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Investigations on Alternative Treatment Approaches in the Unilateral 6-OHDA Lesion Rat Model of Parkinson's Disease

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III. Summary

Parkinson's disease is a severe neurodegenerative disorder. Current pharmacological treatment by dopamine replenishment offers benefits but causes serious side-effects after long-term use. In the present series of studies the 6-hydroxydopamine (6-OHDA) lesion-model of Parkinson's disease was used to investigate alternative treatment approaches, i.e., intranasal (IN) treatment with L-3,4-dihydroxyphenylalanine (L-dopa) and chronic treatment with progesterone. Furthermore, we investigated the features of a test of sensorimotor performance that had not been used in the study of 6-OHDA-lesioned rats before, and extended our investigations to the neuronal mediators of cognitive symptoms seen in Parkinson's disease. In experiment 1, intranasal administration of L-dopa was applied in an animal model of late stage Parkinson's disease and its behavioral effects were assessed. Intranasal L-dopa alleviated sensorimotor deficits in the hemiparkinsonian rats compared to the vehicle-treated group. In experiment 2, chronic progesterone administration was conducted after the injections of 6-OHDA into the striatum to test possible neurorestorative effects. However, the results indicated that under the given experimental conditions progesterone had deleterious effects on turning behavior and the use of limbs. In experiment 3, animals with severe and moderate dopaminergic lesions were used to validate a new test of motor performance on an elevated grid. In severely lesioned animals, L-dopa had beneficial effects, while the moderately lesioned rats showed deficits of sensorimotor performance on the side contralateral to the lesion. The results suggested that the grid test is a sensitive and

useful assessment for hemiparkinsonian rats. In experiment 4, the interaction between the medial prefrontal cortex and the nigrostriatal dopamine-system was studied by using a disconnection procedure. The unilateral 6-OHDA lesion was combined with a contralateral medial prefrontal cortex lesion. It was found that, following such disconnections between the nigrostriatal dopamine-system and the medial prefrontal cortex, rats showed impaired object recognition memory. Thus, the interaction between these two brain regions could play a key role in learning and memory.

IV. Zusammenfassung

Morbus Parkinson ist eine schwere neurodegenerative Erkrankung. Die pharmakologische Behandlung mit Dopamin-Ersatzstoffen bietet Vorteile, verursacht aber bei chronischer Behandlung schwere Nebenwirkungen. In der vorliegenden Reihe von Untersuchungen wurde das 6-OHDA-Läsionsmodell der Parkinson-Krankheit verwendet, um alternative Behandlungsansatze, wie die intranasale Behandlung mit L-Dopa oder die chronische Behandlung mit Progesteron untersucht. Weiterhin wurden die Eigenschaften eines Tests von sensomotorischen Leistungen untersucht, die zuvor nicht bei der Untersuchung von 6-OHDA-lädierten Ratten benutzt wurde, und es wurden die neuronalen Mediatoren kognitiver Symptome der Parkinson-Krankheit untersucht. In Experiment 1 wurden die Verhaltemseffekte einer intranasalen Verabreichung von L-Dopa in einem Tiermodell für die späte Phase der Parkinson-Krankheit getestet. Intranasale L-Dopa reduzierte die sensomotorischen Defizite in den Hemiparkinson-Ratten im Vergleich zur vehikel-behandelten Gruppe. In Experiment 2 wurde, nach den Injektionen von 6-OHDA in das Striatum von Ratten, mögliche neurorestorative Effekte einer chronischen Progesteron-Behandlung durchgeführt. Jedoch zeigten die Ergebnisse, dass Progesteron unter den gegebenen Versuchsbedingungen das läsinons-induzierte Drehverhalten und die Beeinträchtigung beim Gebrauch der Gliedmaßen verstärkte. In Experiment 3 wurden Tiere mit schweren und moderate dopaminerge Läsionen verwendet, um einen neuen Test der motorischen Leistungsfähigkeit auf einem erhöhten Gitter (Grid-Test) zu validieren. In stark lädierten Tieren hatte L-

Dopa positive Effekte, während die moderat lädierten Ratten Defizite der sensomotorischen Leistungen auf der Seite kontralateral zur Läsion zeigten. Die Ergebnisse legen nahe, dass der Grid-Test ein sensitives und nützliches Verfahren zur Testung Hemiparkinson-Ratten ist. In Experiment 4 wurde die Interaktion zwischen dem medialen präfrontalen Kortex und dem nigrostriatalen Dopamin-System durch ein Diskonnektions-Verfahren untersucht. Die einseitige 6-OHDA Läsion wurde mit einem kontralateralen Läsion des medialen präfrontalen Kortex kombiniert. Es wurde festgestellt, dass nach einer solchen Trennung zwischen dem nigrostriatalen Dopamin-System und dem medialen präfrontalen Kortex, Ratten ein beeinträchtes Objektwiedererkennungs-Gedächtnis zeigten. Daher könnte die Wechselwirkung zwischen diesen beiden Hirnregionen eine Schlüsselrolle bei der Lern-und Gedächtnisleistung spielen.

V. Abbreviations

MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
DOPAC	3,4-dihydroxyphenylacetic acid
5-HIAA	5-hydroxyindoleacetic acid
6-OHDA	6-hydroxydopamine
AADC	aromatic L-amino acid decarboxylase
BBB	blood-brain-barrier
CNS	central nervous system
DBS	deep brain stimulation
DA	dopamine
EC	electrochemical detection
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
IN	intranasal
i.p.	intraperitoneal injection
L-dopa	L-3,4-dihydroxyphenylalanine
LID	levodopa-induced dyskinesia
MFB	medial forebrain bundle
mPFC	medial prefrontal cortex
min	minutes
NMDA	*N*-methyl-D-aspartate
NE	norepinephrine

PD	Parkinson's disease
PBS	phosphate buffered saline
PFC	prefrontal cortex
P4	progesterone
5-HT	serotonin
SNc	substantia nigra pars compacta
STN	subthalamic nucleus

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder and is still an incurable progressive disease. In Germany, the prevalence of PD is around 7 per 1,000 when the age is over 55 years (von Campenhausen et al., 2005). Patients with PD suffer from postural imbalance, gait disorder and bradykinesia, which mainly result from the atrophy of dopamine (DA)-ergic neurons in the substantia nigra pars compacta (SNc) (Dauer and Przedborski, 2003; Obeso et al., 2010). Currently, the most effective treatment is still the therapy of DA replenishment by administration of L-3,4-dihydroxyphenylalanine (L-dopa) or other DA agonists. However, PD is not only a motor disorder but also related to many non-motor problems. Sleep abnormalities, constipation, depression and dementia are common in this disease (Obeso et al., 2010). Therapy by DA replenishment cannot alleviate the non-motor deficits and chronic administration of L-dopa induces serious adverse-effects in PD patients. Thus, studies investigating alternative treatments are required.

In order to investigate alternative treatments for PD, it is necessary to have adequate and sensitive behavioral tests and to understand the neuronal circuits involved in the disease. The present series of studies set out to approach these issues.

1.1. Parkinson's disease

PD is a neurodegenerative disease characterized by the manifestation of motor deficits, such as tremors at rest, rigidity in muscles, akinesia and postural instability. James Parkinson was first to describe the clinical features of this disease as "shaking palsy" in his classic 1817 monograph (Parkinson, 2002):

> "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured."

At present, PD is the second most common neurodegenerative disease after Alzheimer's disease and is also strongly correlated with age (Collier et al., 2011). In general, the prevalence increases from 0.5 to 1 percent at age 65 to 69, to 1 to 3 percent at age 80 and older (Nussbaum and Ellis, 2003). The atrophy of DAergic neurons in the SNc and the presence of intraneuronal proteinacious cytoplasmic inclusions, termed "Lewy bodies", are the pathological hallmarks of PD. The deficiency of DA in the nigrostriatal pathway, the DAergic projections from the SNc to the putamen, accounts for the major motor deficits in PD (Dauer and Przedborski, 2003). Thus, replenishment of striatal DA by treatment with a DA precursor or agonist alleviates most of the motor deficits. The administration of the DA precursor L-dopa, in combination with a peripheral dopa decarboxylase inhibitor (benserazide or carbidopa), is still the "gold-standard" therapy in PD (Lang and Lozano, 1998a;

Brooks, 2008). However, the chronic administration of L-dopa causes adverse fluctuations in motor response which is known as levodopa-induced dyskinesia (LID). LID was observed in 20-30% of PD patients for a mean of 20.5 months of receiving DA replenishment (Bezard et al., 2001). Furthermore, up to 80% of patients developed dyskinesia within five years of treatment (Quinn, 1995; Rascol et al., 2000). Both the frequency and the severity of dyskinesia are increased with duration of treatment (Bezard et al., 2001). In addition, L-dopa treatment cannot alleviate non-motor deficits in PD and prevent the progression of the disease. Other therapeutic alternatives, like the subthalamic nucleus -deep brain stimulation (STN-DBS) or infusion of neurotrophic factors, are developing options for treating PD.

In addition to the motor deficits, PD patients also show emotional (Eskow Jaunarajs et al., 2011) and cognitive (Dubois and Pillon, 1997; Kehagia et al., 2010) deficits. Depression has been found in approximately 40% of PD patients (Cummings, 1992), while anxiety disorders occur in approximately 25-35% of this population (Dissanayaka et al., 2010; Goetz, 2010). Furthermore, executive, memory and visuo-spatial functions are commonly affected by PD (Dubois and Pillon, 1997) and the performance of patients with PD in cognitive tasks resembles that seen in patients with prefrontal cortex (PFC) lesions (Taylor et al., 1986; Owen et al., 1992; Kehagia et al., 2010). PD patients also show deficits of temporal processing (Sagar et al., 1988) and recognition memory (Cooper et al., 1993; Stebbins et al., 1999; Whittington et al., 2000). Neuroimaging studies in PD patients have found that the nigrostriatal (Owen et al., 1998; Dagher et al., 2001; Sawamoto et al., 2008) and the mesocortical (Cools et

al., 2002; Mattay et al., 2002) pathways contribute to the cognitive impairments in PD. More specifically, around 31% of PD patients may suffer from dementia (Aarsland et al., 2005), which is a 4- to 6-fold increased risk compared to an age-matched population (Aarsland et al., 2010). These cognitive and emotional impairments may not be solely attributed to dysfunctions of the DAergic systems. As serotonin (5-HT)-ergic, norepinephrine (NE)-ergic and cholinergic neurotransmitter systems are also degenerated in PD (Agid et al., 1987), these factors may play a role in emotionality and cognition. Given that emotional and cognitive deficits in PD have a strong impact on quality of life (Schrag et al., 2000; Den Oudsten et al., 2007; Soh et al., 2011), understanding their mechanisms and the development of selective treatments are highly required.

1.2. Unilateral 6-OHDA lesioned rat model

One of the most common animal models of PD is unilaterally applying 6-hydroxydopamine (6-OHDA) into the nigrostriatal tract in rats (Deumens et al., 2002; Lindgren and Dunnett, 2012). 6-OHDA is a selective catecholaminergic neurotoxin, preferentially damaging DAergic and NEergic neurons. 6-OHDA cannot pass through the blood-brain-barrier (BBB) and thus, has to be injected intracranially for producing catecholamine depletions in the brain. Desipramine, a NE reuptake inhibitor, is usually administrated prior to a 6-OHDA injection in order to protect NEergic neurons. Thereby, a selective DA depletion can be produced. Since DA deficiency in the nigrostriatal tract is one of the pathological hallmarks of PD, 6-OHDA is injected into the SNc (somata), the medial forebrain bundle (MFB; axons) or the striatum (terminals) to produce nigrostriatal DA depletions (Fig. 1). Bilateral injections of 6-OHDA easily induce dysphagia and high mortality, thus, 6-OHDA is generally applied only into one hemisphere to create the hemiparkinsonian rat.

Urban Ungerstedt developed the unilateral 6-OHDA lesioned rat model of PD. Unilateral injections of 6-OHDA into the nigrostriatal tract cause turning asymmetry, i.e., the animal shows significantly more rotations toward the side of the lesion (Ungerstedt, 1968; Ungerstedt and Arbuthnott, 1970; Schwarting and Huston, 1996). When an indirect DA agonist, e.g. amphetamine, is administrated, the lesioned rats show pronounced rotations toward the lesioned side, but when a direct DA agonist, e.g. apomorphine, is administrated, the lesioned rats exhibit contraversive turnings

(Schwarting and Huston, 1996). Whereas amphetamine stimulates release of endogenous DA primarily on the intact side of brain, apomorphine causes direct activation of supersensitive DA receptors on the lesioned side. These drug-induced rotations can be used as a behavioral index for the assessment of the degree of striatal DA depletion (Fuxe and Ungerstedt, 1976; Schwarting and Huston, 1996). Furthermore, the hemiparkinsonian rats show sensorimotor deficits on the body-side contralateral to the lesioned hemisphere. For example, unilateral 6-OHDA lesion-induced impairments in the initiation of stepping movements (Olsson et al., 1995) and grasping of food (Miklyaeva et al., 1994) with the contralateral paw were reported. In a cylinder, the lesioned animals used the contralateral forelimb less than the ipsilateral forelimb to support rearing against the walls (Schallert et al., 2000) and showed more contralateral than ipsilateral foot-slips on a grid (Chao et al., 2012). These sensorimotor deficits of unilateral 6-OHDA lesioned rats mimic, the parkinsonian deficits, like postural instability and bradykinesia (Schallert and Hall, 1988; Johnston et al., 1999).

At different sites of the nigrostriatal tract, injections of 6-OHDA cause different degrees of DA degeneration in rats (Kirik et al., 1998). Injection of 6-OHDA into the MFB leads to severe nigrostriatal degeneration and a striking asymmetry in motor behaviors (Schwarting and Huston, 1996; Deumens et al., 2002; Yuan et al., 2005). This lesion model can be considered appropriate to investigate late-stage PD (Yuan et al., 2005). In contrast, injections of 6-OHDA into the striatum causes a progressive loss of SN neurons (Berger et al., 1991; Ichitani et al., 1991; Sauer and Oertel, 1994),

which is useful as a partial DA depletion model in studies of functional recovery (Kirik et al., 1998). Therefore, different stages of PD can be studied based on the site of unilateral 6-OHDA injections and on the applied dose of the toxin. These properties make the model very useful for testing new treatment approaches, studying neuroprotective/neurorestorative therapeutics and complex electrophysiological as well as neurochemical mechanisms that underlie the observed deficits.

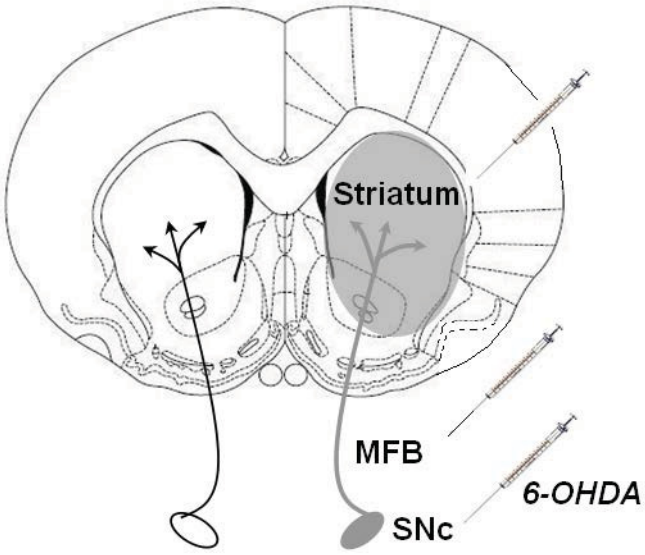

Figure 1. Sketch of a unilateral injection of 6-OHDA into the nigrostriatal tract in rats. 6-OHDA is unilaterally injected into the SNc, the MFB or the striatum to produce hemi-parkinsonian rats. Gray parts indicate DA depletions (Schwarting and Huston, 1996).

2. Methods

This section briefly describes the methods and paradigms used in this dissertation. The detailed description can be found in the methods section of the published studies in the appendix.

2.1. Surgeries

Male Wistar rats were anesthetized with pentobarbital (50 mg/kg, i.p.). They were placed on a heating pad for maintaining body temperature and heads were fixed in a Kopf stereotaxic frame. The scalp was cut and retracted to expose the skull. Holes were drilled above either the right or left MFB (Metz and Whishaw, 2002). 6-OHDA (dose used based on (Monville et al., 2006) was injected into the MFB. This lesion procedure was used for inducing severe nigrostriatal DA depletions as seen in advanced PD. For those animals that received unilateral 6-OHDA injections into the striatum, holes were drilled above the right dorsal striatum (Kirik et al., 1998). 6-OHDA was injected at various coordinate of the dorsal striatum. This procedure leads to a moderate degree of DA depletion and was thus used as an early-stage model of PD.

In experiment 4, a unilateral *N*-methyl-D-aspartate (NMDA) injection into the medial prefrontal cortex (mPFC) was conducted, combined either with a sham-lesion or a unilateral 6-OHDA lesion of the MFB, ipsilaterally or contralaterally. NMDA

was injected into either the right or left mPFC (Lacroix et al., 2002). For sham lesions the same surgical procedures were followed, but phosphate buffered saline (PBS) was injected into the brain. Toxin injections into opposite hemispheres are used to functionally disconnect two brain areas, while an intact circuit is spared in the group with lesions in the same hemisphere. Figure 2 presents an illustration of this disconnection procedure.

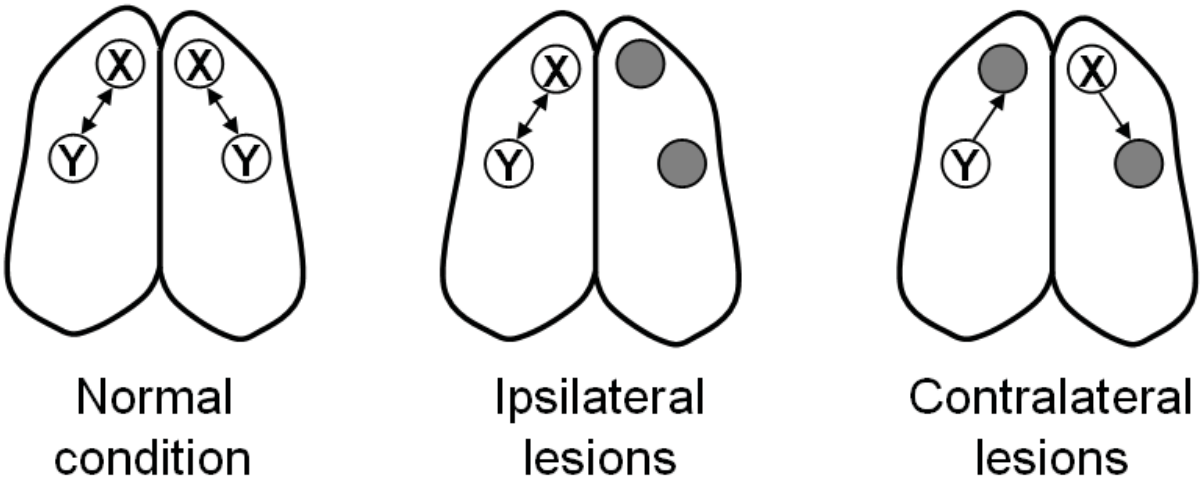

Figure 2. Schematic of a disconnection procedure. For example, two areas, called X and Y in the brain, are interconnected with each other (normal condition). When lesions are made in one hemisphere only, an intact circuit is preserved (ipsilateral lesions). When the same lesions are made in opposite hemispheres, the interconnections are damaged (contralateral lesions). Gray parts indicate lesioned areas.

2.2. Behavioral paradigms

2.2.1. Open-field test

The open-field test is a behavioral paradigm based on animals' spontaneous behavior. Usually a round or square arena is used as the apparatus. The test was introduced in 1932 by Calvin Hall and has been used extensively in behavioral neuroscience since then (Walsh and Cummins, 1976). Distance travelled by the animal can be taken as an general index for locomotor activity, while resident time at different areas of the arena is an index for emotionality (more time spent in the center of the arena reflects lower anxiety) (Prut and Belzung, 2003). In the present projects, a video image analyzing system was used to analyze locomotion, turning and thigmotactic behavior in hemiparkinsonian rats. (Schwarting et al., 1993)

2.2.2. Cylinder test

The cylinder test (Schallert et al., 2000) is a widely used behavioral assessment of forelimb use for hemiparkinsonian rats. The lesioned rats show more ipsilateral than contralateral forelimb uses during rearing against the walls (Tillerson et al., 2001; Woodlee et al., 2008), and this kind of forelimb asymmetry is correlated with the degree of DA depletion (Tillerson et al., 2001) and can be improved by administration of L-dopa or a DA agonist (Lundblad et al., 2002). In the present studies, a transparent cylinder (30 cm in diameter and 45 cm high) and a camera

connected to a DVD-recorder were used. After the animal was placed in the cylinder, the animal's use of its forelimbs while rearing up against the walls was recorded on DVDs for 5 minutes (min). The frequency of the use of limbs ipsilateral (intact) and contralateral (impaired) to the lesion side was assessed.

2.2.3. Grid test

The grid test is a widely used behavioral assessment in many models of neurological diseases, while it has not been used to investigate animals of PD before. There are multiple tests available for the assessment of motor deficiencies of hemiparkinsonian rats, such as drug-induced rotations (Ungerstedt, 1971; Steiner et al., 1985), adjusting steps of the weight-bearing forepaw (Olsson et al., 1995; Chang et al., 1999), food retrieval on a descending staircase (Whishaw et al., 1997; Barneoud et al., 2000), food-grasping (Miklyaeva et al., 1994), the analysis of foot prints (Metz et al., 2005), asymmetrical thigmotactic scanning (Steiner et al., 1988), and forelimb use (Schallert et al., 2000). However, these methods did not prove to be sensitive for less than severe DA depletion (Kirik et al., 1998; Deumens et al., 2002). Given that the motor impairments in the 6-OHDA lesioned model can be very subtle, it is important to have multiple behavioral criteria for such assessments (Metz et al., 2000). Since the grid test is a sensitive test for assessment of sensorimotor functions involved in precise stepping, coordination, and accurate paw placement, it could provide information on the effect of DA depletion in the nigrostriatal system that is hard to

gain from other methods. Furthermore, it has the advantage that it does not include possible confounding factors, like extensive handling of the animals as the adjusting-steps test (Olsson et al., 1995; Chang et al., 1999), or food deprivation like the staircase test (Whishaw et al., 1997; Barneoud et al., 2000). In the present study, a metal square grid was used and a camera connected to a DVD-recorder was located below the grid with an angle of 20-40 degrees. Animals were put on the grid for 5 min and their behaviors were recorded on DVDs. Foot-slips for each limb were assessed. A slip was scored (a) when the paw completely missed a rung and the limb fell between the rung, or (b) when the paw was correctly placed on the rung but slipped off during weight bearing (Ma et al., 2001; Zhang et al., 2002; Starkey et al., 2005).

2.2.4. Spontaneous object exploration

Rodents prefer exploring a novel stimulus more than a familiar one. Several spontaneous object exploration tests exploiting this natural tendency were developed to investigate recognition memories for object, location and temporal order. In the classical design of novel object recognition, rodents are put firstly into an arena, e.g, an open-field, for habituation. Then, two copies of objects are placed in the arena and the animal is free to explore them for a period of time (usually four to five min). After a delay, the animal is put back into the arena with one old object and a novel object presented simultaneously. Normal rodents tend to explore the novel object more

than the old one, suggesting that they remember the object which has been explored earlier (Ennaceur and Delacour, 1988). Comparable procedures are used to test memory for location of objects and temporal order of presentation of objects. To test memory for location, one of the objects remains at the old location, while the other one is moved to a novel location during the test trial. Rodents prefer exploring the object at the novel location more than the one at the old location, suggesting that they remember where they encountered a particular object before (Ennaceur et al., 1997). In a temporal order memory test, two copies of objects are placed into an arena for exploration, then, two copies of a novel object are presented after a delay. After another delay, one of the old familiar objects and one of the recent familiar objects are placed into the arena together. Rodents spend more time exploring the old familiar object than the recent familiar one, indicating that rodents remember the temporal-order by which the objects had been presented (Mitchell and Laiacona, 1998). In a further object-exploration test called object-in-place test (Barker et al., 2007; Barker and Warburton, 2011), four distinct objects are placed in an open-field and rats are allowed to freely explore them for 5 min. After a delay of 5min, they are placed back into the arena with the same objects, while the locations of two of them are interchanged. The time spent exploring the two changed objects is higher than the time spent exploring the two stationary objects by normal rats, suggesting that rats exhibit memory for the original spatial configuration of the different objects (Barker et al., 2007; Barker and Warburton, 2011). Figure 3 represents the four kinds of spontaneous object exploration tests.

Different sets of objects in quadruplicate were used in experiment 4. The assignments of objects for each test were counterbalanced and different sets of objects were used for each test. The heights and diameters of the objects were about 18-34.5 and 8-12 cm, respectively. The objects were made of different materials (plastic, glass, porcelain) and had different colors (white, red, green), shapes (column, square, irregular-shapes) and textures (smooth, rough). As previously described, the animals underwent the four object recognition tests. For the object and spatial recognition tests, the exploration time for each trial was 4 min with a delay of 90 min. For the temporal order memory test, the exploration time for each trial was also 4 min with delays of 30 and 40 min. For the object-in-place test, the exploration time was 5 min in the sample trial, followed by a delay of 5 min, the time for the test trial was 4 min. Object exploration was defined as a physical contact with the object with snout, vibrissae or forepaws. Climbing on the object, or contacting the object with the body but not being oriented toward it, was not included in this measure. Time for exploring objects was recorded and analyzed.

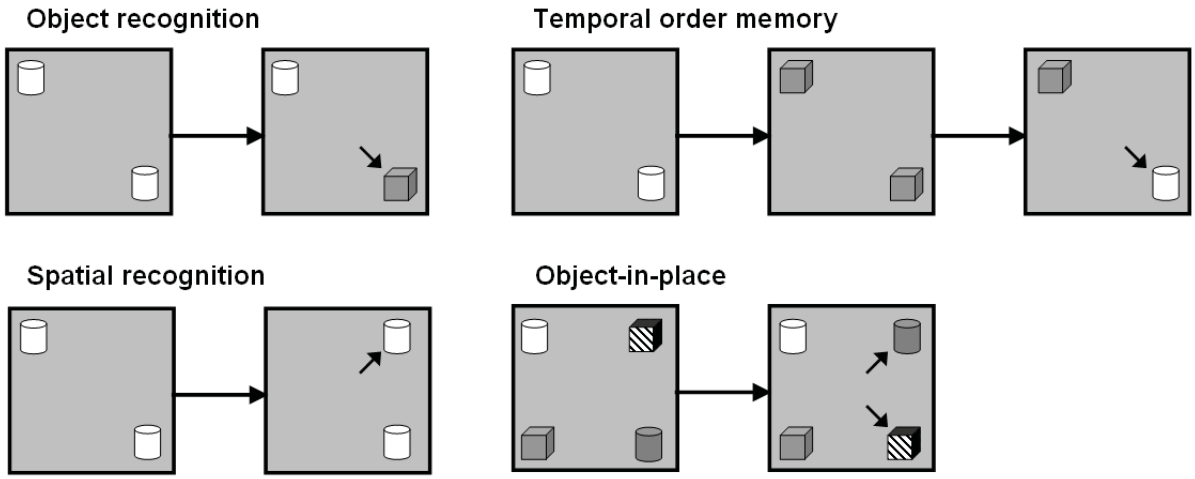

Figure 3. Schematic of representation of four spontaneous object exploration tests. Small arrows indicate the object(s) which normal rodents spend more time exploring than the other one(s).

2.3. Neurochemical analysis

After behavioral tests, the animals were anaesthetized with CO_2, decapitated and their brains immediately excised. Both hemispheres of dorsal striatum were dissected and stored at -80 °C until analysis. The samples were analysed for the content of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA), by means of high-performance liquid chromatography – electrochemical detection (HPLC-EC). Evaluation of the striatal DA content offers the extent of DA depletion by the 6-OHDA injection.

3. Experiment 1: Effects of intranasal administration of L-dopa in hemi-parkinsonian rats

Intranasal (IN) administration offers a method of drug delivery which is non-invasive and bypasses the first-pass metabolism (Hanson and Frey, 2008; Dhuria et al., 2010). Many studies indicate that drugs that can bypass the BBB via the IN route show therapeutic and behavioral effects. For instance, IN insulin administration improved memory in Alzheimer's patients (Reger et al., 2008). IN DA ameliorated attention deficits in an animal model of attention-deficit-hyperactivity disorder (Ruocco et al., 2009), sensitized the turning responses to amphetamine, and increased the use of the ipsilateral forelimb in hemiparkinsonian rats (Pum et al., 2009). The present experiment investigated the effect of IN L-dopa in unilateral 6-OHDA lesioned rats. IN L-dopa or vehicle was administrated in lesioned rats, with or without benserazide pre-treatment, before conducting open-field, cylinder and grid tests. There was no significant group effect in the extent of DA depletion. IN L-dopa reduced ipsilateral turnings and increased contralateral turnings 10-20 min after the administration. This 10-20 min effect was consistent with a peak of DA concentration at around 12 min following IN L-dopa administration (Kim et al., 2009). IN L-dopa also reduced contralateral forelimb-slips on the grid. These effects were found under the saline pre-treatment condition, while no group difference was found when benserazide was pre-treated. There is evidence that benserazide increases striatal DA and DOPAC (de Souza Silva et al., 1997) and decreases aromatic L-amino acid

decarboxylase (AADC) activity in the striatum (Jonkers et al., 2001; Shen et al., 2003). Thus, it is possible that benserazide affected the conversion from L-dopa to DA, and attenuated the effect of IN L-dopa. Statistically, there was no difference between the performance with saline pretreatment and the performance with benserazide pretreatment, suggesting that the influence of such an interaction is negligible. At present, L-dopa administration is usually applied via the oral route in PD patients. However, food may compete with the drug for intestinal absorption (Simon et al., 2004; Muller et al., 2006) and dysphagia, with a highest prevalence as 52% in PD, may reduce bioavailability of L-dopa (Nyholm, 2006). On the other hand, intravenous injection is inconvenient for routine clinical use and is painful for patients. IN administration prevents the disadvantages of systemic administration and offers rapid delivery with non-invasive application. These present results suggested beneficial effects of IN L-dopa in the unilateral 6-OHDA lesioned rats, without requiring the combination with benserazide.

4. Experiment 2: Effects of chronic administration of progesterone in hemi-parkinsonian rats

progesterone (P4) shows neuroprotective effects in the central nervous system (CNS), such as decreasing behavioral abnormalities in rodent models of traumatic brain injury (Shear et al., 2002; Djebaili et al., 2004), spinal cord injury (Thomas et al., 1999) and middle cerebral artery occlusion (Gibson and Murphy, 2004; Sayeed et al., 2007). Furthermore, P4 also exhibits neuroprotective effects against DAergic degeneration induced by 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) (Grandbois et al., 2000; Callier et al., 2001; Morissette et al., 2008) and methamphetamine (Yu and Liao, 2000) in rodents. Thus, we applied P4 in a unilateral 6-OHDA lesioned rat model of PD to investigate its effects. Rats received unilateral 6-OHDA injections into the striatum (Kirik et al., 1998) and were divided into three treatment groups: vehicle, P4 4 mg/kg, and P4 8 mg/kg. The animals were treated daily for 13 days after surgery and behavioral tests in the open-field, the cylinder and on the grid were conducted. No group difference was found in the content of striatal DA (mean depletion of 67%). Chronic P4 8 mg/kg-treated hemiparkinsonian rats showed a higher ipsiversive turning asymmetry, more contralateral hind limb slips, and more asymmetrical use of the forelimbs during rearing behavior. In addition, the high dose of chronic P4 administration increased DA turnover in the lesioned striatum. Thus, negative effects of chronic P4 administration were found in the male hemiparkinsonian rats. In previous MPTP studies, P4 was applied 5 days before until 5 days after MPTP

injection (Grandbois et al., 2000; Callier et al., 2001; Morissette et al., 2008), which may increase the expression of antiapoptotic molecules (Yao et al., 2005) in the CNS before the lesion, while P4 was administrated after the surgery in the present study. Negative effects of P4 were also reported in other studies (Murphy et al., 2000; Goss et al., 2003). The neuroprotective mechanisms of sex steroids for PD are complicated and interact with other steroids, dose and gender. Estrogens had neuroprotective effects in female hemiparkinsonian rats, while no or detrimental effects were found in males (Gillies et al., 2004; Gillies and McArthur, 2010). These findings are incompatible with the hypothesis of positive treatment effects with P4 administration after the onset of PD, but do not rule out possible beneficial effects of P4 when given in early stages of the disease onset.

5. Experiment 3: The grid test as a measure of sensorimotor performance of hemiparkinson

The grid test is a sensitive test for evaluating the sensorimotor coordination of the four limbs in models of neurological disorders such as pyramidotomy (Z'Graggen et al., 1998; Starkey et al., 2005), spinal cord injury (Ma et al., 2001; Onifer et al., 2005; Sandrow et al., 2008), somatosensory cortex lesion (Napieralski et al., 1998; Shanina et al., 2006), and ischemic stroke (Zhang et al., 2002; Lourbopoulos et al., 2008). Behavior of hemiparkinsonian rats on a grid was shown to be suitable for screening of rotational activity (Silvestrin et al., 2009), while the foot-slips on a grid have not been analyzed systematically. Given that bradykinesia, rigidity and postural abnormalities are the primary motor characteristics of PD (Dauer and Przedborski, 2003), observing skilled use of the limbs is likely to provide useful information. In the present study 6-OHDA was injected either into the MFB or the striatum for producing severe and progressive DA degeneration in the nigrostriatal tract, respectively. The group of severely lesioned animals (mean depletion 92%) was divided into an L-dopa and a vehicle treatment group, and were placed on the grid 30 min after the treatment. The L-dopa-treated group showed fewer forelimb-slips than the vehicle-treated group. Animals with moderate DA depletions (mean depletion 54%) showed more contralateral than ipsilateral forelimb-slips. Compared with naïve rats, the hemiparkinsonian rats exhibited more foot-slips. These results suggest that the grid test is a sensitive behavioral assay for sensorimotor deficits in 6-

OHDA lesioned animals, which can be used to study impairments also in animals with moderate DA-depletions.

6. Experiment 4: The functional role of the interaction between the mPFC and the dopamine system

Patients with PD show not only motor deficits but also impairments in learning and memory (Lang and Lozano, 1998a, b; Rodriguez-Oroz et al., 2009). Neuroimaging and neuropsychological studies indicate that the PFC and the nigrostriatal pathway both contribute to the cognitive deficits in PD (Owen et al., 1992; Owen et al., 1998; Dagher et al., 2001; Mattay et al., 2002; Sawamoto et al., 2008). However, little is known about the functional role of the interaction between the PFC and the nigrostriatal DA-pathway. Thus, a disconnection procedure was applied to investigate this hypothesis. Male rats received either a unilateral injection of 6-OHDA into the MFB or a unilateral NMDA lesion in the mPFC, or both these lesions combined in either the same or opposite hemispheres. The circuit is disconnected bilaterally at two different levels when these lesions are applied in opposite hemispheres, whereas an intact circuit is preserved in one hemisphere when these lesions are applied in the same hemisphere (Geschwind, 1965b, a). Spontaneous object recognition, motor and sensorimotor tests were conducted. The three groups with 6-OHDA lesions showed over 90% DA loss in the lesioned striatum, while there was no group difference among them. The group with the combined lesions in opposite hemispheres showed no intact object recognition memory, whereas the group with the same lesions in the same hemisphere did. The groups treated with 6-OHDA showed impairments on temporal order memory, while the groups with

combined lesions showed no intact memories in the object-in-place and spatial recognition tests. Detailed results are presented in Table 1. A disconnection between the PFC and the MFB disrupted object association learning and object recognition memory in a delayed match-to-sample task in monkeys (Easton and Gaffan, 2001; Easton et al., 2001). The interplay between these two brain regions may be involved in information processing during object recognition or in the transmission of memory to the medial temporal lobe (Easton and Gaffan, 2001; Easton et al., 2001). The present result indicates that the forebrain DAergic projections may play a crucial role in these processes. The groups with 6-OHDA lesions exhibited an impairment of temporal order memory, which is consistent with studies in PD patients showing deficits on temporal processing (Sagar et al., 1988). In addition, our results strengthened the association between DA and time perception (Coull et al., 2011; Allman and Meck, 2012). In normal rats, the mPFC is not involved in spatial recognition memory (Barker et al., 2007; Barker and Warburton, 2011), while in the hemiparkinsonian rats the mPFC lesions contributed to the impairment of spatial cognition. This suggests that the mPFC might play a role when DA is deficient in the nigrostriatal pathway. This explanation is in line with studies in PD patients, which showed increases of cortical activity during cognitive tasks (Cools et al., 2002; Monchi et al., 2007), particularly, when the tasks do not require the striatum (Monchi et al., 2007). Thus we concluded that the interaction between the mPFC and the DAergic forebrain pathways, particularly the nigrostriatal pathway, is crucial for

object recognition memory. This finding may account for possible mechanisms that underpin cognitive deficits in PD.

Table 1. Summary of the results of experiment 4. OR = object recognition; TOM = temporal order memory; OP = object-in-place; SR = spatial recognition. Symbols of **+** and **-** mean that the group does or does not show the specific recognition memory, respectively.

	Hemi-PD	Hemi-PD + mPFC Ipsi-	Hemi-PD + mPFC Contra-	mPFC	Sham
OR	**+**	**+**	**-**	**+**	**+**
TOM	**-**	**-**	**-**	**+**	**+**
OP	**+**	**-**	**-**	**+**	**+**
SR	**+**	**-**	**-**	**+**	**+**

7. General discussion

IN L-dopa ameliorated sensorimotor deficits and modulated the turning pattern in hemiparkinsonian rats. Notably, IN L-dopa tended to have more pronounced behavioral effects when it was applied without benserazide pre-treatment. These results suggest that L-dopa can bypass the BBB via the IN route and influence behaviors of hemiparkinsonian rats. Whereas oral administration of L-dopa is confounded by the competition from food for intestinal absorption and dysphagia, a common symptom in PD, IN administration of L-dopa circumvents these factors. Whether IN L-dopa administration may be considered as an adjuvant treatment procedure for PD will depend on the outcome of further studies, particularly on the effects of chronic treatment.

Studies using repeated systemic treatment with the neurotoxin MPTP to lesion the DA-system indicate that P4 has neuroprotective effects, while our results suggest that P4 exacerbated the motor impairments in hemiparkinsonian rats. In previous MPTP studies, P4 was applied for 5 days before until 5 days after MPTP injection (Callier et al., 2000; Grandbois et al., 2000; Morissette et al., 2008). Thus, P4 influenced the CNS before the lesion and showed neuroprotective effects, while in the present study P4 was applied after the lesion and thus, might have lost its neuroprotective effects. Furthermore, the neuroprotective mechanisms of sex steroids against PD are complicated as there are interactions with other steroids, dose, and gender. In hemiparkinsonian rats, estrogens had neuroprotective effects in females, but they

had no effects or exacerbated the neurodegeneration in males (Gillies et al., 2004; Gillies and McArthur, 2010). The present results indicate that P4 is deleterious in male hemiparkinsonian rats when administrated after the lesion, which provides valuable information concerning the role of sex steroids and gender for testing alternative therapies for PD.

The grid test is a suitable and sensitive behavioral assessment for testing the sensorimotor function in the 6-OHDA lesioned animal model of PD. Detecting motor impairments in 6-OHDA lesioned animals with moderate DA depletions (below 80%) has proven to be difficult because of functional recovery taking place (Schwarting and Huston, 1997). Furthermore, most tests were used in animals with over 80% DA-depletion of the nigrostriatal tract (Olsson et al., 1995; Chang et al., 1999; Schallert et al., 2000; Tillerson et al., 2001). In the present study, the animals with moderate DAergic lesions (mean 54%) showed significantly more contralateral than ipsilateral foot-slips on the grid. Therefore, the grid test offers a behavioral phenotype for evaluating moderate DA depletion, which is very useful for developing neuroprotective agents and studying early-stage of PD.

Cognitive deficits in PD have a strong impact on the quality of life (Schrag et al., 2000). Thus, understanding the neuronal circuits that contribute to the impairments of learning and memory is important. Previous findings have claimed that the PFC and the nigrostriatal DA system are both involved in parkinsonian cognition (Owen et al., 1998; Dagher et al., 2001; Cools et al., 2002; Mattay et al., 2002; Sawamoto et al., 2008). Based on these findings, the present study focused on testing the functional

role of the interaction between these two regions, showing that the interplay is critical for object recognition memory. The results shed light on the understanding of the cognitive deficits in PD.

The present results raise some interesting issues concerning the use of the 6-OHDA model. For example, experiment 2 suggests that the interaction between the factors "time since lesion" and "initiation of treatment" might be important for the interpretation of drug effects. In experiment 2, chronic administration of P4 had deleterious effects when applied after the lesion, but P4 shows beneficial effects when treatment was conducted before MPTP injections (Callier et al., 2000; Grandbois et al., 2000; Morissette et al., 2008). Compared with the situation of treating patients with PD with drugs, the present experimental approach can be considered as a more valid assessment of drug action.

There was a significant effect of the interaction between the mPFC and the DA-system in experiment 4. 6-OHDA lesions alone did not induce impairments in object recognition memory but influenced temporal order memory, whereas the additional lesions of the mPFC led to impairments of both memories. Our results suggest that the mPFC plays an important role in learning and memory in animals with DA deficiency. Replenishment of DA by administration of L-dopa does not necessarily ameliorate cognitive deficits in patients with PD (Svenningsson et al., 2012), while in some cases it has deleterious effects (Cools et al., 2003). A hypothesis claims that L-dopa compensates DA in the midbrain DAergic system but overdoses in the PFC, thus, exacerbating cognitive performance of PD patients (Cools, 2006). The

interaction between the PFC and the midbrain DA-system, and the association between the compensation of midbrain DA and the imbalance of DA in the PFC, could be key factors to understand some of the non-motor symptoms of PD. Comparable findings might be expected for the interaction of the midbrain DA system and brain areas related the cognitive and/or emotional processes.

Some limitations are worth noting when taking the unilateral 6-OHDA lesioned rats as a PD animal model. Firstly, this unilateral 6-OHDA lesioned model lacks the pathological characteristic of PD, the Lewy bodies. Secondly, PD is a progressive neurodegenerated disease in which the DAergic neurons are degraded over years, while the 6-OHDA rapidly damages the DAergic neurons in a short period. Thirdly, PD accompanies emotional as well as cognitive impairments, while the unilateral 6-OHDA lesioned animal model does not necessarily result in such deficits. For instance, the unilateral 6-OHDA lesioned rats showed normal emotionality compared to the controls on the elevated plus maze (Delaville et al., 2012). Alternatively, inconsistent results were found in various learning and memory tasks when comparing hemiparkinsonian rats to the controls (Mura and Feldon, 2003; Hritcu et al., 2008; Ciobica et al., 2012). When conducting experiments using the unilateral 6-OHDA lesioned rats as an animal model of PD, these differences should be considered.

Although the administration of L-dopa is still the "gold-standard" therapy in PD (Lang and Lozano, 1998a; Brooks, 2008), it causes severe side-effects, the therapeutic effects are not stable for non-motor deficits, and L-dopa cannot prevent

the progression of PD. Alternative treatments for PD are highly required. The present studies suggest that a single approach for studying the disease is not sufficient to resolve the issue. Therefore, multiple complementary approaches considering a wide spectrum of possible interactions at the behavioral, pharmacological and anatomical levels are needed.

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9. Publications

The following lists are the published studies, on which this dissertation is based.

1. Chao OY, Mattern C, De Souza Silva MA, Weßler J, Ruocco LA, Nikolaus S, Huston JP, Pum ME (2012) Intranasally applied L-DOPA alleviates parkinsonian symptoms in rats with unilateral nigro-striatal 6-OHDA lesions. Brain Res. Bull. 87: 340-345.
2. Chao OY, Huston JP, von Bothmer A, Pum ME (2011) Chronic progesterone treatment of male rats with unilateral 6-hydroxydopamine lesion of the dorsal striatum exacerbates parkinsonian symptoms. Neuroscience 196: 228-236.
3. Chao OY, Pum ME, Li JS, Huston JP (2012) The grid test: assessment of sensorimotor deficits after moderate or severe dopamine depletion by 6-hydroxydopamine lesions in the dorsal striatum and medial forebrain bundle. Neuroscience 202: 318-325.
4. Chao OY, Pum ME, Huston JP (2013) The interaction between the dopaminergic forebrain projections and the medial prefrontal cortex is critical for object recognition memory: Implications for Parkinson's disease. Exp. Neurol.

10. Appendix

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Research report

Intranasally applied L-DOPA alleviates parkinsonian symptoms in rats with unilateral nigro-striatal 6-OHDA lesions

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ABSTRACT

L-3,4-Dihydroxyphenylalanine (L-DOPA) remains the most effective drug for therapy of Parkinson's disease. However, the current clinical route of L-DOPA administration is variable and unreliable because of problems with drug absorption and first-pass metabolism. Administration of drugs via the nasal passage has been proven an effective alternate route for a number of medicinal substances. Here we examined the acute behavioral and neurochemical effects of intranasally (IN) applied L-DOPA in rats bearing unilateral lesions of the medial forebrain bundle, with severe depletion (97%) of striatal dopamine. Turning behavior in an open field, footslips on a horizontal grid and postural motor asymmetry in a cylinder were assessed following IN L-DOPA or vehicle administration with, or without, benserazide pre-treatment. IN L-DOPA without benserazide pre-treatment mildly decreased ipsilateral turnings and increased contralateral turnings 10–20 min after the treatment. IN L-DOPA with saline pre-treatment reduced contralateral forelimb-slips on the grid while no effects were evident in the cylinder test. These results support the hypothesis that L-DOPA can bypass the blood–brain barrier by the IN route and alleviate behavioral impairments in the hemiparkinsonian animal model.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder which is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Loss of SNc neurons leads to striatal dopamine (DA) deficiency, which is responsible for the major PD symptoms such as bradykinesia, resting tremor, muscle rigidity, and postural abnormalities [8]. Due to DAs hydrophilic properties, it cannot cross the blood–brain-barrier (BBB). Thus, the DA precursor, L-3,4-dihydroxyphenylalanine (L-DOPA) is used to replenish DA in PD [2,4]. L-DOPA bypasses the BBB via a saturable transporter and is converted to DA by aromatic L-amino acid decarboxylase (AADC), primarily within the presynaptic terminals of DAergic neurons in the striatum [29]. However, L-DOPA is converted to DA in the periphery as well, and only a small amount of L-DOPA enters the brain [14]. In order to prevent the conversion of L-DOPA into DA in the periphery, AADC inhibitors, such as benserazide or carbidopa, which cannot cross the BBB, are used in combination with L-DOPA [18,19]. L-DOPA combined with carbidopa administration is usually applied via the oral route in PD patients. However, large neutral amino acids contained in food may compete with the drug for intestinal absorption [27,41] and dysphagia, with a highest prevalence as 52% in PD, may reduce bioavailability of L-DOPA [28]. Intravenous infusion of drugs is effective for PD treatment, but is impractical and inconvenient for routine clinical use.

The intranasal (IN) route of administration, which is non-invasive and bypasses first-pass metabolism due to its potential for a direct delivery to the brain, is offered as an alternative to systemic methods of drug application [13,15]. A number of drugs have been shown to by-pass the BBB via IN route and to have therapeutic effects in rodents, nonhuman primates and humans. For instance, IN insulin administration improved memory in healthy volunteers and Alzheimer's patients [1,32,33], IN oxytocin administration reduced stress in monkeys [30] and IN insulin-like growth factor-I (IGF-I) decreased infarct volume and improved neurologic function in stroke models in rats [22,23]. DA seems also to bypass the BBB when applied into the nose and has been shown to have behavioral and neurochemical effects in rats. For example, IN DA administration increased DA levels in the neostriatum and nucleus accumbens [12]. Animals showed an antidepressant-like behavior in a forced-swimming task and higher activity in a familiar open field (OF) following IN DA administration [3]. IN DA also

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reduced activity and improved attention in an animal model of attention-deficit-hyperactivity disorder [34]. In animals with unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal tract, one of the most used animal models of PD [38,39,43], IN DA administration sensitized the turning response to amphetamine, and increased the use of the ipsilateral forelimb [31].

IN L-DOPA has not, so far, been examined in an animal model of PD. Microdialysis studies have shown that IN L-DOPA administration increased extracellular DA in the neostriatum of healthy rats and that this increase is stronger in the hemisphere ipsilateral to the nostril into which L-DOPA was injected [9,10]. Based on this finding, we predicted also an effect of IN L-DOPA in the hemiparkinsonian rat. Thus, the objective of the present study was to characterize the effects of IN L-DOPA administration, with and without benserazide pre-treatment in the rat bearing a unilateral 6-OHDA lesion of the nigro-striatal DA projections. Amphetamine-induced turning behavior in the open field, footslips on a grid and forelimb contacts in a cylinder were analyzed. HPLC-EC was used to quantify contents of DA, and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), as well as 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin (5-HT), in the lesioned and intact striatum.

2. Materials and methods

2.1. Animals

Twenty male Wistar rats (Tierversuchsanlage, University of Duesseldorf, Germany) weighing between 350 and 400 g were used. Animals were housed under standard controlled conditions, with a reversed light–dark rhythm (light off from 07:00 to 19:00). They were grouped 4 or 5 in a cage and water and food was provided *ad libitum*. After arrival, they were given at least 2 weeks before surgery. All experiments were conducted in conformity with the Animal Protection Law of the Federal Republic of Germany and with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Surgery

Animals were anaesthetized with pentobarbital (50 mg/kg; Narcoren, Merial GmbH, Germany, i.p.). The rats were placed in a Kopf stereotaxic frame and the scalp was cut and retracted to expose the skull. A hole was drilled above the right or left medial forebrain bundle (MFB). 6-OHDA (10.5 μg in 3 μl PBS with 0.1% ascorbic acid) was used to damage dopaminergic neurons by a unilateral injection into the MFB (AP: −4.0 mm, ML: ±1.5 mm, DV: −8.5 mm; relative to bregma, flow rate 1 μl/min) [24]. The cannula was left in place for 4 min to prevent reflux. Finally, the scalp was sutured and 70% ethanol was used for disinfection. Behavioral measurements were begun two weeks after the surgery. The dose of 6-OHDA used here was previously shown to create an effective massive DA depletion [26], since behavioral deficits are typically not manifested unless striatal DA is depleted by 80–90% [38,44].

2.3. Apparatus

An open field (OF) (48 cm × 48 cm × 48 cm), which was located in a sound attenuating box (110 cm × 70 cm × 70 cm), was used to measure the animals' turning behavior and locomotor activity. Two red light bulbs provided illumination (luminous density on floor level ~8 lux). A camera was mounted 66 cm above the OF, and connected to a DVD-recorder and a personal computer running the VIAS video imaging software, provided by Dr. Jay-Shake Li, for analyzing turning behavior (counts of ipsilateral or contralateral quarter-turns). A metal square elevated grid (41 cm × 41 cm, high 41 cm, with each grid cell 3.5 cm × 3.5 cm) was used to test the animals' ability of accurately placing limbs during spontaneous exploration in the grid test. The grid apparatus was located in a quiet room under dim lighting. A transparent plastic cylinder (30 cm in diameter and 45 cm high) was used to test the animals' forelimb contacts with the cylinder's wall. The cylinder was located in the same room as the grid apparatus. The apparatus was cleaned with 70% ethanol after each trial.

2.4. Drugs

The drugs used in the present study: amphetamine, L-DOPA, benserazide and castor oil, were purchased from Sigma–Aldrich (Germany). A single amphetamine injection was used to test the effectiveness of the 6-OHDA lesion [16]. The dose of amphetamine used (1.5 mg/kg in saline, injection volume 1 ml/kg; i.p.) was based on its high effectiveness in inducing ipsilateral turning behavior in previous studies [31]. L-DOPA was suspended in castor oil for IN drug administration. The dose of 12 mg/kg of L-DOPA was previously shown to induce contralateral turning in the unilateral 6-OHDA lesioned animal [21,25]. A relatively low dose of benserazide (15 mg/kg in saline, injection volume 1 ml/kg; i.p.) was used for pre-treatment, because higher doses (e.g., 50 mg/kg) directly influenced DA levels in the central nervous system (CNS) [18,40]. Behavioral effects of benserazide pre-treatment followed by L-DOPA were not different when compared to concomitant L-DOPA and benserazide administration in the 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)-treated marmoset [42]. A transferpettor (Brand GmbH, Germany) was used to apply the castor oil for the vehicle group or L-DOPA for the treatment groups into both nostrils (5 μl/nostril). The method for IN drug administration used was previously shown to be effective behaviorally and neurochemically [3,11,34].

2.5. Experimental procedures

A mixed design with two-way factors was used. The animals were distributed into two groups based on the treatment factor (IN L-DOPA or vehicle) with repeated pre-treatment factor (benserazide and saline).

The animals were injected with amphetamine and placed into the OF. Amphetamine-induced turning behavior was measured for 30 min. Animals which showed less than 80% ipsiversive turnings were excluded from the experiment ($n = 5$). Then the animals were divided into two groups: IN L-DOPA ($n = 8$) and vehicle ($n = 7$). After the amphetamine-induced turning test, the following behavioral tests were conducted (Fig. 1A): measurement of L-DOPA-induced turning was performed one week following the amphetamine-induced turning test. Two 30-min habituation sessions were performed on subsequent days. L-DOPA (12 mg/kg) was administrated intranasally and a vehicle group also received IN castor oil. Each animal was tested twice. Half of the animals received an i.p. injection of the peripheral AADC inhibitor, benserazide (15 mg/kg), 30 min before L-DOPA treatment. The other half of the animals was pre-treated with saline (1 ml/kg). On the next day, the animals that had been treated with benserazide were injected with saline and vice versa (Fig. 1B). Immediately after intranasal administration, the animal was placed into the OF for 60 min for measurement of locomotor activity and turning behavior.

The grid test was conducted one week after OF testing. Behavior was recorded 30 min following the same administration of L-DOPA or vehicle as in the OF test, including the pre-treatment. The animal was placed on the grid and allowed to explore it for 5 min. All behaviors were recorded on DVD and analyzed by an experimenter blind to the treatment group. A footslip was scored either when the paw completely missed a rung and, thus, the limb fell between the rungs, or when the paw was correctly placed on the rung, but slipped off during weight bearing [5].

The cylinder test [35,36] was conducted one week after the grid test. Behavior was recorded 30 min following the same administration of L-DOPA or vehicle as in the previous two tests, including the pre-treatment. The forelimb wall contacts and behavior was recorded for 3 min. This test assessed the use of the forelimbs to support the body against the wall of a cylinder. The number of wall contacts made using the ipsilateral (unimpaired), the contralateral (impaired), and both (simultaneous) limbs was recorded on DVDs. Later, an experimenter blind to the treatments scored the animals' forelimb contacts with the wall.

2.6. Neurochemical analysis

At least one week after the cylinder test, the animals were anaesthetized by CO_2 and decapitated and the brains were excised. The left and right dorsal striatum was dissected separately, homogenised, centrifuged, filtered, and stored at −80 °C until analysis. The samples were analyzed for their contents of DA, DOPAC, HVA and 5-HIAA by means of HPLC-EC. 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, Leyden, The Netherlands) was set at 500 mV vs. an ISAAC reference electrode (Antec, Leyden, The Netherlands) at 30 °C.

2.7. Statistics

Two-way ANOVAs were conducted to analyze the distance moved in the OF, foot-slips on the grid for each paw, and forepaw-use asymmetry in the cylinder with the factors pre-treatment (benserazide, saline) and treatment (IN L-DOPA, vehicle). The asymmetry ratio was calculated as the number of ipsilateral observations plus 1/2 the number of "both" observations, divided by the total number of observations (ipsi plus contra plus both) [45]. Repeated three-way ANOVAs with the factors pre-treatment, treatment, and interval were used to analyze turning behavior. This was followed by two-way ANOVAs with the factors treatment and interval for each pre-treatment condition. One-way ANOVAs with the factor treatment at each time point were conducted when appropriate. Independent samples *t*-test were used to compare the vehicle and the IN L-DOPA group when appropriate. The results of HPLC-EC were analyzed by paired sample *t*-tests to compare the values from the lesioned striatum with the values from the intact striatum. The level of significance was $p \leq 0.05$ for all the tests.

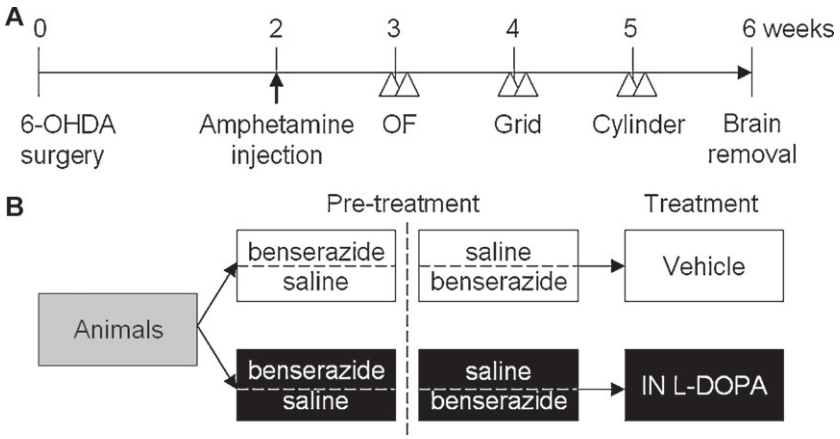

Fig. 1. Experimental design: (A) two weeks after the 6-OHDA lesions, the animals were injected with amphetamine to test the lesion efficiency. One week later, the open field, grid and cylinder test were conducted separately. Each test was administrated twice. The brains were removed at least one week after the cylinder task. Black arrow = injection of amphetamine. Triangles = injection of benserazide or saline 30 min before the treatment. (B) After the amphetamine test, the animals were divided into IN L-DOPA and vehicle groups. Thirty minutes before each trial, half of the animals received benserazide pre-treatment on the first day of each behavioral test, and the same animals received saline pre-treatment on the second day. Conversely, the other half of the animals received saline on the first day and benserazide on the second day.

3. Results

3.1. Neurochemistry

The lesioned striatum was significantly lower in content of DA, DOPAC, HVA than the intact striatum ($p < 0.001$, $p < 0.001$ and $p = 0.004$, respectively). The neurochemical results indicated a severe lesion by the unilateral 6-OHDA treatment. In the lesioned striatum the percentage of DA, DOPAC and HVA was decreased about 97%, 53% and 83%, respectively. Table 1 summarizes the post-mortem results.

3.2. Turning behaviors

IN L-DOPA did not influence the animals' locomotor activity in the OF. There were no overall treatment, pre-treatment or interaction effects on distance moved during 60 min ($p > 0.05$, data not shown).

The analysis of the time-course of ipsilateral turning behavior showed significant effects of treatment [$F(1, 78) = 8.943$; $p = 0.004$] and interval [$F(5, 78) = 12.078$; $p < 0.001$] but no effects of pre-treatment and interactions ($p > 0.05$). Separate two-way ANOVAs were conducted for each pre-treatment condition. There was an effect of interval [$F(5, 90) = 6.994$; $p < 0.001$] but no effects of treatment and interaction ($p > 0.05$) under the benserazide pre-treatment condition (Fig. 2A). Following saline pre-treatment there were significant effects of treatment [$F(1, 90) = 9.315$; $p = 0.003$] and interval [$F(5, 90) = 8.66$; $p < 0.001$] but no interaction ($p > 0.05$). The IN L-DOPA group showed significantly fewer ipsilateral rotations 40–50 min after the treatment when compared to the vehicle group ($p = 0.035$) (Fig. 2B). In addition, the area under curve (AUC) indicated that the animals treated with IN L-DOPA showed fewer ipsilateral turnings than the vehicle-treated animals ($p = 0.05$) (Fig. 2B).

The analysis of the time-course of contralateral turning behavior showed an effect of interval [$F(5, 78) = 4.981$; $p = 0.001$] but no effects of treatment and pre-treatment and no interaction ($p > 0.05$). The following analyses showed that under the benserazide pre-treatment condition no effect of treatment, interval and interaction were found ($p > 0.05$) (Fig. 3A). Following the saline pre-treatment, there was an effect of interval [$F(5, 90) = 2.362$; $p = 0.047$] but no effect of treatment and no interaction ($p > 0.05$). The IN L-DOPA group exhibited significantly more contralateral turnings than the vehicle group 10–20 min after the treatment ($p = 0.034$) (Fig. 3B).

3.3. Skilled walking behavior

For the analysis of the forelimbs slips, there were significant treatment effects [$F(1, 14) = 4.729$; $p = 0.047$ for ipsilateral side; $F(1, 14) = 6.979$; $p = 0.019$ for contralateral side] but no pre-treatment nor interaction effects ($p > 0.05$). There was no significant difference between the vehicle and the IN L-DOPA group when benserazide was administrated as pre-treatment (Fig. 4A). With the saline pre-treatment, the IN L-DOPA emitted fewer contralateral forelimb slips compared to the vehicle group ($p = 0.013$) (Fig. 4B). No significant main effect or interaction was found in the analysis of the hindlimbs slips ($p > 0.05$, data not shown).

3.4. Forelimbs usage preference

There was no treatment effect, pre-treatment effect or pre-treatment × treatment interaction in the analysis of the cylinder asymmetry ($p > 0.05$, data not shown).

4. Discussion

In the present study we investigated the effects of IN L-DOPA, with and without benserazide pre-treatment, on turning-behavior, behaviors on a horizontal grid and in the cylinder test on rats with unilateral 6-OHDA lesion of the nigro-striatal DA projections. IN L-DOPA without benserazide pre-treatment decreased ipsilateral turnings and increased contralateral turnings 10–20 min after the treatment. IN L-DOPA with saline pre-treatment also decreased contralateral forelimb slips during the grid test.

Rats with unilateral 6-OHDA lesions rotate towards the side of the lesion, while unstimulated contralateral rotation is rare [38]. Drugs that act by releasing DA or blocking the DA transporter, like amphetamine, induce ipsilateral behavioral asymmetries [37], while post-synaptic DA agonists, like apomorphine or L-DOPA, induce contralateral behavioral asymmetries [38]. In the present study IN L-DOPA treatment without benserazide pre-treatment decreased ipsiversive turning and increased contraversive turning 10–20 min after the treatment. This 10–20 min effect may reflect the rapid delivery characteristic of IN administration and was consistent with a peak of DA concentration at around 12 min following IN L-DOPA administration [20]. Thus, the turning effects of L-DOPA administration were most likely a result of rapid absorption characteristic of the IN route, rather than through systemic circulation.

Table 1
Post-mortem neurochemistry results of the lesioned and the intact striatum.

	DA	DOPAC	HVA	5-HIAA	DA depletion %
Lesioned	3.85 ± 1.29***	48.50 ± 11.93***	3.51 ± 0.99**	22.42 ± 3.31	96.90 ± 0.79
Intact	127.52 ± 19.65	92.25 ± 11.44	39.76 ± 11.38	31.45 ± 4.18	

Values are expressed as ng/mg wet tissue weight. Data are expressed as mean ± S.E.M. and analyzed by paired *t*-test, $n = 15$.
** $p < 0.01$.
*** $p < 0.001$.

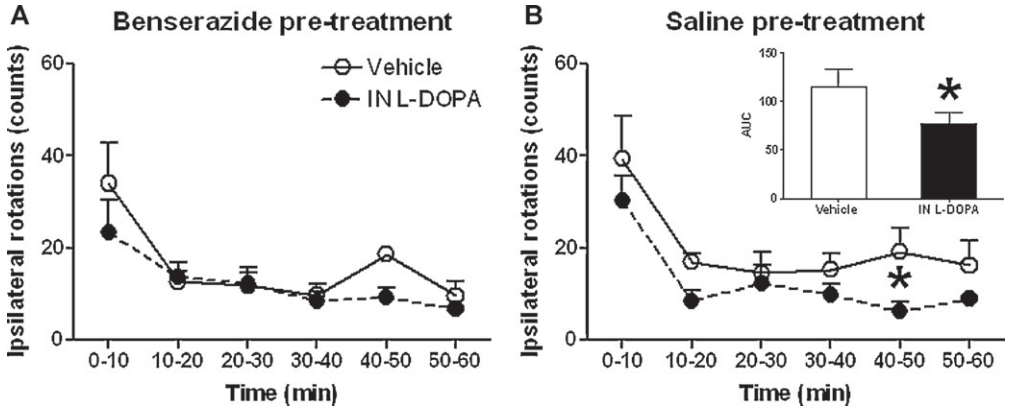

Fig. 2. Time-course of ipsilateral turning behavior following benserazide (A), or saline (B) pre-treatment. IN L-DOPA without benserazide pre-treatment reduced ipsilateral turns 40–50 min after the treatment, while the AUC showed a general reduction when compared to the vehicle group (B). Values are expressed as mean + S.E.M. *$p < 0.05$ compared to the vehicle group.

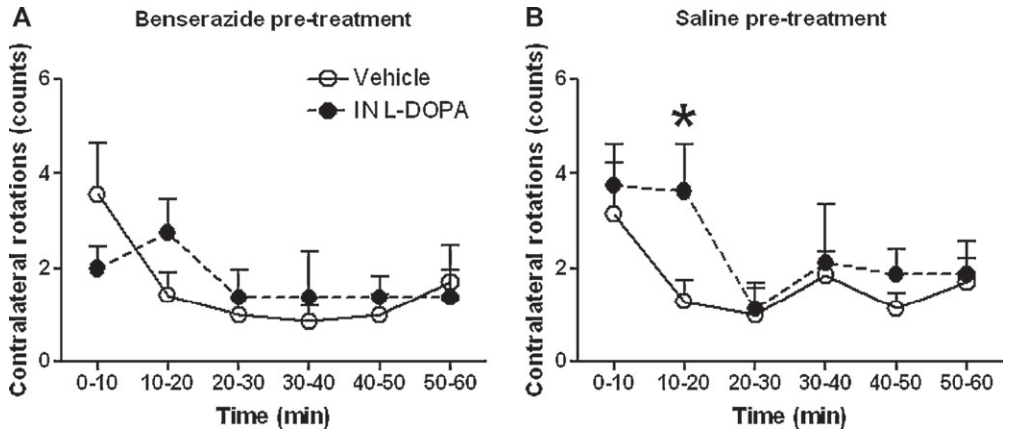

Fig. 3. Time-course of contraversive turning behavior following benserazide (A) or saline (B) pre-treatment. IN L-DOPA without benserazide pre-treatment increased contralateral turns 10–20 min after the treatment (B). Values are expressed as mean + S.E.M. *$p < 0.05$ compared to the vehicle group.

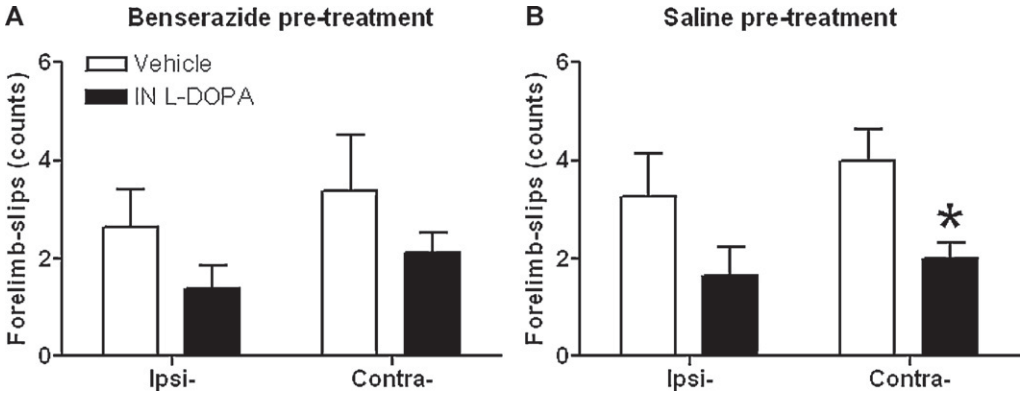

Fig. 4. Forelimb-slips of the hemiparkinsonian rats following the IN L-DOPA or vehicle administration with (A) or without benserazide pre-treatment (B). IN L-DOPA without benserazide pre-treatment reduced contralateral foot-slips (B). Values are expressed as mean + S.E.M. *$p < 0.05$ compared to the vehicle group.

The grid test measures skilled walking and increased foot-slips reflect impaired coordination. Vehicle-treated 6-OHDA lesioned animals showed an increased trend of foot-slips (Fig. 4), while the contralateral forelimb-slips were reduced by IN L-DOPA treatment without benserazide pre-treatment. IN L-DOPA had more sensitized behavioral effects in the contralateral side, which indicates that the L-DOPA administration via the IN route functions primitively in the lesioned hemisphere, thereby ameliorating the contralateral slips. Surprisingly, the animals with the unilateral 6-OHDA lesions showed no significant contralateral footslip preference. One reason may be that animals with contralateral-limb deficits, use the ipsilateral paws more to explore the grid and, thereby, increase the probability of ipsilateral footslips.

In the present study, the behavioral effects of IN L-DOPA were not significant when compared to vehicle-treated animals under the condition with benserazide pre-treatment. It is well known that systemic L-DOPA administration combined with benserazide ameliorates parkinsonian symptoms. This discrepancy with IN L-DOPA administration might be a result of a possible decreased AADC activity induced by benserazide. It is generally thought that benserazide cannot pass through the BBB, but there is evidence that benserazide increases extracellular neostriatal concentrations of DA and DOPAC [10] and a low dose of benserazide (10 mg/kg) decreased AADC activity in the striatum [18,40]. It is likely like that even the relatively low dose of benserazide (15 mg/kg in the present study) can affect the conversion from L-DOPA to DA in the brain. Thus, if benserazide entered the brain, AADC may have been inhibited and the conversion of L-DOPA to DA attenuated. Statistically, there was no different between the behaviors with benserazide pre-treatment and the behaviors with saline pre-treatment, suggesting that this possible influence is minor, however, enough to affect the actions of IN L-DOPA treatment. In a previous study of pharmacokinetic evaluation, IN L-DOPA administration combined with carbidopa showed a higher value of AUC from time zero to infinity when compared to IN L-DOPA administration alone [20]. However, the dose of carbidopa was likely too low (0.63 mg/kg), to influence the conversion from L-DOPA to DA in the brain. The present results do not rule out the beneficial effects of benserazide, but indicate the possibility that the behavioral effects of IN L-DOPA can be expressed without a peripheral AADC inhibitor.

The most common oral route for L-DOPA administration which was absorbed in the proximal duodenum is limited by high-protein diets and gastric factors [27,41]. Although intravenous injection has therapeutic action, it is inconvenient and painful for PD patients. IN administration of drugs provides an alternative method of delivery to bypass the BBB and to directly target the CNS [6,7,17]. Intranasally applied L-DOPA was rapidly absorbed into the brain and showed a higher bioavailability as compared with the oral and intravenous administration [20]. Thus, IN L-DOPA administration could be a useful therapy for a replacement of systemic L-DOPA administration for PD patients [20].

To summarize, it was shown, (A) that IN L-DOPA administration had behavioral effects by decreasing contralateral forelimb-slips, ipsilateral turnings and increasing contralateral turnings 10–20 min after the treatment, but did not influence the postural asymmetry in the cylinder test, and (B) IN L-DOPA administration had more sensitized behavioral effects under the condition without benserazide pre-treatment. These results suggest that L-DOPA can bypass the BBB following IN administration and alleviate motor impairments in animals with unilateral 6-OHDA lesions of the MFB, without requiring the combination of benserazide. It is the first study to show the behavioral effects of IN L-DOPA administration in the hemi-parkinsonian rat. Although IN L-DOPA administration showed mildly contralateral turning behaviors, it effectively decreased the number of contralateral foot-slips. The results of the present study are based on acute application of IN L-DOPA. Further studies employing high solubility of L-DOPA (methyl ester) and chronic IN treatment will decide whether this route of L-DOPA administration may be considered as an adjuvant treatment procedure for PD.

Conflict of interest

The authors declare that they have no competing financial interests.

Acknowledgements

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CHRONIC PROGESTERONE TREATMENT OF MALE RATS WITH UNILATERAL 6-HYDROXYDOPAMINE LESION OF THE DORSAL STRIATUM EXASPERATES PARKINSONIAN SYMPTOMS

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Abstract—Progesterone (PROG) shows neuroprotective effects in numerous lesion models, including a mouse model of Parkinson's disease (PD) induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, the possible beneficial effects of PROG on the behavioral and neurochemical impairments incurred in the hemiparkinsonian 6-hydroxydopamine (6-OHDA) model have not been investigated. Vehicle or PROG (4 mg/kg or 8 mg/kg) was daily applied over 13 days after unilateral injection of 6-OHDA into the dorsal striatum of male rats. Turning behavior, foot slips on a horizontal grid, and forelimb use during rearing in a cylinder were observed on days 4, 5, 9, 10, 13, and 14 postlesion, and then the brain samples were analyzed by HPLC-EC. Chronic 8 mg/kg of PROG administration increased the DOPAC/dopamine (DA) ratio in the lesioned striatum, ipsiversive turnings, and the number of hind limb slips and decreased the symmetrical use of forelimbs. Thus, contrary to hypothesis, the chronic treatment with PROG exasperated rather than alleviated the motor impairments in the hemiparkinsonian rats. Because previous studies with the MPTP model had shown protective effects when PROG treatment was administrated before the lesion, our results do not rule out such potential neuroprotective action with prelesion PROG treatment. However, our results raise the question of possible negative interactions between PROG and parkinsonian symptoms in males.

Key words: Parkinson's disease, progesterone, 6-OHDA, turning behavior, foot slips, forelimb use.

Parkinson's disease (PD) is associated with a loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). The resulting depletion of striatal dopamine (DA) leads to the major motor symptoms of PD, such as bradykinesia, resting tremor, muscle rigidity, and postural abnormalities (Dauer and Przedborski, 2003). These symptoms are reversed by DA replacement therapy using selective DA agonists or L-DOPA, which is still the most effective drug for the treatment of PD (Lang and Lozano, 1998; Brooks, 2008). However, the chronic administration of L-DOPA causes involuntary movements (Obeso et al., 2000; Ahlskog and Muenter, 2001), and DA replacement therapy cannot prevent the progression of PD. Thus, it is necessary to develop new therapies that can delay neurodegeneration or even restore the atrophied DAergic neurons in PD. Several neurotrophic factors have been shown to have neuroprotective and neurorestorative effects in animal models of PD, such as glial cell line-derived neurotrophic factor (GDNF) (Tomac et al., 1995; Gash et al., 1996; Kordower et al., 2000), conserved DA neurotrophic factor (CDNF) (Lindholm et al., 2007), and mesencephalic astrocyte-derived factor (MANF) (Voutilainen et al., 2009). However, neurotrophic factors with large molecular weight cannot cross the blood–brain barrier, and thus intraventricular infusion is necessary and some of these agents also cause side effects in humans (Lie et al., 2004; see Aron and Klein, 2011 for review).

Many studies have indicated a higher prevalence and incidence of PD in men than in women (Kurtzke and Goldberg, 1988; Mayeux et al., 1992; Marder et al., 1996; Baldereschi et al., 2000; Van Den Eeden et al., 2003; Wooten et al., 2004). This gender difference suggests an influence of sex hormones on the development of PD. Consistent with this assumption, estrogens play a neuroprotective role by decreasing the nigrostriatal DA loss induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice (Dluzen et al., 1996; Callier et al., 2001; Ramirez et al., 2003) and 6-hydroxydopamine (6-OHDA) in rats (Dluzen, 1997; Ferraz et al., 2008). Furthermore, increasing evidence suggests that progesterone (PROG) has neuroprotective effects in the CNS (Baulieu et al., 1996; Giachino et al., 2003; Magnaghi et al., 2006; Gonzalez Deniselle et al., 2007; Leonelli et al., 2007; Schumacher et al., 2007a,b). PROG decreased cerebral edema (Roof et al., 1994) and behavioral abnormalities (Shear et al., 2002; Djebaili et al., 2004) in a rodent model of traumatic brain injury, reduced neurological deficits after spinal cord injury (Thomas et al., 1999), and promoted functional recovery after middle cerebral artery occlusion in rodents (Gibson and Murphy, 2004; Sayeed et al., 2007; Cai et al., 2008). In intact male rats, a single dose of PROG increased tissue contents of DA and its metabolites in the striatum in postmortem samples (Di Paolo et al., 1986) and increased the extracellular striatal DA concentration measured by *in vivo* microdialysis (Petitclerc et al., 1995; de Souza Silva et al., 2008). PROG also exhibited neuroprotective effects against DAergic degeneration induced by MPTP (Grandbois et al., 2000; Callier et al., 2001; Morissette et al., 2008) and methamphetamine (Yu and Liao,

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Abbreviations: DA, dopamine; GABA, γ-aminobutyric acid; GP, globus pallidus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OF, open field; PD, Parkinson's disease; PROG, progesterone; SNc, substantia nigra pars compacta; STN, subthalamic nucleus; 6-OHDA, 6-hydroxydopamine.

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2000) in rodents. Thus, there is a substantial body of evidence suggesting that PROG has neuroprotective properties, that is, it attenuates detrimental effects of a manipulation when it is applied before the intervention. However, less is known about possible therapeutic effects of PROG, that is, the effects of a treatment applied following a manipulation.

In the previous studies, neuroprotective effects of PROG were reported when PROG was applied before MPTP administration (Grandbois et al., 2000; Callier et al., 2001; Morissette et al., 2008). However, to our knowledge, there has been no investigation of whether PROG can delay or restore the effects of a selective neurochemical lesion when administrated at the time of lesion onset. Therefore, in the present study, we evaluated the effects of chronic daily treatment with PROG on different tests of motor behavior in rats with a unilateral 6-OHDA lesion of the dorsal striatum. This lesion method was used to produce a progressive lesion (Kirik et al., 1998; Deumens et al., 2002), to be able to test the neuroprotective potential of the PROG treatment.

EXPERIMENTAL PROCEDURES

Animals

Twenty-nine male Wistar rats (Tierversuchsanlage, University of Düsseldorf, Germany) weighing between 250 and 350 g were used. Animals were grouped four or five in a cage and located in a standard environment with controlled temperature and humidity. They were housed under a reversed light-dark rhythm (light off from 07:00 to 19:00 h) and water and food was provided *ad libitum*. After arrival, they were given at least 10 days for adaptation before surgery. Each animal was handled 3 min daily for 3 days before surgery. All experiments were in accordance with the Animal Protection Law of the Federal Republic of Germany and of the European Communities Council Directive (86/609/EEC).

Surgery

Animals were anesthetized with 50 mg/kg pentobarbital (Narcoren, Merial GmbH, Germany; i.p.). The rats were placed in a Kopf stereotaxic frame; the scalp was cut and retracted to expose the skull. Aiming at the dorsal striatum, four holes were drilled and 6-OHDA (7 μg in 1 μl PBS with 0.1% ascorbic acid, flow rate of 1 μl/min) was injected at the following coordinates relative to bregma: AP: +1.3 mm, ML: −2.6 mm, DV: −5.0 mm; AP: +0.4 mm, ML: −3.0 mm, DV: −5.0 mm; AP: −0.4 mm, ML: −4.2 mm, DV: −5.0 mm; and AP: −1.3 mm, ML: −4.5 mm, DV: −5.0 mm (Kirik et al., 1998). The cannula was left in place for 2 min to prevent a reflux. Finally, the scalp was sutured and 70% ethanol was applied for disinfection.

PROG treatment

Animals were randomly divided into vehicle, 4 mg/kg PROG, and 8 mg/kg PROG (Sigma, Steinheim, Germany) treatment groups (n=10, 10, 9, respectively). PROG was dissolved in sesame oil (Sigma). The injection volume was 0.8 ml/kg (s.c.). The animals were treated daily for 13 days, starting 22–24 h after surgery, with the injections being applied between 17:30 and 18:30 h. The doses of 4 mg/kg and 8 mg/kg used in this study were shown to have behavioral or morphological effects in the animal models of traumatic brain injury (Roof et al., 1994; Murphy et al., 2002; Yao et al., 2005), spinal cord injury (Thomas et al., 1999; Labombarda et al., 2006), and middle cerebral artery occlusion (Sayeed et al., 2007; Cai et al., 2008; Ishrat et al., 2009).

Apparatus

A transparent plastic cylinder (30 cm in diameter and 45 cm high) was used to measure animals' use of forelimbs during rearing against the walls (Schallert et al., 2000; Schallert and Tillerson, 2000). The cylinder apparatus was located in a sound-attenuating room. A metal-elevated grid (41×41 cm^2, 41 cm high, with each grid cell 3.5×3.5 cm^2) was used to test animals' ability to accurately place their limbs during spontaneous exploration of the grid. The grid was located in the same room as the cylinder apparatus. An open field (OF; 48×48×48 cm^3), which was located in a sound-attenuating box (110×70×70 cm^3), was used to assess the animals' horizontal activity and turning behavior. Two red light bulbs provided illumination (luminous density on floor level ~8 lux). A camera was mounted 66 cm above the OF and connected to a DVD recorder and a personal computer. The behaviors were automatically recorded by the VIAS video imaging software (Schwarting et al., 1993; Duesseldorf, Germany). Ethanol (70%) was used to clean the apparatus following each trial.

Experimental procedures

All behavioral measurements were conducted between 10:00 and 17:00 h (before the treatments). The experimental procedure is shown in Fig. 1.

Cylinder test. Four, 9, and 13 days after the surgery, the animal was placed into the cylinder and its behavior was recorded on DVD for 5 min. An experimenter blind to the treatments, scored the animal's wall contacts with the ipsilateral (intact), contralateral (impaired), and both limbs. One animal from the vehicle group, which performed no wall contacts, was excluded from the analysis. A cylinder asymmetry ratio was calculated as the number of ipsilateral wall contacts plus 1/2 the number of both limbs' wall contacts, divided by the total number of wall contacts (ipsi + contra + both) (Woodlee et al., 2005).

Grid test. Testing was conducted 90 min after the cylinder test. The animal was placed on the center of the grid and allowed to freely explore it for 5 min. All behaviors were DVD-recorded and analyzed by an experimenter blind to the treatment groups. A foot slip was scored either when the paw completely missed a rung, and thus, the limb fell between the rungs, or when the paw was correctly placed on the rung but slipped off during weight bearing (Baskin et al., 2003; Menet et al., 2003; Starkey et al., 2005).

OF test. Five, 10, and 14 days after the surgery, the animal was put into the OF for 30 min, and the distance it moved and rotational behaviors were analyzed. A single dose of the DA D2/D1 receptor agonist apomorphine (0.25 mg/kg, Sigma; diluted in saline; injection volume 1 ml/kg) (Kirik et al., 1998) was injected s.c. 14 days postlesion to test for contraversive turnings (Ungerstedt, 1971; Schwarting and Huston, 1996). The percentage of the rotational asymmetry was computed as follows: the ipsiversive

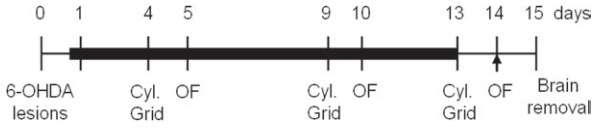

Fig. 1. Experimental procedure of daily progesterone treatments in rats with a 6-OHDA lesion in the dorsal striatum. Twenty-four h after the surgery, the animals received vehicle or PROG administrations daily over 13 days. Behavioral tests were conducted on days 4,5,9,10,13 and14 after the lesion. A single apomorphine injection was used to test for contralateral turnings on day14 postlesion. One day after the apomorphine injection, the animal's brain was removal for neurochemical analysis. Black bar: daily PROG treatments. Arrow: apomorphine injection. Cyl, cylinder test.

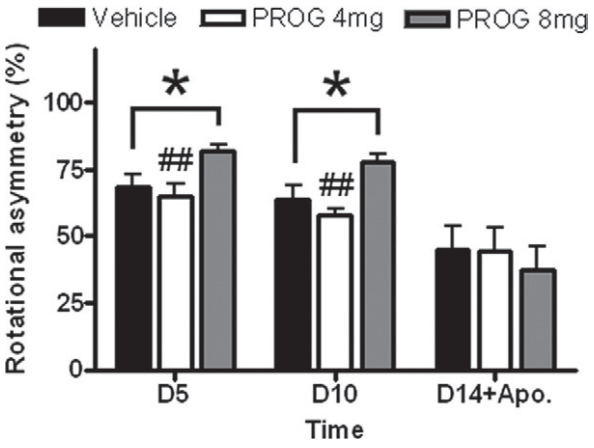

Fig. 2. Effects of daily PROG administrations on spontaneous rotational asymmetry and the asymmetries after an apomorphine injection. The 8 mg/kg PROG treatment increased asymmetry when compared to the vehicle and the 4 mg/kg PROG groups on days 5 and 10 post-lesion. After an apomorphine injection, no group difference was observed. The rotational asymmetry = (ipsilateral turnings / total turnings) × 100. Apo. = apomorphine; D = day. *$P<0.05$ compared to the vehicle group; $^{\#\#}P<0.01$ compared to the 8 mg/kg PROG group.

turning counts were divided by the total turning counts, and then multiplied by 100.

Neurochemical analysis

One day after the test for apomorphine-induced rotations, the animals were anesthetized by CO_2, decapitated, and their brains excised. The left and right dorsal striatum were dissected, homogenized, centrifuged, filtered, and stored at −80 °C until analysis. The samples were analyzed for the content of DA and its metabolites DOPAC and HVA and the main metabolite of serotonin, 5-HIAA, by means of HPLC-EC. 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 500 mV vs. an ISAAC reference electrode (Antec, Leyden, The Netherlands) at 30 °C.

Statistic

Two-way mixed ANOVAs with the factors treatment (vehicle, 4 mg/kg PROG, 8 mg/kg PROG) and time (3 test days) were conducted to analyze the use of the forelimbs in the cylinder test, the foot slips on the grid for each limb, and horizontal activity and turning in the OF. One-way ANOVAs were used to analyze the factor treatment at each time point when appropriate. The area under curve (AUC) of the symmetric forelimb use in the cylinder was analyzed by independent samples *t*-test. To compare the contralateral foot slips with the ipsilateral foot slips, paired samples *t*-tests were used. One-way ANOVA with the factor treatment was used to analyze horizontal locomotion and turning behavior after the apomorphine administration. Paired sample *t*-tests were used to compare the values from the lesioned striatum with the values from the intact striatum. One-way ANOVAs with the factor treatment were applied to analyze the neurochemical contents and their ratios for each side. Fisher's LSD was used for post hoc examination. All the values were presented as mean±SEM, and the level of significance was $P<0.05$ for all the tests.

RESULTS

OF test

The PROG administrations did not affect the horizontal activity in the OF. No effect of treatment and interaction between treatment and time ($P>0.05$), but an effect of time [$F(2,52)=79.85$, $P<0.001$] was found (data not shown). In the analysis of the rotational asymmetry, there was a main effect of treatment [$F(2,26)=3.713$, $P=0.038$] and an effect of time [$F(2,52)=106.646$, $P<0.001$], but no treatment× time effect ($P>0.05$) was found. Subsequent one-way ANOVAs revealed significant effects of treatment 5 days [$F(2,26)=4.808$, $P=0.017$] and 10 days postlesion [$F(2,26)=6.649$, $P=0.005$]. Post hoc tests showed that the 8 mg/kg PROG group had a higher asymmetry score than the vehicle and the 4 mg/kg PROG group 5 days ($P=0.027$, $P=0.006$, respectively) and 10 days postlesion ($P=0.019$, $P=0.001$, respectively) (Fig. 2).

The analysis of the apomorphine test did not indicate significant main effects for locomotion ($P>0.05$; data not shown) and rotational asymmetry ($P>0.05$) (Fig. 2).

Grid test

There were significantly more forelimb slips on the contralateral side in all the groups 4 days [all groups ($P=0.001$); data not shown], 9 days [vehicle ($P<0.001$), 4 mg/kg PROG ($P=0.002$), 8 mg/kg PROG ($P=0.007$); data not shown], and 13 days postlesion [vehicle ($P=0.005$), 4 mg/kg PROG ($P=0.006$), 8 mg/kg PROG ($P=0.002$); data not shown]. In the analysis of the foot slips of the hind limbs, there were significant increases in number of contralateral foot slips in the 4 mg/kg PROG group 4 days ($P<0.001$; data not shown) and 9 days postlesion ($P=0.038$; data not shown). The 8 mg/kg PROG group also showed an increase in number of contralateral slips 9 days postlesion ($P=0.038$; data not shown).

In the analyses of the forelimb slips, there was no effect of treatment and no interaction between treatment

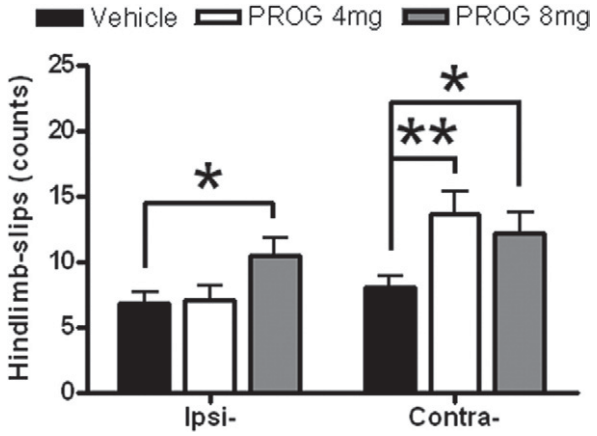

Fig. 3. Chronic PROG administration increased the hindlimb-slips on the grid. On the ipsilateral side, the 8 mg/kg PROG group exhibited more foot-slips than the vehicle group. The 4 mg/kg and the 8 mg/kg PROG groups also emitted more foot-slips than the vehicle group on the contralateral side. *$P<0.05$, **$P<0.01$ compared to the vehicle group.

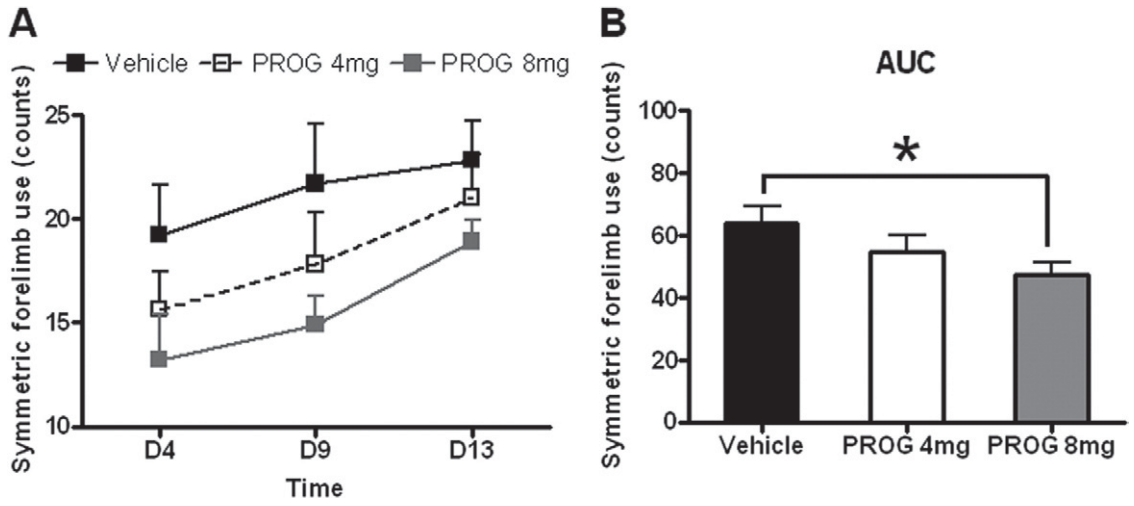

Fig. 4. Use of symmetric forelimbs in the cylinder on the testing days 4, 9 and 13 (A) and the area under the curve - AUC (B) in all the animals. There is a significantly lower symmetric forelimb use in the 8 mg/kg PROG group when compared to the vehicle group (B). D = day. *$P<0.05$ compared to the vehicle group.

and time ($P>0.05$), but significant effects of time [$F(2,52)=12.962$, $P<0.001$ for ipsi-; $F(2,52)=5.616$, $P=0.006$ for contra-] were found. In the analysis of the hind limb slips, there were significant effects of treatment [$F(2,26)=3.436$, $P=0.047$ for ipsi-; $F(2,26)=4.113$, $P=0.028$ for contra-], but no effect of time and no treatment×time interaction ($P>0.05$). The following post hoc tests showed that the 8-mg/kg PROG administration significantly increased hind limb slips on both sides ($P=0.027$ for ipsi-; $P=0.039$ for contra-), whereas the 4-mg/kg PROG treatment significantly increased the number of contralateral hind limb slips ($P=0.009$) (Fig. 3).

Cylinder test

The analyses of the forelimb-use asymmetry indicated no effect of treatment, time, and interaction between treatment and time ($P>0.05$; data not shown). However, there was a decreasing trend for the symmetric forelimb use in the high dose of PROG group (Fig. 4A). Accordingly, analysis of the AUC showed that the 8 mg/kg PROG group used both limbs during rearing less often than the vehicle group ($P=0.031$) (Fig. 4B).

Neurochemistry

Animals injected unilaterally with 6-OHDA into the dorsal striatum were relatively severely depleted of striatal DA in the lesioned side (Table 1). The extent of DA depletion was about 58% in the vehicle group, 68% in the 4 mg/kg PROG group, and 76% in the 8 mg/kg group, whereas no significant group difference was found ($P>0.05$). DA tissue levels were decreased in the lesioned striatum when compared with the intact side in all groups [vehicle ($P=0.001$), 4 mg/kg PROG ($P=0.006$), 8 mg/kg PROG ($P<0.001$)]. DOPAC levels were also reduced in the vehicle and the 4 mg/kg PROG groups ($P=0.013$, $P=0.026$, respectively). There was a significant decrease in 5-HIAA tissue levels in the animals of the 4 mg/kg PROG group ($P=0.009$).

There was no significant effect of treatment on tissue levels of DA, DOPAC, HVA, and 5-HIAA ($P>0.05$). However, there was a treatment effect in the analysis of the DOPAC/DA ratio in the lesioned striatum [$F(2,26)=5.531$, $P=0.01$]. Post hoc tests indicated that the 8 mg/kg PROG group had a higher DOPAC/DA ratio when compared with the vehicle group ($P=0.004$) and with the 4 mg/kg PROG

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

DA depletion	Vehicle($n=10$)		P 4 mg/kg ($n=10$)		P 8 mg/kg($n=9$)	
	58.22±6.69%		67.77±6.77%		76.18±7.45%	
	Intact	Lesioned	Intact	Lesioned	Intact	Lesioned
DA	451.60±60.72**	188.48±36.77	529.34±114.47**	146.26±34.81	381.84±45.81***	101.20±42.46
DOPAC	194.39±45.15*	122.36±36.06	179.79±35.07*	100.99±34.84	192.41±43.27	169.70±61.63
HVA	195.47±40.16	172.17±52.92	330.75±79.46	201.52±52.54	234.93±55.62	265.26±83.16
5-HIAA	40.23±11.27	22.32±6.12	37.32±6.94**	12.09±4.13	24.81±9.23	18.19±5.93

Values are expressed as ng/mg wet tissue weight. DA depletion=(intact DA content−lesioned DA content)/intact DA content, then×100. P, progesterone.

*** $P<0.05$, * $P<0.01$, ** $P<0.001$ compared with the lesioned side.

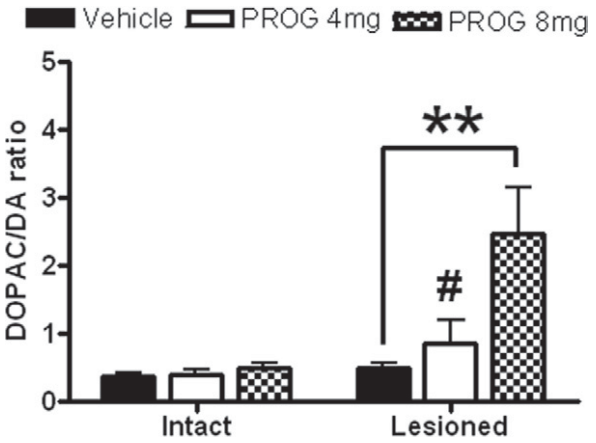

Fig. 5. DOPAC/DA ratio in the intact and the lesioned striatum. The 8 mg/kg PROG group showed a higher DOPAC/DA ratio than the vehicle and the 4 mg/kg PROG groups in the lesioned striatum. $^{**}P<0.01$ compared to the vehicle group; $^{\#}P<0.05$ compared to the 8 mg/kg PROG group.

group ($P=0.017$) (Fig. 5). There was no significant effect of treatment on the ratio of HVA/DA in either hemisphere ($P>0.05$; data not shown).

DISCUSSION

The main results of chronic daily treatment with PROG in animals given unilateral injections of 6-OHDA into the dorsal striatum were as follows: daily administration of 8 mg/kg of PROG enhanced the DOPAC/DA ratio in the lesioned striatum of unilateral 6-OHDA-lesioned rats. The ipsiversive turning asymmetry in the OF and the number of hind limb slips were increased by the 8 mg/kg of PROG treatments, whereas the dose of 4 mg/kg PROG also increased the number of contralateral hind limb slips. In the cylinder test, the symmetric forelimb use during rearing against the walls was decreased by the daily treatment with 8 mg/kg PROG.

Rats with unilateral 6-OHDA lesions rotate toward the side of the lesion (ipsiversive), whereas turning toward the side of the intact (contraversive) side is rare (Schwarting and Huston, 1996). In the present study, there was a PROG-dose-dependent increase in ipsiversive turning behavior on days 5 and 10 after the lesion (Fig. 2), whereas there was no effect on contraversive turning. Neurochemical results showed that in the lesioned striatum, the DOPAC/DA ratio was also increased in a PROG-dose-dependent manner. Thus, the increased turning asymmetry in the 8 mg/kg PROG-treated animals could have been influenced by the increased DA metabolism in the lesioned hemisphere. It is plausible to assume that PROG enhanced DA release in the intact side, thereby affecting the turning asymmetry. However, there was no difference in the DA tissue contents between the vehicle and PROG groups in the intact side, suggesting that the increased turning asymmetry by the PROG treatment was not because of an imbalance of DA release. Possible mechanism will be discussed later.

Daily treatment with 8 mg/kg PROG decreased the use of both forelimbs during wall-supported rearing. Previous studies showed that rats with unilateral 6-OHDA lesions performed fewer wall contacts with both forelimbs when compared with the presurgery baseline, whereas the sham animals did not show such a decrease (Woodlee et al., 2008). Thus, such a decrease in symmetrical forelimb use during rearing after a unilateral 6-OHDA lesion may be regarded as an index for a motor impairment. Consistent with this finding is that PROG administrations increased the number of contralateral hind limb slips on the grid. The 8 mg/kg PROG group showed behavioral effects on both sides. However, the contralateral side was more sensitized by PROG administrations because the low dose of PROG was enough to induce more contralateral slips. All the animals showed more contralateral than ipsilateral forelimb slips. In addition, PROG-treated animals exhibited contralateral than ipsilateral hind limb slips. Our previous findings indicated that L-DOPA treatment effectively reduced number of foot slips of hemiparkinsonian rats on a horizontal grid (Chao et al., unpublished observation), showing that more foot slips on the grid are an index for a motor impairment in the unilateral 6-OHDA-lesioned rats.

There was no effect of PROG-treatment on the contents of DA, DOPAC, HVA, and 5-HIAA in the intact and lesioned striatum. However, daily treatment with 8 mg/kg PROG enhanced the DOPAC/DA ratio in the lesioned striatum, which suggests that the chronic PROG treatment augmented DA turnover in the hemiparkinsonian rats. Previous studies have shown that PROG enhanced spontaneous DA release in the striatum of healthy male rats as measured by *in vivo* microdialysis (Petitclerc et al., 1995; de Souza Silva et al., 2008). Chronic PROG treatment increased DA transporter density in striatum and SNc of ovariectomized rats (Morissette and Di Paolo, 1993). These findings show that there is a cross-talk pathway between PROG and the neurotransmission of DA in the striatum, although the exact mechanism is yet to be determined. The fact that an acute treatment with PROG increased DA release in the striatum of rats (Petitclerc et al., 1995; de Souza Silva et al., 2008), suggests that this treatment has an amphetamine-like action. Such an action would seem to preclude the possibility of PROG being considered as a therapeutic tool for PD, although it is possible that such a psychostimulant-like action could favor recovery of function in other neurological lesion models, as has been reported (Roof et al., 1994; Thomas et al., 1999; Murphy et al., 2002; Yao et al., 2005; Labombarda et al., 2006; Sayeed et al., 2007; Cai et al., 2008; Ishrat et al., 2009). It should be mentioned that an acute injection of PROG also increased DA in the amygdale of rats (de Souza Silva et al., 2008). It is not known how such an effect could interact with DAergic depletion on the striatum, or how it could influence beneficial effects of PROG in other lesion models. However, in the present study, it is unlikely that the effects of chronic PROG treatment could be due to increased release of DA from intact terminals, as all the behavioral tests were conducted before the PROG treatments.

Taken together, the behavioral results point out a clear direction: chronic-applied PROG-treated hemiparkinsonian rats exhibited a higher ipsiversive turning asymmetry, more contralateral hind limb slips, and lower symmetrical use of the forelimbs during rearing behavior. Although chronic PROG administration affects the DA metabolism in the lesioned striatum, this neurochemical change may not be the only factor underlying the behavioral effects. PROG and its metabolites modulate several neurotransmitters including the activity of the γ-aminobutyric acid type A ($GABA_A$) receptor (Schumacher et al., 2000, 2007b). It is well known that the basal ganglia are extensively coordinated by the GABAergic system. Anatomical evidence indicates that the globus pallidus (GP) and SN pars reticulata receive GABAergic input from the striatal medium spiny neurons and the GP pars externa provides a dense GABAergic input to the subthalamic nucleus (STN). In MPTP-treated monkeys, local administration of muscimol, a $GABA_A$ receptor agonist, into areas outside of the motor territory of the STN induced circling and behavioral abnormalities, whereas injections into the sensorimotor region ameliorated motor impairments (Baron et al., 2002). Intrasubthalamic injection of muscimol in rats with a unilateral nigrostriatal lesion exacerbated motor deficits in the forced-step test, but alleviated some motor impairments (Mehta and Chesselet, 2005). Inactivation of STN by a $GABA_A$ receptor agonist relieved parkinsonian symptoms; however, under some conditions aggravated the motor deficits (Baron et al., 2002; Mehta and Chesselet, 2005). In the present study, the motor impairments that were induced by systemic PROG administration might have been a result of an influence on the basal ganglia neural circuit through the GABAergic pathway.

Even though we did not find evidence for a neuroprotective effect of PROG in the hemiparkinsonian male rat, the time point for PROG treatment might be an important factor to consider. In previous MPTP studies, PROG was applied for 5 days before until 5 days after MPTP injection (Grandbois et al., 2000; Callier et al., 2001; Morissette et al., 2008). Thus, PROG influenced the nervous system before the lesion, perhaps by increasing the expression of antiapoptotic molecules, such as B-cell lymphoma (Bcl)-2 (Yao et al., 2005), which showed protective effects against MPTP and 6-OHDA in SNc neurons (Yang et al., 1998; Yamada et al., 1999). In the present study, PROG was administrated after the lesion and thus might have lost its neuroprotective effects. Furthermore, PROG activates PROG receptors, and thus, the expression of ERK (Boonyaratanakornkit et al., 2001; Ballaré et al., 2003). In *in vitro* studies, activation of ERK contributed to the survival of neurons (Xia et al., 1995; Cavanaugh et al., 2006; Lin et al., 2008), but in some other conditions, neural cell death was enhanced by persistent rather than transient ERK activation (Stanciu et al., 2000; Kulich and Chu, 2001). In an animal model of middle cerebral artery occlusion, daily PROG treatment (30 mg/kg) for 4 days exacerbated infarct volumes in the striatum, whereas a single dose did not (Murphy et al., 2000). Chronic PROG treatment (32 mg/kg) also negatively influenced the outcome of traumatic brain injury in rats (Goss et al., 2003). These results are consistent with the present findings that postlesion daily PROG (especially the high dose) administration exacerbated the behavioral impairments incurred by a dorsal striatal 6-OHDA lesion.

A critical factor that may contribute to the ineffectiveness of PROG to ameliorate the lesion-induced deficits could be that treatment was started 24 h after the lesion, which might be already too late to find significant effects. The lesion method used was introduced as a progressive lesion model of PD (Kirik et al., 1998). Walsh et al. (2011) found that the injection of 6-OHDA into the striatum induced a DA depletion in the striatum of about 50%, 6 h after surgery, whereas no significant degeneration of the nigral cells was evident within 72 h. Only after this period, a progression of the lesion extend was observed (Walsh et al., 2011). In the present study, the first treatment was applied 24 h after lesion surgery, which might have been too late to affect the initial strong effects of the toxin, which seem to occur within 6 h (Walsh et al., 2011).

The neuroprotective mechanisms of sex steroids for PD are complicated and interact with other steroids, dose, and gender. Although PROG and estrogens have shown to have neuroprotective effects in preventing striatal DA depletion by MPTP toxicity (Dluzen et al., 1996; Grandbois et al., 2000; Callier et al., 2001; Ramirez et al., 2003; Morissette et al., 2008; see Bourque et al., 2009 for review), under some conditions they may worsen the degeneration of PD. For example, in young PD women, the progression of parkinsonian symptoms was more rapid during pregnancy (relatively higher levels of PROG and estrogens) (Rubin, 2007). In unilateral 6-OHDA-lesioned rats, estrogens had neuroprotective effects in females, whereas they had no effects or even exacerbated the neurodegeneration in males (Gillies et al., 2004, see Gillies and McArthur, 2010 for review). More studies are needed to assess the role of PROG in PD in relation to gender.

In the present study, it was shown that PROG has behavioral as well as neurochemical effects in rats with unilateral 6-OHDA lesions of the dorsal striatum. Chronic PROG administration increased DA turnover in the lesioned striatum and induced a higher ipsiversive turning asymmetry, more hind limb slips, and lower symmetric forelimb use, which are representative of motor impairments in the hemiparkinsonian rats. These findings are incompatible with the hypothesis of positive treatment effects with PROG administration after the onset of parkinsonism, but do not rule out possible beneficial effects of such treatment given in early stages of the disease onset. In fact, the impairing effects of PROG in this study raise the question of possible negative interactions between PROG-related substances and parkinsonian symptoms. It will be interesting to assess the effects of chronic PROG administration in advance of the initiation of the 6-OHDA lesion for the possibility of preventive, rather than ameliorative actions.

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THE GRID-WALKING TEST: ASSESSMENT OF SENSORIMOTOR DEFICITS AFTER MODERATE OR SEVERE DOPAMINE DEPLETION BY 6-HYDROXYDOPAMINE LESIONS IN THE DORSAL STRIATUM AND MEDIAL FOREBRAIN BUNDLE

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Abstract—The present study aims to evaluate the applicability of the grid-walking test in rats with moderate or severe dopamine-depletion incurred by unilateral nigro-striatal 6-hydroxydopamine (6-OHDA) lesions. Striatum samples were analyzed by high pressure liquid chromatography coupled to electrochemical detection (HPLC-EC) after behavioral testing. In Experiment 1, 2 weeks after the injection of 6-OHDA into the medial forebrain bundle, adult Wistar rats were divided into an L-3,4-dihydroxyphenylalanine (L-dopa) and a vehicle treatment group and their behaviors on the grid were compared. The severely lesioned animals (mean dopamine depletion of 92%) did not exhibit behavioral asymmetry in the number of contralateral foot-slips. However, L-dopa administration selectively reduced the number of foot-slips of the contralateral forelimb when compared with the vehicle group. In Experiment 2, 6-OHDA was injected into the dorsal striatum and foot-slips on the grid were analyzed 4, 9 and 13 days following the lesion. The rats with moderate dopamine-depletion (mean depletion of 54%) exhibited more contralateral forelimb-slips on all testing days. Compared with naive rats, hemiparkinsonian rats also showed more forelimb-slips. These results suggest that the grid-walking test should be a powerful and sensitive behavioral assay for sensory-motor deficits in rat models of nigro-striatal dopamine lesions.

Key words: 6-OHDA, grid-walking, foot-slips, medial forebrain bundle, dorsal striatum, hemiparkinsonian rats.

Parkinson's disease (PD) is a progressive, neurodegenerative disorder associated with a loss of dopaminergic neurons in the substantia nigra pars compacta. This degeneration of the nigro-striatal pathway is presumed to be the cause of the defining motor symptoms of PD such as bradykinesia, resting tremor, muscle rigidity, and postural abnormalities (Dauer and Przedborski, 2003). A widely-used animal model of PD applies unilateral injections of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) into the nigro-striatal tract, thereby producing behavioral asymmetries (Ungerstedt, 1976; Schwarting and Huston, 1996a,b). Several behavioral tests have been proposed to assess behavioral asymmetries in these so called "hemiparkinsonian" animals, such as drug-induced rotations (Ungerstedt, 1971; Steiner et al., 1985), adjusting steps of the weight-bearing forepaw (Olsson et al., 1995; Chang et al., 1999), food retrieval on a descending staircase (Whishaw et al., 1997; Barnéoud et al., 2000), food-grasping (Miklyaeva et al., 1994), the analysis of foot prints (Metz et al., 2005), asymmetrical thigmotactic scanning (Steiner et al., 1988), and asymmetrical forelimb use (Schallert et al., 2000). However, various studies report that the measurement of drug-induced rotations seems to be a poor indicator of an impairment of motor function (Chang et al., 1999; Metz and Whishaw, 2002b; Lane et al., 2006; Meredith and Kang, 2006). In tests involving food reward (Miklyaeva et al., 1994; Whishaw et al., 1997; Barnéoud et al., 2000), motivational factors may interact with motor variables. The adjusting-step test requires an experimenter to hold the animal (Olsson et al., 1995; Chang et al., 1999). Furthermore, it is difficult to evaluate motor impairments and behavioral asymmetries when the reduction of striatal dopamine is below 80% (Schwarting et al., 1991; Lee et al., 1996; Kirik et al., 1998; Deumens et al., 2002), wheras the onset of clinical motor symptoms in PD can occur with 60–80% losses of striatal dopaminergic terminals (Kish et al., 1988; Lang and Obeso, 2004). Given that the motor impairments induced by unilateral 6-OHDA-lesions can be very subtle, it is important to have multiple behavioral criteria for such assessments (Metz et al., 2000), particularly to develop sensitive tests for partial dopamine depletions in the hemiparkinsonian rat.

The grid-walking test (or foot-fault test) has been shown to be sensitive for evaluating the sensorimotor coordination of the four limbs in various rodent models of neurological diseases such as pyramidotomy (Z'Graggen et al., 1998; Starkey et al., 2005), spinal cord injury (Ma et al., 2001; Onifer et al., 2005; Sandrow et al., 2008), somatosensory cortex lesion (Barth et al., 1990; Napieralski et al., 1998; Shanina et al., 2006), and ischemic stroke (Zhang et al., 2002; Lourbopoulos et al., 2008). A previous study demonstrated that rotational activity on a grid is suitable for screening hemiparkinsonian rats; however, in that study foot-slips were not tested (Silvestrin et al., 2009). Since bradykinesia, rigidity, and postural abnormalities are the primary characteristics of PD motor symptoms (Dauer and Przedborski, 2003), observing skilled use of

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Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; dSTR, dorsal striatum; HVA, homovanillic acid; MFB, medial forebrain bundle; PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine.

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the limbs is likely to provide useful sensorimotor information on the hemiparkinsonian rat.

The objective of the present study was to evaluate the applicability of the grid-walking test to assess 6-OHDA-lesion induced motor impairments by measuring foot-slips through a grid. To this end, two experiments and one supplementary experiment were conducted; in Experiment 1, we investigated the effects of dopamine depletions incurred by injection of 6-OHDA into the medial forebrain bundle (MFB) on grid-walking. The MFB carries ascending dopaminergic neurons from the substantia nigra pars compacta to the striatum. This lesioned-procedure leads to severe nigrostriatal degeneration and a striking asymmetry in motor behaviors (Schwarting and Huston, 1996a; Deumens et al., 2002; Yuan et al., 2005). The lesion induced can be considered appropriate to investigate late-stage PD (Yuan et al., 2005). In Experiment 2, 6-OHDA was infused into the dorsal striatum (dSTR) in order to (a) determine the effects of a lesion that develops over time and to (b) investigate the influence of a moderate depletion of dopamine on foot-slips (Kirik et al., 1998). The injection of 6-OHDA into the striatum causes a progressive loss of substantia nigra neurons (Berger et al., 1991; Ichitani et al., 1991; Sauer and Oertel, 1994), which is useful as a partial dopamine depletion model in studies of functional recovery (Kirik et al., 1998). In the supplementary experiment, naive rats were also subjected to the grid-walking test to compare their behaviors with the hemiparkinsonian rats.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (Tierversuchsanlage, University of Düsseldorf, Germany) weighing between 250 and 350 g were used. They were grouped four to five in a cage with water and food provided *ad libitum* and they were housed under a reversed light–dark cycle (lights off from 7:00–19:00). The animals were given at least 1 week for adaptation before surgery. All experiments were conducted in conformity with the Animal Protection Law of the Federal Republic of Germany and with the European Communities Council Directive (86/609/EEC).

Experiment 1—MFB lesion

The animals were anesthetized with pentobarbital (50 mg/kg, i.p., Narcoren, Merial GmbH, Germany). Body temperature was maintained at normothermia using a heating pad. They were placed in a Kopf stereotaxic frame, and the scalp was cut and retracted to expose the skull. Holes were drilled above the MFB. 6-OHDA (10.5 μg in 3 μl PBS with 0.1% ascorbic acid) (Monville et al., 2006) was used to damage dopaminergic neurons by a unilateral injection into the right or left MFB (AP: −4.0 mm, ML: ±1.5 mm, DV: −8.5 mm; relative to bregma) (Metz and Whishaw, 2002a) with a 26-gauge steel cannula. The injection was made at a flow rate of 1 μl/min with a 10 μl Hamilton syringe and a micro-infusion pump. The cannula was left in place for 4 min to prevent a reflux. Finally, the scalp was sutured and 70% ethanol was used for disinfection. After the surgery, the rats were returned to their home cage and behavioral tests were started 2 weeks later.

Experiment 2—dSTR lesion

Surgery was carried out as described in Experiment 1. After anesthesia with pentobarbital (50 mg/kg, i.p.), the animals were placed in a stereotaxic frame with a heating pad beneath the body. The scalp was cut and holes were drilled above the dSTR. Then, 6-OHDA (7 μg in 1 μl PBS with 0.1% ascorbic acid) was injected at four coordinates unilaterally into the right dSTR: AP: +1.3 mm, ML: −2.6 mm, DV: −5.0 mm; AP: +0.4 mm, ML: −3.0 mm, DV: −5.0 mm; AP: −0.4 mm, ML: −4.2 mm, DV: −5.0 mm and AP: −1.3 mm, ML: −4.5 mm, DV: −5.0 mm; relative to bregma (Kirik et al., 1998). For injections a 10 μl Hamilton syringe, fitted with a 26-gauge steel cannula, was used. The injection rate was 1 μl/min and the cannula was left in place for 2 min before slowly retracting it. Then, the scalp was sutured and the wound was disinfected with 70% ethanol. The animals were allowed to recover for 3 days and the grid-walking test was conducted on day 4.

Grid-walking apparatus

An elevated metal square grid (41×41 cm^2, with each grid cell 3.5×3.5 cm^2; height: 41 cm) was used (Fig. 1A). The grid apparatus was located in a sound attenuated room with dim lighting. After each trial, 70% ethanol was used to clean the apparatus. A camera connected to a DVD-recorder was located below the apparatus with an angle of about 20–40 degrees (Fig. 1B). Behaviors on the grid were recorded on DVD and were analyzed later by an experimenter who was blind to the experimental design.

Procedures

Experiment 1. Two weeks after the surgery, the animals were given amphetamine (1.5 mg/kg, i.p.) and put into an open-field (48×48×48 cm^3) for 30 min. Their turning behaviors were recorded automatically by VIAS system (Schwarting et al., 1993) provided by Dr. Jay Shake Li (Li and Huang, 2006). Animals exhibiting more than 80% of ipsiversive turning were chosen for the grid-walking test (n=16). Then, the animals were randomly divided into the vehicle (n=8) or the L-dopa treatment group (n=8). Vehicle (castor oil with 0.8 ml/kg volume s.c.) or L-dopa (12 mg/kg with 0.8 ml/kg volume s.c.) were administrated 30 min before the grid-walking test. The animal was placed onto the central part of the grid apparatus and was free to explore the apparatus for 5 min. The behaviors were recorded on DVD, and analyzed afterward.

Experiment 2. For the animals with the dSTR lesion the grid-walking test was conducted 4, 9 and 13 days after the lesion (n=11). The behavioral testing of grid-walking and data acquisition was carried out as described for Experiment 1. Then, their behaviors on the grid were recorded and analyzed later.

Supplementary experiment. Seven naive rats were used for this experiment. All procedures were conducted as described above.

Drugs

Amphetamine, L-3,4-dihydroxyphenylalanine (L-dopa), castor oil, and 6-OHDA were purchased from Sigma-Aldrich (Steinheim, Germany). In Experiment 1, a single amphetamine injection was used to evaluate the lesion extent induced by the 6-OHDA injection (Hudson et al., 1993). L-dopa was suspended in castor oil for s.c. administration. Our previous study showed that the 12 mg/kg dose of L-dopa effectively modulated behaviors in 6-OHDA lesioned rats (Chao et al., in press).

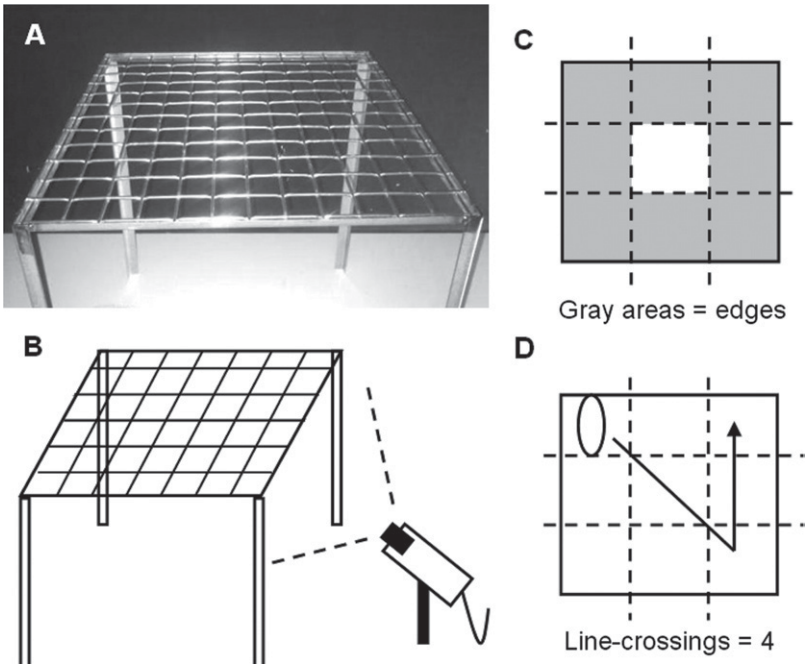

Fig. 1. (A) The grid-walking test apparatus, (B) the relative location between the apparatus and the camera, (C) the definition of the edges of the grid, and (D) example to count line-crossings. (B) The camera, connected to a DVD recorder, was located in front of the grid apparatus and lower than the grid with an angle. Video recorded by the camera was required to capture the whole extent of the grid in order to count foot-slips. (D) The white-oval shape represents an animal. The line and the arrow represent its moving trajectory, and in this example the number of line-crossings is four.

Behavioral analysis

The grid-walking test assesses spontaneous motor deficits and limb movements involved in precise stepping, coordination, and accurate paw placement. A foot-slip was scored (a) when the paw completely missed a rung and (b) the limb fell between the rungs, or (c) when the paw was correctly placed on the rung, but slipped off during weight bearing (Ma et al., 2001; Zhang et al., 2002; Baskin et al., 2003; Menet et al., 2003; Starkey et al., 2005). Counts of foot-slips for right forelimb, left forelimb, right hindlimb and left hindlimb were obtained. Later, the data were recorded as ipsilateral or contralateral to the lesioned side. Total foot-steps were also recorded in Experiment 1. Activities on the grid, latency to move, and time spent around the edges of the grid (Fig. 1C) were also assessed. The apparatus was divided equally into nine areas and line-crossings were recorded (Fig. 1D). In Experiment 2, these indexes were collected on day 13 after the lesion. The number of forelimbs- and hindlimb-slips of the naive and the hemiparkinsonian rats were compared.

Post-mortem analysis

Two weeks after the grid-walking test in Experiment 1 and 15 days after the dSTR surgery, the animals were anesthetized by CO_2, decapitated, and the brains were excised. The left and right dorsal striatum was dissected, homogenized, centrifuged, filtered, and stored at −80 °C until analysis. The samples were analyzed for their contents of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) by means of high pressure liquid chromatography coupled to electrochemical detection (HPLC-EC). 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, Leyden, The Netherlands) was set at 500 mV vs. an ISAAC reference electrode (Antec, Leyden, The Netherlands) at 30 °C.

Statistics

All the values were expressed as the mean±SEM. Paired samples *t*-tests were used to compare the ipsilateral foot-slips with the contralateral foot-slips and the neurochemical contents of the lesioned with the non-lesioned dorsal striatum. One-way ANOVA with the factor Treatment was used to compare the behaviors of the vehicle group with the behaviors of the L-dopa group. The behaviors of the naive rats were compared with the behaviors of the lesioned animals by separate one-way ANOVAs. A repeated one-way ANOVA with the factor TIME was conducted to analyze the foot-slips on the grid in the dSTR lesion experiment. The level of significance was $P<0.05$ for all the tests.

RESULTS

Neurochemistry

In the MFB lesioned animals, the content of dopamine, DOPAC and HVA in the lesioned striatum was significantly decreased when compared with the intact striatum ($P<0.001$, $P<0.001$ and $P=0.003$, respectively; paired *t*-test; Table 1). The mean reduction of striatal dopamine content was 92%. Dopamine and DOPAC were also significantly reduced in the striatum of the animals that had received unilateral 6-OHDA injections into the dSTR ($P=0.001$ and $P=0.009$, respectively; paired *t*-test; Table 1). The mean decrease in striatal dopamine concentration was 54%.

Table 1. Neurochemical results of the lesioned and the intact striatum in the 6-OHDA lesioned rats

	MFB lesion Lesioned striatum	Depletion 92.58% Intact striatum	dSTR lesion Lesioned striatum	Depletion 54.40% Intact striatum
Dopamine	7.98±5.17	116.10±16.29***	186.98±33.30	429.21±59.31**
DOPAC	56.74±11.45	90.04±10.52***	115.34±33.36	181.64±42.78**
HVA	2.44±0.58	27.25±7.17**	180.25±48.54	202.41±36.98
5-HIAA	17.99±3.49	22.37±2.94	23.47±5.66	38.71±10.31

Values are expressed as ng/mg wet tissue weight.

Depletion=(intact dopamine content–lesioned dopamine content)/(intact dopamine content), then ×100.

MFB lesioned rats $n=16$, dSTR lesioned rats $n=11$.

Paired *t*-tests were used to compare the lesioned and the intact striatum (** $P<0.01$, *** $P<0.001$).

Experiment 1

There was no significant difference between the ipsilateral foot-slips and the contralateral foot-slips within the vehicle and the L-dopa treatment groups ($P>0.05$; data not shown for hindlimbs). However, the animals treated with L-dopa showed significantly fewer contralateral forelimb-slips when compared with the vehicle-treated animals [$F(1,14)=8.463$, $P=0.011$] (Fig. 2A). There were no significant differences in line-crossings and foot-steps between the vehicle and the L-dopa groups ($P>0.05$; Table 2). In addition, L-dopa treatment did not influence the time spent around the edges of the grid and latency to move when compared with the vehicle group ($P>0.05$; Table 2).

Experiment 2

The animals with the dSTR lesion exhibited significantly more contralateral than ipsilateral forelimb-slips (Fig. 2B), although not in the hindlimbs (data not shown). This asymmetry was evident 4, 9 and 13 days following the lesion (all $P<0.001$; paired *t*-test). There was a TIME effect in the analysis of the ipsilateral slips [$F(2,20)=9.696$, $P=0.001$], whereas not contralateral slips ($P>0.05$). The slips of the ipsilateral forelimb were increased on day 13 as compared to day 9 ($P=0.001$; paired *t*-test with Bonferroni's correction; Fig. 2B). There was no significant effect in the analyses of hindlimb-slips ($P>0.05$; data not shown).

Supplementary experiment

The hemiparkinsonian rats with both, dSTR or MFB 6-OHDA injections, showed significantly more forelimb-slips than the naive rats (Table 2). The animals with severe dopamine depletion induced by injection of 6-OHDA into the MFB exhibited more forelimb-slips than the naive rats, irrespective of vehicle or L-dopa treatment [$F(1,14)=29.061$, $P<0.001$; $F(1,14)=24.341$, $P<0.001$, respectively]. The vehicle-treated animals spent less time close the edges of the grid when compared with the naive rats [$F(1,14)=6.793$, $P=0.022$], whereas the L-dopa-treated animals did not ($P>0.05$). No significant differences were found in the comparisons of line-crossings, hindlimbs-slips and latency for both MFB lesioned groups compared with the naive rats ($P>0.05$). L-dopa administration also significantly reduced forelimb-slips when

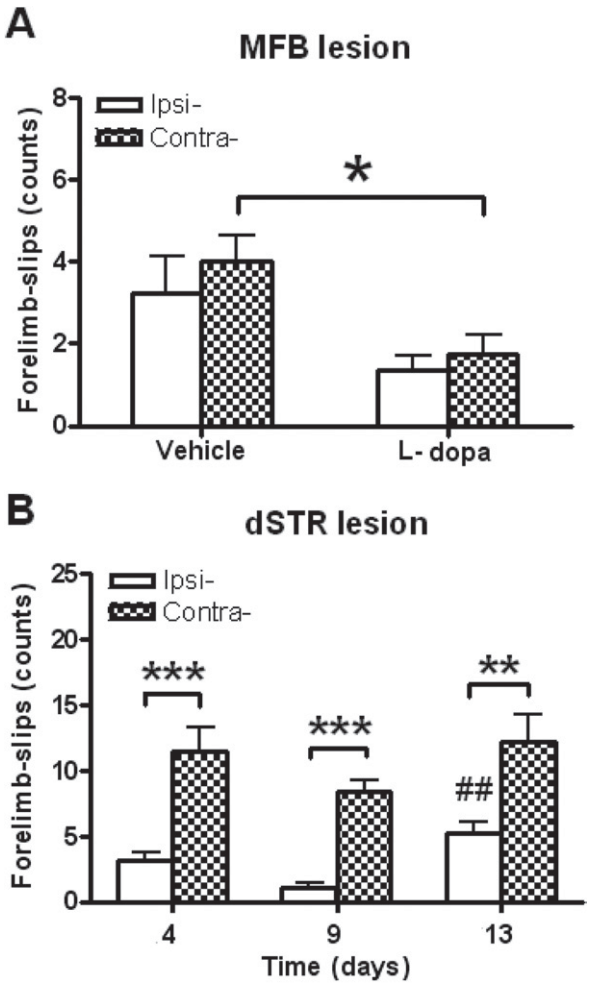

Fig. 2. Slips of forelimbs in the unilateral 6-OHDA MFB lesioned and in the dSTR lesioned rats. (A) The L-DOPA treatment group showed fewer slips of the contralateral forelimb when compared with the vehicle group. * $P<0.05$ compared with the respective vehicle group; one-way ANOVA. (B) The animals exhibited more slips of the contralateral than ipsilateral forelimb 4, 9, and 13 d after the surgery. The animal made more ipsilateral slips on day 13 as compared with day 9. ## $P<0.01$ compared with day 9 after the surgery. ** $P<0.01$, *** $P<0.001$ compared with the ipsilateral slips; paired sample *t*-test.

Table 2. Detailed behaviors of the hemiparkinsonian rats and naive rats on the grid

	Experiment 1 MFB		Experiment 2 dSTR	Suppl. naive
	Vehicle $n=8$	L-dopa $n=8$	$n=11$	$n=7$
Line-crossings	15.25±3.90	18.25±3.39	9.91±0.89**	20.43±2.83
Forelimbs slips	7.25±1.22***	3.13±0.55***†	17.45±2.5***	0.14±0.14
Hindlimbs slips	3.63±1.05	4.25±0.65	5.55±0.81*	2.71±0.84
Time in periphery	198.75±29.41*	255.75±10.82	229.64±22.15	283.43±8.14
Latency	6.50±3.14	7.13±3.89	4.73±2.71	1.43±0.3
Foot-steps	86.13±11.11	110.25±10.02	#	#

Counts of line-crossings, forelimbs slips, hindlimbs slips, foot-steps, latency to move (seconds) and time in peripheral areas (seconds).

Forelimbs slips = summation of the slips of both forelimbs; hindlimbs; slips = summation of the slips of both hindlimbs. Suppl. = supplementary experiment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with control group (one-way ANOVAs); † $P<0.01$ compared with vehicle group (one-way ANVOA). # data not provided.

compared with the vehicle-treated animals [$F(1,15)=9.493$, $P=0.008$], whereas no group differences were found in the analyses of other parameters ($P>0.05$; Table 2).

The animals with partial dopamine depletion induced by 6-OHDA injections into the dSTR exhibited significantly more forelimb- and hindlimb-slips than the naive animals [$F(1,17)=29.746$, $P<0.001$; $F(1,17)=5.37$, $P=0.034$, respectively; Table 2]. The dSTR lesioned rats also showed less activity when compared with the naive rats [$F(1,17)=17.921$, $P=0.001$], whereas no significant difference was found in the comparisons of time spent near edges and latency ($P>0.05$; Table 2).

DISCUSSION

This study describes a simple behavioral test that assesses foot-slips on a grid in rats with severe and partial dopamine-depletions of the nigro-striatal tract. L-dopa administration reduced the number of slips of the contralateral forelimb in the severely dopamine-depleted animals (mean 92%) that had received 6-OHDA-lesions of the MFB. The animals with the moderate striatal dopamine-depletion (mean 54%) expressed an obvious behavioral asymmetry by exhibiting more contralateral than ipsilateral forelimb-slips.

In Experiment 1, there was no difference in the number of foot-slips between the paws ipsi- and contra-lateral to the MFB lesions. It is likely that the severe dopamine reduction strongly affects the contralateral limbs and, thus, the animal depends more on the ipsilateral limb to explore the apparatus, thereby increasing the probability of ipsilateral foot-slips. It could also be that the serve dopamine-depletion influenced not only the ipsilateral- but the contralateral-hemisphere. Previous studies have shown that dopamine content was reduced also in the contralateral striatum 7 days after the unilateral 6-OHDA lesions (Pierucci et al., 2009) and alternations in both hemispheres were found by *in vivo* magnetic resonance imaging in 6-OHDA lesioned rats (Soria et al., 2011). The nigro-striatal afferents include interhemispheric connections (Pritzel et al., 1983; Morgan et al., 1985, 1986), which are likely to be influenced by severe dopamine-depletion animals and, thereby may also incur deficits in the limb usage ipsilateral to the 6-OHDA injection. In the present study L-dopa administration reduced the slips of the contralateral forelimb in the animals with a severely dopamine-depleted nigro-striatal system. The hypothesis that foot-slips on the grid assess motor impairments of hemiparkinsonian rats was supported by the improvement after the replenishment of dopamine by L-dopa treatment. The hemiparkinsonian rats exhibited more forelimbs slips than the naive rats (Table 2), which suggests that the increase in the number of foot-slips was likely because of the degeneration of dopamine neurons in the nigro-striatal pathway. There was a trend for fewer slips of the ipsilateral forelimb after the L-dopa treatment (Fig. 2A), but the treatment led to a more pronounced improvement of the impaired side as compared with the intact side. These results are consistent with previous observations that L-dopa alleviated motor impairments of the contralateral forelimb in hemiparkinsonian rats (Olsson et al., 1995; Chang et al., 1999). Given that total foot-steps and locomotion on the grid were not different between the vehicle and the treatment animals (Table 2), the reduction in contralateral foot-slips seen in the treatment group is likely to be caused by the L-dopa treatment.

The animals with moderate dopamine-depletion of the nigro-striatal tract showed remarkable behavioral asymmetries on the grid. In the hemiparkinsonian animal models, behavioral performance of the ipsilateral limbs (controlled by the intact hemisphere) are usually considered as a within-subject control for the contralateral side (the damaged hemisphere) (Schwarting and Huston, 1996a). The impaired function of the contralateral limbs were usually compared with the ipsilateral limbs in numerous behavioral tests, like limb-use asymmetry (Schallert et al., 2000; Tillerson et al., 2001), adjusting steps (Olsson et al., 1995; Chang et al., 1999), and forelimb placing (Schallert et al., 2000). Detecting motor impairments in animals with moderate dopaminergic lesions (below 80%) has proved to be difficult because of functional recovery taking place (Schwarting and Huston, 1997). However, these tests were used in animals with over 80% dopamine-depletion of the nigro-striatal tract (Olsson et al., 1995; Chang et al., 1999; Schallert et al., 2000; Tillerson et al., 2001). Here we

found that rats with less than 60% dopamine-depletion induced by the infusion of 6-OHDA into the dSTR can show a substantial behavioral asymmetry. The grid-walking test provides a suitable and sensitive behavioral assessment for testing the sensorimotor function of hemiparkinsonian rats, especially when the extent of the lesion is moderate.

We hypothesized that the impairment of the contralateral limb should increase over time. Tyrosine hydroxylase immunoreactivity in the striatum was largely decreased almost immediately after the infusion of 6-OHDA into the dSTR and then declined slowly (Grealish et al., 2008; Walsh et al., 2011). The slow atrophy of dopaminergic fibers taking place after lesion might be too slow to be detectable within 2 weeks of testing. Probably, more time is needed to determine such an increase of impairment parallel to the degeneration of dopaminergic cells caused by 6-OHDA-injections into the dSTR.

The vehicle-treated MFB-lesioned animals spent less time close the edges of the grid when compared with the naive rats (Table 2). Whether this index reflects motor impairments or other parameters, like emotional deficits, is not clear from the present data. The rats with a moderate dopamine depletion showed fewer line-crossings comparing with the naive rats. Since the animals were placed on the grid for the third time (day 13 after the surgery), they could have been habituated to the environment and, thus, showed less exploratory behavior. Nevertheless, both groups of 6-OHDA lesioned animals exhibited more foot-slips than naive rats on the grid (Table 2).

In both experiments, slips of forelimbs were more evident than slips of hindlimbs. According to our observations, there are three kinds of behavioral patterns on the grid: (a) the animal moves with four limbs, (b) the animal does not move, and (c) the animal's hind-limbs are immobile while the forelimbs are used to "explore" the environment. Slips of the forelimbs resulted from moving and exploring, but slips of the hindlimbs were primarily generated by moving. The time involved for moving and exploring was definitely more than that required for moving, thereby increasing the probability of forelimb-slips. Secondly, impairments in forelimb and digit use are the most obvious symptoms after nigrostriatal dopamine loss in rodents, especially when they are linked to sensorimotor integration (Aldridge and Berridge, 1998; Schallert and Woodlee, 2003). The grid-walking test assesses limb movements involved in sensorimotor functions, such as precise stepping, coordination and accurate placement of the paw. Accordingly, we found a significant reduction in foot-slips upon treatment with L-dopa (Experiment 1) and more foot-slips of the contralateral forelimbs (Experiment 2).

The ladder-rung walking test provides a similar behavioral assessment as the present grid-walking test. The ladder-rung apparatus consists of two transparent walls with metal rungs inserted at irregular distances to create a floor, which evaluates skilled walking, coordination of four limbs, limb placing, and stepping (Metz and Whishaw, 2002a,b). Qualitative scales based on observing paw-usage on the floor have also been used (Metz and Whishaw, 2002a,b). Rats with unilateral 6-OHDA lesions of the MFB exhibited more slip-errors of all limbs when compared with the control group (Metz and Whishaw, 2002a); whereas no behavioral asymmetry (higher errors in the contralateral side) was observed (Metz and Whishaw, 2002a,b; Paquette et al., 2009), there was an asymmetry in the scores of paws (Metz and Whishaw, 2002b; Faraji and Metz, 2007). There is a study showing more errors in the contralateral side and no difference in the ipsilateral errors compared with the control group (Klein et al., 2009). Among differences between the ladder-rung walking and the grid-walking tests is that animals are "freer" on the grid than on the ladder in that they show a diversity of behaviors, like grooming, rearing, exploration, and turnings. This difference could lead to different results observed in the hemiparkinsonian rats, for example, evident results in the number of forelimb-slips rather than hindlimbs in the present study as compared with more errors of the hindlimbs than forelimbs on the ladder (Metz and Whishaw, 2002a).

L-dopa administration reduced the number of slips of the contralateral forelimb following severe unilateral dopamine depletion, which validates the applicability of this test upon pharmacological treatment. Furthermore, animals with moderate dopaminergic lesions (about 54% depletion) showed an increase of contralateral foot-slips in the grid-walking test. Our results suggest that the grid-walking test is sensitive to behavioral deficits in two different animal models of PD, and, thus, may provide a sensitive procedure to assess motor changes and within modulation in animals with a lower degree of dopamine depletion.

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Regular Article

The interaction between the dopaminergic forebrain projections and the medial prefrontal cortex is critical for memory of objects: Implications for Parkinson's disease

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ABSTRACT

Neuropsychological and neuroimaging studies have implicated the dopaminergic nigrostriatal pathway and the prefrontal cortex in learning and memory deficits in patients with Parkinson's disease. However, little is known about how these two brain regions interact in the processing of learning and memory. We employed a disconnection procedure to test whether interaction of these regions contributes to performance in various memory tasks. Male rats received either a unilateral injection of 6-hydroxydopamine into the nigro-striatal tract or a unilateral NMDA lesion in the medial prefrontal cortex, or both these lesions combined in either the same or opposite hemispheres. Spontaneous object exploration, spatial working memory, locomotor, emotional and sensorimotor tests were administered. Only the group with both lesions placed in opposite hemispheres failed to show object recognition memory. None of the groups treated with 6-hydroxydopamine showed intact temporal order memory, whereas only the groups that received combined lesions failed to show object-in-place and spatial recognition memory. No differences between groups were found in the spatial working memory test. Our data indicate that locomotor, emotional and sensorimotor factors are not likely to confound the results of the memory tests. Thus, the interaction between the dopaminergic forebrain projections, particularly the nigrostriatal dopamine, and the medial prefrontal cortex is critical for object recognition memory but not for spatial working memory in rats.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by atrophy of dopaminergic neurons in the substantia nigra pars compacta (SNc). The resulting dopamine (DA) deficiency of the nigrostriatal pathway is considered to account for the major motor symptoms in PD (Dauer and Przedborski, 2003). In addition to motor deficits, PD patients exhibit impairments in learning and memory (Lang and Lozano, 1998a, 1998b; Rodriguez-Oroz et al., 2009). Executive as well as visual–spatial functions are affected by PD (Dubois and Pillon, 1997) and the pattern of cognitive impairments in PD resembles that seen in patients with prefrontal cortex (PFC) lesions (Kehagia et al., 2010; Owen et al., 1992; Taylor et al., 1986). In particular, PD patients are deficient in tasks of temporal processing (Harrington et al., 2011; Sagar et al., 1988) and recognition memory (Cooper et al., 1993; Stebbins et al., 1999; Whittington et al., 2000). Learning and memory impairments are also found in animal models of PD, such as in 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys and rats (Da Cunha et al., 2001; Fernandez-Ruiz et al., 1995; Miyoshi et al., 2002; Taylor et al., 1990; Wang et al., 2009) and in genetically modified mice (Freichel et al., 2007; Pham et al., 2010; Zhu et al., 2007). Neuroimaging studies in PD patients suggest that the nigrostriatal (Dagher et al., 2001; Owen et al., 1998; Sawamoto et al., 2008) and the mesocortical (Cools et al., 2002; Mattay et al., 2002) pathways contribute to the cognitive deficits in PD. However, it is not clear whether the cognitive impairments in PD originate from the DA depleted nigrostriatal pathway or from the PFC, which is modulated by the mesocortical DA-pathway, or if they are caused by a disruption of the interaction between these brain regions. Given that both, the nigrostriatal pathway and the PFC, play an important role for cognition in PD patients, knowing the nature of their interaction is of critical importance for understanding learning and memory deficits in this population.

Accordingly, the aim of the present study was to investigate whether the interaction between the DA-depleted nigrostriatal pathway and the medial PFC (mPFC) contributes to learning and memory processing. To this end, male rats received a 6-hydroxydopamine (6-OHDA) injection in one hemisphere and a unilateral *N*-methyl-D-aspartate (NMDA) injection into either the ipsilateral or contralateral mPFC. When these lesions are applied in opposite hemispheres, the circuit is disconnected bilaterally at two different levels, whereas when the lesions are applied in the same hemisphere, an intact circuit is preserved in one hemisphere (Geschwind, 1965a, 1965b). This disconnection paradigm employing the hemiparkinsonian preparation together with the mPFC

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lesion provides the possibility to assess task specific functions of this circuit with the added relevance for understanding mechanisms that underlie PD-related cognitive deficits.

Materials and methods

Animals

Male Wistar rats (Tierversuchsanlage, University of Düsseldorf, Germany) weighing between 250 and 350 g were used. Until surgery they were housed in groups of five animals per cage. They were located in a standard environment under a normal light–dark rhythm (lights off from 19:00 to 07:00). Water and food was provided *ad libitum* except during periods of food restriction for behavioral testing (see below). Each animal was handled for about 3 min per day for three days before surgery. All experiments were in accordance with the Animal Protection Law of the Federal Republic of Germany and of the European Communities Council Directive (86/609/EEC). All efforts were made to minimize suffering and the number of animals used.

Surgery

Animals were anesthetized with 50 mg/kg pentobarbital (Narcoren; Merial GmbH, Germany, i.p.), placed in a Kopf stereotaxic frame, and the scalp was retracted to expose the skull. They received either a unilateral 6-OHDA-injection into the medial forebrain bundle (MFB) (6-OH, n = 13), a unilateral NMDA-injection into the mPFC (NM, n = 6), unilateral lesions of both areas in the same hemisphere (6-OH + NM S, n = 10) or in different hemispheres (6-OH + NM D, n = 16), or sham lesions (n = 8).

Holes were drilled into the skull above the target sites of the MFB and mPFC. 6-OHDA (10.5 μg in 3 μl phosphate buffer saline, pH 7.3, with 0.1% ascorbic acid, flow rate 1 μl/min; Monville et al., 2006) was injected into either the right or the left MFB (AP: −4.0 mm, ML: ±1.5 mm, DV: −8.5 mm; relative to bregma; Metz and Whishaw, 2002) in the 6-OH, 6-OH + NM D and 6-OH + NM S groups. After each injection, the needle was left *in situ* for an additional 4 min to prevent reflux. NMDA (2 μg in 0.2 μl phosphate buffer saline, pH 7.3, flow rate 0.1 μl/min; Lacroix et al., 2002) was injected into either the right or the left 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; Lacroix et al., 2002) in the NM, 6-OH + NM D and 6-OH + NM S groups. After each injection, the needle was left *in situ* for an additional 1 min to allow diffusion. The injections were applied with a 26-gauge steel cannula by a 10 μl Hamilton syringe connected to a micro-infusion pump. In the 6-OH + NM D group, the injection-sites were in opposite hemispheres. In the 6-OH + NM S group, the lesioned sites were in the same hemisphere. The sham group received phosphate buffered saline infusions unilaterally into the MFB and into the mPFC in the contralateral hemisphere. In the 6-OH and NM groups, phosphate buffered saline was injected into the mPFC and the MFB, respectively. The experimental design of surgeries is presented in Fig. 1. After the injections, the scalp was sutured and 70% ethanol was applied for disinfection of the wound. The animals were kept in a single cage until recovery from anesthesia, after which they were housed in the original groups again, except for one cage in which two animals were lost during surgery. Their body weights were recorded.

Apparatus

An acrylic open-field (60 × 60 × 30 cm), located in a sound attenuating room, was used to measure locomotor activity and turning. Dim light (luminous density on the center ~6 lx and ~4 lx in the corners) was used to provide illumination. A camera was mounted 2 m above the open-field and connected to a DVD-recorder. Several distinct geometric symbols were presented on the walls of the room as spatial cues. The behaviors were analyzed by the VIAS software (Schwarting et al., 1993) modified by Dr. Jay-Shake Li. The same open-field was used as an arena for conducting spontaneous object exploration tests (Dere et al., 2007; Ennaceur and Delacour, 1988; Ennaceur et al., 1997; Mitchell and Laiacona, 1998). Different sets of objects in quadruplicate were used. The objects were heavy enough to ensure that the rats could not move them. The assignments of objects for each test were counterbalanced and different sets of objects were used for each test. The heights and diameters of the objects were about 18–34.5 and 8–12 cm, respectively. The objects were made of different materials (plastic, glass, porcelain) and with different colors (white, red, green), shapes (column, square, irregular-shapes) and textures (smooth, rough). An acrylic light–dark box (50 × 35 × 30 cm) and an acrylic elevated-plus-maze (EPM) were used to assess anxiety-related behaviors. The light–dark box and the EPM were located in a sound attenuating room. The light–dark box consisted of two compartments (one being black and the other white), divided by a central barrier with a small passage. The dim light used in the open-field test was applied to provide illumination. The EPM was composed of two open arms (50 × 10 cm) and two walled arms (50 × 10 × 38.5 cm) and a central platform (10 × 10 cm). The two arms of each type were placed opposite to each other (luminous density on the closed arm ~40 lx and ~38 lx on the open arms). A camera was mounted 1.5 m above the apparatus and connected to a DVD-recorder. A radial-arm maze modified as a T-maze was used to assess working memory by a delayed nonmatching-to-place task (Dias and Aggleton, 2000; Murphy et al., 1996). The T-maze consisted of three walled arms (72 × 14 × 20 cm) and at each end there was a food well (diameter 3 cm, depth 1.5 cm). There was a central platform with a diameter of 46 cm. The maze was elevated 50 cm high and illumination was provided by a 36 W white light. A camera was mounted 1.5 m above the maze. Several geometric symbols were provided as spatial cues on the walls. A transparent plastic cylinder (30 cm in diameter and 45 cm high) (Schallert et al., 2000) and a metal elevated grid (41 × 41 cm, high 41 cm, with each grid cell 3.5 × 3.5 cm) were used to measure the use of forelimbs during rearing against the walls and the use of four limbs on the horizontal grid, respectively (Chao et al., 2012). Dim light bulbs were used for illumination (luminous density on floor level ~2 lx). The behaviors of animals in the cylinder and on the grid were captured by a camera and recorded on DVDs.

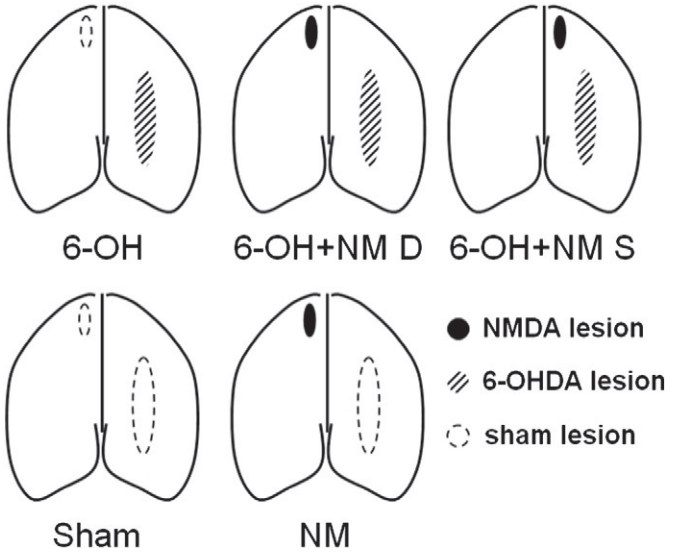

Fig. 1. Schematic of the experimental design. A shape represents a rat brain with two hemispheres. Either right or left sides of lesions are presented in one side.

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Behavioral testing

Behavioral assessments were conducted between 10:00 and 16:00. Ethanol (70%) was used to clean the apparatus after each trial.

Open-field test

Fourteen days after the surgery, the animal was placed into the open-field for 15 min for two consecutive days. The distance traveled, number of center crossings and turning were analyzed.

Object recognition

Two days after the open-field tests, the animal was put into the center of the arena and two copies of an object were placed in two corners of the apparatus. The animal was situated so as to face the opposite wall with no objects present and was free to explore the objects and the arena for 4 min (Barker and Warburton, 2011; Barker et al., 2007). Then, it was returned to its home cage. After 90 min, the animal was returned to the arena for 4 min and one of the previous objects was replaced by a novel one (Fig. 5A). Normal rats prefer to explore a novel object more than a familiar one (Ennaceur and Delacour, 1988).

Temporal order memory

One day after the object recognition test, a test for temporal order memory was conducted. This test was composed of two sample trials and a test trial. The animal was placed into the center of the arena with two copies of an object for 4 min (sample 1). The two objects were located in two corners of the open-field. After 30 min, the animal was put into the apparatus containing two identical novel objects for 4 min (sample 2). After 40 min, one of the objects used in sample 1 and one of the objects presented in sample 2 were placed into the open-field and the animal was allowed to explore the two objects and the arena for 4 min (Fig. 5B). The locations used for placing objects were identical in all the trials. Normal rats tend to spend more time exploring the old familiar object than the recent familiar one (Mitchell and Laiacona, 1998).

Object-in-place

This test involves the integration of object- and spatial information (Barker and Warburton, 2011; Barker et al., 2007). One day after the test for temporal order memory, four different novel objects were presented in the four corners of the arena. Each animal was placed into the center of the open-field and allowed to explore the objects for 5 min and then was returned to its home cage. After 5 min, it was returned to the arena in which the locations of two of the objects were exchanged, while the other two objects remained at the same place (Fig. 5C). The animal was allowed to explore the objects for 4 min. Normal rats explore the displaced objects more than the stationary ones (Barker and Warburton, 2011; Barker et al., 2007).

Spatial recognition

One day after the object-in-place test, the animal was put into the center of the open-field containing two identical copies of an object. All procedures were the same as described for the object recognition test, except that during the second trial one of the objects was placed at a novel location (Fig. 5D). Normal rats spend more time exploring an object in a novel location (Ennaceur et al., 1997).

For all the spontaneous object exploration tests, object exploration was defined as a physical contact with the object with snout, vibrissae or forepaws. Climbing of the object, or contacting the object, but looking around the environment, were not included in this measure.

Light–dark box

The day after the spatial recognition test, the animal was placed into the center of the white compartment facing the black compartment and allowed to explore the box for 5 min. The time spent in the white and black compartments was measured.

Elevated plus maze

Two hours after the light–dark box test, each animal was placed onto the central platform of the EPM facing an open arm and allowed 5 min for free exploration. The time spent in the open and the closed arms was computed (Henniger et al., 2000; Pellow et al., 1985).

T-maze nonmatching-to-place

This task was conducted as described previously (Dias and Aggleton, 2000), with minor modifications. The animals were food-deprived to 85% body weight and maintained at this weight throughout the experiment. Before the task, the animals were given 15 min to explore the whole apparatus in which food pellets were scattered. Then, food pellets were placed into the food wells at the end of both arms. Pre-training was continued until animals ran reliably down the stem of the maze to find food pellets in both arms. Subsequently, a series of seven acquisition sessions, each consisting of six trials, was conducted. Each trial was divided into a "sample run" and a "choice run". Before each trial, one food pellet was placed in each food well, but an acrylic barrier blocked one of the arms. On the sample run, the animal was placed into the start arm and was free to find the food pellet. Since one of the two arms was blocked, the rat could only enter the arm with no barrier. After eating the food, the rat was returned to the start arm. A transparent acrylic board was used to confine the animal in the start area for 10 s and the barrier in the second goal arm was removed during the delay. Then, the board was raised and the animal was free to choose between the two goal-arms of the maze. A choice was scored when the hindlimbs entered one of the two arms. If the animal entered into the arm which was previously blocked, it could eat the food pellet in the well and was then returned to its cage (correct choice). If the animal turned into the arm which was previously entered, it was confined therein for 10 s and obtained no food reward and was then returned to its cage (wrong choice). A random sequence of three correct left and three correct right choices between the two arms was applied. The percent of correct choices was calculated for each session. Animals were free to eat food after completion of this task.

Cylinder test

Two days after the T-maze task, the animal was placed into the cylinder and its behavior was recorded for 5 min. The animal's use of its forelimbs while rearing up against the walls was scored. The frequency of the use of limbs ipsilateral (intact) and contralateral (impaired) to the MFB lesion was assessed.

Grid test

Ninety min after the cylinder test, the animal was placed into the center of the grid and was free to explore it for 5 min. All behaviors were recorded on DVD and analyzed offline. A footslip was scored either when the paw missed a rung and, thus, fell between the rungs, or when the paw was placed onto the rung but slipped off during weight bearing (Chao et al., 2012).

Neurochemical analysis

After the behavioral tests, the animals were anesthetized with CO_2, decapitated and their brains immediately excised. Both hemispheres of dorsal striatum and the nucleus accumbens (NAc), and the non-lesioned side of mPFC 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 and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5-HT) with

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its main metabolite 5-hydroxyindoleacetic acid (5-HIAA), by means of high-performance liquid chromatography-electrochemical detection (HPLC-EC). 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 500 mV *vs.* an ISAAC reference electrode (Antec, Leyden, The Netherlands) at 30 °C.

Histology

The lesioned side of mPFC was immersed in a 10% buffered formalin solution for at least 48 h and then transferred into a 30% sucrose–formalin solution and preserved at 4 °C. Coronal brain sections were cut with a cryostat (50 μm; Leica, Germany). The slices were stained with cresyl violet (Sigma Aldrich, USA) and used to assess the extent of the lesions.

Statistics

Only data of animals with a dopamine-depletion in the dorsal striatum of more than 70% were used for statistical analyses, which led to in the unequal group sizes described above. Repeated two-way ANOVAs with the factor group and days were applied to analyze behaviors in the T-maze and relative body weight. One-way ANOVAs with the factor group were used to analyze behavioral data and neurochemical values. Bonferroni *post-hoc* tests were conducted when appropriate. For the object exploration tests, mixed two-way ANOVAs with the within factor, object, and the between factor, group, were applied (Li and Chao, 2008). Since exploring one object prevents exploring the others, the time spent in exploring each is not independent from each other. Therefore, paired *t*-tests were used for the comparisons (Schable et al., 2012). As the sign of exploring preference is important for the interpretation, one-tailed statistical comparisons were applied (Li and Chao, 2008). One-sample *t*-tests were applied for the cylinder test to compute the comparisons against chance level performance. Paired sample *t*-tests were used to compare turning and foot-slip behavior and the neurochemical values from the lesioned side with the values from the intact side. With the exception of the paired *t*-tests applied in the object exploration tests, other statistical tests were two-tailed comparisons. All tests were with level of significance set at $p \leq 0.05$.

Results

Histology

The extent of the lesions in the mPFC was similar in the 6-OH + NM D, 6-OH + NM S and NM groups that received the NMDA injections. The mPFC lesions extended from the frontal pole and continued rostrally to the genu of the corpus callosum. The dorsal anterior cingulated, the prelimbic and the infralimbic areas were damaged, with minor variability between the groups. A diagram of the lesions is presented in Fig. 2.

Neurochemistry

DA depletion was found in the dorsal striatum in the 6-OH, 6-OH + NM D and 6-OH + NM S groups ($p = 0.011$, $p = 0.003$, $p = 0.001$, respectively). The animals with a DA depletion of less than 70% were excluded from this study. The three groups with the unilateral 6-OHDA injection showed over 90% DA loss in the lesioned striatum (Table 1). One-way ANOVA indicated that there was no significant group effect in the extent of DA depletion ($p > 0.05$). Details are presented in Table 1.

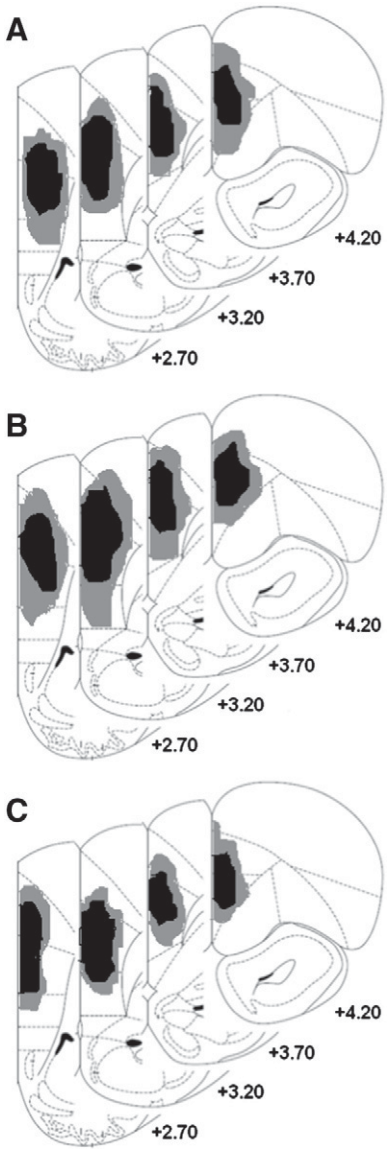

Fig. 2. Diagrammatic reconstructions of the NMDA injected mPFC. It shows that the smallest (black) and largest (gray) lesions in the 6-OH + NM D (A), 6-OH + NM S (B) and NM (C) groups. Either left or right sides of the lesions are presented in the right hemisphere. Numbers show the approximate distance (mm) from bregma.

The between-hemisphere comparisons of DA content of the NAc were significant in the 6-OH, 6-OH + NM D and 6-OH + NM S groups ($p = 0.001$, $p < 0.001$, $p = 0.027$, respectively). There was an average DA depletion of 78% in the three groups, with no significant differences between the groups ($p > 0.05$; Table 1).

In the mPFC, there were significant group differences in contents of DA and 5-HT on the side ipsilateral to the 6-OHDA-lesion ($F_{3,30} = 8.001$, $p < 0.001$; $F_{3,30} = 6.287$, $p = 0.002$, respectively). Significant effects of group were also found in the contents of DA and 5-HT on the contralateral side ($F_{3,30} = 5.017$, $p = 0.006$; $F_{3,30} = 6.488$, $p = 0.002$, respectively). *Post-hoc* tests showed that the NM group had higher DA content as compared to the sham, 6-OH ipsi-, 6-OH contralateral and 6-OH + NM D groups ($p = 0.002$, $p = 0.001$, $p = 0.007$, $p = 0.001$, respectively; Fig. 3A). On the other hand, the 6-OH ipsi-, 6-OH contralateral, 6-OH + NM D and 6-OH + NM S groups had lower 5-HT content when compared to the sham group ($p = 0.007$, $p = 0.003$, $p = 0.002$, $p = 0.004$, respectively; Fig. 3B). No significant difference was found in the comparisons of the ipsilateral and the contralateral sides in the 6-OH group ($p > 0.05$).

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Table 1
Neurochemical data from the dSTR and the NAc in the groups 6-OH, 6-OH + NM D and 6-OH + NM S. DA depletion = (intact DA content − lesioned DA content) × 100. Values are expressed as μg/mg wet tissue weight. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to the lesioned side. dSTR = dorsal striatum.

dSTR	6-OH	n = 12	6-OH + NM D	n = 9	6-OH + NM S	n = 9
DA loss %	94.11 ± 1.65		92.72 ± 3.49		95.98 ± 2.17	
	Intact	Lesioned	Intact	Lesioned	Intact	Lesioned
DA	417.14 ± 137.30*	17.65 ± 7.27	323.15 ± 72.51**	21.77 ± 13.76	489.40 ± 97.40**	12.76 ± 5.24
DOPAC	751.67 ± 330.89	1903.56 ± 1185.71	358.61 ± 111.60	450.24 ± 227.79	115.89 ± 28.97**	9.61 ± 3.15
HVA	190.53 ± 50.70	364.65 ± 233.61	101.37 ± 21.37	66.30 ± 18.89	232.68 ± 92.04	45.08 ± 14.35
5-HT	10.47 ± 2.65	11.86 ± 2.45	8.97 ± 3.69	5.77 ± 1.03	12.62 ± 4.17	7.93 ± 2.06
5-HIAA	21.72 ± 7.86	24.51 ± 4.65	17.99 ± 5.37	11.52 ± 1.70	27.55 ± 8.39	18.08 ± 6.22
NAc						
DA loss %	78.16 ± 7.93		87.53 ± 5.98		70.53 ± 11.50	
DA	173.30 ± 34.34**	43.29 ± 27.20	214.29 ± 30.42***	27.43 ± 14.27	453.88 ± 144.61*	68.88 ± 21.05
DOPAC	897.15 ± 463.35	473.32 ± 248.22	509.98 ± 225.15	369.79 ± 160.39	235.70 ± 98.84	36.61 ± 11.57
HVA	103.63 ± 28.07	326.92 ± 254.26	102.32 ± 18.38	79.20 ± 35.70	297.25 ± 110.69	109.48 ± 27.76
5-HT	18.93 ± 3.31	17.42 ± 4.85	19.78 ± 3.32	21.72 ± 6.90	45.91 ± 21.76	23.29 ± 6.82
5-HIAA	19.86 ± 2.89	25.26 ± 6.49	25.26 ± 4.38	26.20 ± 9.52	65.78 ± 25.75	35.77 ± 5.99

Body weight

There was no significant effect of group ($p>0.05$) and no effect of interaction between time and group ($p>0.05$), but a significant effect of time in the analyses of relative body weight ($F_{3,117}=102.575$, $p<0.001$). The following one-way ANOVAs showed no significant difference at any time point ($p>0.05$; data not shown).

Open-field

There was a significant effect of group in the analysis of distance traveled ($F_{4,39}=5.774$, $p=0.001$). The 6-OH group exhibited significantly less horizontal locomotion when compared to the sham, 6-OH + NM S and NM groups ($p=0.014$, $p=0.024$, $p=0.002$, respectively; Fig. 4A). The analysis of the frequency of center-crossings showed no significant effect of group ($p>0.05$; data not shown).

The 6-OH, 6-OH + NM D and 6-OH + NM S groups performed significantly more ipsilateral than contralateral turnings ($p=0.006$, $p=0.015$, $p=0.05$, respectively), while the sham and the NM groups did not show a significant turning bias ($p>0.05$; Fig. 4B).

Spontaneous object exploration

The 6-OH + NM D group showed an impairment of novel object recognition memory, while the other four groups did not (Fig. 5A). The two-way ANOVA showed that there was a significant effect of object ($F_{1,33}=35.88$, $p<0.001$), while no significant effect of group and effect of the interaction between object and group were found ($p>0.05$). The sham, 6-OH, 6-OH + NM S and NM groups spent significantly more time exploring the novel object than the familiar one ($t_7=3.208$, $p=0.008$; $t_7=2.250$, $p=0.03$; $t_8=5.452$, $p<0.001$; $t_5=2.873$, $p=0.018$, respectively), while the 6-OH + NM D group did not ($p>0.05$; Fig. 5A). No significant effect of group was found in total exploration time in the sample and the test trials ($p>0.05$; Table 2). For the temporal order test, a significant effect of object and an interaction effect between object and group were found ($F_{1,32}=10.847$, $p=0.002$; $F_{4,32}=3.991$, $p=0.01$, respectively), while there was no significant effect of group ($p>0.05$). The three groups of animals with the 6-OHDA injection did not show the preference for exploring the familiar object more than the recent familiar one ($p>0.05$), while the sham and NM groups did ($t_7=5.441$, $p<0.001$; $t_5=4.354$, $p=0.004$, respectively; Fig. 5B). There was no significant effect of group in total exploration time in the sample and the test trials ($p>0.05$; Table 2). In the analysis of the object-in-place task, there were significant effects of object, group and their interaction ($F_{1,29}=29.754$, $p<0.001$; $F_{4,29}=4.979$, $p=0.004$; $F_{4,29}=4.583$, $p=0.005$, respectively). The 6-OH + NM D and 6-OH + NM S groups did not spend significantly more time exploring the changed objects than the stationed ones ($p>0.05$), while the 6-OH, sham and NM groups did ($t_7=2.212$, $p=0.032$; $t_7=4.119$, $p=0.002$; $t_5=6.259$, $p=0.001$, respectively; Fig. 5C). However, there were significant effects of group in the sample and the test trials in the total exploration time ($F_{4,29}=3.742$, $p=0.014$; $F_{4,29}=4.979$, $p=0.004$, respectively). The 6-OH + NM S group spent less time exploring objects when compared to the NM group in the sample trial ($p=0.028$). In the test trial, the 6-OH + NM D and 6-OH + NM S groups

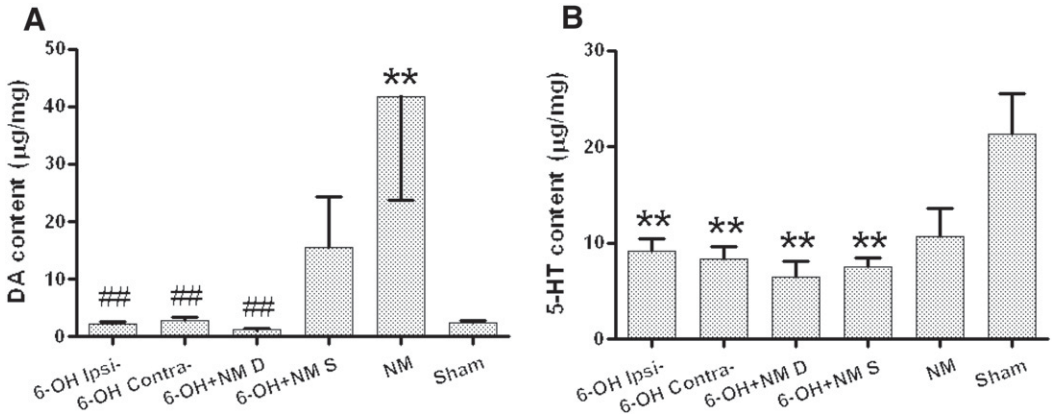

Fig. 3. Neurochemical results of DA (A) and 5-HT (B) contents in the non-lesioned mPFC. The NM group shows a higher DA content when compared to the sham group (A), while the three 6-OHDA-lesioned groups show lower 5-HT contents compared to the sham group (B). $^{**}p<0.01$ compared to the sham group; $\#\#p<0.01$ compared to the NM group.

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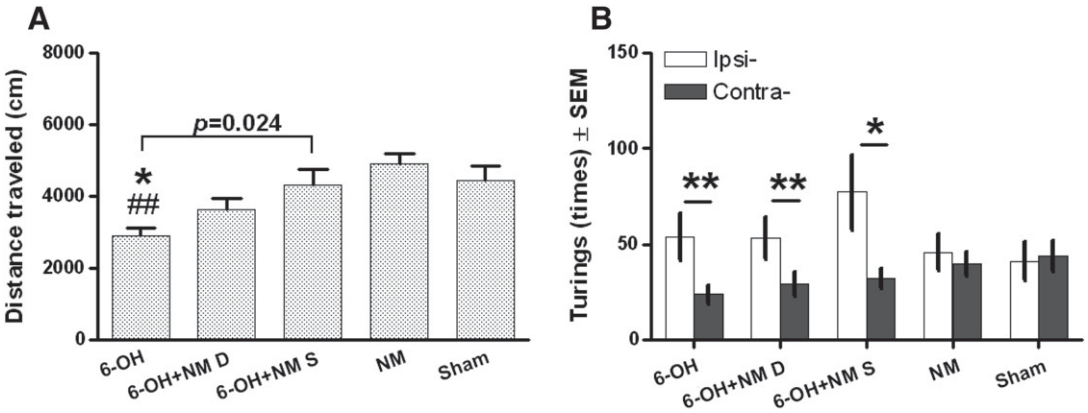

Fig. 4. Locomotion (A) and turning preference (B) in the open-field test. The 6-OH group shows less horizontal activity as compared to the sham, 6-OH + NM S and NM groups (A). $^{*}p<0.05$ compared to the sham group; $^{\#\#}p<0.01$ compared to the NM group. The three groups treated with 6-OHDA show more ipsilateral than contralateral turnings (B). $^{*}p<0.05$, $^{**}p<0.01$ compared to the contralateral side by paired sample *t*-tests.

showed less time exploring objects compared to the sham group ($p=0.048$, $p=0.003$, respectively; Table 2). Finally, there were significant effects of object and interaction ($F_{1,34}=40.88$, $p<0.001$; $F_{4,34}=2.717$, $p=0.046$, respectively), while no significant effect of group was found in the spatial recognition test ($p>0.05$). The sham, 6-OH, and NM groups spent significantly more time exploring the object at the novel location than at the old location ($t_7=5.589$, $p<0.001$; $t_{11}=3.348$, $p=0.035$; $t_5=2.466$, $p=0.029$,

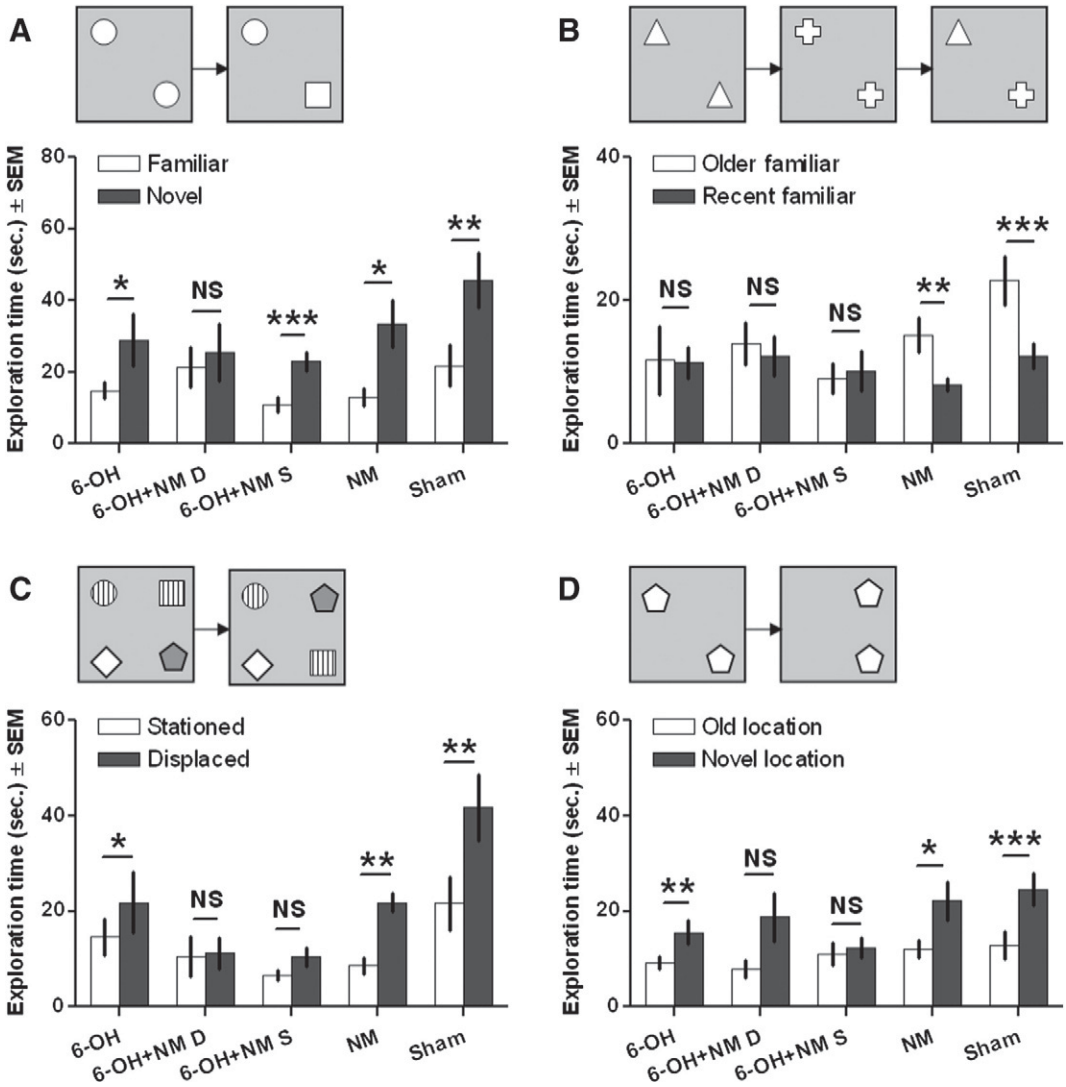

Fig. 5. Schematic of the spontaneous object exploration tests and performance of the experimental groups. Animals showed object exploration in the object recognition test (A), temporal order memory test (B), object-in-place memory test (C) and spatial recognition test (D). NS = non-significance; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared by paired sample *t*-tests.

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Table 2
Total object exploration time (seconds) in sample and test trials of spontaneous object exploration tests. OR = object recognition; TOM = temporal order memory; OP = object-in-place; SR = spatial recognition. S = sample trial; T = test trial. #$p<0.05$ compared to the NM group. *$p<0.05$, **$p<0.01$ compared to the sham group. The used numbers for object recognition test are n = 8, n = 7, n = 9, n = 6, and n = 8; for temporal order memory test are n = 8, n = 7, n = 8, n = 6, and n = 8; for object-in-place memory test are n = 8, n = 4, n = 8, n = 6, and n = 8; for spatial recognition test are n = 12, n = 4, n = 9, n = 6, and n = 8 for the 6-OH, 6-OH + NM D, 6-OH + NM S, NM and sham groups, respectively.

	OR		TOM			OP		SR	
	S	T	S1	S2	T	S	T	S	T
6-OH	49.7 ± 3.9	44.0 ± 8.7	25.4 ± 3.4	23.3 ± 4.6	23.1 ± 6.7	45.8 ± 4.1	36.6 ± 10.0	27.3 ± 3.0	24.8 ± 3.6
6-OH + NM D	59.9 ± 8.3	47.4 ± 10.9	28.9 ± 6.4	23.8 ± 4.9	26.3 ± 5.0	52.4 ± 8.0	21.8 ± 6.7*	23.3 ± 5.3	26.8 ± 5.3
6-OH + NM S	44.4 ± 3.5	34.1 ± 4.2	22.0 ± 2.9	17.9 ± 3.0	19.4 ± 4.2	41.3 ± 2.9#	13.7 ± 2.9**	25.0 ± 2.3	23.4 ± 3.7
NM	62.4 ± 5.7	46.7 ± 7.2	32.3 ± 4.0	30.5 ± 4.2	23.6 ± 3.2	58.8 ± 3.1	30.5 ± 3.0	34.7 ± 3.2	34.4 ± 4.7
Sham	63.5 ± 6.8	67.5 ± 11.1	29.0 ± 2.4	26.7 ± 3.0	35.0 ± 5.0	55.5 ± 2.7	63.5 ± 11.7	25.5 ± 1.6	37.5 ± 5.9

respectively), while the 6-OH + NM D and the 6-OH + NM S groups did not ($p>0.05$; Fig. 5D). No evident effect of group was found in total exploration time in the sample and test trials ($p>0.05$; Table 2). Animals which did not explore all the objects either in sample or test trials were excluded (Table 2).

Emotionality tests

No significant effect of group was found in the analyses of the behaviors in the light–dark box test ($p>0.05$; data not shown). Likewise, there was no effect of group in the analyses of the behaviors on the elevated-plus maze ($p>0.05$; data not shown). Animals which did not explore the elevated-plus maze or drop from the maze were excluded (n = 1 for each sham and 6-OH + NM D groups).

Spatial working memory

There was a significant effect of time ($F_{6,186}=5.077$, $p<0.001$), but no significant effect of group or interaction between time and group ($p>0.05$; data not shown). One-way ANOVAs showed that there was also no significant effect of group at any time point ($p>0.05$). Animals which did not respond to the task on the first day of training were excluded (n = 3 for 6-OH; n = 3 for 6-OH + NM D; n = 2 for 6-OH + NM S groups).

Cylinder test

There was a significant effect of group in the analysis of forelimb usage ($F_{4,36}=4.745$, $p=0.004$). The 6-OH and 6-OH + NM D groups showed higher percent for ipsilateral forelimb preference than the NM groups ($p=0.049$, $p=0.043$, respectively; Fig. 6A). The 6-OH, 6-OH + NM D and 6-OH + NM S groups showed a significant ipsilateral asymmetry in the use of forelimbs ($t_{10}=3.772$, $p=0.004$; $t_7=4.528$, $p=0.003$; $t_7=4.733$, $p=0.002$, respectively; one-sample *t*-test), while the sham and NM groups did not ($p>0.05$; Fig. 6A). Animals which did not explore the cylinder were excluded (n = 1 for each 6-OH, 6-OH + NM D and 6-OH + NM S groups).

Grid test

The 6-OH and 6-OH + NM D groups showed significantly more contralateral than ipsilateral forelimb-slips ($p=0.032$, $p=0.002$, respectively; Fig. 6B), while the sham, 6-OH + NM S and NM groups did not show a significant bias ($p>0.05$). No significant differences were found in the analysis of hindlimb-slips ($p>0.05$).

Discussion

The major finding in the present study was that object recognition memory was impaired by a unilateral lesion of the mPFC combined with a contralateral 6-OHDA lesion of the nigrostriatal pathway, while the same lesions placed into the same hemisphere did not have this effect. Unilateral 6-OHDA lesion of the nigrostriatal pathway only disrupted temporal order memory, while the groups with the combined lesions were totally deficient in memory for temporal order, object-in-place and spatial recognition memory. None of the experimental groups showed impairments in the T-maze non-matching to place task, a test for spatial working memory. The results indicate that the interaction between the mPFC and the DAergic forebrain projections, particularly the nigrostriatal pathway, is essential for the expression in object recognition memory.

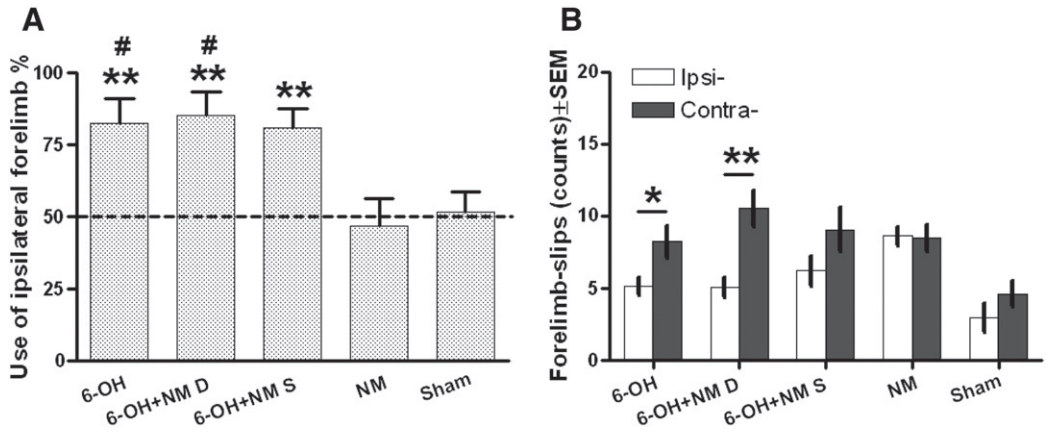

Fig. 6. Forelimbs usage preference of the experimental groups in the cylinder (A) and forelimb-slips of the experimental groups on the grid (B). The three groups treated with 6-OHDA show more uses of ipsilateral forelimb (A). Use of ipsilateral forelimb % = (counts of ipsilateral forelimb/total counts of ipsi- plus contralateral forelimbs) × 100. **$p<0.01$ compared to 50% by one-sample *t*-tests. #$p<0.05$ compared to the NM group. The 6-OH and 6-OH + NM D groups show more contralateral than ipsilateral forelimb-slips (B). *$p<0.05$, **$p<0.01$ compared to the ipsilateral side by paired sample *t*-tests.

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The unilateral 6-OHDA injection into the MFB in the present study not only led to severe depletion of DA (over 90%) in the striatum but also in the NAc (~78%). This pattern is similar to the pattern found in patients with PD where the DAergic nigrostriatal pathway is severely damaged, while the DAergic mesocortical/limbic pathways are also lesioned but less severely (Agid et al., 1993; Hornykiewicz, 1998; Javoy-Agid et al., 1984; Miller et al., 1997; Uhl et al., 1985). Bilateral injections of 6-OHDA into the NAc were reported to impair object recognition memory after a 24 hour but not 5 min delay, while spatial recognition memory was impaired (Nelson et al., 2010). Whether the unilateral loss of DA in the NAc in the present study contributes significantly to the behavioral deficits observed is not clear. In many studies, desipramine, a norepinephrine (NE) reuptake inhibitor, is administrated before a unilateral 6-OHDA injection for preventing lesions of NEergic neurons. Since desipramine was not applied here, 6-OHDA may also have damaged NEergic neurons. Thus, the present results may not be solely due to the lesion of the DA system.

In the mPFC, the unilateral MFB 6-OHDA injection did not affect DA, but decreased 5-HT content, while the unilateral mPFC lesions alone increased the tissue-level of DA, but did not affect 5-HT (Fig. 3). This result is consistent with previous findings on alternations of the 5-HT system in the PFC of parkinsonian rats and PD patients (Chen et al., 1998; Cicin-Sain and Jenner, 1993). Electrophysiological studies also show that the response of mPFC interneurons to 5-HT receptor stimulation is decreased in unilaterally 6-OHDA-lesioned rats (Gui et al., 2010; Zhang et al., 2010). However, the extent of mPFC 5-HT lesions and the alternation of mPFC DA in the present study were not sufficient to have an effect on object recognition memory (Fig. 5A).

It is known from lesion (Kolb et al., 1994; Meunier et al., 1997) and electrophysiological (Xiang and Brown, 2004) studies that the mPFC is involved in recognition memory tasks. More specifically, lesions of the mPFC disrupt acquisition of temporal order and object-in-place memory, but not spatial and object recognition memory (Barker and Warburton, 2011; Barker et al., 2007), whereas the mPFC, particularly the anterior cingulate cortex (Weible et al., 2012), is involved in consolidation of long-term object recognition memory (Akirav and Maroun, 2006). On the other hand, systemic administration of a DA D2 receptor antagonist and apomorphine impairs spatial recognition (Mehta et al., 1999) and item recognition memory (Montoya et al., 2008) in healthy human subjects, respectively. Systemic administration of a DA D1 receptor antagonist reduced the preference for novel objects (Besheer et al., 1999; Clausen et al., 2011), whereas DA D1 receptor agonists improved object recognition memory in rats (de Lima et al., 2011; Hotte et al., 2005). Animals with MPTP injections in the SNc exhibited deficits in object recognition memory (Sy et al., 2010; Wang et al., 2009). Furthermore, object recognition memory was restored in genetically DA-deficient mice by DA replenishment in the striatum by viral rescue (Darvas and Palmiter, 2009, 2010). Anatomically, the basal ganglia are tightly connected to the PFC. Outputs from the basal ganglia to the PFC are extensive and topographically organized (Middleton and Strick, 2002). In monkeys, a disconnection between the fontal cortex and the MFB impaired object association learning and object recognition memory in a delayed match-to-sample task (Easton and Gaffan, 2001; Easton et al., 2001). The interaction between these two regions could play a role in information processing involved in object recognition memory or in the transmission of memory to the medial temporal lobe (Easton and Gaffan, 2001; Easton et al., 2001), and according to our results, the forebrain DAergic projections seem to be crucial for these processes.

Temporal order memory was impaired in the three 6-OHDA-treated groups (Figs. 5B–C). These findings are consistent with human studies showing that PD patients are impaired in tasks of temporal processing (Harrington et al., 2011; Sagar et al., 1988). Administration of a DA D1 receptor agonist enhanced temporal order memory in rats (Hotte et al., 2005), which supports the hypothesis that DA modulates memory containing temporal components and strengthens the association between DA and time perception (Allman and Meck, 2012; Coull et al., 2011).

Rats with the mPFC lesion combined with the DA depletion were impaired in object-in-place and spatial recognition memory, while the hemiparkinsonian rats were not (Figs. 5C–D). The object-in-place memory task involves the presentation of four distinct objects. Furthermore, the relative locations of the objects to each other as well as the relationships between objects and spatial cues are relevant sources of information in this task. Animals with DA depletion combined with lesions of the mPFC showed less exploratory behavior during the test trial (Table 2), which may indicate that they interpreted the altered configuration as the original one and, thus, lost the motivation to explore. The performance of the combined lesion groups in the object-in-place test may also have been confounded by the deficits on spatial recognition (Fig. 5D). Accumulating evidence indicates that striatal DA plays an important role in spatial cognition. For instance, rats with bilateral 6-OHDA injection into the medial striatum preferred to use a response strategy in a place- and response-learning task, suggesting that DA in the medial striatum mediates spatial processing (Lex et al., 2011). Rats with striatal DA depletion also showed impaired spatial navigation (Braun et al., 2012; De Leonibus et al., 2007). In genetically DA-deficient mice restoration of DA in the striatum compensated for spatial memory impairments (Darvas and Palmiter, 2009). In normal animals, the mPFC is not involved in the acquisition of spatial recognition memory (Barker and Warburton, 2011; Barker et al., 2007). However, in the hemiparkinsonian rats the mPFC lesions led to an impairment of spatial recognition memory (Fig. 5D). This suggests that when DA is deficient in the nigrostriatal pathway, the mPFC is activated to participate in information processing. This explanation is consistent with the findings that PD patients showed increased cortical activity when performing cognitive tasks (Cools et al., 2002; Monchi et al., 2007), particularly, when the tasks do not require the striatum (Monchi et al., 2007).

The PFC (D'Esposito et al., 1995; Rowe et al., 2000) and nigrostriatal DA have been implicated in the function of working memory. In humans, striatal DA levels correlated with the activity of the PFC and working memory performance (Landau et al., 2009). Working memory capacity can be used to predict DA synthesis in the striatum (Cools et al., 2008). Animals with lesions of DA neurons in the SNc showed deficits of working memory (Braga et al., 2005), while a disconnection between the mPFC and the striatum impaired performance in a delayed alternation task (Dunnett et al., 2005; White and Dunnett, 2006). The lack of impairment found in the present spatial working memory task may be due to an involvement of another system (*e.g.*, the hippocampus) that is more important for spatial cognition. Taken together, the present results demonstrate that the interaction between the mPFC and the DA-forebrain pathways, particularly the nigrostriatal projection, plays a critical role in memory for objects but not in spatial working memory.

Rats with combined lesions showed no difference in locomotion when compared to the animals with sham lesions (Fig. 4A). On the other hand, the 6-OH group engaged in less locomotor activity not only compared to the sham group but also to the 6-OH + NM S and NM groups. Locomotor and sensorimotor activities may be modulated by a within-hemisphere mechanism between the mPFC and the nigrostriatal pathway (Alexander et al., 1986; Haber et al., 2000). No group difference was found in the two emotional tests, which is in line with the finding that hemiparkinsonian rats and controls show similar emotionality in the EPM test (Delaville et al., 2012). Lesion-induced turning behavior (Fig. 4B) and the sensorimotor deficits (Fig. 6) were not likely to affect object exploration because the 6-OH group still showed object recognition memory and no significant difference was found between the 6-OHDA-treated groups. Furthermore, there were no differences in total time spent in exploring the objects in the sample and test trials, except for the object-in-place test. There were no differences between the animals with the 6-OHDA lesion plus the mPFC lesion and the animals with sham

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lesion in locomotion and emotional behaviors. Thus, it seems unlikely that locomotor, emotional and sensorimotor factors contribute to the findings of the object recognition tests.

The present study provides evidence for a critical role of the interplay between the mPFC and the 6-OHDA-lesioned DA-forebrain pathways in the expression of novel object recognition memory in the rat. These findings also shed light on the possible mechanisms that underlie learning and memory deficits in PD, in-so-far as these involve interaction between the mPFC and the DAergic forebrain projections.

Conflict of interest

The authors declare that there is no interest of conflict.

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11. 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.

Düsseldorf, den 28.02.2013

Yuan-Hsin Chao