Neurochemical mechanisms of motoric, cognitive and affective functions in the hemiparkinsonian rat model

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Summary

Parkinson's disease is a progressive neurodegenerative disorder that affects approximately one percent of the population older than 60 years of age. It is characterized by a continuous loss of dopaminergic cells of the substantia nigra pars compacta, the appearance of Lewy bodies in neural tissue and is accompanied by a variety of characteristic motor impairments. Common symptoms include muscle rigidity, tremor, slowness of movement, as well as gait disturbances and postural instability. Traditionally, Parkinson's disease has been described primarily in the context of motor impairments and disorders of movement. However, there has been a considerable shift in this regard such that so called non-motor symptoms, namely cognitive and neuropsychiatric disorders, are increasingly being recognized as inherent disease entities. Little is known about the participating brain areas and corresponding neurochemical mechanisms involved in this context. Monoaminergic transmitter systems -including dopamine and serotonin- have been linked intimately to diverse cognitive and emotional functions and alterations in these systems likely contribute to the non-motor symptoms accompanied by Parkinson's disease. One set of experiments described here was set out to characterize the involvement of dopaminergic midbrain projections and the serotonergic system of the medial prefrontal cortex in the meditation of memory impairments, anxiety, as well as depression-related disorders associated with Parkinson's disease. To this end, stereotaxic disconnection procedures of the medial prefrontal cortex and the midbrain dopamine system were carried out in hemiparkinsonian rats. It was found that a functional decoupling of the constituents evoked behavioral alterations with respect to memory-, anxiety-, and depression-related measures, indicating a functionally relevant interaction between the medial prefrontal cortex and midbrain dopamine projections concerning cognitive and emotional functions. Moreover, this interplay was demonstrated to involve the serotonergic system of the medial prefrontal cortex, given that serotonin-specifc disconnections resembled the effects of unspecific lesions. These findings highlight the meaning of interactions between defined brain areas and related transmitter systems in the mediation of cognitive and affective alterations accompanied by Parkinson's disease and may be of value as to novel therapeutical implications.

An increasingly applied approach for the treatment of Parkinson's disease is deep brain stimulation of the subthalamic nucleus, a key structure for movement control in the basal ganglia. Despite its proven clinical efficacy, a mechanistic comprehension of the neurophysiological effects induced by deep brain stimulation lags its common application in Parkinson's disease patients. Several mechanisms of action have been proposed in this

context. Another set of experiments was set out to characterize the role of GABAergic and glutamatergic receptors expressed by neurons of the subthalamic nucleus with respect to motor impairments seen in hemiparkinsonian rats. To this end, animals with unilateral lesions of the nigro-striatal tract were injected either with the $GABA_A$-receptor agonist muscimol or the NMDA-receptor antagonist MK-801 (dizocilpine) directly into the subthalamic nucleus. Then, behavioral effects on movement parameters and locomotion were assessed. It was found that the stimulation of $GABA_A$-receptors evoked dose-dependent effects on motor parameters. A lower dose of muscimol counteracted the behavioral asymmetries induced by unilateral dopamine depletion. The administration of MK-801 had no apparent effect on motor parameters. These findings highlight the central role of GABAergic afferents of the subthalamic nucleus in counteracting motor impairments incurred by a loss of dopaminergic midbrain projections and imply conclusions as to the effective mechanisms of deep brain stimulation.

Zusammenfassung

Die Pakinson'sche Krankheit ist ein progredient neurodegeneratives Störungsbild, das in etwa ein Prozent der Population der Menschen über 60 Jahre betrifft. Kennzeichnend hierfür sind sowohl ein fortschreitender Verlust dopaminerger Zellen der Substantia nigra pars compacta, das Vorhandensein von Lewy-Körperchen in nervösem Gewebe, sowie auch eine Vielzahl von charakteristischen Einschränkungen der Motorik. Zu den Leitsymptomen zählen sowohl Muskelsteifigkeit, Zittern, eine allgemeine Verlangsamung der Bewegung, als auch Beeinträchtigungen des Gangbildes und der posturalen Stabilität. Vornehmlich wird die Parkinson'sche Erkrankung auch im Rahmen eben dieser motorischen Einschränkungen und Störungen der Bewegung betrachtet. Neuere Entwicklungen aber haben zu einer dahingehenden Neubewertung geführt, als dass die sogenannten nicht-motorischen Symptome, zu denen unter anderem kognitive Beeinträchtigungen und neuropsychiatrische Störungen zählen, merklich in den Fokus des wissenschaftlichen und klinischen Interesses geraten sind und zunehmend als ebenso krankheitsinhärent angesehen werden. Über die hierbei beteiligten Hirnareale und assoziierten neurochemischen Mechanismen ist jedoch bisher relativ wenig bekannt. Die monoaminergen Neurotransmittersysteme, zu denen auch Dopamin und Serotonin gezählt werden, sind nachweislich sehr eng mit einer Vielzahl kognitiver und emotionaler Funktionen verbunden und eine dahingehende Beteiligung dieser Systeme bei nicht-motorischen Symptomen der Parkinson'schen Krankheit erscheint als sehr wahrscheinlich. Ein Teil der in der vorliegenden Arbeit beschriebenen Experimente ging der Frage nach, inwieweit eine Beteiligung dopaminerger Mittelhirnprojektionen und des serotonergen Systems des medial präfrontalen Kortex bei der Vermittlung von Gedächtnisdefiziten, sowie angst- und depressionsassoziierten Störungen im Rahmen von Parkinsonsyndromen besteht. Hierzu wurden stereotaktische Diskonnektionsprozeduren des medial präfrontalen Kortex und des Dopaminsystems des Mittelhirns in der hemiparkinson'schen Ratte durchgeführt. Die Ergebnisse bestätigten die Annahme, dass eine funktionale Entkopplung beider Größen Änderungen in gedächtnis-, angst- und depressionsassoziierten Verhaltensmaßen hervorbringt und erbrachten somit einen Beleg für die funktionell relevante Interaktion zwischen dem medial präfrontalen Kortex und dem dopaminergen Mittelhirnsystem hinsichtlich kognitiver und affektiver Funktionen. Darüber hinaus wurde auch Evidenz für eine Abhängigkeit dieser Wechselwirkung vom serotonergen System des medial präfrontalen Kortex beobachtet, da die Verhaltenseffekte serotoninspezifischer Diskonnektionen denen von unspezifischen Läsionen ähnelten. Die Befunde heben die Bedeutung einer Interaktion zwischen umschriebenen Hirnarealen und

assoziierten Transmittersystemen in der Vermittlung kognitiver und affektiver Veränderungen im Rahmen der Parkinson'schen Krankheit hervor. Diese Erkenntnisse mögen von Wert für die Entwicklung neuartiger Therapieansätze sein.

Ein zunehmend zur Anwendung kommender Ansatz in der Behandlung der Parkinson'schen Erkrankung ist die tiefe Hirnstimulation des Nucleus subthalamicus, eines für die Bewegungssteuerung zentralen Kerngebietes innerhalb der Basalganglien. Trotz ihrer überaus erfolgreichen klinischen Anwendung sind die von der tiefen Hirnstimulation induzierten neurophysiologischen Effekte Gegenstand anhaltender Diskussion. Ein genaues mechanistisches Verständnis des therapeutischen Wirkens ist noch nicht erschöpfend charakterisiert. Eine Vielzahl möglicher Wirkmechanismen wird aber diskutiert. Weitere in der vorliegenden Arbeit beschriebene Experimente sind der Frage nachgegangen, inwieweit verschiedene Transmittersysteme des Nucleus subthalamicus an der Bewegungssteuerung in den dopamindefizienten Basalganglien beteiligt sind. Hierzu wurde Ratten mit unilateralen Läsionen des nigro-striatalen Traktes entweder der $GABA_A$-Rezeptor-Agonist Muscimol oder der NMDA-Rezeptor-Antagonist MK-801 (Dizocilpin) direkt in den ipsilateralen Nucleus subthalamicus injiziert und die Effekte dieser Manipulationen auf verschiedene Bewegungsparameter wurden erhoben. Es wurde eine dosisabhängige Beziehung zwischen der Stimulation von $GABA_A$-Rezeptoren und motorischen Größen beobachtet. Die Applikation einer geringen Dosis von Muscimol in den Nucleus subthalamicus war hierbei in der Lage die durch die dopaminergen Läsionen hervorgerufenen motorischen Asymmetrien wieder auszugleichen. Die Verabreichung von MK-801 hingegen zeigte keine positiven Effekte auf die motorischen Beeinträchtigungen. Diese Ergebnisse deuten auf die zentrale Funktion GABAerger Afferenzen des Nucleus subthalamicus bei motorische Defizite ausgleichenden Mechanismen im Rahmen der Parkinson'schen Erkrankung hin. Somit mögen diese Mechanismen auch eine bedeutsame Rolle bei der Wirksamkeit der tiefen Hirnstimulation spielen.

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1 Index of abbreviations

5,7-DHT - 5,7-Dihydroxytryptamine
5-HT - Serotonin
6-OHDA - 6-hydroxydopamine
BG - Basal ganglia
DA - Dopamine
DBS - Deep brain stimulation
DOPAC - 3,4-Dihydroxyphenylacetic acid
EPM - Elevated plus maze
FST - Forced swimming test
GABA - γ-Aminobutyric acid
GP - Globus pallidus
GPe - external Globus pallidus
GPi - internal Globus pallidus
HPLC-ED - High-performance liquid chromatography with coupled electrochemical detection
LB - Lewy bodies
L-DOPA - L-3,4-dihydroxyphenylalanine
MFB - Medial forebrain bundle
mPFC - Medial prefrontal cortex
MPTP - *1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin*
MSN - Medium spiny projection neuron
NAc - Nucleus accumbens
NMDA - *N*-Methyl-D-aspartic acid
NMS - Non-motor symptoms
OF - Open field
PD - Parkinson's disease
ROS - Reactive oxygen species
SN - Substantia nigra
SNc - Substantia nigra pars compacta
SNr - Sustantia nigra pars reticulata
STN - Subthalamic nucleus
VTA - Ventral tegmental area

2 Introduction

2.1 Basal ganglia

The basal ganglia (BG) consist of several subcortical nuclei and have been implicated in the processing and mediation of a variety of functions, including perception, movement control, attention, action selection/decision making, as well as learning and memory (Obeso et al., 2014). The BG receive input from virtually all areas of the cerebral cortex and relay this information into other networks which, in turn, provide direct access to behaviorally relevant (motoric) output systems. It is noteworthy that, despite its central role in the generation of behavior, the BG do not dispose of a direct projection to the spinal cord itself. Hence, the principle function of the BG in the modulation of behavior originating in cortical areas is not entirely determined yet. A widely accepted model in this context refers to the BG as a “central selector” which selectively disinhibits one of several competitive inputs from other brain systems that equally seek for entrance into the “final common motor path”, i.e. manifest behavior (Redgrave et al., 1999). A related concept expands this view and ascribes a fundamental role in the mediation of habitual responding and goal-directed behavior, respectively, to the BG (Redgrave et al., 2010). However, there are also other models emphasizing e.g. the pivotal role of the BG in training cortico-cortical connections for automatic behavior (Hélie et al., 2014). The neurotransmitter dopamine (DA) has been intimately associated with BG functioning and seems to play, especially in this context, a prominent role (Haber, 2014). The salience value of a particular input and, in turn, the facilitation of a distinct response driven by this input heavily depends on (preceded) phasic DA signals from the midbrain and, therefore, represents a neural implementation of learning experiences in the sense of operant conditioning.

The BG were associated with movement and motor control from the end of the nineteenth century on, when David Ferrier postulated that the striatum is strongly involved in automatic or sub-voluntary integration (Redgrave et al., 2010). Initially, the BG were thought to reflect a system primarily engaged in the automatic (unintentional) execution of previously learned motor plans (Wilson, 1912; Wilson, 1925; Marsden, 1982). In other words, the primary function (as well as dysfunction) was thought to be exclusively connected to motoric aspects of behavior. During the last decades, several lines of research indicated that the BG are also engaged in the processing of cognitive (associative) and affective (limbic) functions via a variety of distinct, but, parallel circuits

that connect the BG with other cortical and subcortical structures (Alexander et al.,1990). Such a revision also led to an extension concerning the conceptual framework of BG disorders. Alterations with respect to cognitive and affective domains turned into the clinical focus and are increasingly recognized as inherent characteristics of disorders that were formerly associated with mere motor impairments (Svenningsson et al., 2012; Lindgren and Dunnett, 2012). Common deficits seen in the context of e.g. PD feature cognitive impairments and neuropsychiatric comorbidities. Memory impairments, for instance, affect up to 80% of the PD population (Aarsland et al., 2003; Hely et al., 2008). Other disabling constraints frequently observed in PD patients are depressive symptoms, including mood disturbances, apathy and despair, as well as anxiety disorders. Such comorbidities affect up to 20% of the PD population (Reijnders et al., 2008) and often precede motor signs (Merschdorf et al., 2003), indicating their usability as potential diagnostic markers (Wu et al., 2011). The presence of such mood disorders is more pronounced in PD patients as compared to other disabling chronic illnesses (Tandberg et al., 1996), pointing towards PD-specific pathomechanisms being involved. However, there is a considerable lack of knowledge as to the underlying network changes in the parkinsonian brain. The degeneration of midbrain DA neurons seen in PD is accompanied by alterations in diverse brain areas and transmitter systems, including serotonergic transmission in the mPFC (Cicin-Sain and Jenner, 1993; Wang et al., 2009; Wang et al., 2010; Zhang et al., 2011; Gui et al., 2011). These circuits have been associated with the mediation of the aforementioned cognitive and emotional functions in healthy and diseased humans (Xu et al., 2012; Ferrer et al., 2012). Remarkably, preclinical research on the involved circuits and precise neurochemical mechanisms underlying the behavioral expression of the deficits has gained relatively little attention and there are only few animal models focusing on such aspects, although an understanding of the underlying neurochemical pathomechanisms and corresponding circuits involved is crucial in order to develop novel therapeutic strategies for such brain diseases.

2.1.1 Anatomy

The BG comprise at least four distinct structures: the striatum, the pallidum, the substantia nigra (SN) and the subthalamic nucleus (STN). In terms of ontogenesis, the striatum, as well as the pallidum and the STN, derive from the prosencephalon, whereas the SN arises from the mesencephalon. The striatum can further be divided into the caudate nucleus and the putamen. Anatomically, this distinction arises due to the macroscopic separation from

the inspreading internal capsule, a large tract of fibers that traverse the striatum during development. Despite the different spatial organization of the BG and cortical layers per se, distinct cortical areas more strongly project to particular parts of the striatum as to circumscribed others, meaning that there is a conservation of the cortical topography within the BG to a large extend. The vast majority (~95%) of neurons found in the striatum, i.e. the caudate-putamen complex, are “medium spiny projection neurons” (MSNs) which preferentially express γ-Aminobutyric acid (GABA) as neurotransmitter. These cells can also be divided with respect to their projections, i.e. the “direct” and “indirect” pathway. Furthermore, they also differentially express D1- and D2-like receptors. The remaining striatal neurons are interneurons within the striatum and comprise cholinergic large aspiny neurons and GABAergic medium aspiny neurons (Oorschot, 2010). Despite its macroscopical homogenous appearance, the striatum displays characteristic “Patch” and “Matrix” compartments which can be traced by neurochemical markers for calbindin and different opioid receptors (Gerfen and Bolam, 2010). This distinction coincidences with a temporally segregated innervation of distinct DAergic projections during early ontogenesis. The pallidum consists of the the globus pallidus and the ventral pallidum, whereas the globus pallidus can, according to functional aspects, further be divided into an external part (GPe) and an internal part (GPi). Both portions also project GABAergic efferents. Another principal component of the BG is the substantia nigra, which is made up of two physiologically and functionally segregated compartments, called the substantia nigra pars compacta (SNc) and the substantia nigra pars reticulata (SNr). The different compartments not only differ with respect to their projection targets, but also concerning the neurotransmitter synthesized in the respective portion. While the SNr utilizes GABA, the SNc expresses DA and plays a pivotal role in the modulation of overall BG activity. Furthermore, the subthalamic nucleus (STN) displays another BG constituent. The STN holds a strategic position within the cortico-BG-thalamo-cortical loop and expresses glutamate as a neurotransmitter. Due to this excitatory nature, the STN is thought to reflect the driving force of the BG (Charpier et al., 2010). Alterations in STN activity (e.g. resulting from a lack of DAergic innervation) evoke pathological activity patterns (burst firing, as well as abnormal oscillatory activity) that are relayed into the entire BG system (Brown et al., 2004; Kühn et al., 2004, Weinberger et al., 2006).

2.1.2 Circuitry

Excitatory input to the BG derives from the cortex, thalamus and the amygdala and forms

glutamatergic synapses with MSNs of the striatum, the principal structure of entrance to the BG (Gerfen and Bolam, 2010). The corticostriatal projections arise mainly from layer 5 based pyramidal neurons. The MSNs of the striatum form two dissociable pathways within the BG, i.e. the “direct” and “indirect” pathway. While direct efferent projections make contact with the GPi-SNr complex, the indirect pathway targets cells of the GPe. Striatal neurons of the direct pathway express D1-like receptors, while those of the indirect pathway express D2-like receptors. These G protein coupled receptors differentially affect the activity of the enzyme adenylyl cyclase which, in turn, regulates the intracellular concentration of cyclic adenosine monophosphate (cAMP). This second messenger also controls diverse ion channels and, therefore, modulates neuronal excitability. Hence, the direct and indirect pathway display antagonistic constituents with respect tho DAergic stimulation. The main output structure of the BG are the GPi, as well as the SNr. These nuclei project to diverse thalamic targets (centromedian nucleus, ventral anterior, ventral intermedius nucleus), the superior colliculus and the pedunculopontine nucleus which, in turn, project back to the cortex. The specific thalamic projection targets are different between species. In rodents, the principal target is the ventromedial nucleus. In primates the primary projection targets concerning GPi efferents are the ventral lateral, pars oralis and ventral anterior, pars parvocellularis nuclei and the ventral anterior and paralaminar medial dorsal nuclei concerning SNr efferents, respectively. The GPi-SNr complex exerts a tonic inhibition of these targets, thus, inhibiting net cortical excitability. In case of a reduction of this inhibition by the stimulation of the (GABAergic) direct pathway, cortical excitability increases and the conveyance of signals to brainstem nuclei and the spinal cord is facilitated. MSNs of the indirect pathway synapse with the GPe, which tonically inhibits STN activity. In turn, the STN exhibits excitatory inputs into the GPi-SNr complex by glutamatergic synapses. Therefore, activation of the indirect pathway results in an increase of the tonic inhibition of thalamic nuclei and the pedunculopontine nucleus by the GPi and the SNr. The overall result in this context is a decrease in (motor-) cortical excitability and, ultimately, leads to inhibition of behavior. Additionally, a third system can be distinguished as to BG pathways, i.e. the so called “hyperdirect” pathway which originates from collaterals of corticospinal fibers and makes direct contact with the STN, bypassing the striatal pathways. A subtle balance between the pro- and anti-kinetic influence of the BG on movement generation is thought to be overly necessary. Besides the large glutamatergic input into the BG, DAergic projections from the midbrain have a pronounced modulatory impact on the BG pathway activity and, therefore, represent a

rather critical factor in this context (Haber, 2014).

Moreover, recent advances in imaging structural and functional connectivity of the BG in vivo have further shed light upon the detailed organization of afferent and efferent projections of the BG (Lehericy et al., 2004). Applying diffusion tensor imaging, it was demonstrated that the head (or anterior part) of the caudate nucleus is primarily connected to prefrontal areas (medial, ventral and dorsolateral cortex, as well as pre-supplementary motor area), while the tail has connections to cortical areas associated with processing of visual information including inferotemporal cortex. These findings are also in line with evidence from an early study of BG functions that demonstrated differential behavioral effects of head or tail pronounced lesions of the caudate nucleus (Divac et al., 1967). Furthermore, functional connectivity analysis revealed that the (dorsolateral) putamen holds stronger connections to primary cortical areas, while the caudate nucleus is embedded in “higher-order” networks including prefrontal cortex, anterior cingulate and inferior frontal gyri (Postuma and Dagher, 2006). Taken together, these findings also point toward both structural and functional heterogeneity of the striatum. This finding is also of particular interest, given the uneven pattern of DA loss in the striatum of PD patients and it's functional implications (Kish et al., 1988).

2.1.3 Mesencephalic dopamine system

There are four major DA pathways that arise from either the SNc (A9), the ventral tegmental area (VTA; A10) and retrorubral area (A8) of the midbrain or the hypothalamus: (1) the mesolimbic, (2) the mesocortical, (3) the nigrostriatal and (4) the tuberoinfundibular pathway. There are also minor projections from the VTA that directly contact limbic structures, bypassing the nucleus accumbens (NAc). The nigrostriatal pathway originates in the retrobural area and the SNc and is the principle source of DAergic innervation of the striatum. A degeneration of these cells is thought to determine the motoric impairments accompanied by movement disorders. Additional evidence for such an assumption is derived from animal research showing that experimental DA depletion of this projection evokes characteristic motor symptoms including tremor, bradykinesia and rigidity. The mesocortical pathway connects the VTA with frontal and prefrontal cortical areas and also utilizes DA as a neurotransmitter. A lack of innervation in this context is associated with several of the cognitive impairments evident in PD. The mesolimbic pathway connects the VTA and the ventral striatum, i.e. the nucleus accumbens. The NAc is part of a network closely interconnected with limbic structures including amygdala and hippocampus

formation. The mesolimbic pathway plays an important role in the processing of reward-related stimuli and is often related to subjective “pleasure” of a particular stimulus or action. Given it's prominent role in emotion regulation, mood and anxiety disorders occurring in neurodegenerative diseases are discussed to be closely related to degeneration of the mesolimbic pathway.

2.1.4 Functional aspects

Functional aspects of the BG system may roughly be divided into three distinct domains, i.e. (1) motoric, (2) associative/prefrontal and (3) limbic functions (Alexander et al., 1986; Alexander and Crutcher, 1990; Alexander et al., 1990; Parent, 1990; Haber et al., 1995).

2.1.4.1 Motoric domain

Originally, the BG were thought to be solely implicated in the origin of movement disorders (Obeso et al., 2014) and, hence, in motoric functions in general. Such an assumption was extrapolated due to the observation described by Wilson (1914) that lesions of the putamen and GP are associated with dystonia and parkinsonism and that focal lesions of the STN or SNc give rise to hemichorea-ballism, as well as parkinsonism, respectively. Moreover, ablative techniques such as thalamotomy and pallidotomy, as well as stereotaxically induced lesions of the STN were common therapeutic approaches in the treatment of PD before the introduction of DA-substitution therapies in the 1960s. Taken together, these findings determined the conception of the BG as a structure primarily associated with motoric functions. Several hypo- and hyperkinetic movement disorders including PD and dystonia demonstrate characteristic electrophysiological and metabolic alterations in the motor loop of the BG (Grafton and DeLong, 1997; Turner et al., 1998; Ghilardi et al., 2000), which origins in the primary motor cortex (M1), the supplementary motor area (SMA), the premotor cortex (PMC) and the cingulate (ventral medial prefrontal) motor area (CMA). Motor-related signals are then conveyed into the BG, whereby there is a most widely conservation of topographic relations within. The loop is closed by thalamic and brainstem projections that synapse with the original (cortical) areas. In so doing, the motor-loop primarily projects via the ventrolateral nucleus of the thalamus. Activity within the direct pathway leads to a net decrease of the tonic inhibitory influence of the SNr and GPi on thalamic and brainstem nuclei and, therefore, increases cortical excitability and facilitates movement. In contrast, activation of the indirect pathway evokes an increase of the inhibitory tone on thalamic and brainstem nuclei, thus, leading to a reduction of

movement. The coordinated interplay of these two pathways is thought to be essential in physiological movement processing and there is a relationship of movement disorders and imbalance between the two constituents. In particular, afferent DA projections have a major impact on the fine tuning of balance between the pathways. A lack of DAergic (striatal) innervation, therefore, results in sever motor complications as seen in PD. However, it should be noted that there is only very limited evidence demonstrating a direct role of the BG in online motor control. Instead, several models are in favor of a more general function of the motor loop such as learning of automated or habitual motor behaviors (Bar-Gad and Bergman, 2001; Pisani et al., 2005; Doyon, 2008; Graybiel, 2008; Mandali et al., 2015).

2.1.4.2 Associative domain

Initial evidence for an involvement of the BG in cognitive functions (i.e. in particular learning and memory processes, as well as distinct executive functions) derived from studies conducted back in the 1950s. In these set of experiments monkeys were trained on several behavioral tasks (Rosvold et al. 1958; Battig et al., 1960). In one task they had to discriminate between two visual stimuli that were randomly presented in different locations. The monkey had to learn that a distinct stimulus (visual cue) was predictive of a reward despite of it's spatial position. Lesions of the striatum did not affect the monkey's ability to form appropriate associations and to perform correspondingly. In another task, animals were shown where a reward was hidden among several spatial locations and, after a certain delay during which the monkeys were not able to see the location, had to make a decision between the different locations available. In other words, the monkeys had to sustain a mental representation of the correct location in order to be rewarded afterward. Similarly, subjects showed no apparent signs of memory impairments with striatal lesions. However, if animals were challenged to decide between two identical objects presented in two constant locations, whereby the correct choice (location) alternated on each trial between the one and the other, monkeys with lesions of the anterior part of the caudate nucleus exhibited impaired performance in this task. The findings were interpreted as evidence for an involvement of the BG in working memory processes. Further evidence for mnemonic functions of the BG came from findings demonstrating alterations in operant responding after temporal inactivation of the caudate nucleus (Prado-Alcala et al., 1973; Prado-Alcala and Cobos-Zapiain, 1973). Cats were trained to press a lever for a reward. Two groups were introduced: One that was given 15

training sessions and one given 60 training sessions. After establishment of the response, animals were administered potassium chloride intracerebrally into the caudate nucleus that transiently blocked neural activity within that structure. The cats with extended training showed no behavioral difference with respect to baseline performance (i.e. kept on responding to the lever), while animals with only limited training displayed severe impairments in this task. These findings were interpreted as evidence that the BG are initially involved in learning of an operant response and that during the course of overtraining the corresponding associations are forwarded to another memory system. Such an explanation can incorporate the result that animals with only limited training experience did not exhibit the correct behavioral response. This experiment was one of the first to stimulate the hypothesis that the BG are differentially concerned with the control of instrumental behavior. According to this assumption, instrumental or operant behavior can be divided into goal-directed or habitual responding. While goal-directed behavior depends on the relative value of predicted outcomes of different (competing) actions, habitual responding occurs due to the presentation of a stimulus that was constantly paired with reward if a particular response was shown (Balleine and Dickinson, 1998). What kind of association underlies a particular response can be assessed applying appropriate behavioral paradigms. If a response leads to a certain consequence that is of value for the organism, a devaluation of that consequence, in the case of an underlying goal-directed mode of action, evokes a reduction of the response shown. Such a devaluation may be achieved by e.g. prefeeding a hungry animal, if the corresponding response would lead to a food reward. In such a case the animal would no longer show instrumental behavior if the underlying behavioral association would be of a goal-directed type. However, if the animal would persist on responding this would indicate an underlying habitual (or S-R) type of behavior. This behavioral mode may also be assessed by varying stimulus-response contingencies. If the animal keeps on responding, despite the fact that the reward will be delivered even without responding, the underlying process is likely a habitual one (Redgrave et al., 2010). Responses that are initially motivated by goal-directed actions undergo a transition during repeated pairing (over-training) with delivered reward and, therefore, become automatic responses that are under direct stimulus control. Concerning its neural correlates this transition has been described to follow a shift from ventral (accumbal) to dorsal and medial to lateral striatal territories (Belin-Rauscent et al., 2012). A double dissociation was demonstrated utilizing self-administration procedures in rats in this context (Corbit et al., 2012). Taken together, the BG play a very prominent role

in learning- and performance-related behavioral functions. They are discussed to flexibly shift between S-R and A-O behavioral outputs in circumscribed situations and determine what particular response is selected under a variety of competing behavioral options (Redgrave et al., 1999; Redgrave et al., 2010).

2.1.4.3 Limbic domain

The involvement of the BG in processing emotionally relevant stimuli has somehow been neglected (Berrdige et al., 2007), although several studies concerned with affective functions have demonstrated activation during emotional tasks in principle (Peron et al., 2013). An involvement in diverse emotional paradigms was shown in particular with respect to the STN in humans (Péron et al., 2010; Kühn et al., 2005; Huebl et al., 2011) and deep brain stimulation of that structure was observed to generally interfere with emotion processing (Bruck et al., 2011; Drapier et al., 2008). However, STN DBS was also successfully applied in obsessive-compulsive disorders which are also characterized by increased levels of anxiety, while animal studies showed that STN DBS evokes depressive-like behavior (Creed et al., 2013). Moreover, neuropsychiatric disorders are observed quite frequently if the BG lack of dopaminergic innervation (Reijnders et al., 2008; Gallagher et al., 2012; Frisina et al., 2009). A depressive-like phenotype can also be observed after experimental DA depletion in animals (Eskow-Jaunarajs et al., 2010; Zhang et al., 2011), as well as increased levels of anxiety (Espejo, 1997; Jungnickel et al., 2011). Taken together, there is clear evidence for an involvement of the BG in processing emotion-related information. On the other hand, it also seems to apply that the BG are part of a more comprehensive emotion-related network that also includes other cortical and subcortical nuclei including the medial prefrontal and orbitofrontal cortex, amygdala and anterior cingulate (Hamani et al., 2004). Such an assumption is also in line with the observation that BG nuclei hold distinct projections to these structures (Lambert et al., 2012).

2.2 Parkinson syndromes

2.2.1 Historical notes

Parkinson's disease (PD) is a neurodegenerative disorder, which was noticeably described by the british physician James Parkinson. In his famous writing “An Essay on the Shaking Palsy” from 1817, he already reported on characteristic motoric impairments in PD patients comprising resting tremor, abnormal posture and gait, as well as paralysis and decreased muscular strength (Lees, 2007). The profound impact of this early description on subsequent approaches in fields of research and clinic is reflected by contemporary concepts, which still define tremor at rest, rigidity, bradykinesia and postural instability as being the cardinal symptoms in PD (Jankovic, 2008). Researchers like Jean-Martin Charcot and Frederic Lewy further expanded and refined the understanding of the clinical manifestation of the disease, as well as the neuropathological alterations accompanied by PD. Furthermore, the prominent role of dopamine (DA) in the mediation of movement and motor control, as well as it's clinical implications for PD were discovered in the 1950s by leading scientists Arvid Carlsson (Bjorklund and Dunnett, 2007), as well as Hornykiewicz (2006). The introduction of Levodopa (L-DOPA) into clinical routine in the 1960s was another landmark with respect to pharmacological interventions. During the last decades, the remarkable advent of neuromodulative techniques such as deep brain stimulation (DBS) pioneered yet another step in the treatment of PD.

2.2.2 Classification and clinical features

The term *Parkinson syndromes*, or *Parkinsonism*, summarizes several disorders that are characterized by the cardinal symptoms bradykinesia, rigidity and tremor. Additionally, postural instability, flexed posture and freezing of gait have also been closely associated with clinical appearance. The most common form of these syndromes is *Parkinson's disease* (PD) or *idiopathic parkinsonism*. The term refers to the fact that in the vast majority of cases (~75%) a distinct etiology in the individual development cannot be determined explicitly. Other parkinsonian syndromes comprise heritable or familial forms that are due to either autosomal dominant or recessive inheritance. If causal factors are identified that can explain parkinsonian symptoms seen, the term *secondary* or *acquired parkinsonism* is often used. Several determinants are known to display a considerable parkinsongenic potential and may cause parkinsonian symptoms. Yet another class of

Parkinson syndromes is known as Parkinson plus syndromes. Here, there are additional symptoms present clinically. Among the Parkinson plus syndromes there are: multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration, as well as dementia with Lewy bodies.

Parkinson's disease / Idiopathic parkinsonism

The most prominent symptoms observed in PD patients are bradykinesia, rigidity and tremor at rest. These impairments occur when about 80% of striatal dopamine and/or ~50% of dopaminergic neurons in the SNc are degenerated (Fearnley and Lees, 1991). PD affects approximately 1% of the population older than 60 years of age (Rajput, 1992). There are distinct subtypes that can be divided based on the predominant clinical appearance: *akinetic-rigid*, *tremor* or *mixed* type. Bradykinesia refers to a general slowness of movement. Difficulties with fine motor tasks including handwriting or dressing are typically present with respect to daily living activities in PD patients. Rigidity is defined as an increased resistance during passive joint movement. Sometimes this presents as a cogwheel phenomenon. The tremor evident in PD patients is of three to five Hz and is present at rest (Samii et al., 2004). Like bradykinesia and rigidity, an initial asymmetry can be observed that may become less pronounced over the course of the disease. A definite diagnosis of PD can only be achieved post-mortem by means of histopathological confirmation. However, if an improvement of motoric impairments towards DA agonists (incl. L-DOPA) can be observed clinically, the likelihood of a PD diagnosis increases.

Secondary parkinsonism

If a direct causal relationship between motor impairments and circumscribed events can be identified, the term *secondary* or *acquired* Parkinsonism is used to describe the disorder. Several drugs are known for their potential to induce parkinsonian symptoms. Among these are neuroleptic (Jankovic, 1995), antiemetic (Indo and Ando, 1982), antihypertensive and promotility agents (e.g. haloperidol, metoclopramide, reserpine, tetrabenazine etc.). All of these compounds have in common that they act -via diverse mechanisms- as DA antagonist. The symptoms often present symmetrically and usually resolve after medication has stopped. Other forms of parkinsonian symptoms can be due to encephalitis, traumatic head injury, increased intracerebral pressure or (repeated or chronic) vascular alterations affecting the basal ganglia.

Hereditary parkinsonism

In general, PD is not thought to reflect a “classic” disorder of inheritance. Only 15%

percent of patients display a first-degree relative also affected by PD (Payami et al., 1994) and it cannot be ruled out that shared environmental factors may account for these associations (Calne et al., 1987). Furthermore, studies with monozygotic twins show no enhanced concordance rates (Ward et al., 1983). However, several genetic loci have been associated with Parkinsonism that can predispose a subject and it was demonstrated that young-onset variants are more frequently related to genetic susceptibility (Tanner et al., 1999). Two genes related to familial forms are *PARK1* and *PARK2*. The *PARK1* gene codes for a protein named α-synuclein. An intracellular aggregation of this protein can lead to Lewy bodies, a neuropathological hallmark of parkinsonian syndromes. The *PARK2* gene codes for a protein named parkin that is also involved in the breakdown of α-synuclein and, therefore, seem to operate through a common molecular pathway (Dauer and Przedborski, 2003). Several other genes (labeled *PARK3* to *PARK10*) have also been linked to familial PD, but little is known about the associated pathomechanisms involved (Samii et al., 2004).

Parkinson plus syndromes

As a Parkinson plus syndrome, **multiple system atrophy** (MSA) presents clinically with additional impairments that go beyond signs of parkinsonism. MSA manifests with additional cerebellar (Ataxia, intention tremor, impaired oculomotor tracking), autonomic (bladder and blood pressure control, temperature dysregulation) and pyramidal (plantar extension and hyperreflexia) dysfunctions (Gilman et al., 1999). Two distinct forms can be distinguished: MSA-P and MSA-C. Marked postural instability (and hence frequent falls) and less tremor are characteristic for MSA-P, while cerebellar signs are typical for MSA-C (Mark, 2001).

Progressive supranuclear palsy is characterized by speech disturbances, oculomotor impairments (palsy of downgaze), as well as dysphagia. Additionally, patients often exhibit amnestic aphasia and symptoms of behavioral disinhibition, while functions of learning and memory are relatively spared (fronto-executive dementia). Tremor is less prominent within this population and attempts of dopamine substitution often lack of success (Rajput and Rajput, 2001).

Corticobasal degeneration features an asymmetric parkinsonism and can present with dystonia, apraxia, the alien limb phenomenon and sensory extinction of one of two simultaneously presented stimuli (Rinne et al., 1994).

Dementia with Lewy bodies displays an early-onset dementia (early changes in complex

attention and executive function rather than learning and memory) and a variety of neuropsychiatric disorders including hallucinations and delusion (McKeith, 2000).

2.2.3 Degeneration of mesencephalic dopamine system

The most prominent pathological feature of parkinsonism is a loss of neurons in the SNc that can even be identified macroscopically. The characteristic dark appearance of this structure (due to intracellular melanin) presents remarkably diminished in PD patients post-mortem. Another hallmark is the presence of accumulated protein inclusions within dopaminergic neurons termed *Lewy bodies* (LB). There is a characteristic imbalance concerning the affection of nigrostriatal (SNc) vs. mesolimbic (VTA) cell populations, whereby the latter demonstrates less degeneration (Uhl et al., 1985). According to their predominant projections sites, the reduction of striatal DA is, in turn, more pronounced with respect to the putamen than in caudate nucleus, a pattern that can also be demonstrated in individual neuroimaging diagnostics using 6-[^{18}F]-fluoro-L-dopa (F-DOPA) uptake, measured by positron emission tomography (Vingerhoets et al., 1994). Motor impairments are typically observed when striatal DA deficiency exceeds 80%, which roughly corresponds to a degeneration of 50-60% of the respective midbrain DA neurons (Dauer and Przedborski, 2003; Fearnley and Lees, 1991). However, formation of LB can also be observed within presymptomatic stages. According to the Braak staging model (Braak et al., 2004) there is a characteristic progression of α-synuclein accumulation which starts in the medulla oblongata/pontine tegmentum and olfactory bulb/olfactory nucleus and, during the course of the disease, spreads in a topographically defined sequence, whereby the SN follows until neocortical areas are finally affected. This sequence somehow parallels the symptoms present clinically, i.e. initial hypo-/anosmia and depression as far as motor impairments and cognitive loss. Braak stages are subdivided into stages 1-2 (presymptomatic stages), stages 3-4 (developing impairments) and stages 5-6, whereby pathology reaches the neocortex and the disease manifests in all clinical dimensions (Braak et al., 2004). LB develop due to a misfolding of α-synuclein during synthesis that renders the protein susceptible to self-aggregate with other α-synuclein molecules, as well as other proteins including ubiquitin. The physiological degradation of such misfolded proteins by the cell's ubiquitin-proteasome system is severely impaired in parkinsonism and, ultimately, leads to a degeneration of the affected neurons giving rise to manifest clinical symptoms (Braak et al., 2004; Goedert, 2001). LB formation and subsequent neuronal degeneration is not only present in DAergic neurons, but also affects cell

populations that express noradrenaline (locus coeruleus), serotonin (raphé nuclei) and acetylcholine (nucleus basalis of Meynert) as neurotransmitters (Hornykiewicz and Kish, 1987; Jenner et al., 1983; Scatton et al., 1983; Remy et al., 2005). These alterations have also been discussed in the context of cognitive and neuropsychiatric disorders associated with parkinsonism (Xu et al., 2012). Another pathomechanism involved in cell death seen in parkinsonism highlights the role of mtiochondrial dysfunction and oxidative stress. It is not thought to be mutually exclusive as opposed to α-synuclein-based approaches, but to interact with and facilitate protein misfolding and aggregation at several critical levels (Giasson et al., 2000). As regular metabolites of mitochondrial functioning, several oxidants (including hydrogen peroxide and superoxide radicals) arise during physiological cellular respiration. These reactive oxgen species (ROS) form toxic hydroxyl radicals or react with nitric oxide to form peroxynitrite that may cause cellular damage by reacting with the DNA, proteins and lipids of the cell (Dauer and Przedborski, 2003). Evidence for an involvement of mitochondrial dysfunction came from the observation that parkinsonism induced by MPTP exposure (a potent neurotoxin frequently emerging in the illicit synthesis of opioids) is mediated by blockade of the mitochondrial compex I (Nicklas et al., 1987; Langston et al., 1983) and that there are abnormalities in PD (Greenamyre et al., 2001).

Several putative causes are thought to contribute to the development of neuronal degeneration seen in parkinsonism, although a distinct etiology is not known and tarries to be determined. Both environmental and genetic factors seem to play a role in the genesis of PD. Evidence for environmental influences arises from studies demonstrating increased risk for PD with increased exposure to neurotoxins including herbicides, pesticides, metals and solvents (Goldman, 2014; Tanner, 1992). Manifest impairments could be either due to prolonged exposition or to limited exposure initiating a self-perpetuating cascade of deleterious events (Dauer and Przedborski, 2003). However, further evidence for a fundamental role of exogenous compounds in the genesis of parkinsonism was demonstrated in the context of diseases prevention. Here, both tobacco smoking and caffeine consumption (from diverse sources) were shown to be inversely associated with the risk of PD development, therefore, displaying protective properties (Morens et al., 1995; Ross et al., 2000). However, the hypothesis of exogenous compounds being involved in the development of PD is discussed critically as toxin exposure does not account for all variance observed (Lock et al., 2013). Polymorphisms in distinct parkin and synuclein loci probably also contribute to the development of PD (Martin et al., 2001), although a mutual causality seems not to be given (Tanner et al., 1999). Instead, genetic

predisposition likely renders individuals more susceptible to pathogenic influences mediated by defined environmental circumstances (Dauer and Przedborski, 2003).

2.2.4 Non-motor symptoms

Although diagnostic criteria in PD principally refer to motor-related symptoms, the degeneration of midbrain DA projections evokes several other alterations that go beyond impairment of movement preparation, initiation and execution. Among these termed non-motor symptoms (NMS), a variety of neuropsychiatric, cognitive and autonomic dysfunctions can be observed in PD patients (Park and Stacy, 2009). About 90% of all persons affected by PD exhibit a specific configuration of NMS (Chaudhuri et al., 2011). Interestingly, recent evidence suggests that the subjectively perceived quality of life in PD patients is determined by NMS to a greater extend than experienced motor disabilities (Lawrence et al., 2014; Lawson et al., 2014; Bernal-Pacheco et al., 2012). NMS can be subdivided into cognitive impairments (dementia and mild cognitive impairment), neuropsychiatric disorders (depression/apathy and anxiety) and several autonomic complications (hypotension, sleep disturbances, erectile dysfunction, gastrointestinal symptoms, urinary complications). Cognitive decline is a common feature seen in PD and can, according to the criteria of the Movement Disorders Society, affect domains of (complex) attentional functions, working memory, executive functions, as well as (free recall) memory processes (Svenningsson et al., 2012). Depending on the specific criterion applied and the respective study, psychometrically relevant cognitive impairments can be observed in 40 to 80% of the PD population (Aarsland et al., 2003; Hely et al., 2008;). This incidence reflects the prominent involvement of DA in several cognitive domains and specific functions, although not being the only factor (Lindgren and Dunnett, 2012; Chaudhuri and Schapira, 2009; Robbins and Cools, 2014). Compared to age and disability matched populations, PD patients display a significantly higher incidence rate of cognitive impairments and dementia and show a up to sixfold higher risk (Aarsland et al., 2003; Aarsland et al., 2001; Tandberg et al., 1996). The profile of cognitive impairments associated with PD strikingly mimics deficits observed in patients with mPFC lesions (Kehagia et al., 2010; Owen et al. 1992; Taylor et al., 1986). The mPFC is heavily involved in the processing of working memory (Kolb et al., 1974; Larsen and Divac, 1978; Dunnett et al. 1999). Impairments of these functions, mimicking the effects of mPFC lesions, were also demonstrated following selective unilateral DAergic lesions in rats (Foyet et al., 2011; Lex et al., 2011; Ciobica et al., 2012; Chao et al. 2013), thereby, further indicating

interdependence of the mPFC and midbrain DA projections in the mediation of working memory and response control. However, little is known about the precise nature of the arising deficiencies associated with PD in terms of underlying neurochemical mechanisms. The mentioned functions mediated by the mPFC rely on the integrity of DAergic (Florio et al., 1999, Chudasama and Robbins, 2004) and seretonergic (Perez-Vega et al., 2000; Gonzalez-Burgos et al., 2012) innervation of the mPFC and were shown to be impaired by circumscribed focal deafferentation of the respective transmitter systems in the rat mPFC. Such findings highlight transmitter-specific interactions of the mPFC and midbrain DA projections. Lesions of mesocortical DA projections influence not only DAergic transmission in the mPFC (Gratton et al., 1989; Al-Tikriti et al., 1992), but also the serotonergic system. For example, the (unilateral) administration of the neurotoxin 6-hydroxydopamine (6-OHDA) into the MFB leads to a decrease in mPFC 5-HT concentration (Chao et al., 2013), as well as receptor-related functional changes. A reduction in the density of 5-HT_1, 5-HT_2 and 5-HT_3 receptors in the mPFC (Kienzl et al., 1981; Cicin-Sain and Jenner, 1993; Soria-Fregozo et al., 2008), as well as changes of activity patterns in mPFC neurons towards various 5-HT receptor challenges (Wang et al., 2009; Gui et al., 2011; Fan et al. 2011; Zhang et al., 2011; Cao et al., 2007) were observed, indicating altered functional properties of 5-HT neurons after the degeneration of DAergic neurons of the midbrain. These findings concur with observations of alterations of 5-HT-related measures in PD patients in post mortem tissue (Jenner et al., 1983; Scatton et al., 1983) and liquor (Mayeux et al., 1988) levels of 5-HT. Serotonergic transmission has been implicated in memory functions. Disruption of serotonergic transmission by the 5-HT-specific lesion agent 5,7-dihydroxytryptamine (5,7-DHT) impairs spatial working memory (Hritcu et al., 2007; Cassaday et al., 2003; Ricaurte et al., 1993) and object recognition (Lieben et al., 2006) in rats. Given that these memory impairments are also evident in PD and that serotonergic transmission is known to be altered, this points towards a substantial contribution of 5-HT patho-mechanisms within the mPFC to the neuropsychological disturbances seen in PD patients. The mPFC has also been associated with depression and anxiety in humans (Drevets et al., 2008; Lemogne et al., 2012) and rodents (Hamani et al., 2010; Warden et al., 2012). About 20% of the PD population is affected by such comorbidities (Reijnders et al., 2008; Remy et al., 2005) and it is likely that they are a consequence of pathological alterations of the mPFC that result from the degeneration of midbrain DA neurons. The involvement of DAergic innervation of the mPFC has been widely addressed: Unilateral 6-OHDA lesions of the MFB resulted in

impairments in emotional behavior, including increased immobility time in the forced swim test (FST) and reduced time spent in non-walled compartments (Eskow-Jaunarajs et al., 2010; Zhang et al., 2011). DA depletion of the mPFC, as well as MFB-lesions increased anxiety (Espejo, 1997; Jungnickel et al., 2011). The impact of serotonergic manipulations on emotional behaviors has been thoroughly documented in rodents (Carr and Lucki, 2011; Nutt, 2006). Specific 5-HT-Rs differentially mediate antidepressant effects in a variety of animal models of depression (Albinsson et al., 1994; Patel et al., 2004; Marek et al., 2005; Cryan and Lucki, 2000; Dawson et al., 2006; Cryan et al., 1997), indicating receptor-specific mechanisms, rather than 5-HT transmission (levels) per se, to be critically involved.

2.3 The 6-OHDA hemiparkinsonian rat model

The pathological hallmark in PD is a loss of dopaminergic neurons in the midbrain, in particular in the substantia nigra pars compacta, which is accompanied by a marked reduction of available DA in the striatum. Several animal models attempt to mimic this pathophysiological process in order to provide a framework for the scientific study of participating mechanisms and evoked neurophysiological alterations. Two major classes of models can be distinguished: toxin-based models and genetic models (Blesa and Przedborski, 2014). Among the toxin-based models, different substances can be applied. Some compounds induce impairments and changes that are transient and reversible in principle (reserpine, haloperidol), while others lead to chronic depletion (MPTP, 6-OHDA, rotenone, paraquat, methamphetamine). The most frequently applied toxin in rats is 6-OHDA and was introduced and established by Ungerstedt (1971). Due to its impermeability across the blood-brain barrier 6-OHDA is typically administered via intracerebral microinjection. Different sites can serve as application loci. Principally, solutions containing a defined amount of 6-OHDA can be injected into the ascending DAergic midbrain projections (medial forebrain bundle), its terminal projection fields (caudate-putamen complex) or at the level of the corresponding somata (SNc/VTA). The compound is uptaken actively by the dopamine active transporter (DAT) due to its structural similarity to the endogenous ligand DA. Once uptaken into the cell 6-OHDA exerts its neurotoxic effects via three different mechanisms: (1) Generation of reactive oxygen species by auto-oxidation, (2) formation of hydrogen peroxide by MAO activity and (3) direct inhibition of the mitochondrial respiratory chain. Taken together, these mechanisms evoke cytoskeleton disorganization, peroxidation of (membrane) lipids, DNA

damage and a decrease of ATP availability in the affected cell, ultimately, leading to cell death (Blum et al., 2001). Given its structural similarity to other catecholaminergic transmitters (particularly noradrenaline) 6-OHDA is also uptaken by neurons utilizing these transmitters. If a DA-specific preparation is desired, a NA-specific reuptake inhibitor (e.g. desipramine) is applied systemically before the actual 6-OHDA administration. Due to its electroactive properties, 6-OHDA is administered in a solution containing a certain amount of ascorbic acid (0.02-0.2%) to prevent rapid oxidation outside neuronal tissue. Injections can be performed uni- or bilaterally, whereby unilateral lesion offer a variety of advantages. Lesions in both hemispheres may result in pronounced motor impairments that require intensive and elaborate care of the animals in terms of e.g. gavage feeding. However, the use of unilateral lesions is associated with a particular behavioral phenotype that can be exploited experimentally and offers a unique assessment of alterations induced by DA depletion. Because of the emerging imbalance of striatal DA levels the animals show a spatial bias against the side of the lesion in terms of sensorimotor processing.

2.3.1 Physiological effects induced by 6-OHDA

Several measures can serve as an estimate of the degree of DA depletion induced by the administration of 6-OHDA (Schwarting and Huston, 1996a). The most frequently used ones comprise (1) levels of transmitter (and corresponding metabolites) in wet tissue measured post mortem, (2) extracellular niveau as assessed by in vivo microdialysis and (3) amount of tyrosine hydroxylase positive stained neurons. General changes in all measures can be observed within two to twelve days after application, whereas the exact alteration expressed depends on lesion parameters. Principally, partial lesions can be distinguished from total lesions. The latter ones are roughly defined as an irreversible reduction of striatal DA content that is greater than 90%. The amount of 6-OHDA applied and the site of injection strongly determine the degree of depletion, with higher doses and injections aiming at the ascending fibres (MFB) evoking more severe reduction (Francardo et al., 2011). The severity of the lesion (degree of depletion) also determines the temporal development of lesion characteristics. Total lesions are reached within three to five days after toxin application and remain so, whereas less severe depletion can be partially compensated for by an increased amount of DA provided from either (spared) ipsi- or contralateral afferents. Related to the lack of DAergic innervation, postsynaptic DA receptors expressed in striatal neurons also show specific alterations. Generally, increased binding of DA to D2-like receptors can be observed following prolonged denervation

(Schwarting and Huston, 1996a). This happens mostly due to an up-regulation of receptor expression in terms of numbers (Joyce, 1991; Neve and Marshall, 1984) and tends to be rather permanent (Marshall et al., 1989). Such an increase in receptor density has major implications as to exogenously applied DA agonists by means of motor behavior.

2.3.2 Behavioral effects induced by 6-OHDA

The most striking behavioral phenomenon of an unilaterally 6-OHDA challenged animal is its characteristic circling behavior. Animals with a unilateral lesion of the nigrostriatal tract display rotatory movements, either away from the lesioned side (“contraversive turning”) or towards the lesioned side (“ipsiversive turning”), spontaneously or when systemically challenged with different types of DA agonists (Miller and Beninger, 1991; Schwarting and Huston, 1996b). The directionality of turning depends on the type of agonist applied. While substances that actively release DA into the synaptic cleft (e.g. dextroamphetamine and derivates) evoke ipsiversive turning, agents that act directly on postsynaptic receptors (e.g. L-DOPA or apomorphine) induce contraversive circling. This divergence is thought to reflect a relative predominance regarding dopaminergic neurotransmission in the striatum of one hemisphere over the other, resulting from either more transmitter available in the synaptic cleft (amphetamine) or a larger response concerning the transduction of the signal by up-regulated (sensitized) DA-receptors (L-DOPA and apomorphine). This relative imbalance, subsequently, leads to a reversal of the induced ipsilateral motor bias in favor of the opposite laterality, and, ultimately, drives the animal away from the side of the lesioned hemisphere. This behavioral outcome, and the assumed mechanisms associated, represent a mostly validated phenomenon which has been the subject of an extensive line of research dating back to the pioneering work of Ungerstedt in the early 1970s (e.g. Ungerstedt, 1971). Unlike post mortem measures of DA depletion, this type of behavioral criterion can also be used in validating the lesion (and its extend/amount) in vivo.

2.4 Interhemispheric disconnection models

At least two distinct fundamental principles characterize the organization of the brain: *functional segregation* and *functional integration* (Eickhoff and Grefkes, 2011). While there is a considerable amount of specialization of circumscribed brain areas with respect to processing specific aspects related to sensation, transformation and behavioral output, complex and multidimensional demands occurring in a particular situation may only be sufficiently addressed by an active exchange between these specialized constituents. In

other words, nowadays network approaches are preferred in favor of “classical” mapping ideas of a particular function to a distinct area. Since the advent of contemporary neuroimaging (MEG, fMRI, sEEG) and invasive electrophysiological (SUA, MUA, LFP) techniques, task-evoked communication between defined brain areas can be directly assessed. The (task-related) functional connectivity between both cortical and subcortical regions is defined as the temporal coincidence (or co-variance) of spatially distant neurophysiological events (Eickhoff and Grefkes, 2011). Such events include changes in BOLD contrast and electrical potentials. However, there is a major limitation of such approaches, i.e. functional connectivity is primarily correlative in nature. Any parallel changes in two signals may not reflect a direct connection. Correlated activity may rather be the result of additional structures relaying information from the first to the second, or, both signals may be driven by an independent third party (Eickhoff and Grefkes, 2011). An alternative approach in the experimental assessment of networks mediating specific behavioral functions derives from procedures termed *Interhemispheric Disconnection Models*.

Disconnection models aim at a functional disconnection of two distinct brain entities (i.e. defined cortical areas, subcortical nuclei, projection systems or connection fibres) that are thought to constitute or be involved in a neuronal circuit mediating a specific function that can be assessed behaviorally. Principles of disconnection have been described initially by neurologist Norman Geschwind (1965a, 1965b). The underlying rationale of disconnection procedures may be summarized as follows: if there is a functionally relevant interaction between two distinct areas (systems) of the brain, e.g. area A and B, a specific impairment should be found when these two areas are disconnected. If a lesion of area A in one hemisphere, combined with a lesion of area B in the contralateral hemisphere, evokes a larger impairment in a specific function as compared to the same lesions of A and B located in the same hemisphere, it can be concluded that the interaction between A and B is critical for that specific function. The former disconnects two areas at two different levels, while the latter preserves an intact circuit in one hemisphere, capable of compensation of function. Therefore, evidence for a functionally relevant interaction of two brain areas, or systems, can be concluded if there are differential effects of combined ipsi- vs. contralateral (disconnecting) lesions. Although disconnection models display an elegant approach in probing functional circuits in general, it should be noted that special attention has to be be paid to any functional network of interest that is potentially lateralized. In such cases, lesions of the left vs. right hemisphere may yield different functional outcomes.

However, if such lateralization aspects are addressed appropriately in terms of an experimental design that accounts for such differences (e.g. by means of balancing/counterbalancing), valid conclusions concerning functional aspects of circumscribed neural circuits can be drawn.

2.5 Deep brain stimulation

Despite its clinical efficacy and growing number of application in PD patients over the last decades, the neurophysiological mechanisms by which deep brain stimulation (DBS) exerts its therapeutical effects remain poorly understood. The clinical application has, therefore, somehow preceded the scientific understanding of its mechanisms of action (McIntyre et al., 2004). Several modes of action are discussed and are a topic of an ongoing debate. Principally, an electrode placed in a defined volume of neural tissue can affect the activity of adjacent cell somata, afferent projections or even fibres of passage. Thus, not only local effects may be observed but also alterations in downstream targets of the participating network. Depending on the nature of the affected elements, effects on neural activity may either be inhibitory (e.g. stimulation of afferent inhibitory inputs) or excitatory (e.g. direct depolarization by current application) in general.
The modern form of DBS in PD patients has been established since 1987 when Benabid and Pollak and colleagues successfully introduced this stereotactic procedure for the treatment of tremor. Over time, DBS of the STN was developed and virtually replaced older surgical lesion approaches including thalamotomy (Hariz et al., 2010). However, due to the controversial application in psychiatry in the early 20th century, invasive techniques such as DBS are still considered as to meet very strict ethical standards.

2.5.1 Principles

The stimulation electrodes are implanted by means of stereotaxic neurosurgery and can be inserted uni- or bilaterally. At least four contacts per electrode tip are present. Proper localization during the procedure is ensured by radiological tracking and electrophysiological monitoring. Leads are implanted subdermally and connect the electrodes with the pacemaker that is typically implanted at a pectoral level. The stimulation parameters are tuned at defined intervals post operatively to approach the best functional outcome. The stimulator offers variation of the amplitude, pulse width and stimulation frequency of the current applied and it can be run in a mono- or bipolar fashion. Typically, the stimulation parameters applied cover a frequency range from 120-140 Hz, an

amplitude of 2-3 V and a pulse width of 90-110 µs.

Although invasive neuromodulation by means of DBS has become an established surgical intervention in the treatment of PD, the exact neurophysiological alterations underlying improvement in tremor, muscle rigidity, bradykinesia, postural instability and other motor parameters are still not understood and topic of an ongoing debate. The STN is a primary focus of DBS therapy (Fox et al., 2011; Schnitzler et al., 2010; Kleiner-Fisman et al., 2006). The STN acts as a relay station of both in- and output-associated activity in the corticostriatothalamic loop, sending excitatory glutamatergic efferents to the principal output structures of the basal ganglia, i.e. the GPi, the SNr and to the GPe, which, in turn, feeds back inhibitory GABAergic projections to the STN. Because of this glutamatergic nature, the STN has long been considered as the driving force of the basal ganglia (Charpier et al., 2010). Since early ablative techniques including (sub-)thalamotomy and pallidotomy, the STN has become a preferred target in the treatment of PD, especially in prolonged and (DA-) medicaton-refractory states of the disease. The efficacy of STN DBS in PD patients with respect to motor outcome and generalized improvement in quality of life has been studied extensively and was confirmed throughout several clinical trials (Benabid et al., 2009). Therefore STN DBS has become a standard therapeutic intervention while a sufficient comprehension of the underlying mechanism(s) of action is notably lacking. Several suggestions have been proposed by which mechanisms applying electrical current to the STN may contribute to the alleviation of motor impairment (Kringelbach et al., 2010; McIntyre et al., 2004, Dostrovsky & Lozano, 2002). Generally, there are at least four distinct modes of action discussed in the literature: Depolarization blockade (Beurrier et al., 2001), synaptic inhinbition (Dostrovsky et al., 2000), synaptic depression (Urbano et al., 2002) and modulation of pathological (oscillatory) network activity (Montgomery and Baker, 2000; Kringelbach et al., 2010).

2.5.2 Potential mechanism(s) of action

Depolarization blockade

A popular approach concerning supposed mechanisms of action of STN DBS is to argue for actually inducing a net neural inhibition of the stimulated tissue (cell somas) by inducing alterations in the activation of voltage-gated currents that ultimately block neural output (prevent generation of action potentials) surrounding the electrode tip. Such an assumption is in line with the beneficial motor effects observed with subthalamotomy in PD patients (Jourdain et al., 2014; Guridi and Obeso, 2001). However, this “virtual lesion” or

“depolarization blockade” approach (Beurrier et al., 2001) has limitations.

Synaptic depression

Urbano et al. (2002) showed that prolonged high-frequency stimulation of afferent thalamo-cortical fibres evokes a depletion of transmitter that, ultimately, leads to a decrease in output of neurons affected by DBS. It is assumed that a constant stimulation driven at a high rate interferes with physiological transmitter release. As a consequence, there is a decrease in transmitter-mediated postsynaptic stimulation. The net effect resembles an inhibition of the preceding structure induced by DBS.

Synaptic inhibition

Given that microlesions of circumscribed territories of the basal ganglia, in particular (sub-)thalamotomy and pallidotomy, can lead to motor improvement in movement disorders, it has been proposed that electrical stimulation introduced by DBS affects the afferent inhibitory projections arising from the GPe and, hence, result in an increase of GABAergic transmission into the STN, leading to a decrease in STN activity (Feuerstein et al., 2011). In turn, this decrease is thought to attenuate the tonic inhibition of the GPi on the motor territories in the thalamus and, thereby, facilitate movement initiation and performance. Optogenetic experiments have shown remarkable evidence for such an assumption (Gradinaru et al., 2009).

Modulation of (oscillatory) neuronal activity

Considerable alterations in terms of oscillatory activity (both power and coherence) have been demonstrated as a consequence of a lack of midbrain DA innervation of the BG, both in humans (Brown et al., 2004; Kühn et al., 2004, Weinberger et al., 2006) and animal models of PD (Degos et al., 2009; Sharott et al., 2005; Magill et al., 2005).This pathological pattern (increase of power in the beta range) seems also to be relayed into the entire BG system. Studies have been shown that oscillations in the beta range (8-30 Hz) are selectively affected (suppressed) by dopamine replacement strategies (including administration of L-DOPA) and STN DBS (Kühn et al., 2008), thus making it likely for this constituent to be associated with motor dysfunctions. Also, stimulation parameters consisting of low-frequency values (i.e. ~20 Hz) have been shown to further worsen motor outcome (Timmermann et al., 2004; Eusebio et al., 2007). It has been suggested (McIntyre et al., 2004) that STN DBS acts via a modulation of this pattern of oscillations by evoking plastic changes of network activity that rely on mechanisms of either synaptic long term depression (LTD) and/or long term potentiation (LTP).

Another model that emphasizes modulatory mechanisms has been referred to as “neural

jamming” or “noise-masking” (Benazzouz and Hallett, 2000). The conceptual assumption of this approach is that pathological patterns of STN activity, which derive from sustained DA-denervation, are superimposed or substituted with a non-physiological high-frequency signal. Improvement in motor outcome here is thought to reflect the relative advantage of “mere noise” (high frequency) over a pathological input in terms of network integrity. The resulting increase of regularity in GPi output by a reduction of the pathologically miss-information that is constantly generated in the GPi otherwise is discussed as a possible mechanism in this context (Montgomery and Gale, 2008).

3 Methods

In this section, the surgical procedures applied, behavioral tests carried out, as well as the histological and neurochemical analyses performed, are described. A more detailed description of the corresponding methods can also be found in the respective sections of the original papers attached at the end.

3.1 Surgical procedures

Animals

The animals used in the experiments were male Wistar rats that were purchased from Janvier (Le Genest St. Isle, France). They were three months of age on average at the beginning of the experiments and their body weights ranged from 220 to 300g. Rats were housed under standard laboratory conditions (20 ± 2° C; 60% humidity) and under a reversed light-dark cycle (7am to 7pm). Animals were provided food and water ad libitum throughout all stages of the experiments. Behavioral testing was conducted between 8am and 6pm. In experiment 1 (Motor effects GABA STN), animals were housed individually following surgery in standard translucent plastic cages (30 x 20 x 20 cm). In experiment 2 (Effects Disconnection mPFC-MFB), animals were housed conventionally in groups of five in translucent makrolon type 4 cages, except for three days after surgery, where they were housed individually in order to allow for proper recovery. All experiments were in accordance with the European Communities Council Directive (86/609/EEC) and the effective German Law of Animal Protection of 1998 and 2013, respectively, and have been approved by local authorities (Central Institution for Animal Research and Animal Protection, ZETT) and the North Rhine-Westphalia State Environment Agency (LANUV). Subjects were assigned randomly to any of the different experimental groups.

6-OHDA lesions

In order to induce a degeneration of midbrain DA projections, animals were injected intracerebrally with the neurotoxic compound 6-hydroxydopamine (6-OHDA) in a stereotaxic surgery. This agent is taken up actively by the Dopamine Active Transporter (DAT) of dopaminergic neurons and rapidly oxidizes into hydrogen peroxide and free radicals that have cytotoxic properties and induce necrosis of the affected neurons (see also "*Physiological Effects Induced by 6-OHDA"* section for further information). In experiment 1, animals were anesthetized with sodium pentobarbital (60mg/kg, 1ml/kg; Merial, Germany). In experiment 2, anesthesia was induced by isoflurane/oxygen inhalation anesthesia (4% mixture for induction, 2% mixture for maintenance) and animals were also pretreated with desipramine (25mg/kg dissolved in dimethyl sulfoxide, 1ml/kg; i.p.; Sigma-Aldrich, Germany) 45min prior to surgery in order to spare noradrenergic neurons from 6-OHDA toxicity. Prior to surgery, Carprofen (5mg/kg, 1ml/kg; Pfizer, Germany) was administered (i.p.) to prevent inflammatory reactions and to manage postoperative pain. Subjects were mounted on a stereotaxic frame (David Kopf, USA) and the head was sanitized with a 70% ethanol solution. A local anesthetic was injected subcutaneously then, i.e. 0.1 ml of Bucain® (DeltaSelect, Germany) containing 0.25 mg Bupivacaine hydrochloride and an incision was made along the scalp to expose the skull. Skull surface was bleached with 10% hydrogen peroxide solution to localize bregma and lambda. A burr hole was applied and injections aimed at the medial forebrain bundle (AP: -4.00 mm, ML: +1.5 mm, DV: -8.5 mm; relative to bregma and skull surface according to Paxinos and Watson, 1996). Animals then received an unilateral (side of administration, i.e. left vs. right hemisphere, was counterbalanced across all experimental groups in both experiments) injection of 12.5 µg 6-OHDA dissolved in a volume of 3µl 0.9% saline with 0.1% ascorbic acid via a 28-gauge cannula connected to a microinfusion pump (KD Scientific, Boston, USA). The pump was equipped with a 10 µl syringe (Microliter 701, Hamilton, Switzerland) and the 6-OHDA solution was administered with a constant flow rate of 0.5 µl/min. An air bubble was introduced into the tubing to check if there was actual flow occurring. To allow for diffusion and to prevent reflux, the cannula was left in place for another minute after injection. Administration aimed at the MFB since lesions here are known to reliably induce most severe depletion of striatal DA content (Francardo et al., 2011).

Implantation guiding cannulae

In experiment 1, animals were implanted with guiding cannula within the same surgery sessions as the 6-OHDA lesions were induced. Subjects were implanted chronically with guiding cannulae (Model 3260PGA/Spc 8mm, Plastics One, Roanoke, USA) for later intracerebral injections in the awake animal that aimed at the STN (AP: -3.8 mm, ML ± 2.5 mm, DV: -7.6 mm relative to bregma and skull surface). To this end, three additional holes were drilled into the skull 2 mm apart each in the in the lateral, anterior and anterolateral dimension relative to the STN burr hole. Bone screws were mounted in these holes and the pedestal of the guiding cannula (26-gauge, 0.46mm outer diameter, 0.24 mm inner diameter) was fixated with dental acrylic to the skull. After implantation, the skin was sutured and disinfected with a 70% ethanol solution and animals were returned to their home cages.

mPFC lesions

In experiment 2, additional lesions of the mPFC were introduced in parallel with the 6-OHDA lesions of the MFB within the same surgery session. While some experimental groups received lesions of the mPFC within the same hemisphere (ipsilateral groups), other groups were injected in the opposite hemisphere (contralateral groups). The latter approach aimed at a functional disconnection of the respective structures with respect to diverse behavioral readouts (see *Introduction* section for further information on disconnection logic). Animals were either injected with *N*-Methyl-D-aspartic Acid (NMDA) in order to induce unspecific excitotoxic lesions of the mPFC, or, were injected with the 5-HT-specific neurotoxic agent 5,7-Dihydroxytryptamine (5,7-DHT). This compound leads to a retrograde degeneration of afferent 5-HT projections arising from the raphe nuclei and, therefore, induces a 5-HT-specific denervation of the mPFC. Animals were injected at four sites within the mPFC ([1] AP +3.8mm; L ±0.7; DV -3.0mm; [2] AP +3.2mm; L ±0.7; DV -3.0 [3] AP +3.2mm; L ±0.7; DV -2.0 mm; [4] AP +2.7 mm; L ±0.7 mm; DV -3.0 mm; Paxinos and Watson, 1996). Each injection volume was 0.2 µl with a flow rate of 0.25 µl/min. The solutions applied either contained 10mg/ml NMDA, 1mg/ml 5,7-Dihydroxytryptamine creatinine sulfate salt or 10 mg/ml 5,7-DHT, both dissolved in phosphate buffered saline containing and 0.1% ascorbic acid. Infusions were also administered via a microinfusion-pump (KD Scientific, Boston, USA) equipped with a 10 µl syringe (Microliter 701, Hamilton, Switzerland).

3.2 Behavioral testing

Open field

The open-field (OF) consisted of a square arena (48 x 48 x 40 cm) with a video camera connected to an automated analysis system (Ethovision©, Noldus). It was located in a dimly lit room in which white noise (~60 dB) is applied. The OF was differentially illuminated, providing ~6 lx in the center and ~4 lx in the surrounding compartment. The animal was placed in the middle of the arena and was allowed to freely explore it for a certain amount of time.

Dependent variables: Several measures of motoric parameters can be extracted, including horizontal and vertical distance moved, velocity, as well as rotational behavior, a common measure in unilaterally 6-OHDA lesioned rats (Schwarting and Huston, 1996b). Rotational behavior can be assessed by an asymmetry index for quarter turns. This was computed by the ratio of ipsi- or contraversive turns relative to the total number (sum) of ipsi- and contraversive turns, whereas 'ipsi' and 'contra' refer to the side ipsi- or contralateral to the 6-OHDA lesion. Time or frequency in the center compartment can also provide an index of fearfulness.

Amphetamine/L-DOPA Challenge

In hemiparkinsonian rats, there is an imbalance of (striatal) DA content between the affected and non-challenged hemisphere (Schwarting and Huston, 1996b). Despite the lack of dopaminergic innervation, DA receptors are are still expressed and present on the postsynaptic recipient structure. The denervation of these receptors, as induced by the administration of 6-OHDA into the medial forebrain bundle, is accompanied by an increase in D1- and D2-like receptor binding, both concerning an increase in receptor number and receptor affinity (Schwarting and Huston, 1996a). Animals were challenged with 12 mg/kg L-3,4-dihydroxyphenylalanine mehtyl esther hydrochloride (+ 15 mg/kg benserazide hydrochloride) 15 minutes before OF (i.p.). D-Amphetamine hemisulphate salt (1.5 mg/kg; i.p.) was administered immediately prior to testing.

Elevated plus maze

The Elevated plus maze (EPM) provided two opened (50×10 cm) and two walled arms (50×10×38.5 cm), each opposite to each other, forming a plus-shaped maze. The arms were arranged around a central platform (10×10 cm) and the whole apparatus was lifted with respect to the floor to a height of 50 cm. The closed arms were illuminated with 1 lx,

while the open arms were illuminated with 30 lx. Animals were placed on the central platform, facing one of the open arms and were allowed to freely explore the maze for a certain amount of time. Given the aversive or anxiogenic nature of the brighter and less safe open arms, animals tend to prefer exploring the closed arms. The more the open arms are explored, the less anxiety can be assumed. This task is sensitive both to anxiolytic (e.g. diazepam), as well as anxiogenic (e.g. caffeine) treatments.

Dependent variables: Several variables related to fear/anxiety behavior can be measured automatically by the Ethovision© software suite: The time (in seconds) spent in the open and closed arms, number of entries into the open and closed arms (an entry is counted when the animal entered an arm with all four paws), time to first entry into the open and closed arms, as well as the total distance (in centimeters) moved on the apparatus. Other parameters can be tracked manually: Number and duration of rearings on the closed arms, head dips on the open arms (head is lowered beneath the edge of the arm), risk assessments from the closed arms (forepaws and head exit the closed arms; often accompanied by body stretching).

Forced swim test

The forced swim test (FST) apparatus consisted of a Plexiglas cylinder (46 cm height, 20 cm diameter) containing 30 cm of water 24±1 °C). A video camera, connected to a video recorder, was located next to the cylinder providing a front view to record the experiment on video tapes for post-hoc analysis. On the first day (pretest session), animals were exposed to the cylinder for 15 min. At the end of this pretest session, each rat was removed from the water, and placed in a plastic cage under a red light heating lamp for 15 min to allow drying before returning to the home cage. Twenty-four hours later, the animals were re-exposed to the same experimental condition for only 5 min (test session). The FST is thought to induce a depressive behavioral phenotype via principles of learned helplessness (Cryan et al., 2005).

Dependent variables: The duration (in seconds) and frequency of immobility (defined as a lack of motion of the animal, except for movements required to keep its head above the water), as well as swimming and climbing (vigorous movements with forepaw in and out of the water, usually in contact with the walls) was analyzed semi-automatically post-hoc by a trained and experimentally blinded observer.

Object recognition memory test

The arena consisted of an acrylic square arena (60 x 60 x 30 cm) in which different objects

were placed and animals were free to explore these. They were habituated to the empty arena two days before actual assessment for five minutes each. The apparatus was located in a sound attenuating room and illuminated by bulbs mounted above the arena providing light density of 6lx in the center and 4lx in the corners. A video camera was mounted at the ceiling allowing post-hoc analysis of the recorded behavior by a trained and experimentally blinded observer. On the day of testing, two identical copies of a particular object (cylinder-shaped white ceramic vase, textured surface, 30cm height) were introduced to the arena at two out of eight possible locations (sites are randomly chosen for all experimental groups). Animals were allowed to freely explore the objects and any physical contact with the object (snout, vibrissae or forepaws) was assessed with respect to duration and frequency. If animals demonstrated physical contact with the object, but not focusing it, such interactions were not considered as exploration of the object. After this initial trial, animals were returned to their home cages until 90 min passed. Subjects were then re-introduced to the arena which then contained one of the original objects and one animals were not familiar with (curve-shaped translucent glass vase filled with blue marbles, smooth surface, 25cm height). The objects were located in the same locations as in the previous trial. Again, animals were allowed to freely explore the objects. Rats showing intact object recognition memory prefer to explore the new object longer and more frequently than the one from the first trial (Ennaceur and Delacour, 1988). After each trial, the arena and objects were cleaned with 1% acetic acid solution to prevent odor contamination.

Dependent variables:A memory index can be computed according to the formula: $[(ETT_{NO} / ETT_{OO}) / (ETS_{NO} / ETS_{OO})]*100$

"ETT_{NO}" is the exploration time during the test trial for the novel object, "ETT_{OO}" is the exploration time during the test trial for the old object, "ETS_{NO}" is the exploration time during the sample trial for the object located at the same position where in the test trial the novel object appeared and "ETS_{OO}" is the exploration time during the sample trial of the old familiar object. This metric controls for differences in the overall exploration level exhibited by individual subjects. Values above 100 indicate intact object recognition functions beyond chance level, while values ≤100 reflect impaired object recognition.

3.3 Neurochemical and histological analyses

HPLC-ED Assessment of Neurotransmitters

After behavioral testing, brains were analyzed for their content of different

neurotransmitters in the (dorsal) striatum and mPFC. Wet tissue amount was assessed. To this end, animals were anesthetized by $C0_2$ inhalation. They were decapitated and their brains were manually extracted. Tissue samples of the dorsal striatum and the mPFC were taken from both hemispheres. The tissue was weighted and then homogenized with an ultrasonic homogenizer (Microsonic, Germany) in 500µl 0.05N $HCLO_4$. Homogenized Samples were centrifuged at 9000 rpm at 4° C for 20 minutes. Afterwards, samples were filtered and stored at -80° C until neurochemical detection. Several monoaminergic neurotransmitters (NE, DA, 5-HT) and corresponding metabolites (DOPAC, 5-HIAA) were assessed by applying high-performance liquid chromatography with coupled electrochemical detection (HPLC-ED). A ET 125/4, Nucleosil 120-5, C-18 reversed phase column (Machery and Nagel, Germany) was used, which was constantly 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, Netherlands) was set at 500mV vs. an ISAAC reference electrode (Antec, Leyden, Netherlands) and analyses were performed at 30° C.

Histology

In experiment 1, animals were killed by injection of sodium pentobarbital and were transcardially perfused. Perfusion started with phosphate buffered saline followed by a 10% formaldehyde solution. Then, brains were extracted and stored in 10% formaldehyde solution for at least 48 hours. Brains were sliced with a cryostat (Leica, Germany). The slices were stained with cresyl violet (Sigma–Aldrich, USA) for cell bodies and the placement of the guiding cannulae was verified according to the atlas of Paxinos and Watson, 1996.

In experiment 2, the extension of excitotoxic lesions of the mPFC due to NMDA injection was also carried out by means of Nissl staining. Animals were deeply anesthetized by $C0_2$ inhalation, decapitated and the brains were extracted. They were stored in a 10% buffered formalin solution for at least 48h after extraction. Then, brains were stored in a 30% sucrose-formalin solution at 4 C°. After slicing the brains with the cryostat into slices of 50 µm thickness, samples were stained with cresyl violet. The extent of NMDA-induced cell body lesions was determined under a light microscope.

4 Experiments

4.1 $GABA_A$-receptor activation in the subthalamic nucleus compensates behavioral asymmetries in the hemiparkinsonian rat

As outlined above, deep brain stimulation of the subthalamic nucleus (STN DBS) has become a routinely used therapy in the treatment of PD. Despite its well-known and clinically proven efficacy (Benabid et al., 2009), the underlying neurophysiological mechanisms by which STN DBS exerts its therapeutical effects are yet object of an ongoing debate (Deniau et al., 2010). Potential mechanisms that are discussed range from excitatory to inhibitory effects of DBS on STN activity and the role of different transmitter systems and related receptors in this context has received little attention. In an attempt to characterize the involvement of $GABA_A$- and NMDA-receptors in the STN regarding motoric functions in unilaterally 6-OHDA lesioned rats selective agents were injected locally into the STN and motor-related behavioral parameters were assessed in this experiment (Petri et al., 2013). Animals were initially rendered hemiparkinsonian by applying 6-OHDA into the MFB. Afterwards, either the $GABA_A$-receptor agonist muscimol or the NMDA-receptor antagonist MK-801 (dizocilpine) was administered directly into the ipsilateral STN by means of intracerebral microinjections. Then, the effects on turning behavior and locomotion were gauged. Additionally, influences of systemic L-DOPA and D-amphetamine challenges were tested.

There was a dose-dependent effect of intrasubthalamic muscimol administration on turning behavior and locomotion. The higher dose evoked contralateral turning behavior, while the lower dose counteracted the laterality bias induced by unilateral striatal DA depletion to a non-biased occurrence. The administration of MK-801 into the STN had no apparent effect on motor parameters. The findings provide evidence for a prominent role of STN $GABA_A$-receptors in movement processing under conditions of DA depletion. With respect to putative DBS mechanisms, they are also in favor of a conceptual framework that highlights the role of afferent inhibitory STN projections arising from the GPe. The observations also fit other reports that show a secondary role of glutamatergic mechanisms in the mediation of DBS effects (Gradinaru et al., 2009).

Taken together, the experiment yielded evidence for an involvement of $GABA_A$-receptors in the STN in the acute mediation of movement control in unilaterally 6-OHDA lesioned rats. The effects counteracted motor impairments incurred by DAergic denervation of the BG.

4.2 Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats

PD is characterized by a progressive degeneration of midbrain DA neurons determining the appearance of motor impairments. However, it has been widely accepted that besides motoric symptoms accompanied by a degeneration of midbrain DA projections (i.e the nigro-striatal, meso-cortical and mesolimbic pathway) there is also a variety of characteristic cognitive and emotional dysfunctions evident in PD patients. The underlying neurochemical mechanisms contributing to these alterations are less well understood than the ones associated with movement and motor control. This experiment (Petri et al., 2015) set out to determine a functionally relevant interaction between midbrain DA projections and the mPFC in the mediation of cognitive and affective alterations seen in hemiparkinsonian rats, as there is evidence that both systems are involved in circumscribed cognitive and affective functions. To this end, disconnection procedures of the mPFC and the MFB were performed by applying 6-OHDA and NMDA into the respective structures. Furthermore, the involvement of 5-HT in this interaction was addressed by serotonin-specific lesions of the mPFC. Behavioral readouts comprised memory-, anxiety- and depression-related measures.

It was found that a functional decoupling of the mPFC and midbrain DA system had considerable effects on cognitive and affective behavior. Disconnections of the mPFC and MFB showed promnestic, anxiolytic- and antidepressant-like effects, indicating an interaction between these constituents in the control of memory and emotional functions. In addition, 5-HT-specific lesions of the mPFC induced by the administration of 5,7-DHT showed the same pattern of behavioral effects. Thus, it can be concluded that the interaction of the mPFC and the MFB involves serotonergic mechanisms.

Taken together, the experiments demonstrate that a functionally relevant interaction between the mPFC and midbrain DA projections mediate cognitive and affective functions known to be impaired in PD patients. It was also observed that this interplay depends on 5-HT, as serotonin-specific lesions resembled the effects of unspecific excitotoxic lesions. These findings may have therapeutical implications for the treatment of cognitive and neuropsychiatric symptoms accompanied by PD.

5 Summary and discussion

The studies described here were set out to characterize neurochemical mechanisms underlying motoric, cognitive and affective alterations seen in dopamine deficient rats. It was found that (1) stimulation of $GABA_A$-receptors in the STN partially compensates for motoric deficits accompanied by unilateral lesions of the nigro-striatal tract, (2) a functional interaction between the medial prefrontal cortex and midbrain dopamine projections is crucial for memory-, anxiety- and depression-related impairments due to DA depletion and that (3) this interplay is mediated by serotonergic mechanisms in the medial prefrontal cortex.

$GABA_A$-receptor stimulation in the subthalamic nucleus compensates behavioral asymmetries in the hemiparkinsonian rat

The administration of the $GABA_A$-agonist muscimol into the STN ipsilateral to the side of MFB lesions resulted in a dose-dependent reduction of 6-OHDA-induced ipsiversive circling. Higher doses evoked circling behavior opposing this effect, i.e. to induce contraversive circling. These findings parallel the behavioral effects evoked by electrical stimulation of the STN with increasing stimulation intensities in unilateral 6-OHDA lesioned rats (Meissner, 2002; Salin et al., 2002). Thus, comparable mechanisms mediating these similar effects can be assumed. The phenomenon of contraversive circling represents a well-known and extensively studied behavioral response in the unilateral 6-OHDA model of PD and was originally described in the context of stimulation of striatal DA-receptors ipsilateral to the side of DA-depletion (Ungerstedt, 1971). The denervation of these receptors, as induced by the administration of 6-OHDA into the medial forebrain bundle, is accompanied by an increase in D1- and D2-like receptor binding, both concerning an increase in receptor number and receptor affinity (Schwarting and Huston, 1996a). If these up-regulated receptors are challenged with exogenously applied ligands (e.g. with the mixed D1/D2 agonist apomorphine, applied systemically), the amount of DAergic signaling into the striatum is more pronounced in the lesioned hemisphere as compared to the non-lesioned hemisphere. This predominance counteracts the ipsilateral motor bias in favor of the opposite direction and leads the animal to turn away from the side of lesion. Considering the equality of the behavioral phenomenology of apomorphine, STN DBS and the stimulation of $GABA_A$-receptors in the STN, it seems conceivable that all of these manipulations share a common mechanism, i.e. the stimulation of DA-receptors in the caudate-putamen complex ipsilateral to the side of the lesion. Several arguments point

towards such an assumption: (1) It was shown that delivering electrical current to the STN induces an increase in striatal DA concentration ipsilateral to the side of stimulation, accompanied by contraversive circling (Bruet et al., 2001; Meissner et al., 2002) in the hemiparkinsonian rat and that (2) STN DBS is associated with a reduction of DAergic medication in PD patients often by more than 50% (Moro et al., 1999). The latter also indicates a participation of DAergic processes in the mediation of clinical efficacy. In this context, the question emerges what striatal afferents can account for such an increase in extracellular DA levels. Principally, there are two distinct sources conceivable: (1) DAergic nigrostriatal projections (either ipsi- or contralateral) and/or (2) other monoaminergic afferents (e.g. serotonergic terminals, either ipsi- or contralateral). Besides the nigrostriatal projections, the striatum is largely innervated by serotonergic synapses (Parent et al., 2011) and 5-HT neurons display the enzymatic machinery capable for synthesis and release of DA (Carta and Bezard, 2011). A marked hyperinnervation/proliferation of afferent 5-HT fibres from the ipsi- (Berger et al., 1985) and contralateral (Steinbusch et al., 1980) raphe nuclei has been found following 6-OHDA lesions of the ascending nigrostriatal pathway and, moreover, the involvement of striatal 5-HT receptors in motor parameters has been demonstrated in the hemiparkinsonian rat (Dupre et al., 2008; Bishop et al., 2009). Considering the proposed GABAergic mechanisms in the STN, it has been hypothesized that an increase in striatal DA concentration may arise from a disinhibition of SNc neurons (Benazzouz et al, 2000). Although this source is obviously affected by neurotoxins applied in rodent models of PD (or by inherent neurodegenerative processes in PD patients), the fibres spared by the lesion (or degeneration) may account for the release of DA in the denervated striatum. Such a stimulation of up-regulated DA-receptors would then evoke contraversive circling behavior, virtually mimicking the effects of systemically applied DA-agonists. It has been proposed that a disinhibition of DAergic cells of the SNc may be due to a decrease in the activity of SNr neurons, which hold major inhibitory projections to SNc neurons (Grace and Bunney, 1979). SNr cells receive glutamatergic input from the STN, which is known to display pathological (increased) activity in terms of fire frequency and patterns in the DA-depleted state (Ammari et al., 2011). A functional inhibition of this nucleus, either via DBS or pharmacological stimulation of GABAA-receptors, is thought to decrease the pathological 'drive' to SNr neurons and, thereby, to disinhibit SNc cells, resulting in an increase in ipsilateral striatal DA concentrations (Bruet et al., 2001). This concept has also been proved experimentally as STN DBS was shown to decrease SNr activity (Maurice et al., 2003). The DAergic entity of

contraversive circling elicited by STN DBS has also been shown by Bergmann et al. (2004). The authors demonstrated that the STN DBS evoked behavioral response was successfully prevented by a focal pharmacological blockade of (dorsolateral) striatal DA-receptors ipsilateral to the side of DA depletion. More precisely, this effect was observed specifically for the antagonism of D2-like striatal DA-receptors. Although this study was performed in naïve, non-lesioned rats, it never the less allows experimental characterisation of the general mechanisms involved, since the authors demonstrated in other studies (Meissner et al., 2001, 2002) that the behavioral responses of STN DBS observed in the non-lesioned animal resembles those evoked in 6-OHDA lesioned rats. However, there is also evidence that principally question the role of striatal DA as mediating mechanism. As described above, electrical stimulation of the STN increased striatal DA-levels in naïve and 6-OHDA treated rats (Bruet et al., 2001; Meissner et al., 2002) and led to behavioral effects resembling those seen for intrasubthalamic $GABA_A$-receptor activation. This outcome has been reliably replicated by Pazo et al. (2010). These authors were also interested in the neurophysiological underpinnings of this effect and differentially addressed subthalamic $GABA_A$-receptors by applying a $GABA_A$-agonist (muscimol) and an $GABA_A$-antagonist (bicuculline) in unilateral 6-OHDA lesioned animals. The neurochemical effects on the ipsilateral striatum were assessed by microdialysis. Contrary to expectations arising from the mechanistic assumptions outlined above, the administration of muscimol did not evoke an increase in striatal DA levels, but induced a significant decrease. Most interestingly, an increase in striatal DA levels (resembling the effects evoked by electrical STN manipulation) was observed when antagonizing STN $GABA_A$-receptors with bicuculline. These findings contradict the assumption of increased ipsilateral striatal DA concentration as being the origin of the turning response observed for muscimol injections into the STN. Indeed, there are other findings that totally question the participation of the striatum in turning responses evoked by $GABA_A$-agonists at all. The STN sends excitatory glutamatergic projections to the GPi and the SNr, the major output nuclei of the BG. It seems likely, that the effects of STN manipulations are down-streamed to the GPi and the SNr and that functional changes in these structures mediate alterations in motor parameters. The administration of muscimol into the SNr evoked contraversive turning in several studies (Olpe and Koella, 1977; Scheel-Krüger et al., 1977; Martin and Bacino, 1978; Reavill et al., 1979; Thiebot and Soubrie, 1979). There is evidence that this effect is independent of DA, showing no modulation with systemic or striatal DA-antagonists (Haloperidol or cis-Flupenthixol), 6-OHDA induced nigro-striatal degeneration

or kainic acid lesions of the striatum (Arnt and Scheel-Krüger, 1979). In fact, the turning response elicited by muscimol was even present in rats bearing severe lesions of the whole telencephalon (surgical removal of the neocortex, hippocampus, striatum, septal nuclei and the amygdala; Papadopoulos and Huston, 1979).

Beneficial effects on motoric parameters have been demonstrated for lesions of the STN in 6-OHDA treated rats (Burbaud et al., 1995; Piallat et al., 1996; Kafetzopoulos and Papadopoulos, 1983), MPTP treated primates (Hamada and DeLong, 1992) and PD patients (Guridi and Obeso, 2001). Thus, there is considerable evidence that the mechanism by which STN DBS mediates its therapeutical effects is to induce a virtual lesion and, therefore, to prevent the generation of hyperactive burst firing of the STN. Two approaches may account for such an inhibitory effect: *depolarization blockade* or *synaptic inhibition*. There are some limitations as to the *depolarization blockade approach* . As different types of neural elements display variable properties regarding their excitability in terms of “chronaxie” time measurement (defined as “the minimum interval of time required to excite a neural element using twice the intensity that elicits a threshold response”; Kringelbach et al., 2010) and axons (especially extensively myelinated ones) show a much smaller chronaxie (30-200µs) as compared to cell somas (1-10 ms), it is likely that currents applied via DBS electrodes act on an axonal level rather than on neuronal cell bodies. Further, since stimulation of axons (nodes of Ranvier) ultimately leads to initiation of action potentials the prediction of this approach would be incompatible with the *depolarization blockade* hypothesis (McIntyre et al., 2004). The results of the experiments presented are in favor of the synaptic disinhibition approach. The pivotal role of STN afferents, rather than STN neurons per se have been demonstrated by Gradinaru et al. (2009). This group employed optogenetic techniques to achieve control over STN activity. Briefly, light-sensitive sodium-channels were selectively introduced to afferent axons projecting to the STN and driven by a laser diode. The advantage of this approach is comparable to the preparation used in the experiments described here since light-induced alterations are also exclusively attributable to modulations in the targeted structures (afferent axons) as compared to electrical stimulation, which is likely to also affect other neural elements by conduction of current (current spread) and fibres of passage. The results showed robust therapeutic effects by means of correction for the ipsilateral motor bias induced by 6-OHDA. Remarkably, there were no therapeutic effects of introducing light-sensitive chloride-channels directly into cell bodies of STN neurons, indicating the prominent role of afferent influx into the STN in the mediation of motor improvement. Although there was

evidence that the source of a certain number of the afferent fibres originates in cortical layers, the authors also highlight that it is unlikely for all the affected fibres to originate from one source, leaving the possibility to also affect GABAergic afferents arising from the GPe. The results of the present experiment demonstrated further evidence for such an assumption.

Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats

The experiments demonstrated evidence for a functionally relevant interaction between midbrain dopamine projections and the medial prefrontal cortex (mPFC) in the mediation of cognitive and affective functions. Moreover, it was that this interplay involves serotonergic mechanisms. Disconnection effects were observed with respect to memory-, anxiety-, and depression-related behavioral measures. Characteristic impairments in PD patients include cognitive deficits and neuropsychiatric disorders. These examples of non-motor symptoms have a broad impact on the quality of life of people concerned (Lawson et al., 2014; Lawrence et al., 2014) and have gained clinical interest during the last decades (Svenningsson et al., 2012). Comparable devastating chronic diseases are not associated with such deficits, thus a specific involvement of DAergic mechanisms can be assumed since degeneration of midbrain DA projections is considered as a pathological hallmark in PD. The role of DA in the mediation of diverse cognitive and emotional functions is well-known and the mechanistic implementation in the brain has been dissected experimentally in a variety of animal models (Dembrow and Johnston, 2014; Chudasama and Robbins, 2006; Nieoullon and Coquerel, 2003). It was shown that 6-OHDA lesions of the MFB have anxiogenic effects (Santiago et al., 2010; Tadaiesky et al., 2008), mediate memory impairments (Foyet et al. 2011; Chao et al., 2013) and evoke depressive-like behavior (Eskow-Jaunarajs et al., 2010; Zhang et al., 2011). Indications for an involvement of the mPFC in this context arise from observations made in patients with lesions of that structure (Kehagia et al., 2010; Owen et al., 1992; Taylor et al., 1986). These patients exhibit impairments that show a comparable neuropsychological deficit profile as PD patients. The mPFC is innervated by mesocortical DA projections and also holds mutual connections (Patton et al., 2013). It was also shown to be involved in cognitive and affective functions in animal models. Lesions of the mPFC in rats were demonstrated to influence anxiety (De Visser et al., 2011; Pum et al., 2009; Shah et al., 2003), depressive-like behavior (Chang et al., 2014; Klein et al., 2010) and memory functions (Barker and Warburton, 2008, Hannesson et al., 2004; Barker et al., 2007). Furthermore, serotonergic

mechanisms seem to play a pivotal role in this context as serotonin-specific lesions of the mPFC resemble these effects (Perez-Vega et al., 2000; Gonzalez-Burgos et al., 2012). Given that mPFC and MFB lesions evoke similar kinds of functional deficits with respect to memory, anxiety and depression, there is evidence that an interplay between theses structures is crucial for the mediation of these functions under physiological conditions. Moreover, interactions between DAergic afferences and the serotonergic system of the mPFC have also been described on the level of receptor-related mechanisms. Experimental DA depletion of the mPFC evokes differential responses of distinct 5-HT-receptors towards pharmacological challenges as assessed by electrophysiological alterations (Wang et al., 2009a; Gui et al., 2011; Fan et al. 2011; Zhang et al., 2011; Cao et al., 2007). This implies corresponding behavioral alterations and the experiments described here provided evidence fur such an assumption. Another interplay of the serotonergic and dopaminergic systems was described in the context of chronic L-DOPA administration in PD patients. Here, it was demonstrated that prolonged intake can inhibit tryptophan hydroxylase activity and may interfere with physiological aromatic amino acid decarboxylase (Hashiguti et al., 1993; Borah and Mohanakumar, 2007). 5-HT neurons also hold the enzymatic machinery to convert L-DOPA into DA (Navailles et al., 2010; Navailles and De, 2011; Eskow-Jaunarajs et al., 2010). Taken together, these alterations may ultimately lead to a decrease in availability of functional 5-HT which, in turn, can evoke cognitive and emotional disorders. However, contrary concepts have also been proposed. There is evidence that an increase in sprouting of 5-HT terminals, as well as a hyperinnervation of of forebrain 5-HT fibres and a general increase in 5-HT levels occurs following degeneration of midbrain DA projections (Zhou et al., 1991; Smits et al., 2008; Reader and Dewar, 1999; Commins et al., 1989). Thus, a serotonergic hyperinnervation may also account for the functional impairments accompanied by DA deficiency. There are some limitations of the experimental approach utilized in the experiments described here to be considered. Both the mPFC and the MFB have ipsi-, as well as contralateral projections. Thus, an unilateral lesion of the one or the other structure may not result in a complete functional decoupling. However, transcallosal collaterals are way less prominent than ipsilateral projections (Ferino et al., 1987; Pritzel et al., 1983) and any behavioral differences seen between ipsi- and contralateral (disconnecting) lesions point toward a functional interplay of the structures.

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7 Publications

Experiment 1:

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Experiment 2:

Petri, D., de Souza Silva, M.A., Chao, O. Schnitzler, A., Huston, J.P., 2015. Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats. *Manuscript submitted for publication.*

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Research report

$GABA_A$-receptor activation in the subthalamic nucleus compensates behavioral asymmetries in the hemiparkinsonian rat

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HIGHLIGHTS

- Pharmacological STN manipulation influences motor parameters in 6-OHDA-treated rats.
- Compensation of motor asymmetries by activation of $GABA_A$-receptors in the STN.
- Dose-dependent effect of muscimol on turning behavior and locomotion.
- No acute effects of NMDA-antagonism in the STN on motoric behavior.
- GABAergic mechanisms contribute to therapeutical effects of STN manipulations.

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ABSTRACT

The subthalamic nucleus (STN) has a pivotal role in the pathophysiology of Parkinson's disease (PD). Modulation of STN activity (by lesions, pharmacological or electrical stimulation) has been shown to improve motor parameters in PD patients and in animal models of PD. In an attempt to characterize the neurochemical bases for such antiparkinsonian action, we address specific neurotransmitter systems via local pharmacological manipulation of the STN in hemiparkinsonian rats. Here, we have focused on the GABAergic and glutamatergic receptors in the STN. In animals with unilateral 6-hydroxydopamine lesions of the nigro-striatal tract, we administered either the selective $GABA_A$-agonist muscimol (0.5 μg and 1.0 μg), the non-competitive *N*-methyl-D-aspartate (NMDA)-antagonist MK-801 (dizocilpine; 2.5 μg), or vehicle (0.25 μl) into the STN. The effects of GABAergic and glutamatergic modulation of the STN on motor parameters were assessed by gauging rotational behavior and locomotion. Application of muscimol ipsilateral to the side of dopamine-depletion influenced turning behavior in a dose-dependent fashion, with the low dose re-adjusting turning behavior to a non-biased distribution, and the high dose evoking contraversive turning. The administration of MK-801 did not have such effects. These findings give evidence for the involvement of GABAergic activation in the STN in the compensation of motor asymmetries in the hemiparkinsonian rat, whereas *N*-methyl-D-aspartate (NMDA)-antagonism was ineffective in this model of PD.

1. Introduction

The subthalamic nucleus (STN) has a crucial role in the development of motor impairments and abnormal neural activity following degeneration of midbain dopamine (DA) neurons. Several manipulations of the STN (lesions, pharmacological or electrical stimulation) compensate for the deficits associated with parkinsonism. The neurochemical bases of such manipulations remain poorly understood. The present experiment aimed at characterizing the involvement of GABAergic and glutamatergic STN receptors in movement control in hemiparkinsonian rats.

The unilateral administration of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) is a widely applied rodent model of experimental

Abbreviations: 6-OHDA, 6-hydroxydopamine; DA, dopamine; DBS, deep brain stimulation; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; GPe, globus pallidus externa; GPi, globus pallidus interna; HPLC-EC, high performance liqiuid chromatography with coupled electrochemical detection; HVA, homovanillic acid; i.p., intraperitoneal; L-DOPA, L-3,4-dihydroxyphenylalanine; M1, primary motor area; MFB, medial forebrain bundle; MRBD, movement related beta desynchronisation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartate; PD, Parkinson's disease; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

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parkinsonism and is known to alter GABAergic [1,2] and glutamatergic [3] neurotransmission within different nuclei of the basal ganglia (BG), including the STN. The DAergic degeneration of nigro-striatal projections has also been shown to alter the firing pattern [4] and oscillatory activity [5] of several nuclei of the BG. Excessive rhythmic burst-activity and an increase in local field potential oscillations in power and coherence in the beta range (~15–30 Hz) of the STN have been discussed to contribute to the manifestation of motor impairments in animal models of PD [5–7] as well as in PD patients [8–12]. Several manipulations of the STN have been demonstrated to compensate behavioral deficits associated with DA-denervation, both, in PD patients and pre-clinical rodent models, including lesion procedures [13–15], electrical [16] and pharmacological [17,18], as well as optogenetical modulations [19]. Lesions of the STN counteract experimental parkinsonism in monkeys [20]. Electrical high-frequency stimulation of the STN (STN-DBS) alleviates PD motor impairments in monkeys and rats [17,21–24]. GABAergic activation in the STN has antiparkinsonian effects in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys [25,26] and in PD patients [18] and has beneficial effects on oscillatory activity in the DA-depleted BG [27]. Such manipulations of the STN lead to focal and remote changes in various neurotransmitter systems in diverse BG nuclei [28–32].

The STN holds a strategic position within the cortico-BG-thalamo-cortical loop and receives GABAergic inputs from the external part of the globus pallidus (GPe) and glutamatergic afferents from extensive cortical areas associated with motor control. It sends excitatory projections to the GPe, the internal segment of the globus pallidus (GPi) and to the substantia nigra pars reticulata (SNr), using glutamate as its major neurotransmitter. Given the unique role of GABA and glutamate in BG in- and output circuitry, these neurotransmitters have gained special attention in physiological and pathological BG functioning. Unilateral administration of the $GABA_A$-receptor agonist muscimol into the STN evoked contraversive turning behavior (a widely applied behavioral measure in the assessment of unilateral nigro-striatal lesions) in rats with intact nigro-striatal projections [17] and in non-lesioned cats [33], although others have found no effects of subthalamic muscimol injection on turning behavior in non-parkinsonian rats [34]. There is also evidence for the participation of glutamatergic mechanisms in the control of motor behavior by the STN. The involvement of STN NMDA-receptors in turning behavior and other motor deficits incurred by nigro-striatal denervation has been shown with respect to prolonged intrasubthalamic NMDA antagonism. Prolonged (~3 weeks) infusion of MK-801 into the STN immediately after administration of 6-OHDA prevented the development of motor impairments [35]. The acute unilateral injection of MK-801 into the STN led to contraversive dystonic postures when haloperidol was systemically administered afterwards [36]. Intranigral NMDA-antagonism evokes contralateral turning in intact [37] and 6-OHDA treated rats [38]. Furthermore, systemic administration of MK-801 was found to increase contraversive turning behavior evoked by systemically applied dopamine agonists in the unilaterally 6-OHDA lesioned rat [39].

In an attempt to characterize the involvement of subthalamic GABAergic and glutamatergic transmission on motor parameters in a unilateral rodent model of PD, we here administered the selective $GABA_A$-agonist muscimol or the non-competitive NMDA-antagonist MK-801 focally into the STN. Given the role of GABA and glutamate in physiological and pathological movement control, we hypothesized that intrasubthalamic injections of muscimol and MK-801 would compensate for motoric deficits (including turning behavior and locomotion) in the hemiparkinsonian rat. We decided to determine the motoric effects of $GABA_A$-agonism and NMDA-antagonism in the STN in rats experimentally rendered (hemi-)parkinsonian since, to our knowledge, this is the first study to behaviorally characterize the effects of focal pharmacological manipulations of the STN in rats bearing massive unilateral lesions of the nigrostriatal tract.

Turning behavior in the hemiparkinsonian rat is differentially modulated by different types of systemically applied dopamine agonists [40]. To characterize the distinct impact of $GABA_A$-agonism and NMDA-antagonism on pre- vs. post-synaptic DAergic mechanisms, we also challenged animals with L-DOPA and D-amphetamine, respectively, in parallel with the intrasubthalamic administrations.

2. Experimental procedures

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and the effective German Law of animal protection of 1998 and have been approved by local authorities (Central Animal Facility of the University of Düsseldorf and the The North Rhine-Westphalia State Environment Agency). Efforts were made to minimize the number of animals used and to minimize suffering.

2.1. Animals

The subjects were male Wistar rats (weights ranging from 230 to 300 g at the beginning of the experiment), purchased from Janvier (Le Genest St. Isle, France). Following surgery, they were housed individually in standard translucent plastic cages (30 cm × 20 cm × 20 cm). They were kept under standardized laboratory conditions in a temperature (20 ± 2 °C) controlled room with a 12:12 h light–dark cycle starting at 7:00 am and were provided with free access to food and water throughout the experiment. Subjects were randomly assigned to one of the treatment groups. Behavioral experiments were conducted between 08:00 am and 06:00 pm.

2.2. Surgical procedures

2.2.1. 6-OHDA lesions

All surgical procedures were carried out under sodium pentobarbital anesthesia (60 mg/kg; Merial, Hallbergmoos, Germany). Carprofen (5 mg/kg; Pfizer, Berlin, Germany) was applied subcutaneously immediately prior to each surgery session to prevent inflammatory reactions and to manage postoperative pain. Animals were mounted on a stereotaxic frame (David Kopf, Tujunga, CA, USA), the skull was sanitized with 70% ethanol solution, a volume (0.5 ml) of local anesthetics (lidocaine hydrochloride) was subcutaneously administered, and an incision was made along the scalp to expose the skull. The surface was bleached with 10% hydrogen peroxide solution to allow for proper localization of bregma and lambda and burr holes were applied above the MFB and STN, respectively. Additional holes were drilled for fixation of the guiding cannulae with dental acrylic and three bone screws. Screws were mounted 2 mm apart each in the lateral, anterior and anterolateral direction with respect to the location of the STN burr hole. Animals received unilateral (side of lesion was counterbalanced across treatment groups) infusions of 6-hydroxydopamine hydrochloride (12.5 μg/3 μl, dissolved in 0.9% saline with 0.1% ascorbic acid; Sigma–Aldrich, Munich, Germany) via a 28-gauge cannula with a constant flow rate of 0.5 μl/min. Infusions were administered via a microinfusion-pump (KD Scientific, Boston, MA, USA) equipped with a 10 μl syringe (Microliter 701, Hamilton, Bonaduz, Switzerland). Injections were aimed at the medial forebrain bundle (AP: −4.0 mm, ML: +1.5 mm, DV: −8.5 mm; relative to bregma and skull surface according to Paxinos and Watson [41]), since lesions here are known to generate severe dopamine depletion [42]. The cannula was left in place for an additional minute to allow diffusion and to minimize reflux. Animals were given ten to eleven days of recovery after surgery before the effectiveness of the lesions was validated by assessment of amphetamine-induced (1.5 mg/kg; i.p.; in saline) ipsiversive rotational behavior in an open-field (see Section 2.4 for further details). Behavioral asymmetries were observed for 30 min and only animals that exhibited ≥80% quarter turns toward the side of the lesion were chosen to undergo consecutive behavioral motor tests.

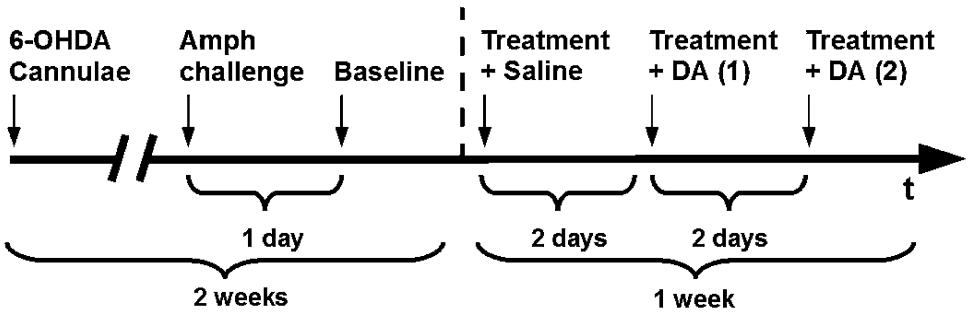
2.2.2. Implantation guiding cannulae

Subjects were also provided with guiding cannulae (Model 3260PGA/Spc 8MM, Plastics One, Roanoke, VA, USA) ipsilateral to the lesion for the acute pharmacological modulation of STN activity (AP: −3.8 mm, ML: +2.5 mm, DV: −7.6 mm, according to bregma and skull surface [41]). Guiding cannulae (26-gauge, outer diameter 0.46 mm, inner diameter 0.24 mm) were 8 mm in length from skull surface, the same length as the injection cannula. The pedestals of the cannulae were fixated with dental acrylic and screws in the skull. Thereafter, the cannulae were sealed with dummies, the retracted skin was sutured and disinfected with a 70% ethanol solution and the animals were returned to their home cages.

2.2.3. Histology

Once behavioral testing was completed the animals were killed with an overdose of sodium pentobarbital and were transcardially perfused, starting with phosphate

A



B

Treatment	Substance	Acute Dose (volume 0.25 µl)	Group Size
Vehicle	Ringer's solution	---	n = 7
NMDA-Antagonist	MK-801	2.5 µg	n = 7
$GABA_A$-Agonist	Muscimol	0.5 µg	n = 7
		1.0 µg	n = 8

Fig. 1. Schematic overview of the experiment (A) time bar representing the course of experimental procedures. DA (1) and DA (2) refer to the systemically applied dopamine agonists administered in a counterbalanced fashion. (B) Substances and corresponding doses used for intrasubthalamic treatment.

buffered saline followed by a 10% formaldehyde solution, before brains were extracted and stored in 10% formaldehyde solution. After slicing the brains with a cryostat (Leica, Germany), slices were stained with cresyl violet (Sigma–Aldrich, USA) and the placement of the guiding cannulae was verified according to the atlas of Paxinos and Watson [41].

2.3. Pharmacological treatments

2.3.1. Drugs

The drugs used were: (+)-MK-801 hydrogen maleate, muscimol (3-hydroxy-5-aminomethyl-isoxazole), L-3,4-dihydroxyphenylalanine methyl ester hydrochloride, benserazide hydrochloride and D-amphetamine hemisulphate salt (Sigma–Aldrich, Munich, Germany). The drugs used for STN administrations (MK-801 and muscimol) were dissolved in sterile ringer's solution. All drugs used for intraperitoneal (i.p.) administrations (L-3,4-dihydroxyphenylalanine methyl ester hydrochloride, benserazide hydrochloride and D-amphetamine hemisulphate salt) were dissolved in sterile phosphate buffered saline (PBS). Administration volume was 0.25 µl for STN administrations and 1 ml/kg for i.p. administrations. The amount applied was 2.5 µg for MK-801, 0.5 or 1.0 µg for muscimol, 12 mg/kg L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (+15 mg/kg benserazide hydrochloride) and 1.5 mg/kg D-amphetamine hemisulphate salt. The doses for the STN injections were chosen according to their reported capability to effectively modulate turning behavior and other motor parameters in animal models of PD [34,36]. It was shown that an intrasubthalamically injected volume of 1 µl muscimol solution, administered with a flow rate of 0.25 µl/min, decreased neural activity extending 1 mm^3 surrounding the application cannula [43]. Given the fourfold smaller volume of the STN injections applied in the present study, it seems unlikely that other targets than the STN were affected by the injections. We chose the $GABA_A$-receptor agonist muscimol in favor of other compounds decreasing net neural activity (i.e. sodium channel blocker including tetrodotoxin and lidocain) since these agents are known to also affect fibers of passage. Such an approach ensures that possible effects on motor parameters by focal STN manipulations may more exclusively be attributable to that structure. The doses for the systemic challenges were selected on the basis of their known actions on motor-associated parameters [44,45].

2.4. Behavioral procedures

PD-related motor impairments induced by the 6-OHDA lesion and behavioral effects of the STN and i.p. treatments were assessed by measuring spontaneous turning behavior in an open field. Quarter turns were registered in an open field (48 cm × 48 cm × 40 cm) situated in a sound attenuating and white-noise masked (60 dB) chamber for 30 min via the Ethovision© software suite (Version XT 8, Noldus Information Technology, Netherlands), which also tracked the total distance moved (horizontal locomotion), including turning behavior. Asymmetry indices for quarter turns were computed by the ratio of ipsi- or contraversive turns relative to the total number (sum) of ipsi- and contraversive turns, whereas 'ipsi' and 'contra' refer to the side ipsi- or contralateral to the 6-OHDA lesion. This was computed for the entire duration of the testing period, and also for 5-min bins, resulting in six bins for each trial and animal. Detection parameters were set to a *center-point to nose-point* detection and the threshold was set to 0.2500 rotations.

The time-course of behavioral testing and the pharmacological treatments applied is depicted in Fig. 1. On the day after the initial amphetamine-challenge and behavioral testing, the subjects were again observed for motor asymmetries in the open-field without any pharmacological intervention to assess a baseline for motor impairments. The order for all behavioral testing was randomized. Thirteen to 14 days after the lesion the animals were given the first of a total of three injections into the STN according to the respective treatment condition (vehicle, MK-801 or muscimol). The injections were given in 2-day intervals. The animals were also treated intraperitoneally with either L-3,4-dihydroxyphenylalanine or amphetamine prior to the second and third STN injections, the order of the injections was counterbalanced for each group. Prior to the first STN injection the subjects were treated with saline. L-3,4-Dihydroxyphenylalanine and benserazide (as well as saline on the first day of STN assessment) were given 15 min prior to STN injections, while amphetamine was administered immediately prior to testing. Motor effects were assessed in the open-field for 15 min post-injection. During drug infusions into the STN the subjects were gently restrained and the administration cannula (model C315IA/SP w/o projection, 8MM, Plastics One, Roanoke, VA, USA) was inserted into the guiding cannula. The tissue was infused with a flow rate of 0.5 µl/min. All injections were performed with a microinfusion syringe pump (KD Scientific, Boston, MA, USA) equipped with a 10 µl syringe (Microliter 701, Hamilton, Bonaduz, Switzerland) connected via a PE-20 tubing to the administration cannula. An air bubble was introduced to the tubing to allow monitoring of the flow. After acute administration the cannula was left in place for 30 s to allow for diffusion of the solution.

2.5. Neurochemical analysis

Neurochemical assessment of the content of DA, and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), was carried out in the dorsal striatum of the lesioned and intact hemispheres. High Performance Liquid Chromatography with electrochemical detection (HPLC-EC; see Chao et al. [46] for further procedural details). The analysis was carried out in 12 rats randomly chosen from a large set of animals with unilateral 6-OHDA injections into the MFB, used in parallel studies [46,47] according to the behavioral

Table 1
Neurochemical assessment of striatal DA-depletion induced by 6-OHDA applied into the MFB.

	DA	DOPAC	HVA	DA depletion %
Lesioned	34.45 ± 15.52*	50.49 ± 14.22**	109.96 ± 50.88	90.97 ± 3.00
Intact	210.50 ± 52.11	98.57 ± 6.91	128.78 ± 40.70	

Values are expressed as ng/mg wet tissue weight. Data are expressed as mean values ± SEM. Paired *t*-test with $n = 12$.
* $P = .001$.
** $P = < .001$.

criterion utilized in the present one (>80% ipsiversive turning during D-amphetamine challenge).

2.6. Statistical testing

Since most of the behavioral data were not normally distributed and did not show homogeneity of variance (Shapiro–Wilk-test and Levene-test, respectively), statistical testing was carried with non-parametric procedures. Between-subject comparisons were done by the Kruskal–Wallis-*H*-test for general effects of the distinct factor levels. If meaningful differences were indicated, group-wise comparisons between the corresponding groups were performed employing the Mann–Whitney *U*-test. Within-subject comparisons were done by the Friedman-χ^2-test for general effects of the distinct occasions of testing. If differences were indicated, condition-wise comparisons were performed employing the Wilcoxon-signed-rank test. All tests were two-sided. A criterion of *P*-values $\leq .05$ was arbitrarily assumed to indicate an effect and, therefore, represents a measure of effect. *t*-Tests for dependent samples were carried out with the neurochemical data.

3. Results

3.1. Neurochemistry

There was a significant reduction in content of DA in the lesioned striatum ($t_{11} = 4.743$, $P = .001$, mean 90.79%, ±3.00 SEM). There was also a significant decrease in DOPAC in the affected striatum ($t_{11} = 5.295$, $P < .001$) with a mean loss of 54.17% (±9.32 SEM). HVA content (mean 58.26%, ±16.16 SEM) did not differ significantly ($t_{11} = .905$, $P = .05$). See also Table 1 for an overview (the mean values presented display averages across individual animals).

3.2. Behavioral asymmetries

3.2.1. Vehicle (control) group

There were differences between effects of sole STN administration as compared to STN + i.p. D-amphetamine and STN + i.p. L-DOPA challenges within this group ($\chi^2_3 = 11.160$ ($n = 7$), $P = .011$). Animals receiving vehicle into the STN showed no change in turning behavior as compared to baseline assessment ($Z = -.943$, $P > .05$). Systemic administration of L-3,4-dihydroxyphenylalanine (12 mg/kg i.p. + 15 mg/kg benserazide i.p.) prior to STN injections did not alter turning behavior ($P > .05$ for all within-group comparisons). Systemic administration of D-amphetamine (1.5 mg/kg) increased ipsiversive turning as compared to acute STN plus i.p. vehicle treatment ($Z = -2.023$, $P = .043$). See also Fig. 2 for an overview of the STN treatments.

3.2.2. NMDA-antagonist (MK-801) group

There were no differences regarding sole STN administration as compared to STN + i.p. D-amphetamine and STN + i.p. L-DOPA within this group ($\chi^2_3 = 6.257$ ($n = 7$), $P > .05$). Since there were differences between the groups in turning behavior (STN + i.p. vehicle: $\chi^2_3 = 13.213$ ($n = 29$), $P = .004$; STN + i.p. L-DOPA: $\chi^2_3 = 16.909$ ($n = 29$), $P = .001$; STN + i.p. D-amphetamine: $\chi^2_3 = 13.888$ ($n = 29$), $P = .003$), post hoc comparisons were carried out. Infusion of the NMDA-antagonist into the STN did not alter the degree of behavioral asymmetry as compared to vehicle-control group ($U = 16.000$, $P < .05$). As compared to the vehicle group, L-DOPA (+STN treatment) increased ipsiversive turning ($U = 7.000$, $P = .026$) in this group.

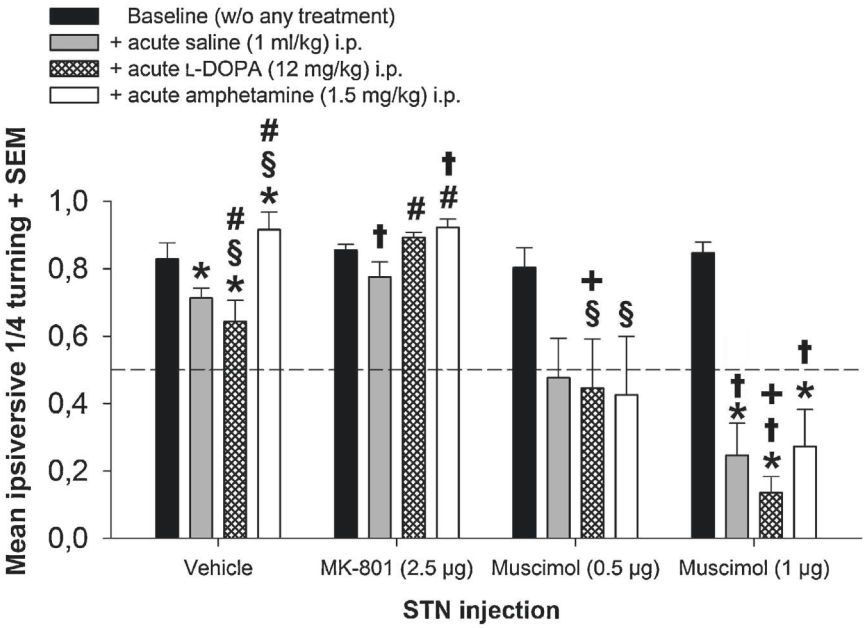


Fig. 2. Effects of injecting MK-801 or muscimol into STN on turning behavior expressed as averaged amount of ipsiversive turning. The dashed line at 0.5 represents an equally distributed amount of ipsi- and contraversive turning as expected to be exhibited by non-lesioned animals. #$P \leq .05$ between vehicle and MK-801 groups. §$P \leq .05$ between vehicle and muscimol 0.5 µg groups. *$P \leq .05$ between vehicle and muscimol 1.0 µg groups. †$P \leq .05$ between MK-801 and muscimol 1.0 µg groups. ✚$P \leq .05$ between muscimol 0.5 µg and 1.0 µg groups.

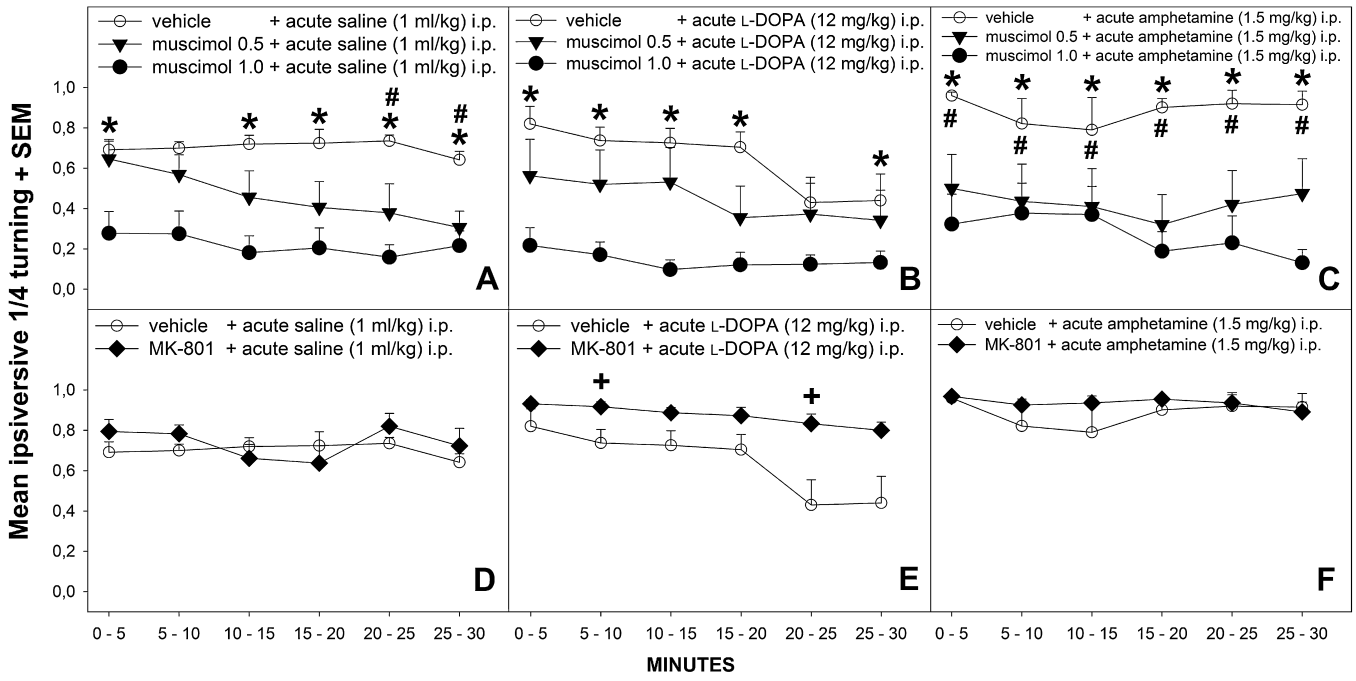


Fig. 3. Time-course of turning behavior for the different STN treatments and systemically applied dopamine agonists. Temporal profile of muscimol applied after systemic administration of saline (A), L-DOPA (B) and D-amphetamine (C). Temporal profile of MK-801 applied after systemic administration of saline (D), L-DOPA (E) and D-amphetamine (F). Note that the vehicle group is the same for A–C and D–F. $^{*}P \leq .05$ between vehicle and high muscimol groups (1.0 μg), $^{\#}P \leq .05$ between vehicle and low muscimol groups (0.5 μg) and $^{+}P \leq .05$ between vehicle and MK-801 groups.

Systemic challenge with D-amphetamine prior to STN administrations had no effects ($U = 17.000$, $P > .05$) as compared to the vehicle group.

3.2.3. $GABA_A$-agonist (muscimol) groups

There were no differences between sole STN administration as compared to STN administration + systemic D-amphetamine and STN administration + systemic L-DOPA within the 0.5 μg group ($\chi^2_3 = 3.857$ ($n = 7$), $P > .05$), but there were effects in the 1.0 μg group ($\chi^2_3 = 9.000$ ($n = 8$), $P = .029$). An increase in contraversive turning was observed within this group for all STN treatments (including systemically applied dopamine agonists) as compared to baseline (vehicle: $Z = -2.380$, $P = .017$; L-DOPA: $Z = -2.521$, $P = .012$; D-amphetamine: $Z = -2.197$, $P = .028$). Since differences between the groups were observed (see above), post hoc comparisons were carried out. As compared to the vehicle-control group there was an induction of contraversive turning in the higher dose group for STN treatment + i.p. vehicle ($U = 3.000$, $P = .002$), for STN treatment + i.p. L-DOPA ($U = .000$, $P < .001$) and STN treatment + i.p. D-amphetamine ($U = 1.000$, $P = .002$). The lower dose group (0.5 μg) showed no difference from the vehicle-control group for STN treatment + i.p. vehicle ($U = 14.000$, $P > .05$) or STN treatment + i.p. L-DOPA ($U = 18.000$, $P > .05$), but for STN treatment + i.p. D-amphetamine ($U = 5.000$, $P = .022$). The higher dose group also showed more contraversive turning than the MK-801 group for all treatments (STN treatment + i.p. vehicle: $U = 2.000$, $P = .001$; STN treatment + i.p. L-DOPA: $U = .000$, $P < .001$; STN treatment + i.p. D-amphetamine: $U = .000$, $P = .001$), while the lower dose group did for STN treatment + i.p. L-DOPA ($U = 5.000$, $P = .011$) but not for STN treatment + i.p. vehicle ($U = 13.000$, $P > .05$) or STN treatment + i.p. D-amphetamine ($U = 10.000$, $P > .05$). There were no differences in the STN treatment between the high and low dose group (STN treatment + i.p. vehicle: $U = 13.000$, $P > .05$; STN treatment + i.p. L-DOPA: $U = 18.000$, $P > .05$; STN treatment + i.p. D-amphetamine: $U = 21.000$, $P > .05$).

3.3. Time-course of behavioral asymmetries

Given the asymmetry indices of the preceding section being averaged over the total time subjects spent in the open-field (30 min), a different approach is to investigate the development of this measure over time. To this end, the total time-span of 30 min was divided into six time bins of 5 min each (Fig. 3). General effects between groups were observed for all respective time bins after STN treatment + vehicle i.p.: 0–5 min: $\chi^2_3 = 10.878$, $n = 29$, $P = .012$; 5–10 min: $\chi^2_3 = 9.311$, $n = 29$, $P = .025$; 10–15 min: $\chi^2_3 = 11.614$, $n = 29$, $P = .009$; 15–20 min: $\chi^2_3 = 11.479$, $n = 29$, $P = .009$; 20–25 min: $\chi^2_3 = 15.955$, $n = 29$, $P = .001$; 25–30 min: $\chi^2_3 = 17.274$, $n = 29$, $P = .001$; after STN treatment + L-DOPA i.p.: $\chi^2_3 = 12.158$, $n = 29$, $P = .007$; 5–10 min: $\chi^2_3 = 15.426$, $n = 29$, $P = .0001$; 10–15 min: $\chi^2_3 = 14.466$, $n = 29$, $P = .002$; 15–20 min: $\chi^2_3 = 15.027$, $n = 29$, $P = .002$; 20–25 min: $\chi^2_3 = 11.975$, $n = 29$, $P = .007$; 25–30 min: $\chi^2_3 = 12.990$, $n = 29$, $P = .005$ and after STN treatment + D-amphetamine i.p.: $\chi^2_3 = 15.583$, $n = 29$, $P = .0001$; 5–10 min: $\chi^2_3 = 8.183$, $n = 29$, $P = .042$; 10–15 min: $\chi^2_3 = 8.399$, $n = 29$, $P = .038$; 15–20 min: $\chi^2_3 = 17.410$, $n = 29$, $P = .001$; 20–25 min: $\chi^2_3 = 14.541$, $n = 29$, $P = .002$; 25–30 min: $\chi^2_3 = 14.899$, $n = 29$, $P = .002$. The muscimol 1.0 μg group displayed more contraversive turning for virtually all (except 5–10 min bin) bins (each $P < .05$) There was an increase in contraversive turning for the 0.5 μg muscimol group compared to the vehicle group after saline administration during the last two bins ($U = 8.000$, $P = .038$ and $U = 4.000$, $P = .007$; Fig. 3A). No effects were observed when applying L-DOPA prior to STN treatment comparing the 0.5 μg muscimol and the vehicle group ($P > .05$; Fig. 3B). However, the high dose of muscimol increased contraversive turning as compared to vehicle, as well as to MK-801, in all bins when prior administered with L-DOPA (each $P < .05$; Fig. 3B). For each bin, there were no differences between the high and low dose muscimol groups (each bin with $P > .05$; Fig. 3B). Both doses of muscimol increased contraversive turning for each bin (except the 10–15 min bin) of the D-amphetamine challenge as compared

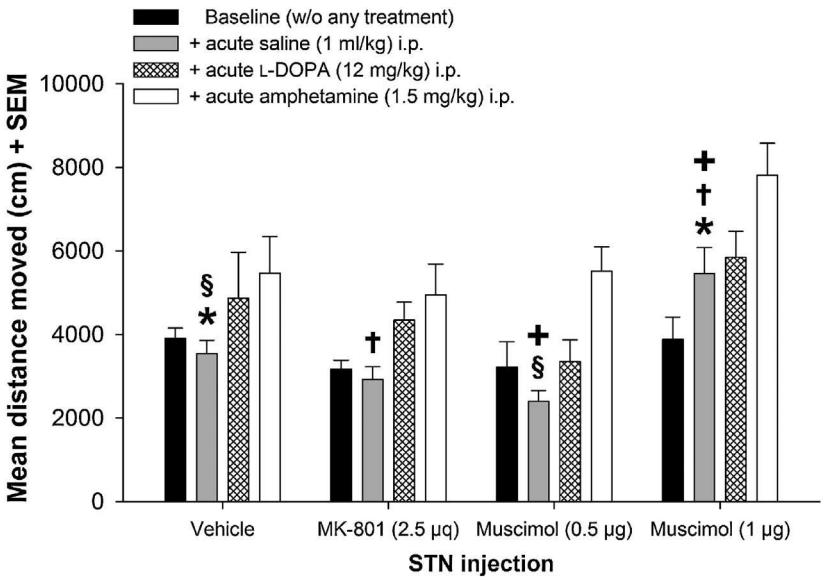


Fig. 4. Effects of the STN treatment on locomotion (distance moved). §$P \leq .05$ between vehicle and muscimol 0.5 µg groups. *$P \leq .05$ between vehicle and muscimol 1.0 µg groups. †$P \leq .05$ between MK-801 and muscimol 1.0 µg groups. ✚ $P \leq .05$ between muscimol 0.5 µg and 1.0 µg groups. Note that for reasons of clarity, only between-group comparisons (and no within-group comparisons) are depicted.

to the vehicle group (each $P < .05$; Fig. 3C). Applying MK-801 did not influence ipsiversive motor bias per se, neither for STN treatment + vehicle i.p., nor for STN treatment + D-amphetamine i.p. as compared to vehicle group (each respective bin with $P > .05$; Fig. 3D). A difference was observed, as compared to vehicle group, for the 5–10 and 20–25 min bin during systemic L-DOPA challenge ($U = 7.000$, $P = .026$ and $U = 8.000$, $P = .038$; Fig. 3E). There were no effects of D-amphetamine as compared to vehicle control group (Fig. 3F).

3.4. Locomotion

There were general effects of the distinct procedures carried out (STN treatment + vehicle i.p., STN treatment + L-DOPA i.p., etc.) within the MK-801 group ($\chi^2_3 = 10.543$, $n = 7$, $P = .014$; see also Fig. 4 for an overview). As compared to baseline, differences in the stimulating effects of L-DOPA were observed for this group ($Z = -.338$, $P = .043$). Except for the vehicle control group, all treatment groups displayed an increase in horizontal activity toward D-amphetamine challenge (MK-801: $Z = -2.366$, $P = .018$; muscimol 0.5: $Z = -2.366$, $P = .018$; muscimol 1.0: $Z = -2.028$, $P = .043$), as compared to the respective baselines. Differences were also seen between acute STN treatment plus i.p. vehicle and acute STN treatment plus L-DOPA within the MK-801 group ($Z = -2.197$, $P = .028$) and the low dose muscimol group ($Z = -2.197$, $P = .028$). Both muscimol groups exhibited an increase with acute STN treatment plus i.p. D-amphetamine as compared to acute STN treatment plus i.p. vehicle ($Z = -2.366$, $P = .018$ in both cases), while within the MK-801 group there were no differences ($P > .05$). Further, both muscimol groups showed a distinct pattern between acute STN treatment plus L-DOPA and acute STN treatment plus D-amphetamine ($Z = -2.366$, $P = .018$ in both cases) with an increase in locomotion for D-amphetamine. Group comparisons revealed general differences only for the acute STN plus i.p. vehicle treatment ($\chi^2_3 = 13.698$, $n = 29$, $P = .003$) but not for the both types of systemically applied dopamine agonists carried out ($P > .05$, respectively). There were differences between the vehicle group and the low dose muscimol group ($U = 8.000$, $P = .038$), the vehicle group and the high dose muscimol group ($U = 10.000$, $P = .040$), the MK-801 group and the high dose muscimol group ($U = 5.000$, $P = .006$) and the high and low dose muscimol group ($U = 3.000$, $P = .002$). See also Fig. 4 for an overview of the treatment effects on locomotion.

4. Discussion

The principal finding of the present experiment was that injections of a $GABA_A$-receptor agonist into the STN ipsilateral to the side of the 6-OHDA lesion influenced turning behavior in a dose-dependent manner, while blocking NMDA-receptors had no effects on motor asymmetries. There was a distinct time-course effect displayed by the low-dose muscimol group in the asymmetry index showing a gradual shift from initial ipsiversive turning, over non-biased turning to contraversive turning. The administration of muscimol into the STN also affected locomotion, with the higher dose increasing the distance moved, whereas administration of MK-801 had no effects on horizontal activity.

4.1. 6-OHDA lesion

The severity of the lesion is reflected by the reduction in content of striatal DA (~91%) and its metabolite DOPAC (~54%). Also the behavioral results indicate that the amount of dopamine-depletion induced was severe, since baseline assessment (without any treatments) of turning behavior demonstrated a strong asymmetrical motor-bias toward the side of the lesion (mean > 0.85 across all treatment groups).

4.2. Role of GABA in the modulation of motor parameters

The application of the $GABA_A$-receptor agonist into the STN ipsilateral to the side of the nigrostriatal lesion counteracted ipsiversive turning behavior induced by the 6-OHDA lesion. This effect was observed in a dose-dependent fashion. The low dose corrected the asymmetry index to an equal, non-biased, value of 0.5. This reflects the anti-akinetic properties of GABAergic stimulation in the STN since this value is expected in non-lesioned animals by chance. The high dose evoked contraversive turning. This reflects dyskinetic effects of GABAergic stimulation in the STN.

Dyskinesias in contralateral limbs, have also been observed upon GABAergic stimulation of the STN in MPTP-treated primates [25] after lesions of the STN in monkeys [48]. Electrical stimulation of the STN in rodent models of PD also evoked contralateral dyskinesias with increasing stimulation intensities [49]. These findings contradict other outcomes showing no effects of applying muscimol into the STN on turning behavior after 6-OHDA injection into the substantia nigra (SN) [34]. The different results may be due to the small doses (0.01 μg and 0.1 μg) and smaller injection volume (0.1 μl) used in the Mehta et al. [34] study. This may account for the blockade of behavioral effects evoked by systemically applied dopamine agonists but may not be sufficient to inherently modulate motor-associated parameters. Such an assumption is also in line with findings by Scheel-Kruger and Magelund [17] showing contraversive turning by STN muscimol injections in non-lesioned rats, whereas Mehta et al. [34] report on mild ipsiversive turning in non-lesioned rats with respect to this manipulation. Also, these authors did not report on an ipsiversive motor-bias associated with the 6-OHDA lesion, indicative of a less severe lesion, as compared to ours. Furthermore, we applied 6-OHDA into the MFB, whereas Mehta et al. [34] injected it directly into the substantia nigra, a protocol known to spare interhemispheric nigro-striatal projections (unlike lesions in the MFB), which are known to account for compensatory processes in the dopamine denervated striatum [50,51]. Such differences related to the lesion procedure may also account for the contradictory results observed and display a valuable new finding in the context of 6-OHDA models of PD.

GABAergic transmission within the BG is known to contribute to the precise and rapid initiation, execution and modulation of movement expression [52,53]. A lack of DAergic innervation, either due to idiopathic nigral degeneration or induced experimentally, has a profound impact on the GABAergic system within the BG. 6-OHDA lesions of the MFB led to an up-regulation of GABA-receptors in several BG nuclei [2] and to a gradual development of an imbalance in GABAergic transmission between the 'direct' and 'indirect' pathway [1]. Furthermore, a proliferation of GABAergic GPe-STN synapses was found following degeneration of midbrain dopamine neurons [54]. These alterations in the dopamine depleted state are discussed to reflect homeostatic compensatory processes, and, therefore, indicate the crucial role of GABA in BG integrity. Prokinetic effects of GABA agonism have also been described in the context of movement related beta desynchronisation (MRBD) in M1, an electrophysiological phenomenon associated with the initiation and duration of voluntary movement [55], as well as in PD patients undergoing administration of a $GABA_A$-agonist into the STN [18]. The STN has been shown to gradually develop pathological activity patterns [4] and oscillatory activity [5] following lesions of nigro-striatal projections. These alterations are presumed to underlie motor impairments seen in PD [5,6] and have been found to be influenced by intrasubthalamic administration of a $GABA_A$-agonist in MPTP-treated monkeys [27]. Furthermore, there is a link between GABA levels at rest and movement-related oscillations in motor-cortices [56]. Several authors also argue for an increase in local GABAergic transmission as the mechanism mediating the therapeutic effects of deep brain stimulation (of the STN or GPi) in PD, a concept referred to as 'synaptic inhibition' [57,58]. Taken together, these findings strongly indicate the pivotal role of GABA in movement control and are in line with the observations made in the present study.

4.3. Mechanisms mediating the effects evoked by stimulating $GABA_A$-receptors in the STN

Several mechanisms could account for how the stimulation of $GABA_A$-receptors may alter motor parameters. One possible mechanism is the functional blockade of STN activity. Taking into account the behavioral similarities evoked by the high dose muscimol and ablation of the STN, i.e. to counteract turning behavior associated with unilateral 6-OHDA lesions [14,15], administration of muscimol into the STN may induce a 'transient chemical lesion'. Muscimol has been shown to virtually abolish STN spiking activity when applied focally [59]. An irregular rhythmic input from the STN into the GPi and SNr is thought to reflect a major correlate of PD pathophysiology [60]. A functional blockade, as evoked by applying a $GABA_A$-receptor agonist into the STN, may correct for irregular (burst) activity of the STN associated with dopaminergic lesions in terms of disrupting the down-stream of this (pathological) information into recipient (output-) structures, i.e. the GPi and SNr [61,62]. Both DA substitution and STN DBS are known to correct for such burst activity [54]. Burst activity of the STN has been discussed to derive from reciprocal GPe-STN projections, whereas $GABA_A$-receptor-mediated de-inactivation of Ca_V1 and Ca_V3 is thought to essentially contribute to this pathomechanism [63]. Recent evidence further supports a pivotal role of GPe-STN connections in the behavioral expression of motor impairments, as a pronounced increase in proliferation of GABAergic GPe-STN synapses was demonstrated following 6-OHDA lesions [54]. The special position of GABAergic mechanisms in the STN regarding generation of motor symptoms seems to come true as evidenced in the present study. Stimulation of STN $GABA_A$-receptors via exogenously applied $GABA_A$-agonists that bypass the inherent pathological interplay of the GPe-STN-loop seems to sufficiently suppress pathological activity patterns. Blocking this pathological drive into the GPi/SNr may, in turn, attenuate the tonic inhibition of these output nuclei on the motor territories in the thalamus and, thereby, facilitate movement initiation and performance [52,53]. Such a mechanism of disinhibition is also in line with our observation that STN administration of the higher dose of muscimol increased locomotion (including turning behavior) compared to the vehicle, the MK-801 and the low dose muscimol group. A similar increase in locomotion after STN $GABA_A$-agonism has also been reported by Williams and Herberg [64] and we interpret this as a measure of anti-akinetic effects of the STN injections.

There is a growing interest in the putative pathomechanism of increased local field potential oscillations in power and coherence in the beta range (~15–30 Hz) of the STN. Such beta oscillations are thought to reflect anti-kinetic constituents [8–12]. It has been proposed that the close reciprocal connection of the GPe and STN contributes to the generation of such oscillations under circumstances of a lack of dompaminergic innervation [54]. The majority of GABAergic input to the STN originates in GPe neurons and displays a pacemaker-like function for STN activity [65,66]. In (experimental) PD, the GPe-STN-loop lacks of appropriate DAergic modulation and generates hypersynchronous rhythmic burst activity that is relayed into the entire cortico-BG-thalamo-cortical circuitry determining excessive inhibitory output of the BG [67]. Stimulating $GABA_A$-receptors in the STN may disrupt this link by de-coupling the hypersynchronous activity. Evidence for such a mechanisms was introduced by Tachibana et al. [27], showing that the administration of muscimol either into the STN or GPe in parkinsonian monkeys decreased abnormal neural oscillations in the BG and was alleviated motor symptoms.

Several remote effects on projection and down-stream neurons have been described in the context of different STN manipulations and may also contribute to the behavioral effects observed. The STN sends excitatory glutamatergic projections to the substantia nigra pars reticulata (SNr). The unilateral administration of muscimol into the SNr induced contraversive circling behavior in naïve and 6-OHDA lesioned rats [68–70]. Systemic administration of muscimol decreased SNr activity [71]. A decrease in STN activity by muscimol injections [25] may, therefore, also induce

a decrease in SNr spiking and thereby explain the contraversive turning observed in the present study. Electrical stimulation of the STN has also been shown to decrease SNr activity [72] and to induce contraversive turning behavior with increasing stimulation intensities [73].

4.4. Lack of motoric effects of NMDA-receptor blocker in STN

It was shown that a systemic administration of MK-801 decreased spiking activity of the STN [74] and potentiated turning behavior evoked by systemically applied dopamine agonists in the unilaterally 6-OHDA lesioned rat [39]. However, we did not observe alterations in motor behavior after acute injections of MK-801 into the STN, which resembles the results of mere systemic MK-801 administration observed by Morelli and Di Chiara [39]. The potentiation of the behavioral effects elicited by systemically applied dopamine agonists are difficult to compare to our findings taking into account a more than two-fold dose of L-DOPA and benserazide applied by Morelli and Di Chiara [39] and that their animals received two L-DOPA challenges within three days which may have led to priming of DAeric receptors and behavioral sensitization. Also, Morelli and Di Chiara [39] observed a pronounced contralateral turning response for mere L-DOPA treatment in 6-OHDA lesioned rats, while this was not the case in the present study. These differences may substantially contribute to the enhancing effects of systemically applied MK-801 on turning behavior observed by Morelli and Di Chiara [39]. The STN not only serves as a key component of the indirect pathway of basal ganglia circuitry, but also functions as a strategic input nucleus of excitatory glutamatergic fibers originating from expansive cortical areas associated with motor-control, known as the hyperdirect pathway. Given that glutamate is the principal neurotransmitter with respect to the STN's efferent and afferent projections, the STN is considered as the driving force of the basal ganglia. Notably, blocking NMDA-receptors in the STN had no effects on motor parameters, although systemic administration of MK-801 decreased STN firing rate [74], prolonged glutamate antagonism prevented motor asymmetries in the unilateral 6-OHDA model [35] and STN administration of MK-801 induced contraversive dystonic postures when haloperidol was systemically administered afterwards [36]. The blockade of STN NMDA-receptors may address a system/mechanism which is not primarily affected by dopamine degeneration (as compared to GABAergic impairments) and may not primarily account for the motor-impairments seen. Therefore, manipulation of these receptor-types is not capable of acute correction for motor-asymmetries as observed in the present study.

4.5. Effects of systemically applied dopamine agonists on turning behavior and locomotion

Since turning behavior is sensitive to (systemic) DAergic manipulations in the 6-OHDA lesioned rat (see Schwarting and Huston [75] for review) and manipulations of STN activity in hemiparkinsonian rats are associated with changes in striatal DA levels (including electrical stimulation [16]), we addressed the question of interactions between intrasubthalamic manipulations and systemically applied dopamine agonists. Specifically, we asked whether targeting either DA-receptors directly (L-DOPA) or indirectly (D-amphetamine) would have different effects on turning behavior and general locomotion. Systemic administration of D-amphetamine emphasized the unilateral motor bias in terms of a further increase in ipsiversive turning, reflecting increased DA concentration in the non-lesioned hemisphere after amphetamine administration that further augments ipsiversive turning [40]. The MK-801 group displayed a similar increase, indicating that applying MK-801 into the STN did not influence the behavioral response induced by D-amphetamine. Neither of the muscimol groups showed such an increase in ipsiversive turning after D-amphetamine challenge compared to STN (+vehicle i.p.) treatment. The latter results should be interpreted with respect to the behavioral responses associated with mere STN (+vehicle i.p.) administration induced contraversive circling. It can be assumed that the opposed behavioral effects of D-amphetamine (as exhibited by the vehicle and MK-801 group) were overshadowed by the effects of intrasubthalamic treatment. According to the standard 'box-and-arrow-model' of basal ganglia circuitry [52,53], both manipulations are likely to equally decrease pallido-thalamic inhibition in the respective hemispheres and, therefore, act competitively by means of directionality of circling behavior, whereas the net amount of disinhibition may have been greater after applying muscimol, thus, driving the animal away from the side of the lesion. Further, no differences in turning behavior were observed within the high and low muscimol groups after STN (+vehicle) treatment compared to STN treatment plus L-DOPA, whereas a synergistic effect in terms of a further increase in contraversive turning (as compared to STN treatment without L-DOPA) may have been expected a priori. Also, no differential effects of the two types of dopamine agonists were observed within these groups, reflecting the predominance of the effects associated with sole STN infusion (+ vehicle i.p.). A clear activating effect of the systemically applied dopamine agonists on horizontal activity was seen in all groups. L-DOPA (+STN administration) led to an increase in the total distance moved as compared to baseline and STN administration (+vehicle i.p.) in the MK-801 and low dose muscimol groups. Although there were no effects of L-DOPA on contraversive turning behavior, the stimulating effect of L-DOPA reflects the increase in DA concentration (probably mediated by the non-lesioned hemisphere) and subsequent increase in locomotion [75]. Both the low and the high dose muscimol groups displayed an increase in locomotion after D-amphetamine as compared to baseline, STN (+vehicle) treatment and STN treatment + L-DOPA, reflecting the potent stimulating effects of this agent on behavior [76]. This increase was also observed in the MK-801 group (as compared to baseline). We did not observe differences between the groups, either for L-DOPA or D-amphetamine treated animals. Taken together, the present data suggest that there are no significant interactions between the manipulation of STN activity and systemically applied dopamine agonists. However, potential interactions on a behavioral level may not be ruled out completely, taking into account ceiling/floor effects determined by mere STN administrations (without systemically applied dopamine agonists).

5. Conclusions

Taken together, the present findings indicate the participation of GABAergic STN receptors in the acute mediation of movement control in unilateral DA-depleted rats. The activation of $GABA_A$-receptors in the STN alleviated motor impairments incurred by a unilateral 6-OHDA lesion. It seems that there is a therapeutic window with respect to stimulating $GABA_A$-receptors in the STN and that an overshoot along this dimension evokes dyskinetic effects in 6-OHDA lesioned rats. Considering the behavioral similarities between GABA-agonism and other manipulations of the STN, the findings also highlight the role of the GABAergic system as a likely neurophysiological correlate contributing to the effects of other therapeutical interventions in PD.

Conflict of interest

The authors declare no competing financial interests.

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Abstract: Parkinson's disease (PD) patients not only exhibit motor impairments, but also characteristic deficits in cognitive and affective functions. Such functions have consistently been associated with the medial prefrontal cortex (mPFC). To determine whether there is an interaction between the MDS and the mPFC underlying the cognitive and emotional deficits seen in rats, we administered a disconnection procedure of these structures by applying lesions to the mPFC (NMDA) and the medial forebrain bundle (6-OHDA) either in the same or opposite hemispheres. The results indicate a functional interaction of the MDS and the mPFC: Disconnection effects on behavior were observed with respect to memory-, anxiety- and depression-related behaviors, as. a disconnection of the mPFC and MDS had promnestic, antidepressant- and anxiolytic-like effects. In order to determine whether this apparent functional circuit between the mPFC and MDS involves serotonergic mechanisms, we also utilized serotonin-specific disconnections of the mPFC by applying the 5-HT-specific agent 5,7-DHT into the mPFC and 6-OHDA into the medial forebrain bundle, again either in the same or opposite hemispheres. The behavioral effects observed here resembled those incurred by the unspecific disconnection of the mPFC, demonstrating a significant contribution of serotonergic mechanisms to the interplay between the MDS and the mPFC. Taken together, these experiments provide evidence for a functional interaction of the MDS and the mPFC in the control of cognitive and affective processes known to be impaired in PD and point towards a prominent involvement of the serotonergic system. A disconnection of the mPFC and the MDS had promnestic, antidepressant- and anxiolytic-like behavioral effects. These findings may impact therapeutic approaches in the treatment of cognitive and neuropsychiatric symptoms seen in PD.

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Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats

Dear Dr. Hirsch and Dr. Powell,

Please consider for publication in *Neuroscience* the enclosed MS by Petri, D et al. entitled "**Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats"**

This MS addresses the interaction between the midbrain DA system and the medial prefrontal cortex in the mediation of cognitive and affective impairments seen in hemiparkinsonian rats. To this end, disconnection procedures of the respective structures were applied. An interplay was found with respect to memory-, anxiety- and depression-related behavior. Furthermore, a contribution of the serotonergic system of the mPFC to these effects was demonstrated given that 5-HT-specific lesions resembled the effects observed with unspecific lesions.

I have read and have abided by the statement of ethical standards for manuscripts submitted to *Neuroscience*.

With kind regards,

Joe Huston

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***Highlights (for review)**

-Functional interaction between mPFC and midbrain DA systems
-Role in cognitive and neuropsychiatric impairments accompanied by Parkinson's disease
-Involvement of serotonergic mechanisms in mPFC
-Disconnection of mPFC and midbrain DA system improves deficits

Serotonergic interaction between medial prefrontal cortex and mesotelencephalic DA system underlies cognitive and affective deficits in hemiparkinsonian rats

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Abstract (272 words)

Parkinson's disease (PD) patients not only exhibit motor impairments, but also characteristic deficits in cognitive and affective functions. Such functions have consistently been associated with the medial prefrontal cortex (mPFC). To determine whether there is an interaction between the MDS and the mPFC underlying the cognitive and emotional deficits seen in rats, we administered a disconnection procedure of these structures by applying lesions to the mPFC (NMDA) and the medial forebrain bundle (6-OHDA) either in the same or opposite hemispheres. The results indicate a functional interaction of the MDS and the mPFC: Disconnection effects on behavior were observed with respect to memory-, anxiety- and depression-related behaviors, as. a disconnection of the mPFC and MDS had promnestic, antidepressant- and anxiolytic-like effects. In order to determine whether this apparent functional circuit between the mPFC and MDS involves serotonergic mechanisms, we also utilized serotonin-specific disconnections of the mPFC by applying the 5-HT-specific agent 5,7-DHT into the mPFC and 6-OHDA into the medial forebrain bundle, again either in the same or opposite hemispheres. The behavioral effects observed here resembled those incurred by the unspecific disconnection of the mPFC, demonstrating a significant contribution of serotonergic mechanisms to the interplay between the MDS and the mPFC. Taken together, these experiments provide evidence for a functional interaction of the MDS and the mPFC in the control of cognitive and affective processes known to be impaired in PD and point towards a prominent involvement of the serotonergic system. A disconnection of the mPFC and the MDS had promnestic, antidepressant- and anxiolytic-like behavioral effects. These findings may impact therapeutic approaches in the treatment of cognitive and neuropsychiatric symptoms seen in PD.

Keywords: Parkinson's disease, anxiety, depression, memory, mPFC, 5-HT

1 Introduction (634 words)

Non-motor symptoms (NMS) accompanied by a degeneration of the midbrain dopamine system (MDS) in Parkinson's disease (PD) include cognitive deficits and neuropsychiatric disorders, such as depression and anxiety (Lawson et al., 2014; Lawrence et al., 2014; Santos-Garcia and Fuente-Fernandez, 2013). Memory impairments affect up to 80% of PD patients (Aarsland et al., 2003; Hely et al., 2008) and 40-50% of them experience affective disorders during the course of their illness, which may even precede manifest motor signs (Mayeux et al., 1984; Merschdorf et al., 2003). The nigro-striatal and meso-cortical dopamine (DA) projections affected by PD are well known to contribute to a variety of distinct cognitive and emotional functions (Lindgren and Dunnett, 2012). These functions have been extensively studied in rodents and demonstrate a crucial role of the ventromedial portion of prefrontal cortical areas in the control of memory, depression and anxiety, both with respect to normal, as well as DA-depleted animals (Chudasama and Robbins, 2006). Many of these functions are also characteristically impaired in PD patients, pointing towards comparable mechanisms involved (Svenningsson et al., 2012). Moreover, the pattern of these functional deficits strikingly resembles that seen in patients with medial prefrontal cortex (mPFC) lesions (Kehagia et al., 2010; Owen et al. 1992; Taylor et al., 1986). This similarity implies an interaction of the MDS and the mPFC in the control of distinct cognitive and affective functions known to be impaired in PD patients. An interaction between two brain areas can be tested directly by disconnecting the respective structures in rodent models (Geschwind, 1956a; 1956b; see also Fig. 1 for disconnection logic). A functional interplay between midbrain dopamine projections and the mPFC in terms of memory for objects was found by using such a disconnection procedure (Chao et al., 2013). Here, we examined the contribution of this network in depression- and anxiety-related functions, as well as the distinct contribution of the serotonergic system in this context. To this end, we disconnected the mPFC and the MDS utilizing 6-OHDA lesions of

the medial forebrain bundle (MFB) and NMDA lesions of the mPFC, respectively, in a first set of experiments. The effects on anxiety-, depression- and memory-related functions were assessed by a variety of behavioral tasks. Given the prominent role of prefrontal serotonin in the mediation of the particular functions assessed (for review see e.g. Puig and Gulledge, 2011 or Pietro and Seamans, 2007), we also examined to which extent the serotonergic system contributes to such a functional interplay of the mPFC and MDS in a separate set of experiments, whereby 5-HT-specific disconnection procedures were applied by local microinjections of 5,7-DHT into the mPFC. Several lines of research demonstrate electrophysiological (Gui et al., 2011; Zhang et al., 2011; Wang et al., 2009), as well as neurochemical (Cicin-Sain and Jenner, 1993; Cunningham et al., 2005) evidence for an interaction of monoaminergic afferents of the mPFC in hemiparkinsonian rats, but the contribution to the observed behavioral impairments in terms of neuropsychiatric and cognitive disorders are yet to be determined. We assume an interaction of 5-HT and DA receptors within mPFC neurons underlying functional impairments seen after DA depletion. A lack of DAergic innervation of the mPFC induces alterations in 5-HT receptors which, in turn, evokes altered 5-HT signal transduction. We hypothesized that this altered 5-HT transmission determines cognitive and emotional dysfunction and that impairments can be modulated by a disconnection of 5-HT inputs into the mPFC.

In experiment 1 we set out to determine a functional interaction between the mPFC and MDS in the control of cognitive and affective functions by comparing the behavioral effects incurred by an excitotoxic lesion of the mPFC ipsilateral to a 6-OHDA-induced lesion of the MFB with alterations induced by excitotoxic lesions of the mPFC contralateral to the side of a 6-OHDA-induced MFB lesion (Disconnection).

In experiment 2 we characterized the involvement of the serotonergic system in this interaction by selective lesions of the serotonergic system in the (contralateral) mPFC of

hemiparkinsonian rats. To this end, we compared the behavioral effects incurred by an 5-HT specific lesion (5,7-DHT) of the mPFC ipsilateral to a 6-OHDA-induced lesion of the MFB with alterations induced by 5-HT specific lesions of the mPFC contralateral to the side of a 6-OHDA-induced MFB lesion (disconnection).

2 Materials and methods

2.1 Animals

The conditions were the same for experiments 1 and 2. Adult male Wistar rats were purchased from Janvier (Le Genest St. Isle, France). Their weights ranged from 220 to 280g at the beginning of the experiments. Animals were housed conventionally (translucent makrolon type 4 cages) in groups of five per cage under a reversed light-dark cycle (lights on from 7pm to 7 am) and under standard laboratory conditions (20°C, 60% humidity). After surgery they were housed in individual cages (translucent 30 x 20 x20cm plastic cages) for three days and then returned to their group cages. They were provided food and water ad libitum throughout the experiments. Behavioral testing was conducted between 8am and 5pm. Assignment of the subjects to any of the respective treatment groups was done randomly. All experiments were in accordance with the Effective Animal Protection Law of the Federal Republic of Germany and of the European Union Directive on the protection of animals used for scientific purposes (2010/63/EU), as well as local authorities. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Surgery

2.2.1 Experiment 1: Effects of disconnecting the mPFC by NMDA lesion and DAergic midbrain projections by 6-OHDA lesion

The purpose of experiment 1 was to examine the possible critical interaction between

mPFC and MDS in the mediation of cognitive and affective deficits found in PD. To this end, we disconnected these structures by applying excitotoxic lesions (NMDA) of the mPFC in either the same or opposite (disconnection) hemisphere as the 6-OHDA-induced lesion of the MFB.

Surgical procedures were carried out under isoflurane/oxygen inhalational anesthesia (4% mixture for induction, 2% mixture for mainmaintenance). Carprofen (5 mg/kg) was applied subcutaneously before surgery to prevent inflammatory reactions and to manage postoperative pain. Animals were mounted on a stereotaxic frame, the scalp was sanitized with 70% ethanol solution, 0.1 ml of Bucain® (DeltaSelect, Germany) containing 0.25 mg Bupivacaine hydrochloride was subcutaneously administered and an incision was made along the scalp to expose the skull. A burr hole was applied above the MFB (AP: -4.0 mm, ML: +1.5 mm, DV: -8.5mm; relative to bregma and skull surface according to the atlas of Paxinos and Watson, 1996). Then, the animals received unilateral infusions of 6-hydroxydopamine (6-OHDA) hydrochloride (4.16mg/ml with 3µl injection volume, dissolved in phosphate buffered saline with 0.1% ascorbic acid; Sigma-Aldrich, Germany) via a 28-gauge cannula with a constant flow rate of 0.5 µl/min. The side of lesion was counterbalanced across all experimental groups. They were then injected at four sites within the mPFC ([1] AP +3.8mm; L ±0.7; DV -3.0mm; [2] AP +3.2mm; L ±0.7; DV -3.0 [3] AP +3.2mm; L ±0.7; DV -2.0 mm; [4] AP +2.7 mm; L ±0.7 mm; DV -3.0 mm; each injection volume 0.2 µl with a flow rate of 0.25 µl/min) with Phosphate buffered saline containing 10mg/ml N-Methyl-D-aspartic acid (NMDA; Sigma-Aldrich, Germany) and 0.1% ascorbic acid. Injections into the mPFC were either applied within the same hemisphere as the MFB lesion (ipsilateral groups) or into the contralateral hemisphere (disconnection groups). See also Fig. 1 for scheme of the disconnection logic.

Infusions were administered via a microinfusion-pump (KD Scientific) equipped with a 10 µl syringe (Microliter 701, Hamilton). Animals were given five to six days of recovery after

surgery. All Animals were pretreated with desipramine hydrochloride (25mg/kg dissolved in DMSO; i.p.; Sigma-Aldrich, Germany) 45min prior to surgery in order to protect noradrenergic projections.

2.2.2 Experiment 2: Effects of 5-HT-specific disconnection of the mPFC by 5,7-DHT and DAergic midbrain projections by 6-OHDA

The purpose of experiment 2 was to examine the possible role of serotonin in the circuit between mPFC and MDS in the control of cognitive and affective domains impaired in PD. To this end, serotonin-specific disconnection procedures of these constituents were applied by injecting 5,7-Dihydroxytryptamine (5,7-DHT), a serotonin-specific toxin, into the mPFC in either the same hemisphere as the 6-OHDA lesion, or into the opposite hemisphere (disconnection).

Surgical procedures were the same as described for experiment 1, except that, instead of NMDA, the respective mPFC was injected with phosphate buffered saline containing either 1mg/ml 5,7-Dihydroxytryptamine creatinine sulfate salt (5,7-DHT; Sigma-Aldrich, Germany; low dose group) or 10mg/ml 5,7-DHT (high dose group).

2.3 Apparatus/behavioral protocols

All behavioral protocols were the same for experiments 1 and 2. An ethanol solution (70%) was used for cleaning the respective apparatus after each animal and trial.

2.4 Open-field

Locomotion and sensorimotor parameters were gauged by the distance moved and turning behavior assessed in an open field (OF) for 15 min. The open field was located in a sound attenuating chamber and consisted of a square arena (48 x 48 x 40 cm) equipped with a video camera connected to an automated analysis system (Ethovision© XT 8, Noldus,

Netherlands) which counted the ipsi- and contraversive turns (ipsiversive refers to the direction turned as to side of the MFB lesion). Detection parameters were set to a center-point to nose-point detection and detection threshold was set to quarter turns. White noise (60 dB) was applied during testing. Asymmetry indices of turning behavior were computed according to the ratio of ipsi- or contraversive quarter turns relative to the total number of turnings (ipsi- and contraversive relative to the hemisphere containing the MBF lesion) emitted by the subject. This index was then transformed, such that a value of zero reflects an unbiased directionality, while any value above or below zero indicates a turning bias in favor of the ipsi- or contralateral direction, respectively.

2.5 Amphetamine-challenge

Animals were challenged with 1.5mg/kg D-amphetamine hemisulphate salt (Sigma-Aldrich, Germany) and then assessed for acute effects on turning behavior in the open field as described above. Subjects were given i.p. injections with an application volume of 1ml/kg and placed into the apparatus five minutes later.

2.6 Elevated plus maze

The elevated plus maze (EPM) consisted of two open and two walled arms with an open roof, arranged around a central platform, with the two arms of each type placed opposite each other. It was located in a sound attenuating room and subjected to white-noise (60 dB). Illumination (<1 and 30 lx on the closed and open arms, respectively) was provided by lights mounted above the maze. Animals were placed on the central platform, facing one of the open arms and were allowed to explore the maze for five min. The time spent in the respective arms and frequency of entries was automatically measured using the Ethovision© software suite connected to a camera mounted above the maze.

2.7 Forced swim test

The forced swimming test (FST) apparatus consisted of a Plexiglas cylinder (46 cm height,

20 cm diameter) containing 30 cm of water 26±1 ℃). On the first day (pretest session), animals were exposed to the cylinder for 15 minutes. Twenty-four hours later, the animals were re-exposed to the same experimental condition for five minutes (test session). The water was changed after every animal. The behavioral parameters assessed were the duration and frequency of immobility (defined as a lack of motion of the animal, except for movements required to keep its head above the water), as well as swimming and climbing (vigorous movements with forepaws in contact with the walls). Behavior was manually tracked by an experienced observer who was blind as to the experimental group assignment.

2.8 Object recognition

Behavioral procedures related to memory functions were carried out in an acrylic square arena (60 x 60 x 30 cm) located in a sound attenuating room. The apparatus was illuminated by bulbs mounted above the arena providing light density of 6lx in the center and 4lx in the corners. A video camera was also mounted above the arena. Animals were given two days of habituation before actual memory assessment by placing them into the arena for five minutes on each day and allowing for free exploration. Object recognition test took place on the following day. They were exposed to the arena again, which now contained two identical copies of a particular object (cylinder-shaped white ceramic vase, textured surface, 30cm height). These were located randomly at two of eight possible locations. Subjects were given five minutes to explore the objects. Any physical contact with the object (snout, vibrissae or forepaws) was counted with respect to duration and frequency by an experimentally blinded observer. If the animal exhibited a physical contact with the object, but was not focusing it, this was not considered as exploration of the object. After this trial, subjects were returned to their home cages and left there for 90 minutes. They were then re-exposed for another five minutes to the arena which now contained one object of the initial trial and one new object (curve-shaped translucent glass

vase filled with blue marbles, smooth surface, 25cm height), each located at the previous locations. Animals were again allowed to freely explore the objects and a memory index was computed based on individual object exploration time. Rats showing intact memory for objects prefer to explore the new object longer and more frequently than the one from the first trial (Ennaceur and Delacour, 1988). After each trial, the arena and objects were cleaned with 1% acetic acid solution to prevent odor contamination. An index for functions of object recognition was computed according to the formula

$[(ETT_{NO} / ETT_{OO}) / (ETS_{NO} / ETS_{OO})]*100$

,whereby "ETT_{NO}" is the exploration time during the test trial for the novel object, "ETT_{OO}" is the exploration time during the test trial for the old object, "ETS_{NO}" is the exploration time during the sample trial for the object located at the same position where in the test trial the novel object appeared and "ETS_{OO}" is the exploration time during the sample trial of the old familiar object. This measure controls for differences in the overall exploration level exhibited by individual subjects. Values above 100 indicate intact object recognition memory above chance level, while values ≤100 reflect impaired object recognition.

2.9 Temporal sequence experiments

After arrival, animals were allowed to adapt to the new laboratory environment for four days. Then they were tested for baseline motoric parameters (turning behavior) in the OF. One to two days later, animals were subjected to surgery and then given five to six days of recovery. Thereafter, they were challenged with D-amphetamine and motoric parameters were gauged in the OF. On the next day, they were tested for anxiety behavior in the EPM. Over the next two days, the FST was administered. Next, they were again assessed for motor behavior in the OF, first without treatment, then, with D-amphetamine. They were then subjected to memory testing on two consecutive days, whereby they were habituated to a novel OF for two days before actual memory testing was carried out. An overview of

the behavioral battery applied is also depicted in Fig. 2.

2.10 Neurochemical analysis

Neurochemical procedures were the same in experiments 1 and 2. The day after the last behavioral testing was conducted, animals were deeply anesthetized by $C0_2$ inhalation, decapitated and brains were extracted. Tissue samples of the dorsal striatum and the mPFC were then dissected from both hemispheres. Samples from NMDA-lesioned mPFC were not collected but histologically analyzed separately in order to assess the extent of unspecific excitotoxic mPFC lesions. The tissue was weighted and then homogenized with an ultrasonic homogenizer (Microsonic, Germany) in 500µl 0.05N $HCLO_4$. Samples were then centrifuged at 9000 rpm at 4° C for 20 minutes, filtered and stored at -80° C until neurochemical assessment. The amount of several monoaminergic neurotransmitters (NE, DA, 5-HT) and corresponding metabolites (DOPAC, 5-HIAA) were measured by applying high-performance liquid chromatography with coupled electrochemical detection (HPLC-ED). The column was an ET 125/4, Nucleosil 120-5, C-18 reversed phase column (Machery and Nagel, Germany), which was perfused for analysis 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, Netherlands) was set at 500mV vs. an ISAAC reference electrode (Antec, Leyden, Netherlands) and set at 30° C.

2.11 Histology

In experiment 1, the extent of NMDA-induced cell body lesions of the mPFC was assessed by Nissl staining. To this end, brains were stored in 10% buffered formalin solution for at least 48h after extraction. Then, tissue was transferred into a 30% sucrose-formalin solution and stored at 4 C°. After slicing the brains with a cryostat (Leica, Germany) into 50

µm slices, samples were stained with cresyl violet and the extent of the lesions was determined under a light microscope.

2.12 Statistics

Statistical testing was carried out by applying independent or paired t-tests. Comparisons were made between the ipsilateral and disconnected (contralateral) lesion groups, given that any differences (either an increase or decrease) between these indicate a functionally relevant interaction of the particular systems in the control of the behavioral functions assessed (see also Fig. 1 for disconnection logic). One-tailed testing was performed and p-values <0.05 were considered as to reflect statistically significant differences.

3 Results

3.1 Experiment 1: Effects of disconnection of the mPFC and DAergic midbrain projections

3.1.1 Neurochemical assessment

Concentration of neurotransmitters (NE, DA, 5-HT), as well as their respective metabolites (DOPAC, 5-HIAA), were assessed as wet tissue weight. Comparison of the 6-OHDA lesioned hemisphere to the non-lesioned side of the ipsilateral group revealed a clear reduction in the content of (dorsal) striatal DA (t_9 = -3.290, p = 0.005), DOPAC (t_9 = -2.669, p = 0.014) and 5-HT (t_{15} = -3.510, p = 0.04). The same decrease was also observed within the disconnected group with respect to striatal DA (t_7 = -1.898, p = 0.05), DOPAC (t_6 = -3.727, p = 0.005) and 5-HT (t_6 = -3.017, p = 0.012) The amount of DA depletion was ~93.4% on average (SEM ± 4.73) across groups. No significant differences were observed with respect to other neurochemical measures in the dorsal striatum, nor were there significant differences between the groups in other neurochemical measures (p > 0.05). Further details on neurochemical data are depicted in Tab. 1. A schematic representation

of the extent of cell lesions in the mPFC is presented in Fig. 3. The extent of depletion of DA confirms the expected effects of this dose of 6-OHDA used to serve as a successful hemiparkinsonian preparation (Schwarting and Huston, 1996).

3.1.2 Turning behavior

A significant directional turning bias (ipsiversive turnings) was induced by the unilateral administration of 6-OHDA into the MFB in both groups baseline vs. post surgery values in the open-field: ipsilateral group: $t_{11} = -5.931$, $p < 0.001$; disconnected group: $t_{11} = -5.289$, $p < 0.001$. No significant differences were found between the groups ($p > 0.05$). Also, there was no respective effect of group on turning behavior after systemic D-amphetamine challenge ($p > 0.05$, data not shown). Turning behavior is depicted in Fig. 4a. Thus, the injection of 6-OHDA led to the expected bias towards ipsiversive turning which characterizes the hemiparkinsonian rat model (Schwarting and Huston, 1996).

3.1.3 Forced swim test

There were significant differences between the ipsilateral and disconnection group concerning the duration of climbing behavior on the second FST day ($t_{21} = 2.087$, $p = 0.025$). The disconnected group showed an increased climbing duration. No significant differences with respect to the frequency or duration of immobility were observed on day two. Furthermore, groups did not significantly differ as to any parameter of the FST on day one ($p > 0.05$). These findings provide evidence for an antidepressant-like effect of a disconnection of mPFC and MDS. See also Fig. 4b for an overview.

3.1.4 Object recognition

Differences were found in the index for object recognition between the ipsilateral and disconnected group ($t_{17} = 2.055$, $p = 0.028$). The disconnection group exhibited superior

object recognition memory, thereby, providing evidence for a promnestic effect of a functional decoupling of a DA-depleted mPFC and MDS. Results are depicted in Fig. 4c.

3.1.5 Elevated plus maze/open-field

Differences were observed between the ipsilateral and disconnection group, both with respect to the duration spent in the open arms (t_{22} = 1.731, p = 0.049), as well as the sojourn time in the center (t_{22} = 1.739, p = 0.048; data not shown). The disconnected group showed an increased duration spent in the open arms, as well as in the center compartment. No differences were observed for the distance moved on the apparatus (p > 0.0.5). Another indicator of anxiety-related behavior was the time spent in the center of the open-field during day one of habituation for the memory paradigms. The mean time spent in the center of the arena during habituation significantly differed between the ipsilateral and disconnected group (t_{18} = 1,734, p = 0.05), whereby the disconnected group spent more time in the center. Taken together, a functional decoupling of a DA-depleted mPFC and MDS, as applied in the disconnected group had anxiolytic-like effects on behavior. Anxiety related measures are depicted in Fig. 4d.

3.2 Experiment 2:Effects of 5-HT-specific disconnection of the mPFC and DAergic midbrain projections

3.2.1 Neurochemical assessment

Methods were the same as described for experiment 1. Within-group comparisons of content of transmitter/metabolites in the striatum revealed a significant reduction in levels of prefrontal DA across all groups between the 6-OHDA-lesioned and non-lesioned hemisphere (t_{43} = -7.464, p < 0.001). The average amount of depletion was ~95.9%. With respect to the content of striatal levels of transmitters/metabolites for the ipsilateral low dose 5,7-DHT group, comparison of the 6-OHDA-lesioned to the non-lesioned side yielded

a clear reduction in the content of DA (t_9 = -2.431, p = 0.02). No significant within-group effects on other transmitters or metabolites were observed (p > 0.05). Within-comparisons of the low dose 5,7-DHT disconnected group showed significant reductions of striatal DA (t_{11} = -2.485, p = 0.02), DOPAC (t_{10} = -1.928, p = 0.042) and 5-HT (t_8 = -4.198, p = 0.02). Within-comparisons of the high dose 5,7-DHT ipsilateral group showed significant reductions of striatal DA (t_{10} = -5.182, p < 0.001) and DOPAC (t_9 = -3.235, p = 0.005). For the high dose 5,7-DHT disconnected group there were also significant reductions of striatal DA (t_{10} = -6.684, p < 0.001) and DOPAC (t_{10} = -4.007, p = 0.001). Disconnection effects, i.e. significant differences between the corresponding ipsilateral and disconnected groups, were observed for the low dose 5,7-DHT groups in level of striatal 5-HT on the lesioned side (t_{19} = 1.867, p = 0.04). There were no other differences between the groups (p > 0.05). Taken together, the expected effects of a 6-OHDA administration (i.e. a significant reduction of DA and related metabolites levels in the striatum) were confirmed by neurochemical assessment, thus, also proving a successful hemiparkinsonian preparation.

Within-group comparisons of transmitter/metabolites in the mPFC revealed significant reduction in levels of prefrontal DA across all groups between the side ipsi- or contralateral to the MFB lesion (t_{41} = -2.823, p = 0.004). The average amount of depletion was ~92.2%. There were also differences in 5-HT (t_7 = 4.224, p = 0.002) and DOPAC levels (t_9 = 2.232, p = 0.03) within the disconnected low dose 5,7-DHT group, as well as the amount of prefrontal NE within the ipsilateral high dose 5,7-DHT group (t_{10} = -1.897, p = 0.04). Furthermore, there were differences in prefrontal DA levels between the ipsilateral and disconnected high dose 5,7-DHT groups (t_{20} = 1.859, p = 0.04), indicating disconnection effects. Summarized, a clear reduction of prefrontal DA was confirmed by neurochemical assessment. Further details on distinct transmitter/metabolites levels can also be found in Tab. 1.

3.2.2 Turning behavior

Significant differences as to directional biases (turning behavior) were observed between the corresponding assessments before (baseline) and post surgery for all groups (low dose 5,7-DHT ipsilateral group: $t_9 = -7.184$, $p < 0.001$; low dose 5,7-DHT disconnected group: $t_{11} = -3.214$, $p = 0.004$; high dose 5,7-DHT ipsilateral group: $t_{10} = -12.291$, $p < 0.001$; high dose 5,7-DHT disconnected group: $t_8 = -10.205$, $p < 0.001$). There were also differences between the high dose 5,7-DHT ipsilateral and disconnected group ($t_{20} = -1.931$, $p = 0.034$) at post lesion assessment but not between the low dose groups ($p > 0.05$). Furthermore, no significant effects of a disconnection were observed after systemic D-amphetamine challenge, neither with respect to the high, nor the low dose groups ($p > 0.05$, data not shown). The results confirm that the injection of 6-OHDA led to the expected bias towards ipsiversive turning which characterizes the hemiparkinsonian rat model (Schwarting and Huston, 1996). A disconnection effect was observed for the high-dose 5,7-DHT groups counteracting the 6-OHDA-induced ipsiversive turning bias. Turning behavior for Experiment 2 is depicted in Fig. 5a.

3.3.3 Forced swim test

No effects of disconnection were found for FST measures when directly comparing the low or high dose 5,7-DHT ipsilateral and disconnected groups. However, if the respective ipsilateral and disconnected groups are pooled, a significant difference was observed in terms of the mean duration of immobility behavior exhibited on the second day of assessment ($t_{42} = 1.880$, $p = 0.034$). The disconnected groups showed less time of immobility, thus providing evidence for an antidepressant-like effect of a 5-HT-specific disconnection of the mPFC. See also Fig. 5b for an overview.

3.3.4 Object recognition

Significant differences were found between the low dose 5,7-DHT ipsilateral and disconnected group in object recognition (t_{19} = -2.362, p = 0.015). Here, the disconnection procedure led to a promnestic effect. Other comparisons yielded no significant differences (p > 0.05). Taken together, the results demonstrate an involvement of the mPFC 5-HT system on object recognition memory. Results are also depicted in Fig. 5c.

3.3.5 Elevated plus maze/open-field

There were significant differences for the high dose 5,7-DHT groups, i.e. the duration in the open arms (t_{21} = -1.726, p = 0.05), while no effects of a disconnection were found between the low dose groups (p > 0.05). The disconnected high group spent significantly more time in the open arms. No differences were observed for the duration and entry frequencies regarding the closed and center compartment (p > 0.05), as well as the distance moved on the apparatus (p > 0.0.5). The mean time spent in the center of the open-field during day one of habituation for the memory paradigms was also significantly different between the high dose 5,7-DHT ipsilateral and disconnected group (t_{20} = -1,746, p = 0.048), but not between the low dose groups (p > 0.05). The disconnected high group spent significantly more time in the center compartment. These data indicate an anxiolytic effect of the manipulation. Anxiety related measures are depicted in Fig. 5d.

4 Discussion (1926 words)

The results of the studies presented here indicate a functionally relevant, causal interaction between the mPFC and the midbrain dopamine projections in the mediation of distinct cognitive and affective functions. The amount of DA depletion incurred by administration of 6-OHDA into the MFB was severe, both with respect to striatal (~95% reduction), as well as prefrontal levels (~92%). Behavioral differences were observed between animals with mesotelencephalic DA deficiency and lesions of the contralateral

mPFC, as compared to animals bearing the lesions within the same hemisphere (ipsilateral). Such disconnection effects were observed with regard to all functional domains assessed, i.e. emotional behavior in terms of anxiety- and depression-like behavior, as well as memory functions. There were no hints as to any potential motoric confounds that may have determined the differences seen concerning cognitive and affective outcomes, as evidenced by comparable locomotor activity between the experimental groups. We also found evidence for a 5-HT-dependent mechanism in this interaction given that a 5-HT-specific deafferentiation of the (contralateral) mPFC resembled the behavioral effects incurred by unspecific excitotoxic lesions of the (contralateral) mPFC. This is in line with the assumption that 5-HT receptor-related changes within mPFC neurons induced by DAergic denervation underlie behavioral alterations in DA-deficient animals, since the functional decoupling of 5-HT afferences counteracted cognitive and emotional impairments.

Over the last decades, it has been widely accepted that besides motoric symptoms accompanied by a degeneration of midbrain DA projections (i.e the nigro-striatal, meso-cortical and mesolimbic pathway), PD patients also exhibit also a variety of characteristic cognitive and emotional dysfunctions. These so-called 'non-motor' symptoms (NMS) involve memory deficits as well as neuropsychiatric disorders, including depression and anxiety disorders (Svenningsson et al., 2012; Bernal-Pacheco et al., 2012). PD patients display a relative high incidence of such impairments as compared to age- and disability-matched populations (Aarsland et al., 2003; Hely et al., 2008; Mayeux et al., 1982; Tandberg et al., 1996). Such findings point towards a functional disruption of mechanisms specific to PD, namely a degeneration of dopaminergic neurons in the midbrain. Indeed, the neurotransmitter DA has been known for decades to be involved in the mediation of distinct cognitive and affective processes. Experimental depletion induces several characteristic deficits with respect to attention, memory and executive functions, as well as

mood and anxiety disorders (for review see e.g. Dembrow and Johnston, 2014; Chudasama and Robbins, 2006; Nieoullon and Coquerel, 2003). The characteristic profile of cognitive and affective dysfunction seen in PD also resembles a pattern of impairments following lesions of the prefrontal cortex (Kehagia et al., 2010; Owen et al. 1992; Taylor et al., 1986). Such a parallel implies a functional interaction between dopaminergic projections and prefrontal cortical areas in the mediation of such functions under physiological conditions. A functional interplay between midbrain dopamine projections and the mPFC in terms of object recognition has been reported (Chao et al., 2013). In the present study, we explore the involvement of this network in depression- and anxiety-related functions and the contribution of the serotonergic system We implemented a preparation focusing on exclusively on dopaminergic mechanisms by the pretreatment with the norepinephrine reuptake inhibitor, desipramine. Prefrontal areas not only receive direct dopaminergic inputs via the mesocortical pathway, but also, directly feed back into the midbrain projections, thereby, forming a distinct circuit (Patton et al., 2013). A disruption of such a network by DA depletion is, therefore, likely to underlie the cognitive and emotional disorders evident in PD patients and here we provide experimental evidence for such an assumption.

All groups showed a characteristic turning bias towards the side of the lesion after 6-OHDA administration. There were no group differences except for a disconnection effect between the ipsi- and contralateral high dose 5,7-DHT groups. Here, a serotonergic deafferentation of the mPFC counteracted the 6-OHDA induced ipsiversive motor bias. This is in line with other findings reporting a participation of the 5-HT system in the development of motoric impairments in PD (Miguelez et al., 2014). There were clear effects of a disconnection of the mPFC on anxiety-related behavior as indicated by an increase in time the contralateral lesion group spent on the open arms in the EPM, as well as in the center compartment of the OF. These findings can be interpreted as an anxiolytic effect evoked by a functional

decoupling of the mPFC from 6-OHDA lesioned DAergic midbrain projections. Behavioral alterations incurred by a 5-HT-specifc deafferentiation of the mPFC resembled these effects. Taking into account that the total distance moved was not different between the groups, motoric confounds that may have determined differences between the respective ipsi- and contralateral groups can likely be ruled out. Anxiogenic effects of 6-OHDA lesions of the MFB have been reported before (Santiago et al., 2010; Tadaiesky et al., 2008). In our interpretation, the data give evidence that a functional decoupling of the mPFC counteracts PD-associated anxiety disorders. The same pattern was also observed with respect to memory functions. Both excitotoxic and 5-HT-specific lesions of the contralateral mPFC led to a partial rescue of object recognition. Thus, these findings provide evidence for a promnestic effect of a functional decoupling of the mPFC in hemiparkinsonian rats. There were also antidepressant-like effects of a functional decoupling of the mPFC and 6-OHDA lesioned DAergic midbrain projections. Both treatments (unspecific and 5-HT-specific lesions of the mPFC) yielded an increase in general behavioral activity. Specifically, an increase in active escape behavior (climbing) was observed with respect to unspecific mPFC lesions, while a decrease in immobility was observed accompanied by 5-HT-specific deafferentation of the (contralateral) mPFC. These results are in line with findings showing a predominant effect of 5-HT-specific modulation (SSRIs) on immobility behavior, while antidepressant treatments targeting noradrenergic mechanisms primarily affect climbing behavior (Cryan et al., 2005).

The mPFC can be divided into a dorsal (dmPFC) and a ventral (vmPFC) region, whereby the vmPFC has most often been described in the context of cognitive and affective functions (Heidbreder and Groenewegen, 2003). Due to its strategic position within networks known to mediate emotion- and cognition-related functions, the mPFC is likely involved in modulating these. The expression of anxiety-related behavior, for instance, is well known to rely on amygdalar mechanisms. The mPFC also has strong projections to

the amygdala complex and can modulate the behavioral expression of anxiety (for review see Maroun, 2012). Manipulations of the mPFC in rats have been demonstrated to influence anxiety-related behavior, whereby the net effect (anxiolytic or anxiogenic) seems to depend on the particular type of manipulation, i.e. whether lesion, inactivation or deafferentiation procedures were applied (De Visser et al., 2011; Pum et al., 2009; Shah et al., 2003). Comparable influences have also been described in the context of affective impairments via projections to other prefrontal areas, the amygdala and hippocampus complex, as well as to parts of the basal ganglia (Drevets et al., 2008; Drevets, 2000). Selective lesions of the mPFC in the rat were shown to evoke depressive-like behaviors (Chang et al., 2014; Klein et la., 2010). Moreover, the mPFC has consistently been implicated in memory functions via projections to the hippocampus complex and the perirhinal cortex (Barker and Warburton, 2011, Hannesson et al., 2004; Barker et al., 2007). There is evidence that all of these functions strongly rely on the integrity of dopaminergic innervation of the mPFC. Dopaminergic lesions in the rat have been reported to impair memory (Foyet et al. 2011) and induce depressive-like and anxiogenic behavior (Santiago et al., 2010; Tadaiesky et al., 2008). Taken together, these lines of evidence point towards similar behavioral effects of mPFC and MFB lesions. Hence, a close interdependence of these constituents seems to underlie the behavioral expression of emotional and memory functions. Here, we found direct support of this assumption, as disconnection effects were observed for all functional domains assessed in the experiments.

Given that the mPFC extends ipsi-, as well as contralateral projections, it may be that the disconnection procedure applied here does not result in an absolute dissociation of the constituents. However, transcallosal projections -in particular with respect to subcortical terminals- are much less pronounced than ipsilateral pathways (Ferino et al., 1987). Moreover, any behavioral change incurred by a contralateral lesion (as compared to an

ipsilateral lesion of the corresponding structure), indicates a meaningful and essential interaction of the structures in terms of behavioral control as was evidenced in the present experiments. We also demonstrated a pivotal involvement of the mPFC's serotonergic system in this context, given that a focal 5-HT-specific disconnection of the mPFC had similar behavioral effects as the unspecific lesions. These findings are in line with other reports demonstrating an interaction of midbrain DA projections and the prefrontal 5-HT system with respect to neurochemical alterations and electrophysiological properties. In general, alterations of 5-HT-related measures in PD patients are observed in post mortem neural tissue levels of 5-HT (Jenner et al., 1983; Scatton et al., 1983), as well as in cerebro spinal fluid (Mayeux et al., 1988). This reduction has been shown to be even more pronounced in depressed PD patients (Kostic et al., 1987; Mayeux et la., 1984) and is associated with a decreased 5-HT_{1A} receptor availability and an increased 5-HT transporter binding in various brain regions (Ballanger et al., 2012). A reduction in the density of 5-HT_1, 5-HT_2 and 5-HT_3 receptors in the mPFC has also been observed following experimental dopaminergic lesions of the MFB (Kienzl et al., 1981; Cicin-Sain and Jenner, 1993; Soria-Fregozo et al., 2008). Furthermore, electrophysiological changes in mPFC neurons in response to 5-HT receptor challenges have been described after 6-OHDA administration into the MFB (Wang et al., 2009; Gui et al., 2011; Fan et al. 2011; Zhang et al., 2011; Cao et al., 2007). These findings point towards alterations in 5-HT transmission and receptor-related changes in the mPFC following DA depletion that underlie functional impairments. A decoupling of serotonergic input counteracted these effects and improved cognitive and emotional deficits. Other functionally relevant interactions of the serotonergic and dopaminergic system in the central nervous system have also been reported. There is evidence that long term DA substitution (as often occurs in PD patients) may alter 5-HT functioning by inhibiting tryptophan hydroxylase and by competing for conversion via aromatic amino acid decarboxylase (Hashiguti et al., 1993;

Borah and Mohanakumar, 2007). Additionally, 5-HT neurons were shown to metabolize L-DOPA into DA, which, in turn occupies vesicular storage and, ultimately, leads to a depletion of synaptically available 5-HT, which is likely to contribute to depressive symptoms (Navailles et al., 2010; Navailles and De, 2011; Eskow Jaunarajs et al., 2010). Taken together, such findings are indicative of altered functional properties of 5-HT neurons evoked by a degeneration of the dopaminergic system. The present studies provide evidence that this interplay is crucial in the mediation of affective and memory functions.

The disconnection of the mPFC resulted in partial restoration of functional integrity with respect to anxiety-, depression-, and memory-related behavior. Thus, a functional decoupling of the mPFC counteracted behavioral impairments induced by dopaminergic deafferentiation of the mPFC. Besides the aforementioned evidence that mPFC neurons display characteristically altered activity patterns after dopaminergic denervation, several lines of evidence also indicate changes in the serotonergic system after experimental DA depletion. There have been structural changes of the 5-HT system described by means of increased sprouting of 5-HT terminals, hyperinnervation of forebrain 5-HT fibres, as well as elevated brain tissue levels of 5-HT (Zhou et al., 1991; Smits et al., 2008; Reader and Dewar, 1999; Commins et al., 1989). Hence, it has been proposed that there is a serotonergic hyperinnervation evoked by DA depletion that determines functional alterations of circumscribed brain regions and, thus, gives rise to disturbances in behavior. This assumption is in line with the data reported here, whereby an experimentally induced modulation of prefrontal 5-HT levels counteracted the 6-OHDA induced behavioral impairments. Such findings provide impetus to focus on the prefrontal 5-HT system as a strategic target in the specific treatment of emotional and cognitive deficits accompanied by a degeneration of dopaminergic projections in PD patients.

5 Conclusions

Taken together, we provide evidence for a behaviorally relevant interaction of the mPFC and the midbrain dopamine projections in the mediation of anxiety-, depression- and memory-related functions that underlie emotional and cognitive impairments seen in PD. Further, we demonstrate a fundamental participation of the serotonergic system in this circuit.

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Figure/Table captions

Figure 1: Schematic representation of the disconnection logic. If a lesion of the medial forebrain bundle (MFB) in one hemisphere, combined with a lesion of the medial prefrontal cortex (mPFC) in the contralateral hemisphere, influences behavior differently than lesions of the MFB and the mPFC located in the same hemisphere, it can be surmised that an interaction between the MFB and the mPFC is involved in that behavioral function. The former disconnects the constituents at two different levels, while the latter preserves an intact circuit in one hemisphere, which may be capable of a functional compensation. Behavior of animals with mesotelencephalic DA deficiency combined with lesions of the contralateral mPFC can be compared to animals bearing the lesions within the same hemisphere. Therefore, a functionally relevant interaction of the MFB and the mPFC can be tested by applying the disconnection procedures presented here.

Figure 2: Temporal sequence of Experiments 1 and 2. OF, open-field; EPM, Elevated Plus

Maze; FST, Forced Swim Test; Amph Chlng, Amphetamine-challenge.

Figure 3: Experiment 1. Schematic representation of NMDA-induced lesions of the mPFC. Coronal sections of largest (light gray) and smallest (dark gray) lesion extents observed. All patterns projected to the left hemisphere. Values represent approximate distance according to bregma. Adapted from Paxinos & Watson, 1998.

Figure 4: Experiment 1. (A) Turning behavior: Mean turning index pre- and post- 6-OHDA application (+ SEM). (B) Depression-related behavior: Mean duration of climbing behavior (+ SEM) on day two of the Forced Swim Test (FST). (C) Memory-related behavior: Mean index of object recognition (SEM). Note that greater values reflect superior memory performance. (D) Anxiety-related behavior: Mean time spent on the open arms of the Elevated Plus Maze, mean time spent in the center compartment of the open field and mean locomotor activity (+ SEM). 'Ips': ipsilateral mPFC lesion group; 'Dis': Disconnected (= contralateral) mPFC lesion group. $^{*}p< 0.05$; n.s., not significant.

Figure 5: Experiment 2. (A) Turning behavior: Mean turning index pre- and post- 6-OHDA application (+ SEM). (B) Depression-related behavior: Mean duration of immobility behavior (+ SEM) on day two of the Forced Swim Test (FST). Pooled values of the respective dosages groups are depicted to the right of the dashed line. (C) Memory-related behavior: Mean index of object recognition (SEM). Note that greater values reflect better memory performance. (D) Anxiety-related behavior: Mean time spent on the open arms of the Elevated Plus Maze, mean time spent in the center compartment of the open-field and mean locomotor activity (+ SEM). Shown are the results for the ipsilateral ('Ips') and disconnected ('Dis') groups that received either a low (0.8µg) or high (8 µg) dose of 5,7-DHT into the respective mPFC, $^{*}p< 0.05$.

Table 1: Results neurochemical assessment of Norepinephrine (NE), Dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), Serotonin (5-HT) and 5-Hydroxyindoleacetic acid (5-HIAA) in the dorsal striatum (dSTR) and medial prefrontal cortex (mPFC) for both hemispheres of the different experimental groups. Values reflect wet tissue content as µg/mg. For the dSTR section, "**int**" refers to the side not challenged with 6-OHDA, while "**les**" reflects content of the 6-OHDA lesioned hemisphere. For the mPFC section (lower part), "**int**" refers to the side where the mPFC was not challenged with 5,7-DHT, while "**les**" reflects transmitter levels of the 5,7-DHT challenged mPFC.

Figure_1
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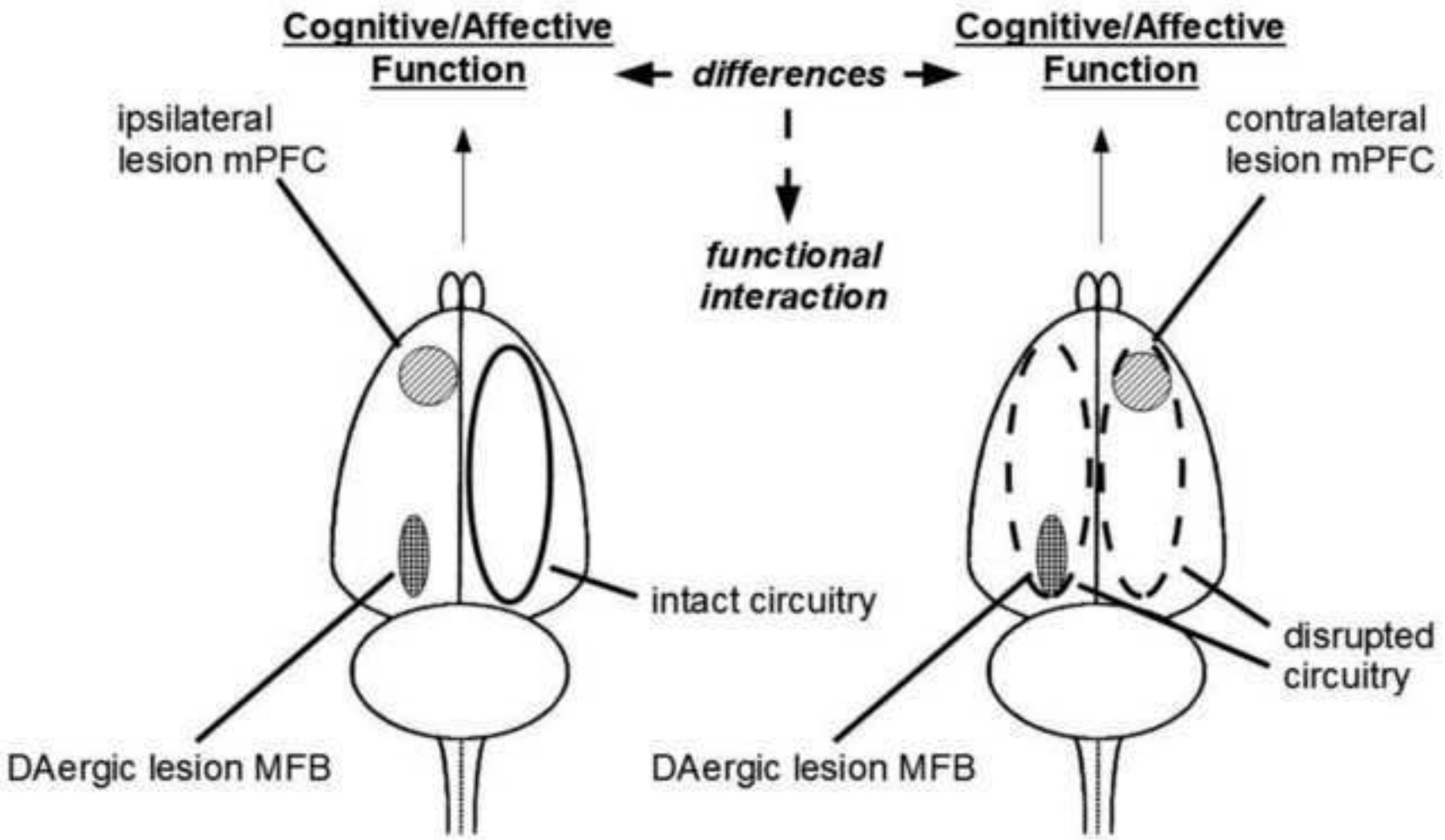

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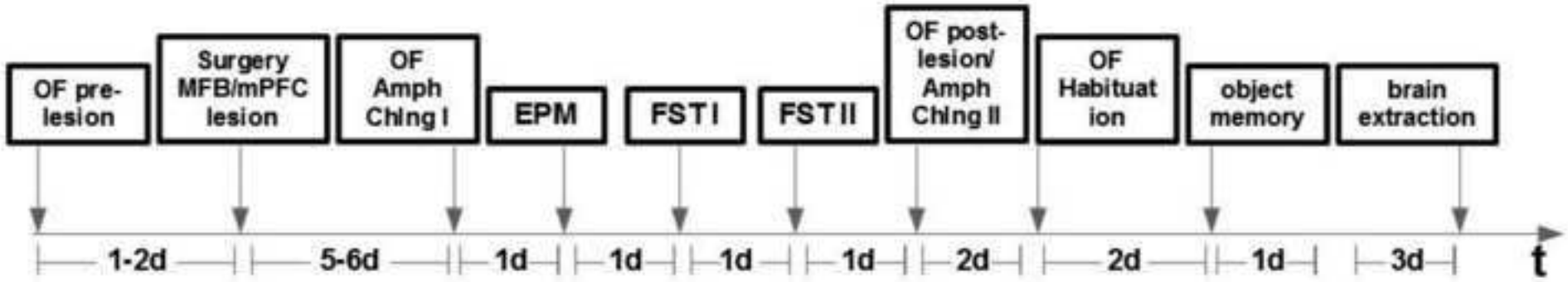

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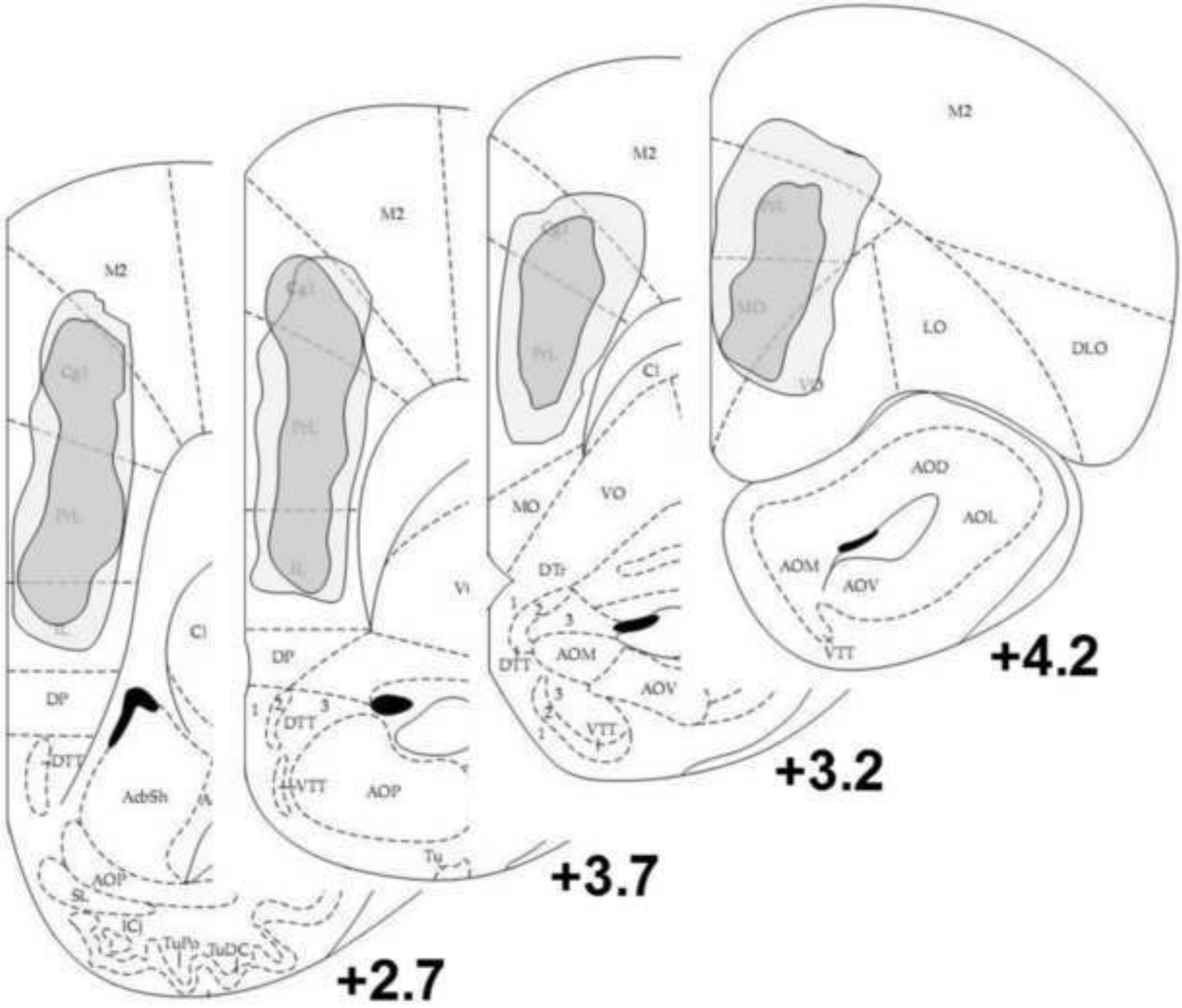

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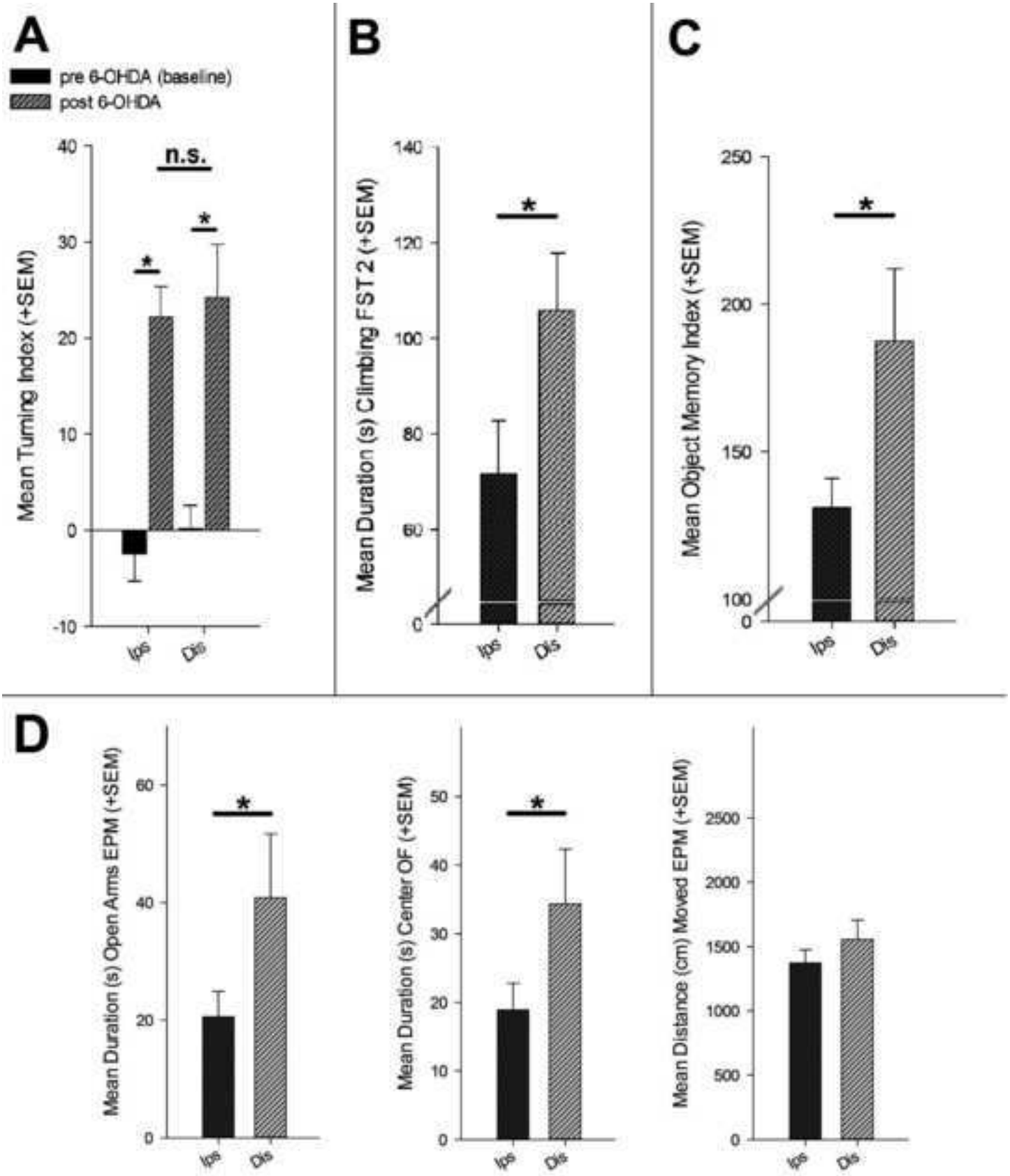

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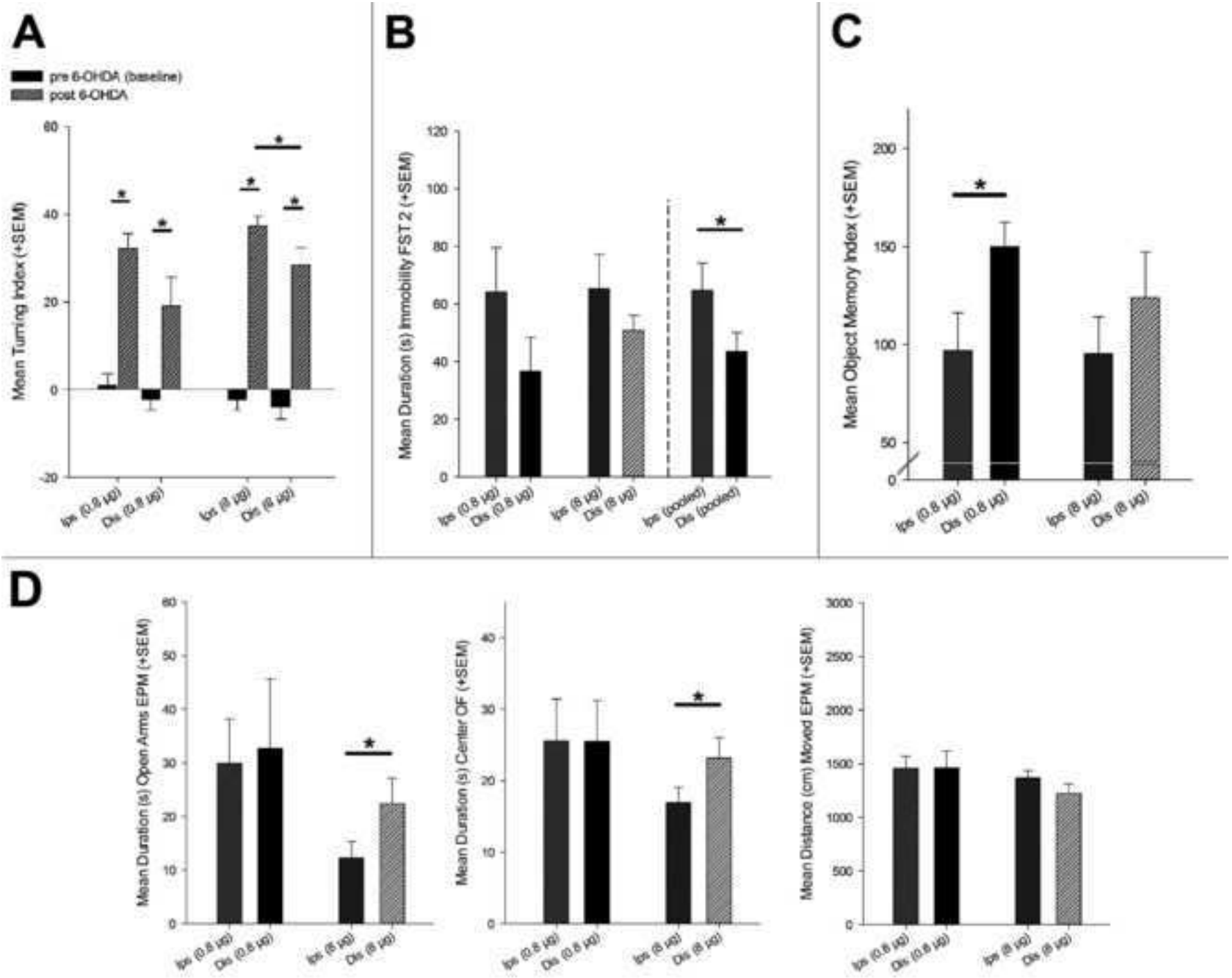

Table_1

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		NMDA Ips		NMDA Dis		5.7-DHT (0.8µg) Ips		5.7-DHT (0.8µg) Dis		5.7-DHT (8µg) Ips		5.7-DHT (8µg) Dis	
		int	**les**	**int**	**les**	**int**	**les**	**int**	**les**	**int**	**les**	**int**	**les**
dSTR	NE	6.6 ±5.62	1.91 ±1.35	1.22 ±0.80	1.48 ±0.93	4.07 ±2.27	1.56 ±1.23	9.53 ±9.03	1.17 ±0.78	2.77 ±1.54	1.33 ±0.56	2.15 ±1.17	0.86 ±0.31
	DA	185.1 ±79.38	63.26 ±59.43	73.90 ±52.44	0.91 ±0.43	195.3 ±96.42	0.76 ±0.29	189.3 ±111.1	1.54 ±1.00	601.5 ±124.4	4.66 ±1.90	592.2 ±97.1	5.97 ±3.11
	DOPAC	57.99 ±20.37	13.12 ±10.85	27.52 ±7.52	1.43 ±0.94	362.5 ±252.2	20.07 ±16.20	40.98 ±8.13	9.25 ±6.64	151.2 ±45.23	10.30 ±2.87	159.1 ±28.05	17.12 ±8.73
	5-HT	26.31 ±2.49	17.80 ±5.08	24.80 ±5.41	10.57 ±3.36	163.3 ±132.1	27.3 ±7.49	32.02 ±6.12	13.17 ±4.38	60.31 ±12.7	60.03 ±18.66	90.85 ±18.11	136.2 ±87.81
	5-HIAA	25.1 ±5.71	24.19 ±8.26	21.04 ±3.10	199.1 ±165.0	271.2 ±223.8	39.98 ±11.59	30.25 ±6.74	27.36 ±12.47	63.60 ±13.21	72.31 ±26.35	74.60 ±13.24	117.8 ±77.62
mPFC	NE	---	---	---	---	1.51 ±0.44	2.45 ±1.35	5.78 ±4.97	6.19 ±4.58	9.49 ±5.97	5.22 ±3.25	12.09 ±6.78	10.47 ±5.38
	DA	---	---	---	---	45.02 ±41.54	2.12 ±0.96	1.12 ±0.67	12.63 ±10.43	25.44 ±13.54	8.91 ±5.27	4.56 ±1.63	39.28 ±19.93
	DOPAC	---	---	---	---	7.75 ±3.89	6.96 ±3.44	1.93 ±1.10	15.93 ±8.58	21.41 ±7.59	15.04 ±7.49	9.05 ±4.78	13.82 ±6.01
	5-HT	---	---	---	---	29.86 ±5.06	30.15 ±8.28	20.94 ±4.21	37.07 ±6.63	35.48 ±7.67	39.25 ±10.42	38.07 ±5.95	39.86 ±5.08
	5-HIAA	---	---	---	---	26.98 ±5.04	23.95 ±6.86	18.23 ±4.79	27.98 ±5.92	52.21 ±4.66	47.88 ±15.77	33.24 ±4.57	43.89 ±9.14