Modeling DISC1-dependent chronic mental illnesses *in vivo* and *in vitro*: generation of a novel DISC1 transgenic rat

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Modeling DISC1-dependent chronic mental illnesses *in vivo* and *in vitro*: generation of a novel DISC1 transgenic rat

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Svenja Verena Troßbach

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Abstract

Chronic mental illnesses (CMIs) such as schizophrenia, bipolar disorder, or recurrent major depression are long-lasting brain disorders, which show considerable phenotypical heterogeneity, even within a single clinical diagnosis. To date, the biological origins of CMIs remain elusive.
Up to the present day, the diagnosis of mental illness patients relies exclusively on a clinical interview and self-reporting of symptoms and is in the absence of distinct biological diagnostic markers often based on exclusion of other disorders. It is conceivable that the existing clinical diagnostic boundaries might not mirror a suspected biology.

One possible method to define a subset of mental illness patients would be to detect the presence of aggregated proteins in the brain that accumulate during the course of CMIs as a result of disturbed proteostasis. Similar to established proteinopathies like the tauopathies or synucleinopathies, Disrupted-in-Schizophrenia 1 (DISC1) protein aggregates have been demonstrated to exist in a subset of *post mortem* brains from CMI patients, termed “DISC1opathies” for DISC1-dependent brain disorders.
DISC1 is the protein product of a key candidate gene for CMI, which was first discovered in a Scottish pedigree with a familial mutation in the *DISC1* gene and a high occurrence of various different clinical phenotypes.

In this cumulative thesis, the interactions between neurotransmitter systems, cognitive impairments, and the disease-associated DISC1 protein were investigated *in vivo*, focused on, but not limited to, three major publications.

In the first study, the role of DISC1 and its multimeric assembly states in the pathophysiology of CMI was investigated. In order to study the biological consequences of dysfunctional DISC1

assembly *in vivo*, the first transgenic rat model of a major mental illness gene was generated, the tgDISC1 rat modestly overexpressing the full-length non-mutant human DISC1 protein. Face validity of the tgDISC1 rat for DISC1opathies was established by comparable protein pathology in terms of DISC1 aggregates and behavioral phenotypes consistent with alterations in dopamine neurotransmission such as amphetamine supersensitivity, hyperexploratory behavior, and rotarod deficits. These findings were corroborated by alterations in the dopamine system of the striatum such as a proportionate increase in high affinity dopamine D2High receptors, elevated dopamine transporter levels, increased clearance of synaptic dopamine, and decreased total dopamine content.

These findings suggest a bidirectional link between DISC1 assembly and dopamine homeostasis and highlight a functional role of DISC1 assemblies in CMI pathophysiology and in causing human DISC1opathies.

In the second study, the value of lymphocytic DISC1 levels as a trait marker for the mental illness schizophrenia was investigated, and, as a major result, reduced DISC1 protein levels in lymphocytes of patients with schizophrenia were discovered. Since the study was also designed to investigate the influence of smoking, a prominent co-morbidity of schizophrenia, it was discovered that smoking itself reduced lymphocytic DISC1 protein significantly, although not to the extent of overriding the trait level.

Finally, to investigate whether nicotine treatment could alter DISC1 assembly status, decreased levels of aggregated DISC1 species in the mPFC of rats sub-chronically treated with nicotine were detected, also indicating an influence of the cholinergic system in DISC1 aggregation.

In the third study, the influence of intranasal application of dopamine (IN-DA) on age-dependent memory decline in aged rats was investigated. In a spatial object recognition paradigm the memory deficit of aged rats could be rescued by IN-DA treatment inducing increased specific exploration of the novel object.

The presented studies advanced the understanding of the role of DISC1 in chronic mental illness, suggesting a physiological function of DISC1 aggregates in the brain that regulates dopamine homeostasis.

Zusammenfassung

Eine Vielzahl an mentalen Erkrankungen ist durch einen chronischen Verlauf gekennzeichnet und umfasst Krankheiten wie Schizophrenie, bipolare oder manisch-depressive Störungen und Depressionen. Diese präsentieren sich mit einer beträchtlichen phänotypischen Heterogenität, bereits innerhalb der Grenzen einer psychiatrischen Diagnose. Gegenwärtig sind die biologischen Ursachen mentaler Erkrankungen ungeklärt.
Bis in die heutige Zeit liegen der Diagnose mentaler Erkrankungen das klinische diagnostische Gespräch und die darin selbst berichteten Symptome des jeweiligen Patienten zugrunde. Gerade durch das Fehlen von spezifischen biologischen Markern ist die Diagnose stets subjektiv und basiert per Definition oft auf dem Ausschluss anderer mentaler Erkrankungen. Es ist daher wahrscheinlich, dass die momentan existierende diagnostische Klassifizierung nicht von der eigentlichen grundlegenden Biologie der verschiedenen mentalen Erkrankungen widergespiegelt wird.

Vorangehende biochemische Studien identifizierten eine Biologie-basierte Subgruppe von psychiatrischen Patienten, die sich durch die Präsenz oder Absenz von aggregierten Proteinen im Gehirn definieren lässt. Diese Protein-Aggregate akkumulieren über den chronischen Zeitverlauf der Erkrankung hinweg als Folge gestörter Proteinhomöostase. In Analogie zu den etablierten Proteinopathien wie Tauopathien und Synucleinopathien konnten Disrupted-in-Schizophrenia 1 (DISC1) Protein-Aggregate in *post mortem* Hirnproben in einer Subgruppe von Patienten mit mentalen Erkrankungen demonstriert werden, die daraufhin als DISC1opathien – DISC1-abhängige Hirnerkrankungen – definiert wurden.
Das DISC1 Protein ist das Produkt eines bekannten und viel untersuchten Kandidatengens mentaler Erkrankungen und wurde erstmals in einer schottischen Familie mit einer familiären Mutation im *DISC1*-Gen beschrieben. Die meisten Träger dieses Gendefekts sind durch eine Vielzahl an klinischen mentalen Phänotypen charakterisiert.

Im Rahmen dieser kumulativen Dissertation wurden die Interaktionen von Neurotransmittersystemen, kognitiven Beeinträchtigungen und dem krankheitsassoziierten DISC1 Protein *in vivo* untersucht, deren Befunde in drei Hauptpublikationen zusammengefasst wurden.

In der ersten Studie wurde die Rolle von assembliertem DISC1 in der Pathophysiologie chronischer mentaler Erkrankungen untersucht. Um die potentiell dysfunktionelle DISC1-Assemblierung und seine biologischen Folgen *in vivo* zu erforschen, wurde das erste transgene Ratten-Modell für mentale Erkrankungen generiert, die tgDISC1 Ratte, die das nicht-mutierte humane DISC1 Protein in voller Länge exprimiert.
Die Validität des tgDISC1 Rattenmodells für humane DISC1opathien wurde zum einen durch eine vergleichbare Protein-Pathologie in Form von DISC1-Aggregaten im Gehirn bekräftigt. Zum anderen wurden spezifische Verhaltensänderungen induziert - eine Supersensitivität gegenüber Amphetamin, Hyperexplorationsverhalten und Defizite im Rotarod-Test - drei Phänotypen, die von der dopaminergen Neurotransmission beeinflusst werden. Des Weiteren wurden explizite Veränderungen im dopaminergen System des Striatums beobachtet, wie eine Zunahme der hochaffinen postsynaptischen Dopamin D2High Rezeptoren, eine Hochregulation des Dopamintransporters, einem erhöhten Rücktransport von synaptischem Dopamin in die Präsynapse und ein reduzierter totaler Dopamingehalt.
Diese Ergebnisse implizieren eine bidirektionale Kopplung von DISC1-Aggregation mit der Dopamin-Homöostase und heben eine potenzielle physiologische Rolle von assembliertem DISC1 in der Pathophysiologie mentaler Erkrankungen und als biologische Grundlage der DISC1opathien hervor.

In der zweiten Studie wurde untersucht, ob der Level an lymphozytärem DISC1 als Charakteristikum oder „trait marker" für Schizophrenie fungieren kann, also prognostischen Wert hat. Hier konnte im Vergleich zu Kontrollprobanden reduzierte Mengen an DISC1 Protein in Lymphozytenproben von Schizophrenie-Patienten detektiert werden. Darüber hinaus wurde der Einfluss von Rauchverhalten, einer Haupt-Komorbidität bei Schizophrenie, auf lymphozytäres DISC1 in Kontrollpersonen analysiert. Hier wurde wiederum eine Reduktion von DISC1 Levels entdeckt, allerdings zu einem weit schwächeren Grad als im direkten Vergleich zu Schizophrenie-Patienten.
Weiterhin wurde ermittelt, ob eine subchronische Behandlung von Ratten mit Nikotin die DISC1-Assemblierung beeinflusst. Die festgestellte Reduktion von aggregiertem DISC1 im präfrontalen Cortex festgestellt weist zusätzlich auf eine Rolle des cholinergen Systems in der Assemblierung von DISC1 hin.

In der dritten Studie wurde der Einfluss von intranasal appliziertem Dopamin auf altersbedingte Gedächtnisdefizite in alten Ratten untersucht. In einem Objektexplorations-Paradigma, das auf die Analyse von räumlichem Objektgedächtnis ausgelegt ist, konnte die Gedächtnisleistung der alten Ratten durch die Gabe von intranasalem Dopamin wiederhergestellt werden und induzierte eine spezifische Exploration des neu lokalisierten Objekts.

Die hier präsentierten Studien haben unser Verständnis bezüglich der Rolle des DISC1 Proteins in der Ätiologie mentaler Erkrankungen deutlich erweitert. Die gewonnenen Erkenntnisse liefern einen neuen innovativen Erklärungsansatz für die molekulare Pathogenese mentaler Erkrankungen. Sie deuten darauf hin, dass DISC1 und assembliertes DISC1 eine physiologische Funktion im Gehirn ausübt und mit der Homöostase des dopaminergen Systems assoziiert sind.

1 Introduction

1.1 Chronic mental illnesses

Chronic mental illnesses (CMIs) include disorders such as schizophrenia and the recurrent affective disorders and are characterized by a progressive, chronic course of disease. Although CMIs are among the most prevalent brain disorders, their biological origins still remain elusive.

1.1.1 Schizophrenia

Schizophrenia (SCZ) is one of the most disabling mental illnesses. Due to the absence of biological markers, the diagnosis of SCZ is still based on clinical interview, the reported symptoms, and behavioral categorization according to the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders 5th Edition, (American Psychiatric Association, 2013)) or ICD-10 criteria (International Statistical Classification of Diseases and Related Health Problems 10th Revision, (WHO, 1992)), the major classification systems of clinical psychiatry.

SCZ exhibits a heterogeneous clinical picture with complex and variable patterns of illness and a variable time course. It is characterized by positive symptoms (e.g. delusions, hallucinations), negative symptoms (e.g. avolition, emotional flattening, poverty of speech, social withdrawal) and a dramatic decline in cognitive abilities. According to the DSM-5, a patient must exhibit at least two of the characteristic symptoms for the diagnosis (see Table 1, Criterion A).

The chronic deteriorating course of SCZ was described by the psychiatrist Emil Kraepelin over 100 years ago, who emphasized the chronic aspects of the illness, especially of the cognitive decline, by the term “dementia praecox” (Kraepelin, 1899).

Criterion A.

Characteristic symptoms

(1) Delusions.
(2) Hallucinations.
(3) Disorganized speech.
(4) Grossly disorganized or catatonic behavior.
(5) Negative symptoms
at least one must be 1, 2, 3

Criterion B.

Social/occupational dysfunction

Reduced level of functioning (work, interpersonal relations, self-care).

Criterion C.

Duration

Continuous signs of the disturbance persistent for at least 6 months.
Period include at least 1 month of symptoms.

Criterion D.

Schizoaffective and major mood disorder exclusion

Criterion E.

Substance/general mood condition exclusion

Criterion F.

Relationship to Global Developmental Delay or Autism Spectrum Disorder

If history of autism spectrum disorder or a communication disorder of childhood onset is known.
Additional diagnosis requires prominent delusions or hallucinations.

Table 1: DSM-5 diagnostic criteria for schizophrenia.
Adapted and shortened from the DSM-5 (American Psychiatric Association, 2013).

Further diagnostic criteria for SCZ in the DSM-5 comprise the exclusion of other mental conditions, indicating that the spectrum of symptoms overlaps with those of other psychiatric conditions and that no single symptom is pathognomonic for SCZ.

The onset of SCZ normally occurs in late adolescence to early adulthood, following a prodromal phase, and the disease course is characterized by repeated relapses and remissions that normally fail to return to baseline functioning.

A complex interplay of genetic factors and environmental insults is considered to form the basis for SCZ. Twin studies have shown that the probability of developing SCZ is about 50 % for one twin if the other twin is affected and that the degree of biological relatedness alters the risk of developing SCZ in relatives of the patient (Gottesman and Erlenmeyer-Kimling, 2001).
Environmental (e.g. season of birth, urban environment, drug abuse) and physiological factors (e.g. pre- or perinatal stress, infection, malnutrition) also contribute, leading to a SCZ lifetime prevalence of about 0.6 % (Saha et al., 2005).

1.1.2 Recurrent affective disorders

The term recurrent affective disorders used in these chapters refers to mood disorders that are characterized primarily by their chronic course and comprises recurring major / persistent depressive (DD) and bipolar disorders (BD).

Patients with DD experience discrete but recurrent prolonged depressive episodes, often with full remissions in between. The symptoms include depressed mood, apathy, social withdrawal, and a feeling of hopelessness in the affected individuals, as well as weight gain or loss, hypo-/insomnia, and fatigue. They range from mild to severe and can be accompanied by, for example, psychotic or anxious features.

BDs refer to a group of disorders that are characterized by recurrent severe mood changes comprising episodes of depression and mania (BD type I) or hypomania (BD type II). During the manic phases the patient experiences a euphoric state of mind, decreased sleep, and flight of ideas that leaves the patient without restraint.
BD cases show a high frequency of misdiagnosis as they usually start with a depressive episode and are therefore easily misdiagnosed as a DD (Bowden, 2005).
The lifetime prevalence is about 3.9 % for BD and up to 16 % for DD (Kessler et al., 2005).

In the Global Burden of Disease report the World Health Organization (WHO, 2008) analyzed the central causes of disability world-wide. Mental illnesses such as SCZ, DD, and BD were among the leading causes of disability, measured in years of life lost to the disease (at position 5, 1, and 7, respectively). This categorization shows how devastating mental illnesses are for the individual as well as for society.

1.2 The dopaminergic system in mental illness

1.2.1 The discovery of dopamine (DA) in mental illness

In 1952, the discovery of the antipsychotic properties of the neuroleptic drug chlorpromazine (Delay et al., 1952) led the way for research strategies targeting the biological basis of SCZ, setting the focus on the primary binding site of neuroleptics (also known as antipsychotics) in the brain. One decade later Carlsson and Lindqvist discovered that chlorpromazine and haloperidol elevate catecholamine metabolite levels in the mouse brain and they proposed a blockade of monoamine receptors by neuroleptic drugs as their mechanism of action (Carlsson and Lindqvist, 1963).

Based on these facts, van Rossum proposed in 1966 the intriguing "dopamine hypothesis of schizophrenia", namely a hyperactivity of dopaminergic neurotransmission in the condition (Seeman, 1987). A consistent line of evidence validated his theory of a connection of SCZ, neuroleptic action, and the dopaminergic system:

After the introduction of neuroleptic therapy for SCZ, Parkinson's disease-like symptoms appeared as clinical side effects of the medication on a regular basis (Faurbye, 1970), suggesting an interference of neuroleptics with dopaminergic neurotransmission.

Further key experiments showed that [^{3}H]haloperidol directly binds to DA receptors in the brain (Seeman et al., 1975). There are five types of DA receptors D1-D5 expressed in the brain (the dopaminergic system will be described in more detail in Chapter 1.2.2). The dopamine D2 receptor (D2R) is considered to be most important of these for neuroleptic action in SCZ as the effectiveness of antipsychotic drugs was strongly correlated with their affinity towards D2Rs specifically (Seeman and Lee, 1975; Creese et al., 1976).

Additional clinical evidence came from observations showing that DA-mimetic psychostimulants such as amphetamine elicit SCZ-like psychoses in mentally healthy subjects (Angrist et al., 1974) and that SCZ patients react in a supersensitive manner towards them (Lieberman et al., 1987), arguing for dopaminergic overstimulation as a possible cause for the positive symptoms of SCZ.

Up until now, the "dopamine hypothesis of schizophrenia" is still the most prominent – although not universal – theory for the biological basis of SCZ and has been continuously refined ever since.

In 1990 Davis et al. postulated a disturbed DA system, namely frontal hypodopaminergia resulting in striatal hyperdopaminergia, as leading to the negative and positive symptoms of SCZ respectively (Davis et al., 1991) (see also next Chapter 1.2.2).

This SCZ-centered view changed in the meanwhile, giving way to the "DA hypothesis of psychosis-in-schizophrenia" (Howes and Kapur, 2009), emphasizing DA dysregulation as a mutual phenomenon in psychosis of mental illness rather than being restricted to SCZ.

Although psychosis is a core feature of SCZ, it also appears in other mental illnesses such as BD or DD (Pearlson et al., 1995; Ohayon and Schatzberg, 2002). In these cases, as well as in patients with high risk for psychoses, a striatal DA elevation was proposed to lead to psychosis (Reith et al., 1994; Howes and Kapur, 2009; Winton-Brown et al., 2014). Therefore treatment with antipsychotics and the subsequent blockade of D2R transmission was effective in reducing psychosis in other mental illnesses including mania or depression with acute mania (Dannon et al., 2006).
Howes and Kapur (Howes and Kapur, 2009) went so far as to presume that when investigating non-psychotic forms of SCZ, most likely no DA abnormalities could be found. Indeed, a proportion of SCZ patients (up to 30 %) are non-responsive to D2R antagonist neuroleptic drugs and discontinue their treatment for that reason (Lieberman et al., 2005).

Increased DA transmission leading to psychosis can be explained either by increased synaptic DA abundance or by an increase in postsynaptic DA receptor density. In mental illness a change in D2R density is controversial. Meta-analyses of imaging studies can at most argue for a modest increase of D2R in SCZ (Kestler et al., 2001), in psychotic but not non-psychotic BD cases (Gonul et al., 2009), but probably no difference in DD patients (Parsey et al., 2001).
Despite the lack of D2R density change, an increase in DA receptor occupancy was detected in SCZ patients (Abi-Dargham et al., 2000). This higher occupancy is seemingly mediated by changes in DA receptor affinity, namely the elevation of the high-affinity state of D2Rs, the so-called D2High receptors (Seeman, 2013).
D2Rs can occur in two states, a low affinity D2Low state which binds DA at micromolar concentrations and a high affinity D2High state that can binds DA even at a nanomolar range (Seeman, 1982). Both affinity states can convert into each other, with the more sensible D2High being the functional physiological state (Seeman et al., 2006).

Repeated administration of psychostimulants that increase DA transmission leads to a gradual increase in behavioral responsiveness. This phenomenon is termed "sensitization" and is reflected by, for example, increased locomotor activation in rodents (Robinson and Becker, 1986). Animal models of psychosis, such as amphetamine-sensitized rats, become supersensitive towards psychostimulants, meaning that after a sensitization and withdrawal phase a low dose of stimulant is sufficient to induce a response.

In analogy to SCZ, in sensitized rodents only a minor change in total DA receptor density, if any, could be detected, as the biological cause for the sensitization of these animals lies in the elevation of the proportions of D2High receptor in the striatum (Seeman et al., 2006; Seeman et al., 2007).

Seeman suggests that D2High receptors are the convergence point of several psychosis pathways, as D2High elevation is a robust phenomenon in several animal models of human psychosis, arguing "down-stream" of Howes and Kapur who instead see DA dysregulation due to several genetic or environmental hits in general as a common pathway for psychosis.

Although evidence has been mounting to suggest a leading role of DA in SCZ or psychosis, it needs to be emphasized that altered DA neurotransmission cannot be seen as the sole underlying mechanism of these disorders. Antipsychotic treatment is effective in treating positive symptoms of SCZ, but fails to alleviate negative symptoms or cognitive deficits (Kirkpatrick et al., 2006a).

Of note, despite administration of neuroleptics some SCZ cases remain resistant to the treatment (Hasan and Wobrock, 2013). Therefore many studies suggest, for example, a role also for the glutamatergic synapse in the etiology of SCZ, which may mainly account for the negative symptoms and cognitive dysfunction (Moghaddam and Javitt, 2012).

1.2.2 The dopaminergic system

In order to understand altered dopaminergic neurotransmission in CMI, the dopaminergic system needs to be explained in more detail. The neurotransmitter dopamine plays an important role in the etiology of mental illness. As it exerts both inhibitory and excitatory functions, dependent on the target cell type, it acts as a neuromodulator of synaptic transmission, influencing pathways implicated in reward and motor control.

The dopaminergic system is highly organized and comprises the midbrain structures substantia nigra pars compacta (SNpc), ventral tegmental area (VTA), dorsal striatum (also known as caudate nucleus and putamen; dStr) and nucleus accumbens (also known as ventral striatum; NAc), which are linked by dopaminergic projections. Due to its unique connections, the dopaminergic system can be divided into four major dopaminergic pathways regulating distinct behavioral functions (Figure 1):

(**a**) the mesocortical pathway: arises from VTA and projects to the frontal cortex

plays a role in concentration, executive motor function and working memory

(**b**) the mesolimbic pathway: arises from VTA and projects to the NAc

plays a role in reward and motivation

(**c**) the nigrostriatal pathway: arises from SNpc and projects to the striatum

plays a role in motor planning and execution

(**d**) the tuberoinfundibular pathway: arises from hypothalamus and projects to pituitary gland

plays a role in hormone secretion

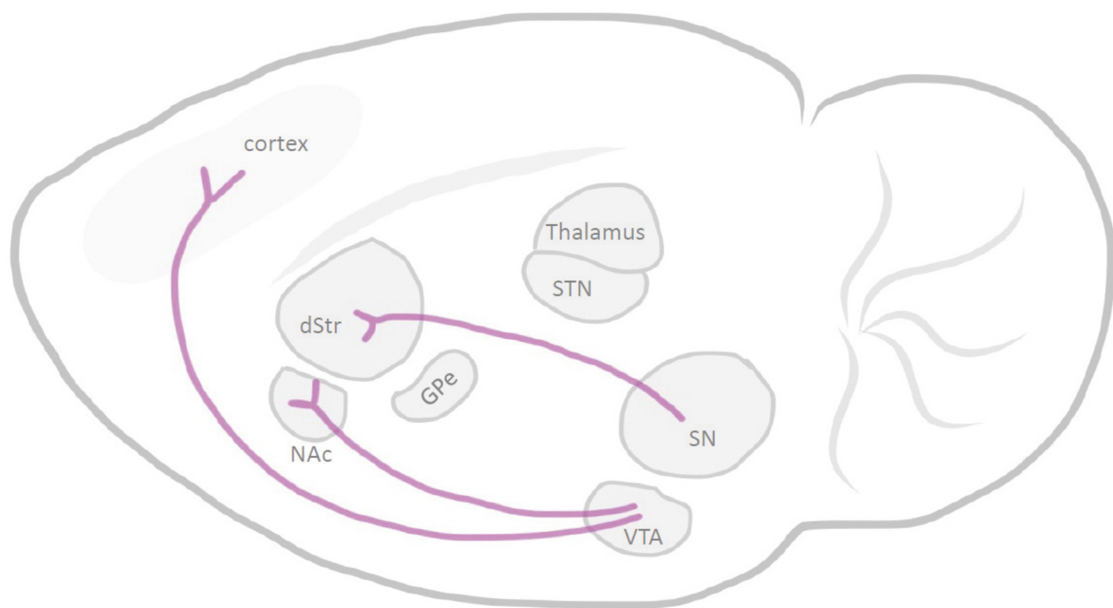

Figure 1: The nigrostriatal, mesocortical, and mesolimbic pathways in the adult rat brain
A simplified scheme of a sagittal view on a rat brain is depicted. The cortex, dorsal striatum (dStr), nucleus accumbens (NAc), substantia nigra (SN), and ventral tegmental area (VTA) form the structural basis of the dopaminergic pathways.

As mentioned in the last chapter, the “dopamine hypothesis of schizophrenia” proposes a frontal hypodopaminergia and striatal hyperdopaminergia to be the basis of negative and positive symptoms of SCZ, respectively (Davis et al., 1991). This frontal hypodopaminergia is caused by hypofunction of the mesocortical pathway, leading to striatal hyperdopaminergia, namely hyperfunction of the mesolimbic pathway.

DA binds to its DA receptors that can be divided into two major groups, the D1 receptor family (comprising the D1 and the D5 subtypes) and the D2 receptor family (covering D2, D3, and D4 subtypes), having diverging molecular properties.

All receptor subtypes are G-protein coupled. Upon activation, the two families have different influences on second messenger systems: the dopamine D1 receptor (D1R) family mainly functions through activating adenylate cyclase, resulting in an increase in cAMP levels, which in turn activates PKA (protein kinase A). The dopamine D2 receptor (D2R) family on the other hand reduces adenylate cyclase activity and cAMP levels and thus increases phospholipase C and protein kinase C instead. All DA receptors are located postsynaptically, with the exception of presynaptic D2Rs that function as autoreceptors, abating DA firing when activated.
DA signaling is terminated by either clearing extracellular DA from the synaptic cleft or by the metabolism of DA. DA can be taken back up into the presynapse, a process that is mediated by the DA transporter (DAT) in the dopaminergic midbrain structures and by the norepinephrine transporter (NET) in the frontal cortex. Once inside the cytosol of the cell, DA ultimately gets either recycled into synaptic vesicles by VMAT (vesicle monoamine transporter) or metabolized, but is located in the cytosol during the transit as a highly reactive molecule (Sulzer et al., 1995). The enzymes degrading DA are COMT (catechol-O-methyl transferase) and the monoamine oxidases MAO-A and MAO-B, which work sequentially to produce the final metabolite HVA (homovanillic acid).

Psychostimulants acting on the dopaminergic system such as amphetamine or cocaine work by reducing the rate of clearance of DA from the synaptic cleft. Cocaine mainly blocks the reuptake of DA by DAT, whereas amphetamine primarily competes with DA for uptake and reverses the direction of DAT transport, making DAT transport DA out of the cell and into the synaptic cleft (Kahlig and Galli, 2003). In parallel, amphetamine also acts on VMAT (vesicular-monoamine transferase) in the membrane of the neurotransmitter vesicle and releases DA into the cytoplasm (Partilla et al., 2006).

1.2.3 Dopamine and the recurrent affective disorders

DA is classically considered to play a role in substance abuse, SCZ, and psychosis, but more evidence for its contribution in other mental illnesses is emerging.
In BD the cycling of manic and depressive phases leaves the patient in a state of affective instability. These phases are characterized by increased dopaminergic transmission in the manic phase and decreased dopaminergic action in the depressive phase, linked to each other by dopaminergic feedback loops (Berk et al., 2007).
Depression is accompanied by anhedonia (the inability to experience pleasure) behavioral despair and loss of motivation. These processes are related to reward behavior and suggest that the dopaminergic mesolimbic system contributes to the pathophysiology of depression and

mood in general. Depression is believed to be caused by decreased monoamines (Serotonin [5-HT], noradrenalin [NA], and DA) in the brain, and most antidepressant therapies target 5-HT and NA transmission (Porcelli et al., 2011). In depressed patients less DA metabolites were detected in the cerebrospinal fluid and in animal models of depression such as “chronic mild stress”, forced swim test (FST) or “learned helplessness” the dopaminergic system is disturbed (Dunlop and Nemeroff, 2007). The clinical routine showed that antidepressant medications also potentiated DA transmission and that drugs which increase DA levels led to mood elevation (D'Aquila et al., 2000).

1.2.4 Dopamine and memory performance in the aged

Alterations of the DA system as part of the etiology of neuropsychiatric disorders are known from conditions such as Parkinson’s disease (PD), Huntington’s disease (HD), and SCZ. The respective patients suffer in addition from cognitive dysfunctions beyond the normal phenomenon of aging (Cropley et al., 2006).

The dopaminergic system is also involved in normal cognitive aging, as mild cognitive impairments during aging are at least partially due to decline in the efficiency of dopaminergic neuromodulation (Li et al., 2001) and stands in contrast to pathological aging as in Alzheimer’s disease (AD) that results from neurodegeneration and synapse loss (Morrison and Hof, 1997).

In aging individuals a reduction of D2R density of around 10 % per decade in the striatum, but also various other brain regions, was observed (Wong et al., 1997) (Kaasinen et al., 2000). This minor decline stands in contrast to, for example, PD in which a loss of about 80 % of the substantia nigra neurons needs to be reached in order to induce PD symptoms. The degeneration also occurs at a much faster rate than in normal aged individuals, leading to the observed increased cognitive impairments (Fearnley and Lees, 1991).

Memory decline in the aged individual is linked to age-related DA deficits in the cortico-striatal network, namely reduced DA transmission to the prefrontal cortex, interrelating disturbances of the dopaminergic system and impaired cognitive functioning (Braver and Barch, 2002; Podell et al., 2012).

A promising solution would be the application of DA to aged subjects in order to restore cognitive function, but a big caveat in DA administration is its inability to cross the blood brain barrier. Fortunately, an alternative route of drug delivery is given via the nasal passage (Illum, 2000).

Intranasal application of DA (IN-DA) enables DA to reach the brain, circumventing the blood brain barrier (Dahlin et al., 2000). Animal studies proved that application of either L-DOPA (de Souza Silva et al., 1997), the precursor of DA, or DA itself (de Souza Silva et al., 2008) led to a long-lasting increase in extracellular DA in the striatum.

Interestingly, this increase in DA was paralleled by behavioral effects. Buddenberg and colleagues (Buddenberg et al., 2008) found an IN-DA stimulated increase in locomotion and antidepressant-like activity in the rat. More evidence for the bioactivity of DA transported via the nose-brain pathway was given by studies in which IN-DA was able to extenuate behavioral asymmetries in a hemiparkinsonian rat model (Pum et al., 2009) and to promote memory and attention in an animal model of attention-deficit hyperactivity disorder (ADHD) (Ruocco et al., 2009; Ruocco et al., 2014).

1.3 Need for biological markers in mental illness

The current diagnoses for CMIs such as SCZ still rely on the clinical interview and evaluation of the self-reported symptoms of the patients. This subjective approach would be improved by enhancing diagnostic accuracy through the application of objective biology-based tests. Unfortunately, no such tests have been developed yet.

Biological diagnostic markers (biomarkers) can be defined as measurable biological indicators that reflect crucial pathophysiological characteristics of one specific illness and can support detection and treatment of psychiatric disorders in patients or aid in the identification of at-risk individuals at preclinical stages, and go beyond the subjective diagnosis of the psychiatrist expert. They can be used in the clinical routine to discriminate diagnoses or, through increased precision in disease diagnostics, even predict a therapeutic strategy and treatment response.

Biomarkers can be related to traits or states of an illness. Trait markers should target the underlying biological basis of a disorder, a state marker on the other hand should reflect a more transient feature, for example the current clinical condition of a patient (Chen et al., 2006).

An example is the P300 component of the event-related brain potential, a change in the electrophysiological activity of the brain that is suggested to be a trait as well as a state marker for SCZ. The P300 wave reflects a cognitive process that occurs after a sensory stimulus and can be measured by attaching electrodes to the skull.

The P300 amplitude is generally reduced in SCZ patients, arguing for its role as a trait marker (Ford, 1999). On the other hand it serves as a possible state marker as the P300 amplitude is negatively correlated with positive symptom severity and is therefore sensitive to changes in the disease course (Higashima et al., 2003).

Up until now, there is a lack of clear, objective biomarkers mainly due to the fact that the origins of mental illnesses are multifactorial, based on genetic predisposition in combination with environmental influences that render potential biomarkers easily unreliable.

Secondly, mental illness patients such as patients with SCZ are not a well-defined patient group, as even within one diagnosis patient subgroups exist. It is therefore quite optimistic to assume that one biomarker could be found that would work, for example, reliably for the entirety of SCZ patients.

To date, several approaches have been attempted in order to identify biomarkers (Table 2), targeting different body fluids by multi-omics approaches or structural and functional brain alterations by imaging techniques.

Tissue		Results (controversial)	Method	Reference
Blood		Haptoglobulin alpha	proteomics	Wan et al. 2007
		ApoA1	proteomics	Huang et al. 2008
CSF		GSK 3β	proteomics	Kozlovsky et al. 2004
		VGF	proteomics	Huang et al. 2006
Urine		Amino acid composition	metabolomics	Yang et al. 2013
		Biopyrrins	metabolomics	Miyaoka et al. 2005
***Ante mortem* brain**				
	Neuroimaging	Increased ventricle size	structural MRI	Shenton et al. 2001
		Cortical gray matter volume reduction	structural MRI	Kikinis et al. 2010
		Prefrontal cortical dysfunction (working memory)	fMRI	Callicott et al. 2000
	In vivo imaging	DA release after amphetamine	PET	Laruelle et al. 1996
		Altered DA neurotransmission	SPECT	Abi-Dargham et al. 2000
	Neurophysiology	P300	meta-analysis	Bramon et al. 2004
	Neuropsychology	Delayed response task, Wisconsin card sorting task	meta-analysis	Allen et al. 2009

Table 2: Potential biomarkers in psychiatry
The search for potential biomarkers in SCZ utilized investigations of the live brain and bodily fluids such as blood, CSF and urine. Several still controversial findings have been published, although none were converted into a biological test. The different approaches have relative advantages and problems compared to one another. Fluids such as blood are easily accessible but exhibit a high molecular complexity. CSF is favourable because of the proximity to the brain, but cannot be easily sampled from acute psychotic SCZ patients and has the problem of a detection limit due to its low protein concentration. Brain imaging has the big advantage of being *ante mortem*, is dependent on a non-invasive method, but is a costly procedure in need of standardized protocols.
Non-comprehensive data were summarized from the following publications:
(Wan et al., 2007), (Huang et al., 2008), (Kozlovsky et al., 2004), (Huang et al., 2006), (Yang et al., 2013), (Miyaoka et al., 2005), (Shenton et al., 2001), (Kikinis et al., 2010), (Callicott et al., 2000), (Laruelle et al., 1996), (Abi-Dargham et al., 2000), (Bramon et al., 2004), (Allen et al., 2009).
Abbreviations: (f)MRI (functional) magnetic resonance imaging, PET positron emission tomography, SPECT single photon emission computed tomography.

Due to the fact that SCZ patients do not represent a homogenous patient group, Kapur et al. (Kapur et al., 2012) claimed there was a need for “stratified medicine” in clinical and biological psychiatry. He proposes to break the vast quantity of CMI patients with various phenotypes down into treatment-relevant subgroups, a process again in need of biological markers that meet the criteria to identify those patient populations. This would simplify treatment even without understanding the multifaceted etiology of an illness and could lead to a type of personalized medicine based on patient group-specific biological features rather than the symptom-based treatment that is favored at the moment.

A biomarker makes most sense in terms of clinical usage, if it can be obtained in a low-invasive manner from easy accessible tissues. Potential sources are body fluids such as urine, blood (whole blood, plasma, or serum), saliva or even cerebrospinal fluid (CSF), which are suitable for research on protein- or RNA-based biomarkers.

Blood cells, mainly lymphocytes, have been widely investigated as possible sources for mental illness markers. RNA- and protein-based studies proposed, for example, VLDLR and ApoER2 mRNA levels as markers for SCZ, histone synthesis and DARPP-32 expression for BD and SCZ, and CREB levels and phosphorylation state in depression (Sourlingas et al., 2003; Suzuki et al., 2008; Torres et al., 2009; Lim et al., 2013). Many of these studies were based on the hypothesis that in CMI changes in brain protein levels could be reflected in peripheral tissues, e.g. blood or lymphocytes.

It is therefore useful to analyze whether key proteins involved in mental illness can serve as biomarkers with the aim of providing biomedical tests for routine clinical practice.

1.4 The biology underlying chronic mental illness - the concept of DISC1opathies

1.4.1 Learning from the neurodegenerative diseases: aggregated proteins in chronic mental illness

CMIs lack a known concrete neuropathological hallmark. In contrast, neurodegenerative diseases exhibit as one common feature the accumulation of aggregated intra- or extracellular protein in the brain. Insoluble protein species were found in several brain disorders, including the presence of Aβ plaque and tau tangle pathology in AD, α-synuclein positive Lewy bodies in PD, superoxide dismutase-1 (SOD1) in amyotrophic lateral sclerosis, polyglutamine-expanded huntingtin in HD and PrP^{Sc} in prion disorders (Taylor et al., 2002).

From familial cases of neurodegenerative diseases it is known that they often result from a mutation in one gene leading to the dysfunction of the respective protein product and its assembly, a process leading to the deposition of protein aggregates in the affected brains. Examples are mutations in APP (amyloid precursor protein) in AD (Goate et al., 1991) or α-synuclein in PD (Polymeropoulos et al., 1997). Interestingly, the causal protein candidates are most often the same for the familial cases as for the sporadic occurring disease (Prusiner, 2001).

Protein aggregation results from protein misfolding (Kopito, 2000) and in a post-mitotic neuron an imbalance in or disturbance of proteostasis may lead to the accumulation of those proteins.

A key study by Leliveld and colleagues (Leliveld et al., 2008) set out to investigate whether disturbances of proteostasis and protein aggregation are also a feature of CMIs due to the chronic-nature of the illnesses, in analogy with the neurodegenerative diseases. They investigated whether aggregation of proteins encoded by CMI candidate genes involved in familial cases of mental illness are also present in brain tissue of sporadic CMI cases. Biochemical purifications of the insoluble fraction of brain material of CMI patients obtained from the Stanley Medical Research Institute (SMRI) Consortium Collection (Torrey et al., 2000) revealed the presence of aggregated proteins in a subset of patients with SCZ, BD and DD (Leliveld et al., 2008; Ottis et al., 2011; Bader et al., 2012b; Bradshaw et al., 2014).

Protein assembly and aggregation might therefore reflect a mutual underlying mechanism in CMI crossing the present illness classifications and may act as a biomarker for a subset of mental illness patients. Interestingly, in about 15 % of CMI cases insoluble species of the DISC1 protein were found, one of the top candidates involved in mental illness.

1.4.2 The DISC1 locus in mental illness

The DISC1 protein and its potential role in chronic mental illnesses were first described in a Scottish pedigree identified a quarter of a century ago. St. Clair et al. (St Clair et al., 1990) investigated a unique Scottish family in which many family members were carriers of a balanced chromosome t(1;11) (q42.1;q14.3) translocation co-segregating with various mental illnesses, ranging from SCZ and DD to BD and anxiety disorders (St Clair et al., 1990; Millar et al., 2000).

The last major clinical report (Blackwood et al., 2001) on this family described a total of 87 family members that were karyotyped, revealing 50 non-carriers and 37 translocation carriers. Psychiatric diagnoses were available from 38 and 29, respectively. From the carriers, 7 had a diagnosis of SCZ, 1 of BD and 10 cases of major recurrent depression. When the clinical diagnoses were considered in combination or solely SCZ cases were analyzed, the genetic linkage was among the highest ever recorded for a CMI family (maximum LOD scores of 7.1 and 3.6, respectively) (Blackwood et al., 2001). There were also 2 cases of adolescent conduct disorder and 1 member with minor depression, but those diagnoses also appeared in the non-carrier group (1 with adolescent conduct disorder, 3 with minor depression, 1 with alcoholism) and are therefore not translocation-dependent.

Interestingly, all translocation carriers exhibited a reduced amplitude of the P300 event-related potential, one of the more reliable trait markers for SCZ (Blackwood et al., 2001) (see Chapter 1.3).

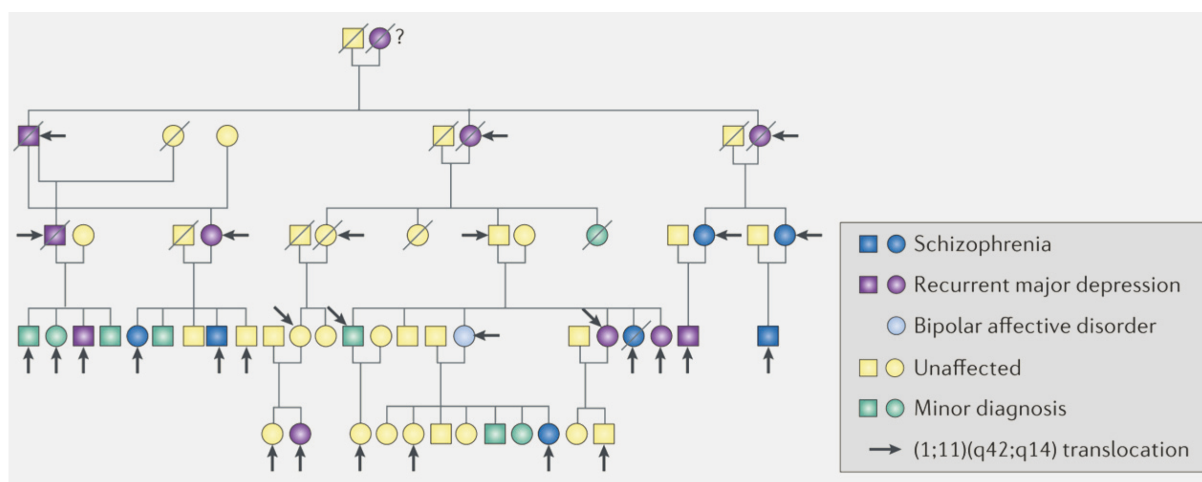

Figure 2: The Scottish pedigree
Adapted from (Brandon and Sawa, 2011).

The described chromosomal translocation led to the disruption of a previously unknown gene that got the memorable name *Disrupted-in-Schizophrenia 1* (*DISC1*), and a non-coding gene on the antisense strand named *DISC2*. The translocation disrupts the *DISC1* gene in intron 8

between exon 8 and 9 and is proposed to lead to the putative expression of a C-terminally truncated protein product composed of the amino acids 1-597 rather than the normal product of 1-854 amino acids (Millar et al., 2000).
In recent years increasing evidence emerged arguing for a fusion protein of truncated DISC1 with DISC1FP1/Boymaw (Zhou et al., 2008; Zhou et al., 2010) that was found to be transcribed in lymphoblastoid cells of affected family members (Eykelenboom et al., 2012). Of note, all carriers are heterozygous for the translocation, they express one intact full-length DISC1 protein and one putative deletion variant (Blackwood et al., 2001).

Given the exhibited variety of mental illness phenotypes caused by the disruption of the *DISC1* locus it is of importance to emphasize that the name Disrupted-in-Schizophrenia 1 is rather misleading as it is not associated exclusively with SCZ, but also with a whole range of mental illnesses.
Subsequent world-wide gene association studies targeting genetic variants executed following the discovery of the Scottish family verified the *DISC* locus in mental illnesses (Chubb et al., 2008; Bradshaw and Porteous, 2012) corroborating it as a vulnerability factor for several mental illnesses in different ethnic backgrounds. This heterogeneity of CMI phenotypes linked to DISC1 highlights its necessity for balancing or regulating mental performance.

1.4.3 DISC1opathies

A consortium of phenotypically and pathologically distinct brain disorders linked to one mutual dysfunctional protein is generally called a "proteinopathy". Biological examples are tauopathies and synucleinopathies, which combine separate diseases on the grounds that they share the same aggregated protein deposit. Tauopathies are pathologically categorized by the presence of filamentous intracellular inclusions of hyperphosphorylated Tau and include several neurodegenerative diseases such as AD, frontotemporal dementia, Pick's disease, progressive supranuclear palsy, and corticobasal degeneration (Lee et al., 2001). Synucleinopathies are accordingly characterized by abnormal accumulation of α-synuclein aggregates, mainly in PD, dementia with Lewy bodies, and multiple system atrophy (McCann et al., 2014).
It was proposed to classify a subgroup of otherwise symptomatically heterogenous CMI patients dependent on the presence of insoluble DISC1 in the brain as one disease entity, termed "**DISC1opathies**", in analogy with the aforementioned proteinopathies (Korth, 2009, 2012). DISC1opathies meet the criteria of a proteinopathy as aggregated DISC1 could be detected in CMI patients (Leliveld et al., 2008). Well characterized brain samples of patients diagnosed with SCZ, BD, or DD were obtained from the Stanley Foundation brain collection (Torrey et al., 2000).

From the n = 15 matched cases per diagnosis, 2 SCZ, 2 BD, and 3 DD patients, but no control brain, exhibited high levels of aggregated DISC1.

A second prominent feature of protein aggregates is their cell-to cell transmission. It occurs in prion disease (Prusiner, 2001) and has also been proposed for proteins of non-prion diseases (Lee et al., 2010) including Aß (Kane et al., 2000), tau (Clavaguera et al., 2009), α-synuclein (Kordower et al., 2008; Desplats et al., 2009), poly-glutamine peptides (Ren et al., 2009; Costanzo et al., 2013), and SOD1 (Munch et al., 2011).
Interestingly, the overexpression of DISC1 in cells led to the presence of aggresomes which are defined as perinuclear protein aggregates probably consisting of smaller aggregated protein species in the cell (Johnston et al., 1998; Kopito, 2000). During cellular investigations, evidence was gathered for cell-to-cell transmissibility as purified DISC1 aggresomes could be taken up by other cells, although with low efficiency (Ottis et al., 2011). Once in the cell, the DISC1 aggresomes could render previously soluble molecules of both DISC1 and other proteins insoluble by recruiting them into the aggregated pool, arguing for a possible physiological gain-of-function for DISC1 aggregates in the cellular system. Additionally recombinant C-terminal DISC1 fragments were, when stereotactically injected into the rat brain, cell-invasive *in vivo* and could be taken up by neurons (Bader et al., 2012a). The same DISC1 fragments were also able to penetrate cells *in vitro* with an uptake rate comparable to that of synthetic oligomeric α-synuclein (Ottis et al., 2011). DISC1 therefore also fulfills the second continuative criterion of a proteinopathy by its cell-to-cell transmissibility.

Consequently, a subgroup of CMIs characterized by aggregated DISC1 fulfills the requirements of a protein-conformational disorder, validating the term “DISC1opathies” for the classification of DISC1-dependent, but otherwise behaviorally heterologous, CMIs. The concept of DISC1opathies now opens the possibility for a biologically defined subcategorization of CMI patients, independent of the clinical diagnosis, and provides an option for the investigation of underlying disease mechanisms or even potentially pharmacological targeting.

1.4.4 DISC1 function in the brain

When discussing the putative role of DISC1 aggregates in CMIs, it is important to also consider the normal role of DISC1 in the brain.

DISC1 is a cytoplasmic protein, expressed throughout the brain in neurons, astrocytes, oligodendrocytes, and microglia (Seshadri et al., 2010), as well as non-neural tissues (Millar et al., 2000; Ozeki et al., 2003; James et al., 2004). DISC1 interacts with a variety of more than 100 proteins (Camargo et al., 2007) and is suggested to act as a molecular hub or scaffolding protein (Yerabham et al., 2013), bridging and guiding intermolecular protein-protein interactions.

In addition, many studies emphasize an important role of DISC1 in neurodevelopment by regulating neuronal proliferation and migration. This is of special interest as, for example, SCZ is believed to be caused by neurodevelopmental disturbances (Lewis and Levitt, 2002; Jaaro-Peled et al., 2009). The connection between DISC1 and neurodevelopment is also reflected by the temporal expression pattern of endogenous rodent Disc1, with peaks at E14 and the late embryonic stages, although robust expression also takes place again from P35 onwards during the lifetime (Ozeki et al., 2003; Schurov et al., 2004; Mao et al., 2009).

The first step in corticogenesis is progenitor cell proliferation. For this, DISC1 interacts with molecules of the canonical Wnt signaling pathway. DISC1 inhibits GSK3β (the glycogen synthase kinase 3 beta) and thereby stabilizes β-catenin, leading to regulation of progenitor proliferation through transcription of Wnt target genes (Mao et al., 2009). The interaction with GSK3β is tightly regulated by DISC1 phosphorylation of a serine residue at position 710 of mouse Disc1 (corresponding to serine 713 in human DISC1). Once phosphorylated, Disc1 loses its interaction with GSK3β, leading to increased β-catenin degradation. Through this action DISC1 is suggested to act as a molecular switch, changing the neuronal program from proliferation to migration (Ishizuka et al., 2011).

Neuronal migration is the next step during corticogenesis and is dependent on remodeling of the cytoskeleton and microtubule dynamics. Partially controlled by the serine phosphorylation, DISC1 triggers neuronal migration by recruiting proteins such as NDEL1 to the centrosome, by functioning as an anchoring molecule (Miyoshi et al., 2004; Bradshaw et al., 2008; Ishizuka et al., 2011).

During adult neurogenesis DISC1 was found to determine proper positioning of the new-born neuron in the hippocampus (Duan et al., 2007), probably through interaction with the AKT/mTOR pathway (Enomoto et al., 2009; Kim et al., 2009). In the matured neuron DISC1 can be found at the postsynaptic density of the synapse (Kirkpatrick et al., 2006b), where DISC1 regulates synapse maintenance by facilitating or blocking several protein-interactions and

signaling molecules dependent on neuronal activity (Hayashi-Takagi et al., 2010; Wang et al., 2011).

In view of the multiple functions of DISC1, it is obvious that the hypothesized deletion of the C-terminal part of DISC1 detected in the Scottish family must have physiological consequences. Studies addressing this question proposed that the truncated mutant form most likely acts as a dominant-negative mutant (Kamiya et al., 2005). As the C-terminal part of DISC1 harbors various interaction domains, many interactions are lost in the truncated mutDISC1, altering the subcellular distribution or inducing dissociation of DISC1 from the centrosome, leading to impaired neurite outgrowth (Morris et al., 2003; Kamiya et al., 2005; Millar et al., 2005). The effect of the mutant form was comparable to those of a knockdown of endogenous Disc1 in disturbing neuronal migration in the developing cortex (Kamiya et al., 2005).

Considering the multi-faced functions of DISC1 in the brain it was proposed to rename DISC1 “Diverse Inhibitor of Signaling Cascades” (Yerabham et al., 2013) as it acts as a wide-range inhibitor of interrelated signaling pathways in the control of neurodevelopmental and adult brain function. The multiple functions of DISC1 especially during neurodevelopment help to explain how it can be involved in a diverse array of different mental illnesses.

1.5 Current animal models relating to a dysfunctional *DISC1* gene

1.5.1 Model description and general phenotyping

Up until now, several laboratories have developed mice expressing dysfunctional DISC1 with the aim of studying behavioral mental illness-related phenotypes in an animal model, as a translational approach. All models so far focused on mutations in DISC1.
DISC1 mouse models can be divided in two groups: missense mutations and deletion variants of the endogenous mouse *Disc1* locus, as well as ectopic expression of human DISC1 variants in the forebrain.

The DISC1 mouse models were generated based on different hypotheses. Four mouse lines were developed expressing C-terminal deletion variants of either the endogenous mouse Disc1 or transgenic human DISC1. They were designed under the premise that the mutant protein functions in a dominant-negative manner by disturbing proper function of the wild type protein in order to simulate the genetic situation of the Scottish family.
The first truncated Disc1 mouse model was described by Koike and colleagues. They discovered a 25 bp deletion in exon 6 in the common 129S6/SvEv laboratory mouse strain, leading to a frameshift, resulting in 13 new amino acids and a premature stop codon in exon 7 of mouse *Disc1* (further described as msDisc1^{129}) (Clapcote and Roder, 2006; Koike et al., 2006). Subsequent studies verified that this naturally occurring deletion is present in all laboratory 129 strains (Clapcote and Roder, 2006).
The mice generated by Shen et al. carry two copies of the endogenous mouse *Disc1* locus, encoding solely for exon 1-8, on a bacterial artificial chromosome (BAC) with a C-terminally fused eGFP tag (msDisc1$^{\Delta 9\text{-}13}$) (Shen et al., 2008).
The mouse developed by Hikida et al. expresses a C-terminally truncated version of human DISC1, as proposed to be present in the Scottish family (Hikida et al., 2007). They chose constitutive expression under the CaMKII promoter, leading to ectopic expression of the transgene in forebrain regions (huDISC1$^{(1\text{-}597)}$).
In contrast to the constitutive expression of the huDISC1$^{(1\text{-}597)}$/Hikida mouse, Pletnikov et al. generated an inducible Tet Off model (Off-huDISC1$^{(1\text{-}597)}$) (Pletnikov et al., 2008). Comparable to the huDISC1$^{(1\text{-}597)}$/Hikida mouse, the truncated huDISC1 is expressed under the CaMKII promoter with the difference that the expression is inhibited by the administration of tetracycline.

Li et al. introduced a C-terminal DISC1 peptide that spans regions important for interactions with proteins responsible for centrosomal location, neuronal migration, and neurodevelopment

(Morris et al., 2003; Ozeki et al., 2003; Brandon et al., 2004; Kamiya et al., 2005). It was predicted to also act in a dominant-negative manner and disrupt DISC1 function comparable to the truncation models.
The animal model is based on a Tamoxifen-dependent Tet On system, expressing the huDISC1 peptide under the CaMKII promoter (On-huDISC1$^{(671-852)}$) (Li et al., 2007).

Clapcote and colleagues did not aim to disrupt the wild type full-length protein function by truncation of Disc1. They phenotyped mice with a missense mutation in mouse *Disc1* under a pleiotropic approach, meaning that a single mutation in one gene may influence multiple traits due to the fact that one gene product interacts with various biological targets.
Treatment with the mutagenic substance *N*-ethyl-*N*-nitrosourea (ENU) resulted in two distinct mouse lines with a point mutation, each in exon 2 of the endogenous mouse *Disc1* locus. The mouse lines were named after the resulting amino acid change Q31L (glycine to leucine at amino acid 31) and L100P (leucine to proline at amino acid 100) (Clapcote et al., 2007).

Kuroda et al. aimed to generate a full-length Disc1-deficient mouse, in which a disturbance of the functional role of Disc1 is achieved by the lack of the full-length protein.
A targeted deletion of exons 2 and 3 of mouse *Disc1* and the insertion of a stop codon in the remainder of exon 2 abolished expression of full-length msDisc1 in homozygous animals (msDisc1$^{\Delta 2-3}$) (Kuroda et al., 2011), although they could not rule out that spliced Disc1 variants lacking exons 2 and 3 could still be generated.

All DISC1 animal models were thoroughly tested for behavioral phenotypes with a focus on behavioral traits of relevance to mental illnesses. The majority of these test results can be divided into three main blocks, namely sensory-motor function, motivation/emotion-related processes and sociability, and memory function (Table 3; categorization of animal behavior modified from the review article by (Lipina and Roder, 2014)). “Sensory-motor functions” summarized in Table 3 comprise open field spontaneous locomotion and prepulse inhibition of acoustic startle response (PPI). “Motivation/emotion-related and sociability” combines social cognition with CMI-related phenotypes such as depression or despair (FST = forced swim test, TST = tail suspension test), anhedonia (sucrose consumption), and anxiety (EPM = elevated plus maze). “Memory and cognition” is divided into working memory (T-Maze, DNMT = delayed non-match to place task), object or spatial reference memory (MWM = Morris water maze, novel object recognition = OR) or associative learning (fear conditioning).

Model	Study	SENSORY-MOTOR FUNCTION						MOTIVATION /EMOTION-RELATED AND SOCIABILITY								MEMORY AND COGNITION					
		locomotion		rotarod		PPI		depression / despair		anhedonia		sociability		anxiety		working memory		object / spatial memory		associative memory / fear conditioning	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Q31L	**Clapcote et al. 2007**	=				↓		↑		↓		↓		=		↓		=			
	Haque et al. 2012	=				=		=		=		↓		↑							
	Shoji et al. 2012	=	n.d.	=	n.d.	=	n.d.	=	n.d.			=	n.d.	=	n.d.	=	n.d.			=	n.d.
	Lipina et al. 2012							↓				↓									
L100P	**Clapcote et al. 2007**	↑				↓		=		=		=		=		↓		=			
	Lipina et al. 2010	↑	n.d.			↓	n.d.														
	Haque et al. 2012	↑ / =				↓		=		=		=		↑							
	Shoji et al. 2012	=	n.d.	=	n.d.	=	n.d.	=	n.d.			=	n.d.	=	n.d.	=	n.d.			=	n.d.
	Lipina et al. 2012	↑				↓															
	Arime et al. 2014	=	n.d.									=	n.d.								
	Walsh et al. 2012	↑																			
msDisc1$^{\Delta2-3}$	**Kuroda et al. 2011**	=	=			=	↓	=	=			=	=	↓ /	↓ /	=	=			=	=
		=				↓		=				↑		↓		=				↑	
msDisc1^{129}	**Koike et al. 2006**	=				=										↓					
	Kvajo et al. 2008															↓	n.d.	=	n.d.	=	n.d.
	Gomez-Sintes et al. 2014	↑	↓			↓	↓	↑	↑							=	=			↓	=
		n.d.				↓		↑								n.d.				n.d.	
	Juan et al. 2014							=	n.d.					=	n.d.	↓	n.d.	=	n.d.		
msDisc1$^{\Delta9-13}$	**Shen et al. 2008**	=						↑													
huDISC1$^{(1-597)}$	**Hikida et al. 2007**	↑	n.d.	=	n.d.	↓	n.d.	↑	n.d.			=	n.d.	=	n.d.	=	n.d.	=	n.d.		
	Ibi et al. 2010					=										=		=			
	Johnson et al. 2013											↓	n.d.								
Off-huDISC1$^{(1-597)}$	**Pletnikov et al. 2008**	↑	=			=	=					↓	n.d.	=	=			=	↓		
	Ayhan et al. 2010							=	↑			↓	n.d.								
	Pogorelov et al. 2012	=	=					=	=											↓	=
	Abazyan et al. 2010							=	n.d.			=	n.d.	=	n.d.						
	Kaminitz et al. 2014	=	n.d.									=	n.d.					=	n.d.		
On-huDISC1$^{(671-852)}$	**Li et al. 2007**	=						↓				↓		=		↓					
tgDISC1 rat	**Trossbach et al. 2016**	=	n.d.	↓	n.d.	=	n.d.	=	n.d.	=	n.d.			=	n.d.			=	n.d.		

Table 3: Behavioral phenotypes of the DISC1 animal models

Table 3: Behavioral phenotypes of the DISC1 animal models

Phenotypes in this table were summarized from DISC1 mouse models described in the literature and the DISC1 rat model presented in this thesis. Behavioral data is summarized concerning three main readouts: sensory-motor function, motivation / emotion-related processes and sociability, and memory function.

n.d. = data not determined or reported for that sex. Red arrows represent an increase, green arrows a decrease, and grey equal sign no difference in the respective behavioral readout in genetically altered compared to control animals. Data is split by sex of the animals. Where data is not split in this manner, then behavior was determined either for both sexes together or no information was provided in the respective publication.

Data was summarized from the following publications: (Clapcote et al., 2007), (Haque et al., 2012), (Shoji et al., 2012), (Lipina et al., 2012), (Lipina et al., 2010), (Walsh et al., 2012), (Su et al., 2014), (Kuroda et al., 2011), (Koike et al., 2006), (Kvajo et al., 2008), (Gomez-Sintes et al., 2014), (Juan et al., 2014), (Shen et al., 2008), (Hikida et al., 2007), (Ibi et al., 2010), (Johnson et al., 2013), (Pletnikov et al., 2008), (Ayhan et al., 2011), (Pogorelov et al., 2012), (Abazyan et al., 2010), (Li et al., 2007; Kaminitz et al., 2014), (Trossbach et al., 2016).

Although the approaches to investigate the role of DISC1 on behavior and the choice of the specific genetic variant are quite divergent in the animal models presented here, it is of interest that some behavioral findings are relatively robust.

One is a deficit in PPI, a measure of sensorimotor gating. It is based on the fact that an animal startles less when presented with a loud auditory stimulus (pulse) if a weak tone (prepulse) is presented milliseconds before the loud tone. A deficit in PPI means that the presentation of the prepulse does not sufficiently reduce the startle reaction following the pulse. Patients with SCZ also have a deficit in PPI, which embodies a SCZ endophenotype, but also occurs in other psychiatric illnesses (Kohl et al., 2013). The PPI deficit represents the SCZ patient's inability to properly filter sensory information from the environment and is a test that can be translated directly from human patients to animals. Regarding the detected PPI deficits in the DISC1 transgenic mouse lines, it has to be pointed out that this phenotype may be consistent, but nonetheless rather weak, as only specific combinations of prepulse/pulse intensities or inter pulse intervals resulted in reduced PPI, whereas many other setups did not. In the msDisc1$^{\Delta 2\text{-}3}$/ Kuroda mouse, for example, only two of four setups induced PPI deficit in the animals and also only in females, not in male mice (Kuroda et al., 2011). For simplification PPI deficits were stated in Table 3 if at least one setup showed genotype differences.

A second more consistent phenotype is hyperactivity in the open field. When placed in a box and allowed to explore the open space, many DISC1-transgenic mice showed increased spontaneous locomotion compared to their controls. This hyperactivity in combination with a PPI deficit led,

for example, to the categorization of the L100P/Clapcote mouse as a "schizophrenic-like" mouse (Clapcote et al., 2007) in contrast to the "depressive-like" Q31L mouse.
Experiments targeting either motivation, sociability, or memory showed inconsistent results between different mouse models. Some models displayed depression-like behavior in the FST or TST, some pointed towards reduced sociability, whereas most groups did not find differences in displayed anxiety. Also the memory tasks showed varying results. A working memory deficit was rather consistent, whereas hippocampal memory formation appeared to be intact.

The combined results of this phenotyping highlight that DISC1 indeed plays a critical role in behavioral control. This gets even more prominent when experiments were conducted to target one major neurotransmitter system intertwined with CMI – the dopaminergic system.

1.5.2 DISC1 mice: alterations of the dopaminergic system

Some DISC1 transgenic animal mouse lines were tested for disturbances in the dopaminergic system (see Table 4), namely alterations in the basic components of the DA system such as synaptic DA levels, DA receptor densities/affinities, or supersensitivity towards stimulants that act on the DA level. For the latter, most studies utilized challenges with amphetamine.
Lipina et al. challenged the SCZ-like L100P/Clapcote mouse with a low dose of amphetamine of 0.5 mg/kg, without prior sensitization, to test whether this mouse line is in analogy to the psychostimulant supersensitivity seen in SCZ patients *per se* sensitized (Lipina et al., 2010). The L100P/Clapcote mice were more active in the open field after stimulation compared to wildtype controls, hinting towards a higher susceptibility for amphetamine.
Challenges with higher doses of amphetamine (>1 mg/kg) that also induce increased locomotion in control animals led to a higher response rate in the L100P/Clapcote mouse (Lipina et al., 2010), the huDISC1$^{(1\text{-}597)}$/Hikida mouse (Jaaro-Peled et al., 2013) and the Off-huDISC1$^{(1\text{-}597)}$/Pletnikov mouse (Ayhan et al., 2011) compared to their respective controls, also indicating supersensitivity.

In some DISC1 transgenic mouse models, the basic elements of the DA system were investigated. Analyses of the basal levels of DA in dopaminergic structures of the brain were inconsistent as measured by microdialysis or *post mortem* neurochemistry. The L100P/Clapcote mouse had comparable DA concentrations in the NAc, dStr, hippocampus (HC), and frontal cortex (FC) of transgenic and control mice (Lipina et al., 2010). The msDisc1$^{\Delta 2\text{-}3}$/Kuroda mouse also had equal baseline DA levels in the NAc (Nakai et al., 2014), whereas reduced DA levels in the NAc were detected in the huDISC1$^{(1\text{-}597)}$/Hikida mouse. The Off-huDISC1$^{(1\text{-}597)}$/Pletnikov mouse displayed,

on the one hand, less DA in cortical regions, but no change was seen in the striatum (Ayhan et al., 2011).

Microdialysis experiments combined with amphetamine challenges were performed in a subset of mouse models and confirmed an increased release of DA in dopaminergic structures of transgenic animals following drug application (L100P/Clapcote (Lipina et al., 2010), huDISC1$^{(1-597)}$/Hikida (Jaaro-Peled et al., 2013), msDisc1$^{\Delta 2\text{-}3}$/Kuroda (Nakai et al., 2014)).

Targeting the basal D2R density, autoradiography data of the striatum revealed a small increase in striatal D2Rs in the huDISC1$^{(1-597)}$/Hikida mouse, whereas the Off-huDISC1$^{(1-597)}$/Pletnikov mouse showed no significant differences of D2Rs in the NAc, dStr, SN or VTA (Pogorelov et al., 2012). Female, but not male, msDisc1$^{\Delta 2\text{-}3}$/Kuroda mice expressed more *D2R* mRNA in the NAc.

In the L100P/Clapcote mouse, Lipina and colleagues looked for DA receptor affinity states by comparing the D2High:D2Low ratio and found an increase in D2High receptors in the L100P mouse compared to controls (Lipina et al., 2010).

Striatal DAT levels were upregulated in the huDISC1$^{(1-597)}$/Hikida mouse, but not in the NAc of the msDisc1$^{\Delta 2\text{-}3}$/Kuroda model.

Niwa and colleagues underwent a different approach by knocking down msDisc1 in the cortex during neurodevelopment by *in utero* electroporation (Niwa et al., 2010). The goal was to shed light on how disturbances in early development affect postnatal brain maturation, as SCZ is thought to be partially of neurodevelopmental origin. Knockdown of DISC1 in neurons of the frontal cortex led to disturbances of the mesocortical maturation in the adult animal, for example reduced extracellular DA in the medial prefrontal cortex (mPFC) and behavioral deficits such as PPI deficit or hypersensitivity to amphetamine.

All these findings show that the dopaminergic system is affected if DISC1 is mutated. It leads to the hypothesis that the DISC1 protein might interact with the dopaminergic machinery to control behavior.

So far, only one recent study has proposed a mechanism of how DISC1 actually alters the dopaminergic system. Su and colleagues could prove a direct interaction between the D2Rs and DISC1 (Su et al., 2014). They could narrow down the interaction domain and used this information to design a peptide that was able to disrupt DISC1-D2R complex formation. In behavioral experiments with the L100P/Clapcote mice they succeeded in restoring the hyperlocomotion and PPI deficit by injection of the interfering peptide. Additionally, the disruption of DISC1-D2R complex prevented amphetamine-induced hyperlocomotion, even in wild type rats.

This study provides a first hint of how DISC1 might induce changes in dopaminergic neurotransmission.

	locomotor reaction to Amphetamine			basal DA levels			changes in DA levels after Amphetamine treatment					D2R changes			DAT levels		
	dose	reaction vs Saline	reaction vs WT	reaction vs WT	region	method	dose	reaction vs baseline	reaction vs WT	region	method	regulation	region	method	regulation	region	method
Lipina et al. 2010	0.5 mg	↑	↑	total =	NAc, Str, HC, FC	PMNC	0.5 mg	↑	↑	Str	MD	↑	Str	D2High			
L100P	1 mg	↑	↑														
Clapcote et al. 2006	2.5 mg	↑	↑	extracellular =	Str	MD											
Jaaro-Peled et al. 2013	1 mg	↑	↑	extracellular ↓	NAc	MD	1 mg	↑	↑	NAc	MD	↑	Str	PET	↑	Str	WB
huDISC1$^{(1-597)}$												↑	Str	AR			
Hikida et al. 2007												↑	Str	qPCR			
Ayhan et al. 2011	1 mg	↑	↑	total =	Str	PMNC											
Off-huDISC1$^{(1-597)}$				total ↓	FC, HC	PMNC											
Pletnikov et al. 2008																	
Pogorelov et al. 2012	escalating	↑	↓									=	NAc, SN / VTA	AR			
Off-huDISC1$^{(1-597)}$																	
Pletnikov et al. 2008																	
Nakai et al. 2014				extracellular =	NAc	MD	2 mg	↑	↑	NAc	MD	↑	NAc	qPCR	=	NAc	WB
msDisc1$^{\Delta 2\text{-}3}$																	
Kuroda et al. 2011																	
Trossbach et al. 2016	0.5 mg	↑	↑	release =	Str, NAc	CV						↑	Str	D2High	↑	Str	WB
tgDISC1 rat				clearance ↑	Str	CV						=	Str, NAc	AR			
				clearance =	NAc	CV											
				total ↓	Str	PMNC											
				total =	NAc	PMNC											

Table 4: DISC1 and dopamine in the DISC1 animal models
The table summarizes DA-related data from DISC1 mouse models described in the literature and the DISC1 rat model presented in this thesis.
Red arrows represent an increase, green arrows a decrease, and grey equal sign indicated no difference in the described readout between genetically altered animals and the respective controls.
Abbreviations: NAc = nucleus accumbens, dStr = dorsal striatum, HC = hippocampus, FC = frontal cortex, PMNC = *post mortem* neurochemistry, MD = microdialysis, PET = positron emission tomography, AR = receptor autoradiography, qPCR = quantitative PCR, WB = Western blot.
Data was summarized from the following publications: (Lipina et al., 2010), (Jaaro-Peled et al., 2013), (Ayhan et al., 2011), (Pogorelov et al., 2012), (Nakai et al., 2014), (Trossbach et al., 2016).

1.5.3 The tgDISC1 rat

A main part of this thesis was set out to investigate the role of full-length non-mutant human DISC1 in behavioral control. The existing human DISC1 mouse models were focused on mutations such as the C-terminally truncated DISC1 variant predicted for the Scottish family. Although this familial mutation was the genetic basis for DISC1 research as a candidate protein for mental illnesses, it needs to be emphasized again that the *DISC1* truncation is unique to this particular family. CMI patients worldwide express the non-truncated full-length protein, so it is of importance to understand how the full-length DISC1 protein contributes to the occurrence of sporadic forms of CMIs.

Therefore a cosmid was designed that, once integrated into the genome, leads to the constitutive expression of full-length human DISC1 under the Syrian hamster PrP promoter. The choice of the PrP promoter was made in order to stay close to the endogenous Disc1 promoter, which shares spatial and temporal expression pattern, but at a stronger expression rate (Manson et al., 1992) (see Chapter 1.4.4).

The decision was made to generate a rat rather than a mouse model, being the first transgenic rat model for a mental illness candidate gene. In recent decades, mice were the models of choice simply because the techniques for genetic manipulations were only possible in that species.

Although a rat has certain disadvantages such as bigger size and therefore housing complications or more time-consuming breeding due to longer maturation time, the advantages predominate. The rat is physiologically, genetically and morphologically closer to humans than mice (Do Carmo and Cuello, 2013). Also, for studies targeting higher cognitive functions, rats serve as the superior model (Papaleo et al., 2012): rats are more intelligent and sociable than mice and are able to learn more complex tasks, and as such, the principles of learning and memory have been studied extensively in that animal. The resulting commonly used tests have mostly been designed in rats and were validated by, for example, pharmacological intervention or lesion studies. Also its larger body and brain size facilitates interventions such as surgery, as well as the sampling of biomarkers such as blood that can be drained repeatedly and in experimentally useful amounts.

1.6 Aims of the conducted studies

In Study I a characterization of the tgDISC1 rat on the neuropathological, biochemical, and behavioral level was performed (for additional data see also Chapter 3). The question was addressed as to whether the tgDISC1 rat shows alterations in the dopaminergic system, as indicated by the dopamine hypothesis of CMIs and the published DISC1 mouse models, and whether it serves as a potential model for studying DISC1opathies in the live animal.

Study II was set out to investigate whether IN-DA treatment could rescue an age-dependent cognitive decline of aged rats in a spatial memory paradigm. In addition, it was tested whether tgDISC1 rats and negative controls responded differentially to IN-DA (for additional data see also Chapter 3.3).

In Study III it was tested whether in lymphocytes derived from SCZ patients and healthy control subjects, *DISC1* RNA or protein levels may serve as a biomarker for SCZ.
The lymphocyte collection included samples from smokers and non-smokers, due to the fact that SCZ patients have an extremely high rate of smoking (70-80 %) compared to other mental illnesses (30-50 %) or the general population (20-30 %) (Hughes et al., 1986; de Leon et al., 1995), allowing question to be addressed as to whether smoking or nicotine abuse could additionally affect DISC1 expression and/or protein levels.
In an animal study the influence of sub-chronic nicotine treatment on DISC1 insolubility in the brain was tested.

2 Synopses of the studies I, II, III

The complete published studies are included in Chapter 9.

2.1 Study I

Misassembly of full-length Disrupted-in-Schizophrenia 1 protein is linked to altered dopamine homeostasis and behavioral deficits.

SV Trossbach*, V Bader*, L Hecher, ME Pum, ST Masoud, I Prikulis, S Schäble, MA de Souza Silva, P Su, B Boulat, C Chwiesko, G Poschmann, K Stühler, KM Lohr, KA Stout, A Oskamp, SF Godsave, A Müller-Schiffmann, T Bilzer, H Steiner, PJ Peters, A Bauer, M Sauvage, AJ Ramsey, GW Miller, F Liu, P Seeman, NJ Brandon, JP Huston and C Korth

*These authors contributed equally to this work.

Molecular Psychiatry (Epub ahead of print)
doi:10.1038/mp.2015.194
accession number: 26754951
impact factor: 15.2

Author's contribution (50 %):

- generation of the transgenic rat
  cloning of the cosmid
  development of founder screen (PCR, qPCR, Southern Blot) for transgene detection
- managing the animal breed in the TVA:
  planning of breeding, screening of rat pups by PCR and/or qPCR

- generation of all animals used for this publication, preparation of brain samples for collaboration partners
- design of experimental setup
- planning, execution and analysis of all behavioral experiments
- preparation of rat brains for Western blots (homogenates and insoluble pellet) and analysis
- generation of DISC1-inducible cell lines: cloning, retroviral infection, and selection
- affinity-purification and analysis of antibody specificity
- co-immunoprecipitations including control experiments
- *post mortem* neurochemistry
- co-writing of the manuscript

The first study was set out to investigate the role of full-length human DISC1 in chronic mental illness in an animal model, by generation of the first transgenic rat of a candidate gene for mental illness, *DISC1*. By expressing full-length human DISC1 in the Sprague Dawley rat (tgDISC1 rat) the effect of DISC1 expression and its propensity to aggregate on the brain, and in particular the dopaminergic system, could be assessed.
I performed a thorough behavioral phenotyping of the tgDISC1 rat, revealing behavioral changes that could be linked to disturbances in the dopaminergic system. I found an intrinsic supersensitivity of the tgDISC1 rat to the DA agonist amphetamine, hyperexploratory behavior in two object exploration paradigms, and a rotarod deficit (further behavioral characterization is presented as additional data in Chapter 3.1). Interestingly, the amphetamine supersensitivity and hyperexploration were two phenotypes that were stable during aging, as I could verify them in 22 months old rats.

To investigate the biochemical origins of these phenotypes, experiments targeting the striatum and the dopaminergic system were conducted through interdisciplinary collaborations. In the dorsal striatum a strong increase in dopamine D2High receptors relative to D2Low receptors without a change in overall DA receptor density was measured. Furthermore an increased clearance rate of extracellular DA, probably caused by the increase in synaptic DAT levels, was detected by collaboration partners. In addition I found a reduction in total DA content by *post mortem* neurochemistry.

The tgDISC1 rats exhibited aggregated DISC1 species in the brain. Interestingly, I could show in biochemical purifications that the insoluble DISC1 species in the tgDISC1 were more prominent

in the dStr, a DA rich brain region, compared to the low DA containing frontal cortex, hinting towards a mechanistic link between DA and DISC1 assembly.
Further animal and cellular studies were conducted, revealing that the transient, cytosolic DA in the cell increased DISC1 assembly. In double-transgenic mouse lines experiencing elevated levels of cytosolic DA, the transgenic DAT-OE mouse overexpressing the DA transporter (Salahpour et al., 2008; Masoud et al., 2015) and the VMAT-DE mouse expressing only a residual amount of VMAT2 (Mooslehner et al., 2001; Caudle et al., 2007), more insoluble DISC1 could be detected in the insoluble pellet.
A challenge with DA *in vitro* increased DISC1 assembly in DISC1-inducible cells and induced the reversible formation of DISC1 aggresomes.

Following sensitization of rats with amphetamine, a process that leads to the depletion of DA in the striatum, the load of endogenous aggregated Disc1 in the dStr was significantly reduced. As shown by pull-down and co-immunoprecipitation experiments DISC1 could directly interact with DAT and aggregated insoluble DISC1 was capable of sequestering DAT into the insoluble fraction.

These findings argue for a bidirectional link between DISC1 assembly and DA homeostasis in which both cytosolic DA increases DISC1 assembly and insoluble DISC1 affects the dopaminergic system. The tgDISC1 rat is therefore a valuable model to investigate full-length, non-mutant DISC1-dependent changes in the dopaminergic system *in vivo*. DISC1 assembly as a function of DA exposure indicates a possible underlying biological mechanism of DISC1opathies, a potential subgroup of CMIs.

2.2 Study II

Intranasal dopamine treatment reinstates object-place memory in aged rats.

SV Trossbach, MA de Souza Silva, JP Huston, C Korth and C Mattern

Neurobiology of Learning and Memory 114 (2014) 231–235
doi: 10.1016/j.nlm.2014.07.006
accession number: 25062646
impact factor: 4.3

Author's contribution (90 %):

- design of experimental setup
- complete execution and data analysis of the study
- co-writing the manuscript

In the second study, the influence of intranasal application of DA on memory performance in aged rats was analyzed. Delivery through the nasal passage bypasses the blood brain barrier, a crucial obstacle in delivering substances to the brain.

Previous studies showed that in adult animals IN-DA treatment led to an increase in extracellular DA in dopaminergic structures, hence proving that the DA indeed reaches the brain (de Souza Silva et al., 2008). This intranasally administered DA was also shown to be behaviorally active as it mediated antidepressant-like action, increased locomotion in the rats, and weakened behavioral asymmetries in a hemiparkinsonian rat model (Buddenberg et al., 2008; Pum et al., 2009).

In older animals an age- and DA-related decline in cognitive performance is inevitable and intranasal application of DA might be able to rescue memory deficits in aged rats. Under control conditions, aged rats showed impaired spatial memory performance in the object place recognition paradigm. More precisely, the rats were not able to distinguish between a displaced and a stationary object. Upon acute pre-trial intranasal application of 0.3 mg/kg DA the rats' learning/memory performance was restored to levels comparable to that of adult animals.

This study was the first to show that IN-DA treatment led to increased memory performance in aged animals, giving rise to the possibility of a low-invasive IN-DA treatment as therapeutic option to target age-related cognitive deficits.

2.3 Study III

Peripheral DISC1 protein levels as a trait marker for schizophrenia and modulating effects of nicotine.

SV Trossbach, K Fehsel, U Henning, G Winterer, C Luckhaus, S Schäble, MA de Souza Silva and C Korth

Behavioural Brain Research 275 (2014) 176–182
doi: 10.1016/j.bbr.2014.08.064
accession number: 25218871
impact factor: 3.6

Author's contribution (80 %):

- design of experimental setup
- Western blot of lymphocyte samples
- lymphocyte preparation and comparative Western blot of Ficoll purified (human and rat) and sorted human lymphocytes
- RNA and cDNA preparation of lymphocyte samples
- qPCR design, validation and analysis
- data analysis and statistics
- preclearing experiment, including recombinant protein expression and purification
- insoluble pellet preparation and analysis of brains from nicotine-treated rats
- co-writing the manuscript

In the third study, the possibility of using lymphocytic levels of DISC1, a candidate protein for chronic mental illnesses, as a trait marker for SCZ was assessed. Up to now no biomarkers are available to assist the clinical diagnosis in psychiatry, mainly due to the multifactorial origins and heterogeneity of CMIs, even between patients with the same diagnosis.

By analyzing lymphocyte samples derived from blood of patients diagnosed with SCZ and matched control subjects I detected significantly lower DISC1 protein levels, but not mRNA levels, in SCZ patients, demonstrating that lymphocytic DISC1 might serve as a trait marker for SCZ.

Due to the fact that smoking is also a prominent comorbidity of SCZ patients, the influence of smoking behavior on DISC1 protein levels was assessed by comparing control smokers and control non-smokers. Nicotine modulated DISC1 levels in control smokers were lower than those of the non-smoking group. Therefore DISC1 may also serve as a state marker for nicotine or substance abuse.

Remarkably, by comparing SCZ smokers and control smokers, the SCZ patients again showed reduced DISC1 protein levels, emphasizing that smoking behavior was not overriding the trait level and narrowing the possible value of DISC1 protein as a SCZ trait marker. These findings implicate that lymphocytic DISC1 protein levels might serve as a potential biomarker for the identification of SCZ patients.

In a subgroup of mental illness patients aggregated DISC1 protein could be detected in *post mortem* brain samples (Leliveld et al., 2008). To investigate whether nicotine might also have an influence on DISC1 insolubility, wild type rats were treated sub-chronically with increasing doses of nicotine. Interestingly, decreased levels of endogenous aggregated Disc1 could be detected in the mPFC of nicotine-treated rats compared to the vehicle-treated animals.

This finding demonstrates that nicotine affects DISC1 assembly, rendering the nicotinic system a potential pharmacological target for DISC1opathies.

3 Further results and discussion

3.1 General behavioral phenotyping of the tgDISC1 rat

A behavioral characterization of the tgDISC1 rat was conducted in order to test the basic pattern of behavior. By targeting sensory-motor function, motivational processes and memory in tgDISC1 rats and controls, I found subtle but DA-related phenotypes of the tgDISC1 rat (Study I and Figure 4 / Figure 5, additional data descriptions in Chapter 5.1).

3.1.1 Assessment of body weight and food / water intake

To test for general differences in physiological parameters, body weight, food and water intake were measured in tgDISC1 rats and negative controls (Figure 3 ABC). No difference in body weight in a time course spanning 2 to 6 months of age could be detected between the genotypes. There was no difference in food intake, but by investigating water intake, a small but significant increase in drinking behavior could be detected in the tgDISC1 rats.

In order to rule out a diabetes phenotype in the tgDISC1 rats, a glucose tolerance test was performed (Figure 3 D). In both animal groups the injection of a glucose solution led to a strong increase in blood glucose levels that normalized to baseline over time. As no differences could be detected between the tgDISC1 and negative control rats, a diabetes phenotype could be ruled out.

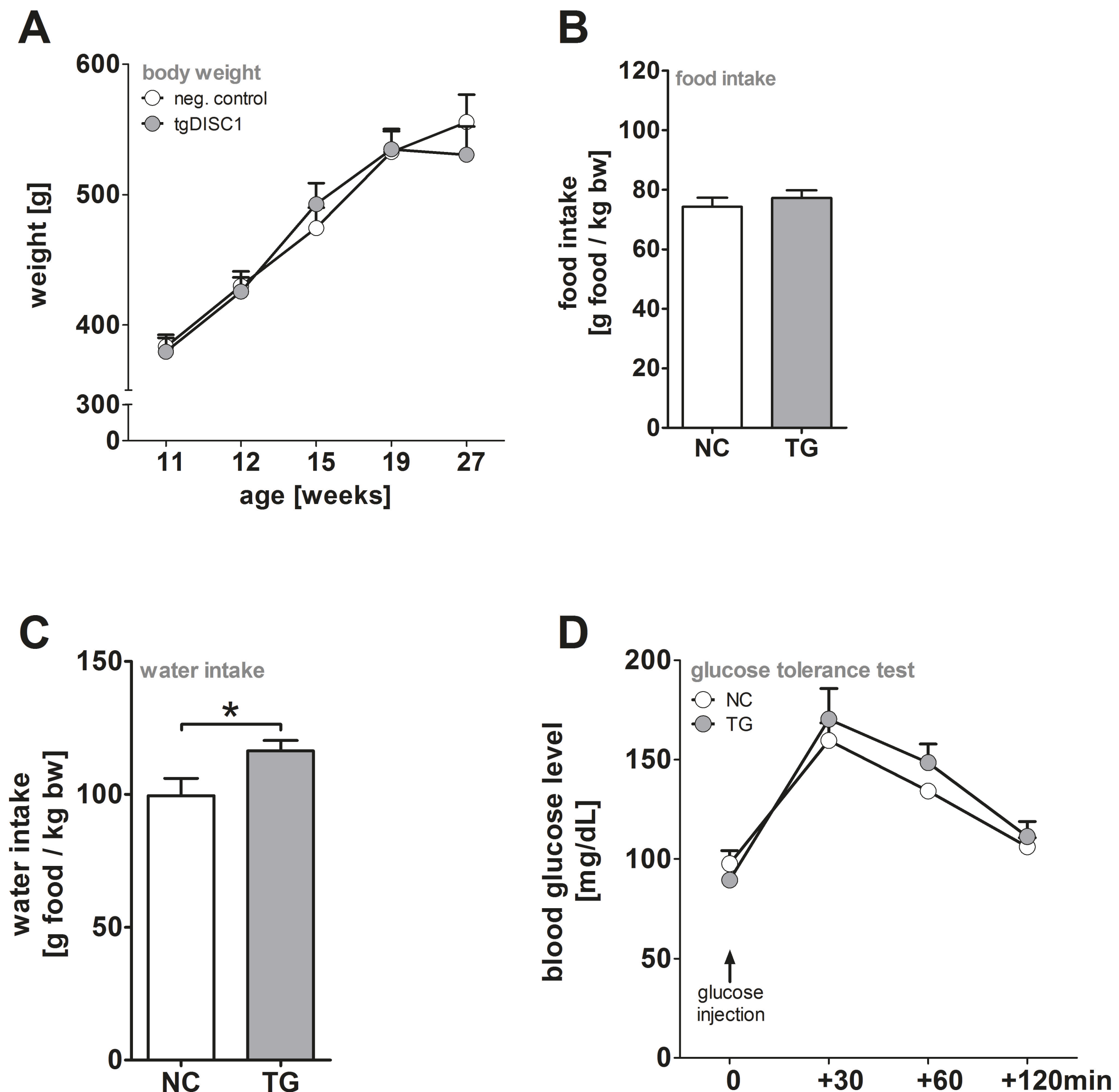

Figure 3: Body weight, food and water intake, and glucose tolerance test
(**A**) No difference in body weight. Over a time course of 2 to 6 months no difference in gaining weight could be detected in tgDISC1 rats and negative controls. (**B**) Comparable food intake. TgDISC1 and negative control rats consumed similar amounts of food pellets normalized to body weight. (**C**) Increased water intake of tgDISC1 rats. When water intake was analyzed the tgDISC1 rats drank slightly more than the control rats (unpaired t-test *p = 0.037). (**D**) No difference in glucose tolerance test. Both, tgDISC1 and negative control rats exhibit a comparable increase and decrease of blood glucose levels over a two hour test period. All means ± SEM.

3.1.2 Sensorimotor function

In experiments directed at determining the sensorimotor function of the tgDISC1 rat, I could not detect differences in spontaneous locomotion (Figure 1 A). For that, I examined general locomotor activity and habituation to an open field on two subsequent days. Normally animals reduce their locomotion over time, within the trial as well as between the two trials, as they adapt to their surroundings. The point that no differences could be detected is important, since alterations in locomotor behavior could have led to unwanted influences on other behavioral paradigms for example immobility time in the FST.

I also investigated sensimotor gating in the rat with the PPI paradigm. As described in Chapter 1.5.1, the PPI test measures the startle reaction of an animal after presentation of a pulse stimulus in relation to the response evoked to the pulse presented milliseconds after a weaker prepulse, whereby sensimotor gating is intact if the prepulse reduces the startle reaction after the pulse (Geyer et al., 2001). With the experimental setups applied (prepulse of 80 db, pulse of 130 db; for further information see Chapter 5.1), I did not detect a PPI deficit in the tgDISC1 rats compared to controls and no difference in the acoustic startle reaction to the different pulse types (Figure 4 BC), although it needs to be emphasized that using other conditions might potentially lead to genotype differences.

The tgDISC1 rats showed deficits in the rotarod task (data presented in Study I). The Rotarod task normally is a measure for motor ability and learning as the rat has to learn to walk on a rotating wheel without falling off. Although a basic neurological testing of the animals revealed no disabilities and all other locomotor data were also comparable to negative control animals, the tgDISC1 rats did not demonstrate a learning curve on the rotarod and failed to acquire the motoric skill.

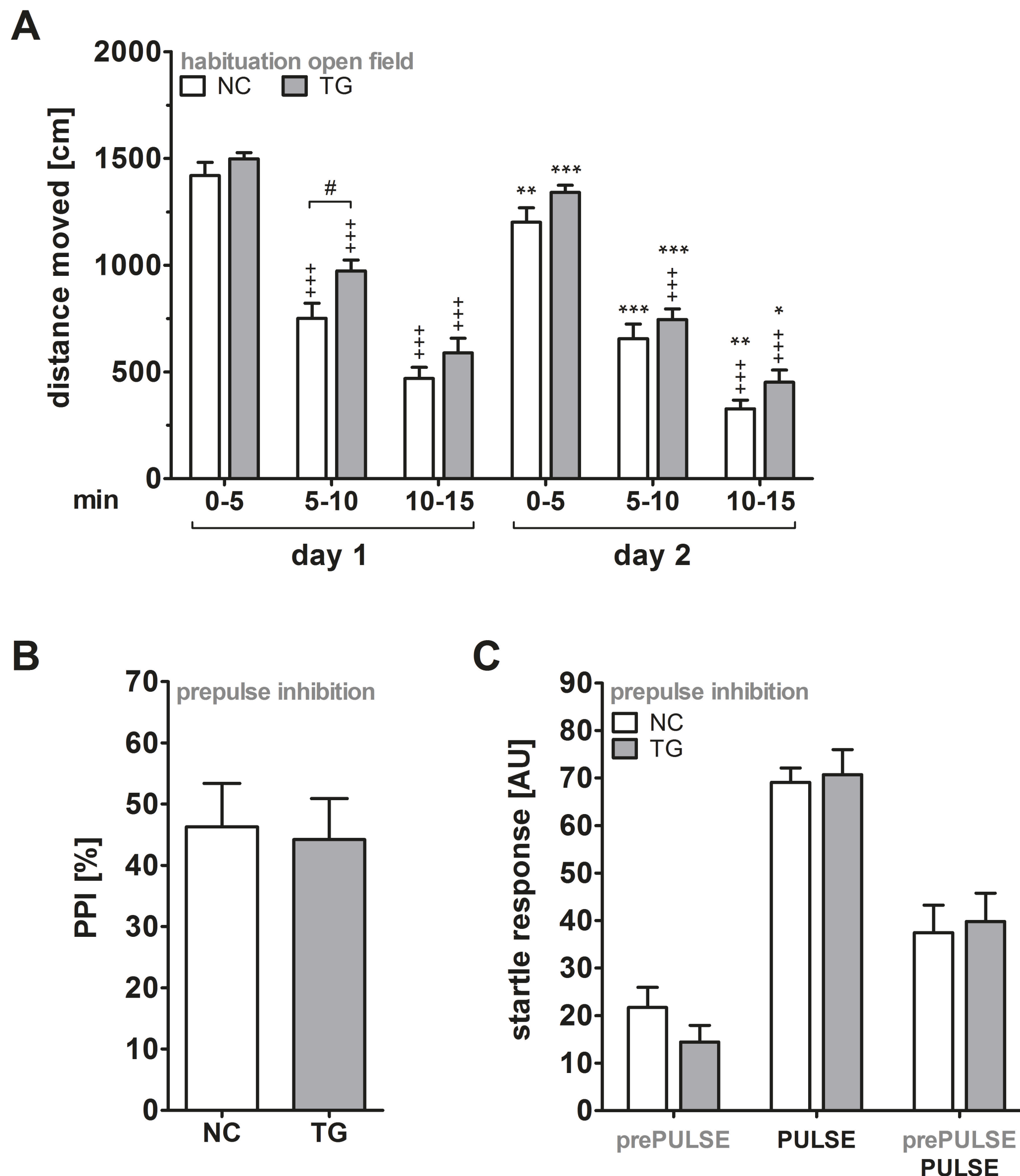

Figure 4: Sensory motor function in the tgDISC1 rat

(**A**) No difference in habituation to a novel environment. In the two-day open field habituation paradigm both, tgDISC1 and negative control rats, habituate to the arena within the trial as well as between two trials. In the 5-10 min time interval of day 1 tgDISC1 rats showed increased locomotion compared to negative control rats ($^{\#}p = 0.019$). (**B**) No PPI deficit in tgDISC1 rats. When confronted with an 80 db prepulse followed by a 130 db pulse, both groups displayed a PPI of startle response compared to the reaction to the pulse alone and no genotype differences could be detected. Negative controls had a PPI of 46.3 ± 7.1 %, tgDISC1 rats of 44.2 ± 6.7 %. (**C**) No differences in startle reactions. Negative controls and tgDISC1 rats showed a comparable startle reaction upon the three different kinds of auditory stimuli, with the startle reaction in answer to the pulse stimulus inducing the strongest reaction.

+ depicts within-trial habituation, * depicts between-trial habituation, # marks genotype differences. All means ± SEM.

3.1.3 Memory function

As included in Study I, spatial and object memory were assessed in three independent novelty preference tasks: the object recognition (OR) task, the object place recognition (OPR) task and the object recognition for temporal order (ORTO) task.

Object exploration paradigms are based on the fact that rats have a natural exploration behavior that favors exploration of a new stimulus over a familiar one, a so called novelty preference. In the object recognition paradigm an animal is expected to explore a newly presented object more extensively than a familiar one (Ennaceur and Delacour, 1988). Spatial object recognition is measured in the object place paradigm in which the rat has to distinguish a stationary object from a spatially displaced one (Ennaceur et al., 1997). In the third test the chronology-dependent memory of object presentation is challenged. The rat has to discriminate a previously presented object of the second trial from the one that was presented in the first trial (Mitchell and Laiacona, 1998).

In accordance with the DISC1 mouse models, the tgDISC1 rats had intact memory formation and no differences were detected between the genotypes (data presented in Study I). Interestingly, when analyzing the duration of object exploration, I found that the tgDISC1 rats spent significantly more time at the novel object then the controls in the OR and OPR task, exhibiting a hyperexploratory behavior.

3.1.4 Motivational processes

Experiments targeting motivational processes were also conducted. In order to mimic human emotions in rats three paradigms were tested: I targeted expression of depressive-like behavior with the FST (Porsolt et al., 1978), anhedonia with the sucrose preference test (Cryan and Holmes, 2005), and anxiety-like behavior with the light-dark preference paradigm (Costall et al., 1989).

In the FST, the rat is forced to stay in a cylinder filled with water that does not allow contact with the bottom during two trials. The rats are therefore forced to swim, float, or try to climb along the outer border of the tank. Increased immobility of the animal in the test trial, namely floating on the water surface, is interpreted as depressive-like behavior as the animal ceases to attempt to flee from the aversive situation. No differences in immobility, swimming, or climbing behavior in the tgDISC1 rat versus wild type could be detected, neither in the sample nor the test trial (Figure 5 ABC).

Sucrose preference was tested in two paradigms, either a 1 h or a 24 h trial, in which the rats could choose between a bottle of tap water or a 1 % sucrose solution. In both setups the animal preferred sucrose over water, again with no genotype differences (Figure 5 D).

As depression is often associated with symptoms of anxiety in humans (Mineka et al., 1998) I tested light-dark preference of rats. In an arena consisting of two adjacent compartments, one made of white and one of black plastic, rats naturally prefer to spend more time in the more comfortable and safe black compartment. As depicted in Figure 5 EF, tgDISC1 rats and negative controls preferred the black over the light compartment without genotype differences.

These experiments concerning motivational processes in the rat could therefore not reveal any differences between tgDISC1 and negative control rats.

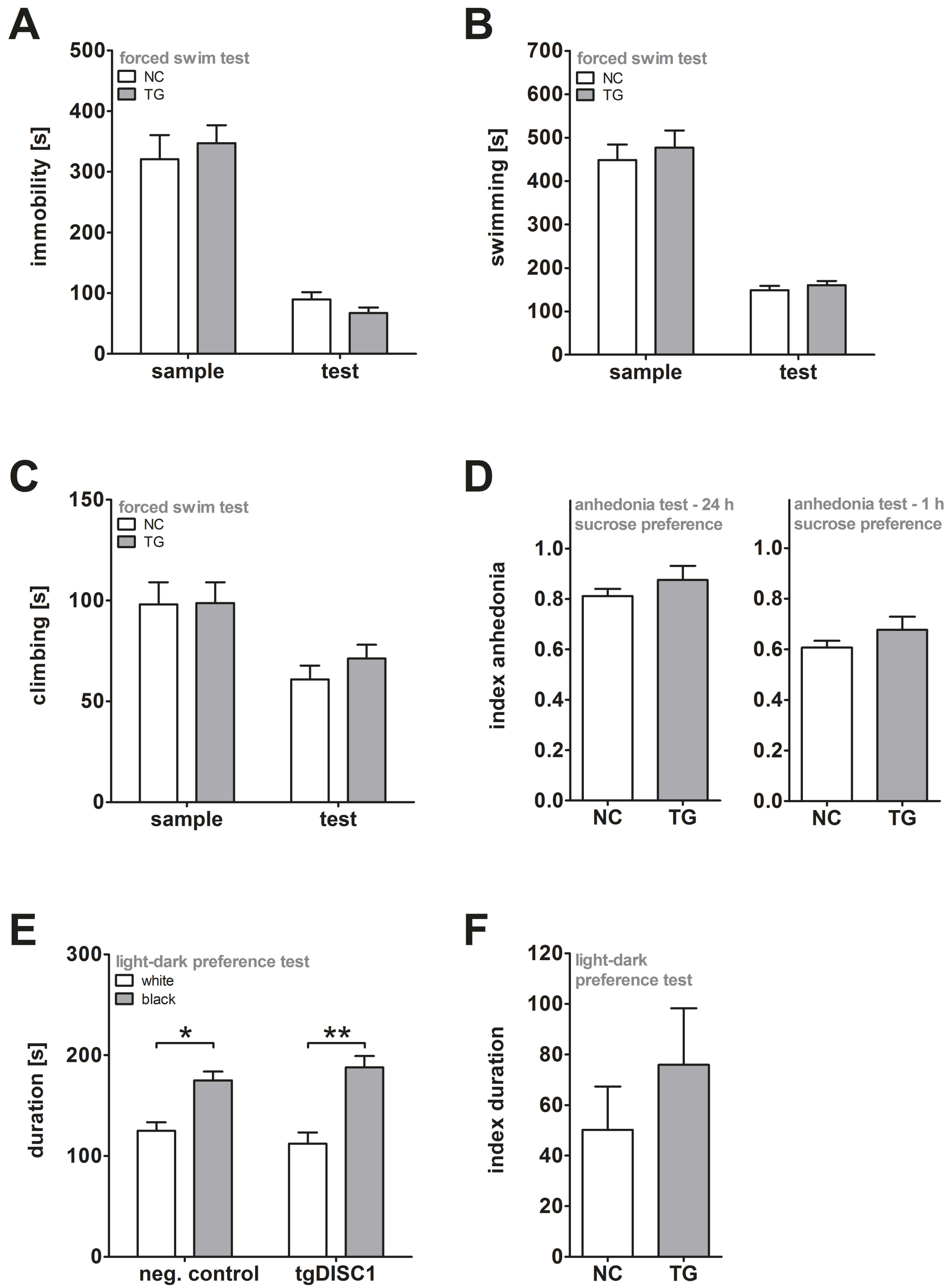

Figure 5: Motivation/emotion-related phenotypes in the tgDISC1 rat

Figure 5: Motivation/emotion-related phenotypes in the tgDISC1 rat
(**A**, **B**, **C**) No depression-like phenotype in the FST. Animals showed no difference in immobility, climbing, or swimming behavior, neither in the sample trial nor the test trial, giving no evidence for a depression-like phenotype in the tgDISC1 rat. (**D**) No anhedonia phenotype in two variants of the sucrose preference tests. Both animal groups preferred sucrose solution over water to the same extent as indicated by the anhedonia index, not supporting an anhedonia phenotype either in the 24 h (left panel) or 1 h trial (right panel). (**E**) No anxiety-like phenotype in the light-dark preference task. Both, transgenic and negative control animals spent more time in the dark compartment of the box, giving no indication of a difference in anxiety level of the animals. (**F**) Anxiety index of duration. Calculation of an anxiety index for time spent in the two compartments also did not reveal differences between the groups. All means ± SEM.

3.1.5 Implications of the behavioral phenotyping

The phenotypic results targeting sensorimotor function, motivation, and memory did not show an overall disturbance in behavior between the tgDISC1 rat and controls. Specifically, subtle changes in behaviors related to the dopaminergic system emerged, getting more pronounced, while the rats' overall behavior is not disturbed.

Nonetheless one has to consider that to a certain degree this could be due to the experimental setups chosen and that a change in those could alter the outcome of the experiment.

It is also striking in the previously published DISC1 mouse models that, even within one mouse model, the outcomes of the behavioral analyses, when modified and/or repeated, were often opposed (see Table 3; as an example (Shoji et al., 2012) versus (Clapcote et al., 2007)). This could be explained by the fact that different studies were performed with male and/or female mice, usage of different genetic donor mouse strains, mixed backgrounds of those or differing numbers of generations of backcrossing to a new genetic background. An additional and important point is that separate labs, with different housing conditions, experimental setups, and experimenter handling the animal, could have a huge influence on mouse behavior.

It needs to be kept in mind that a specific phenotype may be dependent on certain triggers that have yet to be identified, as mental illnesses such as SCZ have a high environmental basis in addition to the genetic predisposition (Gottesman and Erlenmeyer-Kimling, 2001). It is therefore hardly surprising that no overall behavioral disturbance was detected in the tgDISC1 rat. This is also in accordance with the heterogeneity of phenotypical alterations in the Scottish family and sporadic mental illness patients.

This means for the results presented here that a repetition of behavioral paradigms including setup changes (for example altered light intensity in the light-dark preference task, water temperature in the FST, sound volume of prepulse/pulse and inter-stimulus intervals in the PPI to name some possibilities) could push the rats over a certain threshold and change the outcome of the respective experiments. These proposed investigations could not be accomplished within the scope of my PhD thesis, but are in part currently being addressed.

3.2 Behavioral phenotypes of the tgDISC1 rat: alterations in the dopaminergic system

My behavioral findings show that the tgDISC1 rat exhibits subtle, but specific, behavioral phenotypes that are linked to disturbances in the dopaminergic system. Consistent with the amphetamine challenges performed in transgenic DISC1 mice, the tgDISC1 rat also exhibited an increase in horizontal (distance moved) and vertical (rearing) locomotion, when challenged with a sub-threshold dose of amphetamine, indicating an intrinsic sensitization that normally only occurs after chronic amphetamine sensitization.

In this rat, more detailed experiments targeting the DA system and the possible mechanism behind the observed amphetamine hypersensitivity were conducted by collaboration partners (Study I), resulting in the following model:

Under baseline conditions, the release of DA from the presynaptic terminals in the dorsal striatum is the same in tgDISC1 and negative control rats, but the tgDISC1 rats experience a much faster clearance of synaptic DA due to an upregulation of presynaptic DAT. This would probably lead to lower net synaptic DA in the tgDISC1 rat and is also in accordance with lower total striatal DA.

This is most probably compensated for by a near doubling of the portion of D2High receptors, but not in total D2R density, making the synapse more sensitive to synaptic DA. The D2High upregulation is in accordance with the L100P DISC1 mouse, as well as other non-DISC1 related transgenic animal models targeting the DA system, which all show minor changes in total D2R abundance, but drastic changes in D2High receptor content (Seeman et al., 2006; Lipina et al., 2010). Nevertheless, it needs to be validated whether the D2High upregulation merely counterbalances the decrease in synaptic DA or if the tgDISC1 rats exhibit a net increase in D2R activation due to overcompensation.

The connection of DISC1 with the dopaminergic system is further strengthened by a recent publication by Su et al. stating a direct interaction of DISC1 and D2Rs (Su et al., 2014). They observed increased complex formation between DISC1 and D2Rs dependent on D2R activation, which is altered in the striatum of SCZ cases, probably due to the point that SCZ patients exhibit a striatal hyperdopaminergia (Davis et al., 1991).

The increased DAT levels and proportions of D2High receptors give an explanation for the amphetamine supersensitivity observed in the tgDISC1 rat. In wild type animals, a low dose of amphetamine is normally not sufficient to induce a behavioral response, as it is not effective enough to trigger a profound increase in synaptic DA. The situation is different in the tgDISC1 rat: here, amphetamine can induce a strong increase in synaptic DA by reversing DA transport out of the presynapse by the more abundant DAT. Due to increased proportion of D2High

receptors at the postsynapse, DA neurotransmission is further reinforced. This boost in DA neurotransmission could therefore trigger locomotion even following only a low dose of amphetamine. This proposed model of striatal DA signaling at the synapse might explain why the tgDISC1 rat is *per se* amphetamine sensitized.

Both phenotypes, rotarod deficit and hyperexploratory behavior, can possibly also be explained by the detected dopaminergic alterations in the dStr. For that, the dopaminergic circuitry of the dStr needs to first be explained in more detail:

In addition to the aforementioned dopaminergic structures, the substantia nigra pars reticulata (= SNpr), globus pallidus (GP; in humans: GP internal = GPi and GP external = GPe, rodent equivalents: endopenduncular nucleus = EN and GPe), and subthalamic nucleus (STN) also play functional roles in the dopaminergic system (Albin et al., 1989), organizing movement and reward-related behaviors.

The dStr acts as a convergence point receiving glutamatergic input from the cortex and thalamus, as well as dopaminergic input from the dopaminergic midbrain structures. The dStr is mainly comprised of GABAergic inhibitory medium spiny neurons (MSNs, about 95 %) and a minor portion of cholinergic or GABAergic interneurons. The MSNs can be divided into two distinct neuronal populations, one expressing D1Rs and substance P and the other one D2Rs and enkephalin. The afferent projections from other brain areas converge at these striatal neurons, become integrated, and emerge as two major opposing output pathways: the direct and the indirect pathway (Figure 6).

The two distinct populations of MSNs, expressing D1Rs or D2Rs, give rise to the direct/striatonigral and the indirect/striatopallidal pathways respectively, exerting opposing control over motor function (Hikida et al., 2010; Kravitz et al., 2010). A simplified overview of the direct and indirect pathway gives the following circuitry:

Both, the D1R- and the D2R-MSNs become activated by glutamatergic input from the cortex (and thalamus), inhibiting their respective downstream targets.

In the direct pathway activated D1R-expressing MSNs inhibit GABAergic neurons in the SNpr. The phenomenon of inhibition of an inhibitory cell population is called disinhibition, leading to a reduced inhibition of subsequent structures. In this case it results in lowered inhibition of the glutamatergic thalamus that subsequently sends an increased excitatory stimulus back to the cortex.

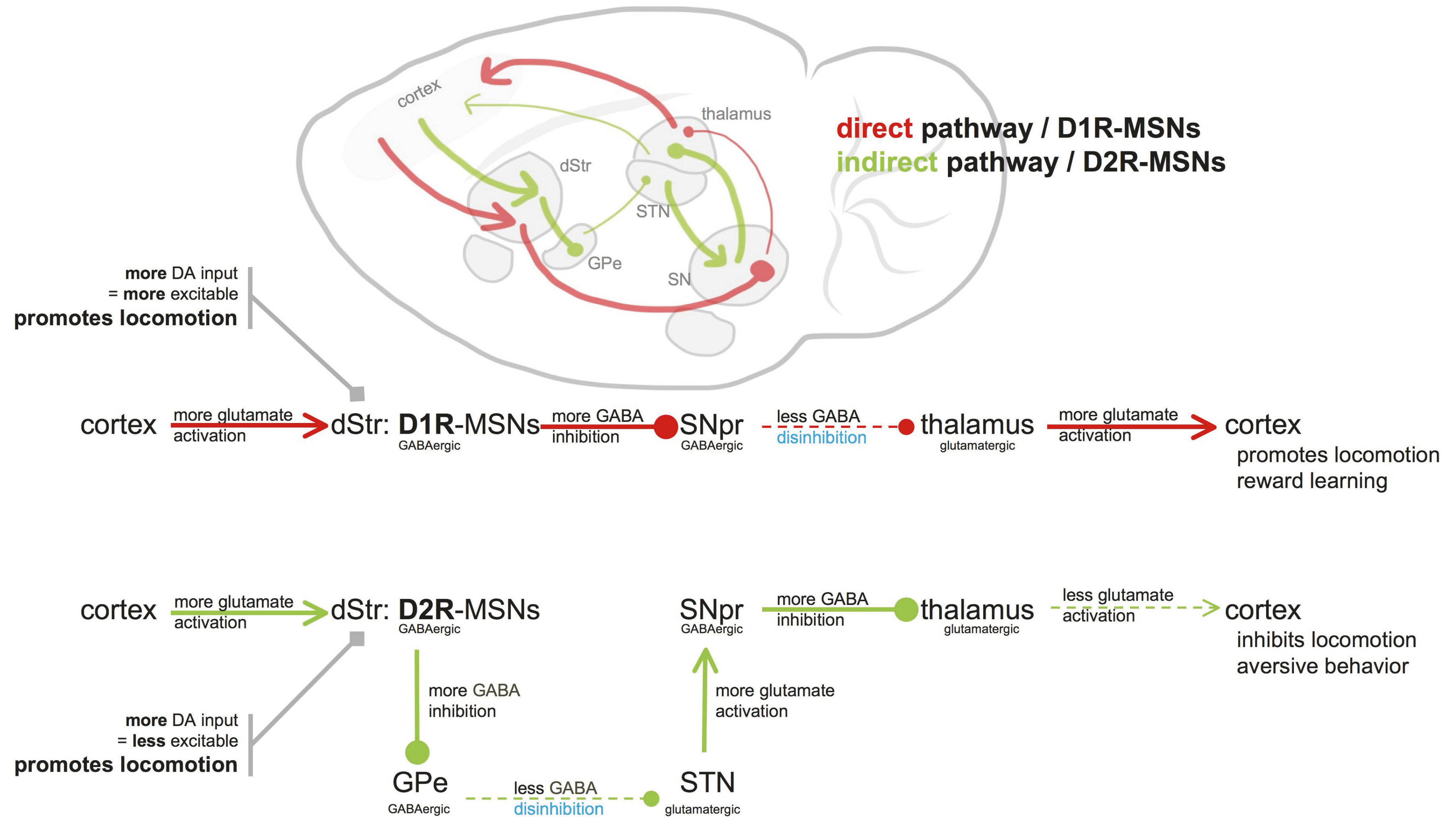

Figure 6: The direct and indirect pathway originating from the dorsal striatum
(**A**) Localization of the structures and projections in the brain. (**B**) Schematic overview of the direct and indirect pathway (for detailed information see text). Arrows represent activation, lines with circles inhibition. Continuous lines mark increased activation / inhibition, dashed lines reduced activation / inhibition.

In the indirect pathway, activated D2R-expressing MSNs inhibit GABAergic neurons in the GPe, leading to disinhibition of STN neurons. These glutamatergic neurons increasingly stimulate the SNpr, activating GABAergic SNpr neurons. This stands in contrast to the inhibition of SNpr in the direct pathway, highlighting the opposing actions of the two circuits on the SNpr. Activation of the SNpr leads to inhibition of the thalamus, which itself sends less glutamatergic signal to the cortex. To summarize, the signaling back to the cortex is increased in the direct pathway and decreased by indirect pathway activation. The activation of the direct pathway promotes movement, whereas the indirect pathway inhibits motor function (Kravitz et al., 2010).
This straight-forward model of corticostriatal circuitry does of course simplify the complex interplay of various cell-types, brain regions and neurotransmitter systems. Of late the general opinion has switched to a more interconnected model stating that both pathways are not exclusive, but are instead partially intertwined (Calabresi et al., 2014; Cazorla et al., 2014).

In addition to the description of the two types of MSNs exerting opposite action on movement control, the dStr is also composed of two anatomical regions which have differing effects on motor performance. It can be subdivided into the dorsolateral striatum (DLS) and the dorsomedial striatum (DMS). The DLS is mainly innervated by the sensorimotor cortex and is thought to be responsible for progressive skill automatization and habit learning, while the DMS is connected to the prefrontal cortex and associative cortex and orchestrates the initial phase of motor skill learning (McGeorge and Faull, 1989; Yin and Knowlton, 2006). Interestingly, this initial stage of motor skill learning is dependent on attention and therefore highly susceptible to interference, which leads to impairments of skill acquisition.
A thorough study conducted by Durieux et al. (Durieux et al., 2012) narrowed this effect down structurally to D2R-MSNs in the DMS. Utilizing cell-type and striatal region-specific ablations they found that D1R-MSNs in the DMS are responsible for increased locomotion and exploratory behavior, whereas the corresponding D2R-MSNs in the DMS decrease both, together with improving initial motor learning. In the DLS, the D1R-MSNs are responsible for general motor skill performance, the stage after initial motor learning.
In the dStr, the glutamatergic input described in the previous paragraphs is highly modulated by dopaminergic input from midbrain dopaminergic structures. As depicted in Figure 6, DA has opposing effects on cortical output by modulating MSN excitability. Increased DA binding renders the D1R-MSNs more excitable, whereas D2R-MSNs become less excitable (Surmeier et al., 2007; Simpson et al., 2010). This has an impact on both the direct and indirect pathways triggered by glutamatergic input from the cortex. More DA thereby strengthens the direct pathway, promoting locomotion and exploration. In contrast, more DA weakens the indirect pathway leading to reduced inhibition of motor behavior.

This means that increased dopaminergic neurotransmission in the dStr leads to an increase in locomotor behavior mediated by both pathways, in analogy with heightened activity in response to amphetamine.
In the case of the tgDISC1 rat, the gathered biochemical data of the striatal dopaminergic system hints towards a subtle amplification in neurotransmission by D2R-MSNs, weakening the indirect pathway. Through this, locomotion and exploration would be promoted, probably mediated by the D2R-MSNs in the DMS, which could then lead to the observed hyperexploration.
This increase in exploration could also give an explanation for the rotarod deficit. In the tgDISC1 rat, the increase in exploratory behavior would disrupt early motor learning as the exploration increases distraction. This course of action could give a plausible explanation for the observed deficit in acquiring the motoric skill on the rotarod.

It is possible to monitor neuronal activity of D1R- and D2R-MSNs by the expression of the neuropeptides substance P and enkephalin, respectively. However, for a change in enkephalin levels, the striatum needs to encounter massive changes in DA content. Nisenbaum and colleagues could detect an increase in enkephalin levels after a DA depletion of about 90 % (Nisenbaum et al., 1996). It is therefore not surprising that our collaboration partners could not detect changes in enkephalin levels in the dStr (Study I).
The subtle dopaminergic changes in the tgDISC1 rat model may be sufficient to induce moderate behavioral and biochemical changes, but is not pronounced enough to alter enkephalin levels. In future studies targeting the neuronal activity of MSNs, expression of immediate-early genes such as c-fos should be checked, which might be more suitable as a marker for activity in the striatum (Curran and Morgan, 1995).

An aspect of this proposed model that remains controversial is the fact that no hyperlocomotion or a PPI deficit was observed, behavioral changes that should parallel the increased activation of striatal D2Rs. Selective D2R agonists such as quinpirone disrupt PPI (Geyer et al., 1990; Peng et al., 1990). As this effect was only visible at doses higher than 0.3 mg/kg, it is possible that the presumably subtle increase in dopaminergic neurotransmission in the tgDISC1 rat was not sufficient to disrupt PPI.
Another idea to explain the lack of hyperlocomotion and PPI deficit in the tgDISC1 rat arises from the recent Su et al. publication (Su et al., 2014). They saw that upon disrupting the complex formation of DISC1 and D2R with an interfering peptide, no hyperlocomotion and PPI deficit was detectable in the L100P mouse anymore. It is not known whether DISC1 assembly changes its ability to bind D2Rs, but if insoluble DISC1 has a loss-of-function and loses its D2R interaction then this effect could explain the lack of increased locomotion and PPI deficit.

3.3 The effect of intranasal dopamine treatment on aged tgDISC1 rats

In addition to object memory experiments in adult animals (Study I), I retested spatial memory performance with the object place recognition task in aged negative control and tgDISC1 rats to assess whether there is a genotype difference in aging-dependent decline of cognitive performance. Additionally I wanted to address the question, whether IN-DA treatment might have a distinct influence specifically on the aged tgDISC1 rat, as the tgDISC1 rats have an altered dopaminergic system. The tgDISC1 rats might therefore react more profoundly to the IN-DA treatment as they appear to be intrinsically DA-sensitized.

The results presented here contain data of control animals already published in (Trossbach et al., 2014), as well as for tgDISC1 rats, including statistical re-analysis of data for genotype effects. The experiment showed that both aged tgDISC1 rats and negative controls were unable to master the memory task under control condition, an effect that could be reversed by IN-DA application in both genotypes (Figure 7, for detailed analysis of the data see Chapter 5.2). Upon IN-DA treatment, tgDISC1 and negative control animals showed increased exploration specifically of the displaced object, comparable to adult rats with intact memory formation (Figure 7 AB). Total object exploration was also analyzed to determine whether IN-DA treatment influences overall exploratory activity. IN-DA treatment reversed a decrease in exploration duration in negative control animals, whereas tgDISC1 rats under control conditions also did not show a change in exploratory behavior comparable to adult animals (Figure 7 C). No influence of IN-DA treatment on general locomotion could be detected (Figure 7 D), arguing for a specific increase in exploration and not overall activity.

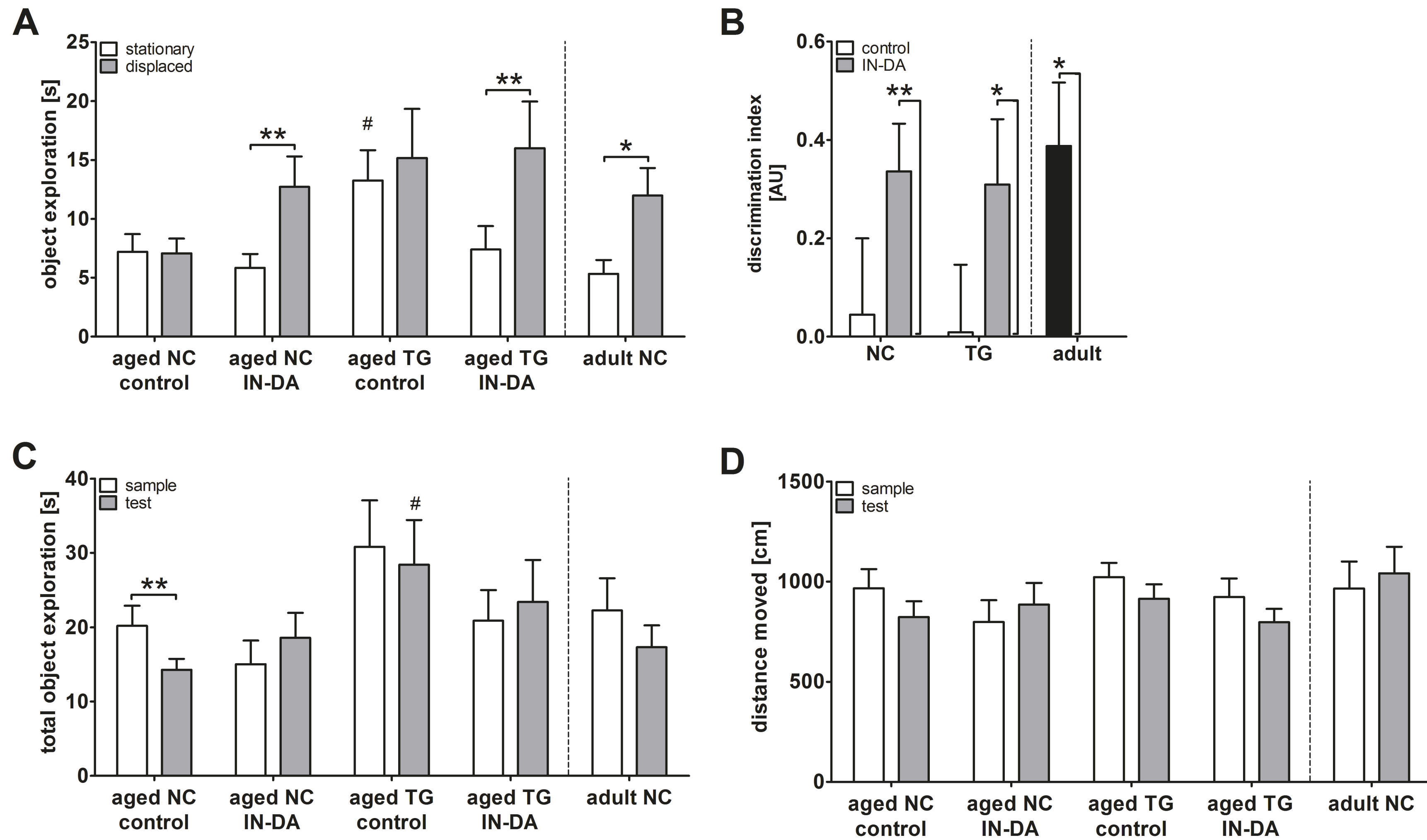

Figure 7: Reinstatement of object-place learning in aged tgDISC1 and negative control rats by intranasal dopamine application

Figure 7: Reinstatement of object place learning in aged tgDISC1 and negative control rats by intranasal dopamine application
Adapted from (Trossbach et al., 2014), here including tgDISC1 rat data (NC n = 11, TG n = 10). (**A**) Object exploration during test trial. Upon IN-DA treatment aged NC and aged TG rats explored the displaced object more than the stationary one, comparable to adult animals. Aged tgDISC1 rats explored the stationary object more than aged negative control rats. (**B**) Discrimination index. In contrast to adult animals, aged NC and TG rats performed at chance level, not exploring the displaced object any more than the stationary one. Both genotypes performed above chance level upon IN-DA administration. (**C**) Total object exploration. Only control rats showed a decrease in total object exploration in the test when compared to the sample trial. Under control conditions, aged TGs explored for longer in the test trial compared to aged NCs. (**D**) Locomotion. No difference in distance moved could be detected in the groups. # depicts differences between genotypes under same treatment condition. Means ± SEM

One important finding in the object place recognition data of the aged rats is that the hyperexploratory phenotype was validated as being stable during aging (part of Study I). Under control conditions, the tgDISC1 rats explored the stationary object more and the difference in total object exploration reached significance in the test trial. Interestingly, increased exploration of the stationary, as opposed to the displaced, object was observed, reflecting the aged animals' inability to properly distinguish objects.
After application of IN-DA on the other hand, the hyperexploration of objects is normalized to the level of NC rats. Arguing in line with the possible explanation for hyperexploration in the adult rats (see Chapter 3.2), the IN-DA treatment should lead to increased bioactive DA in the striatum, reinforcing exploration. A possible explanation could be that in the aged animal the IN-DA treatment was able rescue the memory deficit, but at the expense of hyperexploration, due to the age of the rats. Additionally, IN-DA treatment led in adult animals to an increase in locomotion (Buddenberg et al., 2008) that was not visible in aged animals, which also argues for a specific compensatory effect of IN-DA in terms of memory, but not locomotor activity.

3.4 Lymphocytic DISC1 as a possible trait marker for schizophrenia

Given the need in mental illness diagnostics for reliable biomarkers, the value of peripheral DISC1 levels as a trait marker for SCZ was tested. Study III showed that lymphocytic DISC1 protein levels were significantly decreased in SCZ patients and to a lesser extent reduced by smoking behavior.

It is worth mentioning that the changes in DISC1 protein levels were not observed reflected by changes in mRNA levels, hinting towards posttranslational mechanisms affecting protein half-life. This is not an uncommon observation, as other laboratories have also identified possible SCZ biomarkers that could only be detected as a change in protein, but not mRNA levels in lymphocytes, for example certain transcription factors which act as possible markers for first episode SCZ patients (Fuste et al., 2013).

Two recent studies analyzed DISC1 mRNA expression levels derived from whole blood preparations (Rampino et al., 2014) or lymphocytes (Kumarasinghe et al., 2013) of SCZ and control subjects. The results were seemingly contradictory, with the former reporting reduced DISC1 expression in SCZ and the latter detecting increased DISC1 levels in treatment-naïve schizophrenics. Kumarasinghe and colleagues also tested the effect of antipsychotic treatment on DISC1 expression and found DISC1 to remain upregulated, but otherwise unchanged, 6-8 weeks after drug treatment.

What can be taken from these studies concerning DISC1 as a possible biomarker is that the targeted biomolecule, its source, the experimental setup for detection, and the patient cohort are all of importance and may influence the outcome. In contrast to my experiments, both studies excluded, for example, patients with drug abuse, most likely including nicotine abuse, which could be problematic concerning the huge portion of smoking SCZ patients. In my experiments smoking behavior also has a significant effect on DISC1 protein levels.

As pointed out by Chan et al., a single biomarker may not be sufficient to reach an adequate level of sensitivity and/or specificity to facilitate diagnosis (Chan et al., 2014). Most likely a combination of several markers, each of a small effect size, has to be applied in order to gain significance and be converted into a clinical test. In combination with the idea of stratified medicine for the medication of treatment-relevant subgroups (Kapur et al., 2012), creating a biosignature for subsets of CMI patients by combining multiple specific biomarkers will be a challenge for the next few years.

3.5 TgDISC1 rat, DISC1 assembly, and DISC1opathies

Aggregated protein species are thought to be the result of disturbed proteostasis, caused by changes in cell homeostasis, folding machinery, the proteolytic, or the chaperone system.

One of the best characterized protein assemblies of this kind is the formation of extra- or intracellular amyloid structures. An "amyloid" is defined as a precise assembly of a protein with cross-β-sheet structure, an example being the fibril formation of Aβ in AD (Fowler et al., 2007), but other non-amlyloid assembly variants, as well as amorphous aggregates, are known to occur in nature (Kopito, 2000). The DISC1 aggregates detected in the tgDISC1 rat are not amyloids, as they could not be stained by amyloid-specific fluorescent dyes such as Thioflavin S (Study I).

Recently, a new view in the field of neurodegenerative diseases has emerged which considers the physiological functions of aggregates and amyloids (Fowler et al., 2007), resulting in loss- or gain-of-function (Greenwald and Riek, 2010). Irrespective of the exact nature of the DISC1 assembly the results also argue for a (patho-) physiological function of insoluble DISC1.

Insoluble DISC1 was seen to loose functional protein interactions and was able to co-aggregate previously soluble homo- and heterologous proteins (Leliveld et al., 2008; Ottis et al., 2011; Bader et al., 2012b). Rendering other disease-associated proteins such as dysbindin or CRMP1 insoluble may have downstream effects and be part of the pathology of CMIs. Dysbindin, for example, has been implicated in D2 receptor internalization (Iizuka et al., 2007), linking DISC1 aggregation with the dopaminergic system once again. DISC1 aggresomes could also co-recruit soluble DISC1 which led to disruption of cellular trafficking of mitochondria comparable to silencing of DISC1 in neurons (Atkin et al., 2011; Atkin et al., 2012).

Apart from these experiments, it is not clear at which stage of assembly DISC1 fulfills its physiological function. It is possible that the aggregates are a biologically active assembly state; nonetheless it is no less plausible that the aggregates are merely a biochemically non-toxic - but easily detectable - sink of misfolded proteins and that oligomeric states are of importance, as postulated for the neurodegenerative diseases (Benilova et al., 2012).

Biomedical investigations of DISC1 suggest a specific oligomerization state as the origin for distinct protein-protein interactions. An ordered assembly of DISC1 to octamers, but not higher multimers or aggregates, was needed to arrange binding to its major interaction partner NDEL1 *in vitro* (Leliveld et al., 2009; Narayanan et al., 2011).

The influence of the dopaminergic system on DISC1 insolubility and vice versa could yet be an underappreciated disease mechanism in CMI. Relating to the concept of DISC1opathies and the occurrence of aggregated DISC1 in brains of a subset of CMI patients, the influence of the cholinergic system on DISC1 insolubility was also investigated.

In Study III a small reduction in DISC1 aggregation in the mPFC of nicotine-injected wild type rats could be detected. In analogy with these findings, I injected tgDISC1 rats for 15 days with increasing doses of nicotine (see Chapter 5.3) to see whether the solubility status of the transgenic human DISC1 is also affected by nicotine administration. After dissection of the mPFC and preparation of the insoluble pellet, a strong trend for a nicotine-induced reduction of aggregated human DISC1 was detected (Figure 8).

As the nicotine treatment lasted only 15 days, it is possible that a longer, more chronic treatment could induce significant changes in DISC1 insolubility. It is also unclear whether the nicotine treatment reduced the levels of pre-existing DISC1 aggregates or impeded the aggregation of other, previously soluble, DISC1 molecules.

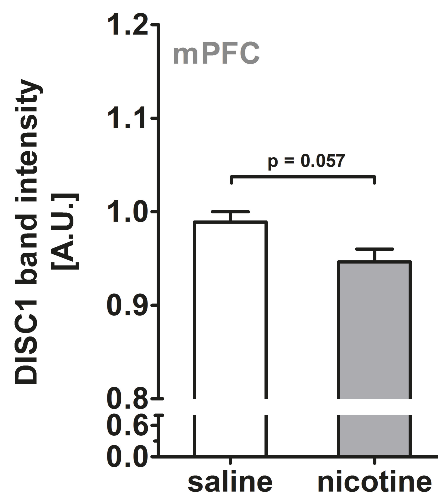

Figure 8: Reduced aggregated DISC1 in the mPFC of nicotine-treated tgDISC1 rats
Nicotine treatment of tgDISC1 rats induced a small but significant reduction in aggregation of the transgenic DISC1 in the mPFC. Means ± SEM

Due to the fact that DISC1 assembly appears to be quite dynamic, at least in terms of the effect of DA and nicotine, DISC1 aggregation does not give the impression of being a mere accumulation of misfolded protein over the progressive course of a mental illness, but argues for a broader value of (patho-) physiological DISC1 assembly, possibly into functional assemblies, for the cell's homeostasis.

4 Conclusion

The novel tgDISC1 rat is the first transgenic rat for one of the most investigated mental illness genes, *DISC1*. It was generated in order to investigate the role of full-length non-mutant human DISC1 in the brain *in vivo* in contrast to a variety of previous studies which focused on mutant DISC1. The results presented in this thesis consolidate the role of DISC1 as a key candidate involved in CMI.
DISC1-dependent CMIs have tentatively been termed DISC1opathies and comprise a subgroup of SCZ, BD, and DD patients that can be characterized by (mis-) assembly and insolubility of DISC1 (Korth, 2012). Given the biological heterogeneity of CMIs and the occurrence of aggregated DISC1 in only 20 % of CMI cases, DISC1opathy patients appear to represent one disease entity, crossing the current clinical diagnostic boundaries (Leliveld et al., 2008). Considering the lack of biological markers in mental illness, DISC1 assembly in the brain provides a first possibility to identify a bio- and pathologically relevant subpopulation of CMI patients.
As shown in this thesis, DISC1opathies can be modelled in the novel tgDISC1 rat modestly overexpressing full-length non-mutant human DISC1. The rat has an overlapping pathophysiology in terms of aggregated DISC1 in the brain and therefore serves as a valid tool to investigate the origin, physiological function, and modulation of DISC1 assembly in CMI and in brain function.

DISC1 modulated DA homeostasis through its assembly state. In the tgDISC1 rat the observed behavioral, neuropathological, and biochemical changes highlight a connection between DISC1, DISC1 assembly, and dopamine homeostasis. Through an increase of aggregation, which was in turn dependent on cytosolic DA, insoluble DISC1 could act as a sensor for DA load, which itself affects the solubility status of DAT. Transgenic human DISC1 caused changes in the striatal dopaminergic machinery, a switch of D2Low to D2High receptors, an increase in the presynaptic DA transporter, and, accordingly, an increased inflow of DA back into the presynapse. These

changes in DA homeostasis could in turn induce the behavioral phenotypes of amphetamine supersensitivity, hyperexploratory behavior and a rotarod deficit.
In addition, although DISC1 assembly is supposed to be highly intertwined with the dopaminergic system, nicotine and therefore the cholinergic system, also affected the solubility status of DISC1. This suggests that the influence of a second major neurotransmitter system, or possibly a combinatory effect of those, to orchestrate DISC1 assembly and downstream (patho-) physiological functions.

DISC1 protein pathology, as a common biological mechanism for multiple CMIs may therefore play a, to date unappreciated, role in the disease etiology of a subset of mental illness patients and thus could be the first step towards a biologically-derived classification of mental illnesses.

5 Additional data and method descriptions

5.1 Phenotyping of the tgDISC1 rat

Animals and behavioral analyses

All experiments were conducted in accordance with the Animal Protection Law and approved by local authorities (LANUV NRW). Male tgDISC1 rats (TG) and negative control rats (NC) were bred in the Animal Facility at the Heinrich Heine University Düsseldorf, Germany. Rats were housed 3 animals per cage under standard laboratory conditions (light from 7 p.m. to 7 a.m.) and free access to food and water. Behavioral testing started at an age of 3-4 months, for the PPI experiments 8 week old rats were used. For more detailed information please see Study I.

Measuring of body weight

To test whether the rats showed differences in body tgDISC1 and negative control rats (n = 12 each) were weighed on a regular basis between 8 a.m. and 10 a.m. A two-way ANOVA revealed no main effect for genotype ($F_{1, 10} = 0.803$, $p = 0.391$).

Measuring of food and water intake

Animal weight was determined before start of the experiment. Animals (n = 12) were single-caged and had free access to food and water. All animals received about 150 g food pellets (ssniff, Germany) and one drinking bottle filled with about 500 mL of tab water. Pellets and bottles were weighed before and after 24 h and were set in relation to the body weight of the respective animal.

Unpaired t-tests revealed that food intake was comparable between groups ($p = 0.473$), whereas tgDISC1 rats exhibited a small increase in water intake ($p = 0.037$).

Glucose Tolerance test

Before start of the glucose tolerance tests rats (NC, TG: n = 6) were deprived of food for 16 h on wood pellet bedding with free access to water.

Before the start of the experiments animals were weighed and the fasting blood sugar level was determined by taking a small blood sample from the tail. Subsequently rats were given an intraperitoneal injection of 1.5 g / kg body weight glucose in PBS (injection volume: 6 mL / kg body weight; both from Sigma-Aldrich, MO, USA). Blood glucose levels were remeasured 30, 60, and 120 min after the injection.

Blood sugar was measured with the ACCU-CHEK Aviva blood glucose meter and the corresponding test strips according to manufacturer's instructions (Roche, Germany).

Analysis by a two-way ANOVA did not reveal genotype differences ($F_{1, 10} = 0.483$, $p = 0.503$).

Sensory-motor function in the tgDISC1 rat

To examine general locomotor activity in tgDISC1 rats, habituation to an open field was examined. The test was carried out in an open-field (40 x 40 x 39 cm; TruScan, Coulbourn Instruments, PA, USA), located in a sound attenuated box on two consecutive days to test for within-trial and between-trial habituation. Locomotion was automatically calculated by the TruScan light beam system (Coulbourn Instruments, Allentown, USA). The rats were placed in an open field for 15 min for each trial, separated by 24 h, and the distance moved was analyzed.

By comparing the distance moved of tgDISC1 rats and negative controls no changes in habituation to the novel environment could be detected, neither within nor between trials.

Analysis of the distance moved in 5 min time windows per day was conducted with a 3-way ANOVA for genotype (NC and TG, n = 12), trial (day 1 and day 2) and time intervals (0-5 min, 5-10 min and 10-15 min). The ANOVA showed main effects for trial ($F_{1, 20} = 60.604$, $p = 0.000$) as well as within-trial time intervals ($F_{2, 40} = 333.780$, $p = 0.000$) and for genotype ($F_{1, 20} = 5.241$, $p = 0.033$). Subsequent two-way ANOVAs for between-trial analyses revealed the following differences for the 0-5 min time interval: trial ($F_{1, 20} = 30.987$, $p = 0.000$), but no genotype effect ($F_{1, 20} = 2.828$, $p = 0.108$); for the 5-10 min time interval: trial ($F_{1, 20} = 13.651$, $p = 0.001$) and genotype effect ($F_{1, 20} = 4.420$, $p = 0.048$); for the 10-15 min time interval: trial ($F_{1, 20} = 15.149$, $p = 0.001$), but no genotype effect ($F_{1, 20} = 3.034$, $p = 0.097$). As there were significances for trials in all time points measured, data was split for genotype and analysed for NC and TG separately. Paired t-test were conducted for each time pair, giving significant between-trial differences of the tgDISC1 rats for 0-5 min ($p = 0.000$), 5-10 min ($p = 0.009$) and 10-15 min time intervals ($p = 0.042$), whereas negative controls only show significant differences in the 0-5 min ($p = 0.007$) and 10-15 min time interval ($p = 0.006$), but not in the 5-10 min period ($p = 0.096$). These results showed that both, tgDISC1 rats and negative controls, habituated to the open field

as they showed less exploration on the second day compared to the first day in the arena for the respective time intervals.
In the second two-way ANOVAs the within-trial habituation of the distance moved was analysed. It revealed differences of time interval and genotype for day 1 (time point: $F_{2, 40}$ = 209.947.704, p = 0.000; genotype: $F_{1, 20}$ = 5.141, p = 0.000). At day 2 only the time points turned out significant ($F_{2, 40}$ = 206.623, p = 0.035), with a strong trend for a genotype effect ($F_{1, 20}$ = 4.062, p = 0.057). Subsequent 1-way ANOVAs for day one revealed a genotype effect for the 5-10 min time window on day 1 (p = 0.019). Paired t-tests for within-trial habituation (0-5 min versus 5-10 min and 5-10 min versus 10-15 min) for each genotype all came out highly significant (all p = 0.000), proving intact within-trial habituation of locomotion next to between-trial habituation.

TgDISC1 and negative control animals were also tested for PPI, a measure of sensorimotor gating.
PPI testing was carried out in the STARTFEAR system by Panlab Harvard Apparatus (Penlab, Spain). Acoustic presentation is controlled by the LE 111 Load Cell Coupler, LE 118-8 Startle & Fear interface and the corresponding STARTLE v1.2.04 software (all: Penlab, Spain). The system is based on the recording and subsequent analysis of the analog signal generated by the startle or movement of the animal in the chamber through a sensible weight transducer system. Acoustic tones are generated by the calibrated sound card of the computer and transmitted into the sound-proof experimental chamber by a loudspeaker. One day before the experiment, animals were presented with the restrainer box in their home cages and had 30 min to explore it. Rats used for this test were 8 weeks old due to the weight and size restriction of the restrainer. The experiment started with a 5 min exploratory period without any acoustic stimuli, followed by the period in which the animal is presented with 10 acoustic pulses of 130 db with a 30 s inter-trial-interval. This period serves to adjust the sensitivity of the load cell unit, so that a proper signal can be detected. For all animals a gain of 4/2000 and a threshold of 7/10 was used in order to be able to compare the startle responses. After this, animals were presented with 1 out of 40 randomized states that were separated by a 29 sec inter-trial-interval. One state lasted 10 ms followed by a 1 sec startle measurement. The 40 states consisted of 10x startling stimuli / pulses of 130 db, 10x weak stimuli / prepulses of 80 db, 10x no-stimulus as measure for basal activity of the animal, and 10x the combination of prepulse and pulse (prepulse-pulse). For that the prepulse of 80 db / 10 ms length is presented 100 ms before the actual pulse of 130 db / 10 ms. During the test period the experimenter has to analyze whether the signal is valid or invalid due to an increase in animal movement independent of the presented acoustic stimulus. These values have to be excluded from the calculation of mean startle reaction of the respective stimulus type. One mean value per stimulation type per animal is used for the calculation of the PPI. The PPI (in percent) is calculated as follows: 100-[(signal of prepulse minus signal of

prepulse-pulse)*100]. A PPI of 60 % represents a 60 % reduction of signal in prepulse-pulse state compared to pulse alone.
With the setup applied no differences in PPI of tgDISC1 rats and controls could be detected (TG, NC: n = 12; unpaired t-test p = 0.837). One-way ANOVA of the three different pulse types did also not reveal any genotype differences in startle responses towards the presented auditory stimuli (prepulse: p = 0.220; pulse: p = 0.795; prepulse-pulse: p = 0.779).

Motivational phenotypes of the tgDISC1 rat

The light-dark preference test and the FST are behavioral paradigms designed to mimic emotions in humans, namely anxiety and depression, respectively. In the light-dark preference test the preference of a rat to stay in the dark compared to the white compartment of a box is measured.

The FST analyzes depressive-like behavior of an animal. As the animal is put into a water tank that does not allow contact to the bottom, animals are forced to either float, swim, or climb. Immobility of the animal is interpreted as depressive-like behavior. Rats (NC, TG: n = 12) were tested on two subsequent days in an acrylic glass water tank (40 cm radius x 1 m) filled with 30 L of 30°C cold water. The filling height in relation to the rim of the tank was chosen so that the animal could neither escape the tank nor touch the bottom of the cylinder. The tank was located in a separate room and a camera was pointed at the side of the tank in order to monitor movement of the rats' hind legs. On day 1, a 15 min sample trial was conducted for each animal, followed by a 5 min test trial on the second day. The parameters immobility, climbing and swimming behavior were recorded manually. After each trial the water was exchanged.
Measure of immobility, swimming and climbing behavior in transgenic and negative control animals revealed no difference between the groups either in the sample trial on day 1 or on the test day, displaying no evidence for behavioral despair. Two-way ANOVA without main genotype effects in in sample and test trial: immobility $F_{1, 22} = 1.151$, $p = 0.295$; climbing: $F_{1, 22} = 1.416$, $p = 0.247$; swimming: $F_{1, 22} = 0.335$, p = 0. 569. Unpaired t-test of test trial: immobility p = 0.147, climbing p = 0.293, swimming p = 0.384.

Anhedonia, the inability to experience pleasure, can be assessed in the rat by changes in sucrose consumption. Two days before the trials, rats were habituated to drink from two bottles in their home cages, one containing tap water, the other a 1 % sucrose solution diluted in tap water (sucrose from Sigma-Aldrich, MO, USA). For the sucrose preference tests, animals were single-caged with free access to food. For the long-term 24 h sucrose preference test all animals (NC, TG: n = 12) received two equally filled bottles with water and a 1 % sucrose solution in randomized positions. Bottle weight was determined before start and after 24 h. The

experimented started at 16 p.m. at the end of the light phase. For the short-term 1 h experiment animals (NC = 13, TG = 16) were water-deprived for 4 h. Afterwards they were presented with the bottles filled with water and 1 % sucrose solution for 1 h. Bottle weight was again determined before and after the test. Sucrose preference, the anhedonia index, was calculated for both test variants on basis of total fluid intake: [g] sucrose / ([g] sucrose + [g] water).
In the 24 h trial, all rats preferred a sucrose solution over tap water with no differences between the genotypes as indicated by the anhedonia index (unpaired t-test $p = 0.304$). Also in the 1 h trial no differences in sucrose preference could be detected between negative control and tgDISC1 rats (unpaired t-test $p = 0.222$).

The light-dark preference test was conducted in a separate light-and sound-attenuated room. Behavior was recorded using an Eyseo Ecoline Standard TV7002 camera (ABUS, Germany) and analyzed with the EthoVision software (EthoVision 3.1; Noldus, Netherlands). After each trial the arena was cleaned with 70 % Ethanol.
Light-dark preference of tgDISC1 rats and negative controls was investigated using a box consisting of two adjacent compartments (1 x 38 x 32 cm, l x b x h), one compartment with dark and the other one with white acrylic walls. For the trial, animals (NC: n = 11, TG n = 12) were placed in the white chamber and were allowed to explore the arena for 5 min. Time spend in each compartment was recorded by the EthoVision software. An anxiety index was calculated: duration in black compartment [s] minus duration in white compartment [s]. To examine whether the tgDISC1 rats display an anxiety phenotype, the animals were tested for location preference in the white or black compartment.
A two-way ANOVA comparing duration in the two compartments showed that both genotypes preferred the light over the dark compartment to the same extent (compartment $F_{1, 21} = 19.429$, $p = 0.000$, genotype $F_{1, 21} = 0.811$, $p = 0.378$; data were split for genotype and paired t-test were used to compare behaviour in the two compartments: NC $p = 0.015$, TG $p = 0.006$). No differences between the groups were detected. Also calculation of an anxiety index did not indicate a genotype effect (unpaired t-test $p = 0.378$).

5.2 The effect of intranasal dopamine application on aged tgDISC1 rats

Reinstatement of object-place learning in aged tgDISC1 by IN-DA

For the comparison of object exploration in the test phase a three-way ANOVA for genotype, object, and treatment was conducted. Main effects were found for genotype ($F_{1, 19}$ = 5.273, p = 0.033) and object ($F_{1, 19}$ = 9.941, p = 0.005), but no treatment effect. Subsequent two-way ANOVAs (genotype/treatment) showed again a genotype ($F_{1, 19}$ = 4.515, p = 0.047), but no treatment effect for the stationary object and no effects for the displaced one. The one-way ANOVA pinpoints the genotype differences to exploration of the stationary object under control condition (p = 0.051), but not after IN-DA treatment (p = 0.498). A second two-way ANOVA (genotype/object) has main effects for genotype ($F_{1, 19}$ = 5.710, p = 0.027) in control condition and object ($F_{1, 19}$ = 20.262, p = 0.000), but not genotype, in IN-DA comparisons. A paired t-test comparing object exploration in the IN-DA condition, separately for NC and TG, revealed differential exploration of objects (NC: p = 0.010; TG: p = 0.008). These results showed that both, aged tgDISC1 and negative control rats, explored the objects to the same extend under control condition. After IN-DA treatment both genotypes started exploring the displaced object more, proving a rescue of place memory in aged rats by IN-DA administration. The only genotype difference was detectable in exploration of the stationary object under control condition.

The discrimination index was calculated: [time spent exploring the object at novel location – time spent exploring the object at old location]/[total time spent exploring the two objects]. An index at 0.0 (chance level) describes equal exploration of both objects, whereas an animal that prefers the displaced, more interesting, object above the stationary object has an index >0.0 (Ennaceur et al., 1997). T-tests against chance level at 0.0 shows significant differences for both IN-DA treated groups (NC: p = 0.006; TG: p = 0.045), but not for the control treated animals (NC: p = 0.778; TG: p = 0.949), strengthening the findings from Figure 7 A that IN-DA treated animals of both genotypes prefer exploring the displaced over the stationary one.

Overall exploratory activity

For the comparison of total object exploration in sample versus test phase a three-way ANOVA for genotype, trial, and treatment was conducted, showing a main effects for genotype ($F_{1, 19}$ = 4.982, p = 0.038). A two-way ANOVA for genotype/trial revealed a main effect for genotype under control treatment ($F_{1, 19}$ = 6.276, p = 0.022), but not IN-DA application. A second two-way ANOVA for genotype/treatment compared the two trials, finding a genotype effect for the test phase ($F_{1, 19}$ = 5.273, p = 0.033). A subsequent one-way ANOVA tracked the genotype difference to the test trial under control condition (p = 0.027). The results indicate that only aged negative control animals reduced exploration duration in the test trial under control condition and that

control-treated tgDISC1 rats explored more during the test trial than the respective NC animals. As the tgDISC1 animals did not exhibit a decrease in overall duration of exploration from sample to test trial in contrast to the negative controls, the IN-DA treatment could therefore not rescue that behavior.

General locomotor behavior

The three-way ANOVA for locomotion in sample versus test trial did not reveal significant differences for genotype, treatment or trial, showing that IN-DA treatment did not merely increase an unspecific locomotor response.

5.3 Influence of nicotine on DISC1 insolubility

For testing the effect of nicotine on the aggregation of endogenous DISC1, male tgDISC1 rats were treated for subsequent 15 days with either vehicle (saline, 1 mL/kg bw injection volume; n = 4) or nicotine solution (n = 4). For the injections (−)-Nicotine hydrogen tartrate salt (Sigma-Aldrich, Germany) was dissolved in PBS in terms of its nicotine content. Animals were weighted daily before the injections. Transgenic animals were treated with either saline or 0.5 mg/kg, followed by 1 mg/kg and 2 mg/kg bw nicotine for 5 days each.

Brain dissection was carried out 24 h after the last injection. The prefrontal cortices were flash-frozen and underwent a biochemical insoluble pellet preparation assay as previously described (Ottis et al., 2013) and DISC1 in the pellet was detected by the mAB 14F2 by Western blot. Densitometric analysis was performed using the ImageJ 10.2 software (National Institute of Health, MD, USA). DISC1 band intensity of the insoluble fraction was normalized to transgene expression in homogenates. The resulting ratio was compared for saline- versus nicotine-treated animals for all brain regions.

Statistical analysis revealed a strong trend for reduced insoluble DISC1 in nicotine-treated tgDISC1 rats (Mann Whitney U-test: $p = 0.057$).

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9 Appendices: studies I, II, III

9.1 Study I

Misassembly of full-length Disrupted-in-Schizophrenia 1 protein is linked to altered dopamine homeostasis and behavioral deficits.

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ORIGINAL ARTICLE

Misassembly of full-length Disrupted-in-Schizophrenia 1 protein is linked to altered dopamine homeostasis and behavioral deficits

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Disrupted-in-schizophrenia 1 (*DISC1*) is a mental illness gene first identified in a Scottish pedigree. So far, DISC1-dependent phenotypes in animal models have been confined to expressing mutant DISC1. Here we investigated how pathology of full-length DISC1 protein could be a major mechanism in sporadic mental illness. We demonstrate that a novel transgenic rat model, modestly overexpressing the full-length *DISC1* transgene, showed phenotypes consistent with a significant role of DISC1 misassembly in mental illness. The tgDISC1 rat displayed mainly perinuclear DISC1 aggregates in neurons. Furthermore, the tgDISC1 rat showed a robust signature of behavioral phenotypes that includes amphetamine supersensitivity, hyperexploratory behavior and rotarod deficits, all pointing to changes in dopamine (DA) neurotransmission. To understand the etiology of the behavioral deficits, we undertook a series of molecular studies in the dorsal striatum of tgDISC1 rats. We observed an 80% increase in high-affinity DA D2 receptors, an increased translocation of the dopamine transporter to the plasma membrane and a corresponding increase in DA inflow as observed by cyclic voltammetry. A reciprocal relationship between DISC1 protein assembly and DA homeostasis was corroborated by *in vitro* studies. Elevated cytosolic dopamine caused an increase in DISC1 multimerization, insolubility and complexing with the dopamine transporter, suggesting a physiological mechanism linking DISC1 assembly and dopamine homeostasis. DISC1 protein pathology and its interaction with dopamine homeostasis is a novel cellular mechanism that is relevant for behavioral control and may have a role in mental illness.

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INTRODUCTION

Disrupted-in-schizophrenia 1 (*DISC1*) is a gene involved in vulnerability to behavioral disorders. It was discovered in a large Scottish pedigree with a chromosomal translocation leading to a 3′ truncation of the *DISC1* gene and, putatively, a C-terminal truncation of the resulting protein.[1] In this family, the translocation is associated with several major clinical diagnoses such as schizophrenia, recurrent major depression and bipolar disorder.[1–3] Subsequent genetic association studies in multiple populations of different ethnicities support the involvement of DISC1 in mental illnesses (reviewed in refs. 4,5). For example, the coding polymorphisms S704C (rs821616) and L607F (rs6675281) in *DISC1* were associated with mental illness and also showed increased DISC1 protein aggregation *in vitro*.[6,7] In parallel, various transgenic or genetically altered mouse models have been developed, either targeting the mouse *Disc1* locus or introducing mutant human DISC1 variants. Missense mutations,[8] deletion variants[9,10] or partial knockout of the endogenous mouse *Disc1* locus[11] were generated. In addition, the dominant-negative truncated form of human DISC1, which is thought to correspond to the truncated *DISC1* gene in the Scottish family, was also induced[12] or constitutively[13] expressed in mouse models. Together these studies have provided evidence of DISC1 being involved in neurodevelopment and behavioral control.[14]

Thus far, DISC1 mouse models have been used to investigate the role of genetically altered or silenced DISC1 in behavioral control rather than the full-length form present in all sporadic cases of chronic mental illness that may, at least in part, underlie the etiology of the disorder.

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The fact that *DISC1* has not yet been identified among the major GWAS hits has raised controversies[15,16] even though it merely indicates that *DISC1* is not targeted by common risk variants. It has been pointed out that the study of rare gene variants may provide valuable insights into disease mechanism. One such example is Alzheimer's disease (AD) where common mutations in the major disease genes *APP* and the presenilins also do not appear in GWAS screens[17] even though APP processing is a critical step in AD pathogenesis.

Genetic association, however, is only one way to address the connection between a disease and its biological cause. Investigations of the protein itself can also validate its role in non-familial forms of a brain disease. For example, protein pathology is a major biological cause for most chronic brain diseases such as AD, frontotemporal dementias or Parkinson's disease in which a dysfunctional proteostatic system leads to the accumulation of disease-specific protein aggregates.[18] Remarkably, in these diseases the same proteins accumulate in sporadic forms as well as familial genetic forms where these proteins are mutated.[18] Furthermore, accumulation of proteins is a controlled process in the cell that is even used to generate functional aggregates in physiological circuitry.[19]

In this study, we asked whether non-mutant, full-length DISC1 could have a role in sporadic chronic mental illness including schizophrenia and recurrent affective disorders. Specifically, we investigated whether protein pathology or misassembly of DISC1 could have a role in causing mental illness. Our initial investigations using biochemical techniques identified insoluble DISC1 in a subset of mental illness patients,[20] leading to both gain and loss of function interactions.[20,21]

Although both cellular and animal studies linked DISC1 to various neurotransmitter systems,[22] including the dopaminergic system,[23–28] the actual role of DISC1 in altering dopamine signaling was not elucidated to molecular detail. Of note, in the rodent and human brains, formation of a functional complex between DISC1 and postsynaptic dopamine 2 receptors (D2R) has been demonstrated.[29]

Here to mimic DISC1 protein misassembly, non-mutant full-length human DISC1 was modestly overexpressed as a transgene in Sprague Dawley rats (tgDISC1 rats). Extensive neurochemical, biochemical and behavioral analyses demonstrate a signature of behavioral phenotypes including amphetamine supersensitivity, hyperexploratory behavior and rotarod deficits. These phenotypes are attributable to: (1) a switch of low affinity to high affinity dopamine D2 receptors and (2) increased clearance of extracellular dopamine due to translocation of the dopamine transporter (DAT) in the dorsal striatum (dStr) of tgDISC1 rats. A reciprocal relationship between DISC1 aggregation and dopamine homeostasis suggests that DISC1 may act as a sensor of cytosolic oxidative stress. Regulation of DISC1 assembly through environmental insults may therefore impact dopamine homeostasis.

MATERIALS AND METHODS

Generation of the DISC1 transgenic rat

Transgenic Sprague Dawley rats were generated by injecting the linearized fragment of the CosSHa.tet vector[30] containing the full-length, non-mutant human DISC1 as transgene with the polymorphisms F607 and C704 into pronuclei of Sprague Dawley rats (contracted to the IBZ University of Heidelberg, Germany). Integration of the transgene in the genome of the resulting litters was confirmed in independent founder animals by PCR and Southern blotting of genomic DNA extracted from tail biopsies. Subsequent breeding with wild type Sprague Dawley rats excluded transgenic founders that did not carry the transgene in their germline. By quantitative RT-PCR of genomic DNA and western blotting of brain homogenate the transgene load as well as expression level was compared between the four resulting stable founder lines.

Transgene detection by PCR and qPCR

Genomic DNA was prepared by digesting tail biopsies in 100 mM Tris pH 8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg ml^{-1} Proteinase K, precipitation with isopropanol and solubilization in pure water.

PCR for the detection of the transgene was performed on the genomic DNA by utilizing the HotStarTaq (Qiagen, Hilden, Germany) and primers binding the transgene promoter region: forward 5′-CTGATCTCCAGAAGC CCAAA-3′, reverse 5′-CAGGCCTATTCCTTGACAGC-3′. For the quantitative real-time PCR the same primers for the transgene were used. For normalization primers targeting the genomic sequence of the housekeeping gene rat beta-actin were designed: forward 5′-GCAACGCGCAGCCACTGTCG-3′, reverse 5′-CCACGCTCCACCCCTCTAC-3′. Real-Time PCR was conducted with the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). PCR conditions: 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 60 °C for 1 min. The data were processed with the corresponding StepOne Software v2.3 (Thermo Fisher Scientific, Waltham, MA, USA) and DISC1 expression was normalized to expression level of beta-actin.

Animals and behavioral analyses

All experiments were conducted in conformity with the Animal Protection Law approved by local authorities (LANUV NRW, Recklinghausen, Germany). Male tgDISC1 rats (transgenic rat; TG), negative controls (NC) and Wistar rats were bred in the Animal Facility at the University of Düsseldorf, Germany. NCs were bred from constantly renewed transgene-negative offspring. Male homozygous tgDISC1 rats of founder line 3, but also line 1, non-transgenic littermates and NCs were used for the experiments presented. Experiments were mainly performed in founder line 3, but main results were validated in founder line 1. Rats were housed three animals per cage under standard laboratory conditions, with light on from 0700 to 1900 hours with food and water provided *ad libitum*. Behavioral testing started at an age of 3–4 months. Animals were tested in a randomized manner, alternating tgDISC1 rats and NCs, without blinding.

If not stated otherwise, then the behavioral experiments were conducted in a separate sound-attenuated room and experiments were performed under dim light. Behavior was recorded using an Eyseo Ecoline Standard TV7002 camera (ABUS, Wetter, Germany) and analyzed with the EthoVision software (EthoVision 3.1; Noldus, Wageningen, The Netherlands). After each trial in each experiment arena and objects were cleaned with 70% ethanol. An $n = 12$ was used for all behavioral analyses if not otherwise stated.

A routine pathology survey using conventional methods, for example, hematoxilin–eosin and cresyl violet staining, did not show any aberrant changes in organs of the central nervous system (brain, myelon), peripheral nervous system (nervus ischiadicus), endocrinum (pituitary gland, thyroid glands and adrenal glands), as well as in peripheral organs (lung, liver, kidney, spleen, pancreas, gut, heart muscle and lymph nodes) upon a routine exam (data not shown).

Novelty preference tasks. The tasks were carried out in an open-field arena (60 × 60 × 30 cm, l × w × h) with cues for orientation. Object exploration was recorded manually, the criterion for exploration being active examination of the presented objects. Two 1.5-l plastic water bottles, one filled with clear water, the other one with purple-colored solution, were used as objects. The day before the novelty preference tests animals were allowed to habituate to the arena for 10 min without presentation of objects. Tests were separated by one week to minimize memory interference between the tasks. Animals that did not explore an object in either sample or test trial were excluded from the analysis (object recognition (OR): NC $n = 2$ animals excluded; object place recognition (OPR): NC $n = 4$ animals excluded).

Object recognition. Animals were allowed to explore the arena with two identical objects for a 5-min sample trial, followed by a second 5-min test trial separated by a 25-min intertrial interval. In the test phase one object was replaced by a new one, keeping the original positions. OR is defined as increased exploration of the novel in contrast to the old object.

Object place recognition. This test consists of a 5-min sample trial with two similar objects, a 25-min intertrial interval and a 5-min test trial. In the test trial animals are presented with one object at the same position as in the sample trial (stationary object) and one displaced object that has been moved to a new location. OPR is defined as increased exploration of the displaced compared with the stationary object.

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Rotarod. For the testing of motor learning, animals were trained to walk on the rotating cylinder of a rotarod apparatus (Accelerating Rota-Rod, Jones & Roberts, for rats 7750, Ugo Basile, Gemonio, Italy). Three trials with a constant pace of 1 r.p.m. separated by a 50-min intertrial interval were performed. In the first trial each animal had 120 s to learn the task and was supported by the experimenter to keep balance until it learned to walk on the wheel for 3 s on its own. In the following trials animals were left alone once they kept balance. Animals that failed to learn to walk on the wheel in the following trials were excluded from the analysis (NC $n=2$, TG $n=1$). The trial was ended when the animal fell off the rotarod. The latency to fall in seconds was measured manually.

Low-dose amphetamine challenge. Testing was carried out in the TruScan open-fields (Coulbourn Instruments, Whitehall, PA, USA), located in sound- and light-isolated chambers (110 × 70 × 70 cm) on two consecutive days for 15 min each. On the first day the rats were tested following an injection of saline (1 ml kg^{-1}; intraperitonial (i.p.)), on the second day their behavior was recorded following the administration of d-amphetamine (0.5 mg kg^{-1}; i.p.; in saline with an injection volume of 1 ml kg^{-1}; Sigma-Aldrich, St. Louis, MO, USA). Locomotion was automatically measured by the TruScan light beam system.

Generation of mice with increased DAT and decreased VMAT2 expression

DAT overexpressing (DAT-OE) mice were generated using BAC transgenesis as previously described.[31,32] In addition, mice with decreased VMAT2 expression (VMAT2-DE) were generated by gene targeting as outlined elsewhere.[33,34] DAT-OE and VMAT2-DE mice were inter-crossed to produce double-transgenic mice (DAT-OE:VMAT2-DE) that simultaneously display high DAT expression and low VMAT2 expression (Masoud and Ramsey, unpublished). DAT-OE:VMAT2-DE mice were used to model accumulation of cytosolic dopamine due to increased dopamine uptake coupled with reduced vesicular storage. All mice have a C57BL/6 background.

Synaptic plasma membrane preparation and DAT levels

Synaptic plasma membrane fractions of the striatum were prepared as previously described.[35,36] Striata were removed from the fresh rat brains, frozen in 2-methylbutane on dry ice and stored at −80 °C until used.

Measurement of D2High

The method for measuring the dopamine D2High receptors in rat striata *in vitro* was performed as reported earlier.[37,38] Striata were removed from the fresh rat brains and stored at −80 °C until usage. The dopamine receptors in the rat striatal tissue were measured with [^{3}H]domperidone (2 nM final concentration; custom synthesized as [phenyl-^{3}H (*N*)]domperidone; 41.4 Ci per mmol; made by Moravek Biochemicals and Radiochemicals, Brea, CA, USA).

Dopamine D2High receptors were best defined by the number of D2 receptors occupied by 100 nM dopamine, as compared with 1 nM dopamine (where dopamine does not occupy any significant amount of D2 receptors; see ref. 38). Therefore, D2High was measured by the amount of [^{3}H]domperidone bound at 1 nM dopamine minus the amount bound at 100 nM dopamine (Y). The specific binding, S, of [^{3}H]domperidone at 2 nM was measured by the amount of [^{3}H]domperidone bound at 1 nM dopamine minus the amount bound in the presence of 10 µM *S*-(-) sulpiride. The percent of D2 receptors in the high-affinity state was defined by (Y/S) × 100%.

Cyclic voltammetry

Rats were sacrificed by rapid decapitation. The head was immediately submerged into oxygenated ice-cold sucrose buffer. Following removal of the brain, coronal slices (350 µm) from NC and TG animals were sectioned with a vibratome and incubated at 32 °C in artificial cerebrospinal fluid (aCSF). DA release was electrically stimulated (1 pulse, 350 µA) using a double-pronged stainless steel stimulating electrode placed into the region of interest. DA release was recorded using a carbon fiber microelectrode, placed in the slice to form an equilateral triangle with the stimulating electrode. A cyclic voltage ramp (−0.4 V to 1.0 V to −0.4 V) was applied to the carbon fiber microelectrode and the resultant background-subtracted current was measured. Application of waveform, stimulus and current monitoring was controlled by TarHeel CV (University of North Carolina), using a custom potentiostat (UEI, UNC Electronics Shop). A 5-stimulation recording survey of four different dorsal striatal and nucleus accumbens sites were taken for each animal with a 5-min rest interval between each synaptic stimulation. Following the experiment, the carbon fiber microelectrode was calibrated using a flow-cell injection system and known dopamine standards. Kinetic constants were extracted using nonlinear regression analysis of release and uptake of dopamine from the extracellular space.

Receptor autoradiography

Animals were decapitated and their brains rapidly removed and immediately frozen in 2-methylbutane (−50 °C) and stored until sectioning. Coronal sections (20 µm) were produced in a cryostat microtome (Leica, Nussloch, Germany), thaw-mounted onto silica-coated object slides and stored at −80 °C until further use.

Dopamine D2 receptors were labeled accordingly with [^{3}H]raclopride (0.56 nM) in 50 mM Tris-HCl (pH 7.4; 45 min at 22 °C) containing 0.1% ascorbic acid and NaCl (150 mM), using the displacer butaclamol (1 µM).

Slices exposed to phosphor-imaging plates for 3 days together with tritium standards. Autoradiograms from phosphor-imaging plates were scanned with a high-performance plate reader and subsequently processed in a blinded manner using image-analysis software (AIDA 4.13; all Raytest, Straubenhardt, Germany).

Neurochemical analysis of *post-mortem* brain tissue

After dissection brain tissues were homogenized with an ultrasonic device in 0.05 M perchloric acid (Janssen, Geel, Belgium) containing deoxyepinephrinehydrochlorid (Sigma-Aldrich) as the internal standard. Dopamine content was electrochemically detected as previously described[39] and analyzed with the Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA).

Rat and mouse insoluble proteome preparation

Transgenic and control mouse brain medial prefrontal cortex (mPFC) and hemispheres of heterozygous TG and NC rats were isolated and weighed. Each tissue piece was homogenized in 2.5% buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 250 mM sucrose, 5 mM GSH, 5 mM $MgCl_2$, 1% NP-40, 0.2% sarcosyl, 2× protease inhibitor, 1 mM PMSF), supplemented with 40 U ml^{-1} DNaseI and incubated for 30 min at 37 °C followed by 16 h at 4 °C.

Next, samples were spun at 1800 × *g* for 30 min at 4 °C. The pellet was resuspended in buffer B (50 mM HEPES pH 7.5, 1.5 M NaCl, 250 mM sucrose, 5 mM EDTA, 5 mM GSH, 1% NP-40, 0.2% sarcosyl, 2× protease inhibitor, 1 mM PMSF) and centrifuged at 1800 × *g* for 30 min at 4 °C. The resulting pellet was washed in buffer C (50 mM Tris pH 8, 250 mM sucrose, 5 mM GSH, 1% NP-40) and spun at 1800 × *g* for 30 min at 4 °C. The pellet was resuspended in buffer D (50 mM HEPES pH 7.5, 5 mM GSH, 1% NP-40, 2× protease inhibitor). Finally, the pellet was resuspended in buffer D´ (50 mM HEPES pH 7.5, 0.2% sarcosyl, 2× protease inhibitor) and spun at 100 000 x *g* for 45 min at 4 °C in an ultracentrifuge (TLA-55 rotor in Optima; Beckman Coulter, Krefeld, Germany). The resulting insoluble pellets were solubilized in 2× SDS-loading buffer and used for western blots. Thus, we define insoluble pellet as what is pelleting after centrifugation in cold ionic detergent.

The preparation of the insoluble proteome of mPFC and dStr of transgenic rats was performed as previously described.[20] Transgenic DISC1 was detected with the huDISC1 specific mAb 14F2 and fluorescent secondary anti-mouse antibody (IRDye 800CW Goat anti-Mouse IgG, LI-COR Biosciences, Lincoln, NE, USA).

Structural MRI analysis and calculation of ventricle size

Structural MRI imaging was performed on a 7.0 tesla small animal Scanner (Bruker BioSpin, Billerica, MA, USA) with a horizontal bore magnet. The system included a 20-cm Gradient and a 29-cm shimm system. For RF transmission a transmit only quadrature coil (inner diameter 86 mm) (Bruker BioSpin) was used. RF reception was carried out with a rat brain 20-mm surface loop coil (Bruker BioSpin). Forty-eight coronal slices (0.156 × 0.156 × 0.5 mm) with a matrix of 256 × 256, FOV 4 cm × 4 cm, were recorded using a RARE sequence: TE 14.370 ms, TR 7080 864 ms, 4 averages, RARE factor of 4, scan duration of 15 min 6 s. During image recording animals were kept under 2% isoflurane. Respiration and body temperature were monitored and controlled throughout the entire

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experiment. For the analysis the Anatomist/ BrainVisa program version 4.3.0 (NeuroSpin, Gif-sur-Yvette, France) was used.

Immunohistochemistry of rat brains

For the immunostaining of transgenic rats animals were perfused with phosphate-buffered saline (PBS) pH 7.4 and sagittal cryo sections were cut on a Cryostat (Leica CM1900; Leica, Germany) and dried for 20 min at RT. The sections were post-fixed with ice-cold 4% paraformaldehyde solution buffered with PBS pH 7.4 (PFA, Sigma-Aldrich, MO, USA). After blocking with antibody diluent (Dako, Hamburg, Germany) sections were incubated with the mAb 3D4 for 16 h at 4 °C in antibody diluent. After two PBS washes the secondary antibody (anti-mouse IgG AlexaFluor594 1:300; Invitrogen) was applied for 1 h at RT. Sections were washed in PBS and in PBS plus 0.05% Tween-20 for 10 min. Subsequently sections were washed shortly in distilled water, 70% ethanol and incubated 2× 5 min in Sudan Black (Division Chroma, Münster, Germany) in 70% ethanol to block autofluorescence. After washing the sections in 70% ethanol and H_2O, they were mounted with ProLong Gold containing DAPI (Invitrogen). All images were collected with a Zeiss Axiovision Apotome.2 confocal microscope (Zeiss, Oberkochen, Germany).

Dopamine-induced DISC1 insolubility in cell culture

Human neuroblastoma SH-SY5Y cells were obtained mycoplasma-free from the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and tested at irregular time intervals for mycoplasma contamination. SH-SY5Y cells expressing inducible full-length human DISC1 were generated using the Retro-X Tet-On Advanced Inducible Expression System (Clontech Laboratories, Mountain View, CA, USA). SH-SY5Y cells were seeded onto glass coverslips, induced for 24 h, treated with 100 μM DA for 24 h and fixed with 4% PFA in PBS. Cells were permeabilized with PBS (plus 0.5% saponine, 5% milk powder, 1% BSA) and incubated with the mAb 14F2 and/or a vimentin antibody (1:100; Cell Signaling, Danvers, MA, USA) and subsequent AlexaFluor594 secondary antibody (Invitrogen) for 1 h in PBS plus 0.5% saponine and 1% BSA was used. Cells were mounted with ProLong Gold with DAPI (Invitrogen). Images were collected with a Zeiss Axiovision Apotome.2 confocal microscope (Zeiss).

Purification of the insoluble proteome of human neuroblastoma (NLF) cells transfected with DISC1 and primary cortical neurons of the tgDISC1 rats were performed as described previously.[6] Primary neurons at DIV 14 were incubated with 50 μM DA for 24 h to induce DISC1 aggregation and then underwent the insoluble proteome purification.

Cryoimmunogold electron microscopy

Cryoimmunogold electron microscopy was performed as described previously.[40]

Co-immunoprecipitation of DISC1 by DAT in striatal membrane preparations and cell lysates

For the co-immunoprecipitation (co-IP) of DAT and DISC1 striata of adult tgDISC1 rats were dissected and the plasma membrane fraction was purified by a sucrose gradient. The striatal tissue was homogenized in buffer A (250 mM sucrose, 50 mM HEPES pH 7.4, 15 mM EDTA pH 8, 3 mM DTT, protease inhibitor). After centrifugation at 500 × *g* for 5 min at 4 °C 1 ml of the supernatant was mixed with 1.7 ml of buffer D (2 M sucrose, 50 mM HEPES pH 7.4, 15 mM EDTA pH 8, 3 mM DTT, protease inhibitor) to gain a final sucrose concentration of 1.34 M (now solution C). The sample was layered on top of 1 ml of buffer D, followed by 1 ml of buffer B (850 mM sucrose, 50 mM HEPES pH 7.4, 15 mM EDTA pH 8, 3 mM DTT, protease inhibitor) and buffer A in 5 ml ultracentrifugation tubes (Beckman Coulter, Krefeld, Germany). The samples were centrifuged for 16 h at 100 000 × *g* at 4 °C (MLS-50 rotor in Optima; Beckman Coulter, Germany). Afterwards the interphase between layer B and C containing the membrane fraction was collected, diluted with 3 volumes of PBS and frozen at − 80 °C for 2 h for precipitation. After thawing and pelleting by centrifugation (14 000 × *g* at 4 °C for 30 min), the membrane fractions were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, supplemented with 0.5 mM DTT and protease inhibitor).

For the co-IP the solubilized membrane fractions (input: one rat striatal hemisphere per IP reaction) were equally divided. One sample did not receive an antibody for capturing, the other one was mixed with 3 μg of a polyclonal antibody raised against DAT (AB2231, Merck Millipore, Darmstadt, Germany) and incubated for 2 h at 4 °C, before addition of 20 μl of Protein A/G magnetic beads (Pierce, Rockford, IL, USA) and further incubation for 16 h at 4 °C. The beads were washed 4× with 1 ml of 50 mM Tris pH 7.6, 150 mM NaCl, 0.05% NP-40 and precipitated proteins were eluted by addition of loading buffer containing 2% β-mercaptoethanol for 10 min without boiling. Subsequently, the samples were used for western blot and membranes were incubated with the 14F2 (detecting huDISC1) and MAB369 antibody (detecting DAT; Merck Millipore).

Primary antibodies

For western blot: actin (rabbit, 1:10 000; Sigma-Aldrich); DAT (rat MAB369, 1:1 000; Merck Millipore); huDISC1 (mouse 14F2, 1:1 000; Korth lab[21]); ratDISC1 (rabbit hu-precleared C-term,1:1 000; Korth Lab[41]); Na/K-ATPase (rabbit, 1:1500; Cell Signaling; the antibody detects the α1 subunit. On the basis of sequence homology, the antibody could also cross-react with α2 and α3 isoforms); Tubulin (mouse, 1:10 000, Sigma-Aldrich). For immunostaining: human DISC1 for ICC (mouse 14F2, 1:250; Korth lab); human DISC1 for IHC (mouse 3D4, 10 μg ml^{-1}; Korth lab[20]); Vimentin for ICC (rabbit D21H3, 1:100; Cell Signaling).

Western blot

For western Blots the Novex NuPAGE SDS-PAGE Gel System (all: Thermo Fisher Scientific) with the corresponding NuPAGE Novex 4–12% Bis-Tris Midi Protein Gels, NuPAGE MES SDS Running buffer and NuPAGE LDS Sample Buffer (4 ×, plus 8% β-mercaptoethanol) or Laemmli loading buffer (4 ×, 200 mM Tris pH 6.8, 40% glycerol, 10% SDS, 0.4% bromphenolblue, 8% beta-mercaptoethanol) was used. For molecular size estimation the PageRuler Prestained Protein Ladder (#26617; Thermo Scientific, MA, USA) was used. As the dyes used for prestaining of the marker proteins influence their electrophoretic mobility, the apparent marker size was calibrated to the PageRuler Unstained Protein Ladder (#26614; Thermo Scientific) according to manufacturer's instructions in the gel system used. Accordingly, all depicted marker sizes have subsequently been adapted to the calibrated sizes.

Densitometric analyses

Band intensities were calculated from luminescent sensitive film (Amersham Hyperfilm ECL; GE Healthcare, Buckinghamshire, UK) using the ImageJ 10.2 software (National Institute of Health, Bethesda, MD, USA). Alternatively fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA; 1:15 000 in PBS plus 0.05% Tween-20) were used and intensities were analyzed on a LI-COR Odyssey CLX and the corresponding Image Studio Version 2.1 software (LI-COR Biosciences).

Statistics

All statistical analyses were performed as indicated using the IBM SPSS Statistic program (Versions 20–22; IBM, Ehningen, Germany) or GraphPad Prism (Versions 4 and 5; GraphPad Software Inc., San Diego, CA, USA).

All data sets were tested for normal distribution based on the expected experimental results and appropriate parametric or non-parametric tests were chosen. An estimate of variation was made for selected analyses. Comparison of two groups with similar variances was done by Student's *t*-test or Wilcoxon non-parametric test. Analyses were two-sided, if not stated otherwise.

Sample size was chosen according to Fisher's exact test and the expected difference between experimental conditions. Animal behavior was analyzed by two-way repeated measures analysis of variances (ANOVAs) with the variables genotype and treatment, object, or trial, thus considering correcting for multiple testing. Significant effects of the independent variables were explored further by splitting the data appropriately and conduction of lower level ANOVAs and *post hoc* comparisons including respective corrections for multiple testing. Paired sample *t*-tests were applied when appropriate.

Appropriate statistical tests and *P*-values are stated in the respective figure legends and/or results section. *P*-values of $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$ were used as significance levels.

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RESULTS

Neuropathology of tgDISC1 rats

Previously, we reported the presence of insoluble DISC1 in biochemically purified fractions from *post mortem* cases of the Stanley Medical Research Institute Consortium Collection (SMRI CC)[42] diagnosed with mental illness.[20,21] To model DISC1 aggregation *in vivo*, we generated a transgenic rat modestly overexpressing non-mutant, full-length human DISC1 under control of the Syrian hamster prion protein (PrP) promoter (tgDISC1 rat). These rats showed about 11-fold higher transgenic human DISC1 expression compared with endogenous rat Disc1 protein levels (Supplementary Figure S1a and c; protein expression rates were measured at P58). We chose to include common variants C704 and F607 as they are frequent in the normal population[43] and have been demonstrated to increase the risk to mental illness[4] and, in biophysical studies, DISC1 misassembly.[6,7] The PrP promoter was utilized for two reasons: first, it provides pan-cellular expression in the brain that has previously been demonstrated for DISC1[44] and, second, it leads to sufficient expression levels for inducing misassembly as successfully demonstrated in non-prion animal models of protein conformational disease.[45,46] The PrP promoter is broadly active at embryonic day E13.5 in the mouse brain[47] and continues to be expressed into adulthood, while the *Disc1* gene has two expression peaks at E13.5 and around postnatal day P35.[48]

Four different founder lines were generated (Figure 1a), and the founder line with the lowest expression level was selected (Founder line #3; Figure 1a and Supplementary Figure S2) in order to avoid artifacts due to strong overexpression. The level of overexpression was estimated to be 11-fold higher than endogenous Disc1 protein (Supplementary Figure S1a, c), however, precise determination is impossible due to dozens of splice forms of both endogenous rodent and human DISC1.[49] The chosen founder line exhibited neuronal, mainly perinuclear DISC1 aggregates throughout the brain and in primary neuron preparations that co-stained with the centrosome marker γ-tubulin (Figure 1b, Supplementary Figure S3a, b). We define DISC1 aggregation or misassembly (a broader term) using the following criteria: (1) insolubility in ionic detergents upon biochemical fractionation;[20] and (2) presence of (perinuclear) inclusion-like structures that may represent cellular accumulation of insoluble material, as observed in some protein conformational diseases like, for example, Huntington's disease.

There was accentuated DISC1 aggregation in dopamine-rich dorsal striatum (dStr; Figures 1b and c) that could not be explained by differences in PrP promoter activity (Supplementary Figure S2b), suggesting that posttranslational mechanisms led to an enrichment of aggregated DISC1. Aggregation of the transgenic DISC1 protein also led to recruitment of endogenous rat Disc1 in the insoluble fraction (Supplementary Figures S2c, d).

No major pathology was observed in peripheral organs or the brain of tgDISC1 rats (data not shown). However, we did detect slightly enlarged ventricles in the brain (Figure 1d), a trait also observed in patients with schizophrenia[50] and some transgenic mouse models expressing mutant DISC1.[10,12,13,26] Of note, the mainly perinuclear DISC1 aggregates were not positive for ThS (Supplementary Figure S4a and b), a marker for amyloid, even though a recombinant DISC1 fragment (598–785 (ref. 6)) injected into the brain of wild-type rats did stain for ThS (Supplementary Figure S4c). This indicates that DISC1 or its fragments are principally able to form amyloid, but not when endogenously expressed in adult or aged tgDISC1 rats.

Dopamine-related behavioral phenotypes of tgDISC1 rats

Similar to patients with schizophrenia,[51] tgDISC1 rats exhibited spontaneous amphetamine supersensitivity. When challenged with a single low-dose of $0.5\,\mathrm{mg\,kg^{-1}}$ d-amphetamine (Figures 2a and b), tgDISC1 rats exhibited increased locomotion and rearing behavior, whereas negative control rats did not. A two-way ANOVA revealed a main effect for treatment (distance: $F_{1,22} = 19.026$, $P < 0.001$; rearing: $F_{1,22} = 43.909$, $P < 0.001$) and genotype-treatment interaction (distance: $F_{1,22} = 6.052$, $P = 0.022$; rearing: $F_{1,22} = 17.706$, $P < 0.001$). A paired *t*-test of locomotion data during saline and amphetamine for the two genotypes revealed that tgDISC1 rats exhibited a large increase in locomotion upon amphetamine treatment (distance and rearing: $P < 0.001$), whereas NCs did not respond (distance: $P = 0.280$; rearing $P = 0.146$). Amphetamine supersensitivity was persistent even in 22-month-old tgDISC1 animals (Supplementary Figures S5a and b). This suggests that disturbance in the dopamine system of tgDISC1 rats is a persistent, life-long trait.

Furthermore, tgDISC1 rats exhibited motor deficits in the rotarod task (Figure 2c). A two-way ANOVA checking for genotype differences in the learning curve of the rotarod task showed a main effect for genotype ($F_{1,19} = 7.534$, $P = 0.013$) and trial ($F_{2,38} = 3.360$, $P = 0.045$). Subsequent *post hoc* unpaired *t*-tests for trials 1, 2, 3 comparing genotypes revealed a significant difference in trial 3 performance ($P = 0.041$), whereas no differences could be detected in the first two trials (trial 1: $P = 0.217$; trial 2: $P = 0.122$). These major behavioral phenotypes were confirmed in a different founder line as well (Supplementary Figure S6).

Spatial and object memory was also tested in tgDISC1 rats using novelty preference tasks. Although both genotypes could distinguish objects in the OR, OPR and object recognition for temporal order paradigms (Supplementary Figure S7), tgDISC1 rats displayed a marked preference towards novel objects compared with controls (Figures 2d and e). This preference was also persistent in old age (Supplementary Figures S5c and d). In the OR task, rats have to identify the new object presented in the test trial. A two-way ANOVA showed main effects for object ($F_{1,20} = 31.110$, $P < 0.001$) and object-genotype interaction ($F_{1,20} = 9.343$, $P = 0.006$). A subsequent one-way ANOVA revealed genotype differences in hyperexploration of the new, but not the old object (old $P = 0.737$, new $P = 0.048$). However, in general both genotypes preferred exploration of the new over the old object (data split for genotype; paired *t*-test: NC $P = 0.045$, TG $P < 0.001$). Comparable behavioral results were detected in the object place recognition task in which the animal has to discriminate between the displaced and the stationary object. Here, the two-way ANOVA showed main effects for both object ($F_{1,18} = 26.118$, $P < 0.001$) and genotype ($F_{1,18} = 6.923$, $P = 0.017$), again highlighting hyperexploration of the more interesting displaced object in tgDISC1 rats (one-way ANOVA stationary $P = 0.128$, displaced $P = 0.019$). Comparisons of exploration time of the stationary and displaced object by a paired *t*-test show that both genotypes investigate the displaced object for longer (NC $P = 0.021$, TG $P = 0.001$).

As amphetamine supersensitivity indicated a disturbance in the dopaminergic system, we performed *ex vivo* neurochemistry and analysis of dopamine receptor densities in the striatum of tgDISC1 rats. Dopamine concentrations were decreased in the dStr (Figure 3a), amygdala and hippocampus (Supplementary Figures S8a and b) of tgDISC1 rats. However, no changes were detected in total D2Rs (Figure 3b), D1 receptors (D1Rs), serotonergic, or glutamatergic receptors (Supplementary Figure S8c) as measured by receptor autoradiography. Surprisingly, when we investigated the affinity state of D2Rs, we found an ~80% increase in high affinity D2 (D2High) receptors in tgDISC1 rats (Figure 3c). A switch from low-affinity D2Low to D2High receptors is a characteristic feature of schizophrenia.[52,53] To investigate whether the changes in D2R affinity state affected signaling in striatal medium spiny neurons (MSNs), we assessed substance P and enkephalin expression as markers of neuronal activity

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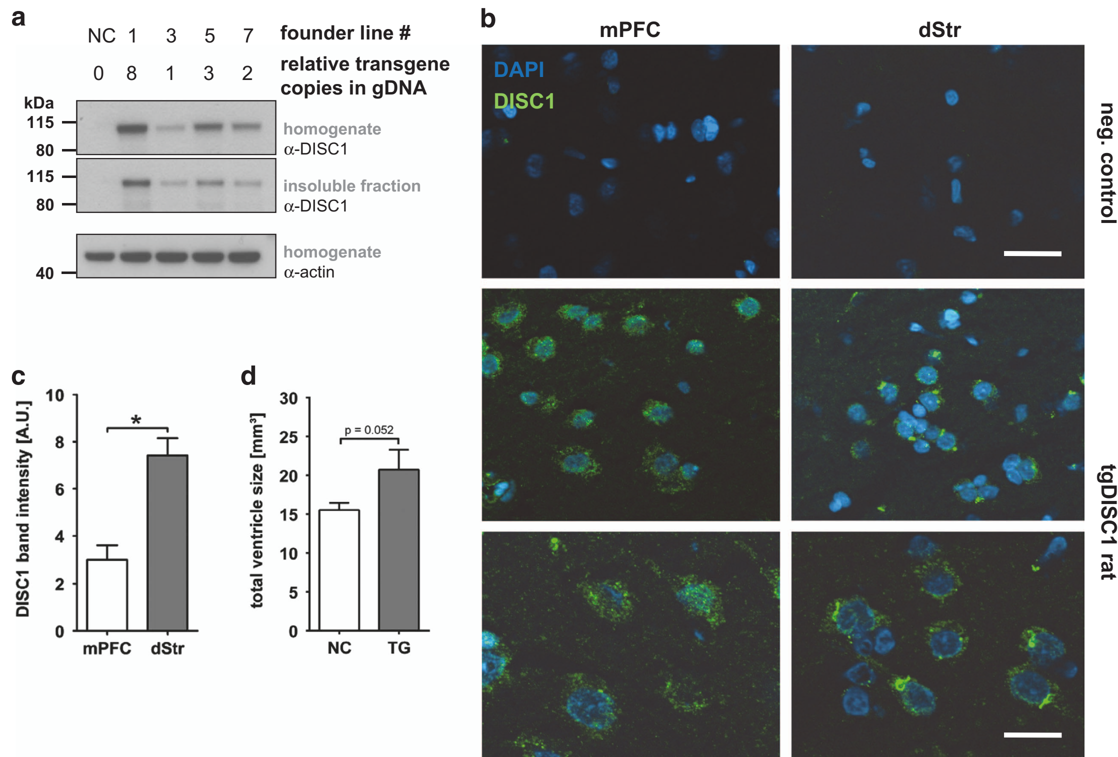

Figure 1. Aggregated DISC1 detected by IHC in brains of the tgDISC1 rat. (**a**) Western blot comparing transgene expression and aggregation in four different tgDISC1 founder lines. Heterozygous rats of founder lines 1, 3, 5 and 7 displayed different levels of full-length human DISC1 transgene expression (homogenate) which were reflected in the insoluble fraction. No huDISC1 could be detected in the negative control animal. Beta-actin was used as loading control in the homogenate. Relative number of transgene copies in founder lines was determined by quantitative Real-Time-PCR of genomic DNA and revealed the least transgene copies in the gDNA of founder 3 (arbitrarily set to one for comparison between founder lines) and a Mendelian-like inheritance pattern upon breeding. Founder line 3 with the weakest DISC1 expression was chosen for further experiments to avoid artifacts of too strong transgene expression. (**b**) Confocal immunofluorescence of striatal (dStr, right panels) and frontal (left panels) cryosections of the transgenic DISC1 rat (middle and in higher magnification in lower panels) and a negative control rat (upper panels). Abundant punctuate, mainly perinuclear staining as evidence for the existence of DISC1 aggregates can be detected that are more frequent and bigger in the striatum. Green: DISC1; blue: DAPI; bar 40 μm (upper two panels), bar 10 μm (lower panels). (**c**) Densitometric analysis of biochemically purified insoluble fraction of the tgDISC1 rat mPFC and dorsal striatum. The tgDISC1 rat ($n=6$) had more aggregated DISC1 in the dStr than in the mPFC in accordance with the IHC. Wilcoxon $^*P=0.028$. (**d**) NMR analysis of ventricle size. The tgDISC1 rat ($n=8$) had a larger total ventricle size than negative controls ($n=10$), namely 20.75 ± 2.5 mm^3 in tgDISC1 rats and 15.54 ± 0.9 mm^3 in negative controls. Unpaired t-test: $P=0.052$. All means ± s.e.m. DISC1, Disrupted-in-Schizophrenia 1; gDNA, genomic DNA; NMR, nuclear magnetic resonance.

in D1R- and D2R-expressing MSNs (Supplementary Figure S8d), respectively. We did not find significant changes indicating that altered dopamine transmission in the dStr was not strong enough to induce changes in substance P or enkephalin expression.

To further characterize the dopaminergic system, we performed fast-scan cyclic voltammetry in striatal slices from tgDISC1 rats and controls. Peak dopamine release was similar between genotypes (Figure 3d) but, interestingly, extracellular dopamine clearance was increased in the dStr of tgDISC1 rats (Figure 3e, see also Supplementary Figure S8e), indicating elevated DAT function. Therefore, using dissected striata, we further investigated the cellular localization of DAT. In tgDISC1 rats, significantly more DAT was translocated to the plasma membrane, likely explaining the observed increase in dopamine clearance (Figure 3f and Supplementary Figure S8f).

Taken together, these findings indicate that dopamine homeostasis is changed in tgDISC1 rats, as evidenced by D2R affinity state and DAT function. Thus, misassembly of full-length human DISC1 is sufficient to cause changes in the dopaminergic system consistent with some biochemical and behavioral symptoms seen in schizophrenia or psychosis.

Molecular and cellular interactions between DISC1 protein assembly and the dopamine system

To further investigate interactions between DISC1 assembly and dopamine on the cellular and molecular level, we generated full-length human DISC1-inducible SH-SY5Y cell lines (Figure 4a). We did not detect different aggresomal sizes in the cell lines expressing either the full-length human DISC1 (S704, L607) or the (C704, F607) variant (Supplementary Figure S9). This suggests that changes in misassembly of DISC1 polymorphisms observed in biophysical studies do not translate to microscopically visible differences. When these cells were induced to drive DISC1 expression and then exposed to a high, but non-toxic concentration of dopamine for 24 h, DISC1 aggresomes were detected (Figures 4a and c; Supplementary Figure S10a). The DISC1 aggresomes were coated with vimentin, a defining marker for aggresomes[54] (Figure 4c, Supplementary Figures S9a and b), which also makes it unlikely that DISC1 is a component of the aggresome machinery itself. Primary neurons and brain slices of the tgDISC1 rat could not be stained to check for vimentin coating of aggresomes, as vimentin is only expressed in proliferating tissues.[55] However, these cellular perinuclear DISC1 aggresomes co-localized with γ-tubulin, but neither with HSP70, nor the

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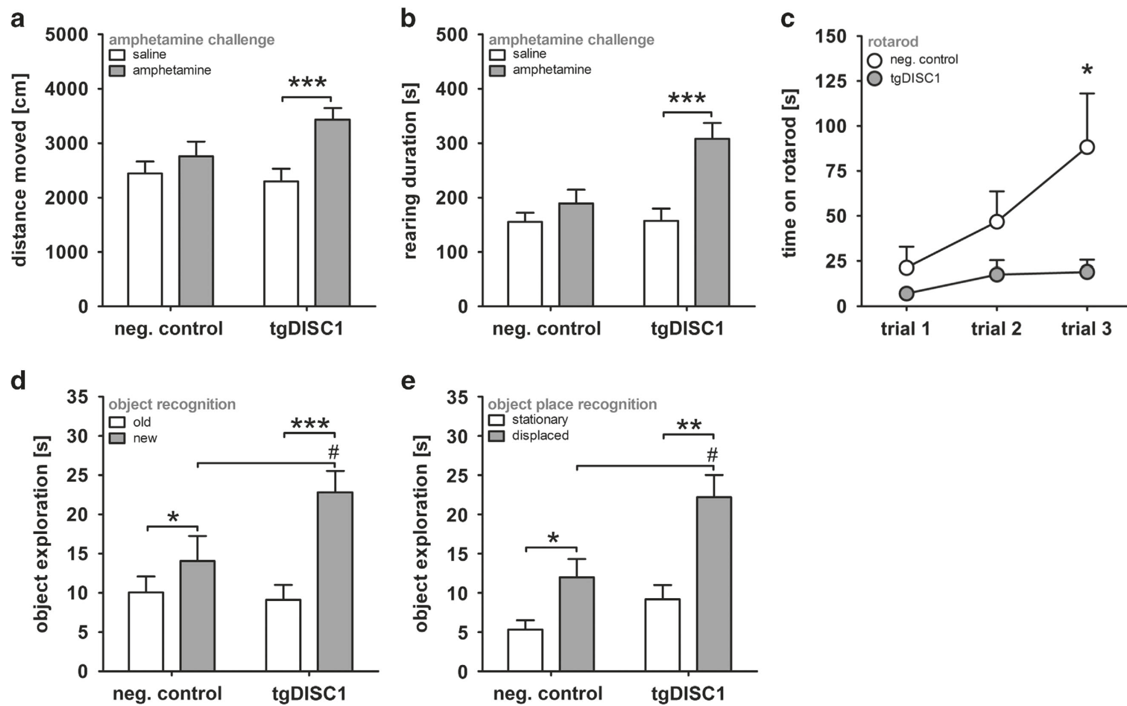

Figure 2. Behavioral phenotypes of the tgDISC1 rat. (**a**) Amphetamine hypersensitivity in the tgDISC1 rat shown by horizontal locomotion. Spontaneous locomotion before (saline; white bars) and after (gray bars) application of a single low dose of d-amphetamine (0.5 mg kg^{-1}, i.p.) is presented. Whereas the d-amphetamine had no significant locomotor effect in the control animals (paired *t*-test: $P = 0.280$, $n = 12$), it led to increased locomotion in the tgDISC1 rat indicating hypersensitivity to d-amphetamine (paired *t*-test $^{***}P < 0.001$, $n = 12$). (**b**) Amphetamine hypersensitivity in the tgDISC1 rat shown by duration of rearing. Only tgDISC1 rats reacted with increased duration of rearing to d-amphetamine treatment (gray bars) compared with saline (white bars; paired *t*-test: negative controls (NC) $P = 0.146$, TG $^{***}P < 0.001$; TG and NC $n = 12$). (**c**) The rotarod task as measure of motor learning ability and attention. Under constant speed of the wheel, the negative control animals showed a significant progressive improvement in walking on the rotarod over three subsequent trials, whereas the transgenics did not display such a learning curve (*t*-test of trial 3: $^{*}P = 0.041$; TG $n = 11$, NC $n = 10$). (**d**) Hyperexploration of tgDISC1 rats in the object recognition task (OR). Comparing duration of exploration of the new vs the old object in tgDISC1 rats and controls during OR test trial (TG $n = 12$, NC: $n = 10$) showed that tgDISC1 rats explored the new object more extensively than the negative controls (one-way ANOVA: $^{\#}P = 0.048$), although both genotypes preferred the new over the old one (paired *t*-test: NC $^{*}P = 0.045$, TG $^{***}P < 0.001$). (**e**) Hyperexploration of the tgDISC1 rats in the object place recognition task (OPR). Comparably, in the OPR task (TG $n = 12$, NC $n = 8$) the tgDISC1 rats explored the displaced object longer than control rats (one-way ANOVA: $^{\#}P = 0.019$). Again, both genotypes favored the displaced over the stationary object (paired *t*-test; NC $^{*}P = 0.021$, TG $^{**}P = 0.001$). All means ± s.e.m. DISC1, Disrupted-in-Schizophrenia 1; i.p., intraperitonial.

oligomer-specific marker A11, or the amyloid marker ThT (Supplementary Figure S10b and c).

This increase in DISC1 misassembly upon exposure to dopamine is consistent with our observation of increased DISC1 aggregation in the dStr (Figures 1b and c), which is the brain region with the highest dopamine content (Supplementary Figure S8b).

These results suggest a bidirectional link between dopamine homeostasis and DISC1 assembly because not only did DISC1 assembly regulate D2R affinity state and DAT function *in vivo,* but also because increased cytosolic dopamine itself augmented DISC1 insolubility. To investigate whether DISC1 could interact in a complex with DAT we performed co-immunoprecipitation experiments. First, using plasma membrane fractions from the striatum of tgDISC1 rats, we demonstrated that DAT antibody co-immunoprecipitated transgenic human DISC1 (Figure 4d). Second, using SH-SY5Y cells that overexpress DISC1 and DAT, we demonstrated co-immunoprecipitation of DISC1 by DAT (Supplementary Figure S11b), as well as subcellular colocalization *in vivo* of the two proteins using immunofluorescence and the Proximity Ligation Assay[56] (Supplementary Figures S12). Furthermore, dopamine-induced DISC1 aggregates (Figure 4a) were also able to sequester DAT into the insoluble fraction (Supplementary Figures S13c and d). Taken together, all these *in vivo* and *in vitro* results converge on the demonstration of a functional complex comprising DISC1 and DAT.

Pulse treatment of DISC1-transfected cell lines and primary neurons of the tgDISC1 rat with a single, high and non-toxic dose of dopamine induced a high-molecular-weight (HMW) band with an electrophoretic mobility of ~200–230 kDa in western blots suggesting a biochemical signature of dopamine-induced DISC1 multimers (Figure 5a and Supplementary Figure S14a). The identity of this band as DISC1 was confirmed by mass spectrometry (Supplementary Figure S14b and c). Dopamine-induced appearance of this HMW band and aggresome-positive cells could not be prevented by addition of D1R or D2R antagonists (Supplementary Figure S15). However, it was partially inhibited by a DAT inhibitor (Supplementary Figure S16) suggesting that these phenotypes were not induced by signaling events but by the presence of cytosolic dopamine.

To further investigate whether conditions that lead to increased cytosolic DA *in vivo* can also induce the HMW DISC1 band, we used transgenic mice that are postulated to have elevated levels of cytosolic dopamine. In particular, we used mice that either overexpress DAT (DAT-OE), underexpress VMAT2 with only ~5% of normal levels (VMAT2-DE) or double-transgenic mice (DAT-OE: VMAT2-DE). Using purified insoluble fractions from the mPFC of these mice, we demonstrated an increase in the endogenous HMW DISC1 band in DAT-OE and DAT-OE:VMAT2-DE brains (Figure 5b), thus confirming cytosolic dopamine-induced aggregation of DISC1 *in vivo*. The identity of this HMW band

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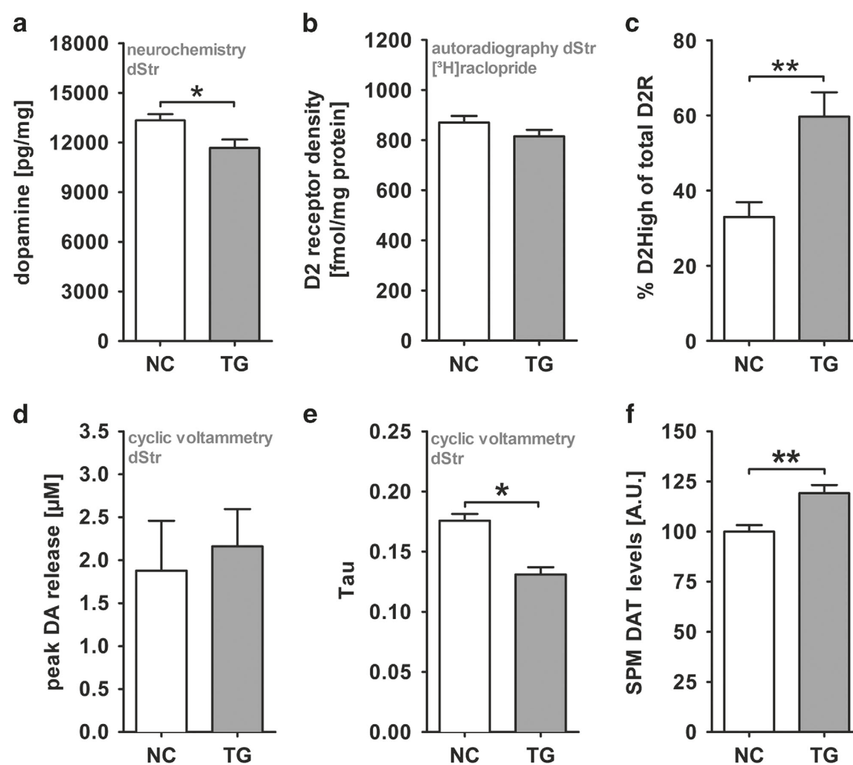

Figure 3. Dorsal striatum and dopamine homeostasis in the tgDISC1 rat. (**a**) Neurochemical quantification of *post mortem* dopamine (DA) in the dStr of tgDISC1 and negative control animals. TgDISC1 rats ($n=12$) had lower levels of DA as compared with negative controls (NCs) ($n=12$). TgDISC1 and negative control rats had 13 337 ± 375 and 11 675 ± 504 pg/mg DA. Unpaired *t*-test $^{**}P=0.005$. (**b**) Total D2 receptor abundance in the dStr. Autoradiography was performed with the D2R specific radioligand [^{3}H]raclopride. No difference in ligand binding and therefore total receptor density could be found in tgDISC1 rats ($n=10$) and controls ($n=10$) in the dorsal striatum. Mean receptor density was 870 ± 26 fmol/mg protein for controls and 815 ± 26 for tgDISC1 rats. Unpaired *t*-test $P=0.156$. (**c**) Elevated striatal D2High receptor portion in tgDISC1 rats. TgDISC1 rats ($n=6$) had an 81% increase in D2High receptor portions compared with negative controls ($n=6$) as measured by [^{3}H]domperidone binding. Binding of the radioligand was challenged with either 1 nM or 100 nM DA, concentrations at which no significant occupation of D2Rs or D2High-specific binding occurs, respectively. Proportions of D2High receptors in relation to total D2 receptors were 33 ± 3.9% in NCs and 59.7 ± 6.5% in TGs. Unpaired *t*-test $^{**}P=0.005$. (**d**) Peak DA release in the dStr of tgDISC1 rats. Fast-scan cyclic voltammetry measurement of DA in striatal slices revealed no difference in the peak release of DA in tgDISC1 rats (2.16 ± 0.43 µM; $n=4$) and controls (1.88 ± 0.58 µM; $n=6$). Mann–Whitney *U*-test $P=0.762$. (**e**) Clearance of extracellular DA in the dStr of tgDISC1 rats. TgDISC1 rats (tau = 0.131 ± 0.006; $n=4$) show increased extracellular DA clearance compared with negative controls (0.176 ± 0.006; $n=6$) as measured by fast-scan cyclic voltammetry. Mann–Whitney *U*-test $^{*}P=0.036$. (**f**) Striatal DAT levels in tgDISC1 rats. Preparation of the synaptic plasma membrane (SPM) and subsequent western blotting revealed a 19% increase in dopamine transporter levels in the dorsal striatum of tgDISC1 rats (NC, TG $n=6$). Densitometric analysis was performed by normalization of DAT to the Na/K-ATPase signal in the preparations. Unpaired *t*-test $^{**}P=0.004$. All means ± s.e.m.

under different conditions was determined for endogenous rat and mouse Disc1 as well as artificially expressed human DISC1 (in tgDISC1 rats) using an overlay of species-specific antibody signals (Figure 5c).

Amphetamine sensitization is a pharmacological model of psychosis.[57] Amphetamine is known to block and even reverse DAT's ability to transport dopamine out of the presynapse,[58] thereby enhancing extracellular dopamine concentrations. Sensitization with amphetamine leads to the depletion of stored dopamine in the striatum and enhancement of dopamine in the typically low dopamine-containing mPFC.[57] We sensitized wild-type rats for 5 days with daily doses of 2 mg kg^{-1} amphetamine, and probed them after a 2-week interval with a single dose of 0.5 mg kg^{-1} amphetamine for behavioral testing of supersensitivity (Supplementary Figure S17a). Afterwards, animals received another high dose of amphetamine and 24 h later the mPFC and dStr were dissected and the insoluble proteome was purified and probed for Disc1. Although we could not detect a difference in Disc1 aggregation in the mPFC, we observed decreased insoluble Disc1 in the dStr upon DA depletion (Supplementary Figure S17b and d). According to these results, the presence of endogenous insoluble Disc1 is correlated with cytosolic DA levels in wild type rats.

DISCUSSION

We present the first transgenic rat model for one of the best-characterized genes implicated in mental illness, *DISC1*. We demonstrate that modest overexpression of full-length human DISC1 causes DISC1 misassembly and a signature of neuropathological, biochemical and behavioral phenotypes involving the dopamine system.

DISC1 is a gene for which mutations or polymorphisms have been associated with chronic mental illnesses like schizophrenia, recurrent major depression, bipolar disorder and autism. This highlights the role of DISC1 as a general vulnerability factor for behavioral control that is not restricted to one specific mental illness, despite its name.[4,5,14] Here, we present a novel mechanism by which full-length DISC1 protein without genetically linked mutations may have a role in a subset of sporadic cases of chronic mental illness, cases where a clear and unambiguous genetic basis cannot be determined. Protein pathology, that is, misassembly of

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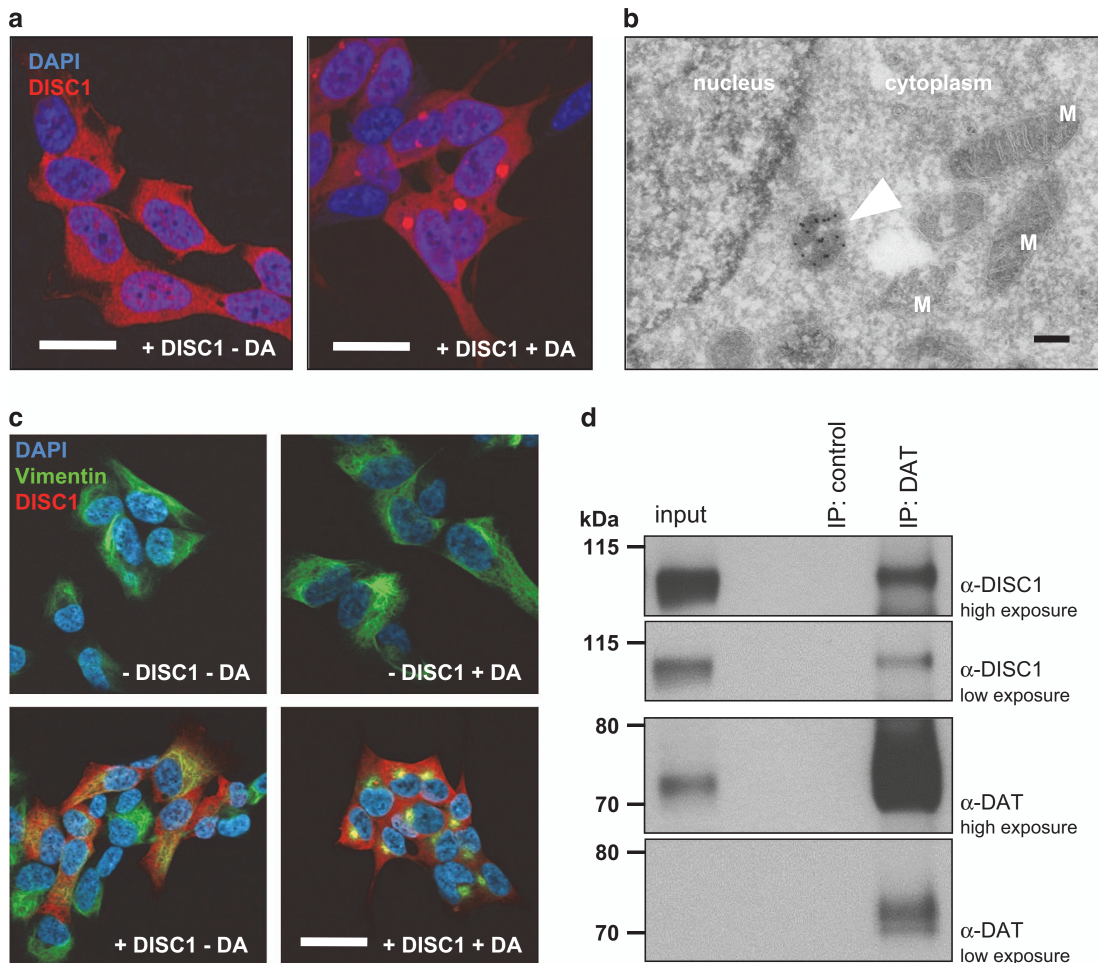

Figure 4. Dopamine-induced DISC1 aggresome formation in cell models. (**a**) Confocal immunofluorescence light microscopy of SH-SY5Y human neuroblastoma cells induced for expression of full-length human DISC1 (S704, L607). DISC1 was diffusely expressed throughout the cytoplasm (left panel). Upon DA treatment (100 μM for 24 h; right panel) the previously diffusely distributed DISC1 built up aggresomes inside the cell. Bar 20 μm. (**b**) Cryoimmunogold electron microscopy for cells from **a**. Arrow marks the perinuclear, immunolabeled dopamine-induced DISC1 aggresome. Bar 100 nm. (**c**) Characterization of dopamine-induced DISC1 aggresomes as shown in **a**. Double-staining shows that DA-induced DISC1 aggresomes (red) are caged by vimentin (green). Bar 20 μm. (**d**) Co-immunoprecipitation of DISC1 by DAT in the tgDISC1 rat brain. In a plasma membrane preparation of the striatum the DAT antibody co-immunoprecipitated transgenic human DISC1. Upper two panels show DISC1 signal, lower two panels DAT signal at two different exposure times. DA, dopamine; DISC1, Disrupted-in-Schizophrenia 1; M, mitochondrion.

a gene product, is an established mechanism in chronic brain diseases like Alzheimer's disease, Parkinson's disease and other neurodegenerative disorders,[18] as well as several non-brain diseases.[59] In addition, protein assembly to large, ordered complexes occurs physiologically and plays important functional roles, including synapse maintenance.[19,60] DISC1 misassembly in tgDISC1 rats led to a signature of biochemical and behavioral phenotypes involving the dopamine system. These phenotypes correspond to phenotypes that are also observed in schizophrenia, such as amphetamine sensitization and the switch from low-affinity to high-affinity D2 receptors.[52] Therefore, we present a novel animal model with high face validity that is relevant for a significant subset of human cases with DISC1-dependent mental illness, tentatively termed DISC1opathies.[61]

In the tgDISC1 rat, we chose to overexpress full-length DISC1 containing the C704 and F607 polymorphisms because they increase aggregation propensity in cell-free *in vitro* systems.[6,7] Both polymorphisms are common alleles that do not predict mental illness as opposed to mutations. Therefore, when evaluating the potential contribution of these polymorphisms on the observed phenotypes, genetic and protein aggregation effects cannot be separated because both phenomena are linked. Furthermore, when expressed in cells, aggregation propensity of the DISC1 protein seems to override effects of polymorphisms (Supplementary Figure S9).

In addition to prominent amphetamine supersensitivity, the hyperexploratory behavior and rotarod deficits can also be related to subtle alterations in the striatal dopaminergic system, which is essential for motor control, reward-related behaviors and exploration. The dStr acts as a convergence point for inputs from the basal ganglia, cortex and thalamus. From the dStr, the direct and indirect pathways arise and exert opposing control over motor function. This is mediated by D1R- and D2R-expressing MSNs that, respectively, promote or inhibit locomotion and exploration.[62] Both pathways are thought to be partially intertwined[63,64] and are affected by dopaminergic input to the dStr. Increased striatal dopamine reduces the excitability of D2R-MSNs and favors locomotion and exploration by weakening the indirect pathway.[65,66] Our results indicate a subtle enhancement of D2R-mediated neurotransmission in tgDISC1 rats: the large (80%) increase in D2High receptors suggests amplification of D2R signaling that is probably not compensated by the modest

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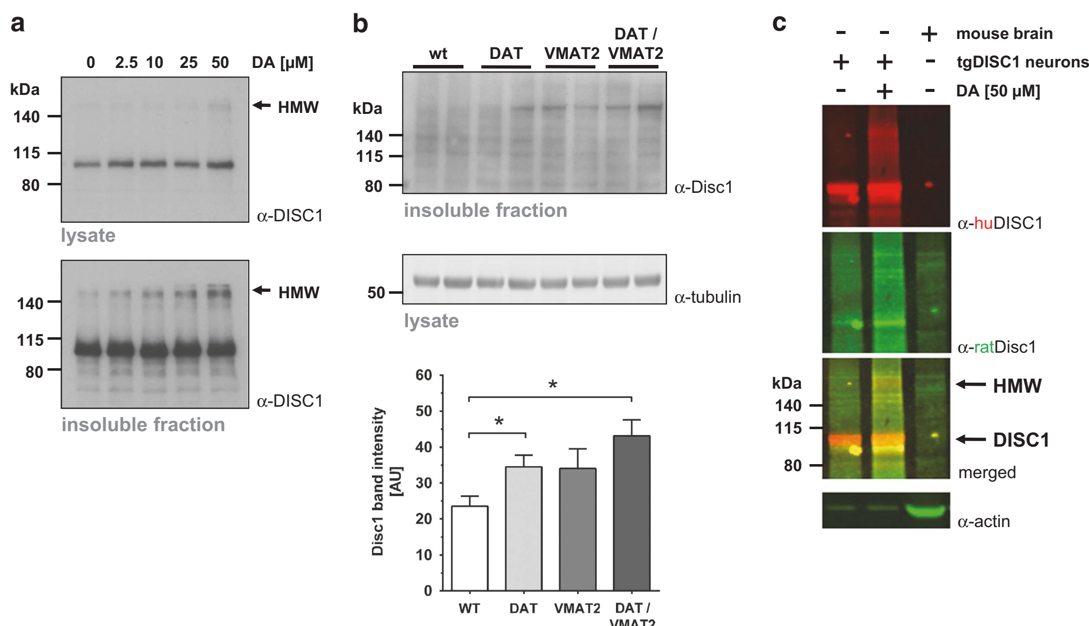

Figure 5. Dopamine-induced DISC1 high-molecular-weight (HMW) bands in cell and animal models. (**a**) Appearance of DISC1 HMW bands upon DA-treatment of cells. Western blot of the lysate (upper panel) or insoluble fraction (lower panel) of NLF human neuroblastoma cells transiently transfected with full-length DISC1 (S704, L607) and treated with DA at indicated concentrations. HMW DISC1-immunoreactive bands appear (arrow) with increasing concentrations of DA. (**b**) Analysis of the purified insoluble fraction of the mPFC of three transgenic mouse lines either overexpressing DAT (DAT-OE), with decreased VMAT2 expression (VMAT2-DE) and double transgenics (DAT-OE:VMAT2-DE). In the western blot (upper panel) a HMW Disc1 signal is visible in all transgenic mouse lines, but not in wild-type mice. As a loading control of the input material for the insoluble fraction preparation homogenates were blotted and incubated with beta-actin on a separate blot. Densitometric quantitation (lower panel) of insoluble HMW Disc1 in the mPFC of transgenic mice shows that while VMAT-DE animals exhibit a trend towards increased Disc1 aggregation in mPFC, in DAT-OE mice Disc1 aggregates were significantly elevated. DAT-OE:VMAT2-DE mice displayed nearly double the amount of Disc1 aggregates in mPFC compared with WT animals (Kruskal-Wallis with one-tailed Dunn's *post hoc* test; DAT-OE: $^{*}P = 0.029$; DAT-OE:VMAT2-DE: $^{*}P = 0.014$; $n = 4$ each). (**c**) Western blots of the purified insoluble fraction showing the HMW band as a common signature of DA-induced aggregation of human, rat, and mouse DISC1. Depicted are the insoluble fractions of primary rat cortical neurons derived from tgDISC1 rats with (+) and without (−) incubation with 50 µM dopamine and of endogenous Disc1 from DAT-OE mouse brain, stained with the huDISC1 specific mAB 14F2 (green) and rodent Disc1 specific polyclonal C-term Ab (red), detecting human transgenic and endogenous mouse DISC1, respectively. Primary neuron samples show a dopamine induction-dependent, HMW immunoreactive band above 200 kDa (upper arrow) for endogenous rat as well as transgenic human DISC1. Also endogenous mouse Disc1 shows HMW bands in the insoluble fraction. The actin control demonstrates equal protein content for the lysate input of primary neurons. All means ± s.e.m.

increase in dopamine clearance by DAT. Overall, this would lead to increased D2R-MSN activation promoting hyperexploration in tgDISC1 rats. This might also explain the rotarod deficits in these animals as motor learning is mediated by D2R-MSNs and can be disrupted by exploratory distraction as shown by cell-type specific striatal lesion studies.[67] Mutations in the *DISC1* gene have been linked to aberrant dopamine-related functions. Amphetamine supersensitivity was observed in mouse mutants and in transgenic mice expressing C-terminal deleted DISC1 (1–597).[23,24,26] Also in these transgenic mice, changes were subtle as similar basal DA levels were detected in the dStr.[23,26] Although the Disc1 (L100P) mutant mouse showed elevated D2High receptors in the dStr,[23] the transgenic mouse expressing C-terminal deleted DISC1 showed more D2R and DAT in the dStr.[24] DA-related behavioral and biochemical phenotypes have consistently been found in *Disc1* mouse mutants and transgenic mice expressing mutant *DISC1* gene. Given that partial *Disc1* knockout or localized Disc1 silencing[25] as well as overexpression of non-mutant DISC1 (as shown here) both lead to DA-related phenotypes, it appears that DISC1 protein expression has to be tightly regulated within a narrow range to maintain functional dopamine homeostasis. Thus, DISC1 integrity as well as expression levels seem to have a critical role in dopamine homeostasis.

Our demonstration of a reciprocal relationship between DISC1 misassembly and the major neuromodulator dopamine was unexpected. Unlike other neurotransmitters, cytosolic dopamine is a highly reactive oxidant[68] and is able to induce or accentuate oxidative damage. The suggested connection between DISC1 assembly, DAT function, and cytosolic dopamine could therefore have an important role in controlling cellular stress. We postulate that DISC1 assembly / misassembly could act as a molecular sensor for cellular oxidative stress. Whether the observed DISC1 aggregates[20,21] are archaic remnants of oxidative stress events should be further explored.

In summary, our investigations reveal significant functions of DISC1 in two major regulators of dopamine homeostasis: D2R affinity and DAT activity. Modest DISC1 overexpression in the tgDISC1 rat causes changes in DISC1 assembly that directly impacts dopamine homeostasis in the absence of genetic mutations. Thus, we have identified a novel mechanism of DISC1 pathology involving protein insolubility that was previously underappreciated as a potential risk factor for mental illness.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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9.2 Study II

Intranasal dopamine treatment reinstates object-place memory in aged rats.

SV Trossbach, MA de Souza Silva, JP Huston, C Korth and C Mattern

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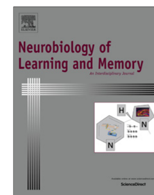

Rapid Communication

Intranasal dopamine treatment reinstates object-place memory in aged rats

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ABSTRACT

Following oral or IV administration, dopamine (DA) cannot cross the blood–brain barrier to a significant extent, but can enter the brain when administered via the nasal passages. Intranasal administration of DA was shown to increase extracellular DA in the striatum, to have antidepressant action and to improve attention and working memory in rats. Here we show that aged (22–24 months old) rats are deficient in an object-place learning task, but that this learning/memory is intact and comparable with that of adult rats upon pre-trial administration of 0.3 mg/kg DA gel into the nasal passages. This result raises the possibility of the therapeutic application of intranasal DA treatment for age-related cognitive disorders.

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1. Introduction

Deficiencies or imbalances in levels of the catecholamine neurotransmitter dopamine (DA) in the brain, which are known to be a hallmark of various neurological disorders, such as Parkinson's disease, also correlate with normal aging-related deficits in cognitive and emotional processes. Humans from the age of 22–80 years exhibit a decline of more than 7% per decade in the dopamine transporter (DAT) in the striatum (Shingai et al., 2014) and aging-related dopamine deficits have been closely linked to normal age-related decline in working memory (Podell et al., 2012). DA is well known to promote learning and memory (e.g.: Da Silva, Köhler, Radiske, & Cammarota, 2012; Ellis & Nathan, 2001; Packard & White, 1989) and its action is strongly implicated in the presumed neural mechanisms of information storage and retrieval, (e.g.: Bethus, Tse, & Morris, 2010; Lemon & Manahan-Vaughan, 2006; Li, Cullen, Anwyl, & Rowan, 2003; Wolf, Sun, Mangiavacchi, & Chao 2004). Especially working memory for spatial objects was shown to be facilitated by DA action in humans (Luciana & Collins, 1997).

Since DA is unable to cross the blood brain barrier (BBB) to a significant extent due to its polar properties, DA agonists or DA precursors which pass the BBB are applied systemically for experimental or therapeutic purposes. However, they also activate peripheral DA receptors and may have side effects, such as the nausea elicited by L-dopa, a DA precursor, commonly administrated in patients with Parkinson's disease (PD). There is evidence that DA can directly enter the brain via intranasal administration (Dahlin, Bergman, Jansson, Bjork, & Brittebo, 2000; Dahlin, Jansson, & Bjork, 2001). Intranasally administered DA (IN-DA) has been shown to be neurochemically and behaviorally active. Application of DA into the nostrils of anaesthetized rats was shown to increase extracellular DA in the nucleus accumbens and dorsal striatum lasting for at least 2 h (de Souza Silva, Topic, Huston, & Mattern, 2008). In the freely moving rat, IN-DA had antidepressant-like action and increased locomotor activity (Buddenberg et al., 2008). Furthermore, behavioral asymmetries in the hemiparkinsonian rat were attenuated with chronic administration of IN-DA (Pum et al., 2009) and with acute intranasal application of L-dopa (Chao et al., 2012).

Administration of IN-DA has also been shown to have memory-promoting action in an animal model of attention-deficit hyperactivity disorder (ADHD), the Naples high-excitability rat. Chronic IN-DA treatment in this rat increased the number of arms visited before the first repetitive arm entry in the Olton radial maze, an

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index of working memory and selective spatial attention (Ruocco et al., 2009).

In the present study, we examine the influence of acute application of intranasal dopamine on an age-related memory deficit in aged rats. Having found aged (21–22 months old) Sprague Dawley rats to be deficient in memory for spatial location of objects, we hypothesized that IN-DA treatment would improve their memory in an object-place recognition test, which assesses the memory for the location of objects by virtue of preferential exploration of displaced vs stationary objects (Ennaceur, Neave, & Aggleton, 1997).

2. Materials and methods

2.1. Animals

Subjects were male Sprague Dawley rats bred at the University of Dusseldorf Animal Facility. They were housed in groups of 1–3/ per cage under a 12 h day/night cycle (light on at 7 a.m.) with food and water available *ad libitum*. Aged (∼21–22 months, $n = 11$) and adult (∼4 months, $n = 8$) animals were used in this study. All experiments were approved by local authorities (LANUV NRW) and performed in accordance with the German Animal Protection Law.

2.2. Open field

An open field (40 × 40 × 40 cm high) was used to assess locomotion and object exploration. It consisted of two adjacent grey and two dark acrylic walls. A black "plus" symbol and a white circle (both 10 cm^2) laminated in a plastic sheet were taped to a grey and a dark wall, respectively, as local cues. Dim overhead illumination was provided by LED lights integrated into the camera with a luminous density of ∼ 6.8 lx in center and ∼ 2.1 lx in the corners. The camera (IR-100 B 3.6 mm, Conrad Electronic, Germany) was placed 40 cm above the center of the arena and connected to the EthoVision system (EthoVision 3.1, Noldus; Netherlands) that automatically tracked locomotion behavior. The animals were habituated to the open field by being allowed to explore it for 10 min prior to experimentation.

2.3. Spatial object recognition test

This test exploits the tendency of rodents to spend more time exploring a familiar object that has been displaced to a novel position than a familiar one that has not been displaced, suggesting that they recognize objects at different locations (Ennaceur et al., 1997). The test consists of a sample trial, during which two identical objects are presented in two of the corners of the open field, and a test trial, during which one of these two objects is now located in a different corner.

Two sets of plastic bottles with distinct colors and shapes served as objects (height 32 cm). The use of the two sets of objects was counterbalanced and their positions were randomized for each trial. The animal was placed into the center of the open field with two identical objects located near two corners of the apparatus and allowed to freely explore objects and arena for 5 min. It was returned to its cage afterwards. After a 25 min inter-trial interval the rat was placed into the open field again for 5 min, with the difference that one of the objects was moved to a new location (the displaced object), whereas the other object occupied the same place as in the sample trial (the stationary object). Object exploration was defined as physical contact with an object with snout, vibrissae or forepaws. If the animal explores the displaced object more than the stationary one, this indicates a memory for the location of the object.

A discrimination index was calculated: [time spent exploring the object at novel location – time spent exploring the object at old location]/[total time spent exploring the two objects]). If animals spend time at both objects approximate equally, the index will be ∼0 (chance level). If animals spend more time in exploring the displaced object than the stationary one, the discrimination index will be >0.0 (Ennaceur et al., 1997). Animals that failed to explore both objects in either the sample or test trial were excluded from the analyses.

The experiments were conducted between 09:00 and 16:00 h. Ethanol (70%) was used to clean the arena and objects after each trial. The distance moved was automatically recorded by the EthoVision software 3.1 (Noldus, Netherlands). Times for object exploration were rated by experimenters blind to the experimental design using the Ethovision software.

2.4. Dopamine treatment

For the aged animals, each rat received IN-DA and its vehicle, in a counterbalanced procedure, with a two weeks wash-out period between each administration. DA hydrochloride (DA-HCl, Sigma Aldrich, Germany) was prepared to a final concentration of 0.3 mg/kg calculated on the basis of DA base. The crystalline DA was suspended in a volume of 10 μL of gel composed of a viscous castor oil mixture (M & P Pharma, Emmetten, Switzerland) immediately before usage and was kept on ice throughout the experiment and protected from light. Per nostril 5 μL of the oleogel was applied with an applicator pipette for viscous liquids (Microman; Gilson, France) and corresponding tips (CP10 Tips, Gilson, France). Drug administration was performed over 3 sec per nostril with an application depth of 2 mm. The dosage was chosen based on studies, showing that IN-DA at 0.3 mg/kg had behavioral effects (Buddenberg et al., 2008). After the application, animals were placed into the open field and the object-place recognition test was conducted. The adult animals did not receive IN-DA, but were administered the object-place memory test.

2.5. Statistics

For statistical calculations the IBM SPSS Statistics 22 program was used. For the aged animals, a 2 × 2 ANOVA with the two within factors "treatment" and "object" or "trial" was conducted to analyze object-place memory and locomotor activity. Subsequent one-way repeated ANOVAs with the factor "treatment" were applied to analyze exploration behavior for the aged animals. Paired sample t-tests were applied when appropriate. For the adult animals, paired t-tests were conducted to analyze the memory test and locomotion. The discrimination indexes were compared to chance-level performance (zero value) by one-sample t-tests. The level of significance was taken to be $p \leqslant 0.05$ for all tests.

3. Results

3.1. IN-DA reinstates object-place learning in aged rats

We assessed the time spent in exploring the stationary vs the displaced object during the test trial. As can be seen in Fig. 1A, the IN-DA treated group, unlike the vehicle group, spent significantly more time exploring the displaced (grey bars) than the stationary object (open bars).

A two-way ANOVA revealed a main effect for objects ($F_{1,20} = 4.519$, $p = 0.046$) and an object-treatment interaction ($F_{1,20} = 14.865$, $p = 0.039$), but not an effect for treatment alone ($F_{1,20} = 1.360$, $p = 0.257$). A paired t-test, comparing time spent at the stationary and the displaced object for each aged group,

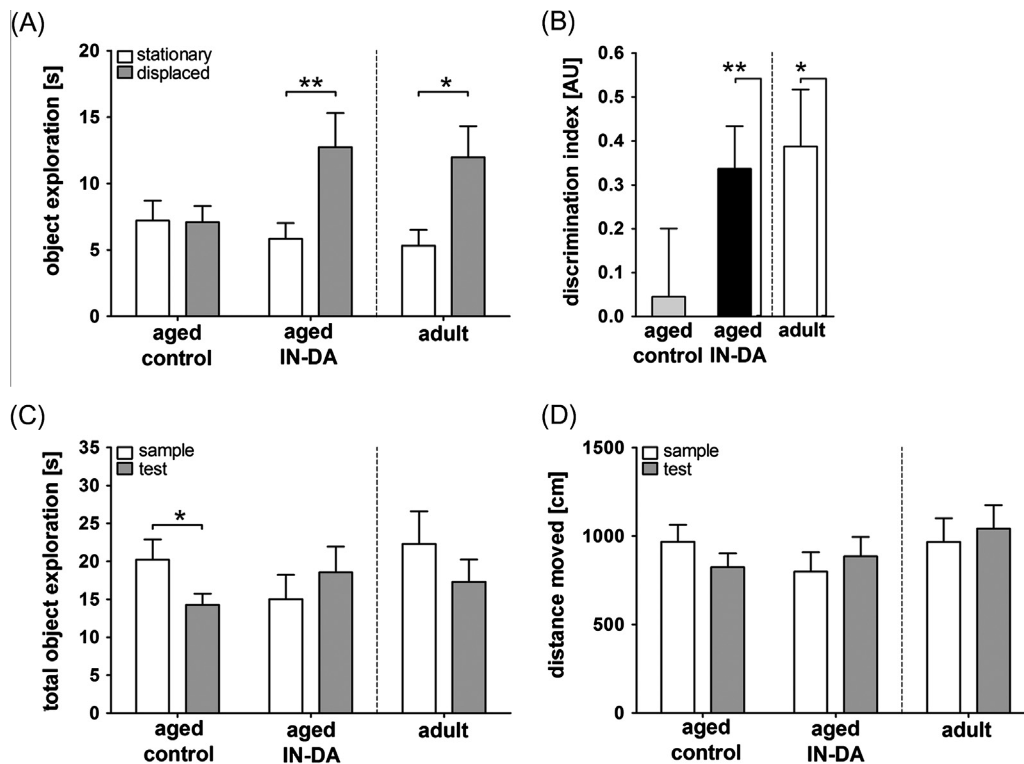

Fig. 1. Intranasal application of DA reinstates object-place learning in aged rats. (A) *Object exploration during test trial*. Only the IN-DA treated aged rats and the adults explored the displaced object more than the stationary one. (B) *Discrimination index of object place recognition*. Chance level is at zero value. Only the adult and IN-DA treated aged rats explored the displaced relative to the stationary one significantly above chance level ($^{*}p = 0.020$; $^{**}p = 0.006$). (C) *Total object exploration*. Only the aged rats treated with control gel showed significantly less overall exploration (both objects combined) in the test trial compared to the sample trial ($^{*}p = 0.048$). (D) *Locomotion during object place recognition task*. None of the groups show a difference in locomotion between sample and test trials. (aged control $n = 11$, IN-DA $n = 11$, adult $n = 8$).

resulted in a significant difference in object exploration for the IN-DA group ($p = 0.010$), but not the control group ($p = 0.958$), indicating a focused investigation of the more interesting object and, therefore, an enhancing effect of IN-DA. These findings are complemented by calculation of the discrimination index (Fig. 1B), which revealed a significant performance above chance level for the IN-DA treated aged animals (black bar; one-sample t-test against zero value: $p = 0.006$) and adult group ($p = 0.021$), but not for the control animals treated with gel (grey bars ($p = 0.705$).

Thus, upon IN-DA treatment, the aged rats behaved comparable to adult rats, which also explored the displaced object more than the stationary one in the test trial and, thus, exhibited intact memory for objects in place.

3.2. Overall exploratory activity

To determine whether IN-DA treatment influenced overall object exploration, the total exploration times in the sample and test trials were analyzed (Fig. 1C). A two-way repeated measures ANOVA for object exploration displayed a trial-treatment interaction ($F_{1,20} = 6.930$, $p = 0.016$), but no main effect of either treatment ($F_{1,20} = 0.438$, $p = 0.516$) or trial ($F_{1,20} = 0.016$, $p = 0.900$). Subsequent paired t-tests revealed a significant reduction in total time of object exploration from sample to test trial in the aged vehicle-treated animals ($p = 0.048$), but not in the IN-DA-treated animals ($p = 0.178$), nor adult rats (paired t-test $p = 0.383$). Thus, IN-DA treatment prevented the decrease in overall amount of exploration during the test trial exhibited by the controls (Fig. 1C).

3.3. General locomotor behavior

The distance moved (Fig. 1D) during the object place recognition test was assessed. In a two-way repeated measures ANOVA no effect of either trial ($F_{1,20} = 0.137$, $p = 0.715$), treatment ($F_{1,20} = 0.210$, $p = 0.651$) or their interaction ($F_{1,20} = 2.192$, $p = 0.154$) were found. This indicates that IN-DA did not simply increase activity of the aged rats, but specifically enhanced exploration of the displaced object in the place-memory task.

4. Discussion

The main result of this study is that intranasal application of 0.3 mg/kg DA rescued the learning of, or memory for the location of, an object in aged (22–24 month old) rats that were shown to be deficient in this kind of spatial learning/memory.

Whether the age-related memory deficiency resulted from the process of encoding, consolidation or retrieval of the spatial information is not known. Also, since we applied the DA shortly before the sample trial and not post-trial, we cannot discern whether the treatment acted on acquisition- or memory-related processes. Nor can we rule out that the treatment was effective by virtue of, e.g. psychostimulant action that enhanced attention to the objects. However, IN-DA has been shown to have neurochemical effects lasting for at least 100 min, suggesting that its action in our study extended into the post-trial period of consolidation (de Souza Silva et al., 2008).

The vehicle-treated control animals, but not the IN-DA-treated group exhibited a decrease in total amount of exploration of objects from sample trial to test trial, suggesting that IN-Da compensated for this decrease in exploration during the test trial. As shown in Fig. 1B, this apparent increase in exploration was limited to exploring the displaced, but not stationary object, which precludes the possibility that the intact expression of memory for objects in space after IN-DA was due simply to an overall behavioral arousal and general increased tendency to explore any object. Unlike in previous studies (Buddenberg et al., 2008;

de Souza Silva et al., 2008), we found no influence of IN-DA on general activity level, perhaps due to the old age of the animals used here, or to the fact that the testing period was only 5 min per trial, whereas in the previous studies the trial duration was 30 min and the animals were more thoroughly habituated to the open field.

The mechanisms that underlie the passage of substances from nose to brain are not well understood, but several modes of transport from the nasal mucosa to brain have been proposed, including routes involving olfactory and trigeminal nerves from nose to brain (Tayebati, Nwankwo, & Amenta, 2013). It has also been shown (Dahlin et al., 2000) that unchanged DA is transferred into the ipsilateral olfactory bulb following unilateral nasal administration of [^{3}H]dopamine to mice and that DA is transferred into the olfactory bulb via the olfactory pathway in rats (Dahlin et al., 2001). Olfactory neurons that express the dopamine transporter (DAT) innervate the nasal mucosa, which also contains the organic cation transporter-2 (OCT-2). There is also evidence that DAT-mediated uptake underlies the absorption of intranasally administered dopamine and that DA metabolism in the nasal mucosa is minimal (Chemuturi, Haraldsson, Prisinzano, & Donovan, 2006).

Little information is available as to the central neural effects of IN-DA and how these could relate to our finding of memory rescue in the aged organism. The increase in extracellular DA in striatum and nucleus accumbens found after IN-DA, suggests that IN-DA increased DA levels in the nigrostriatal as well as mesocorticolimbic system (de Souza Silva et al., 2008). The striatal dopaminergic systems participate in the mediation of memory for objects in space (Chao, Pum, & Huston, 2013). It remains to be determined which DA receptors are responsible for the behavioral effects of IN-DA reported so far and the role played by pre- vs postsynaptic receptors. Imaging studies has revealed a decrease in DAT binding in the dorsal striatum after IN-DA treatment in adult rats administered the same dose as in the present study (unpublished results). Aside from its acute effects on striatal DA levels and DAT binding, it was found that prepuberal chronic treatment with IN-DA led to long-term changes in several amino acids in the brain of wild type rats, including increased soluble L-Asp and decreased membrane-trapped L-Glu and L-Asp in the prefrontal cortex, implicating significant lasting effects on glutamate neurotransmission in the adult animal (Ruocco et al., 2014). In the Naples High Excitability rat, a model of ADHD, chronic prepuberal administration of IN-DA led to long-term changes in excitatory amino acids, NMDA-R1 subunit protein, tyrosine hydroxylase and DAT protein levels in the pre-frontal cortex and dorsal and ventral striatum, together with changes in working memory and indices of selective attention (Ruocco et al., 2014). Glutaminergic mechanisms have been strongly implicated in the control of recognition memory and spatial learning (Morris, Steele, Bell, & Martin, 2013; Warburton, Barker, & Brown, 2013) and in aging-related memory disorders (Burgdorf et al., 2011; Foster 2012; Hara et al., 2012).

The recollection/familiarity detection processes involved in the storage, encoding and retrieval of information as to the location of objects in place are likely to enlist multiple, interconnected neural circuits (Aggleton & Brown, 2006). These processes must involve the complex interplay of many known transmitter systems (e.g. de Souza Silva et al., 2013; Dere, de Souza Silva, & Huston, 2007). Although the hippocampus and associated cholinergic forebrain mechanisms have received the most attention so far (e.g.: Izquierdo & Medina, 1997), DA receptors must also be considered to play significant roles in such processes and their malfunctioning with aging (Shingai et al. 2014) and the present results supplement the evidence for an important role of DA in working memory involving spatial recognition (Chao et al. 2013; Luciana & Collins 1997; Podell et al., 2012).

5. Conclusion

Our finding of an attenuation of an aging-related deficit in the encoding or retrieval of spatial memory by acute application of IN-DA, supplements the earlier report of long-term beneficial effects of IN-DA treatment on attention and working memory in the 8-arm Olton maze in an ADHD rat model (Ruocco et al., 2009; Ruocco et al., 2014). These cognitive-enhancing effects of IN-DA, along with evidence for anti-depressant (Buddenberg et al., 2008) and anti-parkinsonian (Pum et al., 2009) action, bolster the prospect of considering intranasal dopamine application as a therapeutic measure against cognitive and mood-related deficits.

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9.3 Study III

Peripheral DISC1 protein levels as a trait marker for schizophrenia and modulating effects of nicotine.

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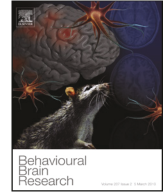

Research report

Peripheral DISC1 protein levels as a trait marker for schizophrenia and modulating effects of nicotine

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HIGHLIGHTS

- Detection of a protein from a major mental illness gene, Disrupted-in-Schizophrenia 1 (DISC1) in lymphocytes of humans.
- Demonstration of decreased levels of lymphocytic DISC1 in patients with schizophrenia suggesting its potential for a schizophrenia biomarker.
- Demonstration that peripheral DISC1 levels are modulated by nicotine but not to the degree of interfering with schizophrenia diagnosis establishing its possible peripheral readout for psychotropic drug effects.
- Demonstration that administration of nicotine leads to decreased aggregated DISC1 in the rat mPFC.

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Drug monitoring

ABSTRACT

The Disrupted-in-Schizophrenia 1 (DISC1) protein plays a key role in behavioral control and vulnerability for mental illnesses, including schizophrenia. In this study we asked whether peripheral DISC1 protein levels in lymphocytes of patients diagnosed with schizophrenia can serve as a trait marker for the disease. Since a prominent comorbidity of schizophrenia patients is nicotine abuse or addiction, we also examined modulation of lymphocyte DISC1 protein levels in smokers, as well as the relationship between nicotine and DISC1 solubility status. We show decreased DISC1 levels in patients diagnosed with schizophrenia independent of smoking, indicating its potential use as a trait marker of this disease. In addition, lymphocytic DISC1 protein levels were decreased in smoking, mentally healthy individuals but not to the degree of overriding the trait level. Since DISC1 protein has been reported to exist in different solubility states in the brain, we also investigated DISC1 protein solubility in brains of rats treated with nicotine. Subchronic treatment with progressively increasing doses of nicotine from 0.25 mg/kg to 1 mg/kg for 15 days led to a decrease of insoluble DISC1 in the medial prefrontal cortex. Our results demonstrate that DISC1 protein levels in human lymphocytes are correlated with the diagnosis of schizophrenia independent of smoking and thus present a potential biomarker. Reduced DISC1 protein levels in lymphocytes of healthy individuals exposed to nicotine suggest that peripheral DISC1 could have potential for monitoring the effects of psychoactive substances.

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1. Introduction

Schizophrenia is a mental illness exclusively diagnosed clinically by the occurrence of positive, negative, and cognitive symptoms. Positive symptoms are, for example, hallucinations or delusions, negative symptoms include affective flattening, lack of motivation, whereas cognitive impairments comprise attention deficits and impaired working memory [1].

One comorbid behavioral characteristic of patients diagnosed with schizophrenia is the high prevalence for nicotine abuse by excessive smoking. Among patients with schizophrenia, 70–80%

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smoke in contrast to 20–30% of the general population and 30–50% in other psychiatric illnesses [2,3]. One attempt to explain this phenomenon has been the self-medication hypothesis of schizophrenia, stating that smoking and thereby nicotine administration serves to reduce particularly negative symptoms [4]. In support of this hypothesis it was shown that nicotine reversed sensory gating deficits, i.e. the inability to process sensory information in patients diagnosed with schizophrenia. This deficit, measured, e.g. by the diminished gating of the P50 auditory-evoked response to repeated stimuli, could be rescued by treatment with nicotine in patients and their non-affected family members [5,6] and thus presents an endophenotype. Nicotine administration or smoking also improved sensorimotor gating in rats [7] and in non-psychiatric human control subjects [8,9]. The sensory gating deficit in schizophrenia is genetically linked to chromosome 15q14 [10], the gene locus of the nicotinic α7 receptor subunit, interrelating schizophrenia and nicotine.

Disrupted-in-schizophrenia 1 (DISC1) is one of the best characterized vulnerability genes for psychiatric disorders. It was first discovered in a Scottish family in which carriers of a balanced chromosome t(1;11) (q42.1;q14.3) translocation are prone to mental illnesses of different clinical phenotypes ranging from schizophrenia and major depression to bipolar disorder, and anxiety disorders [11,12]. The translocation presumably leads to the expression of a C-terminally truncated protein named DISC1. Subsequent gene association studies corroborated the importance of the DISC1 locus in mental illness and substance abuse [13–15], however, importantly, not limited to one clinically defined disease entity, but as a risk factor to several mental illnesses [16–20]. Cell and molecular studies highlight its function as a scaffold protein [21] that interacts with a variety of proteins [22] for integrating cellular mechanisms like developmental control of neuronal migration, neuronal progenitor cell proliferation, signaling pathways, synaptic function, centrosome formation and neurite outgrowth, amongst others [23–26]. DISC1 transgenic animal models have clearly corroborated the key role of DISC1 in behavioral control, such as in working memory, locomotion and sensorimotor gating [27–33]. Our own previous findings in human *post mortem* brain material indicated that a subpopulation of mentally ill patients with schizophrenia or recurrent affective disorders was characterized by the presence of insoluble DISC1 protein in the brain, thus crossing current clinical diagnostic boundaries [34,35]. Subsequent *in vivo* and *in vitro* biochemical analyses investigating DISC1 protein assembly indicated that DISC1 builds oligomers and insoluble aggregates under specific physiological and pathological conditions [26,36,37].

A meta-analysis of several genome-wide association studies of smoking phenotypes indicated linkage of a single-nucleotide polymorphisms (SNP) in the 3′ region of the mental illness candidate gene DISC1 to earlier onset of smoking, although the analysis failed to reach significance due to multiple testing corrections [38].

In the present study we set out to test, whether the DISC1 protein may serve as a trait marker for schizophrenia by assessing DISC1 protein levels in lymphocytes derived from blood samples of patients diagnosed with schizophrenia and control subjects. We also investigated the role of DISC1 as a state marker by examining lymphocytic DISC1 protein levels in healthy smokers versus non-smokers. Plasma cotinine levels, the main metabolite of nicotine in the blood, of patients with schizophrenia and controls were measured in an attempt to correlate lymphocytic DISC1 levels and nicotine intake. Furthermore, to examine, whether nicotine can influence the solubility status of DISC1 in the brain, we measured levels of aggregated DISC1 in rats sub-chronically exposed for 15 days to up to daily 1 mg/kg nicotine.

2. Material and methods

2.1. Subjects and classification

Healthy subjects and patients diagnosed with schizophrenia were investigated as part of a clinical study comparing acute nicotine challenge with placebo in a neuroimaging setting (ClinicalTrial.gov Identifier: NCT00618280) [39]. The schizophrenia patients were clinically stable for more than six weeks. All patients and controls underwent a structured interview (SCID-1), and diagnosis was established according to DSM–IV criteria [40]. 78.4% of patients received antipsychotic monotherapy with either amisulpride, aripiprazole, fluphenazine, olanzapine, paliperidone, quetiapine, risperidone or ziprasidone. A small number of patients were treated with a combination of two antipsychotic drugs: flupentixol/quetiapine, haloperidol/quetiapine, olanzapine/ziprasidone or paliperidone/aripiprazole. Healthy controls were required to have no life-time diagnosis of schizophrenia, illegal drug or alcohol dependence. Exclusion criteria were: concomitant neurological diseases or any other medical condition that was considered as a potential confounder of the study, including a positive drug screen or a history of substance abuse during the last six months prior to participation in the study. In schizophrenia patients, assessment of current psychopathology was conducted with the Positive and Negative Syndrome Scale (PANSS; [41]). Nicotine dependency was verified using the Fagerstrom test (FTND [42]). Study participants were either habitual smokers as defined by a FTND score of >4, smoking 10–30 cigarettes per day or never-smokers who had smoked less than 20 cigarettes in their life-time. The study was approved by the Ethics committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf and the German regulatory agency for drug trials BfArM (Bundesarzneimittelbehörde) and under full consideration of the Declaration of Helsinki. Written informed consent was obtained from each study participant prior to the start of the study.

2.2. Animals

Animal experiments were performed in accordance with the German Animal Protection Law and were authorized by local authorities (LANUV NRW). Adult male Wistar rats were housed three animals per cage under standard laboratory conditions with a reversed day-night cycle and food and water access *ad libitum* (light from 6 am to 6 pm). After arrival in the animal facility, animals were allowed to habituate for two weeks before the injections started. Nicotine and saline treatments were given daily subcutaneously in the afternoon.

2.3. Preparation of lymphocytes from blood

Venous blood (10 mL) was collected in EDTA tubes (Sarstedt, Germany) and centrifuged at $1600 \times g$ for 10 min. The buffy coat between the upper plasma layer and the lower layer of packed erythrocytes was harvested and diluted 1:5 with Phosphate-buffered saline (PBS). The leukocyte suspension was carefully laid on top of the Ficoll-Paque Plus solution (GE Healthcare, Germany). Centrifugation at $600 \times g$ for 20 min resulted in separation of the mononuclear and polymorphonuclear cells into two distinct bands at the interphases. The lymphocyte ring in the upper layer was removed and washed twice with PBS and stored at −80 °C until further processing.

For the preparation of lymphocytes from blood of rats, blood was collected in EDTA tubes (Sarstedt, Germany) and isolation was performed according to manufacturer's instructions with the Ficoll-Paque Premium 1.084 solution (GE Healthcare, Germany). After

isolation the cell pellets were flash-frozen and stored at −80 °C until further processing.

2.4. Measurement of plasma cotinine

Cotinine is the main metabolite of nicotine [43]. Due to its longer half-life of 20 h, in contrast to nicotine which has a much shorter half-life of about 20 min, it is widely used as a biomarker of nicotine exposure. Based on the linear relationship between nicotine and cotinine levels in blood plasma, one can correlate smoking behavior to cotinine content in the blood [44].

Cotinine concentrations were determined using the commercial EIA based kit Inspec II (Mahasan Diagnostika, Germany). Plasma samples which had been stored at −80 °C were processed on 96-well microplates. All samples were diluted in water (1:100) and, in order to prevent blood clotting as the consequence of lowering the EDTA concentration in respect to the subsequent preparation steps, 25 μL of the diluted plasma aliquots were supplemented with 25 μL EDTA (20 mM, pH 7). After the addition of 100 μL enzyme conjugate, the samples were incubated for 30 min at room temperature. After six washing steps, substrate solution was added followed by a second 30 min incubation time. Color processing was stopped and the cotinine concentration which is inversely proportional to the color intensity was measured at 450 nm using the MRX microplate reader (Dynatech Laboratories, Germany). Test reliability was monitored using one negative and four positive calibrators in the concentration range of 0–50 ng/mL.

2.5. Western blot analysis of lymphocytes

Lymphocyte pellets were thawed on ice and immediately lyzed in VRL buffer: 50 mM HEPES (pH 7.5), 250 mM sucrose, 5 mM $MgCl_2$, 100 mM KAc, 2 mM PMSF (all Sigma–Aldrich, Germany), 2× Protease Inhibitor (Roche, Germany) supplemented with 1% Triton X-100, 1 mM PMSF (Sigma–Aldrich, Germany) and 40 U/mL DNaseI (Roche, Germany). After 30 min on ice, the lysate was incubated for 30 min at 37 °C for DNA digestion. Afterwards, the protein content of the lysate was determined using the *DC* Protein Assay Kit (Bio-Rad, Germany) and 30 μg of total protein per sample were loaded onto 10% SDS-PAGE gels. Afterwards the samples were blotted onto a 0.45 μm nitrocellulose membrane (GE Healthcare, Germany) for 16 h at 150 mA. After blotting, the membranes were directly treated with 100 mM KOH for 5 min at room temperature and subsequently blocked with 5% milk in PBS with 0.05% Tween-20 (PBST). DISC1 immunoreactivity was tested with the human DISC1 specific mAB 14F2. Analysis was done blind with regard to diagnosis.

Western blot procedure was identical for rat lymphocytes, but then the ratDISC1 specific polyclonal C-term antibody was used [45].

2.6. Isolation of lymphocytes by flow cytometry

Flow cytometry sorting of freshly isolated lymphocytes from peripheral blood with Ficoll-Paque Plus (GE Healthcare, Germany) was done by Cellsort, the Core Flow Cytometry Facility of the Medical School Düsseldorf, Germany. The resulting pure lymphocyte fraction was lyzed as described above and used for Western blot analysis.

2.7. Preclearing of 14F2 antibody

NHS-Activated Sepharose 4 Fast Flow (GE Healthcare, Germany) was activated with 1 mM HCl (VWR, Germany) according to manufacturer's instructions. Fifty microliter beads were coupled with 1 mg of either BSA (GE Healthcare, Germany) or recombinant DISC1 (amino acids 316-854) protein [34], both dialyzed to 10 mM sodium phosphate buffer (NaPi, pH 8), or buffer only for 16 h at 4 °C. Afterwards, beads were blocked with 100 mM Tris–HCl (pH 8.5) for 2 h at room temperature and washed with hybridoma cell medium (MEM media supplemented with FCS, Pen/Strep, HT supplement, L-Glutamine; all Gibco, Germany). For the preclearing, 2 mL fresh antibody supernatant of 14F2 was mixed with 2 mL of PBS supplemented with 0.05% Tween-20 (Sigma–Aldrich, Germany) and incubated with either NHS beads coupled to BSA, rDISC1 (316-854), or uncoupled and preincubated for 1 h at room temperature. The antibody solution was then centrifuged for 10 min at 2000 × *g* to pellet the beads and supernatant was used as primary antibody on the Western blots.

2.8. Quantitative expression analysis

Total RNA was prepared from human lymphocyte pellets with the RNeasy Mini Kit (Qiagen, Germany). From this preparation, 1 μg total RNA was used as template to synthesize cDNA using the random hexamer primers of the RevertAid First Strand Synthesis Kit (Thermo Scientific, USA) in a total volume of 20 μL. For quantitative Real-Time PCR, the cDNA template was diluted 1:10 and 5 μL were used as template for the qPCR reaction.

Primers targeting huDISC1 spanning exon 5 and 6: forward 5′-ACACCCCACTGAGAATGGAG-3′; reverse 5′-GTTGCTGCTCTTGCTCCTCT-3′ (300 nM each). Primers for the housekeeping gene ARF1: forward 5′-GACCACGATCCTCTACAAGC-3′; reverse 5′-TCCCACACAGTGAAGCTGATG-3′ (300 nM each). PCR conditions for both primer pairs: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min. Real-Time PCR was conducted with the StepOnePlus Real-Time PCR System (Applied Biosystems, Germany) and the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Germany). The data was processed with the StepOne Software v2.3 and DISC1 expression was normalized to expression level of the housekeeping gene ARF1 and set in relation to a control cDNA sample that was used in all individual qPCR runs.

2.9. Nicotine treatment of rats

For testing the effect of nicotine on the aggregation of endogenous DISC1, male Wistar rats were treated for 15 days with either saline (1 mL/kg bw injection volume) or increasing amounts of nicotine. For the injections (−)-Nicotine hydrogen tartrate salt (Sigma–Aldrich, Germany) was dissolved in PBS calculated on the basis of the nicotine component. Animals were weighed daily before the injections. The nicotine group was treated for 5 days with 0.25 mg/kg, the next 5 days with 0.5 mg/kg and last 5 days with 1 mg/kg nicotine subcutaneously. Blood withdrawal and brain dissection was carried out 24 h after the last injection.

2.10. Aggregome assay of rat brain material

For the preparation of the insoluble aggregome of Wistar rats, brain tissues underwent a low-stringency aggregome assay due to the low expression of endogenous rat DISC1.

For the preparation of 10% homogenate, the mPFC was homogenized in ice-cold VRL buffer (see Section 2.5). The homogenate (200 μL) was mixed with 100 μL of buffer A3: 50 mM HEPES pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 100 mM KAc, 15 mM GSH, 2 mM PMSF, 1× PI, 3% NP-40, 0.6% sarcosyl and 120 U/mL DNaseI and rotated overnight at 4 °C to digest the DNA. Next day, 450 μL of a 2:1 VRL:A3 mix and 530 μL buffer B3 (50 mM HEPES pH 7.5, 2.3 M sucrose, 5 mM GSH, 1% NP-40, 0.2% sarcosyl; final sucrose concentration of 1.1 M) was added to the tube. Samples were mixed by vortexing and ultracentrifuged for 45 min at 4 °C and 100,000 × *g* (TLA-55 rotor in an Optima ultracentrifuge; Beckman Coulter).

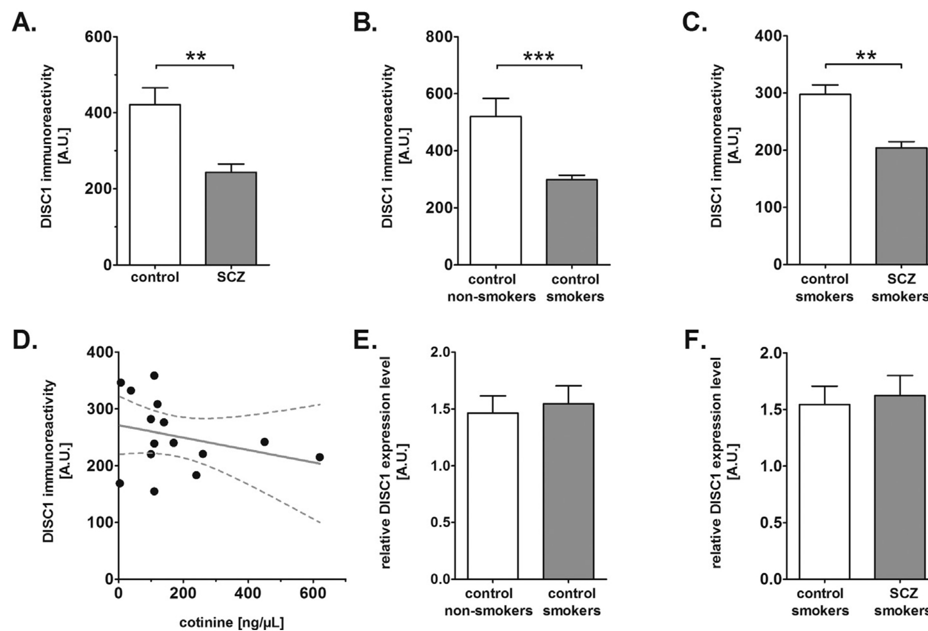

Fig. 1. Altered DISC1 immunoreactivity in lymphocytes of schizophrenic (SCZ) and control patients and the influence of smoking behavior. (A) Comparison of the 100 kDa DISC1 immunoreactive band in lysates of lymphocytes derived from blood of either schizophrenic ($n = 12$) or control patients ($n = 18$). SCZ cases display significantly reduced DISC1 band intensity. Unpaired two-tailed t-test $^{**}p = 0.004$ (means ± SEM). (B) Comparison of DISC1 band intensity in lysates of control group only. Control cases included smokers ($n = 8$) and non-smokers ($n = 10$). Lymphocytes from smoking controls had significantly less DISC1 immunoreactivity than non-smoking controls. Unpaired two-tailed t-test $^{**}p = 0.008$ (means ± SEM). (C) Comparison of the DISC1 protein levels between smoker groups. SCZ smokers ($n = 8$) express less DISC1 than control smokers ($n = 8$). Unpaired two-tailed t-test $^{*}p = 0.037$ (means ± SEM). (D) No significant correlation between DISC1 Western blot signal and cotinine content of plasma derived from smokers. DISC1 immunoreactive signal is plotted against cotinine content in the blood plasma of control and SCZ smokers. Regression line (full line) and 95% confidence interval (dashed line) are depicted. Pearson's correlation coefficient $r = -0.287$, $p = 0.300$. (E) No DISC1 expression changes in lymphocytes from control non-smokers ($n = 10$) and control smokers ($n = 8$). Quantitative Real-Time PCR did not show a difference of DISC1 expression between the two groups. Unpaired two-tailed t-test $p = 0.718$ (means ± SEM). (F) Quantitative Real-Time PCR of lymphocyte samples from control smokers and SCZ smokers. SCZ smokers had the same DISC1 expression as controls. Unpaired two-tailed t-test $p = 0.747$ (means ± SEM).

Buffer B3 was mixed 7:3 with buffer C3: 50 mM HEPES (pH 7.5), 5 mM GSH, 1% NP-40, 0.2% sarcosyl (total sucrose concentration 1.6 M). The pellet was resuspended in 700 μL of this buffer and after addition of another 700 μL the sample was spun again at 4 °C and 100,000 × g. The pellet was resuspended in 1 mL buffer D3: 50 mM HEPES (pH 7.5), 1.5 M NaCl, 5 mM GSH. After another ultracentrifugation, the pellet was washed in 1 mL E3 (50 mM HEPES pH 7.5, 0.2% sarcosyl), ultracentrifuged and the final pellet was dried in a speedvac centrifuge (Eppendorf, Germany). The insoluble aggregome pellet was taken up in 2× SDS-loading buffer, separated by SDS-PAGE and blotted onto 0.45 μm nitrocellulose membrane. Endogenous rat DISC1 was detected with the polyclonal C-term ratDISC1 antibody.

2.11. Densitometric analysis and statistics

Densitometric analysis was performed using the ImageJ 10.2 software (National Institute of Health, USA). For statistical analyses the IBM SPSS Statistic 20 program was used.

3. Results

3.1. Decreased DISC1 immunoreactivity in lymphocytes of patients diagnosed with schizophrenia compared to healthy controls

To investigate DISC1 levels in mononuclear cells from schizophrenic patients and healthy controls, cells were purified on a Ficoll gradient, lyzed and DISC1 was detected by immunoblotting with the monoclonal antibody 14F2 highly specific for human DISC1 [35]. Densitometric analysis blind to clinical diagnosis of samples of the predominant approximately 100 kDa DISC1 immunoreactive band revealed a significant reduction of DISC1 protein in schizophrenia (SCZ) cases compared to mentally healthy controls (Fig. 1A).

We also performed a separate flow cytometry analysis with the goal to identify the cell population within mononuclear cells that was the origin of the DISC1 signal. We identified the main DISC1 signal in a mainly lymphocytic cell population with only minor content of monocytes and granulocytes (data not shown). Western blotting of sorted lymphocytes revealed a strong DISC1 signal (Fig. 2A, left panel) comparable to that of the whole lysates of the Ficoll purified lymphocytes. We, therefore, conclude that the majority of the DISC1 immunoreactivity stems from lymphocytes.

To validate the identity of the approximately 100 kDa immunoreactive band as DISC1, we precleared mAB 14F2 with recombinant human DISC1 (316-854) protein coupled to NHS beads. Subsequent incubation of blots with the precleared antibody supernatant revealed a signal reduction on Western blots both of cell lysates transfected with full length human DISC1, as well as the lymphocytic 100 kDa immunoreactivity (Fig. 2B), indicating that the approximately 100 kDa immunoreactive band on Western blots is a lymphocyte-specific form of DISC1.

3.2. DISC1 protein levels in lymphocytes of mentally healthy smokers

When we investigated DISC1 protein levels in lymphocytes of smokers and non-smokers in healthy controls in a blinded analysis, we observed less DISC1 immunoreactivity in control subjects that were smokers than in non-smokers (Fig. 1B). This could indicate that, to some degree, DISC1 protein levels are modulated by smoking, but not to the extent of influencing the overall effect of schizophrenia itself since the smoking cohort of patients diagnosed

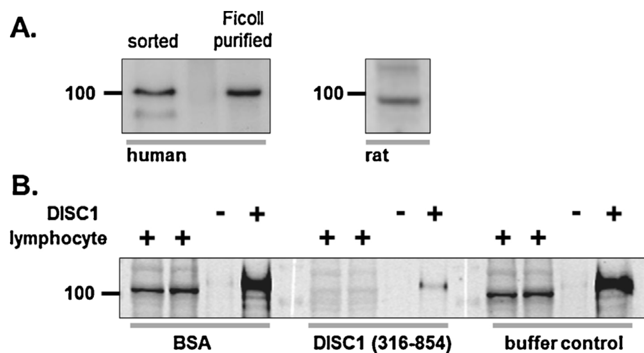

Fig. 2. Validation of the 100 kDa DISC1 immunoreactive band in lymphocytes. (A) Derivation of the 100 kDa DISC1 band from lymphocytes. Flow cytometry sorting of the Ficoll purified lymphocyte fraction from peripheral blood and subsequent Western blotting showed that the DISC1 signal of the mAB 14F2 results exclusively from lymphocytic DISC1 species (left panel). Also in lymphocytes from the rat a 100 kDa endogenous rat DISC1 species can be visualized by the polyclonal C-term antibody (right panel). (B) Reduction of the 100 kDa DISC1 band intensity by preclearing of the mAB 14F2 with recombinant DISC1 protein. Depicted are two different lymphocyte samples, lysates from SH-SY5Y cells without (−) or with (+) expression of human full-length DISC1. Preincubation of the mAB 14F2 with NHS-beads coupled to recombinant DISC1 (316-854) led to a decrease of the 100 kDa band as well as the DISC1 overexpression control, whereas preincubation with NHS coupled to BSA or buffer only did not lead to a diminished signal intensity.

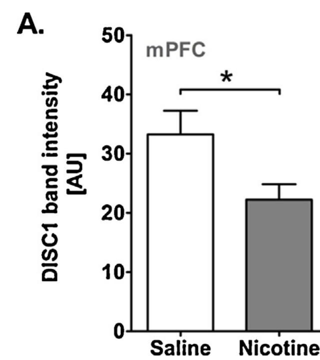

Fig. 3. Nicotine-dependent reduction of DISC1 aggregation in rat brains. Densitometric analysis of the 200 kDa endogenous ratDISC1 species in the mPFC of saline ($n = 6$) and nicotine-treated animals ($n = 7$) detected with the polyclonal C-term rat-DISC1 antibody. Treatment with nicotine leads to significantly reduced aggregation of endogenous DISC1. Unpaired two-tailed t-test *$p = 0.037$ (means ± SEM).

with schizophrenia still had significantly less DISC1 immunoreactivity than control smokers (Fig. 1C).

To investigate the relation between nicotine and lymphocytic DISC1 levels in a more quantitative way, we determined the levels of cotinine in the plasma of the patients at the same time the lymphocytes were taken.

We did not identify a significant correlation between plasma cotinine levels and lymphocytic DISC1 protein levels in either control smokers (Pearson's correlation: $p = 0.072$, $r = -0.664$) or schizophrenic smokers analyzed separately (Pearson's correlation: $p = 0.146$, $r = 0.610$) or by combining the smoking groups (Pearson's correlation: $p = 0.300$, $r = -0.287$), indicating that the difference in DISC1 levels of patients with schizophrenia and healthy controls is not due to a different quantity of nicotine intake (Fig. 1D).

For these same patients, we also determined expression of DISC1 mRNA in lymphocytes by quantitative Real-Time PCR, but did not find significant differences (Fig. 1E and F), neither by comparing control smokers and non-smokers, nor comparing control smokers and SCZ smokers.

3.3. Nicotine-dependent reduction of aggregated DISC1 in rat brains

To investigate the possible modulatory effect of nicotine on DISC1 protein assembly in the brain, adult wild type Wistar rats were treated for 15 subsequent days with either saline or nicotine solution (increasing doses every 5 days up to 1 mg/kg). Preparation of the insoluble aggregome displayed a significant reduction of an endogenous 200 kDa DISC1 species in the frontal cortex in nicotine treated animals compared to vehicle-treated controls (Fig. 3). Although endogenous DISC1 expression and thus, aggregation, is relatively low in rats, systemic nicotine administration had an effect on its aggregation propensity in the brain. When, in the same rats, DISC1 protein levels in lymphocytes were investigated as described above, a prominent 100 kDa band was detectable with the rat DISC1 specific polyclonal C-term antibody (Fig. 2A, right panel), but no reduction in DISC1 protein levels was detected between the two treatment groups (data not shown).

4. Discussion

In this study, we investigated whether peripheral DISC1 protein levels in lymphocytes have the potential to serve as a candidate biomarker of schizophrenia. We found a significant reduction of DISC1 levels in lymphocytes derived from patients, apparently not through a change in expression but rather due to a change in its biological half-life time. In addition, it was found that lymphocytic DISC1 levels differed between mentally healthy smokers and non-smokers independent of the clinical diagnosis schizophrenia.

Our results show that, at least for DISC1 protein levels, lymphocytes can be seen as windows to the brain, mirroring brain disease. A subset of mental illness patients has already been characterized by the presence of insoluble DISC1 protein in *post mortem* tissue [34]. Here we showed that DISC1 levels are also dysregulated in one peripherally accessible tissue, namely blood, of patients diagnosed with schizophrenia.

An interesting observation is that in this investigation, lymphocytes of patients diagnosed with schizophrenia revealed differential DISC1 protein levels whereas in a previous investigation, EBV-immortalized lymphoblasts did not show differences in DISC1 protein levels [46]. This could indicate that yet unidentified factors in the cellular and molecular machinery involved in maintaining cell proliferation are critical for schizophrenia-dependent DISC1 protein levels.

In our study, lymphocytic DISC1 levels are also a state marker, as the amount of DISC1 protein was decreased in control smokers, perhaps due to nicotine administration, although other covariates from smoking cannot be excluded. But this range of modulation did not exceed its value as trait marker for schizophrenia. These findings indicate that, to some extent, administration of the psychoactive substance nicotine is reflected in the peripherally accessible disease marker, DISC1 and, therefore, has a potential use as a marker in monitoring the effects of externally administered psychoactive substances in individuals.

The correlation between cotinine content, the main metabolite of nicotine, and DISC1 protein levels in the blood plasma of schizophrenics and controls was not significant (Fig. 1D), indicating that the intensity of smoking is related to DISC1 levels only to a minor degree. Other smoking- or schizophrenia-related factors, which have yet to be determined, may account for the reduced amount of DISC1. It is also conceivable that decreased DISC1 protein levels are related to a common biological cause for both schizophrenia and nicotine abuse or addiction.

It is also of importance that the difference in DISC1 protein levels was seen solely on the protein level, not on the mRNA level. This result points towards a cellular mechanism affecting

posttranslational modifications of DISC1, its protein clearance or half-life in the cell rather than changes in expression regulation by differential transcription the DISC1 protein. This is consistent with previous reports of differential posttranslational processing that potentially leads to misfolding and/or aggregation of DISC1 [33]. One caveat of our data is that although they were gathered in a blinded fashion, the case numbers are relatively low, so the results of this study need to be validated in a larger cohort.

In the second part of our study corroborating biological effects of smoking or nicotine on DISC1 protein expression and/or posttranslational modifications, we showed that sub-chronic nicotine treatment of rats changed DISC1 insolubility in the rat brain. A subset of mental illness patients were previously characterized by insoluble DISC1 species in *post mortem* brain material [34,35]. The decrease of DISC1 aggregation in the mPFC of wild type rats treated with nicotine demonstrates that insoluble DISC1 assembly can be modulated by administration of small molecules. Although it is not clear whether nicotine actually decreases the existing aggregates or if it prevents its *de novo* assembly, our data show that nicotine decreases the aggregome load in the mPFC.

Other studies have shown that chronic nicotine treatment of rats favors an upregulation of ubiquitin and heat-shock proteins as well as members of the proteasome and chaperone pathways in the mPFC [47,48]. As protein degradation is a dynamic process, aggregated DISC1 protein could be cleared by the cell in a faster rate due to an upregulated proteasomal system to restore the homeostasis of the cell.

Reduction of aggregated DISC1 could also occur by preventing further accumulation of aggregated protein. Nicotine was identified to inhibit the aggregation of proteins prone to self-assembly like Aβ *in vitro* [49,50]. Therefore chronic nicotine might prevent the formation of further DISC1 aggregation rather than increasing the clearance of previously formed aggregated species.

Nicotine treatment of rats did not lead to changes in lymphocytic DISC1 levels as seen in the comparison of smoking versus non-smoking human controls. This suggests that 15 days of exposure to nicotine may not be sufficient and a chronic exposure to nicotine could be crucial to change lymphocytic gene expression or protein half-life. Also, the start of nicotine exposure might influence DISC1 levels, as the human participants normally started smoking during adolescence and were tested as adults, whereas nicotine treatment of the adult rats started after adolescence [51]. Alternatively, these data may support our previous reasoning that a common yet unidentified factor may account for both decreased DISC1 levels and schizophrenia, as well as nicotine abuse that is restricted to human pathophysiology.

Taken together, our results suggest that DISC1 expression in lymphocytes is a potential trait marker for schizophrenia. In addition, it represents a state marker for monitoring the effects of exogenously administered psychoactive substances. The change in lymphocytic DISC1 protein levels emphasizes that, at least for some candidate proteins, lymphocytes can represent a window to the brain and brain disorders. The influence of nicotine on DISC1 protein aggregation in the brain highlights its potential pharmacological reversibility and makes the nicotinic receptors in the brain a potential pharmacological target for DISC1-dependent disease [36].

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