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Behavioral phenotypes of a transgenic rat overexpressing the full-length non-mutant human *DISC1* gene

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I Contents

Ⅱ Abstract

The *Disrupted-in-schizophrenia 1 (DISC1)* gene has been linked to psychiatric disorders such as schizophrenia and major depression. Manipulation of the *DISC1* gene in mice was shown to result in cognitive impairments and alteration of neuroanatomy in the cortex. The transgenic DISC1 (tgDISC1) rat, which overexpresses the human *DISC1* gene, has been reported to have impaired motor learning ability, hyperexploratory behaviors and an alteration of the brain's dopamine(DA)ergic system. Additionally, *DISC1* overexpression decreased the density of DAergic neurons in the substantia nigra and reduced the DAergic fibers in the striatum. In the current study, we addressed the question of whether the *DISC1* gene influences behavioral functions, including attention, memory and emotion, and whether DA is involved in the connection between the *DISC1* and behavioral malfunctions. In Experiment 1, 14-15 months old male tgDISC1 rats and wild type (WT) controls were subjected to the novel object preference (NOP) test and object-based attention test (OBAT) for assessing short- (1 h) and long (24 h)-term memory and attention-like behaviors. The results showed that tgDISC1 rats were deficient in long-term memory and attention-like behaviors. In Experiment 2, a different group of tgDISC1 rats was administered dopamine (3 mg/kg) (IN-DA) or its vehicle intranasally prior to the NOP or OBAT. IN-DA reversed the impairments of cognition in both the NOP and OBAT. In Experiment 3, a further cohort of tgDISC1 rats were used to analyze the neurotransmitters, DA, serotonin, noradrenaline and acetylcholine, via post-mortem analysis in a variety of brain regions. TgDISC1 rats had lower levels of DA in the neostriatum, amygdala and hippocampus, of serotonin in the amygdala, of noradrenaline in the amygdala and of acetylcholine in the neostriatum, nucleus accumbens, amygdala, and hippocampus, but more serotonin in the nucleus accumbens. In Experiment 4, 9-month old tgDISC1 rats and WT controls (n = 12 each) were subjected to the open field, spontaneous alternation behavior test with T-maze and elevated-plus maze in order to assess attention-related and emotional

behaviors. The tgDISC1 rats exhibited locomotor activity comparable to that of the WT rats. However, they displayed abnormal alternating and emotional behaviors. These results suggest that, first, overexpressing the *DISC1* gene leads to a variety of behavioral and neurotransmitter changes that may have relevance to the hypothesized role of the *DISC1* gene in mental disorders. Secondly, IN-DA reversed the impairments of long-term memory and attention-like behaviors in tgDISC1 rats, indicating that DA may play a crucial role in *DISC1*-related behavioral and neural functions.

Ⅲ Zusammenfassung

Das *Disrupted-in-schizophrenia 1 (DISC1)* Gen wurde mit verschiedenen Krankheitsbildern, wie der Schizophrenie und der Depression, in Verbindung gebracht. Studien haben gezeigt, dass die Manipulation des *DISC1* Gens in Mäusen kognitive Defizite und Veränderungen in der Neuroanatomie des Kortex vursacht. Es wurde gezeigt, dass die transgene DISC1 (tgDISC1) Ratte, welche das *DISC1* Gen überexprimiert, Defizite in der motorischen Lernfähigkeit, ein vermehrtes Explorationsverhalten, und auch eine Veränderung im zentralen dopaminergen System aufweist. Zusätzlich führte die *DISC1* Überexprimierung zu einer verringerten Dichte der dopaminergen Neurone in der Substantia Nigra and einer reduzierten Anzahl an dopaminergen Fasern im Striatum. In der gegenwärtigen Studie haben wir die Frage gestellt, ob das *DISC1* Gen verschiedene Verhaltensfunktionen, wie die Aufmerksamkeit, das Gedächtnis, und die Emotionen beeinflusst, und auch, ob Dopamin (DA) die Malfunktionen in den Zusammenhängen zwischen *DISC1* und dem Verhalten vermittelt. In Experiment 1 wurden 14-15 Monate alte tgDISC1 Ratten und Wildtypen (WT) als Kontrolle in einem "Novel Object Preference (NOP)" Test und in einem Test für Aufmerksamkeit (genannt OBAT), welches sich die Exploration von Objekten zu Nutze macht, getestet, um das Kurz- (1 Std.) und Langzeitgedächtnis (24 Std.) und auch Aufmerksamkeitsprozesse zu erforschen. Die Ergebnisse zeigten, dass die tgDISC1 Ratten so wie im Langzeitgedächtnis als auch in den Aufmerksamkeitsprozessen Defizite aufwiesen. In Experiment 2 wurde eine neue Gruppe von tgDISC1 Ratten kurz vor dem NOP oder OBAT entweder mit Dopamin (3 mg/kg) (IN-DA) oder mit Vehikel intranasal behandelt. IN-DA machte die Verhaltensdefizite im NOP and OBAT rückgängig. In Experiment 3 wurde ein weiterer Kohort von tgDISC1 Ratten auf die Neutransmitterkonzentrationen von Dopamin, Serotonin, Noradrenalin und Azetylcholin in verschiedenen Hirnregionen mit einer post-mortem Analyse untersucht. TgDISC1 Ratten hatten weniger Dopamin im Neostriatum,

Hippokampus und der Amygdala, und weniger Serotonin und Noradrenalin in der Amygdala. Zusaetzlich war die Konzentration von Azetylcholin niedriger im Neostriatum, Nucleus Accumbens, Hippokampus und der Amygdala. Die Konzentration von Serotonin war im Nucleus Accumbens jedoch höher. In Experiment 4 wurden 9 Monate alte tgDISC1 und WT Ratten (n = 12 pro Gruppe) einem Offenfeld, dem T-Maze zur Messung des spontanen Wechselverhaltens, und dem Elevated-Plus Maze ausgesetzt, um Aufmerksamkeitsprozesse und emotionales Verhalten zu messen. Die lokomotorische Aktivität der tgDISC1 Ratten ähnelte der der Kontrolltiere. Das spontane Wechselverhalten und das emotionale Verhalten war bei den tgDISC1 Tieren jedoch abnormal. Diese Ergebnisse weisen erstens daraufhin, dass die Überexprimierung des *DISC1* Gens zu verschiedenen Veränderungen im Verhalten und in den Konzentrationen von Neurotransmittern fuehrt, welche ein Relevanz fuer die hypothetische Rolle des *DISC1* Gens bei psychiatrischen Krankheiten haben. Und zweitens hat IN-DA die Defizite von tgDISC1 Ratten im Langzeitgedächtnis und bei Aufmerksamkeitsprozessen rückgängig gemacht, welches darauf hinweist, dass Dopamin eine zentrale Rolle bei Verhaltens- und neuronalen Funktionen spielt, die durch *DISC1* vermittelt werden.

Ⅳ Abbreviations

ACh	acetylcholine
ADHD	attention deficit/hyperactivity disorder
DISC1	disrupted-in-schizophrenia 1
DN	dominant-negative
DA	dopamine
D2R	dopamine 2 receptor
DAT	dopamine transporter
EC	electrochemical detection
EPM	elevated plus-maze
HPLC	high-performance liquid chromatography
IN	intranasal
NA	noradrenaline
NE	norepinephrine
NOP	novel object preference
OBAT	object-based attention test
5-HT	serotonin
SAB	spontaneous alternation behavior
SABt	spontaneous alternation behavior test with T-maze

SNP	single-nucleotide polymorphism
tg	transgenic
WT	wild type

1 Introduction

1.1 General background of *DISC1* gene

1.1.1 From cell to tissue

The link between the *disrupted-in-schizophrenia 1 (DISC1)* gene and psychiatric disorders has been reported since it was cloned and named in 2000 (Millar et al., 2000). The *DISC1* gene is widespread within the brain and peripheral tissues and plays a critical role during brain development (Austin et al., 2004), including neurite outgrowth (Ozeki et al., 2003), neuronal migration (Steinecke et al., 2014) and the regulation of neurogenesis (Ye et al., 2017). DISC1 expression was found in the dentate gyrus of the hippocampus, temporal and parahippocampal cortex in post-mortem human brains (Lipska et al., 2006). DISC1 expression was also found in the dentate gyrus of the hippocampus, cerebellum, cerebral cortex, olfactory bulbs via RAN *in situ* hybridization of adult mouse brains (Miyoshi et al., 2003). In adult and fetal human tissues, DISC1 expression was found in heart, brain, placenta, kidney, pancreas, limb and liver via detecting RNA and protein level (Millar et al., 2000; James et al., 2004). In adult rats, DISC1 proteins were detected in brain, heart, liver and kidney. In adult brains, high densities of protein were detected in the olfactory bulb, the cerebral cortex and hippocampus, and lower expression in the cerebellum (Ozeki et al., 2003). Taken together, these data suggested that the *DISC1* gene plays an important role during brain development and maintenance of the adult brain function.

1.1.2 The Scottish family

The *DISC1* gene was initially found in a Scottish family (Jacobs et al., 1970). Among the 58 members investigated, 29 were translocation carriers of t(1;11)(q42.1,q14.3), which is a breakpoint within the *DISC1* gene. Of these, 21 were diagnosed with mental illnesses (7 with schizophrenia, 1 with bipolar disease, 10 with recurrent major depression and 3 with minor

depression), based on the Diagnostic and Statistical Manual of Mental Disorders criteria by a long-term follow-up study (Blackwood et al., 2001). The results indicated a strong association between the *DISC1* gene and psychiatric disorders. To date, the translocation carriers in this family were shown to have structural changes, including the reduction of cortical thickness in the left temporal lobe, reduction of gyrification in prefrontal cortex, increased activation in the caudate nucleus and reduction of glutamate concentration in the right dorsolateral prefrontal cortex by brain imaging (Thomson et al., 2016). These changes were correlated with general psychopathology, including severity of positive psychotic symptom. Thus, the above findings have indicated the *DISC1* gene as a high impact factor for psychiatric disorders.

1.1.3 DISC1 expression in other populations

Several studies have reported linkage between the *DISC1* gene and psychiatric disorders in other populations. In a genome-wide study, Hovatta *et al.* (1999) found three markers, D1S2141, D1S491 and D1S2891, which were located in the chromosome region 1q22-44, to be positively linked with schizophrenia (Hovatta et al., 1999). Linkage analysis of schizophrenia with markers located in the 1q22–44 region in Taiwanese families revealed that the markers D1S1679, D1S251 and D1S2836 showed positive association (Hwu et al., 2003).

On the other hand, Lipska *et al*. (2006) used quantitative real-time PCR to measure the DISC1 mRNA in post-mortem brain samples (Clinical Brain Disorders Branch, NIMH) and found no difference in the expression of DISC1 mRNA or the risk DISC1 single-nucleotide polymorphisms (SNPs) (hCV219779, rs821597, rs821616) in the hippocampus and dorsolateral prefrontal between schizophrenia patients and controls. However, the results of DISC1 protein immunoreactivity in the hippocampus showed a significant increase in schizophrenia patients (Lipska et al., 2006). Norlelawati *et al.* (2015) examined 11 SNPs within or related in *DISC1* gene (rs821597, rs4658971, rs843979, and rs821616, rs1538979,

rs2812385, rs4658890, rs1407599, rs16854957, and rs2038636, rs2509382) in schizophrenia patients and controls in Malaysia. This case-control study showed that two SNPs (rs4658971 and rs1538979) were associated with schizophrenia. And, rs2509382, which is located at 11q14.3, where the mutual translocation region of the DISC1 translocation [t(1;11)(q42.1; q14.3)] is, showed susceptibility among male schizophrenia patients. The outcome supports the hypothesis that *DISC1* gene is a marker of schizophrenia in Malaysia population (Norlelawati et al., 2015). Despite a few negative results, the accumulating research suggests a positive linkage between the *DISC1* gene and psychiatric illness.

1.2 Behavioral tests in DISC1 mouse models

1.2.1 Behavioral models in the affective domain

For investigating neurobiological dysfunctions underlying psychiatric disorders and developing potential therapeutic strategies, a series of mouse models of *DISC1* gene have been generated through genetic engineering, since the *DISC1* gene is consider a candidate gene for psychiatric disorders, such as schizophrenia and major depressive disorder (Thomson et al., 2016). Various behavioral tests have been used for studying affective and cognitive dysfunctions related to psychiatric disorders (Aleman et al., 1999; Rose and Ebmeier, 2006; Carbon and Correll, 2014).

The forced swim test is commonly used for assessing depressive-like behavior in rodents (Slattery and Cryan, 2012). The predominant behavioral marker is immobility, which means the animal floats in the water without struggling and keeps its head above water. The *DISC1* gene is considered an impact factor in depression according to the study of the Scottish pedigree. The Q31L *Disc1* mutant mouse, exhibited more immobility in the forced swim test (Clapcote et al., 2007). Another mouse which expressed the dominant-negative (DN) of DISC1 (DN-DISC1), also showed high immobility behavior (Hikida et al., 2007), consistent

with the results of the Q31L mice. However, another *Disc1* mutant, L100P, showed no difference in immobility compared to control mice (Clapcote et al., 2007; Cui et al., 2016). Shoji *et al.* (2012) failed to repeat the results of depressive-like behavior in the Q31L *Disc1* mouse (Shoji et al., 2012).

Anxiety, is commonly assessed via the elevated plus-maze test (Walf and Frye, 2007). The primary behavior representing anxiety-like level is the activity in open arm (duration and/or entries). High open arm activity reflects anti-anxiety behavior. Haploinsufficiency mice lacking exons 2 and 3 of the *Disc1* gene, spent more time in the open arms than control mice (Kuroda et al., 2011). However, in the Q31L and L100P mutant mice no such effects were found (Shoji et al., 2012; Cui et al., 2016). The Light/Dark box is another widely used test to estimate anxiety-like behavior in rodents (Takao and Miyakawa, 2006). L100P mutant mice traveled a longer distance in the light chamber and frequently transited between the chambers in comparison with control mice (Shoji et al., 2012). As with indices of depression, most experiments did not find difference between mutants and control animals in anxiety-like behavior. Also, in the study of the Scottish family, few of the members had anxiety issues. These results suggest that *DISC1* gene may not the main risk factor for anxiety.

1.2.2 Behavioral models in the cognitive and social domain

Cognitive dysfunctions and social problems are concomitant with psychiatric disorders (Etkin et al., 2013). Cognitive and social behaviors can also be assessed in rodents. Commonly used tests include the delayed non-match to place task, t-maze forced alternation task and alternation behavior in the Y-maze for assessing working memory, and the water maze, novel place preference test and novel object recognition test for measuring short- or long-term memory. Social interaction and social preference test are used to assess social behaviors.

Briefly, in the delayed non-match to place task, animals should make a choice opposite

to the one from the previous trial after a delay (Aultman and Moghaddam, 2001). Koike *et al.* (2006) generated mice with *mDisc1* gene which abolished full-length protein production. The *Disc1* mutants failed to make correct choices after a 15 sec or 30 sec delay compared to the control mice (Koike et al., 2006). Clapcote *et al.* (2007) employed the t-maze forced alternation task to test working memory in both Q31L and L100P mutant mice. In the training phase, L100P mice needed more training days to reaching the criterion (70% correct). In the test phase, the Q31L and L100P mice made fewer correct choices after 5 sec or 15 sec delays compared to control mice (Clapcote et al., 2007). In addition, female mutant human DISC1 transgenic mice displayed spatial working memory deficits in the water maze test, while the male mutants displayed intact spatial working memory (Pletnikov et al., 2008). However, inconsistent results appeared in the same or different behavioral models. A DN form of a DISC1 transgenic mouse model exhibited no significant difference between transgenic mice and wild type (WT) mice in spatial memory in the Y-maze (Hikida et al., 2007). Q31L and L100P mice showed no differences in alternation behaviors in the t-maze forced alternation task compared to their controls (Shoji et al., 2012). Furthermore, comparable spatial memory in the water maze was found in the L100P mouse model and its controls (Cui et al., 2016).

The results of novel object recognition tests revealed that DN-DISC1 mutant mice displayed similar exploratory behaviors to control mice after either 3h- or 24h-delays (Kaminitz et al., 2014). However, L100P mice spent comparable time exploring a familiar and novel object in the test trial after a 1h-interval; whereas, the WT mice spent more time exploring the novel object. In the novel place preference test, L100P mice also failed to distinguish the object at the new location from the object at the old location in the test trial after 24h-interval (Cui et al., 2016).

Abnormal social behaviors have also been observed in mutant mice (Pletnikov et al., 2008; Kuroda et al., 2011; Shoji et al., 2012; Johnson et al., 2013; Kaminitz et al., 2014).

Male mutant human DISC1 mice exhibited changes in social interaction. They spent more time attacking or biting and less time sniffing unfamiliar mice than controls (Pletnikov et al., 2008). *Disc1* mutant mice which were lacking exons 2 and 3, exhibited increased social interaction compared with control mice (Kuroda et al., 2011). However, in the Q31L and L100P models, animals showed no differences in social interaction between genotypes (Shoji et al., 2012; Cui et al., 2016). In terms of sociability in the three-chamber social interaction test, DN-DISC1 mutant mice displayed no preference between the empty chamber and a stranger mouse (Johnson et al., 2013; Kaminitz et al., 2014), and similar results were found with the Q31L and L100P mice (Shoji et al., 2012). On the other hand, DN-DISC1 mutant mice preferred the familiar mouse to the novel one in the social novelty preference test (Johnson et al., 2013; Kaminitz et al., 2014), while the Q31L and L100P mice exhibited similar preference between the familiar and novel mouse (Shoji et al., 2012). Social problems are common in patients with psychiatric disorders. The above results demonstrated that the mutated *DISC1* gene could lead to abnormal social behaviors.

1.2.3 Behavioral models in the locomotor and sensorimotor domain

Locomotor activity is used to assess psychomotor agitation (Hikida et al., 2007). Transgenic mouse models and point mutation mouse model exhibited hyperactivity in the open field compared to controls (Hikida et al., 2007; Pletnikov et al., 2008; Lipina et al., 2010). However, as in the other behavior tests above, there were inconsistent results. The mutant mice traveled comparable distance to control mice (Koike et al., 2006; Shoji et al., 2012; Kaminitz et al., 2014; Cui et al., 2016).

It is well-known that patients with schizophrenia have prepuls-inhibition deficits (Geyer et al., 2001). The prepulse-inhibition test, as an index of schizophrenia-like behavior, was applied in various DISC1 mutate mouse models. The mutant mice exhibited the deficit consistently (Clapcote et al., 2007; Hikida et al., 2007; Lipina et al., 2010; Kuroda et al.,

2011), although there were a few inconsistent results (Koike et al., 2006; Shoji et al., 2012).

The above studies have led to positive results and some contrary results in affective, cognitive, social, locomotor and sensorimotor behaviors in transgenic DISC1 mice. Nevertheless, multiple factors should be considered, like the different protocols, behavioral tests, conditions, gender and so on. On the other hand, relatively few behavioral studies have so far been carried out in the *DISC1* gene field. Overall, several mutant *DISC1* gene mouse models have exhibited abnormal behaviors in mice and the results tend to support the hypothesis that DISC1 is a risk factor for psychiatric disorders.

1.3 DISC1 rat models

Several studies have attempted to manipulate the *DISC1* gene in rats because of the advantages of rats over mice for neurophysiological studies. In general, the size of rats provides the advantages for detailed brain analysis and the physiology of rats is closer to the human. In terms of behavioral testing, rats are easier to handle and less stressed than mice by human contact (Ellenbroek and Youn, 2016). Based on the susceptibility of the *DISC1* gene to environment stressors (Cash-Padgett and Jaaro-Peled, 2013), in the study of Gamo *et al*. (2013), rats were subjected to knocked down *DISC1* gene in the prefrontal cortex via active viral infusion surgery after they were trained in the delayed alternation spatial working memory task in a T-maze. One-hour restraint stress was performed before postsurgery testing in the T-maze, which resulted in working memory impairment in the *DISC1* gene knockdown group, but not in the other control groups (control, scrambled, and anatomical control groups). Furthermore, reduction of DISC1 expression in the prefrontal cortex in rats significantly reduced the length and density of dendrites in prefrontal cortex. Hence, the *DISC1* gene mutant influenced prefrontal cortex function under the stress condition (Gamo et al., 2013).

In 2016, Trossbach *et al*. (2016) published a novel transgenic DISC1 rat model, which

was also the first one, by injecting the linearized fragment of the CosSHa.tet vector containing the full-length, non-mutant human *DISC1* gene as transgene with the polymorphism F607 and C704 into pronuclei of Sprague Dawley rats. This transgenic rat model overexpressing the *DISC1* gene, resulted in the impairment of motor learning ability (rotarod deficit), hyperexploratory behaviors and amphetamine supersensitivity and an altered dopamine(DA)ergic system, including an 80% increase in high-affinity DA 2 receptors (D2R), an increased translocation of the dopamine transporter (DAT) to the plasma membrane, and a corresponding increase of DA reuptake into the presynaptic terminal (Trossbach et al., 2016). Neuroanatomical data exhibited that *DISC1* overexpression decreased the density of DAergic neurons in the substantia nigra and also reduced the DAergic fibers in the striatum (Hamburg et al., 2016). These studies on rats have provided evidence that abnormal DISC1 conditions are linked to changes in behaviors, morphology and the DAergic system.

1.4 Medication in *DISC1* gene-related deficits

The above evidence has indicated that the *DISC1* gene influences brain functions and cognition, in both rodents and the patients. Clozapine, affects both D1- and D2-like receptors (Josselyn et al., 1997), is an atypical antipsychotic with fewer side effects and more effective in schizophrenia patients who are lacking full response to other antipsychotics (Nucifora et al., 2017). Clozapine is effective not only in patients, but also in different *DISC1* gene mutant models (Niwa et al., 2010; Kuroda et al., 2011; Nagai et al., 2011; Cui et al., 2016; O'Tuathaigh et al., 2017). Acute administration of clozapine rescued the emotional deficits in the elevated-plus maze test (Kuroda et al., 2011), improved deficits in prepulse inhibition, the performance of long-term memory in the novel object recognition test, and normalized the level of DA in the prefrontal cortex in DISC1 knockdown mice (Niwa et al., 2010). Acute treatment of clozapine prevented short-term memory in the novel object recognition test and long-term memory deficits in the novel place recognition test in L100P mutant mice (Cui et

al., 2016), and reversed novelty-induced hyperactivity in the open field in adult neuregulin-1 × DISC1 mutant mice, which is a gene × gene interaction model (O'Tuathaigh et al., 2017). Through repeated treatment with clozapine, cognitive impairments in the novel object recognition test were significantly improved in polyriboinosinic–polyribocytidylic acid-treated DN-DISC1 transgenic mice, which is a model combining both environment and gene factor (Nagai et al., 2011).

Haloperidol, which has been tried to treat DISC1-related deficits in animal models, is a typical antipsychotic and D2R antagonist (Nagai et al., 2011). Acute treatment with haloperidol normalized the hyperactivity in the open field, reversed prepulse inhibition deficits, and significantly antagonized the effect of amphetamine on motor activity in the L100P mutants (Lipina et al., 2010). In addition, haloperidol suppressed the augmentation of MK-801-induced hyperactivity in polyriboinosinic-polyribocytidylic acid-treated DN-DISC1 transgenic mice (Nagai et al., 2011).

Bupropion, an antidepressant, is a dual norepinephrine (NE) and DA reuptake inhibitor (Stahl et al., 2004). It has been shown to reverse depression-like behavior in the forced swim test with acute treatment (Clapcote et al., 2007) and to correct deficient social behaviors (social facilitation, social reward, and social novelty) with chronic treatment (Lipina et al., 2013) in mice with mutation Q31L.

A number of studies have applied other medication to treat DISC1-related deficits in mutant mice. Chronic treatment with valproic acid prevented the emergence of hyperactivity and prepulse inhibition deficits in L100P mutants (Lipina et al., 2012). Two antidepressants, fluoxetine (selective serotonin (5-HT) reuptake inhibitor) and desipramine (NE transporter inhibitor), were found to improve social motivation in Q31L mice (Lipina et al., 2013).

1.5 Dopaminergic system in the DISC1 animal models and application of intranasal dopamine

The DAergic system plays a critical role in mood disorders, including depression and schizophrenia (Diehl and Gershon, 1992; Dailly et al., 2004; Yang and Tsai, 2017), as well as in cognitive functions (Mehta and Riedel, 2006). Evidence has been found for a relationship between depression and the DAergic system in humans and animals. D2R was observed to up-regulate in the basal ganglia/cerebellum in patients with depression in comparison with healthy subjects (D'Haenen H and Bossuyt, 1994). In the forced swim test it was found that the mesolimbic DAergic system had a permissive role in the effect of desipramine (Cervo et al., 1990). Its antidepressant-like effect was reduced after the administration of sulpiride, which is a D2R antagonist, bilaterally into the nucleus accumbens. In positron emission tomographic imaging studies presynaptic DAergic dysfunction in the striatum (Howes et al., 2007) and elevated DA synthesis capacity in the dorsal striatum (Egerton et al., 2013) was found in schizophrenia patients. In addition, many treatments are involved in antidopaminergic mechanisms, supporting the DA hypothesis of schizophrenia (Lang et al., 2007).

Accumulating research has shown that the *DISC1* gene is a risk factor for mental disorders, leading to the dysfunction of the DAergic system in the striatum and other brain regions in humans and rodents. It influences striatal volume (Chakravarty et al., 2012), leads to the DAergic deficits in the subcortical region (Jaaro-Peled et al., 2013), reduces the density of DAergic neurons in the substantia nigra and decreases DAergic fibers in the striatum (Hamburg et al., 2016). It has been found that the *DISC1* gene increased the affinity D2R with amphetamine treatment in the striatum (Lipina et al., 2010) and increased translocation of the DAT to the plasma membrane and a corresponding increase of DA reuptake into the presynaptic terminal (Trossbach et al., 2016). Manipulation of the *DISC1* gene decreased

levels of DA in the cortex (Ayhan et al., 2011) and nucleus accumbens (Lipina et al., 2013), altered the homeostasis of multi-receptor interaction of coincident signaling of DA and glutamate in the nucleus accumbens (Kim et al., 2015), and altered glutamatergic mechanisms through DAergic abnormalities in the prefrontal cortex (Matsumoto et al., 2017). Although mostly researchers employed the mutant male animals, the effects of DISC1 mutants on the DAergic system also appeared in female animals with disruption of exons 2 and 3 of the *Disc1* gene (Nakai et al., 2014). The above evidence, showing a strong link between the *DISC1* gene and the DAergic system, suggests the possibility to develop a therapeutic strategy via the DAergic system for treating DISC1-related deficits. Su *et al.* (2014) proposed that a potential therapy might be to disrupt the D2R-DISC1 complex, since an enhanced D2R-DISC1 interaction seems to lead to the psychotic symptoms (hyperactivity and abnormal prepulse inhibition behaviors) in schizophrenia, and may contribute to antipsychotic-like effects (Su et al., 2014).

Intranasal (IN) application is a non-invasive route for drug delivery, which is widely used in humans and animals, including in the treatment of neurodegenerative disorders (Reger et al., 2008; Danielyan et al., 2011; Craft et al., 2012). Its advantages are to bypass the blood-brain-barrier, avoid the hepatic first pass effect, systemic dilution effect, reduce drug delivery to non targeted sites and allow a higher molecular mass to access the central nerve system (Jogani et al., 2008). In terms of the nose-to-brain transport pathways, two possible pathways seem to be involved. One is delivery onto the olfactory epithelium, leading to transfer into the olfactory bulb and diffusion into the brain. The other one is delivery into the trigeminal network and transfer into the brain (Badhan et al., 2014). Despite its several advantages, there are several limitations, such as the poor permeability for hydrophilic drugs or drugs with molecular weight bigger than 1000 Da and absorption time limited by mucociliary clearance (Grassin-Delyle et al., 2012).

IN application with DA (IN-DA) is a potential therapy for improving cognition deficits and mood disorders. Previous research from this laboratory has shown that:

1. Both IN-DA and intraperitoneal application of DA increased the DAergic activity in the neostriatum and nucleus accumbens and locomotor activity 10 min after treatment. However, the effective dose of IN application was 10 times less than that of intraperitoneal injection (3 mg/kg vs. 30 mg/kg) (de Souza Silva et al., 2008). Furthermore, our group also found that the intranasally applied DA reduced DAT binding in the neostriatum, suggesting an enhanced DA availability (de Souza Silva et al., 2016).

2. IN-DA in a dose of 0.3 mg/kg exerted antidepressant-like activity in the forced swim test (Buddenberg et al., 2008), and rescued object-place memory in aged rats (Trossbach et al., 2014).

3. Chronic IN-DA reduced activity level and improved attention in a rat model of attention deficit/hyperactivity disorder (ADHD) (Ruocco et al., 2009; Ruocco et al., 2014).

1.6 Aims

Given the close relationship between the DAergic system and the *DISC1* gene, we hypothesized that: **Experiment 1:** overexpression of non-mutant full-length human *DISC1* gene would disrupt cognitive functions, including attention and memory processes. Hence, the novel object preference (NOP) test with different delays and object-based attention test (OBAT) were utilized for investigation; **Experiment 2:** the administration of DA via the nose-brain route would alleviate behavioral deficits induced by the overexpression of the human *DISC1* gene, which might be of future relevance for the pharmacological targeting of DISC1-dependent behavioral changes. **Experiment 3:** DA, noradrenaline (NA), 5-HT and acetylcholine (ACh) levels in brain regions, which are relevant for learning and memory (neostriatum, nucleus accumbens, amygdala, hippocampus, frontal cortex) would be influenced by the overexpression of non-mutant full-length human *DISC1* gene. The transmitters were assessed by post-mortem high-performance liquid chromatography (HPLC) analysis. Additionally, **Experiment 4:** manipulating the *DISC1* gene may also influence motor activities or emotional behaviors. Hence, the open field, spontaneous alternation behavior (SAB) test with T-maze (SABt) and elevated plus-maze (EPM) test were performed in this study.

2 Methods

In this section of Experiments 1, 2, and 3, I present a brief summary of material and methods. The detailed description can be found in the published studies in the appendix. Experiment 4 would be expatiated since it has not been submitted.

2.1 Animals

Male transgenic DISC1 (tgDISC1) Sprague Dawley rats and their littermate controls, so-called WT, were bred at the local animal facility (ZETT, Heinrich-Heine University, Düsseldorf, Germany) and were housed in Makrolon cages (Type IV; 60 × 38 × 20 cm) in groups of 2-3 rats. The group numbers and the age in each experiment were: Experiment 1 used 15 WT rats and 15 tgDISC1 rats aged 14-15 months; Experiment 2 used 18 tgDISC1 rats aged 14-15 months; Experiment 3 used 12 WT rats and 12 tgDISC1 rats aged 4-5 months; Experiment 4: 12 WT rats and 12 tgDISC1 rats aged 9 months. Animals had free access to food and water and were placed in an environment with controlled humidity and temperature and reversed light-dark cycle rhythm (lights off from 7:00 to 19:00). After over 2 weeks of habituation to the environment, each rat was handled for 5 min/day for 10 consecutive days. From one week prior to the beginning of the experiments until they finished the non-emotional tests they were given restricted access to food (15 g/rat/day). This procedure was applied to increase the level of alertness in the animals for object exploration and SAB. Animals were excluded if they were sick or explored an object less than 1 sec in any trial. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW and carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the German Law on the Protection of Animals.

2.2 Behavioral testing and neurochemical analysis

2.2.1 Novel object preference test

Rats have a natural tendency to explore a novel object more than a familiar one. From this it can be inferred that they have a memory of the familiar object and, thus, show an explorative bias for the novel one (Ennaceur and Delacour, 1988). The NOP test has been widely employed to assess object recognition in rodents (Dere et al., 2007). Rats were exposed in an open field (60 × 60 × 40 cm) made of wood and painted black, placed in a sound-attenuating chamber. Exposure trial: two identical objects were placed in two of the corners of the apparatus (Fig. 1A). Test trial: after different delays, (1 h for the assessment of short-term and 24 h for the assessment of long term memory), one of the objects was replaced by a novel object, which had shown the same level of preference for exploration in previous studies, while the other one remained in the open field. The delays of 1 h and 24 h were chosen on the basis of pilot studies, where aged WT Sprague Dawley rats had shown intact memory. For each trial, the animal was put into the apparatus, facing a wall that did not contain an object. It was removed from the testing chamber and returned to its home cage during the inter-trial interval. All trials lasted for 4 min.

2.2.2 Object-based attention test

This test was modified from a paradigm described by Alkam *et al.* (2011; 2013), who named it "object-based attention test" and presented evidence that it may be uses as a test for attention-related processes. On the other hand, the task can also be considered an "ultra short-term working memory task" with practically no time lapse between exposure and test trial. The OBAT procedure was identical to the NOP procedure, except that two distinct objects were used in the exposure trial (Fig. 1B) and there was no inter-trial interval. Immediately after the exposure, both objects were removed from the open field. One object identical with the one that had been previously used was placed at the identical position, while the other object was replaced by a novel one. Animals remained in the apparatus during this transition and the test trial commenced at once. Both trials lasted for 4 min. Different sets

of objects were employed for each object exploration test and were counterbalanced for each subject.

2.2.3 Open field

Animals were placed in a rectangular open field (60 × 60 × 40 cm), which was painted black and made of wood, placed in a sound-attenuating chamber. Two spatial-recognized cues (a white circle and a white squire with black stripes) were stuck on different walls. A camera connected to a DVD recorder and computer with the tracking software was hung 2 m above the open field. Four LED lights offered even illumination for 4 corners and the center (~5 lx). Duration was 10 min/rat. The open field was cleaned via acetic acid solution (0.1%) after each animal finished. Experimenters who were blind to the experimental design registered the time of onset and duration of object exploration using Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Behavioral measures included locomotor activities (distance traveled in cm, velocity in cm/s) and center time (s), as well as activities (duration/frequency) of grooming and rearing behavior. These were also analyzed automatically or manually with the Ethovision software. Behavioral studies were conducted between 10:00 and 17:00 h.

2.2.4 Spontaneous alternation behavior test with T-maze

SAB is a measure of exploratory behavior which is involved in the spatial working memory (Spowart-Manning and van der Staay, 2004) and also reflects attention-like behavior (Hughes, 2004). The present protocol followed the previous article (Spowart-Manning and van der Staay, 2004). The T-maze was made of wood (Start arm: 50 × 20 × 30 cm, two goal arms: 40 × 15 × 30 cm). The sliding doors, walls and floor of the apparatus were black. The sliding doors could be operated by the experimenter. The test consisted of 1 forced-choice trial and 14 free-choice trials. In the first trial, the "forced-choice trial", either the left or right goal arm was blocked by closing the sliding door. After the rat was released from the start arm, it

negotiated the maze, eventually entered the open goal arm, and was returned to the start position. There, the rat was confined for 5 sec by closing the sliding door of the start arm. Then, the "free-choice trials" started immediately. During 14 free-choice trials, the rat could choose freely between the left and right goal arm (both sliding doors opened) after opening the sliding door of the start arm. As soon as the rat entered one goal arm, the other goal arm was closed. The rat eventually would return to the start arm, and the next free-choice trial would start after a 5-sec confinement in the start arm. The test was terminated and the rat was removed from the maze as soon as 14 free-choice trials were performed or 30 min elapsed, whatever event occurred first. During the trials, the rat was not handled by the experimenter. The apparatus was cleaned via 70% ethanol to eliminate the odor cues before every rat started. One succeed alternation was defined that the rat entered one goal arm in a trial, then it entered the opposite goal arm in next trial. The percent alternations out of 14 trials (or the total number of free-choice trials if less than 14 free-choice trials were completed) were calculated. This percentage and the total duration (s) were evaluated statistically. All the processes were be recorded by a DVD player.

2.2.5 Elevated plus-maze

EPM has been widely used to measure anxiety-related behavior. The increased activity on the open arm (duration and/or entries) reflects anti-anxiety behavior. The EPM was composed of two open arms (40 × 10 cm), two enclosed arms (40 × 10 cm, surrounded by 40 cm-high walls) and a center platform (10 × 10 cm). Two open and two enclosed arms were installed on opposite sides of the center platform. The arms, walls, center platform and base of EPM were made of black acrylic. The apparatus was 50 cm high from the floor and placed in a sound-attenuating room. Luminous density on the enclosed arms was 11 lx and 3 lx on the open arms. A camera was fixed 1.5 m above the apparatus and connected to a DVD player and a computer. Animals were placed onto the center platform, facing an open arm, and

allowed to explore freely for 5 min. All activities, duration (s), entries, distance moved (cm), were measured by the Ethovision automatically. The apparatus was cleaned by 70% ethanol to eliminate the odor cues after each rat finished.

2.3 Drug

Dopamine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was suspended in gel of a viscous castor oil mixture (M & P Pharma, Emmetten, Switzerland). It was prepared immediately before usage and applied in a dose of 3 mg/kg in a volume of 10 µl of gel. It was kept on ice and protected from light throughout the experiment. Into each nostril, 5 µl of DA or its vehicle was applied to a depth of 2 mm using an applicator pipette for viscous liquids (Microman, Gilson, Villiers le Bel, France) and corresponding tips (CP10, Gilson, Villiers le Bel, France). The drug injection was performed over 8 s/nostril. The dose of DA was chosen on the basis of previous studies, in which the IN application of 3 mg/kg had stimulated DA release (de Souza Silva et al., 2008), and decreased DAT binding in the striatum (de Souza Silva et al., 2016) as well as exerted antidepressant-like effects (Buddenberg et al., 2008) and facilitated object place recognition (Trossbach et al., 2014). Behavioral testing was started 10 min post application. This 10 min duration was also chosen on the basis of the above-mentioned neurochemical and behavioral studies.

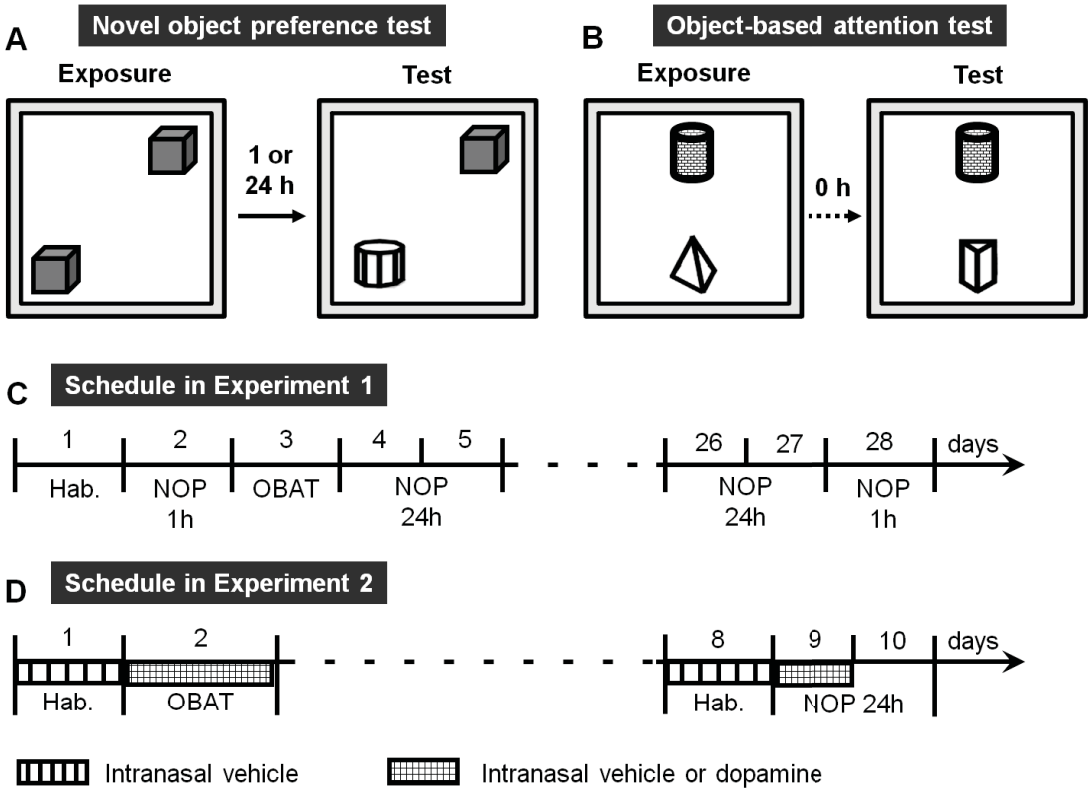

Figure 1. Illustration of the possible location of objects in the open field in tests for novel object preference (NOP) (A) and object-based attention (OBAT) (B). Different sets of objects are applied in these tests. Schedule of the behavioral tests in experiments 1 (C) and 2 (D). The dashed lines represent washout periods. The squares with vertical lines indicate intranasal administration of vehicle and the squares with cross lines indicate intranasal administration of vehicle or dopamine 10 min before testing in the two experimental groups, interchangeably. Abbreviation: Hab., habituation; NOP1h, test with 1h interval; NOP24h, test with 24h interval.

2.4 Experimental procedures

2.4.1 Experiment 1: Time-dependent test for novel object preference in the tgDISC1 rat

Animals were individually placed into the open field for 10 min in order to familiarize with the apparatus. One day after the habituation trial, tgDISC1 and WT groups were subjected to the behavioral tests for examining object memory and attention-related behavior: the NOP1h test, followed by the OBAT test on the next day, and the NOP24h test on the day after. In order to examine the reliability of findings, the NOP1h and NOP24h tests were repeated 20 days later. The experimental procedure is summarized in Fig. 1C.

2.4.2 Experiment 2: Effect of intranasal dopamine on deficits in object-based attention and long-term novel object preference in the tgDISC1 rat

All behavioral protocols here were identical to experiment 1. TgDISC1 rats were randomly divided into two groups (A and B), receiving treatment with either IN-DA (n = 9) or vehicle (n = 9). Habituation: one day before each test, all rats received an IN vehicle application in order to familiarize them with the nasal gel and the application procedure. Ten min later they were placed into the open field for 10 min. This procedure was also considered as a baseline measurement for locomotor, rearing, grooming and center-time behaviors. The OBAT and the NOP24h tests were conducted consecutively, separated by a washout period of 6 days. Group A was administered vehicle on day 2 (OBAT) and DA on day 9 (NOP test), 10 min before the trials. Group B was treated with DA on day 2 (OBAT) and vehicle on day 9 (NOP test), 10 min before the trials. A schematic diagram is presented (Fig. 1D).

2.4.3 Experiment 3: Post-mortem analysis HPLC

Animals were anaesthetized with CO_2 and decapitated. Each brain was dissected and then placed in cold Ringer's solution (B. Braun Melsungen AG, Germany). Neostriatum, nucleus

accumbens, amygdala, hippocampus, frontal cortex and cerebellum were collected for each animal and the content of DA, NA, 5-HT and ACh was assessed via high-performance liquid chromatography with electrochemical detection (HPLC-EC). The separation of monoamines (DA, NA, 5-HT) was achieved by a 125 mm long analytical column filled with Nucleosil C-18 (reversed-phase with 5-µm particle size; Macherey & Nagel, Duren, Germany). The mobile phase was composed of 75 mM NaH_2PO_4, 4 mM KCl, 20 µM EDTA, 1.5 mM sodium dodecylsulfate, 100 µl/l diethylamine, 12% methanol and 12% acetonitrile and adjusted to pH 6.0 using phosphoric acid (de Souza Silva et al., 1997; Pum et al., 2009). The electrochemical detection was performed by the ISAAC reference electrode (Antec, Zoeterwoude, The Netherlands), which was set at 500 mV at 30 °C. The separation of ACh was achieved with a 75 mm long analytical column filled with ChromSpher 5C18 (Merck KGaA, Darmstadt, Germany) and loaded with sodiumdodecylsulfate (Sigma–Aldrich, Saint Louis, Missouri, US). For enzymatic cleavage, an enzyme reactor was connected to the column. The enzyme reactor was filled with LiChrosorb-NH2 (Merck), activated by glutaraldehyde (Merck, Darmstadt, Germany), and then loaded with acetylcholineesterase (Sigma–Aldrich, Saint Louis, Missouri, US). The enzymes were covalently bound to the stationary phase. The enzyme reactor converted ACh to hydrogen peroxide, which was electrochemically detected at a platinum electrode set at a potential of 350 mV. The reference electrode was a ISAAC (Antec, Fremont, California, US). The mobile phase was composed of 1 mM tetramethylammonium chloride and 0.18 M K_2HPO_4 and adjusted to pH 8.0 with KH_2PO_4 (Merck, Darmstadt, Germany) (de Souza Silva et al., 2013). It flowed at the rate of 0.3 µl/min, using a HPLC pump (Merck, Darmstadt). The neurotransmitter content was analyzed with the help of a Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA).

2.4.4 Experiment 4: Effects of overexpressing *DISC1* gene in the open field test, spontaneous alternation behavior test with T-maze and the elevated plus-maze

To assess the influence of overexpressing the *DISC1* gene on locomotor activity, each animal was placed into the open field for 10 min. After a 7-day washout period, animals performed the SABt to assess spatial working memory and attention-like behaviors. Over one month later, the rats were tested for emotional behaviors via EPM.

3 Results

In this section of Experiments 1, 2, and 3, I present a brief summary of results. The detailed description can be found in the published studies in the appendix. Experiment 4 is presented in detail since it has not been submitted.

3.1 Experiment 1: TgDISC1 rats are deficient in long-term novel object memory and object attention

TgDISC1 rats and WT controls were subjected the open field test to estimate their locomotor activity. Distance (cm), velocity (cm/s), time in the center area (s) and durations of grooming and rearing (s) were not significantly different between two groups. Hence, the results of the following tests are unlikely to be confounded by changes in locomotor or emotional behaviors.

In the NOP1h test (both in the first test and the repeated test after 20-day washout) a significant main effect of "object", but not "group" and "object × group" was found. Also, both WT and tgDISC1 groups explored the novel object more than the familiar one, which shows that they remembered the familiar one after the 1h interval. In the NOP24h test, a significant interaction between "object" and "group" and a significant main effect of "object", but not "group" was found, while in the repeated test, there was an "object" effect, but no effect of "group" or "interaction". Similar results were found in two NOP24h tests. The WT group spent more time exploring the novel object than the familiar one; however, the tgDISC1 group spent equal time exploring both objects. It meant that the tgDISC1 group could not distinguish the old from the novel object. Thus, the WT group had intact short- and long-term object memory for novel objects after 1h and 24h-interval, as we expected. The tgDISC1 group had intact short-term memory with an 1h-delay but showed deficient long-term object memory after a 24h interval.

In the OBAT, a significant main effect of "object", but no effect of either "group" or "interaction" was found. In terms of the parameter of memory, the exploration time of each object, the WT group spent significantly more time in exploring the novel object than the familiar one. In the tgDISC1 group, animals did not show a significant difference in exploration time between the novel and familiar object. In summary, the tgDISC1 rats showed deficits in long-term object memory and in object-based attention, while their short-term object memory was intact.

3.2 Experiment 2: Intranasal dopamine application reverses deficits in long-term object memory and object attention in the tgDISC1 rats

In the previous experiment, tgDISC1 rats failed to perform intact long-term object memory and object-based attention. We hypothesized that these behaviors were aberrant due to the known regulation of the DAergic system by DISC1 and that administration of DA to the brain would alleviate these deficiencies. Hence, IN-DA was administrated in the tgDISC1 rats and the OBAT and NOP24h tests were subsequently repeated.

In the OBAT, a main effect of "object", but neither of "treatment" nor of the interaction between "object" and "treatment" was found. Consistent with the results in experiment 1, the vehicle-treated group failed to distinguish between the novel object and familiar object. In contrast, the IN-DA group exhibited memory of the familiar object and spent more time exploring the novel object. There was no significant group difference in the comparison of total time for object exploration between WT and tgDISC1 groups in the test trials. Thus, IN-DA treatment reversed the impairment in object-based attention of the tgDISC1 animals and restored a putative attention deficit.

In the NOP24h, the two-way ANOVA revealed a main effect of "object", but not of "treatment" and the interaction between "object" and "treatment". When the tgDISC1 rats

were treated with vehicle, they spent comparable time exploring the novel object and the familiar one. In contrast, the tgDISC1 rats with IN-DA treatment explored the novel object for a significantly longer time than the familiar one, which indicated intact long-term memory. The total time of exploration of objects was not significant between groups in any of the test trials. In summary, IN-DA administration also reversed the dysfunction of long-term memory in the tgDISC1rats.

3.3 Experiment 3: Neurochemistry of tgDISC1 and WT brains

Monoamines (DA, 5-HT and NA) and ACh were analyzed after the animals were sacrificed via HPLC. DA concentrations were significantly decreased in the neostriatum, amygdala and hippocampus of the tgDISC1 rats relative to WT controls. 5-HT concentrations were decreased in the amygdala of the tgDISC1 rats relative to WT controls. In the nucleus accumbens, 5-HT concentrations displayed the contrary results to the amygdala, which were increased in the tgDISC1 group compared with the WT group. NA concentrations were significantly reduced in the amygdala of tgDISC1 rats compared with the WT controls. ACh levels were significantly decreased in the neostriatum, nucleus accumbens, amygdala and hippocampus of the tgDISC1 rats relative to the WT controls. There was no significant difference between tgDISC1 rats and WT controls in other brain regions in monoamines or ACh.

3.4 Experiment 4: TgDISC1 rats are deficient in alternating behavior and emotional behaviors

Open field

Independent *t*-tests were applied to analyze a variety of locomotor behaviors. The WT group and tgDISC1 group displayed comparable distance, velocity, time in the center, grooming and rearing (duration/frequency) ($P > 0.05$; Table 1), which were consistent with the results of

experiment 1. Time in the center reflects the anxiety level in rodents. WT controls and tgDISC1 rats spent similar duration in the center, which indicates that they had equal anxiety levels in the open field test.

Table 1.

Results of open field test. Values are represented as mean ± SEM. Number per group is 12. In the presented parameters, there were no significant differences between the WT and tgDISC1 groups.

	WT	tgDISC1
Distance moved (cm)	3251.4 ± 108.2	3057.6 ± 117.9
Velocity (cm/s)	5.4 ± 0.2	5.1 ± 0.2
Grooming frequency (no)	5.0 ± 0.8	5.8 ± 0.6
Grooming duration (s)	48.5 ± 15.4	44.3 ± 7.0
Rearing frequency (no)	48.8 ± 4.2	44.3 ± 3.9
Rearing duration (s)	86.1 ± 8.1	77.4 ± 9.6
Time in the center (s)	32.1 ± 6.5	34.3 ± 5.1

Spontaneous alternation behavior test with T-maze

Attention and spatial working memory are two of the components in the SABt (Richman et al., 1986). In the current protocol, animals were subjected to the SABt to examine alternation behavior in one forced-choice trial followed by 14 consecutive free-choice trials in the T-maze. Independent *t*-tests were utilized to evaluate the indexes of the number of alternation, total entries and the duration to complete the 14 free-choice trials. No significantly differences

were found in these parameters ($P > 0.05$; Table 2). Both groups performed comparably on measures of locomotor activity.

Percentage of alternations were measured via one sample *t*-tests in the first free-choice trial and all 14 free-choice trials. The percentage of alternation in the first free-choice was not significantly different compared to chance level in each group (Fig 2A). After the animals completed 14 free-choice trials, the WT controls showed significantly higher alternation percentage, while tgDISC1 rats showed undifferentiated alternation percentage compared to chance level (Fig 2B). In the SABt, animals must remember (using "working memory") the goal arm which has been visited in order to avoid a revisit (Spowart-Manning and van der Staay, 2004; Deacon and Rawlins, 2006). SAB reflects spatial working memory/attention-like in rats. WT controls exhibited intact spatial working memory and attention-like behaviors, but not the tgDISC1 rats. These results implied that overexpressing the *DISC1* gene may influence working memory/attention-like behaviors in the SABt.

Table 2.

Results of spontaneous alternation behavior test with T-maze. Values are presented as mean ± SEM. Number per group is 9. In the presented parameters, there were no significant differences between the WT and tgDISC1 groups.

	WT	tgDISC1
No of alternation	7.8 ± 0.7	5.8 ± 1.2
Total entries	11.3 ± 0.9	9.7 ± 1.5
Duration (s)	1459.8 ± 171.1	1570.8 ± 116.2

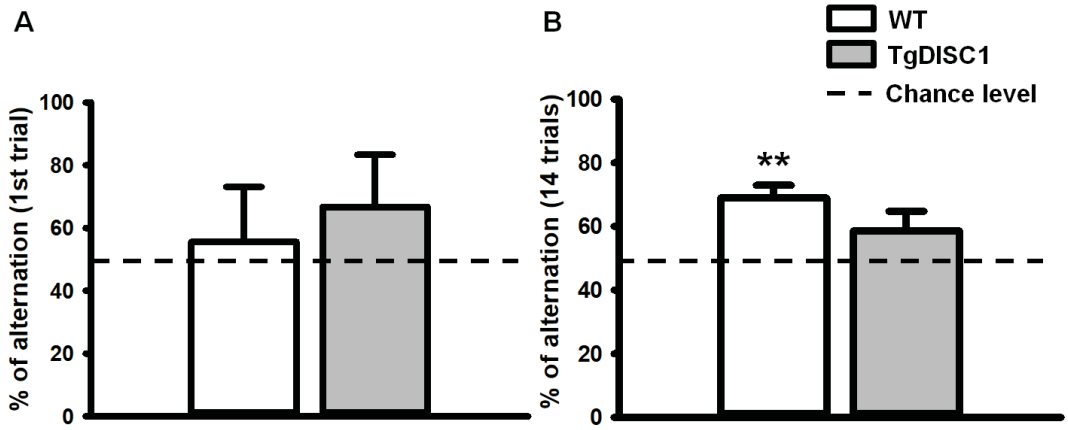

Figure 2. Effects of overexpressing the *DISC1* gene on alternation behavior in the spontaneous alternation behavior test with the T-maze. The WT and tgDISC1 groups showed similar results in the first free-choice trial via one sample *t*-tests (A). However, the WT group showed significant alternation behaviors after 14 free-choice trials compared to 50 (chance level), while the tgDISC1 group did not (B). N = 9 each group. Values represent mean ± SEM. $^{**}p < 0.01$, compared to 50 via one sample *t*-test.

Elevated plus-maze

Independent *t*-tests were employed to analyze the behaviors in the EPM. Time spent in the open arm is a critical index which reflects anxiety level. The tgDISC1 rats spent significantly less time on the open arms compared to the WT rats ($P < 0.05$; Table 3). In the parameters of activity in the closed arms (entries, duration and distance moved), latency of first time into an open arm, total arm entries, total arm distance and the duration in the center were not significantly different between the groups. The difference of time spent in the open arms can be interpreted in terms of differences in anxiety level between WT group and tgDISC1 group. Accordingly, overexpressing the *DISC1* gene increased anxiety-like behaviors in rats in the EPM.

Table 3.

Effects of overexpressing the *DISC1* gene on behaviors in the elevated plus-maze test. TgDISC1 rats showed significantly less time in the open arms compared to the WT rats. Values are presented as mean ± SEM. **p* <0.05, compared to the tgDISC1 group. Number per group is 12.

	WT	TgDISC1
Entries into the closed arms	18.3 ± 1.1	17.8 ± 1.8
Time spent in the closed arms (s)	194.3 ± 9.8	208.0 ± 8.4
Distance run in the closed arms (cm)	1401.5 ± 69.1	1466.5 ± 86.5
Entries into the open arms	6.7 ± 0.8	6.8 ± 1.0
Time spent in the open arms (s)	**51.5 ± 6.0**	**34.4 ± 3.9** *
Distance run in the open arms (cm)	290.1 ± 42.9	201.5 ± 25.3
Latency of first time into an open arm	7.2 ± 3.3	5.5 ± 2.4
Total entries	24.9 ± 1.7	24.6 ± 2.1
Total distance run in all arms (cm)	1964.7 ± 117.0	1923.8 ± 102.8
Time spent in the center	54.2 ± 7.7	57.6 ± 6.9

4 General Discussion

The main findings of the present study are: (1) TgDISC1 rats were deficient in long-term (24 h) object memory and attention-like behavior, but not short-term (1 h) memory, (2) IN-DA application reversed the above deficits in tgDISC1 rats, (3) the post-mortem analyses showed that tgDISC1 rats had lower levels of DA in the neostriatum, amygdala and hippocampus, less 5-HT in the amygdala, less NA in the amygdala and less ACh in the neostriatum, nucleus accumbens, amygdala, and hippocampus, but more 5-HT in the nucleus accumbens, (4) tgDISC1 rats were deficient in alternating behavior and emotional behaviors.

TgDISC1 rats were deficient in long-term object memory and object-based attention. Thus, *DISC1* gene overexpression impaired working memory during a very short delay between exposure and test trials. That is, the *DISC1* gene may affect memory processes in rodents when the animals must respond within a short time window. This is consistent with the findings on humans with one haplotype of the *DISC1* gene, which displayed both, poor short-term visual memory and attention (Hennah et al., 2005). Furthermore, tgDISC1 rats were also impaired in long-term memory. These results suggest that at least two distinct processes are influenced by the overexpression of the *DISC1* gene. Since short-term memory with the 1h-interval was intact, it is a challenge to explain the deficit in the OBAT with no interval between exposure to the objects and the test trial. OBAT can be considered either to evaluate ultra short-term memory and/or attention-like behavior (based on the interpretation preferred by the inventors (Alkam et al., 2011; Alkam et al., 2013). Given that the total exploration time of objects was comparable in both groups in all the test trials, the level of motivation for object exploration should not be a confounding factor here.

Novel object exploration memory is considered to involve the hippocampus and its adjacent cortical areas, including the entorhinal, perirhinal, and parahippocampal cortices. The relevant information required in the NOP includes visual, olfactory and somatosensory

inputs from the perirhinal cortex to the hippocampus (Clarke et al., 2010). The hippocampus plays an important role in NOP (Broadbent et al., 2010; Cohen et al., 2013) and for the consolidation of NOP memory (Chao et al., 2016). The prefrontal cortex is relevant for working memory and attention (Lara and Wallis, 2015) and interacts with the hippocampus for the processing of long-term memory (Simons and Spiers, 2003). Accumulating evidence shows that DISC1 is associated with the prefrontal cortex. Transgenic mice expressing a putative DN-DISC1 had behavioral impairments in prefrontal-dependent tasks (reversal learning and reinforcer devaluation), along with an increase of oxidative stress in the prefrontal cortex (Johnson et al., 2013). Frontal cortical neurons had shorter dendrites and decreased surface and spine density in *DISC1* point mutant mice (Lee et al., 2011). A reduced density of interneurons in the prefrontal cortex was found in DISC1 knockdown mice than the WT (Umeda et al., 2016). Furthermore, in the tgDISC1 rat, interneurons were shifted to deeper layers in the somatosensory cortex (Hamburg et al., 2016). Within this neurobehavioral framework, the function of prefrontal cortex may be disrupted in the tgDISC1 rats. Disrupted prefrontal cortex function may account for both the deficits in long-term object memory and the poor performance in the OBAT. Further studies are required to clarify the contribution of hippocampus and of the interactions between these regions to the cognitive deficits observed in the present study.

The properties of tgDISC1 rats include elevated DAT function and an increase of D2R density in the dorsal striatum (Trossbach et al., 2016). Moreover, in *DISC1*-L100P mutant mice, data also showed connections between the DISC1 and DAergic system, namely, a protein complex composed of D2R and DISC1 facilitated D2R-mediated glycogen synthase kinase-3 signaling. Overactivation of glycogen synthase kinase-3 pathway could lead to the downregulation of D2R internalization (Su et al., 2014). Given the DAergic connections between the dorsal/ventral striatum and prefrontal cortical areas (Haber, 2014), which are

relevant for both motor and cognitive functions, alterations of DA functions are likely to be involved in the behavioral impairments observed in tgDISC1 rats. Therefore, we hypothesized that IN-DA might ameliorate the behavioral impairments in tgDISC1 rats. IN-DA is an effective way to deliver DA into the brain and cerebrospinal fluid (Dahlin et al., 2001), and was found to increase DA levels in the neostriatum and nucleus accumbens (de Souza Silva et al., 2008). IN-DA administration led to reduced DAT binding in the dorsal striatum, which suggests an elevation of synaptic DA activity by this treatment (de Souza Silva et al., 2016). IN-DA in rats also enhanced locomotor activity (de Souza Silva et al., 2008) and had anti-depressant-like effects (Buddenberg et al., 2008). An anxiolytic effect and higher locomotion were found after the mice were treated IN-DA (Kholodar et al., 2013). In aged rats, IN-DA rescued memory impairments in object-place preference test (Trossbach et al., 2014). In an animal model of ADHD, IN-DA application attenuated deficient spatial attention and working memory (Ruocco et al., 2014). Given that the *DISC1* gene influences the DAergic system (Dahoun et al., 2017), treatment with DA may "re-balance" the system and, thus, compensate for DA-related deficits. Hence, we concluded that DISC1 interacts with the striatal DAergic system and, thereby, influences the prefrontal cortex to impede short-term/working memory, attention, and long-term memory deficits.

In our study of the elevated plus-maze, tgDISC1 rats spent less time on the open arms compared to the WT controls, which indicates that they had higher anxiety levels. The amygdala is often discussed in the processes of emotion. Sensory information input pathway to the amygdala is from advanced levels of visual, auditory and somatosensory cortices and the output from amygdala is to a wide range of target regions, including the prefrontal cortex (Salzman and Fusi, 2010) and striatum (Lago et al., 2017). In this framework, the amygdala and the prefrontal cortex interaction is a network to mediate emotion and its influence on cognitive processes. It has been reported that a decrease of DA in the amygdala and striatum

leads to anxiety-like behavior (Choi et al., 2017). The prefrontal cortex has been demonstrated to associate with emotion. Animals or patients with medial prefrontal cortex or orbitofrontal cortex damage demonstrated abnormalities in emotional behaviors or emotional instability (Szczepanski and Knight, 2014). Although the striatum is not a typical region involved in the control of anxiety, a recent review added the striatum into the anxiety map, since the striatum receives information from the amygdala and cortex and is interconnected with the hippocampus and bed nucleus of the stria terminalis, which are the regions related to the anxiety network (Lago et al., 2017). As mentioned above, the function of prefrontal cortex may be disrupted in the tgDISC1 rats. The combination of the prefrontal cortex disruption and lower DA in the amygdala and striatum may account for the higher anxiety of the tgDISC1 rats. However, these results seem to be inconsistent with a previous study. The mice lacking exon 2 and 3 of the *DISC1* gene spent more time in the open arm of the EPM compared to the WT (Kuroda et al., 2011). Since different mutants showed different behavioral characteristics, for example, Q31L showed depressive-like behavior, while L100P exhibited schizophrenic-like behavior (Clapcote et al., 2007), it seems likely that specific DISC1 genetic alterations represent specific behavioral deficits. The inconsistent results between the current study and the previous study in anxiety-like behaviors may be due to the various genetic conditions (overexpression vs. mutant) and the difference of species (rat vs. mouse). The analyses of the emotional involvement of DISC1 deserves further study. Nevertheless, the evidence implies that the *DISC1* gene is related with emotion.

We found that tgDISC1 rats were deficient in object-based attention in the OBAT and alternating behaviors in the SABt. These results connect the *DISC1* gene with attention deficits, which is in agreement with human studies via analyzing the SNPs (Liu et al., 2006; Kayyal et al., 2015). The *DISC1* gene has also been found to be a susceptibility for ADHD (Kayyal et al., 2015) and has also associated with sustained attention deficits in schizophrenic

patients via the continuous performance test, which is a measure of estimating selective and sustained attention (Liu et al., 2006). In rodents, the 5-choice serial reaction time task (5-CSRTT) was developed to measure sustained and selective attention (Robbins, 2002). 5-CSRTT requires training for long time (over months), which becomes a limit and is not suitable for studying the attention-related behavior at younger age. In the current study, OBAT requires one-trial training (exposure trial) and the SABt requires no training. Both tests have been considered to detect attention-like behaviors (Hughes, 2004; Alkam et al., 2011; Alkam et al., 2013). They utilize the natural tendency of rats to explore novel stimuli, such as novel objects or the unexplored goal arm. Of possible relevance is our finding of lower ACh concentrations in the neostriatum, nucleus accumbens, amygdala and hippocampus. The cholinergic system plays a role in cognitive processes, including attention (Ferreira-Vieira et al., 2016). Experimental data using non-human primates have demonstrated that reduced ACh levels in multiple brain regions by the basal forebrain cholinergic neuron lesion leads to attention deficits (Voytko et al., 1994). Based on our findings, the overexpression of the *DISC1* gene in rats seems like a good model to further investigate DISC1-related attention deficits.

Post-mortem analysis revealed lower total DA levels in the neostriatum, hippocampus and amygdala of tgDISC1 rats. However, neurochemical differences were not confined to DA. TgDISC1 rats also had lower ACh levels in the neostriatum, nucleus accumbens, hippocampus and amygdala, which are the major projection areas of the basal forebrain cholinergic neurons (Dani and Bertrand, 2007). The cholinergic neurons of the basal forebrain mainly innervate the hippocampus, which is acknowledged to regulate memory processes (Everitt and Robbins, 1997; Hasselmo, 2006). Eliminating cholinergic neurons in the basal forebrain led to impaired spatial and object recognition memory which was reversed by treatment with acetylcholinesterase inhibitors (Okada et al., 2015). Decreased ACh levels are

known to lead to impairments of memory. The lower levels of hippocampal ACh observed in the present study may be related to the impaired long-term memory in the NOP test. Striatal cholinergic interneurons are targeted by the nigrostriatal DAergic system, and output of ACh from cholinergic interneurons is modulated by DA (Lester et al., 2010). Enhancing endogenous extracellular DA acting on the D1 receptors led to increased striatal ACh release (Imperato et al., 1993), whereas the activation of D2 receptors inhibited the release of ACh in the striatum (DeBoer et al., 1996; Ikarashi et al., 1997). The DAergic and cholinergic systems operate in a dynamic balance. Its disruption can lead to the cognitive impairments. Here, we found significantly decreased levels of hippocampal DA. Disruption of the DAergic system might have impaired cholingeric functions by a variety of mechanism.

In the nucleus accumbens of the tgDISC1 rats, higher levels of 5-HT and lower levels of ACh were assayed, which might indicate an interaction between 5-HTergic and AChergic systems. 5-HT can directly affect cholinergic neurons, resulting in inhibiting the release of ACh by acting on the presynaptic receptors of cholinergic terminals (Gillet et al., 1985). Consistent with DA, 5-HT and NA levels were also reduced in the amygdala, which controls motivated behaviors along with prefrontal cortex and hippocampus (Gruber and McDonald, 2012). These changes of neurotransmitters which were induced by overexpression of the *DISC1* gene in the nucleus accumbens and amygdala may be related to reward-based learning, since in transgenic mice expressing a putative DN-DISC1 had a deficiency in reinforcement devaluation was reported (Johnson et al., 2013). Taken together, both the monoaminergic and cholinergic systems are dysregulated in the tgDISC1 rats, which may be related to the observed behavioral deficits.

5 Conclusion

The overexpression of the full-length non-mutant human *DISC1* gene impaired object long-term memory and ultra-short memory/attention, which was alleviated by IN-DA administration. Aberrant emotional and alternating behaviors were also found in the tgDISC1 rats. In terms of the neurotransmitter systems, DA, 5-HT, NA and ACh were affected in the neostriatum, nucleus accumbens, amygdala and hippocampus in the tgDISC1 rats, suggesting an interaction between the *DISC1* gene and these systems, possibly in relation to the observed behavioral deficiencies.

To date, rare genetic manipulations of the *DISC1* gene are closely linked to various psychiatric disorders, including susceptibility to schizophrenia (Chubb et al., 2008). A functional interaction between the *DISC1* gene and the DAergic system seems to be similar to the DISC1 functions in schizophrenia. Moreover, deficits in attention and working memory correspond to the symptoms of ADHD. IN-DA was shown to ameliorate the attention and working memory deficits in the animal model of ADHD (Ruocco et al., 2014). As a consequence, rather than to assign DISC1 dysfunction to a clinical diagnosis, we consider it a vulnerability factor based on deficient DA homeostasis. Several antipsychotic drugs which affect the DAergic system were also found to alleviate DISC1-related cognitive impairments, like clozapine (Nagai et al., 2011; Cui et al., 2016) and bupropion (Clapcote et al., 2007; Lipina et al., 2013.). Likewise, the administration of DA via the nose-brain pathway reversed the cognitive impairments in the tgDISC1 rats. Thus, the unbalance of DAergic system may be a major cause for the behavioral deficits found in the tgDISC1 rats.

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7 Publications

7.1 Publication in support of the focus of this dissertation

1. **A.L. Wang**, B. Fazari, O.Y. Chao, S. Nikolaus, S.V. Trossbach, C. Korth, F.J. Sialana, G. Lubec, J.P. Huston, C. Mattern, M.A. de Souza Silva, Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human *DISC1* gene, Neurobiology of Learning and Memory 146 (2017) 12-20.

7.2 Other publications by the author

1. F.J. Sialana, **A.L. Wang**, B. Fazari, M. Kristofova, R. Smidak, S.V. Trossbach, C. Korth, J.P. Huston, M.A. de Souza Silva, G. Lubec, Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human *DISC1* Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum, Frontiers in Molecular Neuroscience 11(26) (2018).

2. M.A. de Souza Silva, J.P. Huston, **A.L. Wang**, D. Petri, O.Y. Chao, Evidence for a Specific Integrative Mechanism for Episodic Memory Mediated by AMPA/kainate Receptors in a Circuit Involving Medial Prefrontal Cortex and Hippocampal CA3 Region, Cereb Cortex 26(7) (2016) 3000-9.

3. O.Y. Chao, J.P. Huston, J.S. Li, **A.L. Wang**, M.A. de Souza Silva, The medial prefrontal cortex-lateral entorhinal cortex circuit is essential for episodic-like memory and associative object-recognition, Hippocampus 26(5) (2016) 633-45.

4. O.Y. Chao, **A.L. Wang**, S. Nikolaus, M.A. de Souza Silva, NK(3) receptor agonism reinstates temporal order memory in the hemiparkinsonian rat, Behavioural Brain Research 285 (2015) 208-12.

5. C. Biesdorf, **A.L. Wang**, B. Topic, D. Petri, H. Milani, J.P. Huston, M.A. de Souza Silva, Dopamine in the nucleus accumbens core, but not shell, increases during signaled food

reward and decreases during delayed extinction, Neurobiology of Learning and Memory 123 (2015) 125-39.

8 Appendixes

Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human *DISC1* gene

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Contribution of An-Li Wang (first author)

Experimental design: 50%

Data analysis: 70%

Manuscript writing: 50%

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Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human *DISC1* gene

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ABSTRACT

The *Disrupted-in-Schizophrenia 1 (DISC1)* gene has been associated with mental illnesses such as major depression and schizophrenia. The transgenic DISC1 (tgDISC1) rat, which overexpresses the human *DISC1* gene, is known to exhibit deficient dopamine (DA) homeostasis. To ascertain whether the *DISC1* gene also impacts cognitive functions, 14–15 months old male tgDISC1 rats and wild-type controls were subjected to the novel object preference (NOP) test and the object-based attention test (OBAT) in order to assess short-term memory (1 h), long-term memory (24 h), and attention. *Results*: The tgDISC1 group exhibited intact short-term memory, but deficient long-term-memory in the NOP test and deficient attention-related behavior in the OBAT. In a different group of tgDISC1 rats, 3 mg/kg intranasally applied dopamine (IN-DA) or its vehicle was applied prior to the NOP or the OBAT test. IN-DA reversed cognitive deficits in both the NOP and OBAT tests. In a further cohort of tgDISC1 rats, post-mortem levels of DA, noradrenaline, serotonin and acetylcholine were determined in a variety of brain regions. The tgDISC1 group had less DA in the neostriatum, hippocampus and amygdala, less acetylcholine in neostriatum, nucleus accumbens, hippocampus, and amygdala, more serotonin in the nucleus accumbens, and less serotonin and noradrenaline in the amygdala. *Conclusions*: Our findings show that DISC1 overexpression and misassembly is associated with deficits in long-term memory and attention-related behavior. Since behavioral impairments in tgDISC1 rats were reversed by IN-DA, DA deficiency may be a major cause for the behavioral deficits expressed in this model.

1. Introduction

The *Disrupted-in-Schizophrenia 1* (*DISC1*) gene was identified in a Scottish family with high linkage to various psychiatric disorders (Blackwood et al., 2001; Millar et al., 2000; St Clair et al., 1990). Several association studies have confirmed a role for DISC1 in schizophrenia, bipolar disorder, depression and autism (reviewed in Chubb, Bradshaw, Soares, Porteous, and Millar (2008), Thomson et al. (2016)). In these studies clinical diagnoses were not exclusively related to DISC1 genotypes, indicating that DISC1 should be conceptualized as a general vulnerability factor for mental illness (Brandon & Sawa, 2011; Korth, 2012).

The DISC1 protein has multiple roles in pre- and post-natal neuronal development, consistent with neurodevelopmental theories of mental illness, including regulation of the proliferation and migration of neuronal progenitor cells as well as synapse formation and maintenance (reviewed in Brandon and Sawa (2011)). It acts as a molecular hub that binds and interacts with many proteins, including components of dopamine(DA)ergic neurotransmission such as the DA D2 receptor (D2R) and DA transporter (DAT) (Su et al., 2014; Trossbach et al., 2016;

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Yerabham et al., 2017), thus linking it to the DA system, which is considered key in regulating multiple behavior systems (Dahoun, Trossbach, Brandon, Korth, & Howes, 2017).

The DISC1 gene has also been linked to cognitive processes, including deficits in object recognition (Cui et al., 2016; Ibi et al., 2010; Nagai et al., 2011). Deficits in the novel object- and novel place-preference tests were reversed by clozapine, a D1 receptor agonist, in the L100P mutant mouse model (Cui et al., 2016) and in human dominant-negative DISC1 transgenic mice (Ibi et al., 2010) and a 24 h novel object recognition deficit in a knockdown model of endogenous DISC1 (Niwa et al., 2010). Haloperidol reversed prepulse inhibition (PPI) deficits and blocked amphetamine-induced hyperactivity in DISC1-L100P mice (Lipina et al., 2010). Also, bupropion, a DA and norepinephrine reuptake inhibitor, was reported to ameliorate depressive-like behavior in the forced swim test and a PPI deficit in Q31L mice (Clapcote et al., 2007), underlining the interaction between DISC1 and DAergic systems.

Intranasal (IN) administration of substances is an alternative method for targeting the brain by bypassing the blood-brain-barrier (BBB). DA, which cannot cross the BBB due to its lipophobic properties, can be delivered directly into the brain via the nasal mucosa (Dahlin, Jansson, & Bjork, 2001). Previous studies have shown that IN-DA increased extracellular DA in the striatum (de Souza Silva, Topic, Huston, & Mattern, 2008), had antidepressant-like effects (Buddenberg et al., 2008) and reduced neostriatal DAT binding, suggesting an enhanced DA availability (de Souza Silva et al., 2016). IN-DA attenuated fear responses (Talbot, Mattern, de Souza Silva, & Brandao, 2017) and restored deficient object-place memory in aged rats (Trossbach, de Souza Silva, Huston, Korth, & Mattern, 2014). Furthermore, in a rat model of attention-deficit hyperactivity disorder (ADHD), it reduced hyperactivity and improved attention when administrated during the prepuberal period (Ruocco et al., 2014).

Here, we used the transgenic rat overexpressing the full-length non-mutant human *DISC1* gene (tgDISC1) (Hamburg et al., 2016; Trossbach et al., 2016). In previous studies with this tgDISC1 rat, an 80% increase of high-affinity D2R was observed in the ventral striatum as well as an increased translocation of the DAT to the plasma membrane and a corresponding increase of DA reuptake into the presynaptic terminal (Trossbach et al., 2016). Rats also showed behavioral alterations, such as increased locomotion and rearing upon amphetamine challenge relative to controls (amphetamine supersensitivity) (Trossbach et al., 2016).

The DAergic systems play a key role in reward and learning processes (Guzman-Ramos et al., 2012; Schultz, Apicella, & Ljungberg, 1993; Wise, 2005). DA is a key neurotransmitter in the control of motivation, attention and memory known to degrade with age (Kaasinen et al., 2000; Trifilieff et al., 2013; Wang et al., 1998). Given its close relationship with the *DISC1* gene (Dahoun et al., 2017; Su et al., 2014; Trossbach et al., 2016), we hypothesized that overexpression of non-mutant full-length human *DISC1* gene would disrupt cognitive functions, specifically attention and memory processes. We further hypothesized that the administration of DA via the nose-brain pathway would alleviate behavioral deficits induced by the overexpression of the human *DISC1* gene, which might be of future relevance for the pharmacological targeting of DISC1-dependent behavioral changes.

In the present study, we assessed cognitive function in tests of short-term (1h interval) and long-term (24 h interval) memory with the novel object preference (NOP) test (Ennaceur & Delacour, 1988), and of attention-related behavior with the object-based attention test (OBAT) (Alkam et al., 2011, 2013). We found both memory and attention-deficits in this study. Since we had already observed alterations of DA function in tgDISC1 rats (Trossbach et al., 2016) and IN-DA had repeatedly been found to ameliorate cognitive functions (Ruocco et al., 2014; Trossbach et al., 2014), we then assessed the effect of IN-DA on long-term memory (24h interval) in the NOP test and on attention-related behavior in the OBAT test. In a further group of tgDISC1 rats, *post-mortem* high-performance liquid chromatography (HPLC) analysis was conducted to assess possible changes of DA, noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) levels in a variety of brain regions, which are relevant for learning and memory (neostriatum, nucleus accumbens, amygdala, hippocampus, frontal cortex, cerebellum).

2. Materials and methods

2.1. Animals

TgDISC1 Sprague Dawley rats and their littermate controls, so-called wild-type (WT), were bred at the local animal facility (ZETT, Heinrich-Heine University, Düsseldorf, Germany). For the behavioral studies a total of 33 male tgDISC1 and 15 male WT controls (aged 14–15 months) were housed in Makrolon cages (Type IV; 60 × 38 × 20 cm) in groups of 2–3 rats. Each pair of parents contributed one WT rat and one tgDISC1 rat. A further cohort of 24 animals (aged 4–5 months) underwent post-mortem HPLC analysis of neurotransmitter levels in various brain regions. The age of this cohort, relatively younger than the age used in the behavioral experiments, was chosen to compare the neurochemical changes in the tgDISC1 animals and the age-matched WT. This is a starting step to establish a basic understanding about the *DISC1* gene on neurotransmitter systems in naïve adult animals.

Animals had free access to food and water and were placed in an environment with controlled humidity and temperature and reversed light-dark cycle rhythm (lights off from 7:00 to 19:00). After over 2 weeks of habituation to the environment, each rat was handled for 5 min/day for 10 consecutive days. From one week prior to the beginning of the experiments until the end of the study they were given restricted access to food (15 g/rat/day). This procedure was applied to increase the level of alertness in the animals for object exploration. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW and carried out in accordance with the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and the German Law on the Protection of Animals.

2.2. Apparatus and behavioral testing

A rectangular open field (60 × 60 × 40 cm) made of wood and painted black, was placed in a sound-attenuating chamber. A camera connected to a DVD recorder and computer with the tracking software was hung 2 m above the open field. Two figures (a white circle and a square made of black-white stripes) were attached to the black walls of the chamber as spatial cues. Four LED spotlights (~5 lx) were placed over the open field. Three different sets of objects made of glass or porcelain, with different shapes (cylinder, rectangular or octagonal columns), colors (white, green, transparent), sizes (25–32 cm height, 7–11 cm diameter) and textures (smooth, rough) were used as objects for the animals to explore. All objects weighed over 1.5 kg to prevent being moved by the rats. Acetic acid solution (0.1%) was used to clean the open field and the objects after each trial.

Physical contact with forepaws, snout or vibrissa toward an object was regarded as object explorative behavior. Experimenters who were blind to the experimental design registered the time of onset and duration of object exploration using Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Behavioral measures included locomotor activities (distance traveled, velocity in cm/s) and center time, as well as duration of grooming and rearing behavior. These were also analyzed automatically or manually with the Ethovision software. Behavioral studies were conducted between 10:00 and 17:00 h.

2.2.1. Novel object preference (NOP) test

Rats have a natural tendency to explore a novel object more than a familiar one. From this it can be inferred that they have a memory of the familiar object and, thus, show an explorative bias for the novel one

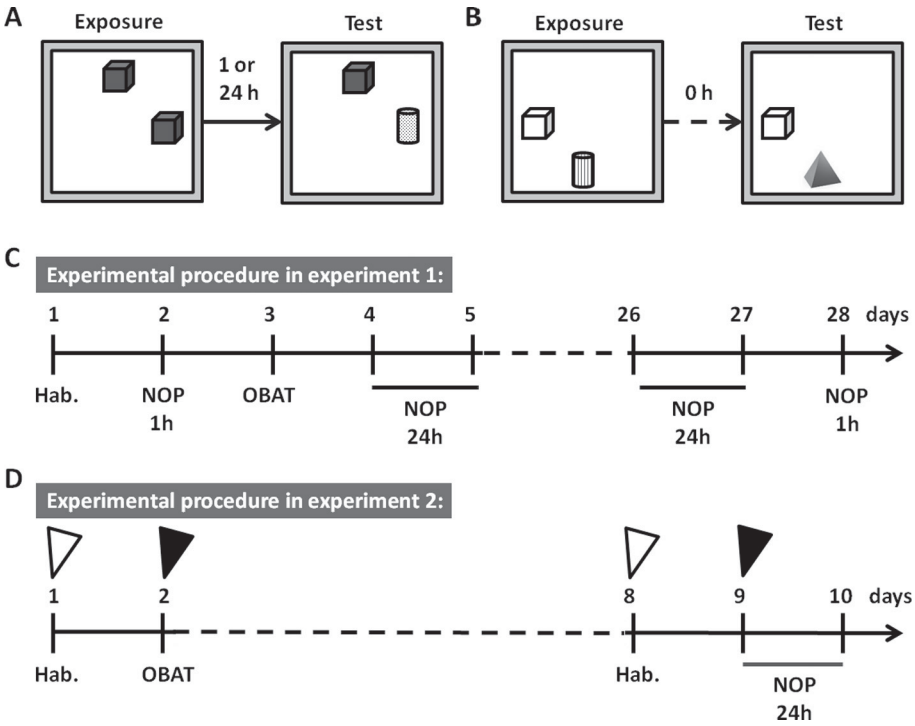

Fig. 1. Schematic representation of possible location of objects in the open field in tests for novel object preference (NOP) (A) and the object-based attention test (OBAT) (B). Different sets of objects are applied in these tests. Schedule of the behavioral tests in experiments 1 (C) and 2 (D). The dashed lines represent washout periods. The white triangles indicate intranasal administration of vehicle and the black triangles indicate intranasal administration of vehicle or dopamine before testing in the two experimental groups, interchangeably. Abbreviation: Hab., habituation; NOP1h, test with 1 h-interval; NOP24h, test with 24 h-interval.

(Ennaceur & Delacour, 1988). The NOP test has been widely employed to assess object recognition in rodents (Dere, Huston, & De Souza Silva, 2007). *Exposure trial*: two identical objects were placed in two of the corners of the apparatus (Fig. 1A). *Test trial*: after different delays, (1 h for the assessment of short-term and 24 h for the assessment of long-term memory), one of the objects was replaced by a novel object, which had shown the same level of preference for exploration in previous studies, while the other one remained in the open field. The delays of 1 h and 24 h were chosen on the basis of pilot studies, where aged wild-type Sprague Dawley rats had shown intact memory. For each trial, the animal was put into the apparatus, facing a wall that did not contain an object. It was removed from the testing chamber and returned to its home cage during the inter-trial interval. All trials lasted for 4 min. In order to examine the reliability of findings, the NOP1h and NOP24h tests were repeated 20 days later.

2.2.2. Object-based attention test (OBAT)

This test was modified from a paradigm described by Alkam et al. (2011, 2013), who named it "object-based attention test" and presented evidence that it may be uses as a test for attention-related processes. On the other hand, the task can also be considered a "ultra short-term working memory task" with practically no time lapse between exposure and test trial. The OBAT procedure was identical to the NOP procedure, except that two distinct objects were used in the exposure trial (Fig. 1B) and there was no inter-trial interval. Immediately after the exposure, both objects were removed from the open field. One object identical with the one that had been previously used was placed at the identical position, while the other object was replaced by a novel one. Animals remained in the apparatus during this transition and the test trial commenced at once. Both trials lasted for 4 min. Different sets of objects were employed for each object exploration test and were counterbalanced for each subject.

2.3. Experimental procedures

2.3.1. Experiment 1: Time-dependent test for novel object preference in the tgDISC1 rat

TgDISC1 and WT controls (n = 15 each) were transported from their colony to a room with dim light, which was next to the testing chamber, and were undisturbed for at least one hour. Then they were individually placed into the open field for 10 min in order to familiarize with the apparatus. One day after the habituation trial, tgDISC1 and WT groups were subjected to the behavioral tests for examining object memory and attention-related behavior: The NOP1h test, followed by the OBAT test on the next day, and the NOP24h test on the day after. The NOP1h and NOP24h tests were repeated 20 days later. The experimental procedure is summarized in Fig. 1C.

2.3.2. Experiment 2: Effect of intranasal dopamine on deficits in object-based attention and long-term novel object preference in the tgDISC1 rat

TgDISC1 rats were randomly divided into two groups (A and B), receiving treatment with either IN-DA (n = 9) or vehicle (n = 9).

Habituation: One day before each test, all rats received an IN vehicle application in order to familiarize them with the nasal gel and the application procedure. Ten min later they were placed into the open field for 10 min. This procedure was also considered as a baseline measurement for locomotor, rearing, grooming and center-time behaviors.

The OBAT and the NOP24h tests were conducted consecutively, separated by a washout period of 6 days. Group A was administered vehicle on day 2 (OBAT) and DA on day 9 (NOP test), 10 min before the trials. Group B was treated with DA on day 2 (OBAT) and vehicle on day 9 (NOP test), 10 min before the trials. A schematic diagram is presented (Fig. 1D).

All behavioral protocols were identical to experiment 1.

2.3.3. Drug

Dopamine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was suspended in gel of a viscous castor oil mixture (M & P Pharma, Emmetten, Switzerland). It was prepared immediately before usage and applied in a dose of 3 mg/kg in a volume of 10 µl of gel. It was kept on ice and protected from light throughout the experiment. Into each nostril, 5 µl of DA or its vehicle was applied to a depth of 2 mm using an applicator pipette for viscous liquids (Microman, Gilson, Villiers le Bel, France) and corresponding tips (CP10, Gilson, Villiers le Bel, France). The drug injection was performed over 8 s/nostril.

The dose of DA was chosen on the basis of previous studies, in which the IN application of 3 mg/kg had stimulated DA release (de Souza Silva et al., 2008), as decreased of DAT binding in the striatum (de Souza Silva et al., 2016) as well as exerted antidepressant-like effects (Buddenberg et al., 2008) and facilitated object place recognition (Trossbach et al., 2014). Behavioral testing was started 10 min post application. This ten-min duration was also chosen on the basis of the above-mentioned neurochemical and behavioral studies.

2.3.4. Experiment 3: Post-mortem analysis HPLC

TgDISC1 and WT controls (n = 12 each) were anaesthetized with CO_2 and decapitated. Each brain was dissected and then placed in cold Ringer's solution (B. Braun Melsungen AG, Germany). Neostriatum, nucleus accumbens, amygdala, hippocampus, frontal cortex and cerebellum were collected for each animal and the content of DA, NA, 5-HT and ACh was assessed via high-performance liquid chromatography with electrochemical detection (HPLC-EC). The separation of monoamines (DA, NA, 5-HT) was achieved by a 125 mm long analytical column filled with Nucleosil C-18 (reversed-phase with 5-µm particle size; Macherey & Nagel, Duren, Germany). The mobile phase was composed of 75 mM NaH_2PO_4, 4 mM KCl, 20 µM EDTA, 1.5 mM sodium dodecylsulfate, 100 µl/l diethylamine, 12% methanol and 12% acetonitrile and adjusted to pH 6.0 using phosphoric acid (de Souza Silva et al., 1997; Pum et al., 2009). The electrochemical detection was performed by the ISAAC reference electrode (Antec, Zoeterwoude, The Netherlands), which was set at 500 mV at 30 °C. The separation of ACh was achieved with a 75 mm long analytical column filled with ChromSpher 5C18 (Merck KGaA, Darmstadt, Germany) and loaded with sodiumdodecylsulfate (Sigma–Aldrich, Saint Louis, Missouri, US). For enzymatic cleavage, an enzyme reactor was connected to the column. The enzyme reactor was filled with LiChrosorb-NH2 (Merck), activated by glutaraldehyde (Merck, Darmstadt, Germany), and then loaded with acetylcholineesterase (Sigma–Aldrich, Saint Louis, Missouri, US). The enzymes were covalently bound to the stationary phase. The enzyme reactor converted ACh to hydrogen peroxide, which was electrochemically detected at a platinum electrode set at a potential of 350 mV. The reference electrode was a ISAAC (Antec, Fremont, California, US). The mobile phase was composed of 1 mM tetramethylammonium chloride and 0.18 M K_2HPO_4 and adjusted to pH 8.0 with KH_2PO_4 (Merck, Darmstadt, Germany) (de Souza Silva et al., 2013). It flowed at the rate of 0.3 µl/min, using a HPLC pump (Merck, Darmstadt). The neurotransmitter content was analyzed with the help of a Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA).

2.4. Statistics

For all object exploration tests, mixed two-way ANOVAs with the within-subjects variable, "object", and the between-subjects factor, "group", were applied. Since the exploration times of objects were not independent of each other, two-tailed paired-sample *t*-tests were used for the comparisons. Independent-sample *t* tests were applied to analyze locomotor and emotional behaviors, as well as neurochemical data. Results were expressed as mean ± SEM and all tests were with the level of significance set at $P < .05$.

3. Results

3.1. Experiment 1: TgDISC1 rats are deficient in long-term novel object memory and object attention

3.1.1. Open field

Distance moved (cm), velocity (cm/s), time in the center area (s) and durations of grooming and rearing (s) were not significantly different between WT and tgDISC1 groups (Table 1). Both groups performed comparably on measures of locomotor activity and emotional behavior (time in the center as measure of anxiety-like behavior) (Prut & Belzung, 2003).

Table 1
Results of open field test in experiment 1. Values are presented as mean ± SEM. Number/group is 14. No significant differences between the experimental groups were detected in the presented behavioral parameters.

	WT	tgDISC1
Distance moved (cm)	2643.2 ± 124.7	2707.3 ± 93.7
Velocity (cm/s)	4.4 ± 0.2	4.5 ± 0.2
Center duration (s)	28.0 ± 5.4	21.6 ± 3.9
Grooming duration (s)	68.3 ± 10.9	44.8 ± 8.6
Rearing duration (s)	66.8 ± 10.5	53.3 ± 6.1

3.1.2. Novel object preference test

In the NOP1h test, a significant main effect of "object" ($F(1, 24) = 22.517$, $P = .0001$), but not of "group" ($F(1, 24) = 0.140$, $P = .711$) and "object × group" ($F(1, 24) = 0.454$, $P = .507$) was found. Paired-sample *t*-tests revealed that both groups spent more time exploring the novel object than the familiar one (WT group: d.f. = 12, $t = -2.878$, $P = .014$; tgDISC1 group: d.f. = 12, $t = -3.833$, $P = .002$; Fig. 2A). In the analysis of the repeated NOP1h test, a two-way ANOVA revealed a main effect of "object" ($F(1,24) = 19.388$, $P = .0001$), but not of "group" ($F(1, 24) = 0.268$, $P = .610$) or "interaction" ($F(1, 24) = 0.401$, $P = .532$). Paired-sample *t*-test showed that the WT group explored the novel object more than the familiar one (d.f. = 13, $t = -2.708$, $P = .018$; Fig. 2B). Accordingly, the tgDISC1 group spent more time exploring the novel object than the familiar one (d.f. = 11, $t = -3.537$, $P = .005$; Fig. 2B). The results indicate that both groups had intact short-term memory for novel objects after a delay of 1 h.

In the NOP24h test, a significant interaction between "object" and "group" ($F(1, 23) = 15.574$, $P = .001$) and a significant main effect of "object" ($F(1, 23) = 10.859$, $P = .003$), but not of "group" ($F(1, 23) = 2.450$, $P = .131$) was found. Paired-sample *t*-test showed a significant difference in exploration time (d.f. = 13, $t = -5.192$, $P = .0001$) between familiar object and novel object in the WT group. The tgDISC1 group explored both objects for an approximately equal time (exploration time: d.f. = 10, $t = 0.468$, $P = .650$; Fig. 2C), showing that they cannot distinguish the old from the novel object. Similar results were found in the repeated NOP24h test. There was an "object" effect ($F(1, 21) = 8.585$, $P = .008$), but no effect of "group" ($F(1, 21) = 0.004$, $P = .949$) or "interaction" ($F(1, 21) = 3.240$, $P = .086$). The WT group explored the novel object longer than the familiar one (time: d.f. = 11, $t = -3.087$, $P = .01$). In the tgDISC1 group, there was no difference in exploration time (d.f = 10, $t = -0.903$, $P = .388$) between the familiar and the novel object (Fig. 2D). In all of the test trials, the total time of exploration of objects was not significantly different between the groups ($ps > 0.05$, independent t-tests, data not shown). Thus, the tgDISC1 rats, unlike the WT controls, did not show intact long-term object memory when the inter-trial interval was extended to 24 h.

3.1.3. Object-based attention test

The ANOVA revealed a significant main effect of "object" ($F(1, 24) = 6.482$, $P = .018$), but no effect of either "group" ($F(1, 24) = 3.016$, $P = .095$) or "interaction" ($F(1, 24) = 2.744$, $P = .111$). The novel object was explored for a longer time (d.f. = 12, $t = -3.626$, $P = .003$) compared to the familiar one in the WT group. In contrast, the tgDISC1 group did not show a significant difference in exploration time between the novel and the familiar object (d.f. = 12, $t = -0.546$, $P = .595$) (Fig. 2E). No significant difference between groups was found in total time of object exploration in the test trial ($p > .05$, data not shown). In summary, these results show that the tgDISC1 animals are deficient in long-term object memory and in object-based attention,

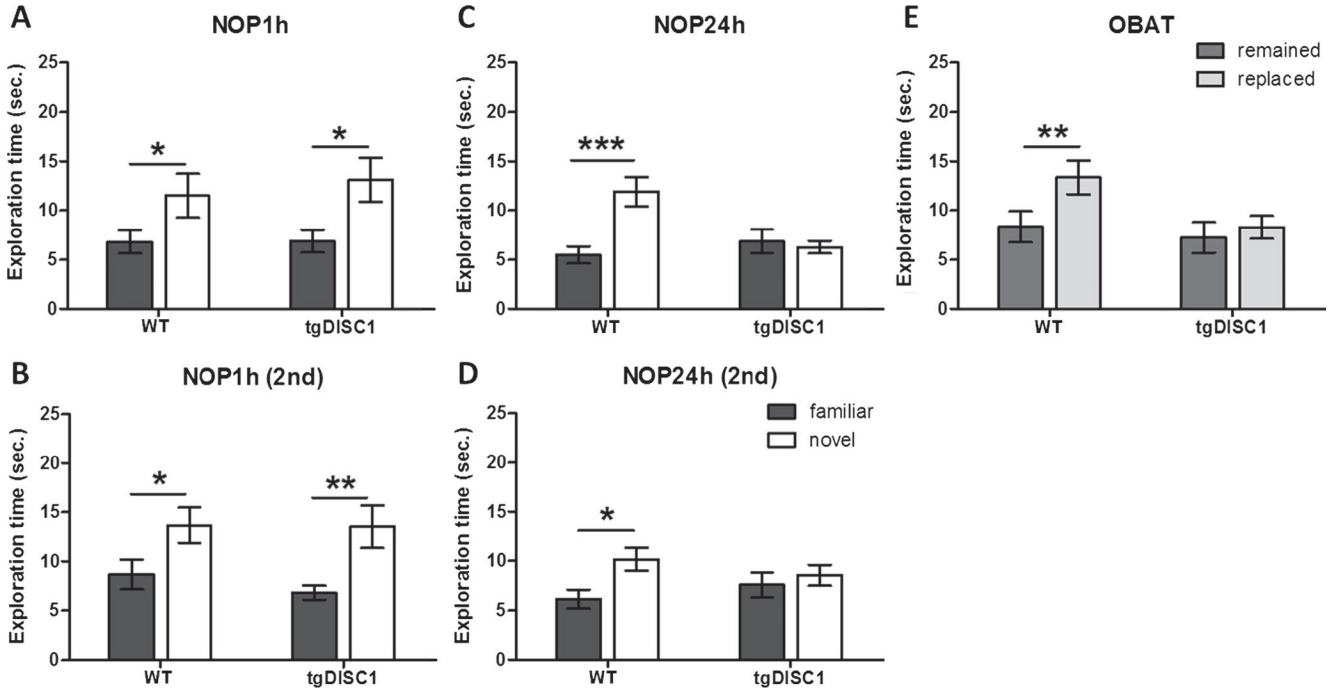

Fig. 2. Object exploration in experiment 1. The tgDISC1 group showed intact short-term memory in the novel object preference (NOP) test with 1 h-interval in the first (A) and second (B) test, but not with the 24 h-interval (C) in the first and in the second test (D), nor in the object-based attention test (OBAT) (E). The wild-type (WT) group showed intact short- and long-term memory and attention-related behavior. N = 12–14 in the WT group. N = 11–13 in the tgDISC1 group. Values represent mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ compared via paired-sample *t*-tests.

while their short-term object memory is intact.

3.2. Experiment 2: Intranasal dopamine application reverses deficits in long-term object memory and object attention in tgDISC1 rats

Since tgDISC1 rats failed to perform intact long-term object memory and object-based attention, we hypothesized that these behaviors were aberrant due to the known regulation of the DAergic system by DISC1 and that administration of DA to the brain would alleviate these deficiencies. Thus, IN-DA was administrated in the tgDISC1 animals and OBAT and NOP24h tests were subsequently repeated.

3.2.1. Object-based attention test

The ANOVA revealed a main effect of "object" ($F(1,15) = 5.205$, $P = .038$), but neither of "treatment" ($F(1,15) = 0.026$, $P = .873$) nor of the interaction between "object" and "treatment" ($F(1,15) = 2.404$, $P = .142$). The vehicle-treated tgDISC1 animals consistently failed to distinguish between the objects corroborating the results of experiment 1. They showed comparable exploration times of the novel and familiar objects (d.f. = 8, $t = -0.528$, $P = .612$). In contrast, the animals treated with IN-DA explored the novel object significantly longer than the familiar one (d.f. = 7, $t = -2.662$, $P = .031$; Fig. 3A). There was no significant group difference in the comparison of total time for object exploration between WT and tgDISC1 animals in the test trial ($p > .05$, data not shown). Thus, IN-DA treatment reversed the impairment in object-based attention of the tgDISC1 animals and restored a putative attention deficit.

3.2.2. Novel object preference test

For the NOP24h test, the two-way ANOVA revealed a main effect of "object" ($F(1,13) = 8.403$, $P = .012$) but not of "treatment" ($F(1,13) = 0.061$, $P = .809$) and the interaction between "object" and "treatment" ($F(1,13) = 1.518$, $P = .240$). When treated with vehicle, the animals showed comparable exploration times of the novel and familiar objects (exploration time: d.f. = 5, $t = -1.137$, $P = .307$; Fig. 3B). In contrast, the animals treated with IN-DA explored the novel object for a significantly longer time than the familiar one (d.f. = 8, $t = -3.164$, $P = .013$; Fig. 3B), and, thus, showed intact long-term object memory. The total time of exploration of objects was not significant between groups in all of the test trials ($ps > 0.05$, data not shown). Thus, IN-DA treatment also reversed the dysfunction of long-term object memory in the tgDISC1 rats.

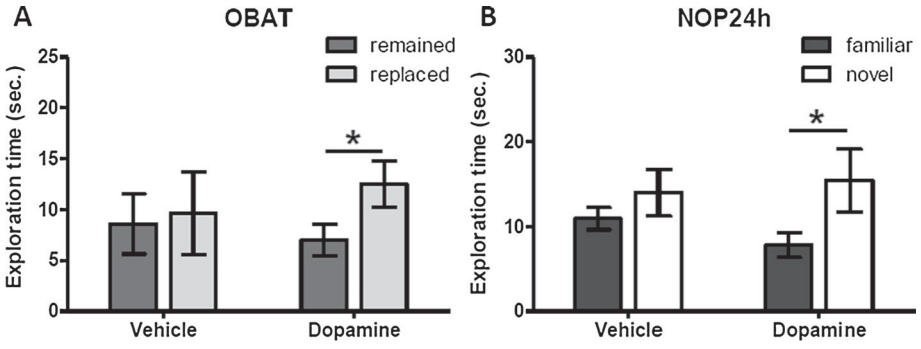

Fig. 3. Effects of intranasal dopamine administration on object explorative behavior in the object-based attention test (OBAT) (A) and novel object preference (NOP) test with a 24 h-interval (B) in tgDISC1 rats. The intranasal dopamine-treated group explored the novel object significantly more in both the OBAT and NOP24h tests; whereas the vehicle-treated group did not. Values represent mean ± SEM. *$p < .05$, compared via the pair-sample *t*-tests.

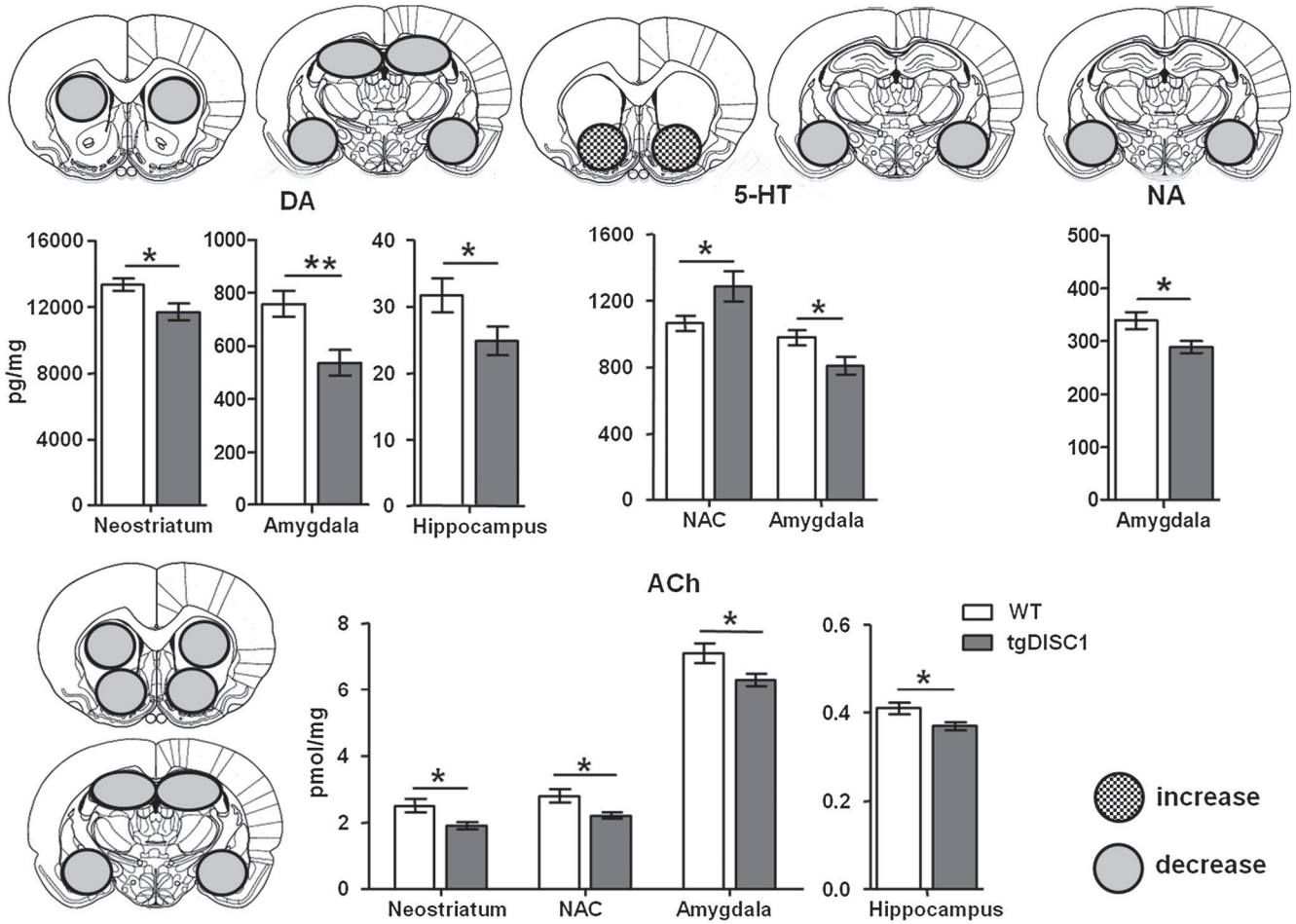

Fig. 4. Neurochemical quantification of dopamine (DA), noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) in the WT and tgDISC1 groups. The mean contents of DA in the neostriatum, amygdala and hippocampus, 5-HT in the amygdala, and NA in the amygdala, was significantly lower in the tgDISC1 group. The mean content of 5-HT in the nucleus accumbens (NAC) was significantly higher in the tgDISC1 group. Lower levels of ACh were found in the neostriatum, NAC, amygdala and hippocampus in the tgDISC1 animals, when compared to WT groups. (The DA results have been published in a previous article.[****9] N = 11–12 each group. Values represent mean ± SEM. *$p \leq .05$, **$p < .01$, compared to the WT group via independent *t*-test.

3.3. Neurochemistry of tgDISC1 and WT brains

3.3.1. Monoamines

DA concentrations were significantly decreased in the neostriatum, amygdala and hippocampus of the tgDISC1 rats relative to WT controls (d.f. = 22, $t = 2.646$, $P = .015$; d.f. = 20, $t = 3.254$, $P = .004$; d.f. = 21, $t = 2.060$, $P = .052$, respectively; Fig. 4) (These results of DA levels have been part of a previous publication (Trossbach et al., 2016).

NA levels were significantly decreased in the amygdala of tgDISC1 rats relative to WT controls (d.f. = 22, $t = 2.553$, $P = .018$; Fig. 4). No significant differences between tgDISC1 rats and WT controls were observed in the other brain regions ($P > .05$; data not shown).

5-HT levels were increased significantly in the nucleus accumbens of tgDISC1 rats relative to WT controls (d.f. = 22, $t = -2.126$, $P = .045$; Fig. 4). In the amygdala, 5-HT concentrations were reduced in tgDISC1 compared with the WT controls (d.f. = 22, $t = 2.442$, $P = .023$; Fig. 4). No significant differences between tgDISC1 rats and WT controls were observed in other brain regions ($P > .05$; data not shown).

3.3.2. Acetylcholine

ACh levels were significantly lower in the neostriatum (d.f. = 22, $t = 2.495$, $P = .021$), nucleus accumbens (d.f. = 22, $t = 2.619$, $P = .045$), hippocampus (d.f. = 22, $t = 2.440$, $P = .023$) and amygdala (d.f. = 22, $t = 2.131$, $P = .045$) of tgDISC1 rats relative to WT controls (Fig. 4). No difference was found in the frontal cortex ($P > .05$; data not shown).

4. Discussion

The main findings of the present study on tgDISC1 rats that overexpress the full-length non-mutant human *DISC1* gene are: (1) TgDISC1 rats were deficient in long-term (24 h) object memory, as well as in attention-like behavior, but not short-term (1 h) memory, (2) in tgDISC1 rats, administration of IN-DA reversed the deficits of long-term object memory and attention-like behavior, and (3) in the tgDISC1 rats post mortem baseline levels of DA were significantly lower in the neostriatum, hippocampus and amygdala. ACh levels were lower in the neostriatum, nucleus accumbens, hippocampus and amygdala. NA was lower in the amygdala, while 5-HT levels were reduced in the amygdala, but elevated in the nucleus accumbens.

In the present study, tgDISC1 rats were impaired in long-term memory for NOP and in attention-related behavior as tested with the OBAT. Thus, it appears that DISC1 overexpression impairs working memory during a very short delay between exposure and test trials, suggesting that the *DISC1* gene may affect memory processes in rodents, when tasks require responses within a short time window. This is consistent with findings on humans with a haplotype of the DISC1 gene, who displayed both poor short-term visual memory and attention (Hennah et al., 2005). The dual effect of the *DISC1* gene - deleterious performance in OBAT (ultra short-term) and long-term NOP test - implies that at least two distinct processes are influenced by the

overexpression of the *DISC1* gene. One deficit involves long-term memory. Since short-term memory at the 1 h interval was intact, the question arises as how to interpret the deficit in the OBAT test with no interval between exposure to the objects and the test trial. This test can be considered either to assess ultra short-term memory and/or attention-like behavior (in accordance with the interpretation preferred by the inventors Alkam et al., 2011, 2013). Given that the total exploration time of objects was comparable in both groups in all the test trials, the level of motivation for object exploration should not be a confounding factor here.

Novel object exploration memory is considered to involve the hippocampus and its adjacent cortical areas, including the entorhinal, perirhinal, and parahippocampal cortices. The hippocampus receives input from the perirhinal cortex, including visual, olfactory, and somatosensory information, which is crucial for object recognition (Clarke, Cammarota, Gruart, Izquierdo, & Delgado-Garcia, 2010). The dorsal hippocampus plays an important role in NOP (Broadbent, Gaskin, Squire, & Clark, 2010; Cohen et al., 2013) and for the consolidation of NOP memory (Chao, Huston, Nikolaus, & de Souza Silva, 2016; Oliveira, Hawk, Abel, & Havekes, 2010; Reger, Hovda, & Giza, 2009). The prefrontal cortex is relevant for visual working memory and attention and interacts with the hippocampus for the processing of long-term memory (Preston & Eichenbaum, 2013; Simons & Spiers, 2003).

There is ample evidence that the DISC1 gene is associated with prefrontal function. Transgenic mice, expressing a putative dominant-negative *DISC1* gene, were impaired in reversal learning and reinforcement devaluation performance, along with increased oxidative stress in the prefrontal cortex (Johnson et al., 2013). Deficient dendrites and a decreased spine density were also found in the frontal cortex of DISC1 mutant mice (Lee et al., 2011). Furthermore, in the tgDISC1 rat interneurons were shifted to deeper layers in the sensorimotor cortex (Hamburg et al., 2016). Thus, it is likely that prefrontal cortex functions are disrupted in the tgDISC1 rat. Disrupted prefrontal cortex function may account for both the deficit in long-term NOP memory and the poor performance in the OBAT test. Further investigations are required in order to clarify the contribution of the hippocampus and of the interaction between these regions to the cognitive deficits observed in the present study.

Previous findings on tgDISC1 rats showed elevated DAT function and an increase of D2R density in the ventral striatum (Trossbach et al., 2016). Moreover, in DISC1-L100P mutant mice, the D2R formed a protein complex with DISC1, facilitating D2R-mediated glycogen synthase kinase-3 signaling and inhibiting agonist-induced D2R internalization (Su et al., 2014). Given the DAergic connections between the dorsal/ventral striatum and prefrontal cortical areas (Haber, 2014), which are relevant for both motor and cognitive functions, alterations of DA functions are likely to be involved in the behavioral impairments observed in tgDISC1 rats. This is underlined by the ameliorative effects of IN-DA. It may be argued that the increased availability of D2 heteroreceptors in the ventral striatum of tgDISC1 rats, firstly, leads to an increased inhibition of substantia nigra/ventral tegmental area, and, thus, to a disinhibition of the prefrontal cortex. Secondly, the increased availability of D2 autoreceptors increases feedback inhibition, which – together with the observed increase of DAT function in tgDISC1 rats – is likely to result in a shortage of extracellular DA. It may be that this deficit is compensated by the administration of IN-DA. After IN-DA delivery, DA was detected in the CSF and brain (Dahlin et al., 2001) and increased DA levels were found in the neostriatum and nucleus accumbens (de Souza Silva et al., 2008). IN-DA administration led to reduced DAT binding in the dorsal striatum, which suggests an elevation of synaptic DA activity by this treatment (de Souza Silva et al., 2016). IN-DA in rats also increased locomotor activity (de Souza Silva et al., 2008) and had anti-depressant-like effects (Buddenberg et al., 2008). An anxiolytic effect and higher locomotion were found in mice with IN-DA treatment (Kholodar, Amikishieva, & Anisimov, 2013). In aged rats, IN-DA compensated memory deficits tested in object-place preference test (Trossbach et al., 2014). Treatment with IN-DA attenuated deficient spatial attention and working memory in an animal model of ADHD (Ruocco et al., 2014). Given that the *DISC1* gene influences the DAergic systems (for review, see Dahoun et al. (2017)), treatment with DA may "re-balance" the system and, thus, compensate for DA-related deficits. A working hypothesis is that DISC1 interacts with striatal DAergic systems and, thereby, influences the prefrontal cortex to impede short-term/working memory, attention and long-term memory deficits.

Post-mortem analysis revealed lower total DA levels in neostriatum, hippocampus and amygdala of tgDISC1 rats. Neurochemical differences, however, were not confined to DA. TgDISC1 rats also had lower levels of ACh in the neostriatum, nucleus accumbens, hippocampus and amygdala, which are major projection areas of the basal forebrain cholinergic neurons (Dani & Bertrand, 2007). The cholinergic system in the hippocampus is acknowledged to regulate memory processes (Everitt & Robbins, 1997; Hasselmo, 2006). The lower levels of hippocampal ACh observed in the present study may be related to the impaired long-term memory in the NOP test. Striatal cholinergic interneurons are targeted by the nigrostriatal dopaminergic system (Pickel & Chan, 1990). DA agonists regulate the inhibition of ACh release in the striatum via D2R on cholinergic neurons (Ikarashi, Takahashi, Ishimaru, Arai, & Maruyama, 1997; Stoof, Drukarch, de Boer, Westerink, & Groenewegen, 1992). Since we also found a significant decrease of hippocampal DA levels, disruption of the DAergic system might have impaired cholinergic functions by a variety of mechanisms.

The higher 5-HT and lower ACh concentrations observed in the nucleus accumbens might indicate an interaction between these two neurotransmitter systems. 5-HT can have direct effects on cholinergic neurons, resulting in their inhibition (Van Bockstaele, Chan, & Pickel, 1996). Along with DA, 5-HT and NA levels were reduced in the amygdala, which controls motivated behaviors along with prefrontal cortex and hippocampus (Gruber & McDonald, 2012; Robbins & Everitt, 1996). The neurotransmitter changes induced by the *DISC*1 gene in nucleus accumbens and amygdala may be related to reward-based learning, since previous studies on transgenic mice expressing putative dominant-negative DISC1 revealed a deficiency in reinforcement devaluation (Johnson et al., 2013). Taken together, both the monoaminergic and cholinergic systems are dysregulated in the tgDISC1 rats, which may be related to the observed behavioral deficits. *Post-mortem* levels of neurotransmitters, as assessed here, indicate changes in the systems involved, but must be interpreted with caution as they do not necessarily reflect extracellular activity.

Further studies are necessary to establish the developmental course of the memory deficits in the tgDISC1 rat as well as to determine whether early intranasal DA treatment can prevent the neurochemical and cognitive deficits.

In conclusion, the overexpression of the human *DISC1* gene impaired object long-term memory and ultra-short memory or attention, which was alleviated by IN-DA administration. In the tgDISC1 rats, DA, 5-HT, NA and ACh levels were affected in neostriatum, nucleus accumbens, amygdala and hippocampus, suggesting an interaction between the *DISC*1 gene and these systems, possibly in relation to the observed behavioral deficiencies.

Rare mutations of the *DISC1* gene have been closely linked to various psychiatric disorders, including susceptibility to schizophrenia (Blackwood et al., 2001; Chubb et al., 2008). A functional interaction between DISC1 and the DA system would seem to be compatible with DISC1 functions in schizophrenia. On the other hand, deficits in attention and working memory correspond to symptoms of ADHD. In fact, IN-DA was shown to alleviate the behavioral ADHD markers, attention and working memory deficit, in an animal model of ADHD (Ruocco et al., 2014). Therefore, rather than to assign DISC1 dysfunction, be it genetic or on the posttranslational level, to a clinical diagnosis, we

consider it a vulnerability factor based on deficient DA homeostasis.

Several antipsychotic drugs, which affect the DAergic system were also found to alleviate DISC1-related cognitive impairments. The DAT inhibitor bupropion improved the antidepressant-like behavior in the forced swim test (Clapcote et al., 2007) and ameliorated social deficits (Lipina, Fletcher, Lee, Wong, & Roder, 2013), while the D1R agonistic clozapine reversed the impairment of short-term object memory (Nagai et al., 2011) and long-term spatial memory (Cui et al., 2016). Likewise, the finding that administration of DA via the nose-brain pathway can restore cognitive functions in the tgDISC1 rat indicates that DA deficiency may be a major cause for the behavioral deficits of the tgDISC1 rat, and that cognitive deficits in DISC1-related disorders, may be ameliorated by administration of IN-DA.

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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human *DISC1* Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Contribution: sacrificed and collected the brain samples, and helped writing the animal section in the manuscript

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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Disrupted-in-schizophrenia 1 (DISC1) is a key protein involved in behavioral processes and various mental disorders, including schizophrenia and major depression. A transgenic rat overexpressing non-mutant human DISC1, modeling aberrant proteostasis of the DISC1 protein, displays behavioral, biochemical and anatomical deficits consistent with aspects of mental disorders, including changes in the dorsal striatum, an anatomical region critical in the development of behavioral disorders. Herein, dorsal striatum of 10 transgenic DISC1 (tgDISC1) and 10 wild type (WT) littermate control rats was used for synaptosomal preparations and for performing liquid chromatography-tandem mass spectrometry (LC-MS)-based quantitative proteomics, using isobaric labeling (TMT10plex). Functional enrichment analysis was generated from proteins with level changes. The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling. DISC1-regulated proteins proposed in the current study are also highly associated with neurodevelopmental and mental disorders. Bioinformatics analyses from the current study predicted that the following biological processes may be activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation. Our findings demonstrate that the effects of overexpression of non-mutant DISC1 or its misassembly has profound consequences on protein networks essential for behavioral control. These results are also relevant for the interpretation of previous as well as for the design of future studies on DISC1.

Keywords: DISC1, proteomics, synapses, animal model, dopaminergic system, axon guidance, striatum

INTRODUCTION

Disrupted-in-schizophrenia 1 (DISC1) is a gene originally identified as a translocation mutation in an extended Scottish pedigree where carriers suffered from diverse mental disorders comprising schizophrenia and affective disorders (Millar et al., 2000). Similarly, the DISC1 haplotype was associated with schizophrenia in a Finnish cohort (Hennah et al., 2003). A second family was later identified with a missense mutation and associated diverse clinical phenotypes (Sachs et al., 2005), and genetic association studies have supported association of DISC1 with mental disorders (Chubb et al., 2008). A role of the DISC1 gene for adaptive behavior was also suggested by various animal studies (Brandon and Sawa, 2011; Dahoun et al., 2017).

The DISC1 protein has features of a scaffold protein (Yerabham et al., 2013) and several subdomains have an intrinsic tendency to form high molecular multimers (Yerabham et al., 2017). Insoluble DISC1 protein has been identified in human post mortem brains with mental disorders (Leliveld et al., 2008), indicating that the DISC1 protein can be subject to aberrant proteostasis *in vivo*. For modeling the effects of aberrant proteostasis *in vivo*, a transgenic rat model overexpressing (approximately 11-fold) the full length, non-mutant human DISC1 gene (transgenic DISC1, tgDISC1 rat) was generated that exhibited perinuclear aggregates throughout the brain, accentuated in dopamine-rich regions such as in the striatum (Trossbach et al., 2016). The tgDISC1 rat exhibited phenotypes such as amphetamine supersensitivity, an increase in D2Rhigh receptors, and dopamine transporter mislocalization and dysfunction consistent with phenotypes observed in schizophrenia (Trossbach et al., 2016). Also, at the neuroanatomical level fewer dopaminergic neurons and projections into the dorsal striatum, as well as aberrant interneuron positioning was observed indicating subtle neurodevelopmental disturbance (Hamburg et al., 2016).

These findings, induced by aberrant proteostasis of the DISC1 protein, leading to its misassembly and perinuclear deposition, suggest an important role of the DISC1 protein and its correct assembly for protein networks involved in adaptive behavior. Such protein networks have been described both, at the protein and the genetic level. At the genetic level, Teng et al. (2017) carried out targeted sequencing of 59 DISC1 interactome genes and 154 regulome genes in psychiatric patients, identifying altered regulation of schizophrenia candidate genes by DISC1. In an attempt to dissect DISC1 function through protein-protein interactions based upon a yeast two-hybrid system along with bioinformatic methods, a comprehensive network around DISC1 was generated (Camargo et al., 2007). Using this iterative yeast two-hybrid system, a framework was provided to explore the function of DISC1, and interrogation of the proposed interactome has shown DISC1 to have protein-protein interactions consistent with that of an essential synaptic protein (Camargo et al., 2007). Current evidence suggests that DISC1 functions as a neuronal intracellular trafficking regulator that includes transport of neurotransmitter receptors, vesicles, mitochondria and mRNA, rendering synaptic regulation vulnerable to DISC1 dysfunction (Devine et al., 2016).

The objective of this study was to identify the proteomic signatures of the tgDISC1 rat model vs. its littermate wild type (WT) control to gain insights onto the DISC1-regulated proteins and downstream synaptic processes and to identify molecular circuitry regulated by relatively modest changes in expression level leading to DISC1 misassembly. Identification of changes in protein networks relevant for behavioral processes would raise the possibility for the DISC1 protein to represent a non-genetic interface with exogenous influences for mental disorders.

There is mounting evidence for a focal role of the DISC1 protein in striatal functions, and particularly on dopamine homeostasis in relation to behavioral changes (Trossbach et al., 2016; Wang et al., 2017). Therefore we chose to select proteins from the synapse-enriched membrane fractions (synaptosomes) from the dorsal striatum for this study. Differential proteomics by isobaric labeling (TMT10plex) enable multiplexed protein identification and quantitative analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This allows the unbiased analyses of approximately 6000 proteins and targets synaptic proteins including receptors, transporters and channels that have been implicated in psychiatric disorders. Combining proteomics and bioinformatics approaches enabled a comprehensive view on the *in vivo* protein changes and the biological functions of DISC1.

MATERIALS AND METHODS

Animals

Previously described tgDISC1 Sprague-Dawley rats and WTs were used in this study (Trossbach et al., 2016). Briefly, full-length, non-mutant human DISC1 as transgene with the polymorphisms F607 and C704 were integrated into the pronuclei of Sprague Dawley rats. Ten male tgDISC1 rats and 10 male WT littermate control rats, aged 14–15 months (ZETT, Heinrich Heine University, Düsseldorf, Germany) were used. One WT rat and one tgDISC1 rat were derived from each pair of parents. The study was carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985), and the German Law on the Protection of Animals. It was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW.

Preparation of Synaptosomal Fractions

Dorsal striata from fresh brains were dissected and stored at −80°C. Synaptosomal fractions from bilateral regions were prepared for individual animals (for tgDISC1 and WT; $n = 10$ each), using a microscale discontinuous sucrose gradient modified from previous protocols (Hahn et al., 2009; Sialana et al., 2016). Collected synaptosomes from 1.25/1.0 M sucrose interface were diluted with 10 mM HEPES, divided into two and pelleted at 15,000× *g* for 30 min. Pelleted synaptosomal samples were reconstituted in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 50 mM TEAB supplemented with protease inhibitors) for LCMS analyses and SDS buffer

(1.5% SDS, 100 mM NaCl, 20 mM Tris supplemented with protease inhibitors) for WB analyses and were sonicated for 1 h. Protein amounts were estimated using the Pierce 660 protein assay or BCA protein assay (ThermoFisher Scientific).

Proteolytic Digestion and Isobaric Labeling

Fifty micrograms of samples were digested with a Trypsin-LysC enzyme mixture (1:100 w/w, Promega) using the filter-aided sample preparation (FASP), as previously described, with minor modifications (Wisniewski et al., 2009). The resulting peptide samples were purified with reversed-phase C18 and labeled with TMT 10-plex according to the instructions supplied by the manufacturer. Two TMT-10plex experiments were performed, with each experiment consisting of five tgDISC1 and five WT animals (n = 10 biological replicates per group). For each TMT experiment, ten isobarically labeled peptide samples were pooled, the peptides separated by high pH reversed-phase LC into 100 time-based fractions and pooled into 25 samples (Gilar et al., 2005). The peptides were vacuum concentrated and reconstituted in 5% formic acid. Details of the procedure are essentially as described previously (Sialana et al., 2016) and in the Supplementary Figure S1.

Liquid Chromatography and Tandem Mass Spectrometry

Samples were injected onto a Dionex Ultimate 3000 system (ThermoFisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Schwerte, Germany). Software versions used for the data acquisition and operation of the Q-Exactive were Tune 2.8.1.2806 and Xcalibur 4. HPLC solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostated autosampler, 10 μL that correspond to 1 μg of the peptide mixture were automatically loaded onto a trap column (PM100-C18 3 μm, 75 μm × 20 mm, ThermoFisher Scientific, Austria) with a binary pump at a flow rate of 5 μL/min using 2% acetonitrile in 0.1% TFA for loading and washing the pre-column. After washing, the peptides were eluted by forward-flushing onto a 50 cm analytical column with an inner diameter of 75 μm packed with 2 μm-C18 reversed phase material (PepMap-C18 2 μm, 75 μm × 500 mm, ThermoFisher Scientific, Austria). For label free quantification (LFQ), the LCMS analyses was performed using a single-shot LCMS approach with 4-h gradient with LCMS parameters as described previously (Stojanovic et al., 2017).

The fractionated TMT10plex labeled peptides were eluted from the analytical column with a 120 min gradient ranging from 5% to 37.5% solvent B, followed by a 10 min gradient from 37.5% to 50% solvent B and finally, to 90% solvent B for 5 min before re-equilibration to 5% solvent B at a constant flow rate of 300 nL/min. The LTQ Velos ESI positive ion calibration solution (Pierce, IL, USA) was used to externally calibrate the instrument prior to sample analysis and an internal calibration was performed on the polysiloxane ion signal at m/z 445.120024 from ambient air. MS^1 scans were performed from m/z 375–1400 at a resolution of 70,000. Using a data-dependent acquisition mode, the 15 most intense precursor ions of all precursor ions with +2 to +7 charge were isolated within a 1.2 m/z window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher-energy collisional dissociation (HCD) cell at 32% normalized collision energy with a fixed first mass at 100 m/z and detected in an Orbitrap mass analyzer at a resolution of 35,000. The dynamic exclusion for the selected ions was 30 s. Maximal ion accumulation time allowed in MS and MS^2 mode was 50 and 100 ms, respectively. Automatic gain control was used to prevent overfilling of the ion trap and was set to 3×10^6 ions and 1×10^5 ions for a full Fourier transform MS and MS^2 scan, respectively.

Protein Identification and Quantification

All MS-MS^2 spectra were searched against UniProtKB/Swiss-Prot rat protein database version v 2016.04.14 (27,815 sequences, including isoforms). In addition, sequences of the human DISC1 protein and 11 isoforms produced by alternative splicing with the polymorphisms F607 and C704 were appended to the rat database. All spectra files were processed in Proteome Discoverer 2.1 (Thermo Scientific, Germany) platform with Mascot using mass tolerances of ±10 ppm and ±0.02 Da for precursor and fragment ions. One missed tryptic cleavage site was allowed. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues, TMT 10-plex labeling of peptide N-termini and lysine residues were set as fixed modification. Thresholds were determined via the target-decoy approach using a reversed protein database as the decoy by imposing 1% false discovery rate (FDR). Label-free quantitation was implemented using the Minora feature of Proteome Discoverer 2.2. The following parameters are used: maximum retention time alignment of 10 min with minimum of S/N of 5 for feature linking mapping. Abundance were based precursor/peptide area intensities. Normalization was performed such that the total sum of the abundance is the same for all sample channels. Imputation was performed by replacing the missing values with random values from the lower 5% of the detected values. For TMT 10-plex labeled samples, relative abundances of proteins were determined from the TMT reporter ions without imputation. Protein abundance ratios were calculated based on unique and razor peptides. Relative protein levels were determined from the sum of the reporter ion intensities per quantitative channel that correspond to each biological animal replicate.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset identifier PXD008123.

Bioinformatics

Quantitative data were analyzed using Perseus statistical package (version 1.5.1.6; Tyanova et al., 2016). Statistical significance of differences in protein levels between the groups were evaluated using a two-sided T-test with $P < 0.05$ (either Student's or Welch's as required). Enrichment of GO annotations were

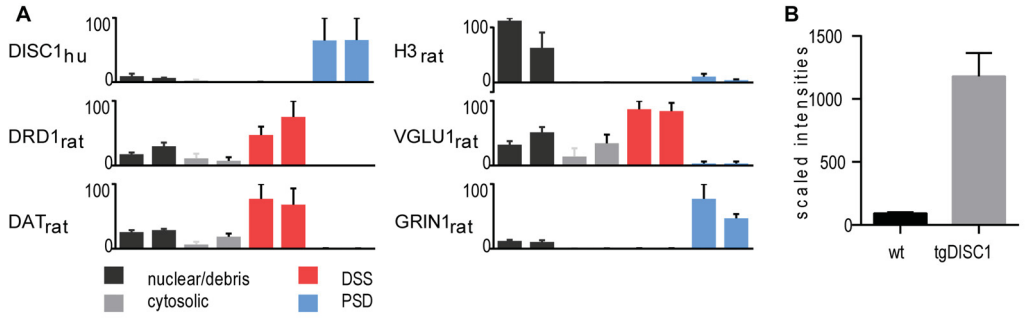

FIGURE 1 | Proteomic profile of the transgenic DISC1 (tgDISC1) fractions. **(A)** Protein levels of representative proteins for the following biochemical fractions of the dorsal striatum: nuclear/debris, cytosolic, detergent-soluble synaptosomal preparation (DSS), postsynaptic density (PSD) preparation. Protein levels of representative synaptic markers were estimated from label-free LCMS analyses. Protein levels of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers are enriched in nuclear/debris, DSS and PSD preparations, respectively. The majority of the human Disrupted-in-schizophrenia 1 (DISC1) protein was enriched in the PSD preparations. The level of overexpression is approximately 10-fold higher than the endogenous DISC1 protein in the whole synaptosomes **(B)**.

performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Bindea et al., 2009; Huntley et al., 2015). To reduce redundancy of GO terms the fusion option was selected. Enriched GO terms (Benjamini–Hochberg P-value < 0.05) are functionally grouped into networks linked by their kappa score level ($\geq$0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled. Pathway analyses on the significant proteins were performed through the use of IPA (Ingenuity® Systems[1]). The differentially expressed genes were categorized to related canonical pathways. Only those experimentally observed or highly predicted molecules and/or relationships from tissues and cells from the nervous system were considered. The top enriched categories of canonical pathways with a P-value $< 10^{-3}$ as well as representative differentially expressed proteins in each canonical pathway is reported. Curated gene-disease annotations were obtained from Comparative Toxigenomics database (Davis et al., 2015). The IPA regulation z-score algorithm was used to predict biological functions that are expected to be activated (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset.

Immunoblotting

The following antibodies were used according to the instructions supplied by the manufacturer: mouse anti-PSD95 (124011, Synaptic Systems), mouse anti-SYP (sc-55507, Santa Cruz Biotechnology), rabbit anti-NMDAR1 (ab32915, Abcam), mouse anti-VGLUT1 (135311, Synaptic Systems), rabbit anti-GAPDH (ab9485, Abcam), rabbit anti-DAT1 (ab111468, Abcam) and mouse anti-huDISC1 (3D4, Korth lab; Ottis et al., 2011). Immunoblot data were normalized to corresponding whole-lane densitometric volumes of protein-stained membranes (Welinder and Ekblad, 2011). Immunoblotting conditions were as previously described (Sialana et al., 2016) and antibody dilutions are provided in the Supplementary Table S1.

[1]www.ingenuity.com

RESULTS

In the current study, a high-throughput proteomic approach was employed to generate a comprehensive view of the *in vivo* protein changes in striatal synaptosomes of the tgDISC1 rat model (experimental workflow, Supplementary Figure S1). Methodologically, tissue fractionation was initially performed on the dorsal striatum of tgDISC1 rats to determine the subcellular expression of tgDISC1 and which enrichment steps would be employed in this study (Phillips et al., 2001; Sialana et al., 2016). Dorsal striata of tgDISC1 rats were fractionated into nuclear/debris, cytosolic, detergent soluble synaptosome (DSS) and postsynaptic density (PSD) preparations. LCMS-based proteomic analyses of the biochemical fractions resulted in the identification and LFQ of 5002 protein groups (Supplementary Data 1). Distribution of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers enriched in nuclear/debris, DSS and PSD preparations is given in **Figure 1A**. Although DISC1 was observed in all preparations, the majority of the human DISC1 protein was enriched in the Triton-X100-resistant PSD fractions. This is in agreement with previous immunoblotting studies of DISC1 in adult rats (Hayashi-Takagi et al., 2010). We have previously shown that dopaminergic pathways are modulated in the striatum of the tgDISC1 rat (Trossbach et al., 2016). Taking into account that dopamine receptor 1 and the dopamine transporter were highly enriched in the DSS preparations (**Figure 1A**), it was decided to study the *whole synaptosome* for quantitative proteomics experiments. Immunoblots of postsynaptic (GRIN1 and PSD95) and presynaptic (VGLU1 and SYP) proteins show enrichment of synaptosomal proteins on the biochemical fraction (Supplementary Figure S2). The level of overexpression is approximately 10-fold higher than

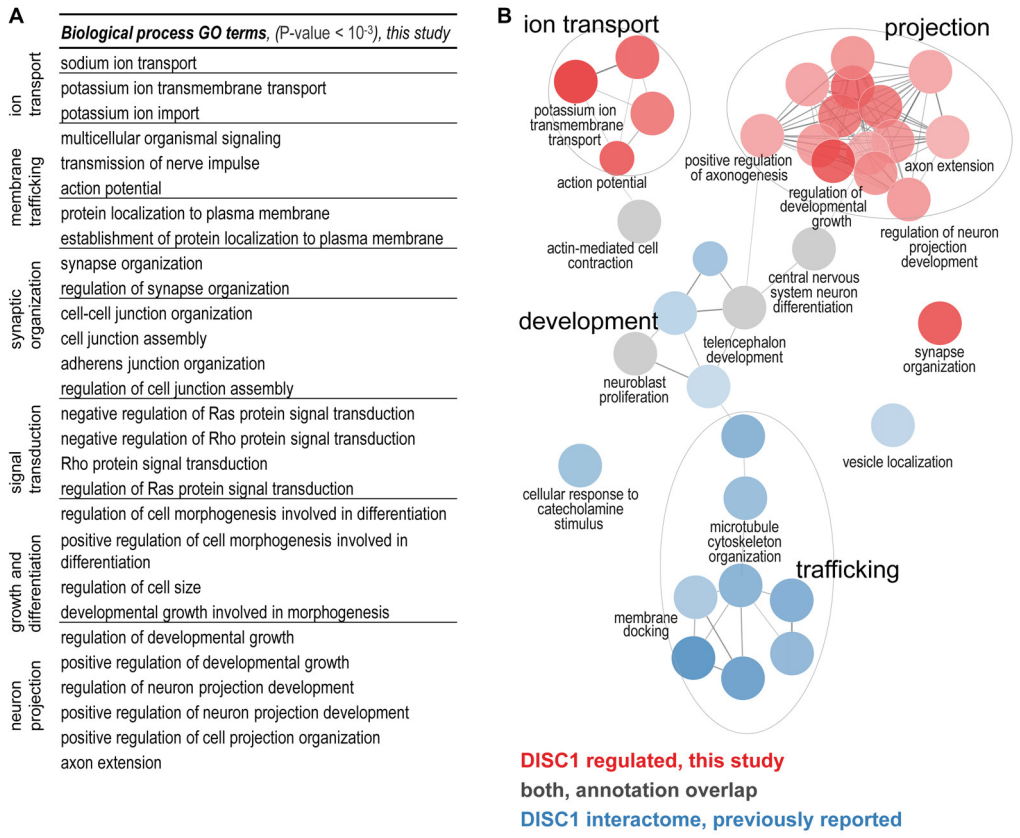

FIGURE 2 | (A) Biological processes regulated by DISC1. Enriched GO terms (*P*-value $< 10^{-3}$) are functionally grouped. The synaptic components terms as well as membrane trafficking, ion transport, synaptic organization and neurodevelopment processes are well represented. **(B)** Comparison of the DISC1-regulated proteins and the previously reported interacting proteins. In the current study ion transport, projections and synaptic organization were novel findings. Developmental processes from previous studies were confirmed (gray GO enrichment analyses was performed using ClueGO. Enriched GO terms (Benjamini–Hochberg *P*-value $< 10^{-3}$) are functionally grouped into networks linked by their kappa score level (≥0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled.

the endogenous DISC1 protein in the whole synaptosomes (**Figure 1B**).

DISC1 Regulated Proteins—Proteomic Profiling of Striatal Synaptosomes

An expression proteomics experiment was performed to identify the proteins potentially regulated by DISC1. Synaptosomal fractions of bilateral dorsal striata of 10 wt and 10 tgDISC1 rats using TMT10plex were analyzed in two separate 10-plex experiments (5 tgDISC1 and 5 WT). In total, 7227 protein groups were identified (Supplementary Data 2) including 252 receptors and 672 transporters/channels. Out of the 6153 quantifiable protein groups, 213 proteins were statistically different between the tgDISC1 and WT rats (Supplementary Table S2, Supplementary Data 3). Protein levels were considered statistically different between groups when $P \leq 0.05$ using a two-sided *T*-test (either Student's or Welch's as required). Given the large number of comparisons made and the possibility of Type 1 error, the *p* values given cannot be interpreted in terms of "significance", but rather as "measures of effect".

As we used a good number of biological replicates for TMT-based proteomics (10 animals per group), we opted to use *T*-test that performs "individual proteins-based" hypotheses test (*T*-test) rather than a background "all-proteins-based" hypothesis test (FDR). TMT-based proteomics experiments are sensitive and precise but quantification is known to undergo ratio compression (Ow et al., 2011). The values from FDR corrections depend on effect size; smaller differences yield higher P-corrected (q-values); thus only two proteins passed the corrected thresholds. An additional filter is applied when enrichment analyses (GO annotation, IPA) is employed. Slight differences in the levels of multiple proteins should cluster relevant processes and the proteins from the top enriched processes/pathways are of higher emphasis (Pascovici et al., 2016).

Immunoblotting analyses of DAT1, GRIN1 and DISC1 of WT and tgDISC1 indicated that the direction of fold differences measured by TMT-proteomics and western blotting (Supplementary Figure S3) was consistent.

A

Canonical Pathways	P-value	Proteins
Signaling by Rho Family GTPases	4.57E-06	MYL6, ARHGEF4, RAF1, ROCK1, GNAZ, FGFR3, DES, PIK3CA, ARPC1B, CDH20, GNAS, ITGA3
Axonal Guidance Signaling	1.07E-04	MYL6, LINGO1, RAF1, ABLIM2, EPHA6, ROBO2, ROCK1, GNAZ, FGFR3, SEMA7A, PIK3CA, ARPC1B, GNAS, ITGA3
RhoGDI Signaling	2.57E-04	MYL6, ARHGEF4, ROCK1, GNAZ, ARPC1B, CDH20, GNAS, ITGA3
Ephrin Receptor Signaling	2.69E-04	RAF1, EPHA6, ROCK1, GNAZ, ARPC1B, GNAS, ITGA3, GRIN1
Actin Cytoskeleton Signaling	3.47E-04	MYL6, ARHGEF4, RAF1, ROCK1, CSK, FGFR3, PIK3CA, ARPC1B, ITGA3
Gαq Signaling	8.71E-04	RAF1, ADRA1A, ROCK1, CSK, FGFR3, PIK3CA, GNAS
Dopamine-DARPP32 Feedback in cAMP Signaling	9.33E-04	CACNA1A, ADCY3, KCNJ4, KCNJ11, GNAS, KCNJ12, GRIN1

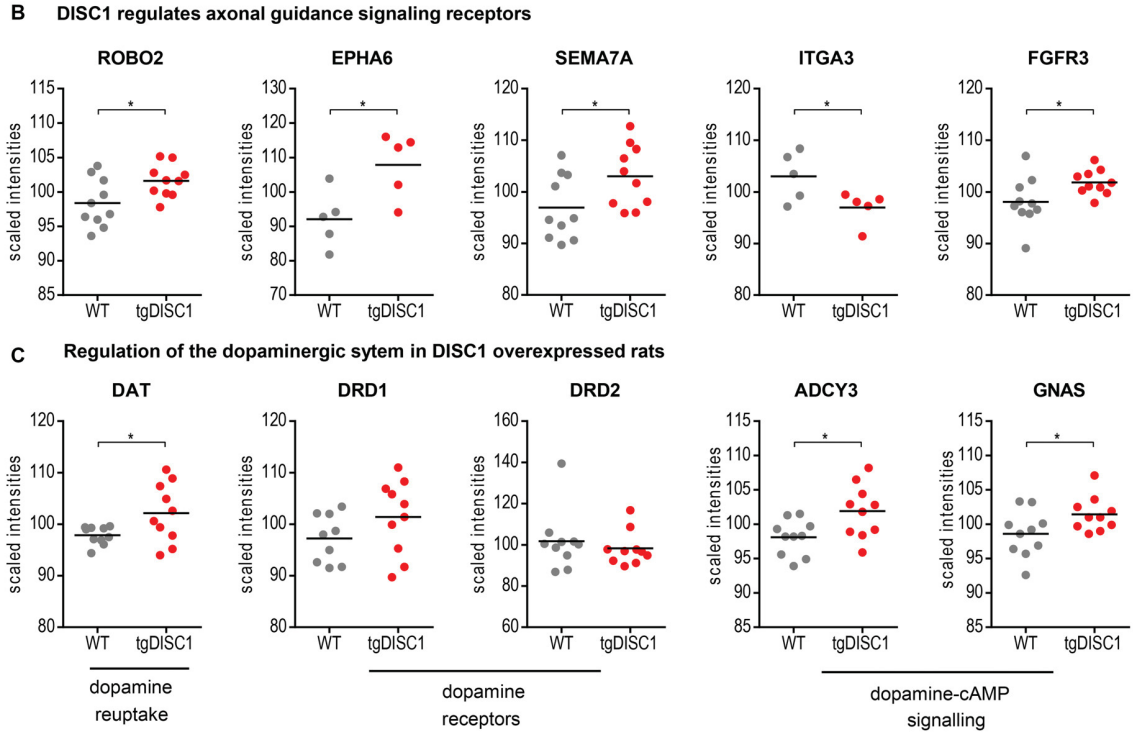

FIGURE 3 | Pathways regulated by DISC1. Significantly enriched canonical pathways (Fishers' exact test, $P < 10^{-3}$, IPA) of the proteins altered in by tgDISC1 rats in the dorsal striatum **(A)**. Representative proteins from the dopaminergic **(B)** and axonal guidance signaling pathway **(C)** are shown. Values represent $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared using two-sided *T*-tests.

Functional Classification of Proteins Modulated in tgDISC Rats

The biological functions of the 213 proteins with highly different protein level changes between wildtype and tgDISC1 rats were explored using GO enrichment analyses. Enrichment of synaptic components such as axons, dendritic spines, membrane rafts, neuron projection membrane, and the ion channel complex were revealed (Supplementary Table S3, Supplementary Figure S4). The voltage gated ion channels were the major protein classes represented (Supplementary Table S4). The results suggest that the modest overexpression of the full-length human DISC1 alters proteins linked to synaptic processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment (**Figure 2A**).

Functional Comparison of the DISC1 Regulated Proteins to Known Interacting Proteins

To determine the biological functions unique to DISC1 regulated proteins, we performed enrichment analyses for the

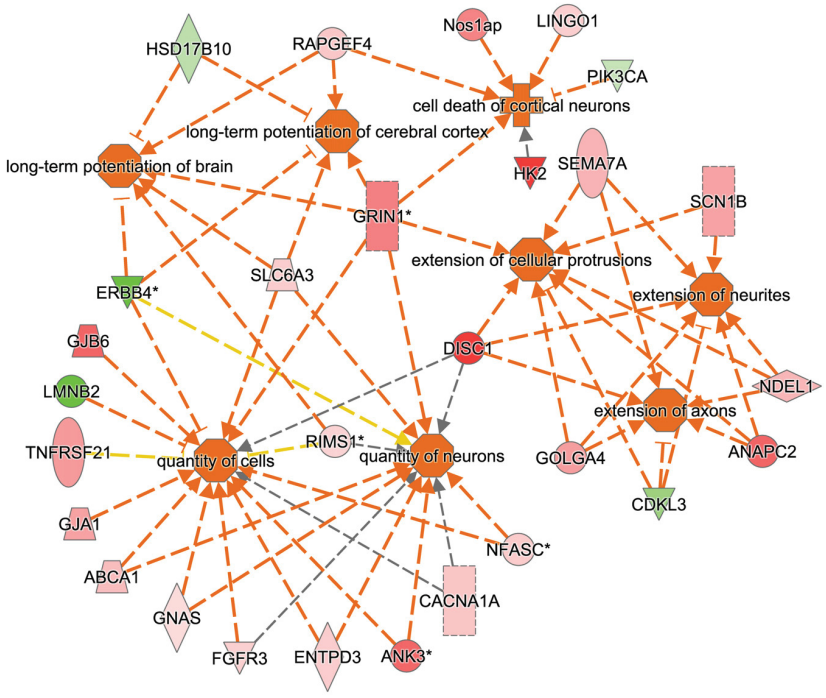

FIGURE 4 | Predicted biological functions of the tgDISC1 regulated proteins as evaluated by IPA. The IPA regulation *z*-score algorithm was used to predict the activation of biological functions in tgDISC1 rats relative to wild type (WT) according to our proteomics data. The network displays functional interactions between proteins (z-score ≥ 2 and p-value ≤ 0.05). Dashed lines indicate direct or indirect interactions. Proteins up-regulated in tgDISC1 rats are colored in shades of red; proteins down/regulated are colored in green.

DISC1 regulated proteins in comparison to previously reported interacting proteins (Camargo et al., 2007; Boxall et al., 2011; Bradshaw and Porteous, 2012; Thomson et al., 2013) as compiled by a recent study (Teng et al., 2017). Using ClueGO, 36 biological processes with strong enrichment ($P < 10^{-6}$) were revealed (**Figure 2B**; Supplementary Figure S5). The clusters of biological processes exclusive to the proteins regulated by DISC1 include: "regulation of neuron projection development", "positive regulation of axonogenesis", "action potential/potassium ion transport and synapse organization". Terms associated with microtubule development and neuronal transport were highly represented in the DISC1-interacting proteins. Biological processes such as "CNS differentiation" and "telencephalon development" were enriched in both, DISC1 regulated and interacting protein data sets.

Prediction of Canonical Pathways and Biological Function

To investigate the molecular mechanisms modulated by DISC1, data were analyzed through the use of Ingenuity Pathway analysis (IPA; Ingenuity® Systems[2]). The differentially expressed proteins were categorized to related canonical pathways. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling (Fisher's exact test, $P < 10^{-3}$, **Figure 3A**, Supplementary Figure S6). Only robustly predicted or experimentally observed molecules and/or relationships from tissues and cells from the nervous system were considered. Receptors from the axonal guidance signaling and the dopamine-DARPP32 feedback from the cAMP signaling canonical pathway are illustrated in **Figures 3B,C**).

The IPA regulation *z*-score algorithm was used to predict biological functions that are expected to be activated in tgDISC1 rats rather than in wildtype (positive z-score) according to own proteomics data (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset. From the expression data of the regulated proteins, the following processes are predicted to be activated: "activation regulation of cell quantities", "neuronal and axonal extension", "long term potentiation" and "apoptosis" (**Figure 4**, Supplementary Table S5).

Annotation of the DISC1 altered protein levels revealed that 54 proteins are associated with mental disorders and/or nervous system diseases as implemented by the Comparative Toxicogenomics Database (CTD; Davis et al., 2015). Disease-gene associations were based on genomic, transcriptomic and proteomic studies on the sequence variation

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and expression changes associated with brain diseases and disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) include: neurodevelopmental disorders, autistic disorders, schizophrenia spectrum, anxiety disorders, substance-related disorders (e.g., cocaine) and intellectual disability (**Table 1**). In particular, the schizophrenia-associated proteins including dopamine transporter 1 (SLC6A3), receptor tyrosine-protein kinase erbB-4 (ERBB4), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), membrane associated guanylate kinase WW and PDZ domain containing 2 (MAGI2) and regulator of G-protein signaling 12 (RGS12) were also regulated by DISC1 (Mateos et al., 2006; Silberberg et al., 2006; Xu et al., 2011; Koide et al., 2012; Guipponi et al., 2014; Jaros et al., 2015; Zhang et al., 2015; Li et al., 2017).

DISCUSSION

By the use of quantitative proteomics of synapse-enriched membrane (synaptosome) fractions of the dorsal striatum of the tgDISC1 rat, we have identified novel protein networks and signaling pathways regulated by an increase of non-mutant DISC1 expression or DISC1 misassembly. These results suggest that the DISC1 protein and its disturbed proteostasis can have an effect on mental disorder-relevant protein networks independent of genetic mutations. Likely, multiple exogenous or endogenous factors other than overexpression could lead to a failure of DISC1 proteostasis, such as exposure to high dosages of dopamine or other oxidants, making DISC1 protein an oxidation "sensor" (Atkin et al., 2012; Trossbach et al., 2016).

In the tgDISC1 rat, an about 11-fold overexpression, leading to DISC1 misassembly, changed proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment is observed. Furthermore, dysregulation of DISC1 potentially modulates pathways including actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling associated with the synaptic pathologies. DISC1-regulated proteins are also highly associated with neurodevelopmental disorders, autistic disorder, schizophrenia spectrum, anxiety disorders, substance-related disorders and intellectual disability (**Figure 5**).

Previously known DISC1-protein interactors have been reported to modulate synaptic processes. The current study revealed that DISC1 regulates an array of synaptic proteins and processes that complements previous protein interaction results (Supplementary Figure S7). Proteins that were previously reported to interact with DISC1 (Millar et al., 2003; Camargo et al., 2007) were also modified in the current study in the tgDISC1 rat. These include microtubule proteins pericentrin (PCNT), GRIP1 associated protein 1 (GRIPAP1), microtubule associated protein 1A (MAP1A), nudE neurodevelopment protein 1 (NDEL1) and microtubule-actin crosslinking factor 1 (MACF1) that are involved in neuronal cytoskeleton organization and membrane transport processes.

Dysregulation of DISC1 was reported to modulate glutamatergic and dopaminergic systems as previously reviewed (Hayashi-Takagi et al., 2010; Ramsey et al., 2011; Dahoun et al., 2017). Own results herein show that NMDAR1 is increased in the striatum of the tgDISC1 rat. A relationship between NMDAR1 and DISC1 has been shown, as knockdown and antagonists of NMDAR1 reduced numbers of synapses and synaptic DISC1 mainly in the striatum (Ramsey et al., 2011). Further, the DISC1 interactor GRIPAP1 is increased in the tgDISC1 rat. GRIPAP1 controls the AMPA receptors/GRIP-complex transport to the synapse by NMDA receptor activation (Ye et al., 2000).

As shown by MS, dopamine transporter levels were highly increased in the tgDISC1 rats, consistent with own previous studies by immunoblotting (Trossbach et al., 2016). Whereas levels of dopamine receptors 1 and 2 were not significantly altered, pathway enrichment analyses (**Figure 3C**) suggest that proteins (e.g., ADCY3, GNAS) from the dopamine-DARPP32 feedback of the cAMP signaling canonical pathway, may be involved in modulation of the known dopaminergic deficits in tgDISC1. Adenylate cyclase ADCY3 as a downstream effector of dopaminergic pathways catalyzes the formation of cAMP in response to G-protein signaling.

TABLE 1 | Disease-protein association of the DISC1 regulated proteins.

Disease name	*P*-value	Proteins
Neurodevelopmental disorders	1.02E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Mental disorders	5.11E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GC, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, KLHL5, LINGO2, MAGI2, RGS12, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Autistic disorder	3.40E-05	ASIC2, CADM1, DISC1, GJA1, KCNMA1, RIMS1, ROBO2, TCN2
Schizophrenia spectrum and other psychotic disorders	4.10E-04	DISC1, GC, GRIN1, MAGI2, RGS12, SLC6A3
Anxiety disorders	2.53E-02	MAGI2, SLC6A3
Cocaine-related disorders	1.74E-02	GRIN1, KLHL5, SLC6A3
Intellectual disability	2.52E-02	ANK3, DISC1, GNAS, GRIN1, KCNA2, SLC4A4
Psychotic disorders	1.13E-02	GRIN1, SLC6A3
Schizophrenia	1.62E-03	DISC1, GC, MAGI2, RGS12, SLC6A3
Substance-related disorders	3.98E-02	GNAS, GRIN1, KLHL5, LINGO2, SLC6A3

Gene-disease associations on the DISC1 regulated proteins were implemented in the Comparative Toxicogenomics Database, CTD. Fifty-four DISC1-regulated proteins are associated with mental disorders and/or nervous system disease disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) are illustrated.

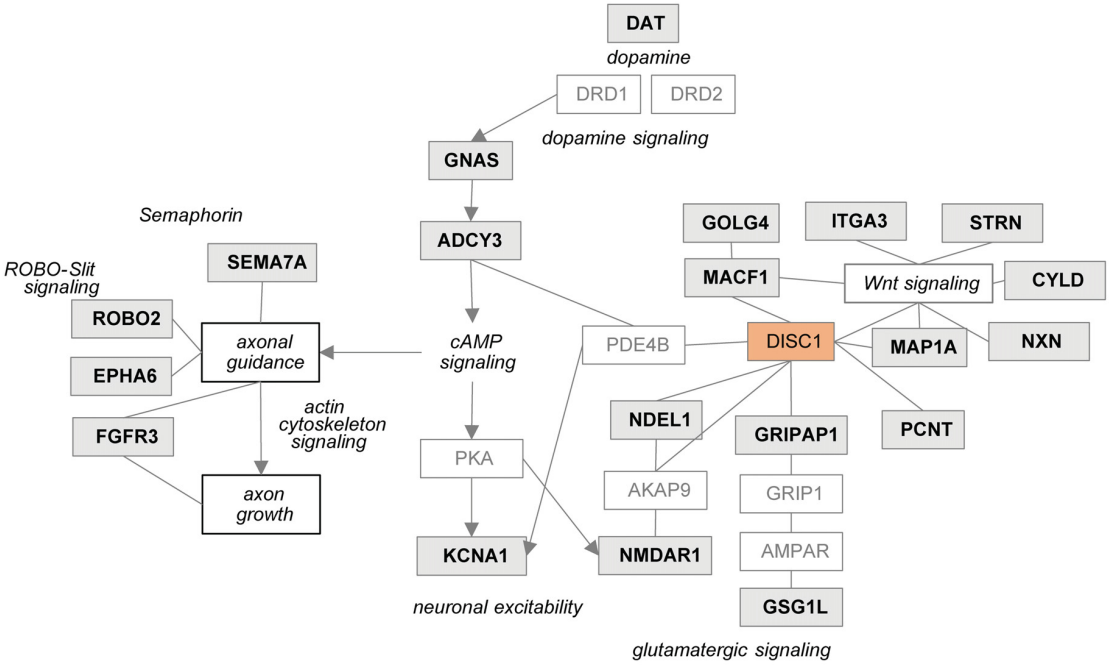

FIGURE 5 | Potential relationship between the DISC1 regulated proteins and synaptic processes. Proteins regulated in tgDISC1 rats from this study are marked in gray boxes.

The protein level changes of this enzyme along with the corresponding G-protein GNAS observed herein supports previous studies proposing dysregulation of cAMP signaling by DISC1 (Millar et al., 2005; Kvajo et al., 2011; Crabtree et al., 2017).

In a mouse Disc1 mutant model, functional reduction of Kv1.1/KCNA1 was proposed to contribute to alterations in neuronal excitability and short-term plasticity. Reduction of this channel was accompanied by reduced phosphodiesterase 4 activity and elevated cAMP levels in the PFC of *Disc1* mutant mice (Crabtree et al., 2017). Interestingly, in our DISC1 overexpressing transgenic model, we found an increase of this and several proteins in the voltage-gated potassium channel complex suggesting potential dyregulation of electrophysiological synaptic functions (Supplementary Figure S8).

Current data also revealed that proteins associated with axonal guidance pathways were altered by DISC1 overexpression: the axonal guidance receptors semaphorin 7A (SEMA7A), EPH receptor A6 (EPHA6), roundabout receptor 2 (ROBO2), fibroblast growth factor receptor 3 (FGFR3) and integrin subunit alpha 3/very late activation protein 3 receptor, alpha-3 subunit (ITGA3) were shown to be modulated by DISC1 (**Figure 3B**). The leading edge of the axons contains receptors that sense guidance cues and aid in the navigation and migration of axons. The attraction or repulsion of cues promotes or decreases active actin polymerization, resulting in axonal extension or retraction by triggering the actin cytoskeleton signaling and Rho-GTPase pathways, as also proposed in the current pathway enrichment analysis (reviewed in Dent et al., 2011; Spillane and Gallo, 2014; Van Battum et al., 2015). The receptor SEMA7A stimulates axonal growth through integrins and MAPK signaling (Pasterkamp et al., 2003). The roundabout receptor 2, ROBO2 is the main receptor from the Slit-Robo pathway, that is involved in axon guidance and which is also associated with DISC1-interacting proteins SRGAP2 and 3 (Camargo et al., 2007). The Ephrin receptor signaling pathway, predicted to be regulated by DISC1, is critical for embryonic development and known as a mediator of axon guidance (Kvajo et al., 2011).

In perspective, alterations of these developmental pathways and processes could explain the subtle neurodevelopmental phenotypes in the tgDISC1, where the substantia nigra (SN) contains fewer dopaminergic neurons (DA), fewer projections into dorsal striatum, and a shift in the parvalbumin-positive interneurons (Hamburg et al., 2016). DA homeostasis deficiency and the proposed disturbed dopaminergic signaling could explain the observed decrease of DA neurons in the SN. The disturbed axonal guidance signaling could lead to the reduction of the projections into the dorsal striatum and the shift of the parvalbumin-positive interneurons. As protein profiles were obtained from adult tgDISC1 rats, it would be interesting to follow up by studying the profiles in the developing brain to reveal the etiopathology effects of DISC1 which exceeds the scope of this study.

Bioinformatics analyses from the current study predicted that the following biological processes were activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation

(**Figure 4**). These results may be relevant for interpretation of previous as well as for the design of future studies on DISC1.

CONCLUSION

Our results suggest that overexpression and/or aberrant DISC1 proteostasis can lead to profound changes in protein networks relevant for mental disorders or endophenotypes and may signify a role for the DISC1 protein alone—in the absence of mutations—in behavioral and neural processes and disorders. DISC1 expression levels likely have to be controlled in a narrow expression window in order to execute adaptive behavior. These findings make the DISC1 protein and its posttranslational modifications a molecular convergence point or sensor for environmental interactions such as oxidative stress. The findings also strongly support the earlier literature indicating involvement of the dopaminergic systems, particularly in the dorsal striatum in functional properties of the DISC1 protein.

AUTHOR CONTRIBUTIONS

FJS, SVT, CK, JPH, MASS and GL conceived and designed the experiments. FJS, A-LW, BF and MK performed experiments. FJS and RS collected data and processed them. FJS, CK, JPH, MASS and GL interpreted the results. FJS, CK, JPH, MASS and GL wrote the article. CK, JPH, MASS and GL revised the intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00026/full#supplementary-material

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Evidence for a Specific Integrative Mechanism for Episodic Memory Mediated by AMPA/kainate Receptors in a Circuit Involving Medial Prefrontal Cortex and Hippocampal CA3 Region

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ORIGINAL ARTICLE

Evidence for a Specific Integrative Mechanism for Episodic Memory Mediated by AMPA/kainate Receptors in a Circuit Involving Medial Prefrontal Cortex and Hippocampal CA3 Region

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Abstract

We asked whether episodic-like memory requires neural mechanisms independent of those that mediate its component memories for "what," "when," and "where," and if neuronal connectivity between the medial prefrontal cortex (mPFC) and the hippocampus (HPC) CA3 subregion is essential for episodic-like memory. Unilateral lesion of the mPFC was combined with unilateral lesion of the CA3 in the ipsi- or contralateral hemispheres in rats. Episodic-like memory was tested using a task, which assesses the integration of memories for "what, where, and when" concomitantly. Tests for novel object recognition (what), object place (where), and temporal order memory (when) were also applied. Bilateral disconnection of the mPFC-CA3 circuit by N-methyl-D-aspartate (NMDA) lesions disrupted episodic-like memory, but left the component memories for object, place, and temporal order, per se, intact. Furthermore, unilateral NMDA lesion of the CA3 plus injection of (6-cyano-7-nitroquinoxaline-2,3-dione) (CNQX) (AMPA/kainate receptor antagonist), but not AP-5 (NMDA receptor antagonist), into the contralateral mPFC also disrupted episodic-like memory, indicating the mPFC AMPA/kainate receptors as critical for this circuit. These results argue for a selective neural system that specifically subserves episodic memory, as it is not critically involved in the control of its component memories for object, place, and time.

Key words: AMPA receptor, CA3, episodic-like memory, medial prefrontal cortex, NMDA receptor

Introduction

Episodic memory refers to the recollection of an event in a particular time and place (Tulving 2005) and in animal models has been defined as an integrated representation of discrete memories for object (what) in place (where) and time (when) (Clayton and Dickinson 1998; Kart-Teke et al. 2006). Conceptually, episodic memory could be a specific and separated mnemonic process or it could merely be a consequence or sum of its component memory systems. One approach to answer this question is to ask whether impairments in episodic memory result from deficits in one or more of the individual component memories, or from a deficiency in the integration of these components, per se. If the integration of the individual component memories into episodic memory are merely a consequence of their sum (due to their simultaneous encoding), then a deficit in episodic memory would be expected to be accompanied by deficits in one or more of the component mnemonic information. In contrast, deficient episodic memory in face of intact memory components would suggests episodic memory to engage a process separated from its composing factors. Concomitantly, one can also ask whether episodic memory requires unique separate neural substrates or if it is dependent on the integrity of the individual neural

mechanisms that subserve memory processing for "what," "where," and "when."

The perirhinal cortex (PRC), hippocampus (HPC), and medial prefrontal cortex (mPFC) are crucial for processing information as to what, where, and when (Hannesson et al. 2004; Barker et al. 2007; Barker and Warburton 2011). Moreover, the interaction between mPFC and HPC was found to be essential for temporal order memory, but not for object memory, nor for memory of its location (Barker and Warburton 2011; DeVito and Eichenbaum 2011). Also, electrophysiological (Leutgeb et al. 2005; Komorowski et al. 2009; Neunuebel and Knierim 2014), neuroimaging (Cabeza et al. 2002; Hassabis et al. 2007), lesion (Ergorul and Eichenbaum 2004; Fortin et al. 2004), and genetics (Place et al. 2012) studies indicate the HPC to be a structure critical for the formation of episodic memory. Particularly, the HPC subregion CA3, with its specific anatomical structure forming a self-feedback circuit, has been proposed to be an autoassociative network for processing episodic memory (Treves and Rolls 1994; Rolls and Kesner 2006). Human neuroimaging findings showing higher neural activation of the CA3/dentate area when episodic memory is encoded (Eldridge et al. 2005), along with the failure of CA3 lesioned rats to exhibit episodic-like memory (Li and Chao 2008), support the critical role of CA3 region in episodic memory. In addition to the HPC, the PFC is also involved in the processing of episodic or episodic-like memory (Blumenfeld and Ranganath 2007; Spaniol et al. 2009; Li et al. 2011). Neuroimaging (Schott et al. 2011; Bonnici et al. 2012), electrophysiological (Watrous et al. 2013), and lesion (Barker and Warburton 2011) studies implicate interacting neural circuits between the PFC and medial temporal lobe, especially the HPC, in the control of episodic memory (Aggleton and Brown 2006).

In the present study, we asked whether an integrating mechanism that determines episodic memory exists independent of those that underlie the component memories for what, where, and when. We used an episodic-like memory test which assesses the integrated memory for object identity (what), object place (where), and temporal order of object presentation (when) (Kart-Teke et al. 2006), plus novel object recognition, object–place and temporal order tests to measure memory for these components individually (Dere et al. 2007). We targeted the circuit comprising the mPFC and hippocampal CA3 as a possible critical substrate for episodic memory. Initially, we found that disconnection of the mPFC and CA3 via contralateral N-methyl-D-aspartate (NMDA) lesions prevented the expression of episodic-like memory, but left the component memories for object, place, and temporal order intact. Since recent studies have implicated the participation of ionotropic glutamate receptors of the mPFC in object recognition memory (Barker and Warburton 2008, 2015), we sought to test whether glutamate receptors in the mPFC are critical components of this circuit. Accordingly, we injected AMPA/kainate- and NMDA receptor antagonists into the mPFC together with a contralateral CA3 NMDA lesion and tested the influence on episodic-like memory.

Materials and Methods

Animals

Male Wistar rats (Tierversuchsanlage, University of Düsseldorf, Germany) weighing between 250 and 300 g were used. They were grouped 5 per cage (60 × 38 × 20 cm) and housed under standard conditions under a reversed light–dark rhythm (light off from 07:00 to 19:00). They had free access to food and water through the whole studies. Each animal was handled for 3 min per day for 5 days before behavioral testing. All experiments were in accordance with the Animal Protection Law of Germany and of the European Communities Council Directive (86/609/EEC).

Drugs

NMDA was used as a neurotoxin for cell body lesions. The competitive NMDA receptor antagonist D-(–)-2-amino-5-phosphonopentanoic acid (AP-5) and the competitive AMPA/kainite receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were dissolved in 0.9% saline in the concentrations of 5 and 1 mg/mL, respectively. Drug solutions were prepared in the volume of 0.5 mL and preserved at –80°C until use. Drugs were purchased from Sigma Aldrich (Steinheim, Germany).

Surgery

Animals were anesthetized with pentobarbital (Narcoren; Merial GmbH, Germany, 50 mg/kg, i.p.), and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). A heating pad beneath their bodies was used to maintain normothermia. Incisions were made and the scalp was retracted to expose the skull. Holes were drilled into the skull over the mPFC and HPC subregion CA3. For the disconnection, NMDA solution was microinjected into the target sites with a 10-μL Hamilton syringe connected to a CMA 100 microinfusion pump. Surgical details are described below.

The disconnection approach relies on the assumption that, if a function is dependent on a neural interaction between 2 areas of the brain, then a unilateral lesion/inactivation of one area combined with a unilateral lesion/inactivation of the other in the opposite hemisphere, disconnects the circuit bilaterally at 2 different levels and thus, leads to a functional deficit. The same lesion/inactivation of the areas located in the same hemisphere spares an intact circuit in the contralateral hemisphere, in which case the function may be preserved in part (Geschwind 1965, 1965).

Experiment 1: Disconnection of the mPFC-CA3 Circuit

Animals received a unilateral NMDA injection into the mPFC combined with an ipsi- or contralateral NMDA injection into the hippocampal CA3 region. Thus, they were assigned into 2 experimental groups: mPFC + CA3 ipsilateral lesions ($n = 7$) and mPFC + CA3 contralateral lesions ($n = 7$). The neurotoxic agent NMDA (2 μg dissolved in 0.2 μL phosphate buffer saline, flow rate 0.1 μL/min) was injected into either the right or the left mPFC, counterbalanced within groups (coordinates relative to skull at bregma: AP = 3.0 mm; ML = ± 0.7 mm; DV = –3.0 mm; AP = 3.0 mm; ML = ± 0.7 mm; DV = –4.0 mm; AP = 4.0 mm; ML = ± 0.7 mm; DV = –3.0 mm) with a 26-gauge steel cannula. The cannula was left in situ for an additional 1 min to prevent reflux. Within the same surgical session, NMDA (2 μg dissolved in 0.1 μL phosphate buffer saline, flow rate 0.1 μL/min) was also injected into 3 locations of the ipsi- or contralateral hippocampal CA3 region (AP = –2.8 mm; ML = ± 3.0 mm; DV = –3.9 mm; AP = –3.3 mm; ML = ± 3.4 mm; DV = –3.9 mm; AP = –4.1 mm; ML = ± 4.2 mm; DV = –4.0 mm). After each injection, the needle was left in place for additional 2 min before retracting it. Then, the scalp was sutured and the wound was disinfected with 70% ethanol. The animals were allowed to recover for 2 weeks.

Experiment 2: The Role of Glutamatergic Receptors in the mPFC-CA3 Circuit

The surgical procedure was identical to experiments 1, except that the animals received a unilateral NMDA injection into the

CA3 area and were implanted with an injection cannula (diameter 0.7 mm; length 16 mm) aiming at the contralateral mPFC (AP = 3.0 mm; ML = ± 0.7 mm; DV = −3.0 mm). A stainless steel thread (26-gauge, protruding 0.1 mm from the tip of the guide cannula) was inserted to prevent occlusion. They were secured to the skull by 2 stainless steel screws (2.6 mm diameter) and dental cement. The animals were allowed to recover for at least 7 days, before being assigned into AP-5 ($n = 7$) or CNQX ($n = 7$) injection groups.

Behavioral Testing

A black-acrylic open field (60 × 60 × 30 cm) was used to assess object exploration. Illumination was provided by 2 LED lights (luminous density on the center ~8 and ~6 lx in the corners). A camera was mounted 2 m above the arena and connected to a computer and a DVD recorder. The open field was located in a sound-attenuated room and geometric figures (29 × 21 cm, black-white stripe and black circle in white background) were pasted around the room as spatial cues.

Different sets of objects in quadruplicate made of different material (glass and ceramic), shape (rectangle and irregular shapes), color (white and blue), and size (25–32 cm height and 7–11 cm diameter) were applied in the spontaneous object exploration tests. The objects were heavy enough to prevent a displacement by the animals. Different sets of objects were used for each object exploration test and the assignments of objects were counterbalanced for each subject. Object exploration was defined as a physical contact with the object with snout, vibrissae, or forepaws. Climbing on the object, or touching the object while looking around the environment, were excluded from this measure. Object exploration times were registered by experimenters blind to the experimental design with the Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Behavioral measurements were conducted between 10:00 and 17:00 h. Acetic acid solution (0.1%) was used to remove odor cues after each trial.

Episodic-like Memory Test

Animals were habituated to the open field by placing them into the center and allowing exploration for 10 min for 3 consecutive days. One day after the third habituation trial in the open field, the memory test was conducted according to the procedure described in detail elsewhere (Kart-Teke et al. 2006). This test was composed of 2 sample trials and 1 test trial, lasting 5 min each and with an intertrial interval of 1 h. Each animal was placed into the center of the open field, in which 4 copies of an object occupied 4 of 8 possible locations along the walls. The locations for placement of objects were randomly selected. One hour later, animals were again put into the arena into which 4 copies of a novel object were placed. Two of them were randomly placed at 2 of 4 possible locations, which were once occupied during the first sample trial, while the other 2 objects were randomly placed at 2 of 4 novel locations (i.e., locations that were not previously occupied). After another delay of 1 h, each animal was put into the arena containing 2 copies of the object from the first sample trial, "old familiar," and 2 copies of the object from the second sample trial, "recent familiar" objects. One object of each type was presented at a randomly chosen location where it was previously located during the respective sample trial. The other object of the same type was placed at a randomly chosen location, which was not previously occupied during the respective sample trial. Therefore, the test trial contained one old familiar object at a stationary location (OFS), one old familiar object at a displaced location (OFD), one recent familiar object at a stationary location (RFS), and one recent familiar object at a displaced location (RFD; Fig. 2A). Previous studies showed that rats explore the OFS object more than the OFD one along with exploring the RFD object more than the RFS one (Kart-Teke et al. 2006; Li and Chao 2008). This behavioral pattern suggests that there is an interaction between "place" and "recency," which indicates that rats show the ability to simultaneously integrate "what–where–when" components (see also "dependent variables" section below).

Object–Place Recognition and Novel Object Recognition Tests

One day after the previous test, animals were tested for their memories for recognizing locations and objects. Each rat was placed into the open field where 2 copies of an object were located in the 2 corners of the apparatus. Animals were free to explore the objects and the arena for 4 min and, then, were returned to their own cage. One hour later, each animal was placed into the center of the arena where the same objects were presented. Whereas one of the two objects was displaced at a novel location, the other one remained at the same location. Each animal had 4 min to freely explore and, then, was returned to their cages. Rodents spend more time exploring the displaced object more than the one at the same location, suggesting that they recognize the displacement and, thus, show memory for place (Ennaceur et al. 1997). After a delay of 1 h, each animal had 4 min to explore the arena where 2 objects were presented. This time, one object was replaced by a novel one while the other one remained the same. These 2 objects were placed at the previously encountered locations and the location for replacing the novel object was counterbalanced. Rodents prefer exploring the novel object more than the old one, indicating that they can recognize the old object (Ennaceur and Delacour 1988).

Temporal Order Memory Test

One day after the previous tests, a test for temporal order was applied. This test was composed of 2 sample trials and 1 test trial. The animal was placed into the center of the open field where 2 copies of an object were presented in the 2 corners of the arena (sample 1). Each rat was free to explore the objects and the arena for 4 min. After a delay of 1 h, the animal was put into the apparatus with 2 copies of a novel object for 4 min (sample 2). One hour later, one of the objects used in sample 1 and one of the objects applied in sample 2 were placed together in the arena (test trial). Each animal was allowed to freely explore the objects and the apparatus for 4 min. The places used for locating objects were identical in all the trials. During the intervals, the animals were returned to their cages. Rodents explore the object previously encountered in sample 1 more than the recent presented object from sample 2, indicating a memory for recency (Mitchell and Laiacona 1998).

Microinjection Procedure

In experiment 2, rats were habituated to manual restraint 3 min per day for 3 consecutive days before testing. Fifteen minutes prior to the test trial for episodic-like memory, animals were restrained manually and the thread in the guide cannula was removed. The infusion cannula (26-gauge, protruding 0.05 mm from the tip of the guide cannula) was connected to a 10-μL Hamilton syringe with a flexible polyethylene tube (PE-10). A CMA 100 microinfusion pump was used to infuse 0.5 μL of AP-5 or CNQX solutions into the brain with a flow rate of 0.5 μL/min. The infusion cannula was left in situ for additional 1 min for diffusion. The microinjection procedure was completed within 5 min.

Only the episodic-like memory paradigm was conducted in this experiment.

Dependent Variables

For all the tests, the length of time the objects were explored during the test trial was taken as dependent variable. In objet exploration paradigms, rodents explore a novel object longer than an old one (Ennaceur and Delacour 1988), a displaced object longer than a stationary one (Ennaceur et al. 1997), and a previously encountered one (more distant in the past) longer than a more recently encountered object (Mitchell and Laiacona 1998). In the episodic-like memory paradigm, rats explore the OFS object longer than the RFS one, the RFD longer than the RFS one, but they explore the OFS longer than the OFD one (Kart-Teke et al. 2006). This exploration pattern indicates that rats show concomitantly memory for when (the temporal order of objects presentation), what (a particular object) was encountered where (the location of objects), since there is an inversion in the pattern of exploration between the OFS × OFD and RFS × RFD objects. This suggests that rats exhibit an integrated memory by combing what–where–when information into an episodic-like memory (Kart-Teke et al. 2006). For statistical comparison between the ipsi- and contralateral disconnection groups, 3 ratios were calculated:

Integration ratio = (time exploring the OFD object – time exploring the OFS object)/(time exploring the OFD object + time exploring the OFS object).

Where ratio = (time exploring the RFD object – time exploring the RFS object)/(time exploring the RFD object + time exploring the RFS object).

When ratio = (time exploring the OFS object – time exploring the RFS object)/(time exploring the OFS object + time exploring the RFS object).

All of the animals explored all of the arranged objects during the sample and test trials. Thus, none of them had to be excluded from data analysis.

Histology

After completion of behavioral testing, each animal was euthanized with an over dose of pentobarbital (80 mg/mL, i.p.). Animals were then perfused intracardially with 0.9% phosphate buffer saline, followed by 10% formalin, and the brains were extracted. Then, the brains were stored in 30% sucrose formalin at 4°C until processed. Coronal brain sections were cut with a cryostat (50 μm; Leica, Germany) and mounted onto presubbed glass slides. The slices were stained with cresyl violet (Sigma Aldrich, USA) to determine the extent of the lesions and the site of cannulae placement.

Statistics

Mixed two-way ANOVAs with "object" as the within factor and "group" as the between factor were applied to analyze times for object exploration in all the tests. If significant effects of object and/or "object × group" were found, the following tests were applied. For the episodic-like memory test, two-way ANOVAs with the 2 within factors place and recency were conducted for each group separately. Paired *t*-tests were then used within each group when a significant effect of place × recency" interaction was found. One-sample *t*-tests were then used for the ratios to compare with chance-level performance (the zero value). To compare group differences, independent *t*-tests were used to analyze the ratios. For the NOR, OPR, and TOM tests, paired *t*-tests were also applied within each group when appropriate. As the preference for the direction of exploring is important for the interpretation, the paired and one-sample *t*-tests were used as one-tailed comparisons. All other statistical tests were two-tailed comparisons. The level of significance was set at $P < 0.05$.

Results

Histology

Similar lesion extents were found in the mPFC and dorsal hippocampal CA3 area among different groups. For the mPFC lesions, the dorsal anterior cingulate, the prelimbic, and infralimbic areas were mainly damaged and also part of the secondary motor cortex. For the hippocampal subregion lesions, dorsal CA3 area was primarily damaged, while part of the primary somatosensory cortex and parietal association cortex were also influenced. The sites for cannula implantation were targeted at the medial PFC in the animals in experiment 2. One animal was excluded because of incorrect lesions ($n = 1$ from the AP-5 injection group). Details are shown in Figure 1.

Behaviors

Experiment 1: Disconnection of the mPFC-Hippocampal CA3 Prevented Episodic-like Memory, but Not Memories for What, Where, or When, Individually

There were significant effects of object ($F_{3,36} = 4.14$, $P = 0.013$) and object × group interaction ($F_{3,36} = 8.497$, $P < 0.001$), but not of group ($P > 0.05$) in a 2 (groups) × 4 (object) mixed two-way ANOVA. The following within factors 2 (place) × 2 (recency) ANOVA revealed a significant interaction effect ($F_{1,6} = 20.835$, $P = 0.004$), but no effects of place and recency ($P > 0.05$) in the ipsilateral lesion group. For the contralateral lesion group, there was a significant effect only of place ($F_{1,6} = 10.987$, $P = 0.016$), but neither of recency nor place × recency interaction (both $P > 0.05$). Paired *t*-tests indicated that the ipsilateral lesion group explored the OFS object more than the OFD one ($t_6 = 2.503$, $P = 0.023$), the RFD object more than the RFS one ($t_6 = -4.84$, $P = 0.0015$), and the OFS more than the RFS ($t_6 = 2.42$, $P = 0.026$; Fig. 2*B*). This characteristic pattern of object exploration based on a distinct spatial-temporal context expresses the integration of what–where–when information into episodic memory (Kart-Teke et al. 2006).

In the comparison of the ratios, the animals with an ipsilateral lesion had significantly lower "integration" and higher where ratios than those with the contralateral lesion ($P = 0.005$ and $P = 0.011$, respectively), while no group difference was found in the comparison of the when ratios ($P > 0.05$; Fig. 2*C*). Whereas the ipsilateral lesion animals exhibited negative integration ($t_6 = -3.117$, $P = 0.01$) and positive where and when ratios ($t_6 = 8.736$, $P < 0.001$; $t_6 = 2.777$, $P = 0.016$, respectively) compared with zero, the contralateral lesion group did not ($P > 0.05$). Hence, the contralateral lesion animals did not exhibit the integrated memory of what–where–when (namely, episodic-like memory), while the ipsilateral lesion group did.

In the analyses of object exploration in the OPR, NOR, and TOM tests, there were significant effects of object ($F_{1,12} = 31.225$, $P < 0.001$; $F_{1,12} = 51.454$, $P < 0.001$; $F_{1,12} = 21.474$, $P = 0.001$, respectively), but not group and object × group ($P > 0.05$). Both the ipsilateral and contralateral lesion groups explored the displaced object more than the stationary one in the OPR test ($P = 0.0025$ and $P = 0.0065$, respectively), the novel object more than the old one in the NOR test ($P = 0.002$ and $P < 0.001$, respectively), and the old-familiar object more than the recent-familiar one in the TOM test ($P = 0.0125$, $P = 0.006$, respectively; Fig. 2*D*). This indicated

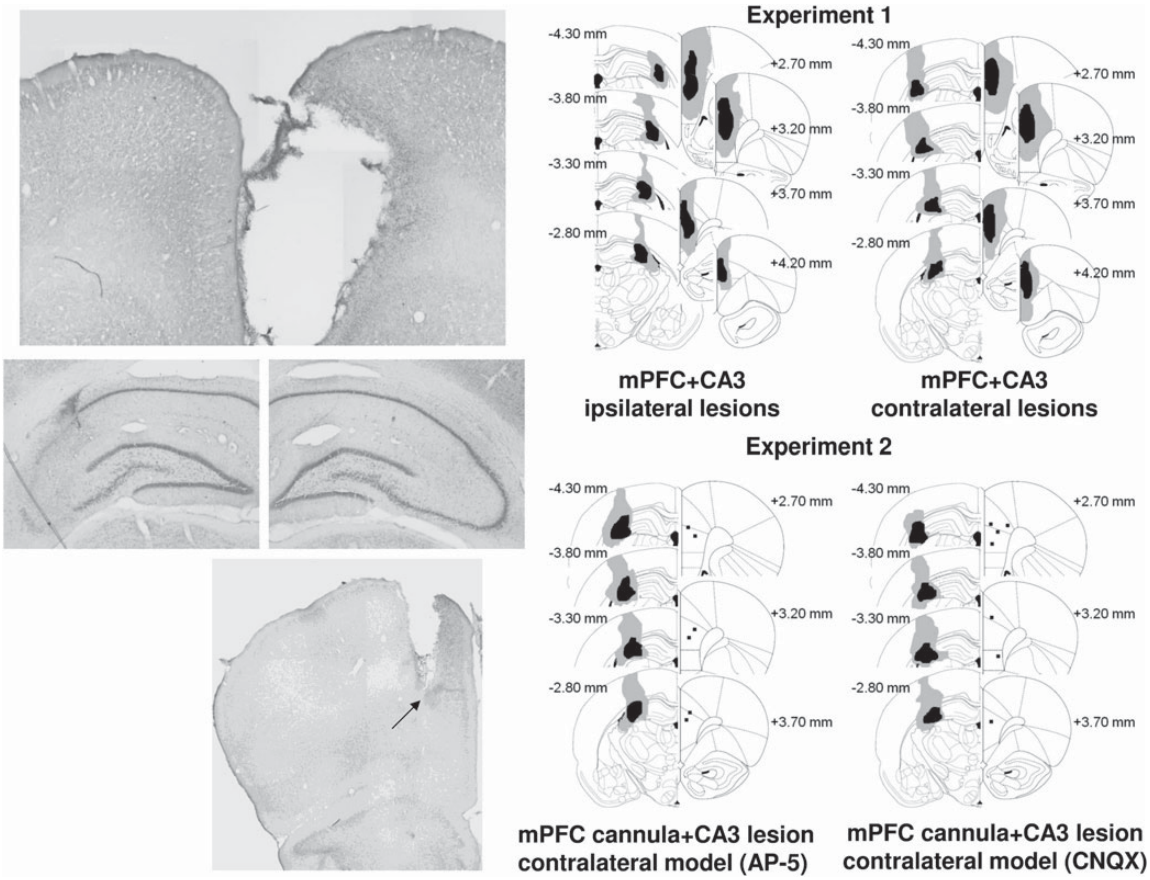

Figure 1. Left: Representative photomicrographs of lesions in the medial prefrontal cortex (top), CA3 region (middle), and injection cannula tract (bottom). The middle part contains an intact side (right) and a lesion side (left) of the dorsal hippocampus CA3 region from the same section. The arrow in the bottom photo indicates the tip of an implanted cannula. Right: Schematic diagram of extent of lesions and sites of injection. The maximal and minimal extent of the lesions is depicted in black and gray, respectively. Both the right and left lesions are presented in one hemisphere. Numbers indicate approximate distance (mm) relative to bregma. Black squares in experiment 2 represent the centers of the tips of the implanted cannulae.

that both groups had intact memory for OPR, NOR, and TOM. Finally, there were no significant group differences in the total times the objects were explored in any of the test trials (*P*s > 0.05; Table 1). Thus, the disconnection of the mPFC-CA3 circuit left the individual memories for what, where, and when, per se, intact, but impaired episodic-like memory.

Experiment 2: The Functional Circuit of mPFC-CA3 Is Dependent on the AMPA but Not NMDA Receptors in the mPFC for Integrating the What–Where–When Components

The 2 (group) × 4 (object) mixed two-way ANOVA revealed significant effects of object ($F_{3,33} = 4.986$, $P = 0.006$) and group × object ($F_{3,33} = 6.801$, $P = 0.001$) but not group ($P > 0.05$). The 2 × 2 ANOVAs with 2 within factors place and recency showed that a significant effect of "place × recency" interaction ($F_{1,5} = 123.789$, $P < 0.001$) was found, but no effects of place and recency ($P > 0.05$) in the AP-5 injection group. In contrast, there were significant effects of place in the CNQX injection group ($F_{1,6} = 8.11$, $P = 0.029$, respectively), but no effects of recency and place × recency interaction ($P > 0.05$; Fig. 3A). The animals with AP-5 injection preferred to explore OFS object more than OFD one ($t_5 = 6.991$, $P < 0.001$), RFD object more than RFS one ($t_5 = -4.536$, $P = 0.003$) and OFS object more than RFS one ($t_5 = 4.092$, $P = 0.0045$; Fig. 3A). Thus, the animals exhibited episodic-like memory.

There was no group difference in total exploration times of objects ($P > 0.05$; Fig. 3B). Thus, application into the mPFC of CNQX, but not AP-5, disrupted episodic-like memory in the context of disconnecting the mPFC-CA3 functional pathway.

Discussion

The interhemispheric disconnection of the mPFC and dorsal hippocampal CA3 subregion prevented episodic-like memory, but left the memories for what, where, and when, per se intact. The NOR, OPR, and TOM tests were conducted to test the hypothesis that the observed deficits in episodic-like memory could be caused by impairments of the individual what, where, or when memory components. Our results suggest that the establishment of an integrated episodic memory requires neural processes that are distinct from those that establish memory for its components. Therefore, we hypothesize that episodic memory requires the integrity of not only individual neural systems for processing information about objects and their location in "place" and "time," but also an independent neural mechanism responsible for the "integration" of these component representations into a memory system. If the integration of the individual component memories into episodic memory were merely a consequence of their sum (due to their simultaneous encoding), then a deficit in episodic memory would be expected to be accompanied by deficits in one or more of the component memory systems. Conversely, deficient episodic memory in face of intact memory components, for which we have provided evidence, suggests episodic

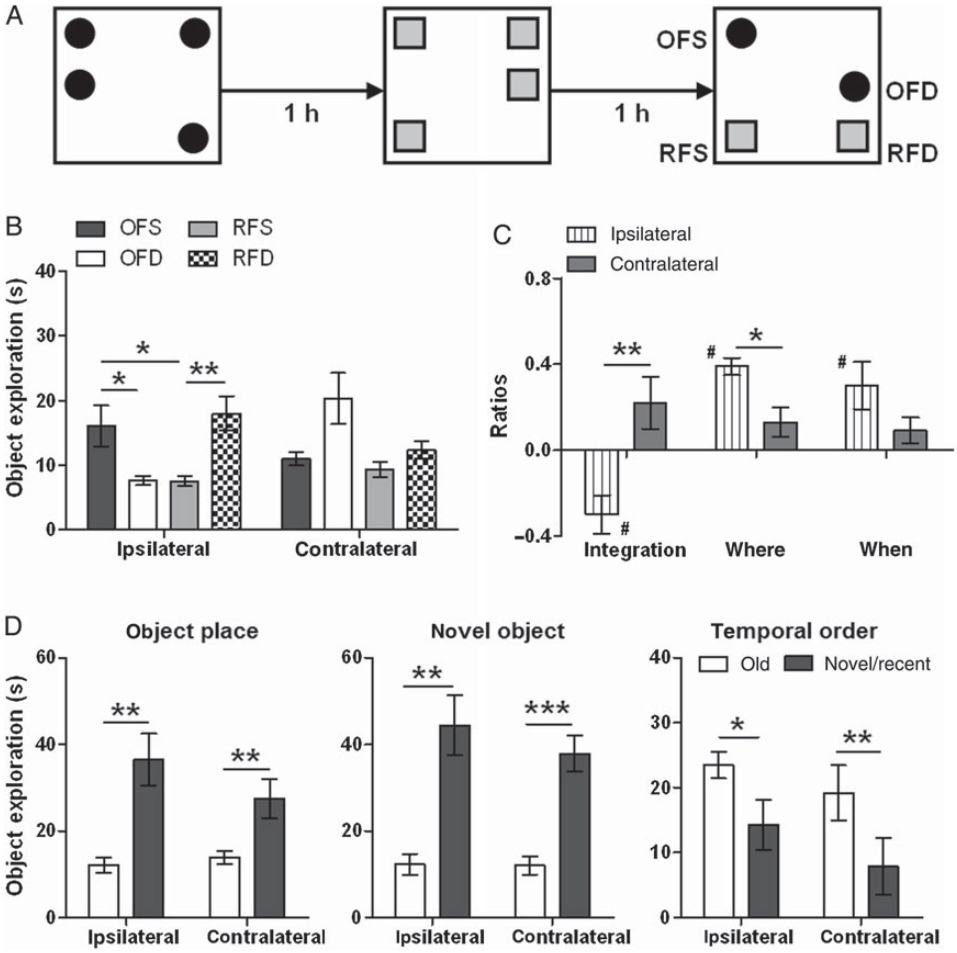

Figure 2. Experiment 1: Effects of ipsilateral and contralateral (disconnection) lesion of mPFC and CA3 region on episodic-like memory and object recognition tests. (*A*) Episodic-like memory paradigm: Two sets of objects were used (represented as black circles and gray squares, counterbalanced for sample trials 1 and 2). In the test trial, rats explore the displaced circle less, but the displaced square more, than the respective stationary objects. OFS, old-familiar stationary object; OFD, old-familiar displaced object; RFS, recent-familiar stationary object; RFD, recent-familiar displaced object. Locations for placing objects are semirandomly selected based on the test design (see Materials and Methods). (*B*) The mPFC + CA3 ipsilateral lesion rats exhibited this pattern (which reflects episodic memory), while the mPFC + CA3 contralateral lesion rats did not. (*C*) The mPFC + CA3 ipsilateral lesion rats had lower "integration" and higher "where" ratios than the contralateral lesion group. (*D*) Both groups showed intact memories for place of object (where), object identity (what), and temporal order (when). Values are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the group; $^{\#}P < 0.05$ compared with zero value.

Table 1 Total duration of object exploration (seconds) in test trials of spontaneous object exploration in experiment 1 (mean ± SEM)

		ELM	OPR	NOR	TOM
mPFC + CA3	Ipsi-	49.29 ± 4.36	48.60 ± 6.79	56.66 ± 7.71	37.80 ± 5.29
mPFC + CA3	Contra-	53.06 ± 5.59	41.34 ± 5.37	49.89 ± 5.06	27.11 ± 5.47

ELM, episodic-like memory; OPR, object–place recognition; NOR, novel object recognition; TOM, temporal order memory; ipsi-, ipsilateral lesions; contra-, contralateral lesions.

memory to engage a meta-system that is separate from its composing factors.

Our data also provide evidence that episodic-like memory is dependent on a functional interaction between the mPFC and CA3 area, but that object and spatial recognition, and temporal order memory of previously encountered objects are not dependent on such a functional interplay. Whereas the PRC, HPC, and mPFC are proposed to be cardinal neural substrates for the distinct what, where, and when memory systems (Hannesson et al. 2004; Barker et al. 2007; Barker and Warburton 2011), convergent findings suggest an interacting system between the PFC and HPC for the processing of episodic memory (Schott et al. 2011; Bonnici et al. 2012; Watrous et al. 2013). Here, we provide evidence that the neural circuits comprising the mPFC and CA3 region is inclusively, but not exclusively, an integral part of such a network.

Inspired by the anatomical features of the recurrent collaterals of the HPC CA3 area, several computational models have proposed that the CA3 acts as an autoassociation network for the formation and storage of episodic memory (Treves and Rolls 1994; Knierim et al. 2006; Rolls and Kesner 2006). Consistent with these models, higher neural activation of the CA3/dentate area was found during episodic memory formation imaged by high-resolution functional magnetic resonance (fMRI)

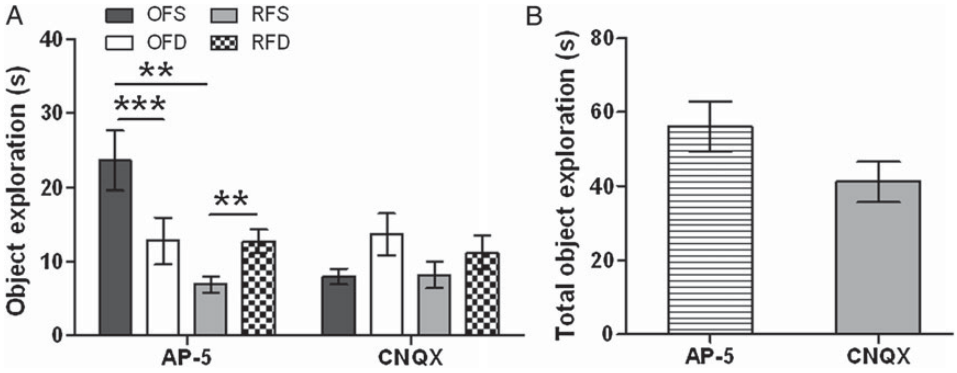

Figure 3. Experiment 2: Effect of AP-5 or CNQX injection into the mPFC of animals with a contralateral lesion of the CA3 area (disconnection model). (*A*) The AP-5 injection group explored OFS more than OFD, and RFD more than RFS. This opposite exploration pattern suggests the existence of an interaction between the "where" and "when" components. In contrast, the CNQX-injected rats did not show this exploration pattern. (*B*) No significant group difference in total time of object exploration in the test trial was found in animals administered the glutamate antagonists into the mPFC. Values are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the group.

(Eldridge et al. 2005). The CA3/dentate area in patients with mild cognitive impairment exhibited higher activity, suggesting that the episodic memory deficit in this population was related to changes of this area (Yassa et al. 2010). In rats, CA3 lesion disrupted episodic-like memory (Li and Chao 2008) and pharmacological inactivation of the CA3 area was found to disrupt episodic retrieval (Zhou et al. 2012).

Evidence for the involvement of the PFC in episodic encoding and retrieval is also provided by neuroimaging studies (Blumenfeld and Ranganath 2007; Spaniol et al. 2009). In episodic memory tasks, aged humans scanned by fMRI showed stronger PFC activity as compared with young ones, implying that the PFC compensated for age-related cognitive deficits (Cabeza et al. 2004; Rajah and D'Esposito 2005). Rats with mPFC lesions showed disrupted retrieval of a specific what–where–when" contextual event in an episodic-like memory test featuring fear conditioning (Li et al. 2011). In an episodic-like memory paradigm, similar to the one we applied here, mice with lesion of the mPFC exhibited a memory deficit akin to the loss of the integration ratio we describe here, indicating that the mPFC is responsible for memory of where an event happened (DeVito and Eichenbaum 2010). Taken together, these results indicate that the CA3 area and mPFC are likely to be cardinally involved in episodic memory.

Anatomically, the HPC CA3 subregion indirectly projects to the mPFC through the HPC CA1 area (Thierry et al. 2000; Vertes 2006). The mPFC sends projections back to the HPC via other regions, such as the nucleus reuniens (NRs) (Xu and Sudhof 2013) and the lateral entorhinal cortex (LEC) (Apergis-Schoute et al. 2006). Both the mPFC–NR–HPC and the mPFC–LEC–HPC circuits might participate in episodic memory, as suggested by evidence that the NR modulates contextual memory (Xu and Sudhof 2013) and that the LEC is critical for associations of object/context (Wilson, Langston et al. 2013; Wilson, Watanabe et al. 2013). A recent review also proposed that the LEC provides information to the HPC as to what happened in the environment (Knierim et al. 2014). Long-term potentiation may provide a mechanism for connecting the HPC and PFC (Laroche et al. 2000), while electrophysiological findings suggest that the mPFC biases object–location memory retrieval from the HPC (Navawongse and Eichenbaum 2013). The EC has been considered to process a more "general" memory representation of interrelated information, while the HPC enhances the information in "detail" further through pattern separation and pattern completion processes (Morris and Frey 1997; Viskontas et al. 2009). Since, the LEC anatomically projects to both the CA1 and CA3 areas, while the CA3 projects to CA1 via the Schaffer collaterals (van Strien et al. 2009), one can hypothesize that global information for object/context associations from the LEC reaches the CA1/CA3 regions and then, integrative detailed information, processed by CA3, is sent to the CA1. Hence, CA1 should also contain an episodic memory representation, as electrophysiological studies evidently showed that CA1 (Takahashi 2013) or CA1/CA3 (McKenzie et al. 2014) neurons respond in a specific manner to episodic-like memory traces. Recent studies have shown higher CA1 neural activation in processing spatial and nonspatial memory representations, while CA3 activation is mainly related to the processing of spatial information (Beer et al. 2013; Beer et al. 2014). The CA1 region, which is not principally recruited by spatial-related information like the CA3, may play a role in processing mnemonic information in a broader extent. When facing different environments, rodent CA1 neurons fired with a substantial overlap across environments, whereas CA3 neurons showed little overlap (Leutgeb et al. 2004). When the locations of proximal and distal cues in a single room were rotated, CA3 place fields remained coherent, whereas a remapping appeared in CA1 neurons (Lee et al. 2004). Thus, small or large geometrical context alternations may determine the way CA1 and CA3 neurons respond (Vazdarjanova and Guzowski 2004). The interplay between the mPFC and HPC, in terms of the comparative neural properties of CA1 and CA3, in the processing of episodic memory is still unexplored.

In experiment 1, a potential confounding could be that the contralateral lesioned rats exhibited intact memory in the OPR, NOR, TOM tests but were impaired in the episodic-like memory test because the environment was "simpler" concerning the amount of information (2 objects instead of 4) and would be less demanding in terms of attentional efforts or processing complexity, especially because the mPFC is known to be involved in attentional processes (Cassaday et al. 2014; Riga et al. 2014). Although one cannot completely rule out the possibility that, in our results, the contralateral lesioned rats exhibited control-level performance in the OPR, NOR, TOM tests because less attentional efforts are involved, it is very unlikely that the lesion, per se, would have caused such deficit, since in both ipsilateral and contralateral disconnection groups, only one mPFC (right or left) was lesioned, but the ipisilateral disconnected group exhibited intact memory in the OPR, NOR, TOM, and episodic-like memory tests. Likewise, unilateral mPFC lesion did not impair performance in OPR, NOR, TOM, and object-in-place tests

(Chao et al. 2013). Other factors, e.g., motor, perceptual, or motivational functions, can likely be excluded as potential confounds because the total durations of object exploration in all the test trials were not significantly different. Finally, animals with sham lesion of the mPFC and CA3 showed comparable episodic-like performances to nonlesioned ones (Li and Chao 2008; Li et al. 2011).

Our findings also show that the critical functional interplay between the mPFC and CA3 area that permits an integrated episodic memory was found to rely on AMPA/kainite, but not NMDA receptors in the mPFC (Fig. 3). When CNQX was injected into the contralateral, but not into the ipsilateral mPFC before the test trial, episodic-like memory could not be observed, implicating a role for AMPA/kainate receptors in the recall of the previously learned information. These findings are compatible with previous studies showing that microinjection of CNQX, but not AP-5, into the mPFC prior to the probe trial compromised previously and newly learned paired-association memories (Tse et al. 2011). Also, the lack of effect of the pretest trial AP-5 injection in preventing the expression of episodic-like memory is corroborated by findings in an object exploration paradigm for testing memory of object–place associations, in which microinjection of AP-5 into the mPFC of rats had no effect when administrated before the test trial (Barker and Warburton 2008, 2015). Selective roles for hippocampal NMDA and AMPA receptors have also been proposed for associative memory encoding and retrieval, respectively (Morris 2006). In a paired flavor-place memory paradigm, the injection of CNQX, but not of AP-5, into the HPC prior to testing impaired performance (Day et al. 2003; Bast et al. 2005). The underlying mechanisms could be that CNQX infusions influenced fast synaptic transmission, while AP-5 infusions did not (Day et al. 2003; Bast et al. 2005). AMPA receptor insertion was also found to contribute to neural plasticity driven by experiences (Takahashi et al. 2003).

Our results 1) delineate a neural circuit that encompasses the mPFC AMPA/kainite receptors and the CA3 region as critical components of a system that determines the integration of memories for object, place, and temporal order into episodic memory and 2) imply that this integrative system for episodic memory is distinct from those employed in the representation of its component memories.

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Notes

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The medial prefrontal cortex-lateral entorhinal cortex circuit is essential for episodic-like memory and associative object-recognition

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The Medial Prefrontal Cortex—Lateral Entorhinal Cortex Circuit Is Essential for Episodic-Like Memory and Associative Object-Recognition

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ABSTRACT: The prefrontal cortex directly projects to the lateral entorhinal cortex (LEC), an important substrate for engaging item-associated information and relaying the information to the hippocampus. Here we ask to what extent the communication between the prefrontal cortex and LEC is critically involved in the processing of episodic-like memory. We applied a disconnection procedure to test whether the interaction between the medial prefrontal cortex (mPFC) and LEC is essential for the expression of recognition memory. It was found that male rats that received unilateral NMDA lesions of the mPFC and LEC in the same hemisphere, exhibited intact episodic-like (what-where-when) and object-recognition memories. When these lesions were placed in the opposite hemispheres (disconnection), episodic-like and associative memories for object identity, location and context were impaired. However, the disconnection did not impair the components of episodic memory, namely memory for novel object (*what*), object place (*where*) and temporal order (*when*), *per se*. Thus, the present findings suggest that the mPFC and LEC are a critical part of a neural circuit that underlies episodic-like and associative object-recognition memory. © 2015 Wiley Periodicals, Inc.

KEY WORDS: associative memory; entorhinal cortex; episodic memory; object recognition; prefrontal cortex

INTRODUCTION

The construction of episodic memory requires complicated interregional processing in the brain, involving the interaction between the prefrontal cortex and hippocampus (Eichenbaum, 2000; Aggleton and Brown, 2006; Ferbinteanu et al., 2006; Schott et al., 2011; Watrous et al., 2013). Recently, we have shown that disconnecting the medial prefrontal cortex (mPFC) and CA3 hippocampus subregion disrupts episodic-like memory (ELM) an integrated what-where-when episode, but not the separate individual component memories for *what*, *where* and *when*, indicating that the integration of distinct spatiotemporal information depends on the mPFC-CA3 circuit (de Souza Silva et al., in press). However, the interplay between the mPFC and hippocampus in episodic memory processing likely involves additional brain areas. Although the hippocampus directly projects to the mPFC (Thierry et al., 2000; Vertes, 2006), the information from the mPFC returning to the hippocampus traverses other regions, such as the nucleus reuniens (Xu and Sudhof, 2013) and the lateral entorhinal cortex (LEC) (Apergis-Schoute et al., 2006). The role of these "delay stations" in the processing of episodic memory remains unclear.

Particularly, the LEC provides input/output to the hippocampus (Kerr et al., 2007). Electrophysiological studies suggest that the firing properties of LEC neurons are distinctly different from those of the medial entorhinal cortex (MEC) (McNaughton et al., 2006; Moser et al., 2008; Yoganarasimha et al., 2011; Zhang et al., 2013). The LEC provides item information, such as of an object (Hargreaves et al., 2005; Deshmukh and Knierim, 2011) and past experience of an absent object (Tsao et al., 2013) to the hippocampus (Knierim et al., 2014). Rats with lesions of the LEC also had deficits in object recognition in relation to location/context, place and object identity, but contradictory results have also been reported (Van Cauter et al., 2013; Wilson et al., 2013a,b). On the other hand, the LEC is reciprocally connected with the prefrontal cortex (Jones and Witter, 2007; Agster and Burwell, 2009), where neurons respond to the information about object, time and place (Ninokura et al., 2004; Hok et al., 2005; Weible et al., 2009). Rodents with lesions of the mPFC showed deficits in ELM (DeVito and Eichenbaum, 2010; Li et al., 2011) and human neuroimaging and lesion studies also provide evidence for the involvement of the prefrontal cortex in episodic memory (Blumenfeld and Ranganath, 2007; Spaniol et al., 2009).

The present study set out to directly examine whether the mPFC–LEC circuit is critical for the expression of ELM. Since the mPFC and LEC have been implicated in object-related and place information, we also conducted

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Abbreviations: ELM, episodic-like memory; LEC, lateral entorhinal cortex; MEC, medial entorhinal cortex.

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tests for object recognition and spatial working memory. We applied a disconnection procedure, which can provide information as to whether specific behavioral functions are dependent on the information processing between two neural substrates (Geschwind, 1965). Incorporating the findings of the critical role of mPFC-CA3 circuit in ELM (de Souza Silva et al., in press), we suggest that the mPFC-LEC-hippocampus circuit, as a whole, processes ELM. Whereas the interaction between the mPFC and hippocampal CA3 subregion integrates what-where-when information, the interplay between the mPFC and LEC processes associations between different objects.

MATERIALS AND METHODS

Experiment Design

Subjects (see below) were randomly divided into two groups: the unilateral lesion group ($n = 10$) with a unilateral NMDA lesion in the mPFC plus a unilateral LEC lesion in the same hemisphere and the contralateral (disconnected) group ($n = 10$) in which the same lesion procedure was conducted but the two lesion sites were at the opposite hemisphere (details see surgery section). Then, the animals were habituated to an open field, followed by a series of object exploration tests for: ELM, object place preference, novel object preference, temporal order memory, novel object preference (distinct objects), object-location preference and object-context preference (see below; Fig. 1). After these tests, an elevated plus maze test was conducted to measure animals' anxiety-like behavior and finally, the animals were food-deprived and trained in a radial-arm maze to assess their spatial working memory.

Subjects

Male Wistar rats weighing between 250 and 350 g were used as subjects (Tieversuchsanlage, Duesseldorf University, Germany). Animals were kept in a standard environment under a reversed light–dark rhythm (lights off from 07:00 to 19:00), five per cage. Food and water were available *ad libitum* except during periods of food restriction for a behavioral test (see below). Before behavioral testing, each animal was handled for 3 min per day for five days. All procedures were in accordance with the European Communities Council Directive (86/609/EEC) and the Animal Protection Law of Germany. Every effort was made to reduce the number of animals used and to release their stress during the whole study.

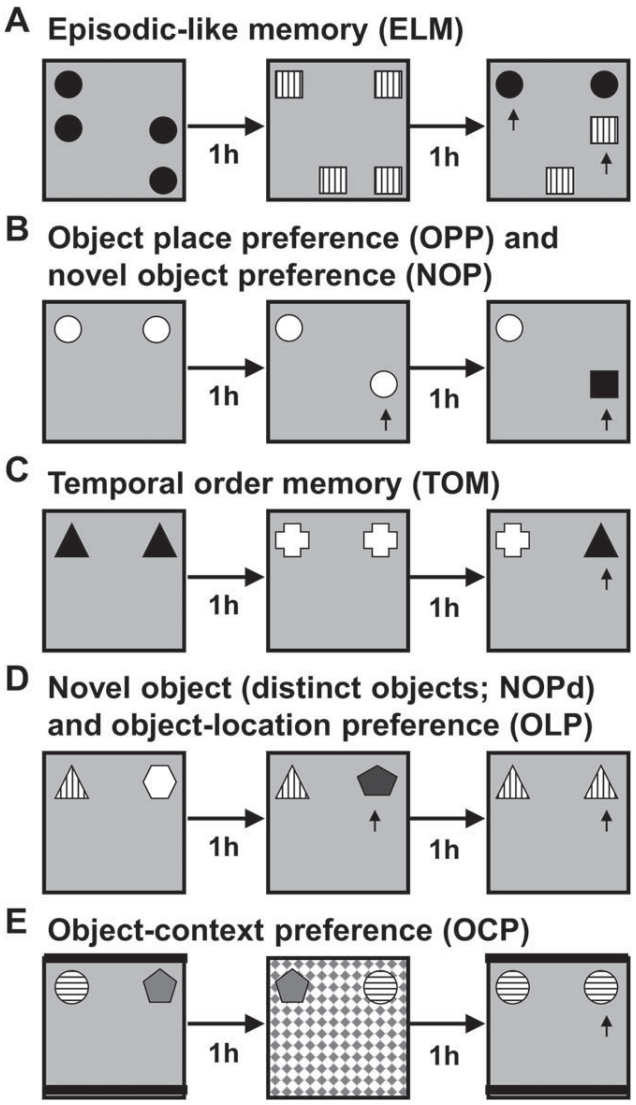


FIGURE 1. Schematic diagrams of spontaneous object exploration tests for (A) ELM, (B) object-place preference and novel-object preference, (C) temporal-order memory, (D) novel-object preference (distinct objects) and object-location preference, and (E) object-context preference. Arrows pointed to an object indicate the one that was expected to be explored more by normal rats. ELM = episodic-like memory; OPP = object place preference; NOP = novel object preference; TOM = temporal order memory; NOPd = novel object preference (distinct objects); OLP = object-location preference; OCP = object-context preference.

Surgery

Animals were anesthetized with 4.5% isoflurance mixed with oxygen by the air rate of 500 ml/min through a calibrated vaporizer (Föhr Medical Instruments GmbH, Germany). They were placed above a heating pad to maintain normothermia and adjusted in a stereotaxic frame (David Kopf, Tujunga CA). Bupivacaine 0.1 ml (Bucain; DeltaSelect GmbH, Germany, 2.5 mg/ml) was injected subdermally as a local anaesthetic. An incision was made and the scalp was retracted to expose the skull. Isoflurance was then maintained at 2% with the rate of 300 ml/min throughout the surgery. Animals received a unilateral mPFC lesion combined with a unilateral LEC lesion either in the same hemisphere ($n = 10$) or in the opposite hemisphere ($n = 10$). Holes were drilled into the skull above the sites of the unilateral mPFC and LEC. The right or the left targets for drilling were counterbalanced within each group. N-methyl-D-aspartate (NMDA; Sigma-Aldrich, Steinheim, Germany) (2 μg dissolved in 0.2 μl phosphate buffer saline, pH 7.3; flow rate 0.1 μl/min) was injected into the unilateral mPFC (AP = +3.0 mm, ML = ±0.7 mm, DV = −3.0 mm;

AP = +3.0 mm, ML = ±0.7 mm, DV = −4.0 mm; AP = +4.0 mm, ML = ±0.7 mm, DV = −3.0 mm; relative to bregma) and the unilateral LEC (AP = −6.6 mm, ML = ±5.0 mm, DV = −8.0 mm; relative to bregma) with a 26-gauge steel cannula. After each injection, the cannula was kept *in situ* for at least 1 min to prevent reflux. A CMA 100 micro-infusion pump with a 10 µl Hamilton syringe via a tube connected to the cannula was used for injections. Then, the scalp was sutured and 70% ethanol was applied to disinfect the wound. Behavioral testing was begun 8 days later.

Apparatus

A black acrylic **open field** (60 × 60 × 30 cm) was used for the spontaneous object exploration tests and to measure locomotor activity. Illumination was provided by LED lights with ∼7 lx in the center. A camera was mounted 2 m above the open field and connected to a DVD-recorder for observation. The arena was located in a sound-attenuating room where several distinct geometric figures were pasted on the walls as spatial cues. Distinct sets of objects in quadruplicate differing in material (glass, porcelain), shape (column, rectangle, irregular shapes), color (white, blue, green), texture (smooth, rough) and sizes (18–34.5 cm height, 8–12 cm diameter) were used in tests for object exploration. All the objects were heavy enough to ensure that the animals could not displace them. For each object exploration test, distinct sets of objects were used and the assignments of objects were counterbalanced. Two acrylic grey plates (60 × 30 cm), which can be installed on the walls of the arena, and a rough rubber floor (60 × 60 cm) were applied to change the context of the arena in a following test (see below). An acrylic **plus-maze**, consisted of two open arms (50 × 10 cm), two walled arms (50 × 10 × 38.5 cm) and a central platform (10 × 10 cm), was elevated for 50 cm and also used to assess anxiety-like behavior (∼6 and ∼24 lux in the dark and white compartment, respectively). A camera was mounted 1.5 m above the elevated plus-maze and connected to a DVD player. A **radial-arm maze** was used to assess spatial working memory using a delayed nonmatching-to-sample task. The maze was consisted of a central platform (46 cm diameter) connected with eight arms (72 × 14 × 20 cm), each contained a food well in the end (3 cm diameter; 1.5 cm depth), in an octagon shape. There were eight doors (14 × 20 cm), which can be presented or removed, between the central platform and each arm to open or block the passage. The maze was elevated 50 cm high and a camera was mounted 2 m above. Several geometric images were provided as spatial cues pasted on the walls around the maze. Behavioral measurements were registered by experimenters blind to the experimental design with the Ethovision software 3.1 (Noldus, Wageningen, Netherlands).

Behavioral Testing

Behavioral tests were conducted between 10:00 and 16:00. Acetic acid (0.1%) was used to mask odor cues after each trial in tests of object exploration. Ethanol (70%) was applied to clean the radial-arm maze, light–dark box and elevated plus maze after each trial.

Open-field test

Eight days after the surgery, the animal was placed into the open field for 10 min per day for three consecutive days. The distance traveled was automatically recorded by the Ethovision software 3.1.

Episodic-like memory test

One day after the open-field test, an ELM test was conducted. Details for performing this test were described elsewhere (Kart-Teke et al., 2006). In brief, this test was composed of two sample trials and one test trial (Fig. 1A). Four copies of objects were placed at different locations in the arena (sample 1). Animals were free to explore them for 5 min. One hour later, four novel copies of objects were placed at different locations (sample 2). Again, animals were allowed to freely explore the arena and the objects for 5 min. After another one hour, two objects used in sample 1 trial and two objects from sample 2 trial were placed together in the open field (test trial). Importantly, one of the two objects for each set was placed at an old location, while the other one was placed at a novel location, respective to their previously presented trial (Fig. 1A). Animals were then given 5 min to explore this environment. The times for exploring each presented object were recorded and three ratios were calculated of the ELM test as follows:

$$\text{Interaction ratio} = (\text{Time at old displaced object} - \text{Time at old stationary object})/(\text{Time at both objects}).$$

$$\text{Where ratio} = (\text{Time at recent displaced object} - \text{Time at recent stationary object})/\text{Time at both objects}).$$

$$\text{When ratio} = (\text{Time at old stationary object} - \text{Time at recent stationary object})/(\text{Time at both objects}).$$

The exploration pattern indicative of ELM leads to a negative value for the "interaction" ratio and positive values for "where" and "when". Note that the "where" and "when" ratios represent the degree of memory for associations between objects and their previous "space" and "time" arrangements (DeVito and Eichenbaum, 2010), not simply spatial recognition or temporal order memory, *per se*.

Object place and novel object preference tests

One day after the ELM test, tests for spontaneous object exploration for object place and object identity were conducted (Fig. 1B). Two copies of objects were placed in the arena and the animals were free to explore them for 4 min. One hour later, the same objects were used, while one of the objects was moved to a novel place. The animals were given 4 min to explore them. Non-lesioned rodents spend more time exploring the displaced object than the one at the old location, suggesting that they have a memory for where an object was located (Ennaceur et al., 1997). After one hour, one of the applied objects was randomly replaced by a distinct novel one (Fig. 1B). Again, the animal was allowed 4 min of exploration.

Rodents explore the novel object more than the old one, indicating that they have a memory for the old object (Ennaceur and Delacour, 1988).

Ratios were computed for the object place preference and novel object preference tests:

Object place preference ratio = (Time at displaced object − Time at stationary object)/(Time at both objects).

Novel object preference ratio = (Time at novel object − Time at old object)/(Time at both objects).

Positive values of the ratios indicate that animals have intact memories for object place (where) and object identity (what).

Temporal order memory test

Three days after the previous tests, a temporal order memory test was applied. This test was composed of two sample trials and one test trial, separated by a delay of one hour. Two copies of objects were placed in the arena and the animals were allowed to freely explore them for 4 min (sample 1). Then, two copies of novel objects were placed at the same locations as in the sample 1 in the open field (sample 2). Four min was given for the animals to explore. In the test trial, one of the objects from sample 1 and one from sample 2 were presented. Their locations were identical as in the sample trials (Fig. 1C). The animals had 4 min to explore them. Rats prefer to explore the earlier-presented familiar object more than the recent familiar object, suggestive of a memory for the temporal order of presentation of objects (Mitchell and Laiacona, 1998).

The temporal order memory ratio was calculated and positive values of this ratio indicate the animals have an intact memory for temporal order (when).

Temporal order memory ratio = (Time at old familiar object - Time at recent familiar object)/(Time at both objects).

Novel object preference (distinct objects) and object-location tests

One day after the temporal order memory test, two modified tests based on the novel object preference test were conducted. Two different objects were placed in the arena and the animals were allowed to explore them for 4 min. One hour later, one of the applied objects was randomly replaced by a novel one and 4 min of exploration were allowed again (Fig. 1D). The nature of this test is similar to the novel object preference test, except that the sample trial involves an association between two different objects (not like in the novel object preference test, where two identical objects were used). In this paradigm rats explore the novel object more than the old one, as in the novel object preference test (Akirav and Maroun, 2006). After one hour, the novel object was replaced by a copy of the old object that was previously presented. The animals were free to explore the two now identical objects for 4 min (Fig. 1D). Rats explore this object at the location where a different object was previously placed more than the other identical one (Wilson et al., 2013a). This test examines whether animals recognize the location that was once occupied by a particular object (an association of object-location).

The ratios were computed for the novel object preference (distinct objects; NOPd) and object-location preference tests:

NOPd ratio = (Time at novel object − Time at old object)/(Time at both objects).

Object-location preference ratio = (Time at object with the location of distinct object − Time at object with the location of identical object) /(Time at both objects).

Positive values of the ratios indicate that animals have intact memories for object identity and object-location with regard to an association between distinct objects.

Object-context preference test

Two days after the previous tests, an object-context preference test was conducted to assess the memory for associations of object-location in distinct contexts. Two different contexts were applied in this test. One of the contexts was made by installing two grey plates on opposite walls of the arena. The other context was made by placing a rough rubber floor in the arena. The animals explored the open field either with the walls of different colors (two black and two grey) or with the rough floor. The order for applying the two contexts was counterbalanced. Two distinct objects were placed in the arena with one of the contexts (sample 1) and 4 min was allowed for exploration. One hour later, the same objects were used, but their locations were reversed and placed in the arena with the other context (sample 2). The animals had 4 min to explore. After one more hour, the context used in the sample 1 trial was applied and two copies from one of the two distinct objects (randomly chosen) were presented (Fig. 1E). The animals, again, had 4 min for exploration. Normally, rats prefer to explore the object at the location in which it was not positioned during sample 1 with the same context, even though it is in the same location as during sample 2 with a different context (Wilson et al., 2013a). This preference indicates that rats have a memory for the location of objects in association with context.

The object-context preference ratio was computed and positive values of this ratio indicate the animals have an intact memory for associations of location of objects in context.

Object-context preference ratio = (Time at object with the context of distinct object − Time at object with the context of identical object) /(Time at both objects).

For all the object exploration tests, times for exploring the objects were measured. The definition of object exploration

was physical contact with the object with snout, vibrissae or forepaws. Other behaviors, such as climbing of the object, or contacting it while looking around the environment, were excluded from this measure. For the applied ratios, values which are not significantly different from zero imply deficient memory.

Elevated plus-maze test

One day after the object-context preference test, the animal was placed onto the center platform, facing an open arm and was free to explore the maze for 5 min. The time spent in the open and closed arms was measured.

Spatial working memory test in a radial-arm maze

One day after the previous tests, the animals underwent food restriction and maintained at around 80% body weight until the completion of this test. Before training, they were habituated to the radial-arm maze with all eight-arms accessible for 10 min per day for two consecutive days. Food pellets (45 mg each; BioServ® Dustless precision pellets) were spread around on the apparatus (16 pellets for day 1 and 8 pellets for day 2) and also in the food wells (2 pellets each) in the habituation sessions. One day later, the training session was begun. Each training trial was composed of a learning phase and a test phase, separated by a 5 min delay. In the learning phase, four randomly selected arms were blocked and food pellets were located in the food wells of the accessible four arms (two pellets each). The animal was placed on the center of the radial-arm maze and allowed to retrieve food within 5 min. Then, it was returned to the home cage. In the test phase, all 8 doors were opened, while only the previously blocked arms contained food. The animal was placed into the center platform again and maximally 5 min was given to retrieve food. The training trial was conducted once per day, daily over 4 days. An error was defined by a visit into an arm that contained no food pellets.

Histology

After behavioral testing, the animals were euthanized with 0.6 ml pentobarbital (Narcoren; Merial GmbH, Germany, 80 mg/ml, i.p.). They were then perfused with 0.9% phosphate buffer saline followed by 10% formalin. Their brains were excised and immersed in a 10% formalin solution for 24 h and then transferred into a 30% sucrose-formalin solution. Brains were preserved at 4°C until processed. A cryostat (50 μm; Leica, Germany) was used to cut coronal brain sections and cresyl violet (Sigma Aldrich, USA) was used to stain the slices for the assessment of the extent of the lesions.

Statistics

Repeated two-way ANOVAs with the within factor "time" and the between factor "group" were used to analyze the performances in the open-field and radial-arm maze tests. For the object exploration tests, repeated two-way ANOVAs with the within factor "object" and the between factor "group" were used. In the ELM test, two-way ANOVAs with the two within factors "place" and "recency" were applied for each group separately and followed by paired *t*-tests when appropriate. Paired *t*-tests were also used for the other object exploration tests for within group comparisons. One-way ANOVAs with the between factor "group" were applied to analyze behavioral performance, including the comparisons of the ratios in the object exploration tests. One sample *t*-tests were applied to compare the ratios in the object exploration tests with zero (chance level). Since the directions of effect were indicated a priori (see above the hypothesis for each object exploration test), one-tailed comparisons were applied in object exploration tests for the paired and one sample *t*-tests. All the other statistics were two-tailed comparisons and the significant level was set as $P < 0.05$.

RESULTS

Histology

The lesion extents were mainly limited to the mPFC and LEC in the ipsi- and contra- lesion groups (Fig. 2). Animals with inaccurate lesions were excluded from this study ($n = 4$ and $n = 3$ for the ipsi- and contra- lesioned groups, respectively). For the mPFC lesions, the dorsal anterior cingulated, the prelimbic and parts of the infralimbic areas were damaged. Both groups showed similar, with minor variation, lesion extents.

Open-Field Test

A significant effect of "time" ($F_{2,22} = 3.84$, $P = 0.037$), but not "group" and "time × group" ($P > 0.05$) was found in the analysis of distance travelled in the open field. The following one-way ANOVAs revealed no significant "group" effect for each day ($P > 0.05$). Thus, the ipsi- and contra- lesion groups exhibited similar horizontal locomotor activity.

Episodic-Like Memory (ELM)

There were significant effects of "object" ($F_{3,33} = 10.869$, $P < 0.001$) and "group × object" interaction ($F_{3,33} = 3.802$, $P = 0.019$), but not of "group" ($P > 0.05$) in the analysis of times in object exploration in the ELM test trial. As effects of "object" and "group × object" interaction were found, further tests for analyzing the object explorations within each group were conducted. For the ipsi- lesioned group, there was a significant "place × recency" interaction effect ($F_{1,5} = 32.646$, $P = 0.002$), but no "place" and "recency" effects ($P > 0.05$) analyzed by the two-way ANOVA. The following paired *t*-tests revealed that the ipsi-lesion group explored the old familiar stationary object more than the old familiar displaced one ($P = 0.0325$), the recent familiar displaced object more than the

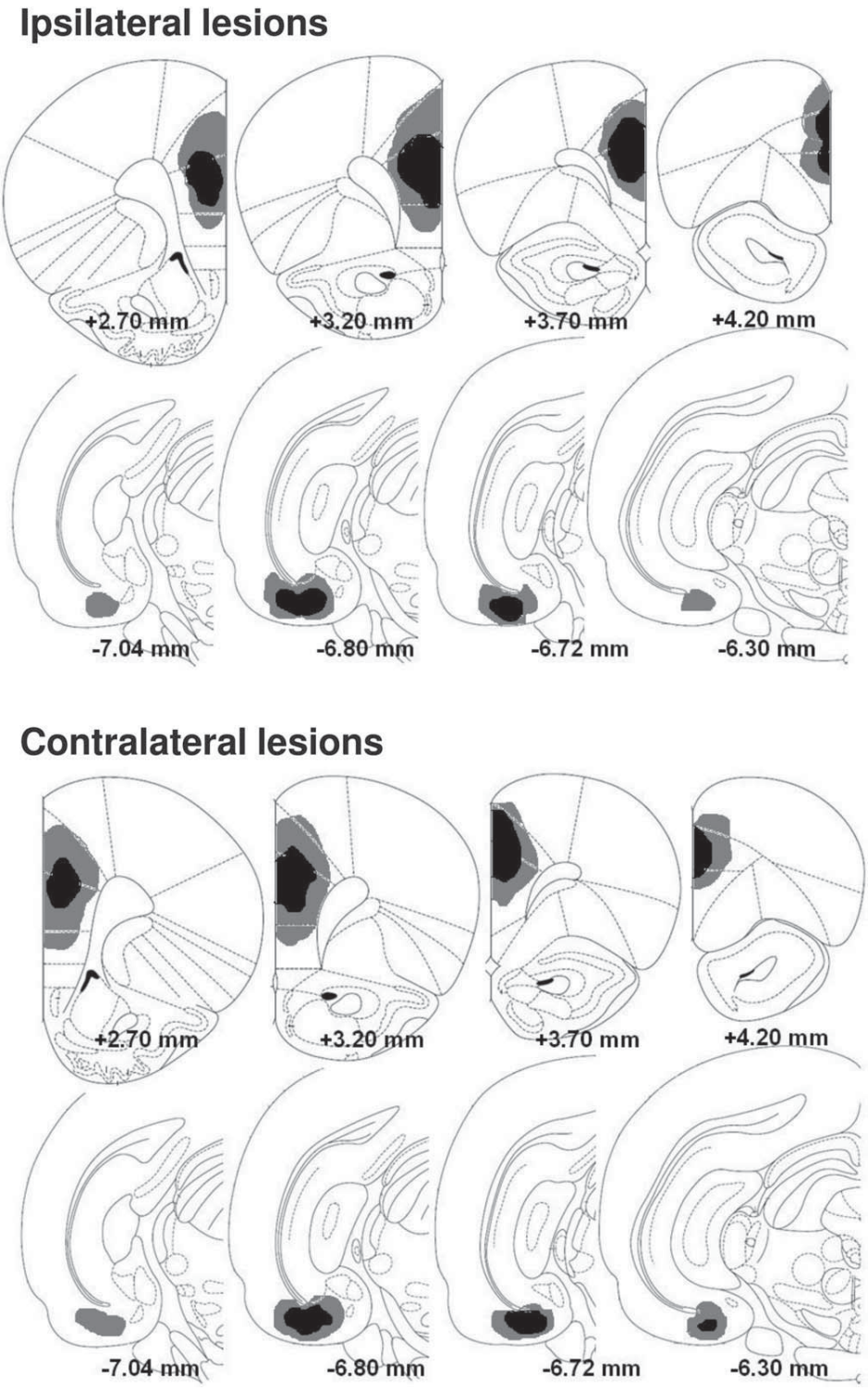


FIGURE 2. Histology of unilateral lesions of the medial prefrontal cortex and lateral entorhinal cortex. The smallest (black) and largest (grey) lesion extents are presented. Numbers indicate the approximate distance from bregma. All the unilateral lesions, irrespective of left or right sides of the lesions, are shown in the half hemisphere.

recent familiar stationary one ($P = 0.001$) and the old familiar stationary object more than the recent familiar stationary one ($P = 0.0195$; Fig. 3A). Hence, the animals exhibited ELM, as evidenced by expressing an integrated memory for *where* and *when* information (exploring the old-stationary object more than the old-displaced one, while exploring the recent-displaced object

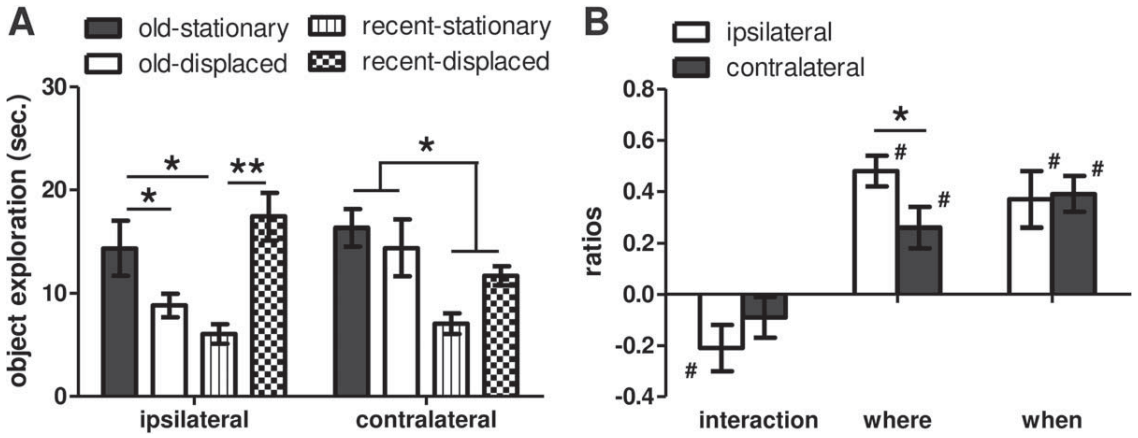


FIGURE 3. Results of the ELM test in (A) time spent in exploring objects and (B) group comparisons of the ratios. The ipsilateral lesioned group exhibited the exploration pattern that defines ELM, i.e., they explored the stationary object more than displaced one if they were presented earlier, while simultaneously exploring the displaced object more than the stationary one if they were presented recently. However, the contralateral lesioned (disconnection) group did not show this pattern. *$P<0.05$, **$P<0.01$ paired *t*-tests (A). The ipsilateral lesioned group showed a higher "where" ratio than the contralateral lesioned group. #$P<0.05$ compared to zero. *$P<0.05$ compared to the group (B). Values are presented as mean ± SEM.

more than the recent-stationary one). On the other hand, a significant effect of "recency" effect ($F_{1,6}=8.638$, $P=0.026$), but not of "place" and "place × recency" ($P>0.05$), was found in the analysis for the contra-lesioned (dissociation) group (Fig. 3A). The contra-lesioned animals did not exhibit the object exploration pattern as the ipsi-lesioned group showed and, thus, they failed to show a complete ELM. Hence, disconnecting the mPFC-LEC circuit prevented the intact expression of ELM.

In the comparisons of the ratios of the ELM test, the ipsi-lesioned group showed significant higher values of the "where" ratio ($F_{1,11}=4.966$, $P=0.048$) than the contra-lesioned group (Fig. 3B). Whereas the ipsi-lesioned group had significant negative "interaction" and positive "where" and "when" ratios compared to zero ($t_5=-2.467$, $P=0.0285$; $t_5=8.462$, $P<0.001$; $t_5=3.298$, $P=0.011$, respectively), only positive "where" and "when" ratios were found in the contra-lesioned group ($t_6=3.492$, $P=0.0065$; $t_6=5.223$, $P=0.001$, respectively; Fig. 3B). No significant difference was found in the analysis of the total times of object exploration in the sample and test trials (Table 1). Hence, a circuit comprising the mPFC and LEC is involved in the processing of the memory for relationships between objects and their places into an episode.

Object Place Preference

There was a significant "object" effect ($F_{1,11}=46.754$, $P<0.001$), but not "object × group" and "group" effects in the analysis of object exploration in the OPP test. This suggested that the two groups did not differ significantly in this test. Both the ipsi- and contra-lesioned groups explored the displaced object more than the stationary one ($P=0.0035$, $P=0.001$, respectively; Fig. 4) and showed positive values of the object place preference ratio ($t_5=5.285$, $P=0.0015$; $t_6=9.844$, $P<0.001$, respectively; Fig. 5), indicating that they had intact memory for object place. There was no group difference in the comparison of this ratio ($P>0.05$) and no significant difference in the total times for object exploration of the sample and test trials (Table 1). Thus, the disconnection of the mPFC-LEC circuit did not influence OPR (*where* memory).

TABLE 1.

Total Times (Seconds) Spent in Object Exploration in the Sample and Test Trials of the Spontaneous Exploration Tests

		Ipsilateral lesions	Contralateral lesions
ELM	Sample 1	76.4 ± 9.4	82.5 ± 6.8
	Sample 2	46.5 ± 7.2	63.1 ± 4.0
	Test	46.7 ± 5.1	49.5 ± 3.7
OPP	Sample	43.1 ± 4.7	42.4 ± 2.5
	Test	30.9 ± 4.2	31.1 ± 3.7
NOP	Test	35.3 ± 6.2	32.0 ± 3.1
TOM	Sample 1	38.3 ± 4.3	36.1 ± 4.9
	Sample 2	35.9 ± 2.9	37.3 ± 4.8
	Test	28.5 ± 4.6	33.5 ± 3.8
NOPd	Sample	34.4 ± 1.6	34.8 ± 3.0
	Test	28.7 ± 4.0	24.1 ± 2.6
OLP	Test	23.2 ± 2.7*	15.5 ± 1.9
OCP	Sample 1	28.8 ± 2.4	24.2 ± 2.7
	Sample 2	19.8 ± 2.5	22.2 ± 2.3
	Test	27.1 ± 3.5	20.0 ± 2.5

Designs are presented in Figure 1.
ELM = episodic-like memory; OPP = object place preference; NOP = novel object preference; TOM = temporal order memory; NOPd = novel object preference (distinct objects); OLP = object-location association; OCP = object-context association.
Values are presented as mean ± SEM.
*$P<0.05$, compared to the contra-lesion group.

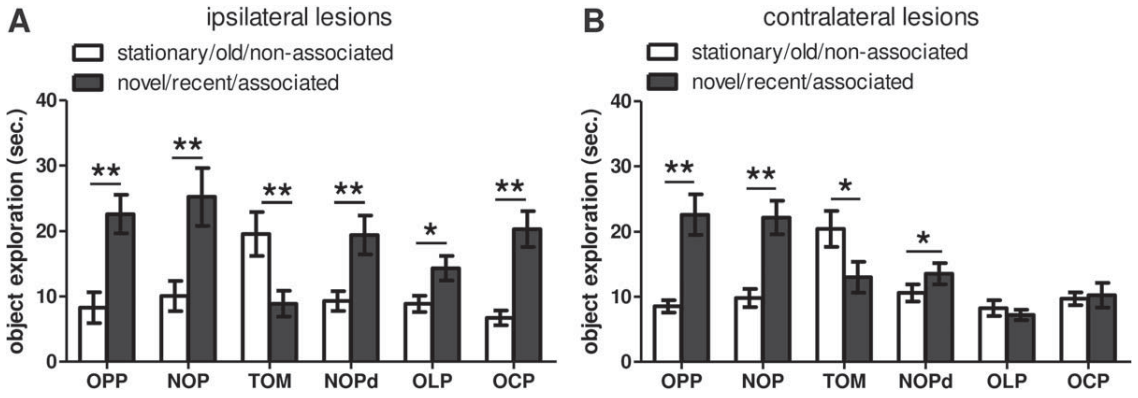


FIGURE 4. Times for object exploration in the object recognition tests for the (A) ipsi- and (B) contra- lesioned animals. The ipsi-lesioned group showed intact memories assessed by these tests, while the contra-lesioned animals had deficits in memories tested by the OLP and OCP tests. OPP = object place preference; NOP = novel object preference; TOM = temporal order memory; NOPd = novel object preference (distinct objects); OLP = object-location preference; OCP = object-context preference. Values are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ paired t-tests.

Novel Object Preference

Similarly, a significant effect of "object" ($F_{1,11} = 42.027$, $P < 0.001$), but not "object × group" and "group" ($P > 0.05$) was found, suggesting that there was no difference between the ipsi- and contra- lesioned groups in the test of novel object preference. Both the ipsi- or contra- lesioned animals explored the novel object more than the old one ($P = 0.003$, $P = 0.002$, respectively; Fig. 4). They, thus, showed positive values of the novel object preference ratio ($t_5 = 6.415$, $P < 0.001$ for the ipsi- group; $t_6 = 6.046$, $P < 0.001$ for the contra- group; Fig. 5). Both groups had intact memory for object identity. No group differences in this ratio ($P > 0.05$) and the total times engaged in object exploration in the test trial were found (Table 1). Thus, the processing of novel object preference (*what* memory) did not require the communication between the mPFC and LEC.

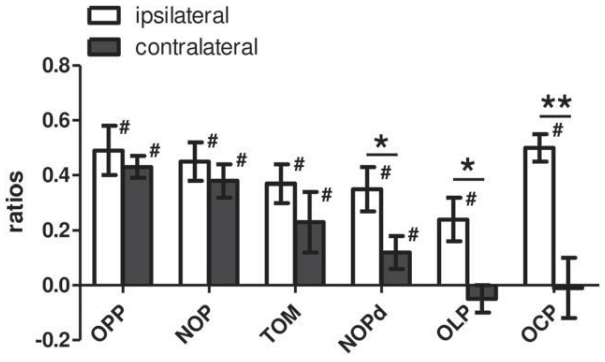


FIGURE 5. Ratios of the ipsi- and contra-lesioned animals in the object recognition tests. Significant group differences were found in the NOPd, OLP and OCP tests. OPP = object place preference; NOP = novel object preference; TOM = temporal order memory; NOPd = novel object preference (distinct objects); OLP = object-location preference; OCP = object-context preference. Values are presented as mean ± SEM. #$P < 0.05$ compared to zero. *$P < 0.05$, **$P < 0.01$ compared to the group.

Temporal Order Memory

There was a significant effect of "object" ($F_{1,11} = 14.616$, $P = 0.003$), but no "object × group" and "group" ($P > 0.05$) for the temporal order memory analysis. Both the ipsi- and contra- lesioned groups explored the old familiar object more than the recent familiar object ($P = 0.009$, $P = 0.0385$, respectively; Fig. 4). Both the ipsi- and contra- lesioned animals exhibited positive values of the temporal order memory ratio ($t_5 = 4.957$, $P = 0.002$; $t_6 = 2.195$, $P = 0.0355$; Fig. 5). No difference in this ratio was found between the two lesioned groups ($P > 0.05$). Thus, all animals exhibited intact memory for temporal order of object presentations. There was also no significant difference in the total time engaged in object exploration of the sample and test trials (Table 1). Thus, the mPFC-LEC circuit was also not found to be critical for the expression of temporal order memory (*when* memory).

Novel Object Preference (Distinct Objects; NOPd)

There were evident effects of "object" ($F_{1,11} = 21.522$, $P < 0.001$) and "object × group" ($F_{1,11} = 6.508$, $P = 0.027$), but not "group" ($P > 0.05$) as analyzed by the two-way ANOVA for the NOPd test. Both groups explored the novel object more than the old one ($P = 0.005$ for the ipsi- group; $P = 0.047$ for the contra- group; Fig. 4). Positive values of the NOPd ratio were found in the ipsi- ($t_5 = 4.539$, $P = 0.003$) and in the contra- lesioned groups ($t_6 = 2.039$, $P = 0.044$; Fig. 5). However, a significant group difference was found in that the ipsi- lesioned group had higher ratios than the contra- group ($F_{1,11} = 5.548$, $P = 0.038$; Fig. 5). Thus, the disconnection of the mPFC-LEC circuit attenuated novel object recognition when two distinct objects were applied during the encoding phase. No significant difference was found in the analysis of the total times for object exploration of the sample and test trials (Table 1).

Object-Location Preference

Significant effects of "group" ($F_{1,11} = 5.924$, $P = 0.033$), "object" ($F_{1,11} = 5.368$, $P = 0.041$) and "object × group" ($F_{1,11} = 11.497$, $P = 0.006$) were found. The animals with the ipsi- lesion spent more time exploring the object in the location that was previously occupied by the different object than the one that was previously occupied by the same object ($P = 0.0135$; Fig. 4A). However, the contra- lesioned group explored the two objects relatively equal ($P > 0.05$; Fig. 4B). These results led to positive values of the object-location preference ratio for the ipsi- group ($t_5 = 2.913$, $P = 0.0165$) and significant higher values of the ipsi- than the contra- lesioned groups in the comparison of this ratio ($F_{1,11} = 9.66$, $P = 0.01$; Fig. 5). Thus, the disconnection of the mPFC-LEC circuit negatively influenced memory for location of an object, while the ipsi- lesioned group overall explored more than the contra-lesioned animals ($F_{1,11} = 5.924$, $P = 0.033$; Table 1).

Object-Context Preference

There were significant "object" ($F_{1,11} = 24.548$, $P < 0.001$) and "object × group" ($F_{1,11} = 20.923$, $P = 0.001$) effects, but no "group" ($P > 0.05$) effect in the analysis of the two-way ANOVA. The ipsi- lesioned animals explored the object when its location was changed in context more than the object whose location was consistent in context ($P = 0.001$; Fig. 4A). The contra- lesioned animals did not show any preference for the two objects ($P > 0.05$; Fig. 4B). Hence, the ipsi- lesioned group showed positive ($t_5 = 9.412$, $P < 0.001$) and higher ratios than the contra- lesioned group ($F_{1,11} = 16.88$, $P = 0.002$; Fig. 5). Disconnection between the mPFC and LEC also disrupted the associative memory of object-location in contexts. No significant difference was found in the total times for object exploration in the sample and test trials (Table 1). Thus, the interaction between the mPFC and LEC was found to be critically involved in the expression of memory for an object-context association.

Anxiety-like Behavior in the Elevated plus Maze

There was no significant effect of "group" in the analyses of behavior on the elevated plus maze ($P > 0.05$). The ipsi- and contra- lesioned groups spent similar length of time (in seconds) on the central platform (58.9 ± 9.0; 81.0 ± 7.8, respectively) and the open (37.7 ± 14.0; 40.4 ± 12.7, respectively) and closed arms (203.4 ± 21.3; 178.6 ± 18.4, respectively).

Spatial Working Memory in the radial-Arm Maze

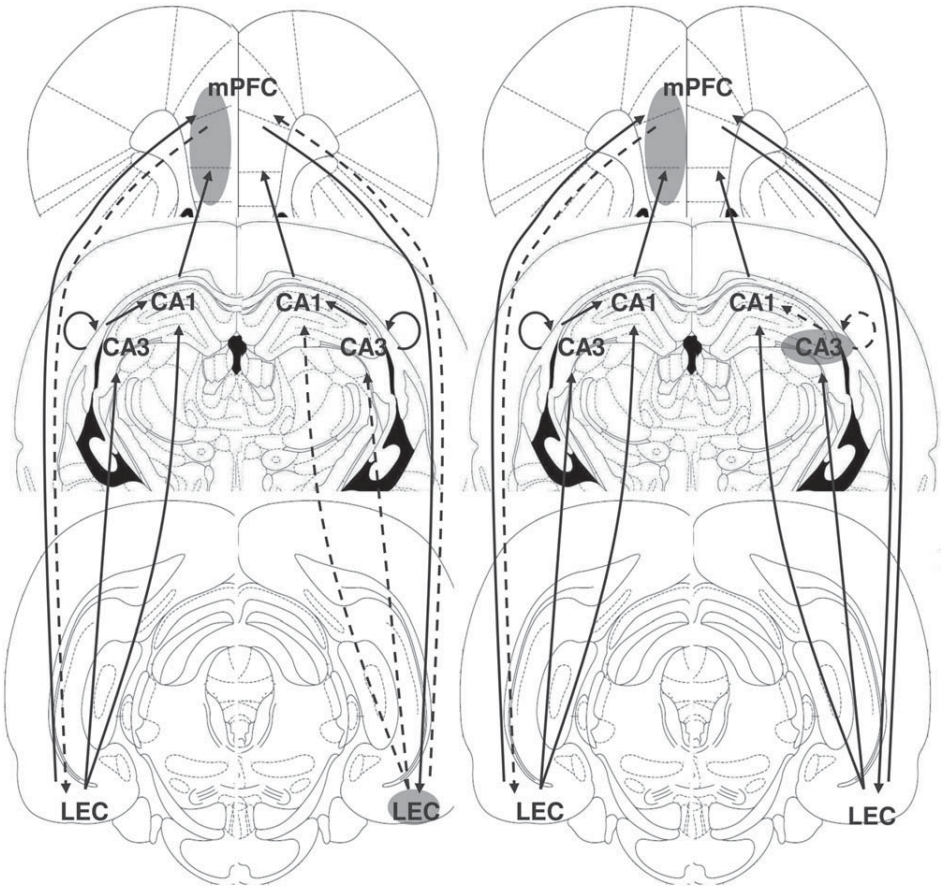
No significant effects of "time," "group" and "time × group" were found in the analysis of the counts of errors in the radial-arm maze test ($P > 0.05$; data not shown). The averaged errors for both groups were 5.23 ± 0.92 at day one and 3.0 ± 0.69 at day four. The ipsi- and contra- lesioned animals were not different in this test. Thus, the disconnection did not influence the spatial working memory applied here, at least not in the early learning period.

DISCUSSION

Disconnecting the mPFC-LEC circuit prevented the expression of intact ELM, but not the individual *what*, *where* and *when* memory components, the integration of which defines a "what-where-when" ELM (Figs. 3–5). Similar results were found by disconnecting the mPFC–hippocampal CA3 area (de Souza Silva et al., in press), suggesting that the mPFC-LEC-CA3 pathway functions together to process ELM. One could surmise that the mPFC-LEC circuit provides general interrelated *item* information and communicates with the hippocampus for the expression of episodic memory (Figs. 5 and 6).

Anatomically, the LEC is interconnected with the mPFC (Jones and Witter, 2007; Agster and Burwell, 2009) and hippocampus (Kerr et al., 2007). Electrical stimulation of the entorhinal cortex strongly inhibits mPFC pyramidal neuron activity (Valenti and Grace, 2009), while it promotes neurogenesis in the hippocampal dentate gyrus and facilitates memory for escape from the water-maze (Stone et al., 2011). Moreover, lesion of the LEC leads to impaired hippocampal CA3 area neural firing when shapes or the color of the environment are altered (Lu et al., 2013). Thus, the LEC acts like a modulator, influencing both the mPFC and hippocampus. The entorhinal cortex has been proposed to process "general" information to the hippocampus, which further improves the information via pattern separation and pattern completion processes (van Strien et al., 2009). When the information processing between the mPFC and LEC was disconnected, ELM was disrupted, while the animals showed positive *where* and *when* ratios (Fig. 3B). Disconnecting the mPFC and CA3, led to impaired *where* and *when* ratios (Fig. 6). Hence, the mPFC-LEC and mPFC-CA3 circuits can be considered to have distinct and hierarchical roles in constructing memory representations to less-detailed and integrative episodic information, respectively (Fig. 6). This perspective is consistent with the concept that episodic memory is mainly processed in the hippocampus, while the entorhinal cortex offers "global" supplementary information (van Strien et al., 2009; McKenzie et al., 2014).

Disconnection of the mPFC–LEC also attenuated the memory for the relationship between objects and their places (the "where" ratio) in the ELM paradigm (Fig. 3B). LEC neurons, unlike the MEC ones that fire for location in the environment and across different environments (Hafting et al., 2005; Fyhn et al., 2007; Solstad et al., 2008), fire for non-spatial features, such as an object (Deshmukh and Knierim, 2011; Tsao et al., 2013). This functional dissociation has also been found by human imaging studies, namely that the LEC and MEC are preferentially involved in the retrieval of non-spatial and spatial stimuli, respectively (Schultz et al., 2012; Reagh and Yassa, 2014). Lesion of the MEC was also shown to impair spatial



Interaction	no	no*
where	yes*	no*
when	yes	no

FIGURE 6. Schematic of anatomical connections focusing on the medial prefrontal cortex (mPFC), lateral entorhinal cortex (LEC) and hippocampus (CA1 and CA3 subregions). Arrows indicate direction of information processing. Dashed lines indicate disrupted projection. Gray areas indicate damaged regions. The results are based on the results of the present disconnection study and those of a previous one (de Souza Silva et al., in press). Either disconnecting the mPFC-LEC or mPFC-CA3 prevented ELM, but to different extent. The disconnected mPFC-CA3 circuit totally impaired ELM, while the *where* and *when* ratios were spared in the mPFC-LEC disconnected animals. These results suggest that a) the mPFC-LEC-CA3 pathway could function together as a whole circuit to process episodic memory and b) the mPFC-LEC and mPFC-CA3 circuits are distinct and hierarchical in the processing of episodic information. The mPFC-LEC circuit is mainly responsible for associations between different objects, while the mPFC-CA3 circuit processes the integration of what-where-when information (de Souza Silva et al., in press). *Interaction*, *where* and *when* indicate the three ratios used in the ELM paradigm. "Yes" and "no" indicate whether or not the animals exhibited the memory, respectively (positive values over the zero). Asterisk indicates a difference between the ipsilateral and contralateral lesioned groups.

navigation, but to spare object place preference (Hales et al., 2014). However, recent studies indicate that the MEC also processes non-spatial odor-based recognition memory in recollection (Sauvage et al., 2010) and that the LEC processes object-related spatial information (Deshmukh and Knierim, 2011; Beer et al., 2013; Sauvage et al., 2013; Van Cauter et al., 2013). Likewise, the mPFC has been demonstrated to be responsible for spatial goals (Hok et al., 2005), remote spatial memory (Teixeira et al., 2006) and memory for objects in place (Weible et al., 2009). As the mPFC and LEC are anatomically combined (Jones and

Witter, 2007; Agster and Burwell, 2009), it is plausible that their interplay is involved in encoding associations between objects and places (Fig. 3B), but not spatial recognition itself (Figs. 4 and 5, object place preference results).

Disconnecting the mPFC–LEC strongly influenced NOPd (novel object preference - distinct objects) and object-location preference, but preserved novel object and object place preference, *per se* (Figs. 4 and 5). A key difference between NOPd/object-location preference and novel object/object place preference is whether distinct or duplicate objects were presented in the sample trials used for memory encoding (Fig. 1). When distinct objects were used, animals could remember information not only about items *per se*, but also about associations between items (e.g. a relative left-right relationship between two different objects). Since the disconnected animals showed intact novel object preference and object place preference, the deficits found in the NOPd and object-location preference tests are not likely a result of impaired memory for identifying an object and its place, especially given the controls employed (identical number of used objects, environment, times for encoding, delay and test). Thus, the mPFC–LEC circuit appears to be critically involved in the memory for associations between distinct objects. This conclusion is strongly compatible with a) studies showing that lesions of neither the mPFC (Mitchell and Laiacona, 1998; Barker et al., 2007; Barker and Warburton, 2011; Cross et al., 2013) nor the LEC (Van Cauter et al., 2013; Wilson et al., 2013a,b) influence novel object preference when duplicate objects were used in the sample trial, whereas the test is disrupted when distinct ones were applied (Akirav and Maroun, 2006; Van Cauter et al., 2013). It is also consistent with the findings from pharmacological inactivation of the hippocampal lateral perforant path, that recognition of object identity and object place were impaired when distinct objects were encoded (Hunsaker et al., 2007), and b) electrophysiological studies showing that neurons of both the mPFC (Weible et al., 2012) and LEC (Tsao et al., 2013) respond in the location where an absent object was formerly placed (reflecting a memory for object-location), which is congruent with the present findings (Figs. 4 and 5; object-location preference results). However, the contra-lesion group exhibited decreased overall duration of exploration in the object-location preference test (Table 1), which could be a consequence of the memory deficit in encoding the association between two distinct objects (Figs. 4 and 5; NOPd results). Thus, the explanation concerning object-location recognition should be considered with care. Overall, our results indicate a functional interaction between the mPFC and LEC in the processing of memory for associations between distinct objects.

The disconnected mPFC–LEC animals did not exhibit intact memory for object-in-context (Figs. 4 and 5). Whereas two distinct objects were applied as samples in the object-context preference test, the findings may be confounded by the deficits in the association between distinct items (maybe also object-location). Thus, whether the mPFC–LEC circuit is involved in the associative memory between objects and contexts *per se* is not clear. Whereas evidence shows that the LEC is crucial for this type of memory (Wilson et al., 2013a,b), novel context discrimination is dependent on the MEC, but not LEC (Hunsaker et al., 2013), while contextual memory tested by fear conditioning is independent of the MEC (Hales et al., 2014).

In the present study, we did not include a sham lesion group to compare with the performance of the behavioral effects of the ipsi- and contralateral lesions. In the case of a disconnection procedure, as used here, the ipsilateral lesion is the essential control, rather than a sham lesion, as differences between the ipsilateral and contralateral groups are used to interpret a functional interaction between brain regions. Such a control is sufficient when the ipsilateral lesion group exhibits "intact" memory performance in all the tests applied, as found here (positive ratios; Fig. 5). One can argue that there could be potential impairments in the ipsilateral lesion group as compared with a sham lesion group. However, such possible deficits in the ipsilateral lesion group would not compromise the main findings here, as the "disconnection" effect, rather than "unilateral mPFC+LEC" lesion effect, is the primary concern. Locomotor, emotional and motivational confounding factors can likely be excluded because both, the ipsi- and contralateral lesioned groups were comparable in the open field, elevated plus-maze tests and in the total time spent exploring objects (except the object-location preference test; Table 1).

We did not find a group difference in the spatial working memory test under the 5 min delay condition. Pharmacological inactivation of the mPFC prior to test, but not to sample, influenced performance in this test under a 30 min delay (Seamans et al., 1995; Floresco et al., 1997). Based on a similar test for choosing one baited arm out of two, it was suggested that the mPFC and hippocampus process spatial memory in parallel (Lee and Kesner, 2003; Churchwell and Kesner, 2011), but that the role of the hippocampus becomes more prominent when the inter-trial-interval is increased from seconds to minutes (Lee and Kesner, 2003). On the other hand, lesions of the lateral perforant path (Ferbinteanu et al., 1999) or LEC (Van Cauter et al., 2013) did not impair spatial navigation in the water maze. Applying short (seconds) vs. long (e.g. 30 min) delays in future studies might clarify whether the mPFC–LEC pathway is crucial for spatial working memory.

Limitations of the present study need to be mentioned. In general, the permanent lesion approach has been shown to influence other brain regions besides the target site, and lesion-induced plasticity or physiological activity may compensate behavioral performance over time. To overcome these drawbacks, electrophysiological recording or temporal pharmacological inactivation of intracranial injection can be used. However, these methods are beyond the scope of this study as we set out to firstly test whether a functional interaction between the mPFC and LEC exists. Although we cannot exclude such compensatory factors, a disconnection study which focuses on identifying critical functional interactions between two brain regions applying permanent lesion can provide essential information. The approach applied here also cannot address questions, such as which brain region, the mPFC or LEC, initiates the interaction. Another issue is that due to the sequence of the conducted tests earlier experiences may influence subsequent ones. Although in each test we used different sets of objects to minimize possible confounds, order effect cannot be entirely ruled out.

The present results indicate a critical interaction between the mPFC and LEC in the processing of associative object recognition and hippocampus-dependent episodic memory. These data together with previous results (de Souza Silva et al., in press) suggest that the mPFC–LEC–hippocampus (CA3) network could be a selective circuit for the expression of episodic memory as defined by the integration of information as to "what", "where" and "when". The entorhinal cortex has been proposed to offer global information to the hippocampus, while the hippocampus further integrates the information (van Strien et al., 2009; Beer et al., 2013). The LEC, with input to the mPFC and hippocampus from its superficial layers, receives output from the mPFC and hippocampus to its deep layers (Jones and Witter, 2007; Agster and Burwell, 2009). This suggests that a role of the LEC may be that of a comparator (Kloosterman et al., 2003), as its neural activities synchronize with the mPFC and hippocampus during temporal-association memory (Takehara-Nishiuchi et al., 2012). The mPFC-hippocampus circuit has been found to be responsible for memory of *when* and object-in-place, but not for *what* and *where* memories (Barker and Warburton, 2011). The mPFC biases neural representations of the hippocampus in rule-based object associations (Navawongse and Eichenbaum, 2013) and retrieves item/place source information (Dobbins et al., 2002; Slotnick et al., 2003). Taken together (Fig. 6), the mPFC-hippocampus network in the control of episodic memory involves, inclusively but not exclusively, the LEC, via which the mPFC selects related item/place sources to cooperate with the hippocampus for retrieving an episode (Dobbins et al., 2002; Navawongse and Eichenbaum, 2013; Watrous et al., 2013). In this network, the interaction between the mPFC and LEC processes information about associations between distinct objects (probably object-location and -context), while the mPFC-CA3 circuit processes more detailed and integrated episodic information (de Souza Silva et al., in press). Thus, the mPFC-LEC and mPFC-CA3 circuits are likely to play distinct roles in the processing of episodic memory in function and hierarchy (Eichenbaum et al., 2012; de Souza Silva et al., in press).

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NK(3) receptor agonism reinstates temporal order memory in the hemiparkinsonian rat

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Research report

NK_3 receptor agonism reinstates temporal order memory in the hemiparkinsonian rat

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HIGHLIGHTS

- Hemiparkinsonian rats show deficits for temporal order but not novel object memory.
- Senktide, a NK_3 receptor agonist, reinstates temporal order memory in this model.
- Senktide may influence prefrontal cortex and hippocampus in this memory.

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ABSTRACT

Animals treated with unilateral 6-hydroxydopamine (6-ODHA) injections, an animal model of Parkinson's disease, exhibit deficits in memory for temporal order, but show intact novel object recognition. Since senktide, a potent neurokinin-3 receptor (NK_3-R) agonist, has been shown to have promnestic effects in the aged rat and to alleviate scopolamine-induced impairment, the present study aimed to assess possible promnestic effects of senktide in the hemiparkinsonian rat model. Animals received unilateral 6-ODHA microinjections into the medial forebrain bundle. Two weeks later, they were randomly assigned to treatment with vehicle, 0.2, or 0.4 mg/kg senktide. Temporal order memory and place recognition tests were conducted, locomotor activity and turning behavior were assessed in the open field and anxiety-related behavior was measured in the light–dark box. Treatments were administered 30 min prior to behavioral testing with an interval of seven days between tests. The animals treated with 0.2 mg/kg senktide exhibited temporal order memory, unlike the vehicle-treated group. No significant treatment effects were found in the open field and light–dark box. Administration of 0.2 mg/kg senktide may influence the prefrontal cortex and hippocampus, leading to compensations for deficits in memory for temporal order.

1. Introduction

Patients with Parkinson's disease (PD) can suffer deficits in visuo-spatial and executive deficits [1], such as impairments in temporal processing [2,3]. Rats with unilateral 6-hydroxydopamine (6-OHDA) lesion, a widely used animal model of PD [4,5], are deficient in temporal order memory, but show intact novel object recognition [6]. Thus, this animal model can be used to examine such deficits primarily induced by striatal dopamine (DA) depletion, which may simulate parkinsonian states.

The neurokinin-3 receptors (NK_3-R) have been implicated in processes underlying learning and memory and are widely expressed throughout the mammalian brain, including the prefrontal cortex (PFC), midbrain and hippocampus [7–9]. NK_3-R knockout mice show impairments in tests of conditioned avoidance learning and in the Morris water maze [10]. Systemic administration of senktide, a potent NK_3-R agonist, was shown to improve episodic-like memory in mice [11] and aged rats [12], to attenuate scopolamine-induced spatial working memory impairment in mice [13–15] and to ameliorate scopolamine-induced deficits in recognition memory for object, place and temporal order in rats [16]. Furthermore, the NK_3-R-coding gene *TACR3* has been found to be correlated with cognitive performance in elderly humans [17]. There is a possible role of NK_3-R in learning and memory via cholinergic modulation, since NK_3-R are expressed on cholinergic neurons [18] and senktide application increases extracellular acetylcholine (ACh) in the striatum [19], hippocampus [12,17,20], amygdale and PFC [12,17].

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Based on the above findings, we hypothesized that the NK_3-R agonist senktide could also ameliorate the impairment of temporal order memory in the 6-OHDA-lesioned animals. In addition, we assessed turning behavior as well as locomotor activity and anxiety-related behaviors.

2. Materials and methods

2.1. Subjects

Thirty male Wistar rats (Tierversuchsanlage, University of Düsseldorf, Germany) weighing between 250 and 350 g were investigated. They were housed in groups of five animals per cage (60 cm × 38 cm × 20 cm) in a standard environment under a reversed light–dark rhythm (light off from 07:00 to 19:00) with free access to food and water. Each animal was handled for 3 min per day for three days before surgery. All experiments were in accordance with the Animal Protection Law of Germany and of the European Communities Council Directive (86/609/EEC).

2.2. Surgery

Animals were anaesthetized with 50 mg/kg pentobarbital (Narcoren; Merial GmbH, Germany, i.p.) placed in a Kopf stereotaxic frame with a heating pad beneath its body for temperature maintenance at 37 °C. Neurotoxic lesions were inflicted as previously described [21]. All rats received single injections into either the right or the left medial forebrain bundle (MFB; stereotaxic coordinates, AP: −4.0 mm, ML: ±1.5 mm, DV: −8.5 mm; relative to bregma [22]) in a counterbalanced fashion. 6-OHDA (10.5 μg in 3 μl phosphate buffer saline with 0.1% ascorbic acid) was injected with a 26-gauge steel cannula using a 50 μl Hamilton syringe connected to a micro-infusion pump. The flow rate was 1 μl/min. The cannula was left in place for an additional 4 min to allow for diffusion. Finally, the scalp was sutured and 70% ethanol was applied for disinfection. Then, the rats were transferred back to their home cage and allowed to recover for 2 weeks.

2.3. Drugs

The NK_3-R agonist, senktide ([succinyl-Asp^6-Me-Phe^8]SP_{6-11}; Bachem, USA), was diluted with 5% dimethylsulfoxide in phosphate buffer saline. Animals were randomly assigned to the following treatments groups: vehicle (n = 10), 0.2 mg/kg (n = 10) or 0.4 mg/kg senktide (n = 10). Injections were applied subcutaneously (s.c.) in a volume of 1 ml/kg body weight. Treatments were administered 30 min prior to the first sample trial of each behavioral test. An interval of 7-days between experiments was applied. The doses used here were based on previous studies showing significant effects on object recognition tests [16].

2.4. Behavioral testing

An acrylic open field (60 cm × 60 cm × 30 cm) was used for assessments of locomotion and object exploration. Illumination was provided by four LED lights (luminous density on the center ~7 lx and ~3 lx in the corners). A camera was mounted 2 m above the arena and connected to a computer and a DVD recorder.

An acrylic light–dark box (50 cm × 35 cm × 30 cm) was used to measure anxiety-related behavior as previously described [6]. The light–dark box consisted of a white and a black compartment, separated by a central barrier with a small passage. Dim light was applied by two LED lights. Behaviors were registered with a camera mounted 1.6 m above the apparatus and connected to a DVD recorder. All apparatuses were located in sound-attenuating rooms.

Different sets of objects in quadruplicate of different material (plastic, glass, ceramic), shape (rectangle, circular, irregular-shapes), surface texture (plain, grooved), color (green, white, red) and height (18–34.5 cm) were applied in the spontaneous object exploration tests. These tests were conducted in the same open field used for the assessment of locomotor activities. The objects were heavy enough so that the animals could not displace them. Different sets of objects were applied for each object exploration test and the assignments of objects were counterbalanced for each test. Object exploration was defined as a physical contact with the object with snout, vibrissae or forepaws. Climbing of the object and contacting the object while looking around the environment were excluded. If an animal failed to explore both objects either in sample or test trial, it was excluded from the analyses. Animals were habituated to the open field for 10 min on the day prior to the beginning of the object exploration tests. Behavioral measurements were conducted between 10:00 and 16:00. Ethanol (70%) was used to clean the apparatuses after each trial.

2.4.1. Open-field test

Fourteen days after surgery, the animal was placed into the center of the open field. Behavior was registered for 30 min. Distance traveled, duration of stay in the center of the area and turning behaviors were analyzed automatically by the Ethovision software (Noldus, Wageningen, The Netherlands).

2.4.2. Temporal-order object-recognition test

The temporal-order object-recognition test comprised two sample trials and a test trial with a 30 min interval between trials. The animal was placed into the center of the arena with two identical objects for 4 min (sample trial 1). After the interval, the animal was placed into the apparatus, which now contained two copies of novel objects, for 4 min (sample trial 2). After another 30-min interval, the rat was placed into the arena for 4 min, which now contained one of the objects used in sample 1 and one of the objects presented in sample 2 (test trial). The positions of objects used in all the trials were identical. Object exploration times were rated by experimenters blind to the experimental design using the Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Normal rats spend more time exploring the old familiar object than the recent familiar one, suggesting that they recognize objects at different time points [23]. A discrimination index (=[time spent exploring the older-familiar object – time spent exploring the recent-familiar object]/[total time spent exploring the two objects]) was calculated. The discrimination index is employed in order to reduce the influence of individual differences. It is considered a more suitable measure of recognition in conditions, where the overall locomotor activity of the animals may be altered, as the case in 6-OHDA lesioned animals [24] or under pharmacological challenge [25].

2.4.3. Spatial object recognition

Seven days after the temporal-order object-recognition test, the animal was placed into the arena for testing spatial object recognition. The animal was free to explore the objects and the arena for 4 min (sample trial). Then, it was returned to its home cage. After 1 h, the animal was placed into the open field with the two previously explored objects, but one at the old location and the other at a novel location. Object exploration times were rated by experimenters blind to the experimental design using the Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Normal rats explore an object at the novel location more than an object at the old place, suggesting that they recognize objects at different locations [26]. A discrimination index (=[time spent exploring the object at novel

Table 1
Neurochemical results of contents of DA, DOPAC, HVA, 5-HT and 5-HIAA in the intact and the lesioned striatum.

DA depletion	Vehicle ($n=5$) 90.87 ± 3.57%		SENK 0.2 mg/kg ($n=7$) 87.14 ± 5.50%		SENK 0.4 mg/kg ($n=7$) 85.85 ± 2.36%	
	Intact	Lesioned	Intact	Lesioned	Intact	Lesioned
DA	111.65 ± 36.12*	9.18 ± 3.08	238.09 ± 36.85**	20.52 ± 3.88	136.20 ± 44.32*	13.93 ± 2.58
DOPAC	27.45 ± 3.52**	9.94 ± 1.26	61.78 ± 15.00*	12.75 ± 2.97	48.10 ± 11.24*	15.65 ± 1.41
HVA	122.03 ± 24.34	78.96 ± 12.76	133.83 ± 17.30*	87.29 ± 20.63	154.80 ± 29.79	146.14 ± 23.64
5-HT	5.45 ± 1.88	4.47 ± 1.01	6.75 ± 0.76**	4.36 ± 0.41	6.15 ± 1.38	6.83 ± 1.22
5-HIAA	33.07 ± 8.35	24.66 ± 6.14	39.15 ± 4.84**	23.81 ± 3.21	35.04 ± 7.72	36.00 ± 3.35

Values are expressed as ng/mg wet tissue weight. DA depletion = (intact DA content − lesioned DA content)/intact DA content, then × 100. *$p<0.05$, **$p<0.01$ compared to the lesioned side. SENK = senktide.

location − time spent exploring the object at old location]/[total time spent exploring the two objects]) was computed.

2.4.4. Light–dark box

Seven days after the spatial object recognition test, the animal was placed into the light–dark box for 5 min. The rat was placed into the white compartment facing the black compartment. Time spent in the two compartments and times of crossing the central passage were automatically measured by the Ethovision software.

2.5. Neurochemical analysis

At least one week after the final behavioral test, the animals were anaesthetized with CO_2, decapitated and their brains immediately excised. Both hemispheres of the dorsal striatum were dissected. Tissue samples were homogenized in 500 μl of 0.05 N perchloric acid and centrifuged at 9000 rpm for 20 min at 4 °C. Then the samples were filtered and stored at −80 °C until analysis. The samples were analyzed for the content of DA, DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-HT) and the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), by means of high-performance liquid chromatography-electrochemical detection. The column was an ET 125/4, Nucleosil 120-5, C-18 reversed phase column (Macherey & Nagel, Germany) perfused with a mobile phase composed of 75 mM NaH_2PO_4, 4 mM KCl, 20 μM EDTA, 1.5 mM sodium dodecylsulfate, 100 μl/l diethylamine, 12% methanol and 12% acetonitrile adjusted to pH 6.0 using phosphoric acid. The electrochemical detector (Intro, Antec, The Netherlands) was set at 530 mV vs. an ISAAC reference electrode (Antec, Leyden, The Netherlands) at 30 °C.

2.6. Statistics

One-way ANOVAs with the factor "group" were applied to analyze behavioral and neurochemical results. Discrimination indexes were analyzed within each group by one-sample *t*-tests to compare to chance level performance (zero value). Given that the direction of the exploring preference can be hypothesized to be positive [23,26], one-tailed comparisons were applied. All statistical tests were two-tailed comparisons except the one-sample *t*-tests used for discrimination indexes. The level of significance was set at $p<0.05$.

3. Results

3.1. Neurochemistry

There were significant differences in the content of DA comparing the intact side with the lesioned side in the vehicle, 0.2 and 0.4 mg/kg senktide groups ($p=0.042$, $p=0.001$, and $p=0.027$, respectively). Animals with a DA depletion of less than 55% were excluded from the present study ($n=5$, $n=3$, $n=3$ for the vehicle, 0.2 and 0.4 mg/kg senktide groups, respectively. DA depletion = [DA content in the intact side − DA content in the lesioned side]/[DA content in the intact side], then × 100). In the remaining animals there was an averaged DA depletion of 87.65% (standard deviation: ±2.32%). DOPAC content was also decreased on the lesioned side in the vehicle, 0.2 and 0.4 mg/kg senktide groups ($p=0.004$, $p=0.01$, and $p=0.04$, respectively). Decreases of the contents of HVA, 5-HT and 5-HIAA were found in the 0.2 mg/kg senktide group ($p=0.03$, $p=0.006$, and $p=0.002$, respectively). However, none of the group comparisons yielded significant results ($p>0.05$). Details are presented in Table 1.

3.2. Open-field test

One-way ANOVAs showed no effect of "group" in the analyses of distance traveled, duration of stay in the center area and turning behavior ($p>0.05$; Table 2). There was also no significant "group" difference in the analyses of behaviors in the habituation trials ($p>0.05$; data not shown).

3.3. Temporal-order object-recognition test

The discrimination index of the 0.2 mg/kg senktide group was positive, suggesting that the rats of this treatment group explored the old familiar object longer than the more recently presented object ($t_6=2.885$, $p=0.014$), which was not the case for the two other treatment groups ($p>0.05$; Fig. 1A). There was no significant effect of "group" in the analysis of discrimination indices ($p>0.05$). No significant effect of "group" was found in total exploration time in the sample and the test trials ($p>0.05$; Table 3).

3.4. Spatial object recognition

The vehicle- and the 0.4 mg/kg senktide-treated groups showed positive discrimination indices ($t_4=2.645$, $p=0.029$ and $t_6=2.454$,

Table 2
Behaviors in the open-field test.

	Distance moved (cm)	Center duration (s)	Turning index
Vehicle	7228.59 ± 1918.49	56.30 ± 14.52	0.67 ± 0.08
SENK 0.2 mg/kg	8375.18 ± 749.41	92.38 ± 21.16	0.57 ± 0.04
SENK 0.4 mg/kg	7288.74 ± 303.29	72.51 ± 10.69	0.61 ± 0.05

Turning index = ipsiversive quarter turns/(ipsi- plus contraversive quarter turns). SENK = senktide.

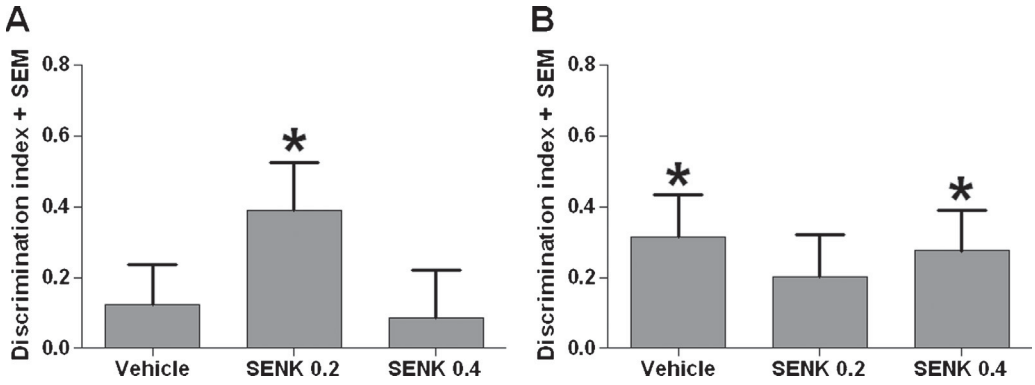

Fig. 1. Discrimination indexes for temporal order (A) and spatial recognition (B). Only the senktide 0.2 mg/kg treated group showed a positive index for temporal order recognition (A). The vehicle and 0.4 mg/kg senktide groups exhibited positive values for object location recognition (B). $^{*}p < 0.05$ compared to zero value. SENK = senktide.

$p = 0.025$, respectively), suggesting that they spent more time exploring the object, which had been moved, compared to the object, which had been left in place. This was not the case in the 0.2 mg/kg senktide-treated group ($p > 0.05$; Fig. 1B). There was no significant effect of "group" in the analysis of discrimination indices ($p > 0.05$). There was also no effect of "group" in total exploration times in the sample and the test trials ($p > 0.05$; Table 3).

3.5. Light–dark box

There was no significant "group" difference in the analyses of time spent in the white compartment and the frequency of crossing between the compartments ($p > 0.05$; data not shown).

4. Discussion

The main finding of the present study is that 6-OHDA-lesioned rats treated with 0.2 mg/kg senktide exhibited intact temporal order memory, unlike the vehicle injected control group.

Animals with unilateral 6-OHDA injections into the MFB show behavioral asymmetries such as ipsiversive turning [5] and contralateral sensorimotor deficits [27]. Given that hemiparkinsonian rats can show novel object recognition [6], the asymmetrical behavioral deficits in these animals were not sufficient to negatively influence object exploration and memory in this test. The extent of DA lesion as well as effects on motor and anxiety-related behavior were similar between groups and, thus, were not likely to confound the deficits incurred in the memory tasks.

Patients with PD exhibit deficits in learning and memory tasks involving temporal processing of information [2,3], which suggests that DA is involved in such learning. Neuroimaging studies indicate that abnormal cortical–striatal activation accounts for impairments of temporal processing in PD patients [3]. Similarly, temporal order memory is impaired in 6-OHDA-lesioned animals [6], while structural [28] and neurochemical [29] alternations of the PFC are observed in this model. The results are in line with the perspective of the medial PFC is critical for temporal order memory [23,30]. Since replenishment of DA in patients with PD does not improve deficits of time perception [3], it is important to search for other pharmacological interventions for treating deficits of temporal processing in PD, particularly targeting the striatal-PFC circuit.

Consistent with previous studies of promnestic effects of senktide in aging and scopolamine-induced deficit models [12–14,16], the deficit in temporal order memory in the 6-OHDA-lesioned animals was also compensated by administration of senktide (Fig. 1A). NK_3-R agonism has been shown to exert promnestic effects in both mice [11,13–15] and rats [12]. An involvement of NK_3-R in cognitive functions is also supported by the deficits in learning and memory exhibited by NK_3-R knockout mice [10]. Moreover, the NK_3-R-coding gene *TACR3* is correlated with cognitive performance in elderly humans [17]. Senktide was also shown to enhance memory in deficit models, e.g. in aged rats [12,17] and in mice treated with scopolamine-induced impairment of spatial working memory [13,14]. The beneficial effects of senktide could be due to its effects on the release of ACh in the PFC and hippocampus [12,17,20], which are brain-regions involved in a neural circuit responsible for temporal order memory [30].

There are few studies assessing motor effects of senktide in the rat model of PD. Microinjection of senktide into the SN decreased ipsiversive rotations of rats with unilateral 6-OHDA lesions [31]. The distribution of NK_3-R in the anterior striatum implies their action on DAergic terminals [32]. It seems that the decreases of ipsiversive rotations induced by senktide appear in partially [31,33] but not in severely DA-depleted rats [33]. In hemiparkinsonian rats primed with L-3,4-dihydroxyphenylalanine (L-DOPA), systemic administration of the NK_3-R antagonist SB222200 increased L-DOPA-elicited contraversive turning, suggesting that NK_3-R may play an inhibitory role in this condition [34]. It is worth noting that substance P (SP), which also binds to the NK_3-R, may have neuroprotective properties in the 6-OHDA lesion model of PD. Chronic administration of SP decreased ipsiversive turning in rats with partial DA depletion either treated after [33] or before the lesion [35,36]. As, however, other studies reported contradictory findings [37,38], the role of NK_3-R in the recovery from motor deficits requires further investigation.

Table 3
Time for object exploration in the sample(s) and test trials (s).

	Temporal order			Location recognition	
	Sample 1	Sample 2	Test	Sample	Test
Vehicle	39.44 ± 8.39	41.10 ± 8.36	29.77 ± 6.04	29.23 ± 7.09	30.60 ± 7.53
SENK 0.2 mg/kg	50.06 ± 3.70	43.38 ± 5.77	32.07 ± 7.86	29.55 ± 3.91	23.67 ± 3.73
SENK 0.4 mg/kg	38.67 ± 5.58	35.99 ± 5.03	35.53 ± 5.70	29.72 ± 3.33	20.84 ± 3.41

The used numbers for the object exploration tests are $n = 4$, $n = 7$, $n = 7$ in the temporal order recognition; $n = 5$, $n = 7$, $n = 7$ in the location recognition for the vehicle, senktide 0.2 and 0.4 mg/kg groups, respectively. SENK = senktide.

In conclusion, the lower dose of the NK_3-R agonist senktide had positive effects on temporal-order recognition in rats with midbrain DA depletion. This effect could be due to ACh release in the medial PFC and hippocampus which are brain areas crucial for processing of temporal order memory [23,30].

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Dopamine in the nucleus accumbens core, but not shell, increases during signaled food reward and decreases during delayed extinction

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Dopamine in the nucleus accumbens core, but not shell, increases during signaled food reward and decreases during delayed extinction

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ABSTRACT

Microdialysis studies in rat have generally shown that appetitive stimuli release dopamine (DA) in the nucleus accumbens (NAc) shell and core. Here we examined the release of DA in the NAc during delivery of reward (food) and during extinction of food reward in the freely moving animal by use of *in vivo* microdialysis and HPLC. Fifty-two male Wistar rats were trained to receive food reward associated with appearance of cue-lights in a Skinner-box during *in vivo* microdialysis. Different behavioral protocols were used to assess the effects of extinction on DA and its metabolites.

Results Exp. 1: (a) During a 20-min period of cued reward delivery, DA increased significantly in the NAc core, but not shell subregion; (b) for the next 60 min period half of the rats underwent immediate extinction (with the CS light presented during non-reward) and the other half did not undergo extinction to the cue lights (CS was not presented during non-reward). DA remained significantly increased in both groups, providing no evidence for a decrease in DA during extinction in either NAc core or shell regions. (c) In half of the animals of the group that was not subjected to extinction, the cue lights were turned on for 30 min, thus, initiating extinction to cue CS at a 1 h delay from the period of reward. In this group DA in the NAc core, but not shell, significantly decreased. Behavioral analysis showed that while grooming is an indicator of extinction-induced behavior, glances toward the cue-lights (sign tracking) are an index of resistance to extinction.

Results Exp. 2: (a) As in Exp. 1, during a 30-min period of cued reward delivery, DA levels again increased significantly in the NAc core but not in the NAc shell. (b) When extinction (the absence of reward with the cue lights presented) was administered 24 h after the last reward session, DA again significantly decreased in the NAc core, but not in the NAc shell.

Conclusions: (a) These results confirm the importance of DA release in the NAc for reward-related states, with DA increasing in the core, but not shell subregion. (b) They provide first evidence that during the withholding of expected reward, DA decreases in the NAc core, but not shell region. (c) This decrease in DA appears only after a delay between delivery of reward and extinction likely due to it being masked by persisting DA release. We hypothesize the decrease in extinction-induced release of DA in the NAc core to be a marker for the despair/depression that is known to accompany the failure to obtain expected rewards/reinforcers.

1. Introduction

The absence of or loss of rewards or reinforcers hold a major role in the development of depression in humans and impact the onset and maintenance of psychiatric diseases, especially depressive disorder (Leventhal, 2008). In humans, the loss of reward falls into the category of stressful life events encompassing, for instance, loss of employment, partnership and health (Hammen, 2005). Behavioral conceptualizations of depression emphasize that depressive symptoms arise when positive reinforcement decreases or is withheld (extinction) and that there is a relationship between

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avoidance behavior and depression that is largely explained by the mediating role of reduced positive reinforcement (Ferster, 1973; Lewinsohn, 1974; Manos, Kanter, & Busch, 2010; Martell, Addis, & Jacobson, 2001). Cognitive and behavioral avoidance increases the risk for depression by escalation of further losses of reinforcers; e.g., it was shown that the degree of cognitive and behavioral avoidance was positively related to severity of depression (Carvalho & Hopko, 2011).

Two types of animal models of extinction-induced depression (EID) have recently been established, based on the extinction of either negatively or positively reinforced behaviors (for review see Huston, De Souza Silva, Komorowski, Schulz, & Topic, 2013): The *extinction of negatively reinforced escape behavior* in the Morris Water Maze induced despair-like behaviors, such as an increased frequency and duration of immobility, which were attenuated by chronic antidepressant treatment (Schulz, Buddenberg, & Huston, 2007a; Schulz, Huston, Buddenberg, & Topic, 2007b; Schulz, Topic, De Souza Silva, & Huston, 2004). Extinction of water-maze escape was also accompanied by changes in monoamines neurotransmitters, neurotrophins and HPA-related stress markers, indicative of a depression-like state (Huston, Schulz, & Topic, 2009; Schulz et al., 2004; Topic, Oitzl, Meijer, Huston, & De Souza Silva, 2008b; Topic et al., 2008a).

The *extinction of positively reinforced behaviors* (food reward) in rats was shown to incur despair-like behaviors, such as an increase in distance from the former source of reinforcement, rearing and biting behavior, which was attenuated by treatment with antidepressant drugs, thus providing an animal model for extinction-induced depression (Huston, Van Den Brink, Komor woski, Huq, & Topic, 2012; Huston et al., 2013; Komoro wski et al., 2012). The withholding or reduction in reward in rats also induces anxiety, which is well known to be comorbid with depression (Manzo, Donaire, Sabariego, Papini, & Torres, 2015; Papini, Fuchs, & Torres, 2015).

The nucleus accumbens (NAc) plays a focal role in the neural organization that controls responses to aversive and rewarding events and has been proposed to contribute importantly to the pathophysiology, symptomatology and etiology of depression (Nestler & Carlezon, 2006). The NAc and its dopaminergic inputs from the ventral tegmental area (VTA) of the midbrain have long been considered to serve as a central anatomical substrate of behaviors related to motivation, reward, and hedonia (Koob & Le Moal, 2001; Wise, 1998). Moreover, it has been shown that antidepressant treatments can alter dopaminergic activity in the VTA or its targets (Nestler & Carlezon, 2006), and that experimental manipulation of dopaminergic transmission in the VTA–NAc pathway can regulate depression-like behavior in animal models (Nestler & Carlezon, 2006). Dopaminergic abnormalities within the "limbic" areas of the brain have been observed in several animal models of depression (Dremencov et al., 2005; Kram, Kramer, Ronan, Steciuk, & Petty, 2002; Zangen, Nakash, Overstreet, & Yadid, 2001). Thus, there is growing evidence that specific molecular pathways in the VTA–NAc, which were originally implicated in the action of addictive drugs and of natural rewards, also subserve depression and antidepressant action (Nestler & Carlezon, 2006).

The NAc is divided into at least two anatomically and functionally distinct subregions, the core and the shell (Zahm & Brog, 1992), and both regions receive a strong and functionally important (Blackburn, Pfaus, & Phillips, 1992; Ikemoto & Panksepp, 1999; Salamone, 1994) dopaminergic (DA) innervation from the ventral tegmental area (VTA). They have distinct input sources and output targets, with the core projecting mainly to motor structures and the shell projecting to more limbic regions, suggesting that the subregions may mediate different behavioral processes. While the afferent projections of the core region mainly arise from the substantia nigra pars compacta and lateral portions of the VTA, the shell region receives inputs from the posteromedial VTA (Brog, Salyapongse, Deutsch, & Zahm, 1993; Ikemoto, 2007; Voorn, Jorritsma-Byham, van Dijk, & Buijs, 1986). The shell of the NAc has been attributed a role in the acquisition and expression of incentive motivation while the core compartment is thought to be involved mainly in the motor expression of motivated behavior (Bassareo & Di Chiara, 1999a,b; Berridge & Robinson, 1998; Brauer, Haeuber, & Arendt, 2000; Zahm & Brog, 1992).

The aim of the present study was evaluate the effect of the extinction of free signaled food reward on the release of dopamine in the NAc shell and core subregions of rats by *in vivo* microdialysis. Many studies have demonstrated an increase in DA release in the NAc in relation to the presentation of conventional rewards, such as food (Bassareo, De Luca, & Di Chiara, 2002; Bassareo & Di Chiara, 1997, 1999a,b; Hajnal & Norgren, 2002; Hajnal, Smith, & Norgren, 2004; Hernandez & Hoebel, 1988; Roitman, Stuber, Phillips, Wightman, & Carelli, 2004) and a variety of self-administered drugs (Anselme, 2009; Di Chiara, 2002; Di Chiara & Bassareo, 2007; Di Chiara et al., 2004; Hernandez & Hoebel, 1988). However, there is sparse literature on the effects of withholding an expected reward (extinction) on DA levels in the NAc. We hypothesized that DA in the NAc during extinction would behave in a reciprocal direction to its response to delivery of reward. Thus, we measured DA and its metabolites during presentation of free signaled food delivered on a time schedule and also during withholding of the food reward during presentation of the conditioned light cue (discriminative stimulus). The main experimental questions posed were: A. Does DA increase in NAc core vs NAc shell during the period of signaled free reward presented on a fixed time schedule? B. Does DA change during non-reward (extinction) when only the signal (conditioned stimulus) is presented?

2. Methods

2.1. Subjects

Fifty-two (weight: 307.6 ± 6.1 g, mean ± S.E.M.) male Wistar rats were obtained 2 weeks prior to the start of experiments from the local breeding facility (Tierversuchsanlage, University of Düsseldorf, Düsseldorf, Germany). They were kept in Makrolon cages (Type IV) in groups of five/cage with food and water provided ad libitum and maintained under a reversed 12-h light/dark cycle (lights on: 19:00 h) in a temperature- (22 ± 2 °C) and humidity-controlled room. After 10 days of familiarization to the new environment, they were submitted to a food deprivation (15 g/animal/day) schedule, which was continued until the end of the study. Moreover, they were marked on the tail with ink for identification and handled over 3 days prior to the beginning of the experiment. The weight of the animals was monitored 3 times per week and they had water freely available. After implantation of guide-cannulae they were housed individually. The study was carried out according to the German Law of Animal Protection and approved by the regional administration authority (Bezirksregierung Düsseldorf).

2.2. Apparatus and procedure

An rectangular operant chamber (72 × 28 × 34 cm), made of dimmed Plexiglas, with floor covered by a black rubber mat, with an open top, was situated in a dark, sound-attenuating box, containing a masking white noise generator. Infra-red cameras (Conrad Elektronik) were mounted on the top and side of the chamber to allow observation of behavior from two perspectives. The hardware modules (Coulbourn Industries®) were placed on one of the short end-walls. The food cup with an integrated

photodetector and the food magazine were fastened in the middle of the wall. The photodetector gathered the frequency and duration of access the food cup. A light bulb with a partially open hood, offering slight diffuse illumination (~1 lux), was placed at the top of the wall, whereas a triple cue lamp (green, yellow, red) was located next to the food cup. For the training session, an additional hand-switch was connected to the magazine, allowing experimenters manually to shape the animals' behavior toward the feeder. All modules were connected to a computer via a link box from PanLab® and controlled by the related PackWin® 2.0.01 Software. Apart from the variables measured by the PackWin® Software, automatic tracking of the animals' behavior was done via center-point detection plus manually by an experienced, blinded rater with Ethovision XT 8® (Noldus®). Experiments in the operant chamber began after 3 days of food deprivation. On every test day, the animals were brought to the behavioral laboratory one hour prior to start of tests and thereafter taken back to the husbandry room for feeding.

2.3. Surgery

Animals were deeply anesthetized with a mixture of ketamine hydrochloride (90.0 mg/kg, Ketavet, Pharmacia GmbH, Berlin, Germany) and xylazine hydrochloride (8.0 mg/kg, Rompun, Bayer, Leverkusen, Germany) and fixed in a Kopf stereotactic frame. Two guide-cannulae with a thread on the top (14 mm, 22 G, stainless steel) were implanted, aimed at the nucleus accumbens shell (AP: +1.2 mm, ML: ±0.8 mm, DV: −6.2 mm) and the nucleus accumbens core (AP: 1.2 mm, ML: ±2.8 mm, DV: −5.8 mm, 10° angle tilted laterally) in the left and right hemisphere respectively. Each animal had one guide-cannulae implanted in the left and the other in the right hemisphere. Laterality was counterbalanced within each experimental group, such that half the animals of a particular group were implanted in the right core and left shell region and vice versa. Coordinates were taken relative to bregma, according to the atlas of Paxinos and Watson (1986). They were fixed to the skull with two screws (stainless steel, $d = 1.4$ mm) and dental cement. After the surgery the animals were given carprofen (5 mg/kg, Rimadyl, Pfizer, GmbH, Berlin, Germany) subcutaneously. After surgery they were housed individually and allowed to recover for at least 5 days before beginning of microdialysis experimentation.

2.4. Microdialysis procedure

Experiments were conducted between 9:00 am and 6:00 pm. The dialysis probes (2.0 mm active membrane length) of a concentric design (for construction details see: Boix, Sandor, Nogueira, Huston, & Schwarting, 1995) were inserted through the guide-cannulae and fixed to the thread. Next, they were connected to syringes attached to a microdialysis pump (CMA 100) and perfused at a flow rate of 2.0 µl/min with artificial cerebrospinal fluid containing Na^+ 146 mM, K^+ 4 mM, Ca^{2+} 2.2 mM and Cl^- 156 mM. After a stabilization period of 2 h, the samples were collected every 10 min into vials containing 5 µl of 0.05 M HClO4, in which 100 pg of deoxyepinephrine (Experiment 2) or 3,4-dihydroxybenzilamine (Experiment 1) was dissolved; these served as an internal standard for chromatographic analysis. The first three (Experiment 2) or six (Experiment 1) samples were taken as baseline; then the animals were submitted to the different procedures according to the protocols.

2.5. Analysis

Immediately after collection, the samples were analyzed for the content of dopamine (DA) and its metabolites 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA), using high performance liquid chromatography. For the separation, a 125 × 2 mm reversed phase column (120-5 C18; Macherey Nagel, Düren, Germany), perfused with a mobile phase composed of 9.5 mM chloroacetic acid, 0.53 mM sodium octyl sulfate, 5 mM ethylenediaminetetraacetic acid, 4 mM KCl, with 6% v/v acetonitrile, and 0.8% v/v tetrahydrofuran, pH adjusted to 3.25 using 6 M NaOH solution, was used. Quantification was performed by amperometric detection (Intro; Antec, Leyden, Netherlands) with the potential set at +530 mV vs an ISAAC reference electrode (Antec) at 28 °C. The limit of detection was 1 fmol per sample for DA at a signal-to-noise ratio of 2:1.

2.6. Histology

After completion of the last sampling interval, the animals were administered an overdose of pentobarbital (Narcoren, Merial GmbH, Hallbergmoos, Experiment 2) or placed in a chamber with CO_2 (Experiment 1) and transcardially perfused with phosphate-buffered saline, followed by 10% formalin. The brains were removed and stored in formalin (10%) until they were sliced on a cryotome, stained with Cresyl Violet and examined under a light microscope for probe localization by comparing the slices to plates from Paxinos and Watson (1986). Only animals with correct probe placed within the boundaries of the NAc shell and NAc core were considered for data analysis.

Two different experimental protocols were used (Fig. 1).

2.7. Experiment 1: DA in the NAc core vs shell during reward and extinction

The purpose of this experiment was to assess the response of dopamine in the NAc core and shell (a) during a 20-min period of cued food reward delivery, (b) during a subsequent period of extinction (with cue conditioned stimulus (CS) present in the absence of during 60-min period of non-reward), and (c) during a 1-h delayed 30-min extinction period (with introduction of CS to group that formerly did not undergo extinction to CS).

This protocol (Fig. 1) allows for a direct comparison between the effects of presence vs non-presence of the conditioned cue lights (discriminative stimuli), i.e. extinction vs non-extinction of appetitive behavior to the conditioned light stimuli. All animals first received signaled reward followed by cessation of reward delivery, during which in one group the cue lights were presented (extinction), whereas in two other groups the cue lights were no longer presented (non-extinction). In one of the two latter groups the cue lights were then again presented (extinction) after 60 min.

Pre-dialysis training: The following training regime (Fig. 1) was employed in order to simulate the time course of the procedure used in the subsequent microdialysis experiment: the subjects received one habituation session lasting 20 min on the first day, to allow free exploration of the apparatus without presentation of cue lights. On the second day, the subjects received a 30 min long training session. During this trial, approach or manipulation of the food cup was reinforced by a single food pellet (BioServ® Dustless precision pellets) delivered to the food cup. In this trial, food pellets delivery was controlled manually via a hand-switch connected to the food magazine. For the following 5 days of acquisition trials we used a variable interval schedule (i.e. the cue-lights turned on every 57, 72, 87, 102, or 117 s for 3 s (VI: 60–120 s) and two food pellets were delivered immediately at the onset of the cue-lights), in which the cue-lights and food delivery were paired 22 times during the 30-min reinforcement procedure. The animals were placed into the operant chamber 1 h before and removed 10 min after the reinforcement procedure (total trial duration: 1 h 40 min). After the last acquisition trial, the animals were submitted to stereotactic surgery, had a recovery

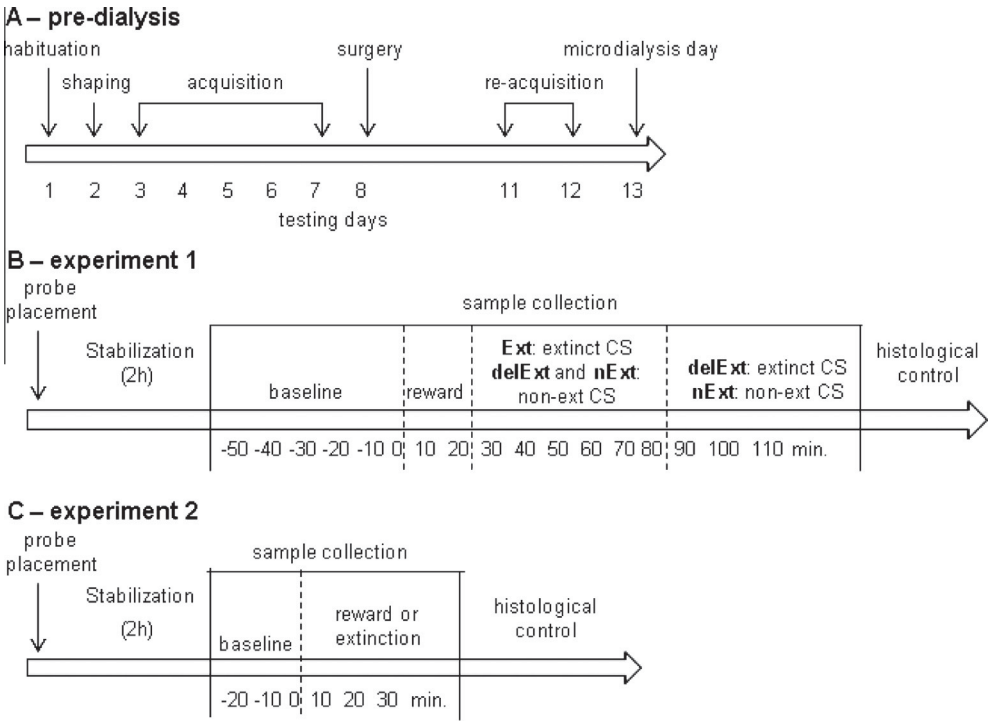

Fig. 1. Timeline of protocol: A. pre-dialysis. B. Microdialysis Experiment 1. C. Microdialysis Experiment 2.

time of five days and underwent reacquisition trials (third and fourth days after the surgery), as during acquisition.

Microdialysis: On the day of the microdialysis procedure, the animals were randomly divided into three groups (Fig. 1). After collecting the baseline sample, the treatment were applied: during the first 20 min the cue-lights and food delivery were paired 15 times using a variable interval schedule (VI: 60–120 s) for all groups. Then, in group one **– extinction condition** (Ext, $n = 12$): the cue-lights were presented (50 times) without food delivery during 1 h using a variable interval schedule (VI: 60–120 s). For group two **– non-extinction followed by extinction condition** (delExt, $n = 8$): (*a*) the animals were kept in the operant chamber for 1 h without cue lights being turned on and then were subjected to one session of 30 min, during which the cue lights were presented (25 times, VI: 60–120 s) without food delivery *(extinction condition)*. The animals from the third group **– non-extinction condition** (nExt, $n = 10$) (*b*) stayed in the chamber for 1 h 30 min without cue lights.

The following categories of behavior were extracted: *distance moved* (cm), *number of beam-breaks in the food cup* (goal tracking) and *mean distance to the food cup* were scored using the Ethovision® software (Noldus®) The *frequency and duration at the food cup* (a contact was counted manually when the animal entered food cup with snout or touched it with forelimbs), the *frequency and duration of rearing*, *grooming* and *glances/rears toward the cue-lights* (sign tracking) were recorded manually by an experienced and blinded observer from the image obtained using two infra-red cameras, one mounted on the top and one on the side of the chamber, which allowed observation of behavior from two perspectives.

2.8. Experiment 2: DA in the NAc core vs shell during reward and delayed extinction

The main purpose of this experiment was to confirm the finding of experiment 1 that DA levels decrease in the NAc core, but not NAc shell if there is a delay between the period of reward presentation and the onset of non-reinforcement (extinction). To test this hypothesis, the animals received pre-dialysis training to expect signaled reward exactly as in experiment 1. However the last day of training preceded dialysis by 24 h, hence, leading to a 24 h delay between acquisition training and extinction.

Another group which received 30 min of signaled reward was added simply to confirm that DA increases in the NAc core during presentation of reward. Thus, in the reward group the cue lights were paired with food reward and in the extinction group the cue lights were presented in the absence of reward.

Protocol. The first two days (habituation and shaping) were the same as in experiment 1. Over the next 5 days, the animals were placed into the operant chamber for a 1 h period without reward, followed by a 30 min period of signaled food reward. During the first 2 trials, the cue lights turned on every 57 s for 3 s (fixed interval FI: 60 s) and two food pellets were delivered immediately at the onset of the cue lights. From the third until the fifth day of these daily trials a 15–90 s variable interval schedule was applied (i.e. the cue-lights turned on every 12, 27, 42, 57, 72 or 87 s for 3 s (variable interval VI: 15–90 s) and two food pellets were delivered immediately at the onset of the cue-lights); thus, the cue lights and food delivery were paired thirty times during each session. The animals were removed from the operant chamber one hour after the end of the reinforcement procedure; thus, the total acquisition trial lasted 2.5 h (1 h adaptation, 30 min reinforcement procedure, 1 h time out from reward). The days following the last day of acquisition, the animals were submitted to stereotactic surgery for implantation of the guide-cannulae. Then, they had a recovery period of five days before the microdialysis procedure. On the third and fourth days they were submitted to reacquisition trials according to the protocol applied in the last three acquisition trials before guide-cannulae implantation (Fig. 1A). On the day of the microdialysis procedure (Fig. 1C), the animals were randomly assigned to the different groups: after the baseline samples were collected, the cue lights were presented (thirty-two times; VI: 15–90 s) without coincident food delivery (extinction group, $n = 12$) or with coincident food delivery (reward group, $n = 9$) during 30 min.

2.9. Statistical analysis

The behavioral data were separated into blocks, depending on the experimental phase, e.g. acquisition, re-acquisition and extinction, and analyzed by two-way repeated measures analysis of variance (ANOVA), followed by one way repeated-measure ANOVAs for each of the time periods and variables, when justified. For the acquisition and re-acquisition periods, the ANOVAs included only the factor "time/trials" as within-subject factor. For the extinction condition, "treatment" was added as the between-subject factor. When indicated, independent *t*-tests were performed in order to compare two groups.

The neurochemical data were expressed as percentage of the mean of six (Experiment 1) or three (Experiment 2) baseline samples taken as 100%. Two-way ANOVAs with repeated measures were calculated with the factors "time" (17 or 12 time points) and "treatment" (extinction/no extinction or extinction/reward). When appropriate, this was followed by independent *t*-test to compare two groups. The groups with dialysis probes in NAc core and shell were analyzed separately. After histological analysis for probe placement some animals were considered only for NAc shell or only for NAc core or for both, resulting in different ns per group.

Behavioral activity, neurotransmitter and metabolites levels were also assessed using within group one-way ANOVAs for repeated measures (12- and 17-time intervals) followed by Fisher's LSD-test vs baseline samples.

The significance level (two-tailed) was set at $p \leqslant 0.05$.

3. Results

3.1. Experiment 1: DA in the NAc core vs shell and behaviors during reward and extinction

3.1.1. Main experimental questions

A. *Reward:* Does DA increase in NAc core vs NAc shell during the period of signaled reward?

B. *Extinction:* Do DA levels differ between the group that underwent extinction (Ext), with CS light presented during non-reward and the group in which the CS was not presented (Non-Ext)?

C. *Delayed extinction:* What is the influence on DA levels in NAc core and shell regions when extinction is delayed by 1 h? See also Fig. 1 for an overview.

3.1.2. Acquisition and reacquisition (pre-dialysis)

Repeated-measures ANOVAs were performed for number of beambreaks in the food cup (goal tracking) over the five acquisition and two reacquisition days. Only the general development over the trials was of interest, since the subjects were not yet assigned to treatment groups. Over the trials the number of beam-breaks significantly decreased (from 429.96 ± 33.94 to 311.57 ± 25.96) to a stable performance level ($F(6,90) = 6.520$, $p = 0.0001$). Thus, in the first trials the animals visited the food cup in an irregular pattern, independent of the light signal for delivery of food, but over trials the number of beam-breaks decreased, indicating that the subjects learned the contingency of the cue-light and the subsequent food reward. There was no significant difference between the groups that were later used for the Extinction- and Non-Extinction groups.

3.2. A. Does DA change during the period of reward and during immediate extinction?

3.2.1. DOPAMINE in NAc Core vs NAc Shell

The number of animals per group considered for statistical analyses were: NAc core: Ext, $n = 7$; nExt, $n = 14$; NAc shell: Ext, $n = 7$; nExt, $n = 13$.

The location of the dialysis membranes in the NAc core and shell is shown in Fig. 2. There was not prevalence of side, right or left, in either NAc core or shell.

Two-way ANOVAs for repeated measures were performed on the DA levels from −50 to 80 min (including baseline, reward and extinction periods) for the NAc core and NAc shell groups:

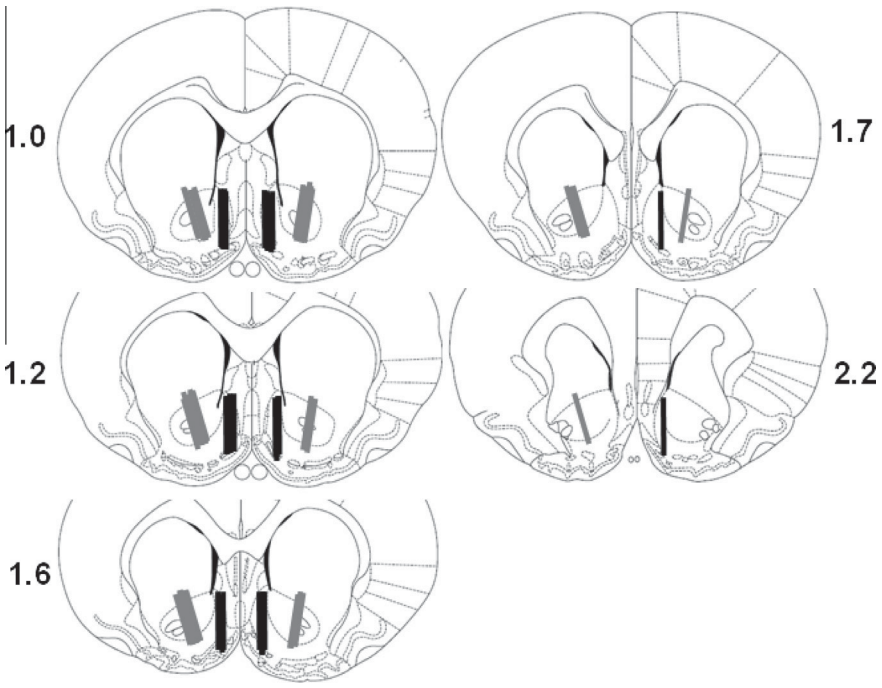

Fig. 2. Schematic representation of the placement of the dialysis probes in the core and shell of the NAc. The gray bars represent the localization of the dialysis membranes in the NAc core, and the black in the NAc shell. Numbers indicate distance from bregma (in mm) (Paxinos & Watson, 1986).

The ANOVAs yielded significant effects of *time* in the NAc core ($F(13,234) = 4.038$, $p = 0.0001$) and in the NAc shell ($F(13,234) = 1.786$, $p = 0.046$). (There was no effect of *group* in either area (Core: $F(1,18) = 0.862$, $p = 0.365$; Shell: $F(1,18) = 0.240$, $p = 0.630$) nor a *time* × *group interaction* (Core: $F(13,234) = 1.072$, $p = 0.384$; Shell: $F(13,234) = 0.735$, $p = 0.727$).

One-way repeated measures ANOVAs were performed to compare the individual time samples against the baseline samples in each group:

In the **NAc core** (Fig. 3A), there was a significant overall increase of DA ($F(13,78) = 2.206$, $p = 0.017$) in the **Ext group**. Post hoc tests confirmed significantly increased DA at 10 min (during the reward period) and at 30, 40, 60 and 70 min (the extinction period) (all $p < 0.05$).

In the **nExt group** there was also a significant increase of DA ($F(13,156) = 2.685$, $p = 0.002$). Post-hoc tests confirmed significant increases in DA at 10 and 20 min (during the reward period) and at 30 and 50 min (during the post-reward period).

In the **NAc shell** (Fig. 3B), ANOVA did not indicate any significant changes of DA in groups Ext ($F(13,78) = 1.418$, $p = 0.170$) and nExt ($F(13,156) = 0.932$, $p = 0.521$).

In summary, (a) During the reward period, DA increased significantly in the NAc core, but not shell subregion; (b) During the post-reward period, DA remained significantly increased in both, the group that underwent extinction and in the group that did not undergo extinction to the signal CS. (c) During extinction, the DA levels did not differ significantly between the groups that underwent extinction (with the CS light presented during non-reward) and the groups that did not (CS not presented), providing no evidence for a decrease in DA during immediate extinction in either NAc core or shell regions.

3.3. B. Effects of delayed (1 h delay) extinction on DA levels in NAc core and shell regions

For this purpose we split the groups that did not undergo initial extinction during the 30–80 min period into one that now underwent delayed extinction for 30 min by presenting the CS cue without reward (delExt) and a non-extinction group that continued to be tested without the CS presented (nExt). NAc core: nExt, $n = 7$; delExt, $n = 7$. NAc shell: nExt, $n = 7$; delExt, $n = 6$) (Fig. 3A and B, 80–110 min).

Two-way repeated measures ANOVA performed on the groups delExt and nExt for samples taken from 80 to 110 min revealed only a significant *time* × *group interaction* in the NAc core ($F(3,36) = 3.042$, $p = 0.040$): (There were no significant effects of *time* (Core: $F(3,36) = 0.348$, $p = 0.791$; Shell: $F(3,30) = 0.899$, $p = 0.453$), of *group* (Core: $F(1,12) = 2.609$, $p = 0.132$; Shell: $F(1,10) = 0.843$, $p = 0.380$) nor *time* × *group interaction* in the NAc shell ($F(3,30) = 1.140$, $p = 0.349$)).

One-way repeated measures ANOVAs, were performed to compare the 90–110 min samples with the last sample before the treatment (80 min) in each group: In the **NAc core** (Fig. 3A) there was a significant effect of *group* ($F(3,18) = 3.336$, $p = 0.043$) in the delExt group, confirmed by a significant decrease in DA at 100 min (post-hoc test, $p < 0.05$). There was no effect of *group* in the nExt group ($F(3,18) = 0.919$, $p = 0.452$).

In the **NAc shell** (Fig. 3B) there were no significant effects for *group* in either the delExt ($F(3,12) = 2.690$, $p = 0.093$) or nExt groups ($F(3,18) = 0.199$, $p = 0.896$).

Thus, dopamine significantly decreased during extinction in the NAc core when there was a 1 h delay between the periods of signaled reward and presentation of the signal in absence of reward (extinction).

3.4. DOPAC

3.4.1. Reward and immediate extinction

Two-way ANOVAs for repeated measures were performed on the DOPAC levels from −50 to 80 min (including baseline, reward and extinction periods) for the NAc core and NAc shell groups: The ANOVAs yielded significant effects of *time* in the NAc core ($F(13,247) = 2.154$, $p = 0.012$) and in the NAc shell ($F(13,234) = 4.705$, $p = 0.0001$). (There was no effect of *group* in either area (Core: $F(1,19) = 2.984$, $p = 0.100$; Shell: $F(1,18) = 0.102$, $p = 0.753$). There was a significant *time* × *group interaction* in the NAc core ($F(13,247) = 2.099$, $p = 0.015$), but not the NAc shell ($F(13,234) = 0.730$, $p = 0.733$).

One-way repeated measures ANOVAs were performed to compare the individual time samples against the baseline samples in each group:

In the **NAc core** (Fig. 3C), there was a significant overall increase of DOPAC ($F(13,169) = 4.308$, $p = 0.0001$) in the **nExt group**. Post hoc tests confirmed significantly increased DOPAC at 10 and 20 min (during the reward period) and at 30, 40, 50, 60, 70 and 80 min (the post-reward period) (all $p < 0.05$). In the **Ext group** there was no significant increase of DOPAC ($F(13,78) = 1.236$, $p = 0.271$).

In the **NAc shell** (Fig. 3D), there was a significant overall increase of DOPAC ($F(13,78) = 2.482$, $p = 0.007$) in the **Ext group**. Post hoc tests confirmed significantly increased DOPAC at 10 and 20 min (during the reward period) and at 30, 40 and 50 min (the extinction period) (all $p < 0.05$).

In the **nExt group** there was also a significant increase of DOPAC ($F(13,156) = 3.958$, $p = 0.001$). Post-hoc tests confirmed significant increases in DOPAC at 20 min (during the reward period) and at 30, 40, 50, 60 and 80 min (during the non-extinction period).

3.4.2. Delayed extinction

Two-way repeated measures ANOVA for the groups delExt and nExt for samples taken from 80 to 110 min revealed no significant effects of *time* (Core: $F(3,36) = 0.587$, $p = 0.627$; Shell: $F(3,30) = 0.686$, $p = 0.568$), of *group* (Core: $F(1,12) = 0.011$, $p = 0.919$; Shell: $F(1,10) = 2.799$, $p = 0.125$) nor a *time* × *group interaction* (Core: $F(3,36) = 0.170$, $p = 0.916$; Shell: $F(3,30) = 0.421$, $p = 0.739$).

One-way repeated measures ANOVAs, were performed to compare the 90–110 min samples with the last sample before the treatment (80 min) in each group: In the **NAc core** (Fig. 3C) there was no significant change in the delExt group ($F(3,18) = 0.144$, $p = 0.932$), nor in the nExt group ($F(3,18) = 0.702$, $p = 0.563$). Also in the **NAc shell** (Fig. 3D) there were no significant effects in either the delExt ($F(3,12) = 0.138$, $p = 0.935$) or nExt groups ($F(3,18) = 0.948$, $p = 0.438$).

3.5. HVA

3.5.1. Reward and immediate extinction

Two-way ANOVAs for repeated measures were performed on the HVA levels from −50 to 80 min (including baseline, reward and extinction periods) for the NAc core and NAc shell groups. The ANOVAs yielded significant effects of *time* in the NAc core ($F(13,247) = 7.734$, $p = 0.0001$) and in the NAc shell ($F(13,221) = 4.210$, $p = 0.0001$). (There was no effect of *group* in either area (Core: $F(1,19) = 4.155$, $p = 0.056$ (borderline); Shell: $F(1,17) = 0.467$, $p = 0.503$). There was a significant *time* × *group interaction* in the NAc core ($F(13,247) = 2.142$, $p = 0.013$), but not the NAc shell ($F(13,221) = 0.693$, $p = 0.769$).

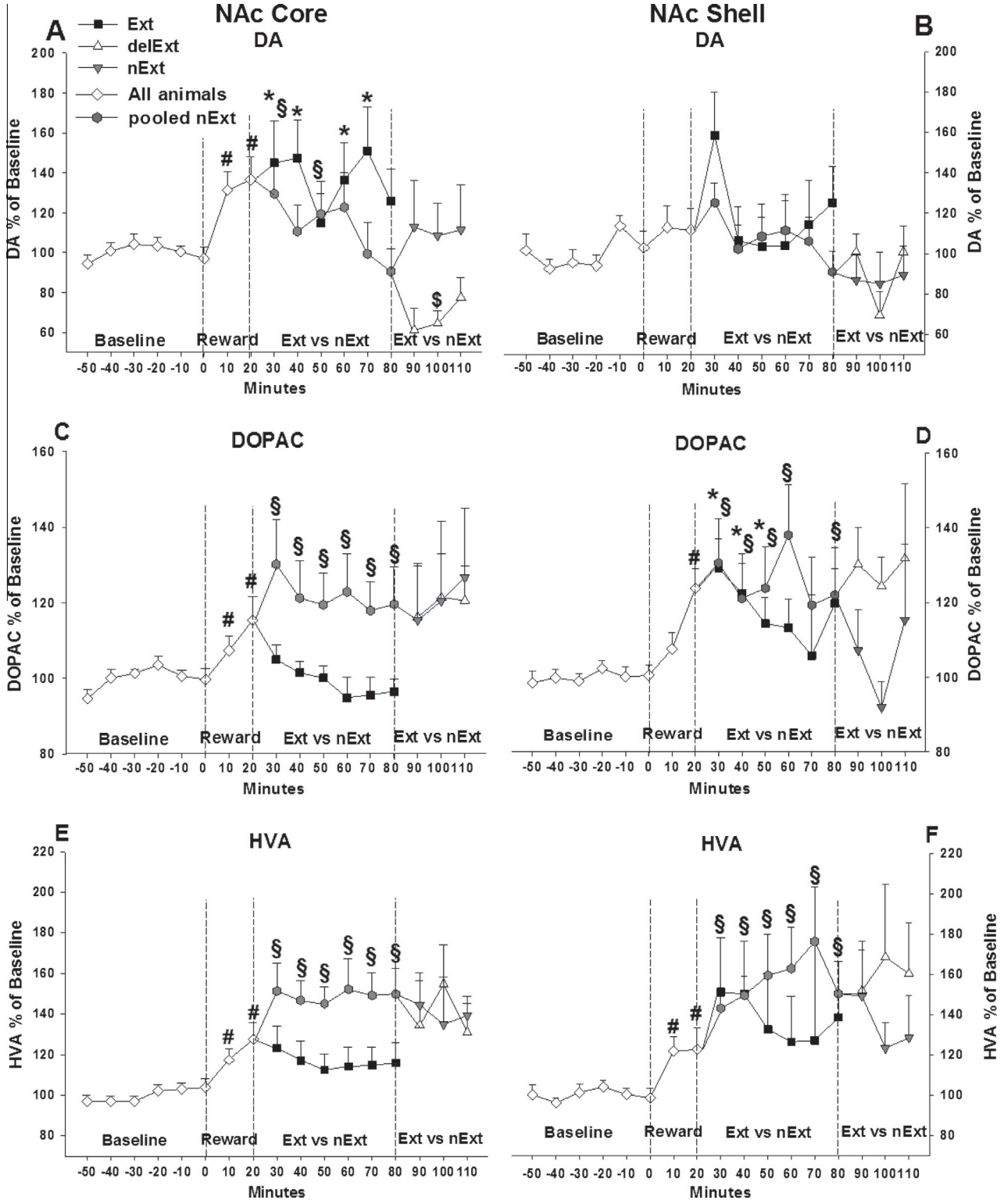

Fig. 3. Levels of DA, DOPAC and HVA in the NAc core and shell expressed as mean (+SEM) per cent of baseline, with the mean of six baseline taken as 100%. Sample size: NAc core: Ext, $n = 7$; delExt, $n = 7$; nExt, $n = 7$; NAc shell: Ext, $n = 7$; delExt, $n = 6$; nExt, $n = 7$; $^{*}p < 0.05$, comparing with the Ext baseline; $^{\S}p < 0.05$, comparing with the pooled nExt baseline; $^{\#}p < 0.05$, comparing with the pooled group "all animals" baseline; $^{\$}p < 0.05$, comparing with the delExt eighth sample. Groups were pooled in the figure whenever submitted to the same procedure.

One-way repeated measures ANOVAs were performed to compare the individual time samples against the baseline samples in each group:

In the **NAc core** (Fig. 3E), there was a significant overall increase of HVA ($F(13,169) = 10.617$, $p = 0.0001$) in the **nExt group**. Post hoc tests confirmed significantly increased HVA at 10 and 20 min (during the reward period) and at 30, 40, 50, 60, 70 and 80 min (the non-extinction period) (all $p < 0.05$). In the **Ext group** there was no significant increase of HVA ($F(13,78) = 1.742$, $p = 0.068$) (borderline).In the **NAc shell** (Fig. 3F), there was a significant

overall increase of HVA ($F(13,156) = 5.100$, $p = 0.001$) in the **nExt group**. Post hoc tests confirmed significantly increased HVA at 20 min (during the reward period) and at 30, 40, 50, 60, 70 and 80 min (the post-reward period) (all $p < 0.05$).

In the **Ext group** there was no significant increase of HVA ($F(13,65) = 1.216$, $p = 0.289$).

3.5.2. Delayed extinction

Two-way repeated measures ANOVA for the groups delExt and nExt for samples taken from 80 to 110 min revealed no significant effects of *time* (Core: $F(3,36) = 0.654$, $p = 0.586$; Shell: $F(3,30) = 0.987$, $p = 0.412$), of *group* (Core: $F(1,12) = 0.001$, $p = 0.988$; Shell: $F(1,10) = 0.081$, $p = 0.752$) nor *time* × *group interaction* (Core: $F(3,36) = 0.762$, $p = 0.523$; Shell: $F(3,30) = 0.329$, $p = 0.804$).

One-way repeated measures ANOVAs, were performed to compare the 90–110 min samples with the last sample before the treatment (80 min) in each group: In the **NAc core** (Fig. 3E) there was no significant change in the delExt group ($F(3,18) = 1.589$, $p = 0.227$), nor in the nExt group ($F(3,18) = 0.303$, $p = 0.823$). Also in the **NAc shell** (Fig. 3F) there were no significant effects in either the delExt ($F(3,12) = 0.1044$, $p = 0.409$) or nExt groups ($F(3,18) = 0.835$, $p = 0.492$).

3.5.3. Metabolites summary

In the **NAc core**, both DOPAC and HVA levels increased during the reward period and remained elevated during the post-reward period only in the group that did not undergo extinction to the CS. In the **NAc shell** DOPAC increased during the reward period and remained elevated during the post-reward period in both, the group that underwent extinction to the CS and the group that did not undergo extinction. HVA also increased during the reward period and remained elevated during the post-reward period, but only in the group that did not undergo extinction to the CS.

3.6. Behaviors during microdialysis

The behavioral measures were analyzed during the reward condition (samples 10–20 min), the immediately subsequent extinction condition (samples 30–80 min) and the delayed extinction condition (samples 90–110 min) (Fig. 4):

Rearing: Analyses of the groups Ext and nExt (−50 to 80 min) using two-way repeated measures ANOVA showed that over *time*, the **duration** ($F(13,338) = 12.885$, $p = 0.0001$) and **frequency** ($F(13,351) = 16.005$, $p = 0.0001$) of **rearing** changed significantly, but there was no *time* × *group* interaction (duration: $F(13,338) = 0.915$, $p = 0.537$; frequency: $F(13,351) = 0.584$, $p = 0.867$) nor effect of *group* (duration: $F(1,26) = 1.979$, $p = 0.171$; frequency: $F(1,27) = 0.752$, $p = 0.393$).

One-way repeated measures ANOVA, performed to compare 10-min blocks of behavior against the baseline showed that in **group Ext** there was an increase of **duration of rearing** ($F(13,143) = 6.993$, $p = 0.0001$) at 10 and 20 min (reward period) and 30 and 40 min (extinction post-reward period) and **frequency of rearing** ($F(13,143) = 7.837$, $p = 0.0001$) at 10 and 20 min (reward period) and 30, 40 and 60 min (extinction post-reward period) (all $p < 0.05$). In the **group nExt** there was an increase of **duration** ($F(13,195) = 5.571$, $p = 0.0001$) at 10 and 20 min (reward period) and 30 and 40 min (post-reward period) ($p = 0.05$) and of **frequency of rearing** ($F(13,208) = 7.953$, $p = 0.0001$) at 10 and 20 (reward period) and 30 min (post-reward period) (all $p < 0.05$).

Two-way repeated measures ANOVA of the groups delExt and nExt from the time 80 to 110 min showed no effect of *time* (duration: $F(3,48) = 2.098$, $p = 0.113$; frequency: $F(3,48) = 2.132$, $p = 0.109$), of *group* (duration: $F(1,16) = 0.275$, $p = 0.607$; frequency: $F(1,16) = 0.361$, $p = 0.556$) nor *time* × *group interaction* (duration: $F(3,48) = 1.164$, $p = 0.333$; frequency: $F(3,48) = 0.809$, $p = 0.495$). One-way repeated measures ANOVAs performed to compare the 90–110 min with the last time before the treatment (80 min) showed no significant change in the **delExt** (duration: $F(3,21) = 0.489$, $p = 0.693$; frequency: $F(3,21) = 1.345$, $p = 0.287$) and in the **nExt** (duration: $F(3,27) = 2.162$, $p = 0.116$; frequency: $F(3,27) = 1.549$, $p = 0.225$).

Thus, the rearing measures increased during the reward period and continued to be higher during the post-reward period in both groups, but persisted longer in the extinguished group. Hence, frequency and duration of rearing are indices of extinction-induced behaviors.

Grooming: Analyses of the groups Ext and nExt (−50 to 80 min) using two-way repeated measures ANOVA showed that over the *time*, the **duration** ($F(13,351) = 4.808$, $p = 0.0001$) and **frequency** ($F(13,351) = 4.086$, $p = 0.0001$) of **grooming** changed, but there was no *time* × *group interaction* (duration: $F(13,351) = 1.494$, $p = 0.117$; frequency: $F(13,351) = 0.917$, $p = 0.535$) nor effect of *group* (duration: $F(1,27) = 1.433$, $p = 0.242$; frequency: $F(1,27) = 0.590$, $p = 0.449$).

One-way repeated measures ANOVA, performed to compare 10-min blocks of behavior against the baseline showed that in the **group Ext** there was an increase in the **duration of grooming** over baseline ($F(13,143) = 3.504$, $p = 0.0001$) at 10, 20 min (reward period) and 30, 40, 50, 60 and 70 min (extinction post-reward period) and of the **frequency of grooming** ($F(13,143) = 2.717$, $p = 0.002$) at 30, 40 and 50 min (extinction post-reward period) (all $p < 0.05$). In the **group nExt** there was an increase in **duration** ($F(13,208) = 2.303$, $p = 0.007$) at 20 (reward period) and 30 and 40 min (post-reward period), but no change in the **frequency of grooming** ($F(13,208) = 1.314$, $p = 0.207$).

Two-way repeated measures ANOVA of the groups delExt and nExt from the time 80 to 110 min showed no effects of *time* (duration: $F(3,48) = 2.374$, $p = 0.082$; frequency: $F(3,48) = 0.558$, $p = 0.646$), of *group* (duration: $F(1,16) = 1.465$, $p = 0.244$; frequency: $F(1,16) = 1.892$, $p = 0.188$) nor *time* × *group interaction* (duration: $F(3,48) = 1.035$, $p = 0.385$; frequency: $F(3,48) = 1.199$, $p = 0.320$). One-way repeated measures ANOVAs performed to compare the 90–110 min with the last time before the treatment (80 min) showed no significant change in the **delExt** (duration: $F(3,21) = 2.337$, $p = 0.103$; frequency: $F(3,21) = 1.124$, $p = 0.362$) and in the **nExt** (duration: $F(3,27) = 1.748$, $p = 0.181$; frequency: $F(3,27) = 0.849$, $p = 0.479$).

In summary, an increase in frequency of grooming during the immediate post-reward period only appeared in the group submitted to extinction. Hence, the frequency of grooming is an extinction-induced behavior.

Duration and frequency at the food cup: Analyses of the groups Ext and nExt (-50 to 80 min) using two-way repeated measures ANOVA showed that over *time*, the **duration** ($F(13,351) = 33.875$, $p = 0.0001$) and **frequency** ($F(13,35\ 1) = 52.5\ 37$, $p = 0.0001$) **at the food cup** changed, but there were no *time* × *group interaction* (duration: $F(13,351) = 0.671$, $p = 0.7\ 91$; frequency: $F(13,351) = 0.310$, $p = 0.990$) nor effect of *group* (duration: $F(1,27) = 0.110$, $p = 0.742$; frequency: $F(1,27) = 0.377$, $p = 0.544$).

One-way repeated measures ANOVAs showed that in the Ext group the **duration at the food cup** increased over baseline ($F(13,143) = 17.019$, $p = 0.0001$) at 10, 20 min (reward period) and 30 and 40 min (post-reward period) and the **frequency at the food cup** increased ($F(13,143) = 22.835$, $p = 0.0001$) at 10 and 20 (reward period) and 30, 40, 50 and 60 min (post-reward period) (all $p < 0.05$). In the **group nExt** there was an increase in **duration** ($F(13,208) = 19.775$, $p = 0.0001$) and **frequency at the food cup** ($F(13,208) = 31.182$, $p = 0.0001$) at 10 and 20 min (reward period) and 30 and 40 min (post-reward period) (all $p < 0.05$).

Two-way repeated measures ANOVA of the groups delExt and nExt from the time 80 to 110 min showed no effect of *time*

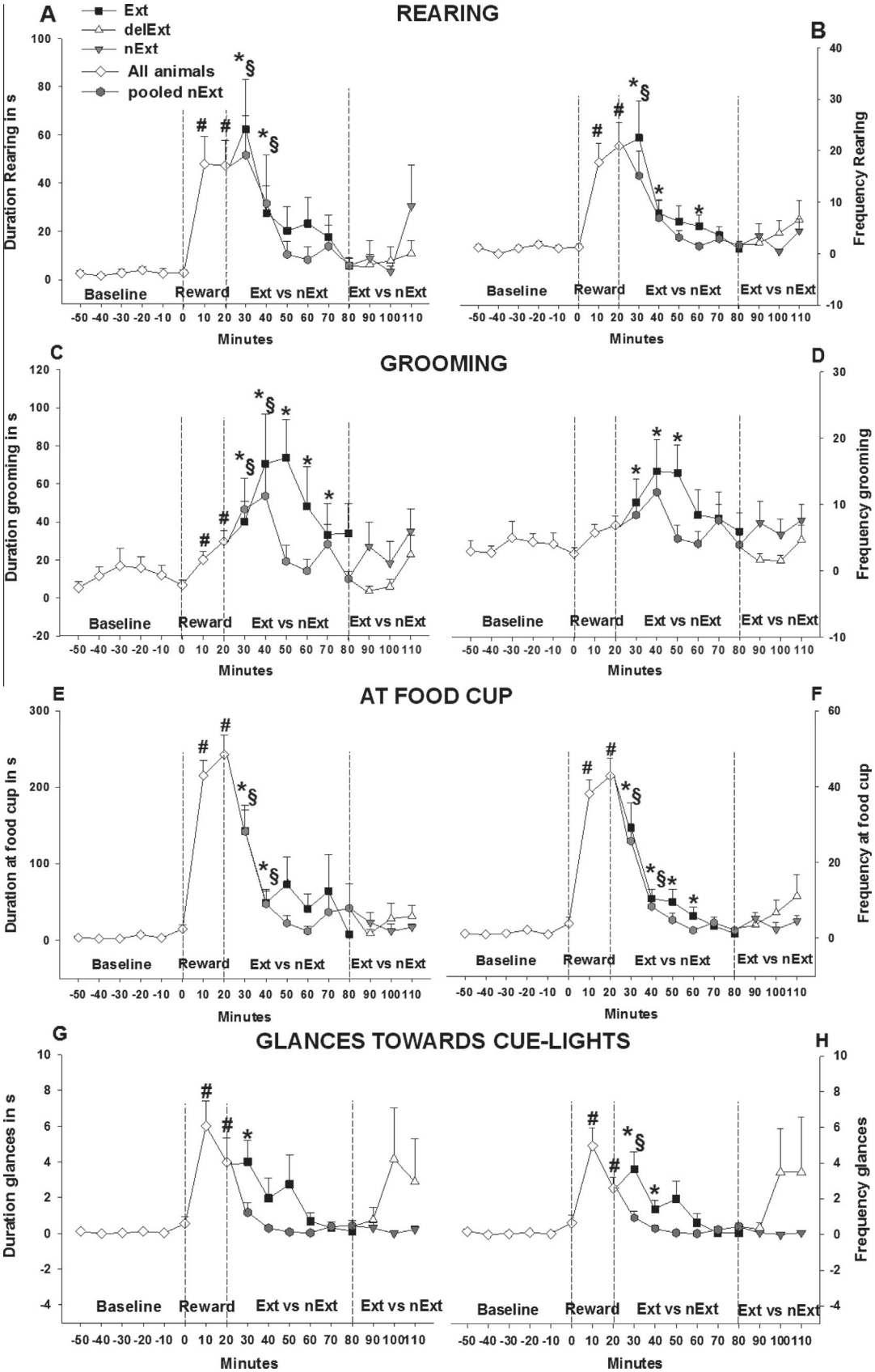

Fig. 4. Duration and frequency of rearing (A and B), grooming (C and D), permanence at the food cup (E and F) and glances toward the cue-lights (G and H). Data are mean (+SEM). Sample size: Ext, $n = 11–12$; delExt, $n = 8$; nExt, $n = 9–10$. $^{*}p < 0.05$, comparing with the Ext baseline; $^{\S}p < 0.05$, comparing with the pooled nExt baseline; $^{\#}p < 0.05$, comparing with the pooled group "all animals" baseline. Groups were pooled whenever submitted to the same procedure.

(duration: $F(3,48) = 0.545$, $p = 0.646$; frequency: $F(3,48) = 2.661$, $p = 0.059$), of *group* (duration: $F(1,16) = 1.058$, $p = 0.319$; frequency: $F(1,16) = 0.971$, $p = 0.339$) nor a *time × group interaction* (duration: $F(3,48) = 0.730$, $p = 0.539$; frequency: $F(3,48) = 1.754$, $p = 0.169$). One-way repeated measures ANOVAs performed to compare the 90–110 min with the last time before the treatment (80 min) showed no significant changes in the **delExt group** (duration: $F(3,21) = 0.530$, $p = 0.667$; frequency: $F(3,21) = 2.096$, $p = 0.131$) and **nExt group** (duration: $F(3,27) = 0.288$, $p = 0.834$; frequency: $F(3,27) = 1.324$, $p = 0.287$).

Thus, the duration and frequency at the food cup decreased over time during the immediate post-reward period in the extinction and non-extinction groups, but persisted much longer in the extinction group, showing the course of behavioral extinction.

Duration and frequency of glances toward the cue-lights (sign tracking): Analyses of the groups Ext and nExt (−50 to 80 min) using two-way repeated measures ANOVA showed an effect over *time* for the **duration** ($F(13,351) = 10.123$, $p = 0.0001$) and **frequency** ($F(13,351) = 15.239$, $p = 0.0001$) **of glances toward the cue-lights**, and also a *time × group interaction* for **frequency** ($F(13,351) = 1.796$, $p = 0.042$), but there was no *time × group interaction* for **duration** ($F(13,351) = 1.282$, $p = 0.222$), nor an effect of *group* (duration: $F(1,27) = 2.163$, $p = 0.153$; frequency: $F(1,27) = 2.682$, $p = 0.113$).

One-way repeated measures ANOVA showed that in the **group Ext** the **duration of glances toward the cue lights** increased ($F(13,143) = 4.578$, $p = 0.0001$) at 10 min (reward period) and 30 min (extinction post-reward period) and **frequency of glances** increased ($F(13,143) = 5.926$, $p = 0.0001$) at 10 min (reward period) and 30 and 40 min (extinction post-reward period) (all $p < 0.05$). And in the **group nExt** there was an increase of **duration** ($F(13,208) = 6.380$, $p = 0.0001$) at 10 and 20 min (reward period) ($p < 0.05$) and of **frequency of glances** ($F(13,208) = 12.167$, $p = 0.0001$) at 10 and 20 (reward period) and 30 min (post-reward period) (all $p < 0.05$).

Two-way repeated measures ANOVA of the groups delExt and nExt from the time 80 to 110 min showed no effect of *time* (duration: $F(3,48) = 1.392$, $p = 0.257$; frequency: $F(3,48) = 1.544$, $p = 0.215$), of *group* (duration: $F(1,16) = 1.906$, $p = 0.186$; frequency: $F(1,16) = 1.896$, $p = 0.188$) nor *time × group interaction* (duration: $F(3,48) = 2.271$, $p = 0.092$; frequency: $F(3,48) = 1.986$, $p = 0.129$). One-way repeated measures ANOVAs performed to compare the 90–110 min with the last time before the treatment (80 min) showed no significant change in the **delExt** (duration: $F(3,21) = 1.524$, $p = 0.238$; frequency: $F(3,21) = 1.396$, $p = 0.272$) and in the **nExt** (duration: $F(3,27) = 0.572$, $p = 0.638$; frequency: $F(3,27) = 1.588$, $p = 0.215$).

Thus, duration of glances at the cue lights extended into the post-reward session only in the extinction group, indicating this measure to be an index of resistance to extinction. Its time-dependent decline serves as a measure of behavioral extinction.

The behavioral effects can be summarized as follows: 1. During the **Period of signaled reward** there was an increase in rearing, grooming, time at food cup and glances at cue lights (sign tracking). 2. During the **Immediate post-reward period** (a) *glances at cue lights (sign tracking)* decreased over time in both, the extinction and non-extinction groups, but were maintained at a higher level in the extinction group in which the cue lights were presented, (b) *grooming* increased over time in the group submitted to extinction and, thus, is an extinction-induced behavior and (c) rearing decreased over time comparably in both, the extinction and non-extinction groups. 3. During the period of 90–110 min, grooming decreased (but not significantly) in the group submitted to delayed extinction. This group also glanced more often at the cue lights, which were introduced during this period to establish extinction to the CS.

3.7. Experiment 2: Dopamine in the NAc core vs shell during reward presentation and delayed extinction

The purpose of this experiment was (a) to confirm the finding of experiment 1 that DA levels decrease in the NAc core, but not NAc shell if there is a delay between the period of reward presentation and the onset of non-reinforcement (extinction) and (b) to confirm whether DA increases in the NAc during presentation of reward.

Pre-dialysis acquisition and reacquisition: Repeated-measure ANOVAs were performed on mean distance to the food cup over the course of the 5 acquisition and 2 reacquisition days. As subjects were not yet assigned to any treatment groups, only the general development over the trials was of interest. The mean distance to the food cup decreased significantly over days ($F(6,72) = 2.528$, $p = 0.028$), showing that the subjects had learned the contingency of the cue-light and the consecutive food reward. There were no significant differences between the groups that were later split into a reward and extinction groups.

3.7.1. DA and metabolites – NAc Core vs NAc shell

The number of animals per group considered for statistical analyses were: NAc core: extinction, $n = 8$; reward, $n = 8$; NAc shell: extinction, $n = 9$; reward, $n = 6$.

The location of the dialysis membranes in the NAc core and shell is shown in Fig. 2.

3.7.2. DOPAMINE

The mean basal levels of DA were 5.73 ± 0.60 pg in the NAc core and 6.79 ± 0.85 pg in the NAc shell. Basal values of DA were comparable between groups ($p > 0.05$). Two-way ANOVA for the **NAc core** showed a significant time × group interaction ($F(5,70) = 3.347$, $p = 0.009$) and an effect of group ($F(1,14) = 14.960$, $p = 0.002$), but no effect of time ($F(5,70) = 1.036$, $p = 0.403$). Thus, the DA levels were significantly elevated in the reward group as compared to the extinction group (Fig. 5A). For the **NAc shell** it did not show any main effects (time: $F(5,65) = 1.002$, $p = 0.424$, time × group interaction: $F(5,65) = 2.092$, $p = 0.078$ or group: $F(1,13) = 3.133$, $p = 0.100$) (Fig. 5B).

For the **NAc core**, **extinction group**, one-way repeated measures ANOVA, performed to compare the treatment samples against the baseline samples, showed a significant decrease of DA ($F(5,35) = 2.199$, $p = 0.038$, one tailed). Pre-planned post-hoc comparisons (Fisher's LSD-test vs baseline samples) confirmed significant decreases in DA at 10 and 20 min (all $p < 0.05$).

One-way repeated measures ANOVA on the **reward group** showed a significant increase of DA ($F(5,35) = 2.189$, $p = 0.039$). Post-hoc test confirmed a significant increase at 30 min ($p < 0.05$).

In summary, the absence of reward during presentation of the cue lights (extinction) led to a significant decrease of DA in the NAc core, but not in the NAc shell. During the period of cued reward delivery, DA levels increased significantly in the NAc core, but not in the NAc shell.

3.7.3. DOPAC

The mean basal levels of DOPAC were 1145.70 ± 46.08 pg in the NAc core and 952.60 ± 70.67 pg in the NAc shell. Basal values of DOPAC were comparable between groups ($p > 0.05$). Two-way ANOVA in **NAc core** yielded a main effect of group ($F(1,14) = 5.420$, $p = 0.035$), but no effects of time ($F(5,70) = 0.408$, $p = 0.841$) and time × group interaction ($F(5,70) = 1.383$, $p = 0.241$) (Fig. 5C). In the **NAc shell** there were effects of group ($F(1,13) = 6.024$, $p = 0.029$), time ($F(5,65) = 5.291$, $p = 0.0001$) and time × group interaction ($F(5,65) = 4.423$, $p = 0.002$) (Fig. 5D).

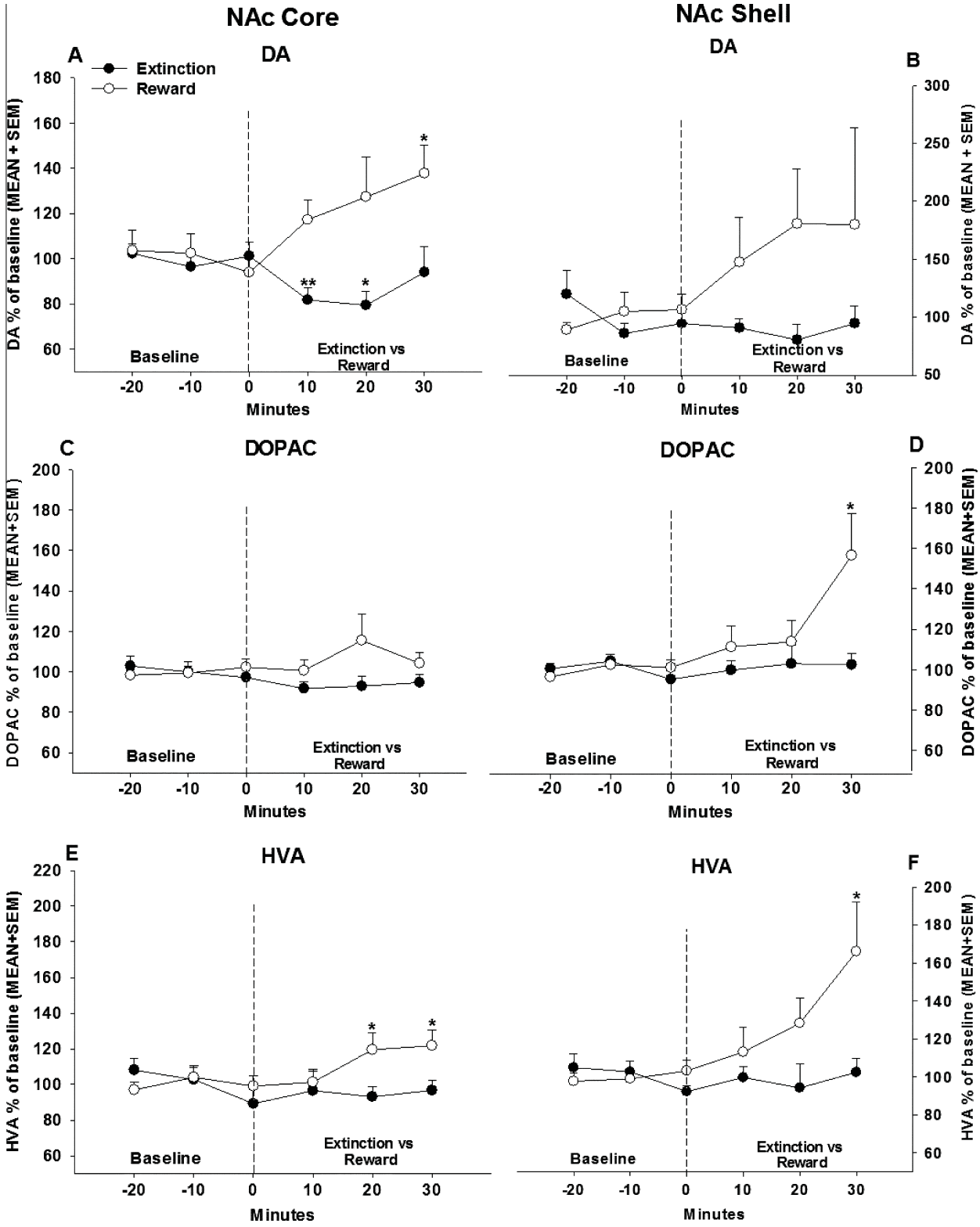

Fig. 5. Levels of DA, DOPAC and HVA in the NAc core and shell expressed as mean (+SEM) per cent of baseline, with the mean of three baseline taken as 100%. Sample size: NAc core: extinction, $n = 8$; reward, $n = 8$; NAc shell: extinction, $n = 9$; reward, $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.001$ comparing to baseline samples.

Thus, in both subregions DOPAC was significantly higher in the reward compared to the extinction group.

One-way repeated measures ANOVA comparing treatment with baseline showed no significant changes of DOPAC in the **NAc core** in the extinction group ($F(5,35) = 0.887$, $p = 0.5$) nor in the reward group ($F(5,35) = 0.90$, $p = 0.492$). It showed a significant increase ($F(5,25) = 4.337$, $p = 0.006$) of DOPAC in the **NAc shell** in the **reward group,** confirmed with post-hoc test at 30 min ($p < 0.05$), but no significant changes in the extinction group ($F(5,40) = 0.517$, $p = 0.762$).

In summary, DOPAC levels significantly increased only in the NAc shell, in the reward group relative to baseline.

3.7.4. HVA

The mean basal levels of HVA were 447.60 ± 35.02 pg in the NAc core and 206.09 ± 20.76 pg in the NAc shell. These did not differ between groups ($p > 0.05$). Two-way ANOVA yielded in the **NAc shell** an effect of time ($F(5,65) = 3.808$, $p = 0.004$), of group ($F(1,13) = 7.443$, $p = 0.017$) and a time × group interaction ($F(5,65) = 3.714$, $p = 0.005$) (Fig. 5F). In the **NAc core** there was a significant effect of group ($F(1,14) = 4.451$, $p = 0.0053$), but no effect of time ($F(5,70) = 0.935$, $p = 0.464$), nor a time × group interaction ($F(5,70) = 1.702$, $p = 0.146$) (Fig. 5E).

One-way repeated measures ANOVA comparing treatment with baseline showed that in the **NAc core** of the **reward group** HVA increased significantly ($F(5,35) = 2.496$, $p = 0.049$), as confirmed by post-hoc test at 20 and 30 min (all $p < 0.05$), but did not change in the **extinction group** ($F(5,35) = 0.600$, $p = 0.700$). In the **NAc shell** of the **reward group** HVA also increased significantly ($F(5,25) = 3.845$, $p = 0.010$) (confirmed by post-hoc test at 30 min ($p < 0.05$)), but not in the extinction group ($F(5,40) = 0.528$, $p = 0.754$).

In summary, the levels of HVA in both, NAc core and NAc shell, increased during cued reward delivery.

4. Discussion

In experiment 1 we found that during a 20-min period of free cued food delivery, DA increased significantly in the NAc core, but not shell subregion. This increase persisted into the subsequent 60-min post-reward period in both subgroups – a group that was subjected to extinction (no reward with CS cue lights presented) and also in a group that did not undergo extinction (CS not presented during non-reward). Thus, these data provide no evidence for a decrease in DA during extinction in either NAc core or shell regions. Instead, they indicate that the increase in DA outlasted the period of reward presentation. However, when there was a 1 h interval between the reward and the extinction periods, DA in the NAc core, but not shell, decreased during the 30-min extinction period, providing first evidence that DA in the NAc core decreases during extinction, but only when there is a (1 h) delay between reward and extinction.

Experiment 2 confirmed the finding of experiment 1, showing that during a 30-min period of cued free reward delivery, DA levels increased significantly in the NAc core but not in the NAc shell. More important, it also confirmed that when there was an interval between the reward and extinction (in this case 24 h), DA significantly decreased in the NAc core, but not in the NAc shell as in experiment 1.

These findings of an increase in DA release during signaled reward only in the NAc core, is in agreement with other microdialysis studies in the rat. Although it was shown that appetitive taste stimuli release DA in both the NAc shell and core subregions (Bassareo & Di Chiara, 1997; Hajnal et al., 2004), the responsiveness of DA in the NAc shell was shown to differ from that in the core, as it is dependent upon the hedonic valence (appetitive or aversive) (Bassareo et al., 2002) and relative novelty of the taste stimuli (Bassareo & Di Chiara, 1997, 1999a; Bassareo et al., 2002). Bassareo et al. (2002) showed that NAc shell DA release is stimulated by unfamiliar appetitive tastes and that NAc shell DA responsiveness habituates after a single exposure to palatable food in a taste-specific manner (Bassareo & Di Chiara, 1997, 1999b; Bassareo et al., 2002). On the other hand, in the NAc core taste stimuli were found to release DA independent of their positive or negative hedonic valence, and not to show single-trial habituation (Bassareo & Di Chiara, 1997; Bassareo et al., 2002). Bassareo et al. (2015) investigated the role of response-contingency and sucrose-related cues on DA transmission in NAc shell and core, showing that repeated feeding of sucrose induced habituation of NAc shell DA responsiveness, since sucrose feeding increased dialysate shell DA only on the first day of feeding but not on the following days. The habituation of DA responsiveness to feeding of palatable foods was also shown for salty and sweet food (Bassareo & Di Chiara, 1997, 1999b). Our finding of increased DA levels in the NAc core, but not in shell during cued reward delivery, is in accordance with these studies, since in our protocol the animals had undergone considerable acquisition training with the food pellets prior to the microdialysis sessions and, thus, can be considered to have habituated to their taste and novelty. Ambroggi, Ghazizadeh, Nicola, and Fields (2011) presented findings of high relevance for our results, namely a dissociation of core and shell contributions to discriminative stimulus task performance. Inactivation of the NAc core, but not shell, decreased responding to a reward-predictive cue and inactivation of the shell, but not core, significantly increased responding to the reward-signaling cue. In addition, a reward-predictive cue elicited more frequent and larger magnitude responses in the NAc core than in the shell and more NAc shell neurons selectively responded to a non-rewarded stimulus.

Differential functional roles of the core and shell subregions of the ventral striatum have been described in terms of value coding and prediction (expectation) monitoring. As mentioned above, the shell region may mediate the direct hedonic value of a stimulus. Uncued application of food-related stimuli evoked an increase in shell DA levels (Bassareo & Di Chiara, 1997, 1999a; Roitman, Wheeler, Wightman, & Carelli, 2008), while bitter tasting quinine solution transiently decreased DA in the shell (Roitman et al., 2008; Wheeler et al., 2011). Importantly, an increase in DA was not observed in the core region. The same pattern also applies to effects induced by uncued drug administration. Cocaine application significantly increased DA in the shell (Aragona et al., 2008). Different functional aspects have been attributed to the core portion. Transients in DA have been shown to reflect cue-associated expectations of outcome, i.e. prediction errors (Rescorla & Wagner, 1972; Schultz, 2010; Sutton & Barto, 1981). Differences in DA levels in the core are greatest if the difference between the expected and actual outcome is greatest (Bassareo & Di Chiara, 1997, 1999a,b; Bayer & Glimcher, 2005; Day, Roitman, Wightman, & Carelli, 2007; Fiorillo, Tobler, & Schultz, 2003; Morris, Arkadir, Nevet, Vaadia, & Bergman, 2004; Nakahara, Itoh, Kawagoe, Takikawa, & Hikosaka, 2004; Satoh, Nakai, Sato, & Kimura, 2003; Tobler, Fiorillo, & Schultz, 2005). In the current study, no differences were seen with respect to the shell region, as in both conditions (delExt vs nExt) rewards were not provided, i.e. no hedonic value coding could have occurred. However, differences in core DA were observed, reflecting differences in cue-associated predictions. The experienced and, in turn, anticipated loss of reward predicted by external stimuli has been described as a key component in EID (Huston et al., 2013) and the current data provide evidence that modulation of core, and not shell, DA levels mediates this mechanism. In line with this, findings from human neuroimaging demonstrated that the receipt of reward enhances, whereas reward omission reduces activity in the NAc, amygdala, and prefrontal cortex, (Breiter, Aharon, Kahneman, Dale, & Shizgal, 2001; Delgado, Nystrom, Fissell, Noll, & Fiez, 2000; Knutson, Fong, Adams, Varner, & Hommer, 2001; O'Doherty et al., 2001). The NAc exhibits greater activation to rewarded, relative to non-rewarded trials and appears to be sensitive to violations in expected reward outcomes (Spicer et al., 2007). These results are in concert with our finding of reduced DA release during the withholding of food reward in the NAc core. Concerning the differential roles of D1- and D2-like DA receptors in this context, it has been shown that pharmacologic antagonism of D1-like DA receptors blocks cue-induced reinstatement of operant

behavior for food rewards that underwent extinction (Ball, Combs, & Beyer, 2011).

DA decreases only during delayed extinction. There are various possible explanations for the finding that the delayed, but not immediate withholding of reward resulted in the decrease in NAc core DA: It is possible that immediately after reward presentation the prevailing conditioned "incentive" stimuli (in the case of the non-extinction group, the conditioned "context" stimuli of the chamber; in the case of the extinction group, the conditioned light CS plus the conditioned context CSs) maintained increased dopaminergic stimulation in the NAc, whereas after a delay between reward and extinction, this persisting dopaminergic activity has dissipated and can no longer override or mask the decrease in DA that marks extinction of responses to a signaling CS or "discriminative stimulus". Such an explanation would presume that the increase in DA release that accompanies reward availability, outlasts the period of reward either in a time dependent manner related to post-consummatory action of the reward or in dependency of the ability of conditioned "incentive" cues to still initiate appetitive behavior (i.e. as long as extinction to the CS is not complete). In either case, it would be important to distinguish between immediate and delayed extinction in a theoretical and empirical account of the processes involved in the withdrawal of expected reward. We know of no relevant literature that directly deals with such a distinction. However, Francois et al. (2014) found that in the NAc (subregion not specified) brain tissue oxygen levels increased during presentation of a rewarded cue, both during acquisition and the first days of extinction, indicating that the NAc remains activated for as long as a "rewarded" conditioned CS triggered appetitive behavior during early extinction trials in accordance with our current results.

Behavioral analyses of experiment 1 showed that the frequency and duration of rearing increased during the reward period and continued to be higher during the post-reward period in both groups, but persisted longer in the group subjected to extinction. In addition, an increase in frequency of grooming during the immediate post-reward period only appeared in the group submitted to extinction. Both, extinction-induced grooming and rearing behaviors have been shown to be influenced by treatment with antidepressant drugs (Huston et al., 2013; Topic, Kröger, Vildirasova, & Huston, 2012). Others studies also found grooming to be influenced by antidepressant treatment (D'Aquila et al., 2000a; Yalcin, Belzung, & Surget, 2008). Rearing behavior has associated with motivational state, attention and arousal level and emotionality (Carnevale, Vitullo, & Sadile, 1990; Sadile, 1995). Grooming has been interpreted as a displacement activity that functioning to reduce stress (Spruijt, van Hooff, & Gispen, 1992) and, thus, is a behavioral marker of stress in rodents (e.g. File, Mabbutt, & Walker, 1988; Kalueff & Tuohimaa, 2005; Kyzar et al., 2011; Nakazato, 2013). Generally, grooming behavior has been consistently associated with changes in DA tone, e.g. as a results of DA agonism (Schwarting & Huston, 1996). The mesolimbic dopamine system has been shown to be involved in grooming (Prinssen, Balestra, Bemelmans, & Cools, 1994) and whereas dopamine D1/D5 receptor agonists elicit an grooming (Watchel, Brooderson, & White, 1992), dopamine D2 receptor agonists reduce this behavior (Ferrari, Pelloni, & Giuliani, 1992). However, in potentially stressful environments (novelty, handling, injections of saline, restraints, situations paired explicitly with aversive events), it is thought to reflect an unspecific stress response and, moreover, can provide an index of fear- related behavior (Gispen, 1982; Tinbergen, 1952). Violations of outcome predictions activate the HPA axis and, thus, can evoke stress-related behaviors. Our data support such an assumption, as this stress response was only seen in the group that immediately underwent extinction.

We also found that the duration and frequency at the food cup decreased over time during the immediate post-reward period in the extinction and non-extinction groups, but persisted much longer in the extinction group, marking the course of behavioral extinction. Moreover, the duration of glances at the cue lights (sign tracking) extended into the post-reward session only in the extinction group, indicating this measure to be an index of resistance to extinction to the cue CS (and not to the context compound CS). Its time-dependent decline serves as a measure of behavioral extinction. Thus, behavioral analysis indicated that while grooming and rearing are indices of extinction-induced behavior, glances toward the cue-lights are an index of resistance to extinction.

Extinction, dopamine and depression: Various studies have indicated the involvement of the NAc in cognition and response to aversive and rewarding stimulus in animal models of depression and the NAc is thought to contribute importantly to the pathophysiology, symptomatology and etiology of depression (Nestler & Carlezon, 2006). Imaging studies indicate that depressed patients compared to healthy control subjects exhibit less activation of the NAc in reward-related performance (Pizzagalli et al., 2009). Similarly, depressed patients showed lower activity in the ventral striatum when reappraising rewards in a reversal-learning task, but not when reappraising punishments (Robinson, Cools, Carlisi, Sahakian, & Drevets, 2011). Depressed patients were also found to have difficulty in maintaining positive mood after a reward, in association with an inability to sustain activity in the accumbens over time (Heller et al., 2009), suggesting that decreased activity within the dorsal PFC may reduce reward sensitivity of the NAc (Disner, Beevers, Haigh, & Beck, 2011). Various substances which increase dopaminergic transmission have also been shown to have antidepressant properties in animal studies, as well as to increase availability of DA in the NAc (Willner, 1997). In addition to the availability of DA as a neurotransmitter, also changes in the sensitivity and number of dopamine receptors in the NAc have been shown to have a role in the genesis of depression (D'Aquila et al., 2000b). Previous studies on rats, using either operant extinction paradigms as applied in the present work or extinction of escape behavior from a water maze, have demonstrated that the withholding of expected food reward or expected escape reward leads to behavioral and physiological changes that can be interpreted as being expressions of depression/despair (for reviews see Huston et al. (2009, 2013). Operant Extinction-induced depression-related behaviors have been described in three different paradigms, one employing food-reinforced lever pressing (Huston et al., 2012), the other using a cued fixed-time food-delivery schedule as used in the present study (Komorowski et al., 2012), and one using extinction of run-way behavior (Topic et al., 2012). The extinction-induced behaviors reported include an increase in distance from the former source of reinforcement, rearing, grooming and biting behavior, all of which were attenuated by treatment with antidepressant drugs. In addition to evoking so-called extinction-induced depression-related behaviors, the withholding of reward or decreasing its magnitude have been shown to be accompanied by behavioral indices of anxiety, which is often co-morbid with major depression in humans (for review see Papini et al., 2015). The present experiments were carried out to assess the hypothesis that operant extinction is accompanied by a decrease in release of DA in the NAc; i.e., that during the withholding reward, DA would behave in a direction opposite to its response to presentation of reward. The results provide evidence for such an action of DA, which decreased selectively in the NAc core, but only when sufficient time had elapsed between availability of rewarding stimulation and its absence. Such a delay in the decrease in DA efflux as a marker of despair/depression makes sense in terms of our hypothesis insofar as it would be expected that a state of despair would only set in when

conditioned incentive stimuli have lost their ability to generate goal-driven operant behavior or when hedonic consequences of reward have totally dissipated. It remains to be seen whether antidepressants that block the reuptake of neuronal DA can reverse the decrease in extinction-induced DA efflux along with the behavioral markers of EID. We have shown that intranasally administered dopamine has antidepressant-like properties in both, the forced swimming task (Buddenberg, Topic, de Souza Silva, Huston, & Mattern, 2008) as well as in the operant extinction-induced depression paradigm (unpublished results), suggesting that the subtype of depression that is a consequence of reward withdrawal can be attenuated by dopamine substitution.

5. Conclusions

This work confirms the importance of dopamine release in the nucleus accumbens for reward-related states. It provides first evidence that during the withholding of expected reward, DA release decreases in the NAc core, but not shell region, and shows that this decrease in DA appears only after a delay between delivery of reward and extinction. This decrease in DA release may reflect response of the DA neurons to outcome prediction error (Schultz, 2010). We hypothesize that the decrease of DA in the NAc core may also be a marker for the despair/depression that is known to accompany the loss of expected rewards/reinforcers (Huston et al., 2013). The directionality of cause and effect in such a relationship remains obscure. Therefore, it would be important to know whether experimentally induced decreases in NAc core DA release result in "emotional" behaviors that are known to accompany reward withdrawal (extinction) or whether the change in DA release is a consequence of the emotional responses to extinction.

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9 Declaration

Die hier vorgelegte Dissertation habe ich selbständig und nur unter Verwendung der angegebenen Literaturquellen angefertigt. Diese Arbeit wurde in der vorgelegten oder ähnlichen Form bei keiner anderen Institution eingereicht. Zudem erkläre Ich, dass Ich bisher keine erfolglosen Promotionsversuche unternommen habe.

Dusseldorf, den 22.03.2018.

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