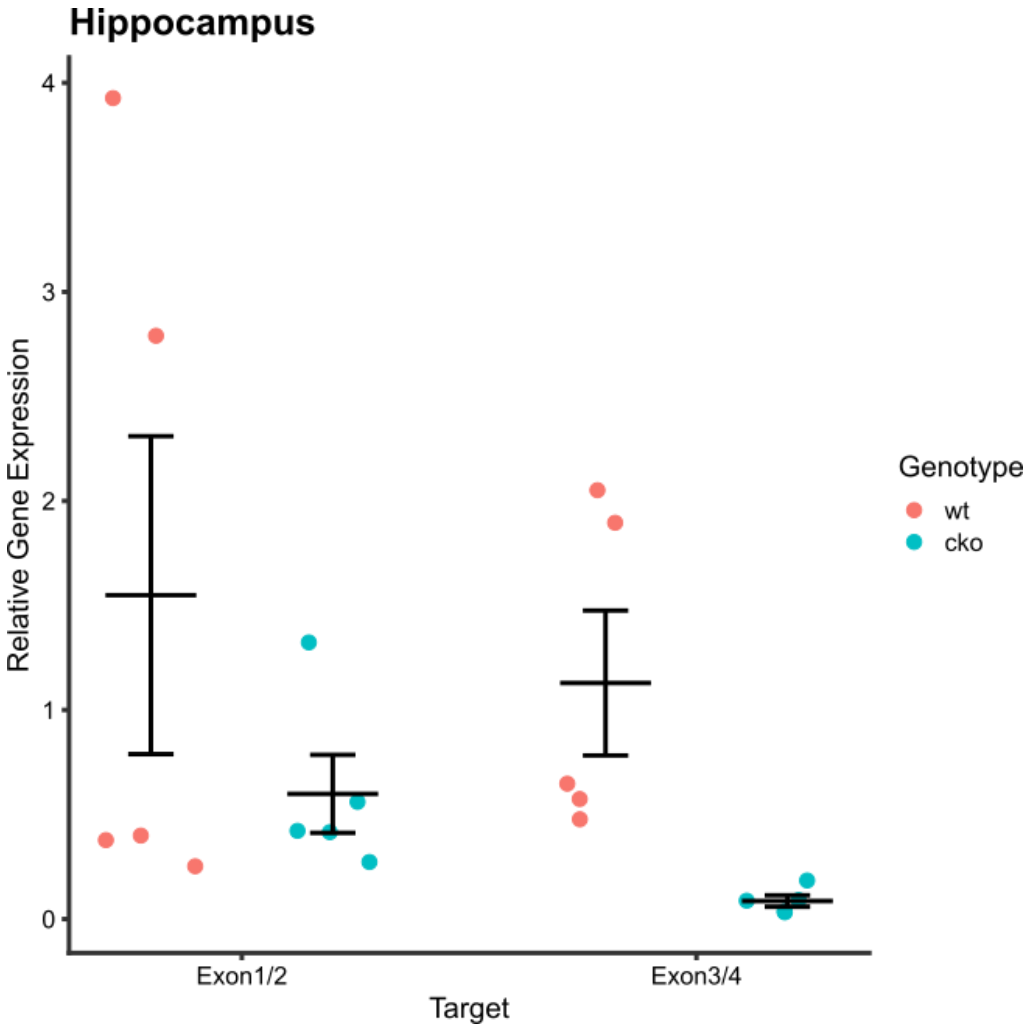

Figure 29: Relative gene expression of exon 1/2 and exon 3/4 in hippocampus of Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat.
Relative gene expression; wt, wildtype; cko, conditional knockout, *n* = 5 wt/5 cko.

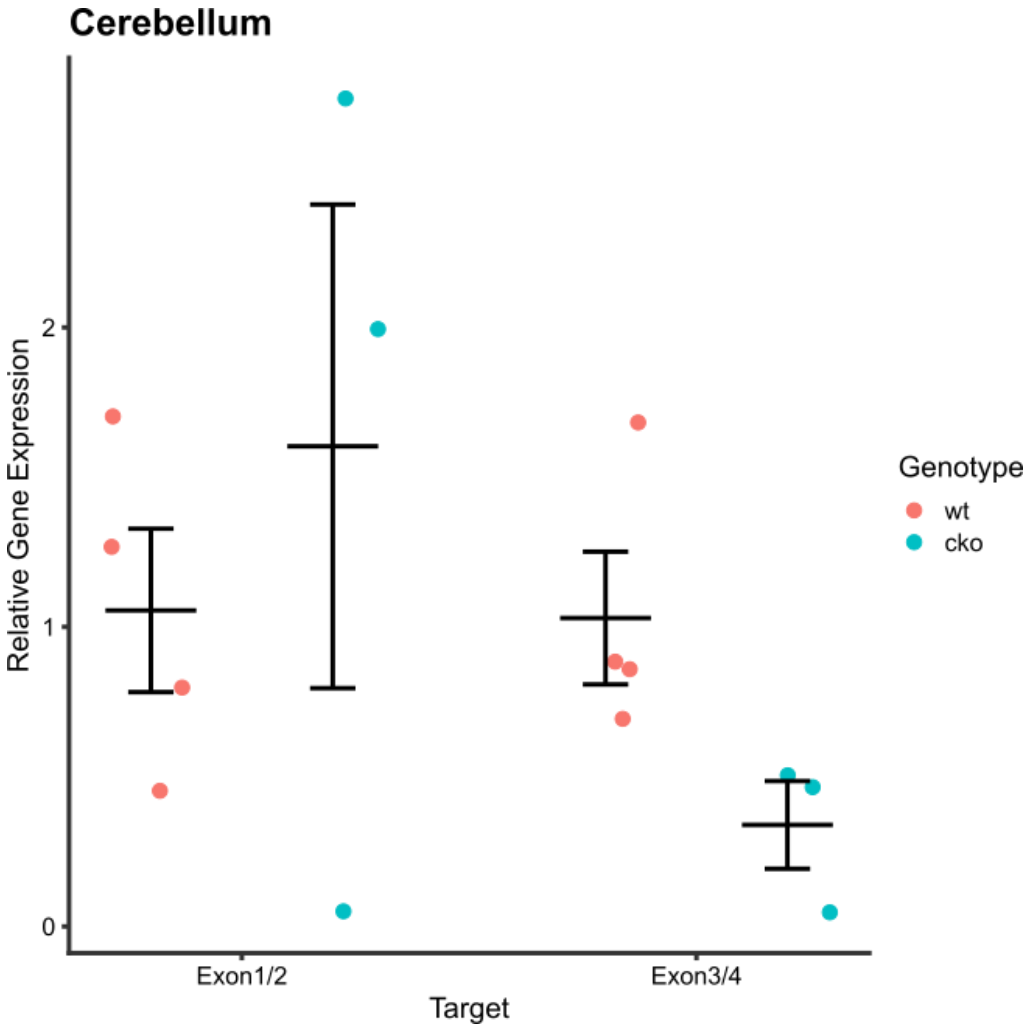

Figure 30: Relative gene expression of exon 1/2 and exon 3/4 in cerebellum of Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat.
Relative gene expression; wt, wildtype; cko, conditional knockout, n = 4 wt/3 cko.

2.2.2 Tcf4-4-fl × Emx1-Cre

The relative gene expression of three samples of medial prefrontal cortex, three samples of hippocampus, four samples of cerebellum from conditional knockout mice and the relative gene expression of three samples of medial prefrontal cortex, three samples of hippocampus, three samples of cerebellum from wildtype mice was measured. Figure 31.

Groups	Tissue samples		
	Medial prefrontal cortex	Hippocampus	Cerebellum
Conditional knockout mice	3	3	4
Wildtype mice/	3	3	3
Total	6	6	7

Figure 31: Experiment design: knockout validation Tcf4-4-fl × Emx1-Cre. Samples of the medial prefrontal cortex (cko: three, wt: three), hippocampus (cko: three, wt: three), cerebellum (cko: four, wt: three).

Two samples of wildtype mice and one sample of a conditional knockout mouse showed a similar expression level of exon 2 in the medial prefrontal cortex (~1.25 RGE). The relative expression level of exon 2 in the third wildtype sample was lower (~0.6 RGE) and the relative gene expression level of two conditional knockout samples were higher (~2.0 RGE, ~2.25 RGE). The relative gene expression level of exon 4 in wildtype mice showed a high distribution between the samples (~0.5 RGE, ~0.75 RGE, ~2.5 RGE). In conditional knockout mice the relative gene expression level was ~ 0.1 – 0.3 RGE. Figure 32.

The relative gene expression level of exon 2 in the hippocampus is similar between the two different genotypes (~1 RGE), except of one conditional knockout sample (~2.8). Three samples of wildtype mice and one sample of conditional knockout mice showed a similar relative gene expression level of exon 4 (~0.5 – 0.75 RGE). One sample of a conditional knockout mouse showed a lower expression level (~0.1 RGE). One sample of each genotype showed a higher expression level of exon 4 compared to the other samples (~4.5 RGE, ~4.8 RGE). Figure 33.

There is one conditional knockout sample with a very high relative gene expression

level of exon 4 (~24 RGE) in the cerebellum. The other samples show a relative gene expression level of ~1 – 2 RGE in exon 1/2 and exon 3/4 in the cerebellum. Six samples of wildtype mice show a relative gene expression level of ~1. Four samples of both genotypes (wt: one samples; cko: three samples show a relative gene expression level of ~1.5 RGE and three samples of both genotypes (wt: one sample; cko: two samples) show a relative expression level of ~2 RGE. Figure 34.

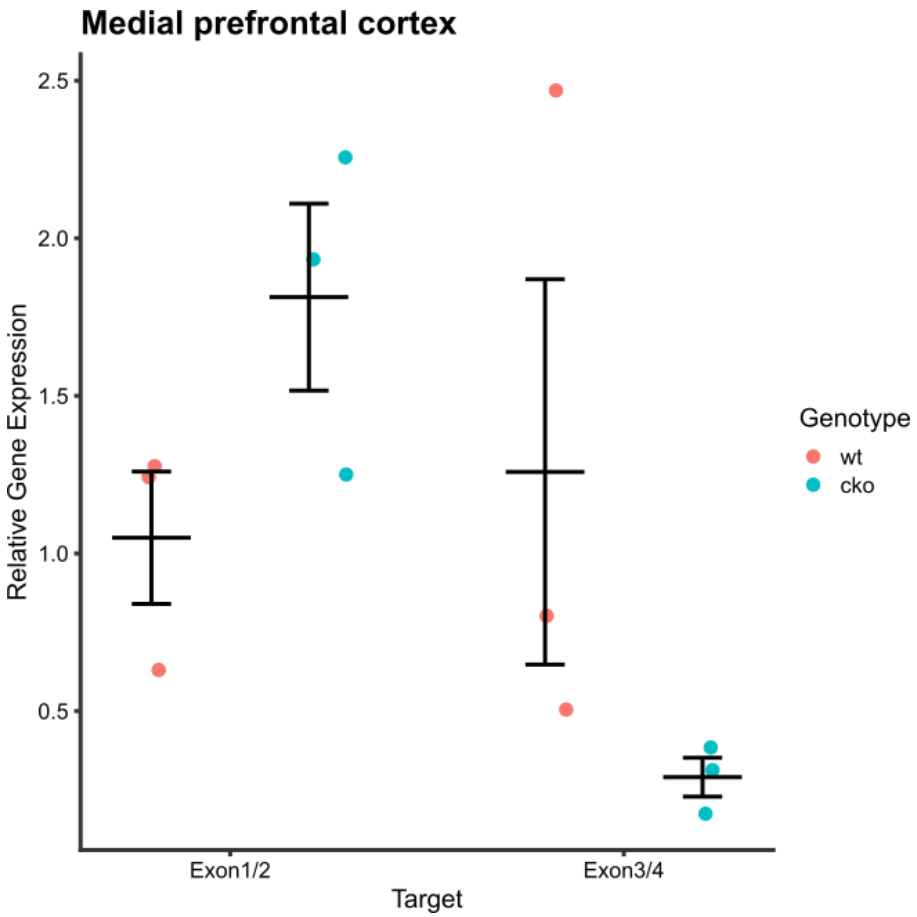

Figure 32: Relative gene expression of exon 1/2 and exon 3/4 in medial prefrontal cortex of Tcf4-4-fl × Emx1-Cre mice/offspring of mice subjected to poly(I:C) injections.
Relative gene expression; wt, wildtype; cko, conditional knockout, *n* = 3 wt/3 cko.

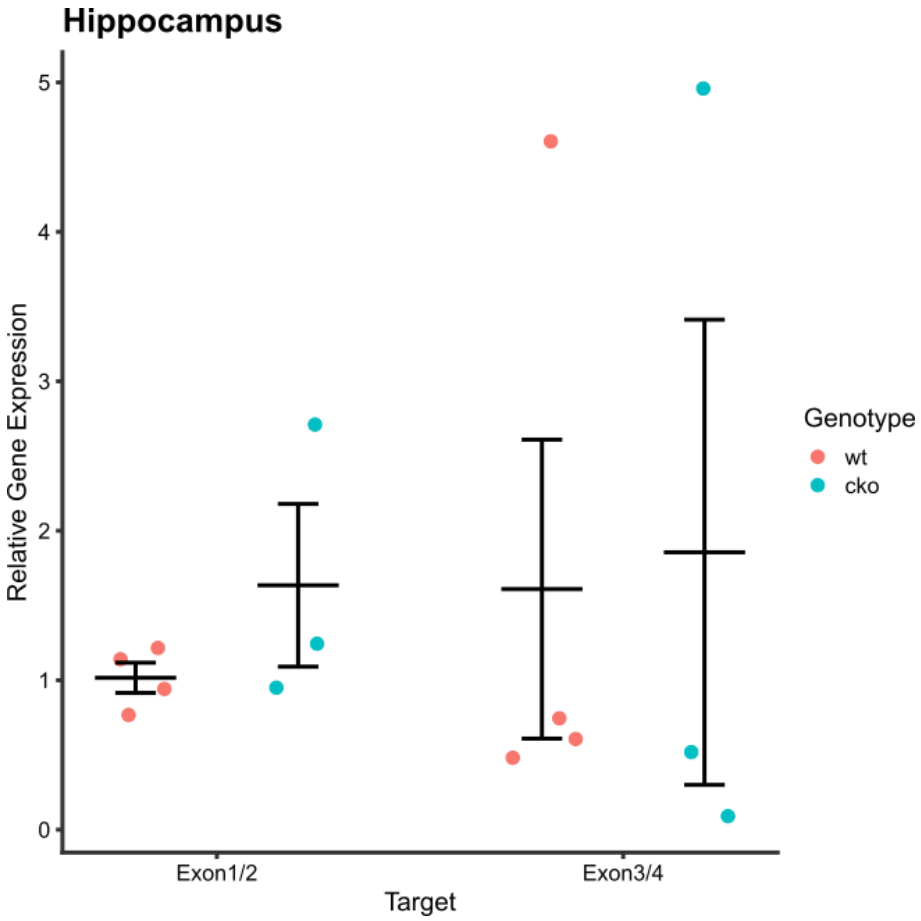

Figure 33: Relative gene expression of exon 1/2 and exon 3/4 in hippocampus of Tcf4-4-fl × Emx1-Cre mice/offspring of mice subjected to poly(I:C) injections.
Relative gene expression; wt, wildtype; cko, conditional knockout, n = 3 wt/3 cko.

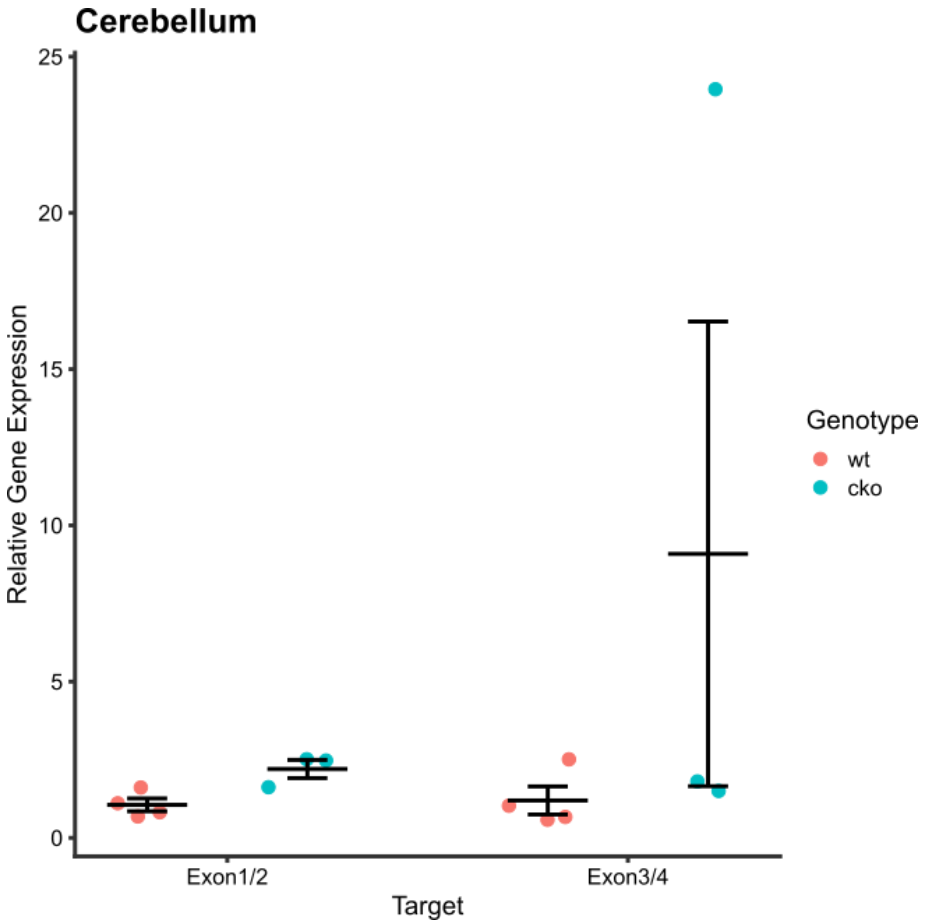

Figure 34: Relative gene expression of exon 1/2 and exon 3/4 in cerebellum of Tcf4-4-fl × Emx1-Cre mice/offspring of mice subjected to poly(I:C) injections. Relative gene expression; wt, wildtype; cko, conditional knockout, *n* = 4 wt/3 cko.

V. DISCUSSION

For behavioral profiling of schizophrenic mouse models, the psycop test battery was used. The standardized experiment design improves the comparability of behavioral experiments in our and other labs interested in psychiatric disease models. Especially animal experiments are often not reproducible (COLLINS & TABAK, 2014). A consistent design of behavioral experiment and analysis can lead to more true effects (IOANNIDIS, 2005).

1 Behavioral profiling of Tcf4-4-fl × Camk2a-Cre mice

The experimental design included four arms consisting of the groups wt/social defeat, wt/no stress, cko/social defeat, and cko/no stress with 15 animals per group. The effect size $d \geq 0.8$ was used for calculation of the group sizes to avoid false findings caused by smaller effect sizes (IOANNIDIS, 2005). Including smaller effects would also increase the number of animals. This would be in contrast to the 3R principle that aims the replacement of animal experiments in general, refinement of living conditions in an experimental setup and reduction of the number of experimental animals (RUSSEL & BURCH, 1959). Due to the lower number of available animals, the experiment had to be split in two runs. The social defeat arm including 25 wildtype mice and 17 conditional knockout mice subjected to social defeat was done first, assuming the strongest behavioral effects. However, I observed no behavioral phenotypes in Tcf4-4-fl × Camk2a-Cre mice subjected to social defeat. Because we could not observe behavioral effects for the combination of two risk factors, we did not continue with the missing no-stress arms of the originally planned 4-arm design and stopped the experiment at this stage.

1.1 Genetic risk factor

According to genome-wide-associations studies, variants in the *TCF4* gene locus are associated with an increased schizophrenia risk (STEFANSSON *et al.*, 2009; GOES *et al.*, 2015; TRUBETSKOY *et al.*, 2022). An increased level of TCF4 combined with social defeat leads to a disturbed spatial learning in mice (D BADOWSKA *et al.*, 2020). The behavioral consequence of a loss-of-function of *Tcf4* in mice was analyzed in this thesis. In this loss-of-function model, the TCF4 level in the forebrain was reduced by crossing Camk2a-Cre mice with Tcf4-4-fl mice. The Tcf4-4-fl line is carrying two loxP sites with the target region in between.

This target region (here exon 4) will be excised by the Cre recombinase under the control of the Camk2a promoter. This deletion leads to a premature stop codon and nonsense-mediated mRNA decay. Just the smaller isoforms of TCF4 will be expressed (SEPP *et al.*, 2011). The Cre-mediated reduction of TCF4 level is expected to start at PD20 and to occur in pyramidal neurons of the hippocampus, neocortex, striatum, and amygdala (MINICHIELLO *et al.*, 1999). The 42 experimental mice (17 conditional knockout mice and 25 wildtype control mice) were subjected to social defeat and tested in our standardized test battery. This test battery includes the open field test, the y-maze, reversal learning paradigms, sucrose preference, activity tracking during night and day, the prepulse inhibition test, the tail suspension test, and the fear conditioning test. The postnatal knockout of *Tcf4* did not affect behavioral patterns that are covered by these tests. The relative gene expression of *Tcf4* is not constant during development: The *Tcf4* mRNA levels in the mouse medial pallidum, the progenitor of the hippocampus, increase from embryonal day (ED) 12.5 until ED 17.5 and then decrease again. At PD7, the hippocampus is fully developed and the *Tcf4* expression levels decrease and stays constant (WANG *et al.*, 2020). That leads to the conclusion that a reduction of the TCF4 level in pyramidal neurons from postnatal day 20, is not affecting cognitive functions. Less specific knockout models of *Tcf4* have been reported to lead to behavioral phenotype, for example in heterozygous full knockout of *Tcf4* in mice. This heterozygous knockout of *Tcf4* targeted all body cells, started before implantation in the uterine wall, and led to impairments of working memory and spatial learning in the Morris water maze (D BADOWSKA *et al.*, 2020). For future research on the impact of TCF4 on cognitive functions and schizophrenic symptoms, the tested conditional knockout model should be modified, for example by using earlier recombination time points.

1.2 Environmental risk factor

As a second hit, the resident-intruder paradigm was chosen in order to increase the genetic effects on behavioral patterns. In wildtype mice and mice overexpressing *Tcf4*, this paradigm led to a reduced sucrose preference, reduced first and serial reversal learning performance, problems in contextual and cued memory, less rotations and increased center time in the open field test, increased number of choices in the y-maze, and a reduced activity compared to wildtype mice not subjected to social defeat (VOLKMANN *et al.*, 2020). Previous studies in our lab

showed a reduced exploratory behavior, more depressive like behavior and impaired cognition in the fear conditioning test in wildtype mice subjected to social defeat (DM BADOWSKA *et al.*, 2015). The concept of social defeat by confronting a male mouse with an older aggressive male in its home cage has been used since 1979 (MICZEK, 1979). This stress paradigm is not only used in schizophrenia models, it is also established for other psychiatric disorders, such as post-traumatic stress disorder (SCHÖNER *et al.*, 2017) and depression (CARNEVALI *et al.*, 2017). The described social defeat paradigm is limited to male mice, so the female litter mates cannot be used. An alternative social defeat model used odorants and pheromones of male urine applied on the fur of female experimental mice to trigger the residents (VAN DOESELAAR *et al.*, 2021).

2 Behavioral profiling of Tcf4-4-fl × Emx1-Cre mice

The experiment was planned as a four arm experiment with 16 animals per group and two backup animals per group. The distribution of the four groups differs compared to the planned group size. 14 wildtype offspring of dams subjected to vehicle only, 16 wildtype offspring of dams subjected to poly(I:C), 18 conditional knockout offspring of dams subjected to vehicle only, and 16 conditional knockout offspring of dams subjected to poly(I:C). The four arm design was used in order to analyze more variables in parallel, e.g. genotype and environmental factor. The 30 wildtype animals were compared to the 32 conditional knockout mice, and the 32 offspring of dams subjected to vehicle only were compared to 32 offspring of dams subjected to poly(I:C). The group sizes were chosen because the poly(I.C) paradigm was not used in our lab, so medium effect sizes were targeted.

2.1 Genetic risk factor

Because a postnatal induced knockout of exon 4 of *Tcf4* did not lead to significant behavioral effects, another *Tcf4* loss-of-function model was tested which targets the expression of *Tcf4* during embryonal development. Instead of the Camk2a-Cre mouse line, the Emx1-Cre mouse line was crossed with Tcf4-4-fl mice. This particular Cre driver line was chosen because it was active earlier during development: The Cre recombinase is affecting 91% of the neurons located in cerebral cortex and hippocampus at ED 12.5 (GUO *et al.*, 2000). The expression of *Tcf4* during hippocampus development starts to raise at embryonal day 12.5 until ED 17.5 (WANG *et al.*, 2020). This earlier knockout of *Tcf4* in hippocampal and

cortical neurons indeed led to a behavioral phenotype. The learning flexibility of the conditional knockout mice was improved compared to their wildtype littermates (p = 0.001). Other parameters modeling the cognitive performance were not affected. There were no significant differences in alternations in y-maze, in first reversal learning paradigm, and in cue and contextual memory in fear conditioning test measured. In other studies the social memory, and contextual memory in fear conditioning of Tcf4-4-fl × Emx1-Cre mice was impaired (WANG *et al.*, 2020). These findings are not mutually exclusive –the problems in contextual memory might lead to an equal investigation of all corners, because they do not remember the correct water bottle from the day before, so they reach the learning criteria earlier. In general, the gene dosage of *Tcf4* seems to affect the cognition in different ways. Mice overexpressing *Tcf4* showed a reduce flexibility learning in the serial reversal paradigm (VOLKMANN *et al.*, 2020). Opposite findings for *Tcf4* polymorphisms have been described for attention processes (ZHU *et al.*, 2013).

The mean speed in the open field test was reduced in conditional knockout mice of *Tcf4* (p = 0.047). A reduced mean speed can be caused by slower speed in general or by an increased time of immobility. Further analysis showed, that conditional knock out mice of *Tcf4* are spending more time immobile in the open field than the wildtype control mice (p = 0.00558). Together with alternations in y-maze and the activity measured in the intellicage, the mean speed in open field test is a parameter for activity. In the intellicages the general activity through night and day is measured. There were no significant differences between conditional knockout mice and wildtype control mice, so the reduced activity seems to be caused by a novel environment. In y-maze test, the environment is unfamiliar for the experimental animals, but the conditions are more comfortable and for mice, because of reduced light (30 lx) and narrow arms. In contrast to the y-maze, conditions in open field test are more aversive. The area is wide and the light is brighter (1600 lx). The novelty-induced reduced activity of conditional knockout mice can be interpreted as less explorative and more anxious-like behavior in a new and aversive environment. In contrast, *Tcf4* transgenic mice subjected to social defeat showed an increased mean speed (VOLKMANN *et al.*, 2020). The trigger in both genetic models seems to be the new environment. Anxiety disorders are a possible comorbidity of schizophrenia. The prevalence for the different subgroups like obsessive-compulsive disorders, social phobia, generalized anxiety disorder,

panic disorders, and post-traumatic stress disorders is extremely heterogenic in schizophrenic patients (ACHIM *et al.*, 2011). The correlation between schizophrenia and anxiety disorders was underrepresented, because of a strict separation between the mental disorders. According to the third version of the diagnostic and statistic manual of mental disorders an anxiety disorder is only diagnosed if it is not caused by schizophrenia or another axis 1 disorder (AMERICAN PSYCHIATRIC ASSOCIATION & AMERICAN PSYCHIATRIC ASSOCIATION. WORK GROUP TO REVISE DSM-III, 1987; Stefano PALLANTI *et al.*, 2013). None the less, anxiety in schizophrenia should be more focused in clinical research, because the anxiety disorder increases the risk of relapse and suicide attempts (GARAY *et al.*, 2015). The lack of suitable treatment increases the need of efficient preclinical drug screening (S. PALLANTI *et al.*, 1999; DOLD *et al.*, 2013). The analyzed Tcf4-4-fl × Emx1-Cre mouse line could be used in preclinical research as an anxiety model.

2.2 Environmental risk factor

Injection of poly(I:C) during pregnancy can cause different behavioral phenotypes in the offspring depending on the time point of injection. The model mimics a viral infection during pregnancy, which leads to an activation of the maternal immune system. Infections during pregnancy and the resulting rise of immunoglobulins has been reported as a potential risk factor for schizophrenia in humans (BUKA *et al.*, 2001; BABULAS *et al.*, 2006). According to the literature, the dose, the administration route and the point of time of the injection are different in individual studies. In most publications describing maternal immune activation (MIA) as a schizophrenia model in mice used GD17 in the third trimester of pregnancy (BITANIHIRWE *et al.*, 2010; LABOUESSE *et al.*, 2015; DA SILVEIRA *et al.*, 2017; LUONI *et al.*, 2017). Behavioral characterization of the offspring showed various behavioral phenotypes, but consistently a reduced social interaction as well as reduced sucrose preference (BITANIHIRWE *et al.*, 2010; DA SILVEIRA *et al.*, 2017). These findings could not be replicated for Tcf4-4-fl × Emx1-Cre mice in our hands: The offspring of dams injected with poly(I:C) showed no significant differences compared to offspring of mice injected with vehicle only. In the prepulse inhibition test at an intensity of 75 dB we observed a nominally significant difference ($H_{(1,51)} = 4$, $p = 0{,}05$), but this was not significant after FDR-adjustment. An effect of poly(I:C) injection on the offspring of injected dams on prepulse

inhibition has been described (GONZALEZ-LIENCRES *et al.*, 2016; KOBAYASHI *et al.*, 2021).

The inconstant behavioral effects in other studies and the lack of significant differences in this study makes it necessary to review the method. For further experiments a validation of the activation of the maternal immune system should be done to proof the effects of poly(I:C) administration in pregnant dams. One option would be the analysis of white blood cells in blood samples of pregnant dams 24 h after poly(I:C) injection (DA SILVEIRA *et al.*, 2017). Adjusting the dosage of poly(I:C) and the time point of injection may lead to stronger behavioral effects.

The poly(I:C) model used in this study could not affect so many behavioral patterns, compared to the social defeat paradigm in previous studies in our lab (DM BADOWSKA *et al.*, 2015; VOLKMANN *et al.*, 2020; STEPHAN *et al.*, 2022). For future experiments, the already established social defeat paradigm may be a better choice as an environmental risk factor. The used paradigm can be extended to female mice by using male urine in order to trigger the aggression of the male residents (VAN DOESELAAR *et al.*, 2021).

3 Conditional knockout of *Tcf4*

In order to validate the tissue and cell-specific knockout of *Tcf4* in excitatory neurons in the forebrain, the relative gene expression of exon 3/4 and exon 1/2 in medial prefrontal cortex, hippocampus, and cerebellum was measured. The assumption was that the relative gene expression of exon 1/2 shows no differences between conditional knockout mice and wildtype mice, because in previous studies the relative gene expression of exon 1/2 in prefrontal cortex and hippocampus was not affected by a heterozygous knockout of *Tcf4* (D BADOWSKA *et al.*, 2020). After Cre-mediated heterozygous knockout of *Tcf4* in both conditional knockout mice the relative gene expression of exon 3/4 can be maximally reduced by 50%. The deletion of exon 4 leads to a premature stop codon, which activates the nonsense-mediated mRNA decay. A quick degradation of the full knockout mRNA is expected. The cerebellum should not be affected by both knockouts, so a similar relative gene expression in both genotypes was expected.

Unfortunately, the analysis of the relative gene expression showed such a high variability in general, that no clear conclusions could be drawn. The qPCR was used

for analysis of the transcriptome. We did not analyze the level of the synthesized proteins. In order to validate the conditional knockout on a protein level, other methods, such as Western blot and immunohistochemical staining can be used. In the conditional knockout mice of the Tcf4-4-fl × Camk2a-Cre line the relative gene expression level of exon 3/4 was reduced in the cerebellum. This region should not be affected by both Cre lines. This could indicate a lack of specificity in the Camk2a-Cre line.

4 Behavioral profiling of Bmal1-ko mice

The experiment design included two arms separated by the gene dosage with 15 animals per group. The effect size $d \geq 0.8$ was used for calculation of the group sizes to avoid false findings caused by smaller effect sizes (IOANNIDIS, 2005). *BMAL1* is part of the cellular circadian clock (BUNGER *et al.*, 2000) which is often affected in psychiatric diseases (MANSOUR *et al.*, 2006). In schizophrenic patients, *CRY1* and *PER2*, two genes of the circadian core clock, have been reported to be expressed arrhythmically (JOHANSSON *et al.*, 2016). Previous studies on heterozygous Bmal1-ko mice showed that the mice spent more time in the center in the open field test and traveled a longer distance (SINGLA *et al.*, 2022). In studies using homo- and heterozygous knockout mice, behavioral differences were limited to homozygous knockout mice. A full knockout of *Bmal1* lead to arrhythmic and generally reduced activity in mice, while heterozygous knockout mice and wildtype mice still showed rhythmic activity (RAKAI *et al.*, 2014; PARK *et al.*, 2015).

Interestingly, in our behavioral analysis, no significant differences in the open field test were measured, but an anhedonic phenotype was observed: The heterozygous Bmal1 knockout mice had a lower sucrose preference compared to the wildtype control group ($p = 0.02$). This is consistent with other studies focusing on homozygous Bmal1 knockout mice (LELIAVSKI *et al.*, 2014). Anhedonia occurs in schizophrenic patients as a negative symptom (MORAN *et al.*, 2022). The symptom affect the reward circuit. Current positive emotions are not inhibited, but the ability of reporting past emotions is impaired (WHITTON *et al.*, 2015). Pharmacological treatment of anhedonia is limited. Improvement of negative symptoms was only observed in secondary negative symptoms in acute patients, but not in primary negative symptoms (ALEMAN *et al.*, 2017). The observed reduced sucrose preference of *Bmal1*-ko mouse line can be used for further

preclinical drug screening in order to find compounds rescuing this part of negative symptoms.

VI. ZUSAMMENFASSUNG

Charakterisierung ausgewählter Mausmodelle für Schizophrenie: Tcf4-4-fl × Camk2a-Cre/social defeat, Tcf4-4-fl × Emx1-Cre/poly(I:C) und Bmal1-ko

Die psychiatrische Erkrankung Schizophrenie weist ein breites Spektrum an Symptomen auf, darunter Wahnvorstellungen, Bewegungsstörungen, Halluzinationen, Motivationsstörungen, verminderte Kontaktfreudigkeit und kognitive Beeinträchtigungen (NATIONAL INSTITUTE OF MENTAL HEALTH (NIMH), 2022). Mit den derzeit verfügbaren Medikamenten können nicht alle Symptome behandelt werden. Außerdem ist die Behandlung nicht bei jedem Patienten erfolgreich. Die empfohlenen Neuroleptika können laut der American Psychiatric Association zu starken Nebenwirkungen führen und sowohl die Motorik als auch den Stoffwechsel beeinträchtigen (JM DAVIS & CASPER, 1977; MELTZER, 1997; LEUCHT *et al.*, 2009, 2023; SANDERS & GILLIG, 2012; AMERICAN PSYCHIATRIC ASSOCIATION, 2020).

Ziel dieser Dissertationsarbeit war es, die Auswirkungen bestimmter Risikogene und Umweltfaktoren, die mit Schizophrenie in Verbindung gebracht werden, in spezifischen Mausmodellen zu analysieren.

Es wurden drei verschiedene Kombinationen von genetischen und umweltbedingten Faktoren ausgewählt: Ein postnataler Knockout des Gens *Tcf4* in Kombination mit einer sozialen Unterwerfung als Stressparadigma. Es wurden 17 Tiere mit einem konditionalen Knockout und 25 Wildtypkontrolltiere getestet. Außerdem ein embryonaler Knockout von *Tcf4* in Tieren, deren Mütter Poly(I:C) injiziert wurde. Hier wurden vier Gruppen verwendet, 14 Wildtypnachkommen von Müttern, denen NaCl als Vehikel injiziert wurde, 16 Wildtypnachkommen von Müttern, denen poly(I:C) injiziert wurde, 18 Tiere mit konditionalen Knockout, deren Müttern NaCl als Vehikel injiziert wurde und 16 Tiere mit konditionalen Knockout, deren Müttern poly(I:C) injiziert wurde. Ein heterozygotes Knockout-Modell des Gens *Bmal1* bestehend aus 17 heterozygoten Knockouts und 19 Wildtypkontrolltieren.

Tiere mit einem embryonalen Knockout von *Tcf4* zeigten eine beeinträchtigte Kognition und Aktivität. *Tcf4* wurde an Tag 12.5 der Embryonalentwicklung durch Kreuzung der Linien Tcf4-4-fl und Emx1-Cre ausgeschaltet (GUO *et al.*, 2000).

Der heterozygote konditionale Knockout von *Tcf4* führte zu besseren Leistungen im Flexibilitätslernen ($p = 0.001$). Im Vergleich zeigten Mäuse, die *Tcf4* überexprimieren, hier eine schlechtere Leistung (VOLKMANN *et al.*, 2020). Die mittlere Geschwindigkeit, gemessen im Open-Field-Test, war bei Tcf4-4-fl × Emx1-Cre-Mäusen reduziert und kann als ängstlicheres Verhalten interpretiert werden ($p = 0.047$).

Ein anhedonie-assoziierter Phänotyp wurde bei Bmal1-ko-Mäusen beobachtet. Die Vorliebe für Saccharose war reduziert ($p = 0.02$). Diese verringerte Affinität zu Saccharosewasser wurde auch in anderen Studien beschrieben (LELIAVSKI *et al.*, 2014)

Insgesamt wurden in zwei der drei analysierten Schizophreniemodelle Verhaltensunterschiede beobachtet. Spezifische RDoC-Domänen waren betroffen, wie Kognition, Arousal und positive Valenz. Die Modelle decken Teile des Symptomenkomplexes der Schizophrenie ab. In weiteren Experimenten kann die Tcf4-4-fl × Emx1-Cre Mauslinie für die Entwicklung angstlösender Medikamente verwendet werden. Die Bmal1-ko Mauslinie kann zur Identifizierung von Substanzen verwendet werden, die auf ein beeinträchtigtes Hedonieverhalten abzielen. Diese potenziellen Ansätze für nachfolgende präklinische Studien könnten zu einem individuelleren Behandlungsansatz bei psychiatrischen Störungen beitragen (DELISI & FLEISCHHACKER, 2016).

VII. SUMMARY

The psychiatric disorder schizophrenia has a broad spectrum of symptoms including delusions, disordered movements, hallucinations, disturbed motivation, reduced sociability, and cognitive impairments (NATIONAL INSTITUTE OF MENTAL HEALTH (NIMH), 2022) With currently available drugs, not all symptoms can be treated and the treatment is not effective in every patient. The recommended neuroleptic treatments, according to the American Psychiatric Association, can have strong side effects and can affect motor function and metabolism (JM DAVIS & CASPER, 1977; MELTZER, 1997; LEUCHT *et al.*, 2009, 2023; SANDERS & GILLIG, 2012; AMERICAN PSYCHIATRIC ASSOCIATION, 2020). In order to develop compounds, which are better tolerated by patients and target the cause of schizophrenia, the focus of research shifted to the pathomechanisms of schizophrenia. Therefore, a working group of the National Institute of Mental Health started in 2010 to elaborate Research Domain Criteria (RDoC) based on the underlying pathophysiology of schizophrenia (CUTHBERT, 2022).

The aim of the thesis was to analyze the effect of certain risk genes and environmental factors associated with schizophrenia in specific mouse models.

Three different combinations of genetic and environmental risks were chosen: A postnatal knockout of *Tcf4* in combination with social defeat as a stress paradigm. 17 conditional knockout mice and 25 wildtype mice were tested. An embryonal knockout of *Tcf4* in the offspring of dams subjected to poly(I:C) injections. Four groups were tested, 14 wildtype offspring of dams subjected to vehicle only, 16 wildtype offspring of dams subjected to poly(I:C), 18 conditional knockout offspring of dams subjected to vehicle only, and16 conditional knockout offspring of dams subjected to poly(I:C). A heterozygous knockout model of Bmal1including 17 heterozygous knockout mice and 19 wildtype mice.

Animals with an embryonal knockout of *Tcf4*, showed impaired cognition and locomotor activity. *Tcf4* was knocked down at ED 12.5 by crossing Tcf4-4-fl mice with Emx1-Cre mice (GUO *et al.*, 2000). The heterozygous conditional knockout of *Tcf4* led to better performance in the serial reversal learning paradigm (p = 0.001). Mice, overexpressing *Tcf4* showed a worse performance in serial reversal

learning (VOLKMANN *et al.*, 2020). The mean speed, measured in open field test, was reduced in Tcf4-4-fl × Emx1-Cre mice and can be interpreted as more anxious-like behavior ($p = 0.047$).

Anhedonia-associated behavior was observed in Bmal1-ko mice. The sucrose preference was reduced ($p = 0.02$). This lower addiction to sucrose water is described in other studies as well (LELIAVSKI *et al.*, 2014).

Taken together in two of the three analyzed mouse models, behavioral differences associated with schizophrenic symptoms were observed. Specific RDoC domains were affected, like cognition, aerousal and positive valence. The models are covering parts of the symptom complex of schizophrenia. In future experiments the Tcf4-4-fl × Emx1-Cre mice can be used for specific drug screening targeting anxious-like behavior. Heterozygous knockout mice of Bmal1-ko can be used to identify compounds that are able to rescue anhedonic behavior. These potential targets for subsequent preclinical studies may contribute to a more individualized treatment approach in psychiatric disorders (DELISI & FLEISCHHACKER, 2016).

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