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Modulation of Adult Neurogenesis in the Olfactory Bulb in an Acute Mouse Model of Parkinson's Disease

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Meiner Familie

I. Introduction

1 Parkinson's disease

1.1 History of Parkinson's disease

Motor signs of Parkinson's disease (PD) were reported the first time by James Parkinson (**Fig. 1**) in 1817, Royal College of Surgery, London. In his manuscript, *An Essay on the Shaking Palsy*, he described the clinical symptoms of 6 patients.

Fig. 1. James Parkinson *(1755-1824)*
(The figure extracted from
http://viartis.net/parkinsons.disease/history.htm)

Among 6 patients, he observed that tremor, rigidity, and walking problems were major complaints. Their age was 50-70 years. The onset of tremor of 2 of the patients in their upper limbs was at the age of 50 years. Parkinson also found a patient, who had difficulties to initiate his first step while intending to walk. After interviewing these patients, he noticed that this disease is progressive and that there is no prospective of relief (Parkinson, 2002). Later, Wilhelm von Humboldt, Jean-Martin Charcot and Alfred Vulpian added more clinical features after James Parkinson's first clinical description of these patients. The term "Parkinson's Disease" was the first time used by Jean-Martin Charcot and he was also the first physician to introduce the alkaloid atropine to treat PD patients. In 1912 Friedrich Heinrich Lewy described inclusion bodies in postmortem tissue, later named “Lewy bodies” as the pathological hallmark of Parkinson’s disease. High doses of atropine to treat PD patients were very effective in the 1930s, but serious side effects were noticed thereafter. Atropine

was used for decades before the discovery of L-DOPA. In 1958, Arvid Carlsson defined the role of dopamine (DA) in the basal ganglia in reserpine treated rabbits. Oleh Hornykiewicz and Herbert Ehringer discovered the dopamine deficiency in PD patients in 1960. Oleh Hornykiewicz, Walther Birkmayer, Patrick Mcgeer and George Cotzias started to treat patients with L-DOPA after 1961. In 1969 the use of L-DOPA in combination with a dopa-decarboxylase-inhibitor was introduced. Till today, L-DOPA is the most important drug (gold standard) in treating PD patients.

1.2 Etiology and pathology of Parkinson's disease

Up to 5-10 % of the patients suffer from the disease before the age of 40, but the incidence increases steeply with age after the age of 60 (Wirdefeldt et al., 2011). The estimated incidence is about 160 per 100 000 person-years at the age of 65 years, in comparison to the range of 1.5 to 22 per 100 000 person-years in all age groups, and men are more frequently affected than women (Hirtz et al., 2007). Furthermore, the estimated number of PD subjects in most nations will be more than doubled within the next 20 years (Dorsey et al., 2007).

1.2.1 Etiology

Even though the etiology of PD is not well understood, both genetic susceptibility and environmental factors are very likely to be involved in the pathogenesis of the disease.

Genetic studies have identified 11genes on 16 loci (PARK1 – PARK16) associated to PD. Two more loci – not yet associated to PD - have been discovered recently. These genes can be divided up in dominantly inherited mutations and recessively inherited mutations; The former include SNCA (PARK1/ PARK4), PARK3, UCH-L1 (PARK5), LRRK2 (PARK8), Omit/HtrA2 (PARK13), and the latter include Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), ATP13A2 (PARK9), PLA2G6 (PARK14) and FBXO7 (PARK15) (Farrer, 2006). However, familial inherited PD accounts only for about 10-15% of all cases, 85-90% of PD patients are sporadic.

Occupational and environmental toxin exposure is one of the risks to induce PD. For example, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin) was indicated to cause chronic parkinsonian symptoms by damaging DA neurons in humans (Langston and Ballard, 1983). Natural toxins, such as rotenone, paraquat and dieldrin, are seen clinically to induce parkinsonian syndromes. Animal studies show that these natural toxins cause mitochondrial dysfunction, increased oxidative stress and aggregation of alpha-synuclein (a-syn) that result in a pathological alteration of the DA system (Wirdefeldt et al., 2011). Living style is also related to the incidence of PD. There is evidence that smoking and coffee consumption have a

negative association as risk factors of PD (Bronstein et al., 2009). Nicotine intake decreased the risk of PD proved in clinical studies (Tanner et al., 2002; Hancock et al., 2007) and experimental studies showed an anti-fibrillogenic activity against a-syn (Ono et al., 2007; Hong et al., 2009). Caffeine is a non-specific adenosine receptor antagonist (Schwarzschild et al., 2006) and drinking coffee was statistically associated with a decreased risk of PD (Ross et al., 2000). Despite nicotine and caffeine seem to provide compelling neuroprotective effects, more clinical and biomolecular studies are needed.

1.2.2 Pathology

Another pathological hallmark of PD is the loss of DA neurons in the substantia nigra (SN), which causes DA deficiency in the striatum. In postmortem studies, 50% to 77% of DA neurons are lost (Fearnley and Lees, 1991; Hirsch et al., 1988), leading to a 80% reduction of striatal DA content (Marsden, 1990). Among SN neurons, neuromelanin-pigmentated neurons are degenerating in PD. A 66% to 83% loss of pigmented neurons has been shown (Hirsch et al., 1988; Pakkenberg et al., 1991). The total number of pigmented neurons in PD patients shows a significant correlation with the duration of the disease process while the SN volume is equally maintained (Ma et al., 1997; Pakkenberg et al., 1991). In comparison to age-related control subjects, the loss of DA neurons is reduced by 64 % in the ventral SN, and the rate of neuronal loss of PD is exponential. In the first decade after disease onset, neuronal loss is 10 times greater than that in age-matched controls. Moreover, distinct regions of neuronal loss in the SN can be distinguished from aging (Fearnley and Lees, 1991) and neuronal loss is not only determined spatiotemporally but also topographically.

1.3 Therapy of Parkinson's disease

Since DA was synthesized the first time by George Barger and James Ewens in 1910 in London, little progress was made in the next 30 years until Peter Holtz discovered AADC (aromatic-L-amino-acid decarboxylase, also called L-DOPA decarboxylase), which metabolizes L-DOPA to DA (**Fig. 2**). AADC in brain provides the formation of exogenous DA, as L-DOPA is able to cross the blood brain barrier (Fahn, 2008). In the early 1960s, Arvid Carlsson demonstrated that L-DOPA makes reserpinized akinetic rabbits immediately mobile. Moreover, he showed that the highest DA concentration is in the SN.

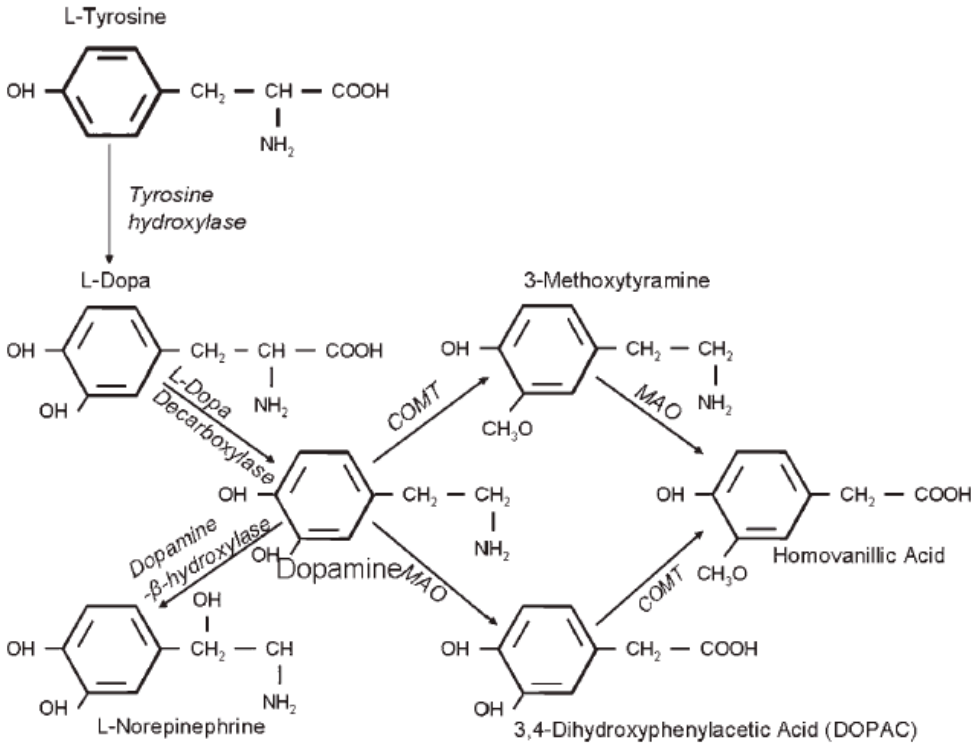


Fig. 2. Biochemical pharmacology of dopamine. (Adapted from Fahn et al. 2008.)

1.3.1 Drug treatment

L-DOPA ($_L$-3,4-dihydroxyphenylalanine) and DA agonists

High doses of L-DOPA were given in the 1960s to treat PD patients until to 1969, then a combination with a peripheral dopa-decarboxylase-inhibitor was thus allowing to reach effectively brain L-DOPA level. L-Dopa is still considered as the gold standard in the management of the disease today. The point in time to start L-DOPA treatment is still a matter of debate. Fahn speculated that early use of L-DOPA supplies sufficient DA to activate DA neurons and their receptors, which can release the burden while remaining DA neurons try to compensate the partial neuronal loss. On the other hand, Cohen suggests that more exogenous DA might cause unnecessary free radicals that damage remaining DA neurons, therefore delayed use of L-DOPA would be favorable (Fahn, 2008). The results of a clinical study of the Parkinson Study Group showed that an earlier treatment of L-DOPA is beneficial for PD patients. However, FP-CIT-SPECT data in the same study contradicted the clinical results (Fahn, 2008). Recently, our group showed a correlated downregulation of striatal FP-CIT signal and TH positive immunoreactivity of the striatum and the SN, in an acute model of 6-OHDA lesioned mice after L-DOPA treatment, and the results suggested long term L-DOPA treatment did not lead to DAergic overstimulation and the ensuing adaptive changes in the nigro-striatal pathway (Depboylu et al., 2013).

Chronic L-DOPA monotherapy can relieve motor symptoms effectively, however, motor complications, such as fluctuation and dyskinesia, are a major challenge of L-DOPA therapy.

A number of DA agonists have been developed to accompany L-DOPA to provide more stable DA concentrations in the brain. Dopamine agonists can also be used as monotherapy in the early stage of the disease to delay the introduction of L-DOPA. However, the agonists are usually less effective than L-DOPA and require the addition of L-DOPA within a few years. There are ergot derivatives and nonergoline agonists. As some of the ergot derivatives have shown severe side effects, like valvular fibrosis of the heart, nonergoline agonists are the first line drugs, e.g. pramipexole and ropinirole. DA agonists extended "on" state and reduced "off" state in combination with L-DOPA. Dyskinesia is also reduced by a combination of pramipexole, ropinirole or bromocriptine with L-DOPA. Besides assisting L-DOPA therapy, side effects of DA agonists require more consideration. Nausea, vomiting, postural hypotension, hallucination, somnolence and impulse control disorders are often seen in these patients (Antonini et al., 2009).

MAO-B (monoamine oxidase-B) inhibitor

The action of MAO is to deaminate monoamines (**Fig. 2**). There are two types of MAO, the genetically distinct isoforms MAO-A and MAO-B. MAO-B is located dominantly in glial mitochondria and available to metabolize DA released into synaptic cleft (Fahn, 2008). Non-selective MAO inhibitors were first designed to treat depression, however, due to the risk of the "cheese effect" (when tyramine is not degraded by MAO-A present in the gut and acts as a false transmitter to cause hypertension and other complications) these drugs are no longer available. Later the irreversible MAO-B inhibitor, Selegiline (formerly named deprenyl), was invented and provides a mild symptomatic effect (Fahn, 2008). One of Selegiline's metabolites, L-methamphetamine, inhibits the reuptake of extracellular DA, and indirectly elevates DA concentration in the brain (Magyar, 2004). Moreover, Selegiline delayed the need for L-DOPA treatment compared to the placebo treated group (Pålhagen et al., 2006). Two studies on Rasagiline, another MAO-B inhibitor, suggested a potential disease modifying effect in addition to its symptomatic benefit. However, it has not been possible to confirm a neuroprotective effect of both Selegiline and Rasagiline in clinical trials.

1.3.2 Surgery

The surgical procedure of thalamotomy was accidently developed in the 1940s. It had a surprisingly good effect on tremor. After discovering L-DOPA in the 1960s, surgery was abandoned. In the last 25 years, surgical treatment is again a clinical treatment option of PD because of three major reasons: Urgent need for treatment of side effects from drug therapy, more knowledge of DAergic circuitry from primate models, and the fast developing technique of image tools, such as MRI and CT scan (Rascol et al., 2011).

Deep brain stimulation (DBS)

David Marsden once said, DBS is the second miracle in the treatment history of PD after the L-DOPA invention. The dysregulated basal ganglia circuitry results from hyperactivity of the globus pallidus internus (GPi), which is mediated by a loss of inhibitory input and by an enhanced excitatory input via indirect DA pathways in primates (Rascol et al., 2011). The principle of DBS is to reduce the hyperactivity in the GPi or subthalamic nucleus (STN) by

applying high frequency stimulation (HFS) with the implantation of intracranial electrodes. Indeed, DBS is dramatically effective on motor symptoms of PD. Moreover, patients can get up to a 50% dose reduction of L-DOPA, whereby fluctuation and dyskinesia decrease (Rascol et al., 2011). However, the cost of surgery and hardware, patient selection, surgical complications, and psychosocial maladjustment need further evaluation.

Cell transplantation and gene therapy

Transplantation of human fetal mesencephalic DA cells or stem cell-derived DA cells into putamen have not yet been established in clinical routine use in the treatment of PD. However, cell replacement is possibly feasible. Standardization of transplanted cells, knowledge to rebuild the DA pathway, prevention of tumor-genesis, and appropriate patient selection are required (Lindvall and Kokaia, 2010). As in PD a number of different neurons degenerate in the peripheral and central nervous systems, cell replacement might only be beneficial for some of the symptoms (Poewe, 2009). Besides cell replacement, gene therapy might become a useful tool to treat the disease. Stereotaxic injection of adeno-associated virus (AAV) carrying the glutamic acid decarboxylase (GAD) gene in the STN or aromatic amino acid decarboxylase (AADC) in the putamen showed small but encouraging results in clinical pilot studies. Whether the delivery of neurturin as an analogon of glial derived neurotrophic factor (GDNF) will become a treatment option, needs to be evaluated in further clinical trials.

1.4 From pathology to pre-clinical symptoms

Alpha-synuclein (a-syn) was first discovered in missense mutations in familial PD and it constitutes the major component of Lewy bodies (LB) and Lewy neurites (LN). These mutations cause a duplication or triplication of a-syn (Polymeropoulos et al., 1997; Spillantini et al., 1997). Braak and his colleagues performed immunohistochemistry of LB in postmortem studies to describe the stage of LB pathology in PD. The Braak staging scheme suggests that the initiation of LB pathology is in the dorsal nucleus of the vagal nerve, medulla oblongata and OB. In stage 2, LB and LN involve also the caudal raphe nuclei and reticular formation. LB pathology ascends dorsally and spreads gradually to the whole brain with disease progression. LB pathology in the SN is not found before stage 3 or 4, when motor symptoms start to develop. In the late stages, LB and LN pathology spread into specific nuclei of the thalamus and many prefrontal association fields (Braak et al., 2003).

Given the fact that premotor symptoms are widely accepted the Braak staging scheme provides a plausible framework to explain the observed evolution of the clinical features of PD (Kalaitzakis et al., 2009). The evidence of premotor symptoms preceding motor signs results from postmortem studies of incidental Lewy body disease (iLBD). Tyrosine hydroxylase (TH) staining shows intermediate levels of iLBD in the striatum and epicardial nerve fibers compared to controls and PD, and similar results in neuronal density of the SN. It is proposed that iLBD would very likely develop into PD if patients lived longer (Dickson et al., 2008). Even though the loss of SN neurons and DA deficiency in STR are hallmarks of PD, increasing evidence indicates that the pathological changes of this neurodegenerative disease occur widespread in the peripheral and central neural systems, affecting the dopaminergic, noradrenergic, serotoninergic and cholinergic system (Lim et al., 2009). The clinical symptoms, such as depression, hyposmia, constipation and rapid eye movement sleep behavior disorder (RBD) are therefore considered as premotor symptoms when the degeneration is not yet affecting the SN. Premotor symptoms can serve as risk factors or predicators for PD and could strengthen the search for potential biomarkers. Biomarkers for PD could enable us to apply disease-modifying therapies that prevent or stop disease progression before motor deficits occur in the future.

1.5 Olfactory dysfunction in PD

Olfactory dysfunction was reported the first time in PD patients in 1975 (Ansari and Johnson, 1975). Today, hyposmia is known as one of the premotor symptoms that can predate motor signs by several years and persist with the progression of PD. However, the cause of olfactory dysfunction in PD remains to be determined. It will require more information from basic and clinical research in order to understand and dissect the biological mechanisms underlying olfactory dysfunction in PD patients

1.5.1 The olfactory system

Offspring reproduction, maternal behavior, emotional responses, aggression, food selection and individual recognition are major functions of the olfactory system in mammals (Shipley and Ennis, 1996). When odor molecules (odorants) bind to receptors of olfactory sensory neurons (OSN) in the olfactory epithelium, the signals pass to the olfactory cortex via the main olfactory bulb (OB). In the OB, glomeruli are the units that refine signals from the OSN in the periglomerular cell layer (PGL). The signal is transmitted to output neurons such as mitral and tufted cells (M/T cells), confining conduction with local intrabulbar circuitries and centrifugal inputs (cholinergic, acetylcholinergic and serotoninergic projections from higher brain centers). The final processing occurs in the primary and accessory olfactory cortex corresponding to the piriform and the entorhinal cortex, respectively (Buck, 2000), and connecting to other neural systems in order to execute relevant functions (Carleton et al., 2002; Shipley and Ennis, 1996).

The other olfactory system, the vomeronasal organ (VNO), was found to play a role in social interaction in mammals. The VNO is enclosed in bony capsules on each side of the ventral nasal septum and connected by a narrow duct (Buck, 2000; Sanchez-Andrade and Kendrick, 2009). Non-volatile odorants such as pheromonal information in urine, skin and reproductive secretions are recognized by the VNO that transmits signals by projecting to mitral cells in the accessory olfactory bulb , where signals are processed and carried to the olfactory cortex. The function of main OB and accessory OB are partially overlapping, being responsible for the conduction of volatile and non-volatile social scents (Sanchez-Andrade and Kendrick, 2009; Zufall and Leinders-Zufall, 2007).

1.5.2 Sensory transduction in the OB

The topographical arrangement reveals that a given OSN projects to a given subset of glomeruli in the PGL (**Fig. 3A-B**) (Carleton et al., 2002; Buck, 2000). The signal relay in the OB is divided into two parts: the first part of the signal relay follows M/T cells receiving excitatory input from axons of the OSN and information via dendro-dendritic synapses with periglomerular cells of other glomeruli (**Fig. 3A**). There are two types of inhibitory transduction in the glomerulus. GABA mediated dendro-dendritic synapses inhibiting M/T cells and DA-mediated depression of synaptic transmission between mitral cells and OSN (**Fig. 3B**) (Aungst et al., 2003); the second part of the signal relay occurs in the deeper part of the OB. The dendrites of M/T cells extend into the external plexiform layer and construct reciprocal dendro-dendritic synapses with granular cells (mainly GABAergic neurons) in the granular cell layer (**Fig. 3A**). The lateral inhibition and negative feedback from GABA-mediated granular cells are used to restrain synaptic information in order to mediate transduction to M/T cells (Carleton et al., 2002; Hsia et al., 1999; Wachowiak and Shipley, 2006).

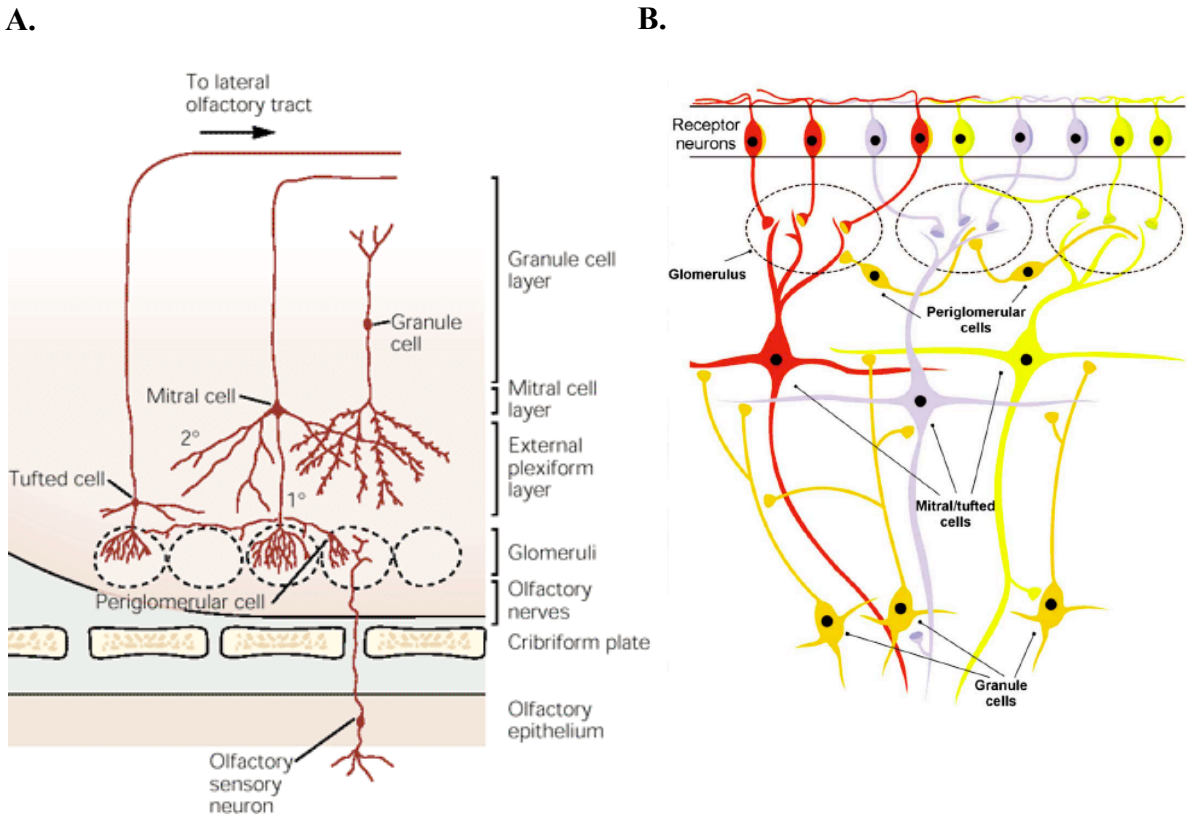


Fig. 3. (A) The olfactory bulb receives signals from olfactory sensory nerves *(Adapted from Shepherd and Greer 1990)*. **(B)** Synaptic transmission in the olfactory bulb *(Adapted from Carleton et al. 2002)*

1.5.3 Clinical and experimental studies

Clinical studies

Olfactory dysfunction is usually referred to hyposmia or anosmia. Olfactory detection, identification and discrimination can be evaluated by the "University of Pennsylvania Smell Identification Test" (UPSIT), another way to assess olfactory function would be the "Sniffing Sticks Test". More advanced methods, such as olfactory event-related potentials, diffusion-weighted magnetic resonance imaging (MRI) and biopsy are also currently used in clinics (Lang, 2011).

It has been shown that olfactory dysfunction is related to a central neural deficit. There was no correlation with proteins collected from the olfactory epithelium and hyposmia in PD patients (Witt et al., 2009). A population-based, longitudinal study demonstrated a clear relationship between olfactory dysfunction and iLBD pathology. Moreover, it has been shown that impaired olfactory function is often preceding the manifestation of motor deficits in PD by at least 4 years (Ross et al., 2008). There is evidence that DA imbalance in the brain is not the only reason to cause olfactory dysfunction. For example, a positive correlation of hyposmia and cholinergic degeneration was shown in a study combining ^{11}C-methyl-4-piperidinyl propionate acetylcholinesterase brain positron emission tomography (PET) and UPSIT (Bohnen et al., 2010). Some reports suggested that the dysregulation of certain subtypes of neurons in the OB might also cause hyposmia in PD. LB pathology involves certain heterogeneous neurons in the OB, such as glutamate, calcium-binding, and P-positive cells that are relatively vulnerable to neurodegeneration (Ubeda-Bañon et al., 2010). An increased number of DAergic neurons was found in the PGL of PD in postmortem studies (Huisman et al., 2004; Mundiñano et al., 2011).

Animal studies

The use of animal models in PD is an important, alternative way to explore unknown disease-related bio- or physiological mechanisms, in order to provide information regarding pathological alterations and therapeutic effects. Due to ethical issues it would not be possible to apply experimental procedures to patients.

A transgenic mouse of a-syn overexpression using a Thy1-promoter showed some deficits of olfactory function. Thy1 a-syn overexpressing mice spent more time to find buried food pellets and spent less time to explore new scents compared to control, suggesting an

impairment of olfactory function (Fleming et al., 2008). Another transgenic mouse of conditional a-syn overexpression under a tetracycline-controlled transactivator driven by CaMK-promoter showed a massive accumulation of a-syn in the OB and a decrease of neurogenesis in the OB (Marxreiter et al., 2009). Olfactory deficits were observed in two mouse models with a depletion of DA D_2 receptors and the vesicular monoamine transporter 2 (VMAT2), respectively (Taylor et al., 2009; Tillerson et al., 2006). In a knockout mouse model of Class VI alcohol dehydrogenase (Adh4) reduced smell ability was accompanied by alterations of DA metabolites in the OB (Belin et al., 2011). An intravenous administration of MPTP showed no impairment of olfactory function (Doty et al., 1992a), an intranasal infusion of low dose MPTP decreased the number of DA neurons and reduced the DA level in the SN and OB (Prediger et al., 2009). A number of reports showed consistent histological findings that the number of DA neurons in the PGL of the OB was increased in toxin-induced animal models (Belzunegui et al., 2007; O'Keeffe et al., 2009b; Winner et al., 2006; Yamada et al., 2004), as found in the human OB (Huisman et al., 2004). An upregulation to overcome DA deficiency in toxin-induced animal models and PD patients would be a reasonable theory to explain the pathological condition.

2 Adult neurogenesis

The subventricular zone (SVZ) in the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) supply neural progenitor cells (NPC) throughout the entire life of mammals. The OB is one of the few organs to receive newborn cells. NPC are renewing and replacing old cells in the adult OB. They migrate from the SVZ to the OB via rostral migratory stream (RMS). Despite the fact that adult-born neurons derived from NPC in the OB were described in a number of studies recently (Doetsch et al., 1999; Gage, 2000; Imayoshi et al., 2008), the impact on olfactory function is however poorly understood.

2.1 History of adult neurogenesis

Until the 1960s a widely accepted opinion was, that everything was fixed, and immutable in the central nervous system. Once neurons die, they cannot be regenerated. This concept was from Santiago Ramón y Cajal published in 1913. In the 1960s, Joseph Altman and colleagues used ^{3}H-thymidine to label cells in the rodent brain and found a generation of newborn cells in the OB and hippocampus (Altman, 1969; Altman and Das, 1965). It was the first time that neurogenesis in the adult brain was proposed. However, Altman only showed morphological evidence of labelled cells. Kaplan and his colleagues confirmed that these labelled cells were newborn neurons by electron microscopy (Kaplan and Hinds, 1977). Nottebohm and coworkers further proved functional aspects on newborn neurons of the songbird learning new songs in spring. This observation suggested that neurogenesis occurs in the adult brain and that these newborn neurons replaced dying neurons (Nottebohm, 2004). In the past 40 years, adult-born neurons were studied and identified by high resolution image analysis, functional electrophysiology, genetic application of green fluorescence protein (GFP) and co-expression of other cellular makers. Until today, it is well accepted that NPC reside mainly in the SVZ of the lateral ventricle and the SGZ of the dentate gyrus in adult mammals, also including humans (Suh et al., 2009). In neurogenic niches of the central nervous system, proliferating cells migrate to a given destination to further differentiate into neurons and glia.

2.2 Neurogenesis in the adult brain

In the SVZ-RMS-OB system, NPC reside and proliferate in the SVZ (**Fig. 4**). They migrate en route to the OB tangentially where they differentiate into different interneurons passing

through the RMS. In the GCL of the OB, most NPC differentiate into GABAergic interneurons; whereas a small proportion of NPC become distinct interneurons in the PGL (**Fig. 4**) (Gage, 2000; Ming et al., 2011). The ablation of dividing cells by administration of anti-mitotic agents or genetic manipulation confirmed the relationship of two populations of different cell subtypes (Doetsch et al., 1999; Suh et al., 2009). Radial and non-radial morphology separates them as B cells and C cells (the transient state of A cells that migrate towards the OB) in the SVZ, respectively (**Fig. 4.**). The B cells have unique radial processes and a ramified structure at the end. The B cells also expresses GFAP, Nestin, Blbp and Sox2. The C cells have no radial processes and express Nestin and Sox2. It was shown that C cells in the SVZ were negative for GFAP, but positive for Dlx2, Mash1 and EGFR (Suh et al., 2009).

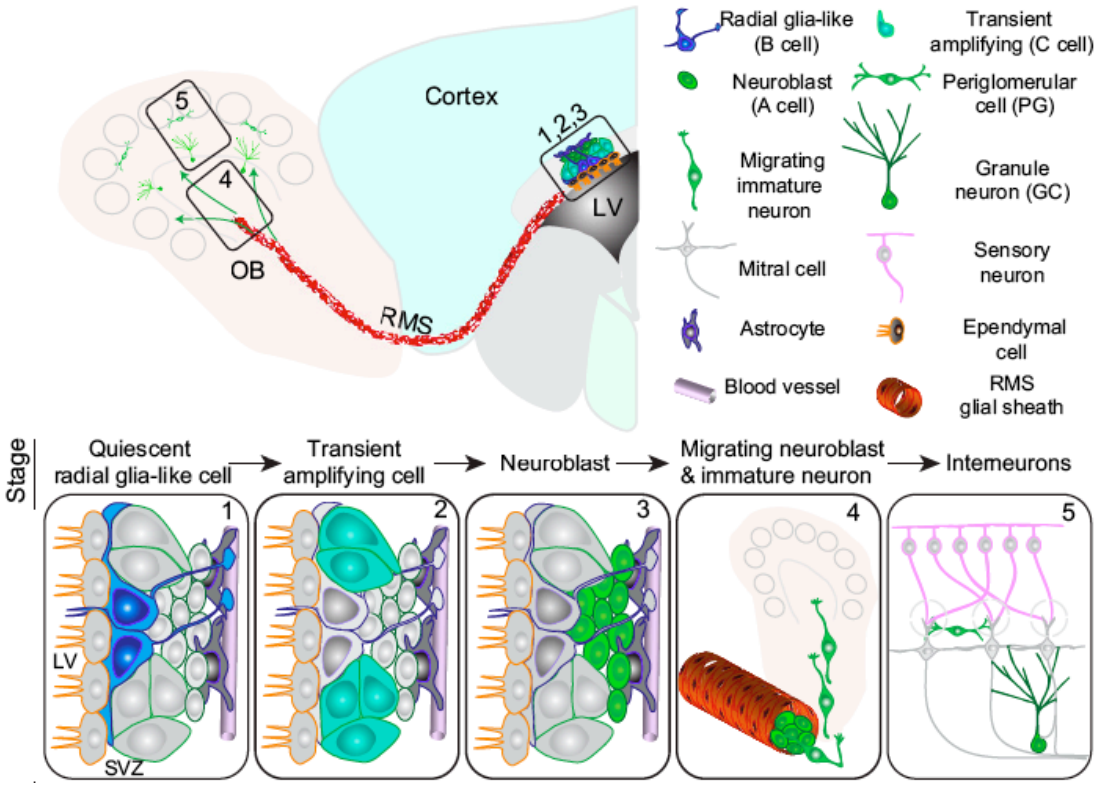


Fig. 4 The adult neurogenesis of the SVZ-RMS-OB. (modified from Ming. et al. 2011.)

2.3 Regulation of adult neurogenesis

The regulation of adult neurogenesis indicates a number of factors that directly or indirectly determine the unidirectional process of NSC, which consists of cell proliferation, survival, phenotypic differentiation and cell death (Kempermann, 2011). The regulatory factors are numerous and heterogeneous. These complex factors are related to the progeny of NSC which raises two hypotheses regarding their characteristics: (1) NSC are homogenous but can be regulated by environmental or extrinsic factors, (2) NPC are heterogeneous with their potency intrinsically specified (Suh et al., 2009). Eventually, cell-fate decision may have an impact on functional behavior.

2.3.1 Dopaminergic control

Dopamine is secreted by DA neurons in the SN, ventral tegmental area (VTA) and hypothalamus. DA is an effective neurotransmitter governing the proliferation of NPC in the SVZ (Freundlieb et al., 2006). DA receptors are expressed in the NPC of the SVZ (Young et

al., 2011). Moreover, the DAergic projections from SN and VTA regulate the survival and the proliferation of NPC (Freundlieb et al., 2006). D1- and D2-like receptors are located on NPC. In animal models of PD, a downregulation of the proliferation in the SVZ after dopamine depletion was shown (Baker et al., 2004; Höglinger et al., 2004; Winner et al., 2009; Winner et al., 2006). Also, a species related regulation was reported for D3 receptors (Baker et al., 2005). The administration of L-DOPA and DA agonists reversed the downregulation caused by DA depletion in the nigrostriatal pathway (O'Keeffe et al., 2009a; O'Keeffe et al., 2009b). An impaired proliferation resulted in a reduced number of NPC and this reduction was reversible by exogenous DA via the activation of EGFR receptors (Höglinger et al., 2004; O'Keeffe et al., 2009b). The proliferation, migration, apoptosis, integration and differentiation of NPC is modified by DA in the SVZ-RMS-OB system.

2.3.2 Intrinsic transcriptional factors

How intrinsic regulators, such as transcriptional factors (TF) involved in the regulation of cell fate, determine adult-born neurons to become dopaminergic is still poorly understood. Pax6 and Dlx2 are recently proposed as determinant TF for the specification and differentiation of NPC towards dopamine neurons in the PGL (Brill et al., 2008; Hack et al., 2005), and an upregulation of Pax6 was shown in the SVZ following dopamine depletion (Winner et al., 2006). Meis2 and Er81 are also expressed in a subtype of dopamine neurons (Allen et al., 2007). The internal fate of a newborn neuron is decided in advance even before they reach the PGL (Baker et al., 2001). Therefore, a single TF responding to environmental cues might interact with other TF when a physiological or pathological alteration is engaged. There is increasing evidence that TF might play an important role determining cell fate of NPC and phenotypic differentiation in the OB.

2.4 Adult neurogenesis and olfactory function

A potential function of newborn neurons in the OB is the maintenance of olfactory circuitries, sharping sensory perception, and supporting olfactory memory and learning (Lazarini and Lledo, 2011). The methods used to challenge newborn neurons are genetic manipulation, anti-mitotic agents and irradiation. However, variant results are reported in distinct experimental paradigms and different behavioral approaches. On one hand, there has never been shown a direct role of adult neurogenesis in odor discrimination (Breton-Provencher et al., 2009; Imayoshi et al., 2008; Lazarini et al., 2009); on the other hand, newborn neurons seem to be

necessary to perform olfactory learning tests, for example, non-associated (animals learn about the relationship between two different stimuli) and associated odor discrimination (animals learn about the relationship between a stimulus and animals' own behavior) (Lazarini et al., 2009; Mouret et al., 2009; Rochefort et al., 2002; Sakamoto et al., 2011). Once NPC are selected to survive by learning, newborn neurons could change the physiology of the OB network. Furthermore, newborn neurons might retain a memory trace of the previous olfactory experience during an associative learning task (Lazarini and Lledo, 2011). In different olfactory learning tasks, it was shown that non-operant associative olfactory learning (pairing of a neutral stimulus with a reward) is independent of newborn neurons in the OB. In contrast, operant associative olfactory learning (acquisition of a specific behavior for getting the reward) increased the survival of newborn neurons and maintained the long-term memory (Lazarini and Lledo, 2011; Mandairon et al., 2011). Nevertheless, the relationship between the degree of adult neurogenesis and the complexity of discrimination learning tasks is not yet clearly identified.

II. Aim of the study

In this project, we hypothesized that NPC can be modulated by direct (L-DOPA) or indirect (Selegiline) dopaminergic stimulation, in an unilateral 6-OHDA mouse model of PD, which might provide a possible explanation of olfactory dysfunction frequently observed in PD patients.

The goal of the study was to understand the modulation of cell survival and differentiation in the OB after anti-parkinsonian treatment with L-DOPA and/or Selegiline. In the OB, the cell fate of NPC might determine the generation of dopamine interneurons in the PGL after medication. Furthermore, we generated different animal models by 6-OHDA lesion and/or olfactory bulbectomy to further analyze the correlation of olfactory discrimination and dopamine depletion. We also examined if chronic Selegiline treatment can reverse hyposmia in a mouse model of PD.

III Materials and methods

1 Materials

1.1 Chemicals

2% Rompun	Bayer, Leverkusen, Germany
2M HCL	Roth, Karlsruhe, Germany
30% H_2O_2	Roth, Karlsruhe, Germany
3,3´-diaminobenzidine (DAB)	Serva, Heidelberg, Germany
6-hydroxydopamine (6-OHDA)	Sigma, Steinheim, Germany
ApopTag	Millipore, Temecula, USA
(Apoptosis *In Situ* Detection Kit)	
Ascorbic acid	Merck, Darmstadt, Germany
Avidin-biotin-peroxidase	Vector laboratories, Burlingame, USA
Boric acid	Sigma, Steinheim, Germany
BrdU	Sigma, Steinheim, Germany
Chromium (III) potassium sulfate	Roth, Karlsruhe, Germany
Desipramine	Sigma, Steinheim, Germany
Ethyl glycerol	Merck, Darmstadt, Germany
Formamide	Sigma, Steinheim, Germany
Gerlatin	Merck, Darmstadt, Germany
Glycerol	Acros Organics, NJ, USA
Isopropanol	Roth, Karlsruhe, Germany
Isofluran	Baxter GmbH, Unterschleißheim, Germany
Ketamin	Intervet, Neumünster, Germany
L-DOPA (Madopar LT)	Roche, Grenzach-Wyhlen, Germany
Normal goat serum	Vector Laboratories, Burlingame, USA
Normal donkey serum	Millipore, Billerica, USA
Paraformaldehyde	Roth, Karlsruhe, Germany
Selegiline (R-(-)-l-deprenyl)	Sigma, Steinheim, Germany
Sodium citrate	Sigma, Steinheim, Germany
TUNEL dilution buffer	Roche Diagnostics, Indianapolis, USA

1.2 Expandable materials

0.2 µm filter	Semadeni, Ostermundigen, Switzerland
Bepanthen	Bayer, Leverkusen, Germany
Betaisodona salbe	Mundi-Pharma GmbH, Limburg, Germany
CryoPure tubes	Sarstedt, Nümbrecht, Germany
Gloves (size M, powder free)	NOBA GmbH, Wetter, Germany
Polyvinyl alcohol mounting medium	Sigma, Steinheim, Germany
Needle (24G and 27G)	BD, Drogehede, Ireland
Microsyringe, stainless needle, 33G	WPI, Sarasota, USA
Microscope slides	Menzel-Gläser GmbH, Braunschweig, Germany
Mounting gel	Corbit-Balsam, Kiel, Germany
Cover slips	Menzel-Gläser GmbH, Braunschweig, Germany
Pipette tips (10µl, 100µl and 1000µl)	Eppendorf AG, Hamburg, Germany
Syringe (1ml, 5ml and 10ml)	B/Braun, Melsungen, Germany
Tissue freezing medium	Leica, Nussloch, Germany
Wooden stick	OBI, Marburg, Germany

1.3 Buffer and solutions

0.2M Phosphate buffer	230 g $Na_2HPO_4 \bullet 2H_2O$
	52.44 g $NaH_2PO_4 \bullet 2H_2O$
	10 L distilled water
0.1M Phosphate buffer saline	9 g NaCl
	1 L 0.1M Phosphate buffer
30 % Sucrose	15 g Sucrose
	0.1 M PB 500 ml
8 % Paraformaldehyde (PFA)	20 g PFA
	241.66 ml distilled water at 60 °C
Anti-freezer solution	5.18 g $Na_2HPO_4 \bullet 2H_2O$
	1.57 g $NaH_2PO_4 \bullet 2H_2O$
	300 ml Ethylglycerol
	300 ml glycerol
	400 ml distilled water
SSC buffer	0.3 M NaCl

	0.03 M Sodium citrate
Borate buffer (pH=8.5)	15.45 g Boric acid
	450 ml distilled water
	5 ml 10 N NaOH
Gelatin Buffer	13 g Gelatin
	0.18 g Chromium (III) potassium sulphate
	300 ml distilled water

1.4 Antibodies

Primary antibodies

List of Antibodies	Marker	Dilution	Source
Rabbit anti-TH	DA Neuron	1:1000	Thermo Scientific, Rockford, USA
Rat anti-BrdU	Base Analogue	1:500	Serotec,Düsseldorf,Germany
Mouse anti-NeuN	Neuron	1:1000	Millipore, Billerica, USA
Mouse anti-PCNA	Proliferation	1:500	Dako, Glostrup, Denmark
Mouse anti-digoxingennin	Apoptosis	1:500	Abcam, Cambridge, UK

Secondary antibodies

List of Antibodies	Dilution	Source
Biotin-SP-conjugated donkey anti-rabbit	1:1000	Jackson ImmunoResearch Laboratories Inc., West Grove, USA
Biotin-SP-conjugated donkey anti-mouse	1:1000	Jackson ImmunoResearch Laboratories Inc., West Grove, USA
Biotin-SP-conjugated donkey anti-rat	1:1000	Jackson ImmunoResearch Laboratories Inc., West Grove, USA
Cy™3-conjugated goat anti-mouse	1:1000	Jackson ImmunoResearch Laboratories Inc., West Grove, USA
CY™5-conjugated donkey anti-rabbit	1:1000	Jackson ImmunoResearch Laboratories Inc., West Grove, USA
Alexa 488 goat anti-rat	1:1000	Invitrogene™, Eugene, USA

1.5 Experimental animals

A total number of 80 male C57BL/6 mice (Charles River, Sulzfeld, Germany), 10 weeks old in the beginning of the experiment, were used. The animals were housed in standard cages with *ad libitum* access to food and water at 23ºC with a 12:12 h light/dark cycle. All

experiment procedures were approved by the appropriate institutional governmental agency (Regierungspräsidium, Giessen, Germany).

1.6 Software and equipment

Cryostat microtome	Leica CM3050 S, Nussloch, Germany
Digital camera	Olympus E-330, Japan
Digital Luxmeter	Voltcraft, MS-1500, Taiwan
Electric Driller	Proxxon, Nierbach, Germany
Halogen light (300 longlife)	Schölly Fiberoptic GmbH, Germany
HPLC BAS Liquid Chromatography	Bioanalytical system, West Lafayette, USA
HPLC computer system	Chromeleon™, Dionex, Germany
Image J	http://rsbweb.nih.gov/ij/
Light microscope	Nikon Microphot-FX, Tokyo, Japan
Laser confocal Microscope	Zeiss Axiovert 200M, Jena, Germany
Pipetus	Hischmann Laborgeräte, Eberstadt, Germany
Pump controller	Micro4™, WPI, Sarasota, USA
Prism 5.0c	GraphPad Software, San Diego, USA
Stereotaxic frame	Kopf Instruments, Tujunga, CA
StereoInvastigator software	v8, MicroBrightField, Magdeburg, Germany
Thermometer	neoLab, Heidelberg, Germany
Ultra Basic pH meter	Denver Instrument, Göttingen, Germany
Ultra turrax	IKA, Bachofer, Reutlingen, Germany
Viewer II video system	Viewer II, Bioserver, Bonn, Germany
Water bath (GFL 1083)	Kobe, Marburg, Germany

2 Methods

2.1 Histological study

Experimental design

In a first set of experiments, 9 animals were perfused at three weeks post an unilateral 6-OHDA injection in order to verify a decreased proliferation of NPC in the SVZ following dopamine depletion (Höglinger et al., 2004). Then, the following 5 groups of animals were established including: I. Normal control animals without treatment (n=11); II. 6-OHDA-lesioned animals without treatment (n=11); III. 6-OHDA-lesioned animals treated with L-DOPA (n=11); IV. 6-OHDA-lesioned animals treated with Selegiline (n=12); and V. 6-OHDA-lesioned animals treated with a combination of L-DOPA and Selegiline (n=11). On day 19 to 21 following the lesion, 5-bromo-2´deoxyuridine (BrdU), was injected intraperitoneally (i.p.) every 12 hours (6 injections), before drug treatment was initiated for 4 weeks. At the end of the treatment (7 weeks post 6-OHDA lesion), 4 animals of each group were taken for measurement of the dopamine concentration in the OB by high performance liquid chromatography (HPLC), while the others were perfused with 4% paraformaldehyde (PFA) for histological analysis (**Fig. 5**).

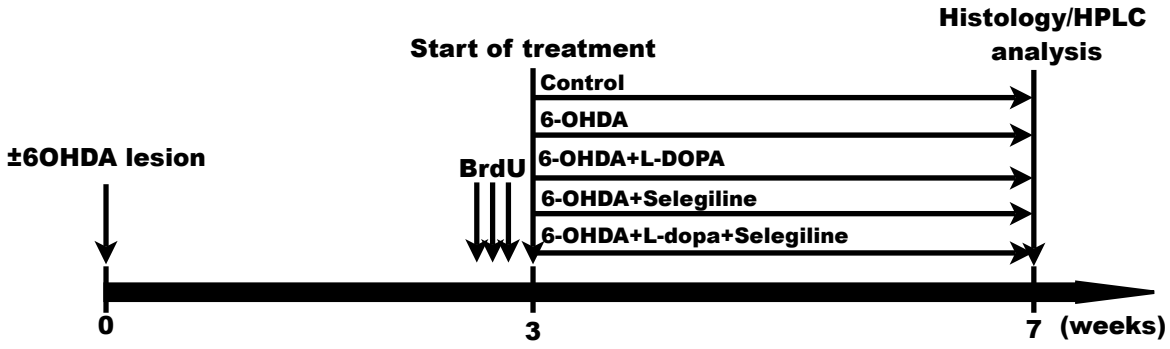


Fig. 5. Experimental timeline

Unilateral 6-OHDA lesion

Mice were deeply anesthetized by a mixture of ketamin (80 mg/kg) and 2% xylazin (4 mg/kg diluted in 0.9% NaCl), and placed in a stereotaxic frame. The noradrenergic system was protected from 6-OHDA toxicity by an i.p. injection of desipramine (25 mg/kg dissolved in 0.9% NaCl) 20 minutes before surgery. A total amount of 4 µg 6-OHDA, dissolved in 2 µl 0.9% NaCl/0.02% ascorbic acid was injected into the SN over 10 min (flow rate of 200 nl/min) by pump. After the injection, the needle stayed in the brain for 5 more minutes before it was slowly retracted. All injections were performed using a microsyringe, stainless needle (33G). The coordinates of the injection were antero-posterior: +3.1 mm, medio-lateral: +1.2 mm, and dorso-ventral: -4.2 mm, relative to bregma using a flat skull position.

BrdU labeling

To label proliferating NPC, mice received an i.p. injection of BrdU (50mg/kg dissolved in 0.9% NaCl; Sigma). Animals received an injection twice a day for 3 consecutive days just before the start of drug treatment.

Pharmacological treatment

L-DOPA (1 mg/ml), and Selegiline (R-(-)-l-deprenyl) (0.1 mg/ml) were both administered via drinking water over 4 weeks. Drinking water was renewed every second day and the bottles were covered by aluminium foil to avoid rapid oxidation of L-DOPA. In order to achieve a sufficient intake of the drugs, all groups were water deprived for 12 hours every day (from 6 pm to 6 am), and allowed to drink during the following 12 hours. Pilot studies showed that animals (n=5) drank approx. 3 ml of water (3.2 ± 0.1 ml) per day using this 12 hours of water deprivation protocol (Depboylu et al. 2013).

Histology

Mice were perfused transcardially with 0.1M PBS solution followed by 4% PFA in 0.1M PB (pH 7.4) using a pump at a rate of 15 ml/min. The brain was carefully removed and postfixed in 4% PFA, and transferred in 30% sucrose and stored at 4°C. Coronal sections were cut at 30µm in 10 series using a cryostat microtom at -20°C, the sections were stored at -20ºC in

cryosolution (1:1:3 volume ratio of ethyl glycerol, glycerol and 0.1M PB buffer) until further processing.

Immunohistochemistry

Free-floating sections were washed three times in 0.1M PB to remove the cryoprotectant, thereafter quenched with 3% H_2O_2 and 10% methanol dissolved in 0.1M PB buffer. The sections were then preincubated in 5% normal donkey serum and 0.3% Triton X-100 in 0.1M PB buffer for 30 min, and incubated over night at 4°C with primary antibodies (rabbit anti-TH, 1:1000; rat anti-BrdU, 1:500; mouse anti-NeuN, 1:1000; mouse anti-PCNA, 1:500; and mouse anti-Digoxigenin, 1:500). On the second day, sections were incubated in biotinylated species-specific secondary antibodies (donkey anti-rabbit/-rat/-mouse, 1:1000) or specific fluorochrome secondary antibodies (Cy3-conjugated goat anti-mouse; Cy5-conjugated donkey anti-rabbit, 1:1000) for one and two hours at room temperature, respectively. The sections treated with biotinylated antibodies were further incubated for one hour in avidin-biotin-peroxidase solution. The protein was finally visualized by a 0.1M PB solution containing 5% 3,3´-diaminobenzidine (DAB) and 0.02% H_2O_2. The DAB stained sections were mounted on gelatin-coated glass slides, dried, and coverslipped using mounting gel. For fluorescence staining the sections were rinsed, mounted on gelatin-covered glass slides and directly coverslipped by polyvinyl alcohol mounting medium.

Detection of BrdU-labeled nuclei

Before incubation with the primary antibody, sections were incubated at 65ºC with a mixture of formamide and SSC buffer (1:1 by volume; 0.3M NaCl plus 0.03M sodium citrate) for 2 hours, followed by 10 min incubation in SSC alone. Then, sections were incubated with 2M HCl, at 37ºC for 30 min. In order to neutralize the pH, sections were incubated 4 x 5 min with borate buffer (0.1M boric acid, pH=8.5).

TUNEL staining

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was used to detect apoptotic cells by enzymatically labeling 3'-OH termini of broken DNA in apoptotic cells. In Situ Detection Kit and modified protocols described previously were used for TUNEL labelling (Kuhn et al., 2005). Briefly, free-floating sections were first rinsed 3 x 10 min in 0.1M PB to

remove the cryoprotectant. They were then incubated in ascending and descending isopropanol solutions. After rinsing in 0.1M PB, the sections were incubated with TUNEL dilution buffer for 2 min, followed by a one-hour incubation at 37°C with terminal deoxynucleotidyl transferase (TdT, 1:3 dilution with TUNEL buffer, in order to reduce the background). Sections were finally incubated using "stop buffer" for 10 min at room temperature and rinsed in 0.1M PB solution.

DA concentration measurement

To assess dopamine concentration in the OB, animals were sacrificed by rapid cervical dislocation one hour after the last drug treatment. The OB was removed from the skull, and dissected on an ice-cold petri dish. The tissue was homogenized in 300 µl 0.4N perchloric acid, and centrifuged at 20000 rpm for 1 min using ultra turrax. Following a 13000 g centrifugation for 20 min at 4°C, the supernatant was passed through a 0.2 µm filter. The dopamine concentration was finally measured in the final volume by HPLC with an electrochemical electrode. The data were analyzed by a HPLC computer system.

Morphological analysis

Striatal TH+ fiber density measurements

In order to determine the extent of dopamine denervation following the 6-OHDA lesion, mean optical density was measured in the striatum. Briefly, images were captured using a digital camera (Olympus E-330) from four selected planes: 0.74 mm, 0.62 mm, 0.5 mm and 0.14 mm, relative to bregma, and analysed using Image J software version 1.43r for Mac OS X platform (http://rsbweb.nih.gov/ij/). The striatum was outlined as previously described (Carlsson et al., 2011b).

Determination of the number of TH+, PCNA+ and BrdU+ cells in the SN, SVZ and OB by stereologic analysis

TH+, PCNA+ and BrdU+ cells were counted in the SN, SVZ and OB using StereoInvastigator software at 40x magnification. A fractionator probe was established for each section. Stereologic analysis was performed under blinded condition on coded slides. The criterion for counting an individual immunoreactive cell was the presence of its nucleus either within the counting frame or touching the upper and/or right frame lines (green), but not touching the lower and/or left frame lines (red). The total number of TH+ profiles in the SN and the number of PCNA+ profiles in the SVZ, respectively, was then determined by the StereoInvestigator program. The entire SN was delineated as previously described (Ries et al., 2006). The SVZ was included in the rostral-to-caudal axis from 1.0 mm to 0.1 mm relative to bregma. In order to assess the number of BrdU+ cells in the GCL and PGL and TH+ cells in the PGL of the OB, 6 coronal sections were used corresponding to 5.18 mm, 4.88 mm, 4.58 mm, 4.28 mm, 3.98 mm and 3.68 mm relative to bregma. The outline of the GCL is shown in

Fig. 10A, corresponding to the region inside the white dotted line, including the internal plexiform layer, but not the external plexiform layer and the mitral/tuft cell layer. The outline of the PGL is shown in **Fig. 11A**, as the region between the white dotted lines, corresponding to the area between the external plexiform layer and olfactory nerve layer. In order to estimate the number of TUNEL+ nuclei in the OB, 6 sections were used corresponding to 4.88 mm, 4.58 mm, 4.28 mm, 3.98 mm and 3.68 mm and 3.38 mm relative to bregma. Here, the total number of TUNEL+ cells in the OB was counted.

Co-localization of TH-, BrdU- and NeuN-positive cells in the OB

In order to evaluate the frequency of neuronal and dopaminergic differentiation in the OB, 50 BrdU+ cells of the GCL and PGL, respectively, were randomly selected from the same six sections as described above. These were analyzed for co-localization with either NeuN (neuronal differentiation) or TH (dopaminergic differentiation) using Laser confocal microscopy at 63x and 1 airy pinhole size. BrdU/NeuN double-labelled cells were evaluated in both the PGL and GCL, while BrdU/TH labelled cells were only evaluated in the PGL. Finally, the number of newly generated neurons and dopaminergic cells in the GCL and PGL were calculated as number of BrdU+ cells multiplied by the differentiation frequency.

Statistics

Multiple comparisons among groups were performed by one-way ANOVA followed by Tukey's post hoc analysis. The difference between two groups was analyzed by t-test. All data are presented as mean ± SEM. Significances were considered at $p < 0.05$ for all tests.

2.2 Behavioral study

Surgery

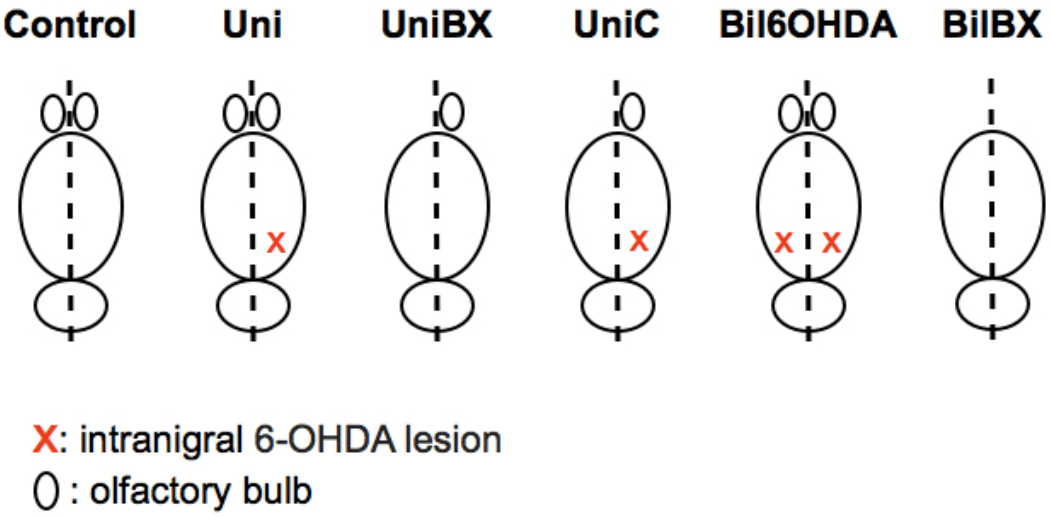
Bulbectomy

Male, 10 weeks old C57BL/6 mice were used for unilateral or bilateral bulbectomy. The mice were deeply anesthetized by a mixture of ketamin and 2 % xylazin, as described before. The head was placed in a stereotaxic frame. The skull above the OB was carefully removed. Then, the OB was carefully removed by surgical instruments either on one side (Left side) or bilaterally.

Experimental design

In total 6 different experimental groups of animals were used in the study: Control, unilateral 6-OHDA injection (Uni), unilateral bulbectomy (UniBX), unilateral 6-OHDA injection plus contralateral bulbectomy (UniC), bilateral 6-OHDA injection (Bil6OHDA) and bilateral bulbectomy (BilBX) (**Fig. 6A**). The behavioral tests started at 3 weeks post surgery. The tests included novel open field (NOF) assessment and olfactory discrimination test (ODT), and were performed at 3 weeks and 12 weeks post op, after 12 weeks of Selegiline treatment and 4 weeks after Selegiline withdrawal (**Fig. 6B**).

A.



B.

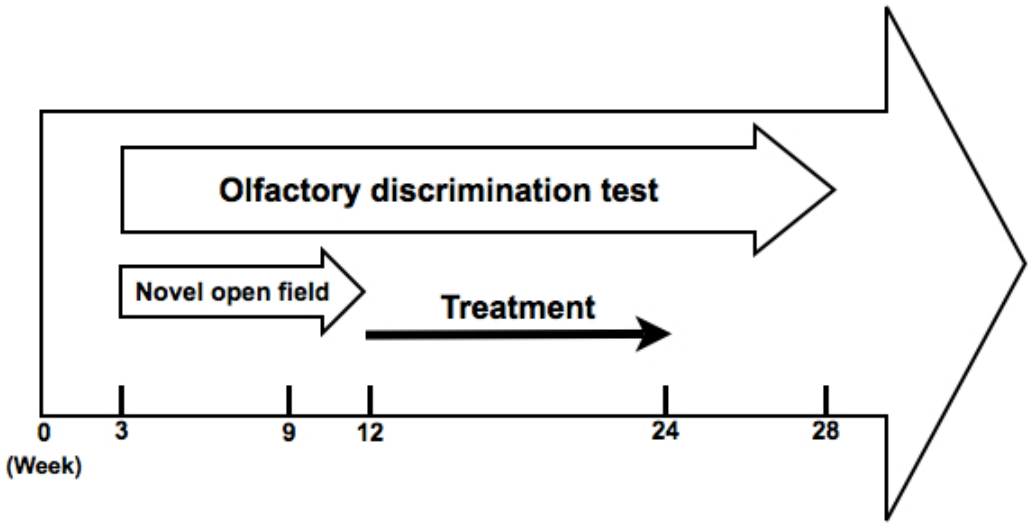


Fig. 6. **(A)** Guideline of six different models. **(B)** Timeline of the behavioral test. X-axis indicates the number of weeks. The black arrow shows Selegiline treatment from week 12 to week 24.

Behavior Test

Novel Open Field (NOF)

The novel open field test was performed in a dark room at 21 ± 1.0 °C. The size of each arena was 50 x 50 x 40 cm under a light intensity of 2.2-2.5 Lux. In order to allow animals to habituate with the room, they were brought and stayed in the room 20 min before the test. In every test trial, 8 animals randomly selected were tested for 30 mins. In between every trial, the arena was cleaned by 70 % ethanol to remove any scents. A Viewer II video system and software program were used to record the behavior of the animals in arena. The center of the area (36.5 cm x 36.5 cm) was delineated in the program. The parameters used were the tracklength and the time spent in the central area of the arena.

Olfactory Discrimination Test (ODT)

The test was described before with slightly modifications (Fleming et al., 2008). Briefly, wooden blocks (2.5 cm x 2.5 cm x 2.5 cm) were incubated in animal cages for 5 days before the test. During this time period, the bedding of the animal cages was not renewed in order to make wooden blocks carrying the animal's scent. On the day of the test, the wooden blocks were removed and put into a clean plastic bag sealed and labeled with corresponding animal number. Each animal was tested to discriminate the own odor from novel odors (male odor of a different colony or female odor) in the open field. To avoid any disturbances during the test wooden blocks were set up in the open field before the start. Then, animals were placed in the other half of the arena covered by a small lid **(Fig. 7)**. Each animal had to perform a total of 6 trials (5 min per trial) and 5 min intervals. In the first trial, two blocks carrying own odor were placed in the arena for habituation, and the animal was released by opening the lid from the other side to explore the entire arena **(Fig. 7)**. In trial 2 to 5, animals explored a block carrying their own odor and one carrying a novel male's odor. In the 6th trial, a female's odor was replaced by the block carrying the other male's odor. All wooden blocks with novel odors were randomly selected from sealed plastic bags. Time spent on both wooden blocks were recorded and further analyzed.

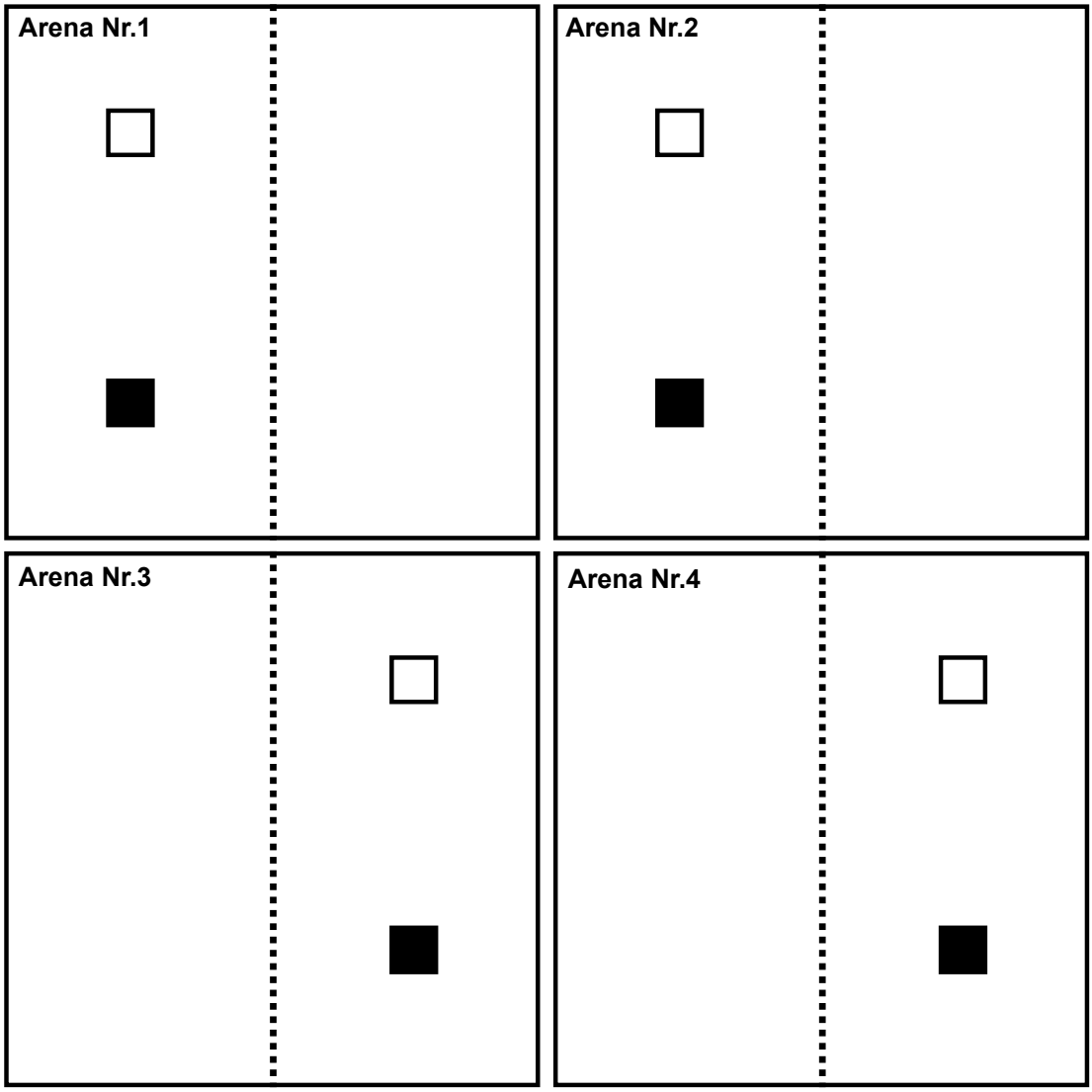


Fig. 7. Position of the wooden blocks in the novel open field. Dashed lines represent the midline of the arena. Open box: control block (own odor); Closed box: experimental block (novel male or female odor)

IV Results

1 Histological study

1.1 Effect of L-DOPA and Selegiline treatment on the nigrostriatal dopaminergic system following 6-OHDA lesion

At 7 weeks after a unilateral 6-OHDA injection into the SN, a histological analysis of the extent of the lesion was performed by TH-immunohistochemistry in all animals. TH-staining revealed an 88% loss of dopamine neurons in the SN on the lesioned side of non-treated mice (compared to the intact side). Similarly, a significant reduction of TH+ fibers was observed in the striatum of these animals on the 6-OHDA injected side (30.1 ± 7.4% of intact side) as measured by optical density.

L-DOPA (1 mg/ml) and/or Selegiline (0.1 mg/ml) were given orally via drinking water for 4 weeks, starting at 3 weeks post lesion. Neither L-DOPA nor Selegiline treatment resulted in a significant change of the number of TH+ neurons in the SN as compared to non-treated animals. Also TH+ fiber density in the striatum of the lesioned side did not show a significant difference compared to non-treated animals. There was also no effect on the number of TH+ neurons in the SN or TH+ fiber density in the striatum when the two drugs were combined (**Fig. 8**).

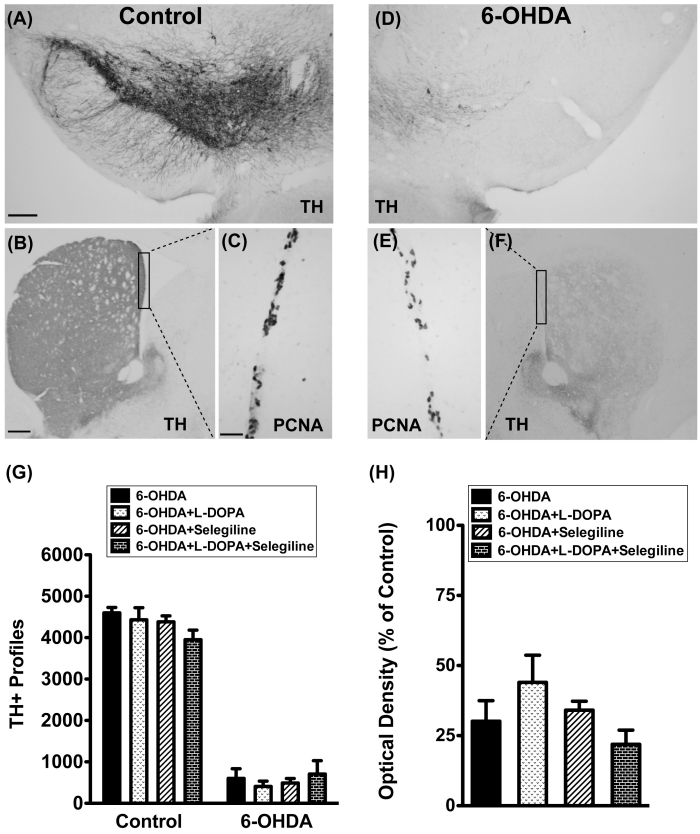


Fig. 8. TH and PCNA immunoreactivity in the nigrostriatal system and SVZ. At 7 weeks post 6-OHDA injection, there was a significant loss of TH+ cells (One-way ANOVA followed by Tukey`s post-hoc test, n = 6-8, $p < 0.001$) and fibers on the lesioned side (**D, F, G, H**), as compared to the intact side (**A, B, G, H**). A loss of PNCA immunoreactivity was observed in the SVZ on the lesioned side, as compared to the intact side (**C, E**). Scalebar in A and B represents 500 μm and applies to A, B, D, F; scalebar in C represents 50 μm and applies to C, E.

1.2 Effect of L-DOPA and Selegiline on NPC proliferation in the SVZ

Evaluating proliferation of NPC in the SVZ, a significant decrease of about 25% (3623 ± 178 PCNA+ cells) was shown on the lesioned side compared to the intact side (4736 ± 229) 3 weeks after 6-OHDA injection. Similar to the result at 3 weeks, at 7 weeks post 6-OHDA lesion the proliferation of NPC in the SVZ showed a significant decrease to 76.2 ± 3.5% of the intact side (**Fig. 9A, B, F)**. Following four weeks of L-DOPA and/or Selegiline treatment, the number of PCNA+ cells in the SVZ was no longer significantly reduced in the treatment groups (L-DOPA: 93.8 ± 3.4% of intact side; Selegiline: 92.1 ± 4.3%; L-DOPA + Selegiline: 83.9 ± 3.8%) as compared to non-lesioned control animals (103 ± 7.5%; **Fig. 9A, C-F**).

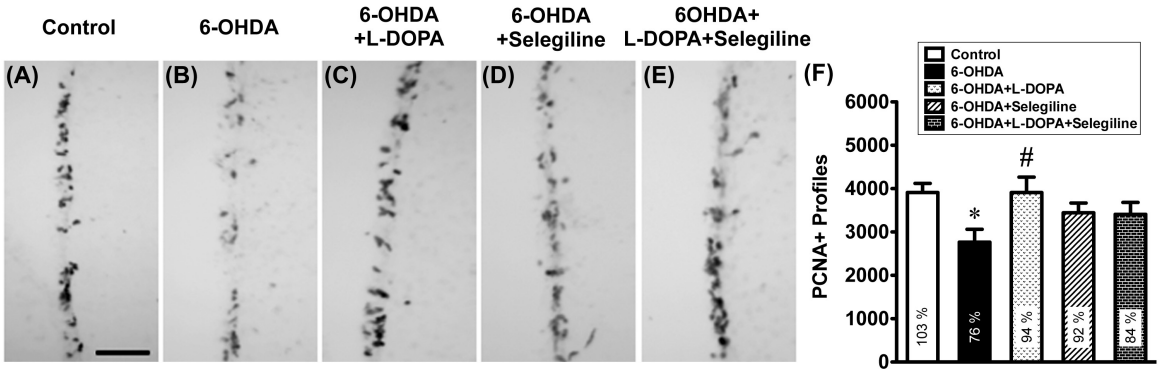


Fig. 9. PCNA expression in the SVZ following 4 weeks of L-DOPA and/or Selegiline treatment in 6-OHDA lesioned mice. The 6-OHDA lesion caused a significant decrease in PCNA+ cells compared to controls at 7 weeks after 6-OHDA (**A, B, F**). Following administration of L-DOPA and/or Selegiline, the cell number was no longer significantly different from control (**C-F**). Number of cells on the lesioned side is represented in each column (in % of intact side). Scalebar in A represents 50 µm. (F: One-way ANOVA, n = 5-6, * $p < 0.05$, compared to control side; # $p < 0.05$, compared to 6-OHDA)

1.3 Effect of L-DOPA and Selegiline on dopamine level in the OB

Following 6-OHDA lesion, the dopamine content in the OB was decreased from 0.49 ± 0.03 ng/mg tissue in control animals to 0.13 ± 0.02 ng/mg (**Table 1.**). Treatment with L-DOPA as well as Selegiline, however, increased the level to normal (0.50 ± 0.08 ng/mg and 0.42 ± 0.07 ng/mg, respectively). When combining the two drugs, the dopamine level was significantly increased compared to non-lesioned controls (0.93 ± 0.18 ng/mg, *$p<0.05$;* **Table 1.**). These

HPLC data show that L-DOPA and Selegiline were effectively administered via drinking water.

Groups	**Dopamine (ng/mg tissue)**
Control	0.49 ± 0.03
6-OHDA	0.13 ± 0.02
6-OHDA + L-DOPA	0.50 ± 0.08
6-OHDA + Selegiline	0.42 ± 0.07
6-OHDA + L-DOPA + Selegiline	0.93 ± 0.18*

Table 1. Dopamine concentration in the OB following 6-OHDA lesion and subsequent L-DOPA and/or Selegiline treatment (One-way ANOVA followed by Tukey´s post-hoc test, n = 4-6 per group, * $p < 0.05$, compared to control).

1.4 Modulation of NPC in the GCL of the OB following 6-OHDA lesion and subsequent L-DOPA and Selegiline treatment

Following dopamine depletion, the number of NPC in the GCL of the OB, as identified by BrdU immunostaining, was significantly decreased from 36960 ± 1414 cells in the control group to 28099 ± 1184 cells in the 6-OHDA group. The administration of Selegiline further enhanced this effect (20693 ± 1010), while in the L-DOPA group there was no significant decrease (33095 ± 2651; **Fig. 10A-E, G**), as compared to control animals. The combination of the two drugs showed an intermediate effect with a BrdU+ cell number of 26856 ± 2332 (**Fig. 10F, G**).

In order to further investigate the neuronal differentiation of the NPC in the GCL, 50 BrdU+ cells per group were randomly selected and sequentially analyzed for the expression of the neuronal marker NeuN by confocal microscopy (**Fig. 10H**). The percentage of BrdU and NeuN co-localizing cells was not changed significantly in any of the groups, as compared to control (**Table 2**). The total number of BrdU+/NeuN+ cells, however, varied in relation to the changes in the number of BrdU+ cells (**Fig. 10I**).

	GCL	PGL	
Groups	**BrdU+ / NeuN+**	**BrdU+ / NeuN+**	**BrdU+ / TH+**
Control	95.4 ± 1.7	90.0 ± 2.7	16.9 ± 1.4
6-OHDA	94.3 ± 1.2	90.5 ± 2.5	40.1 ± 2.6*
6-OHDA + L-DOPA	95.6 ± 3.9	96.8 ± 1.4	47.4 ± 5.8*
6-OHDA + Selegiline	90.8 ± 5.1	97.6 ± 1.0	18.8 ± 2.5#
6-OHDA + L-DOPA + Selegiline	94.8 ± 1.0	87.6 ± 3.2	36.2 ± 2.3*

Table 2. Percentage of BrdU+/NeuN+ in GCL and PGL, and BrdU+/TH+ in PGL (One-way ANOVA followed by Tukey`s post-hoc test, * $p < 0.01$, compared to control; # $p < 0.0001$, compared to 6-OHDA).

The number of adult-born neurons in the group treated with L-DOPA (31367 ± 4482) was not significantly different from the control group (35753 ± 4312). In contrast, the number after Selegiline treatment (18817 ± 3371) was significantly lower than that of the lesioned group without treatments. In addition, the number showed no significant change after treatment with a combination of L-DOPA and Selegiline (23659 ± 3367) compared to the lesioned animals without treatment (**Fig. 10I**).

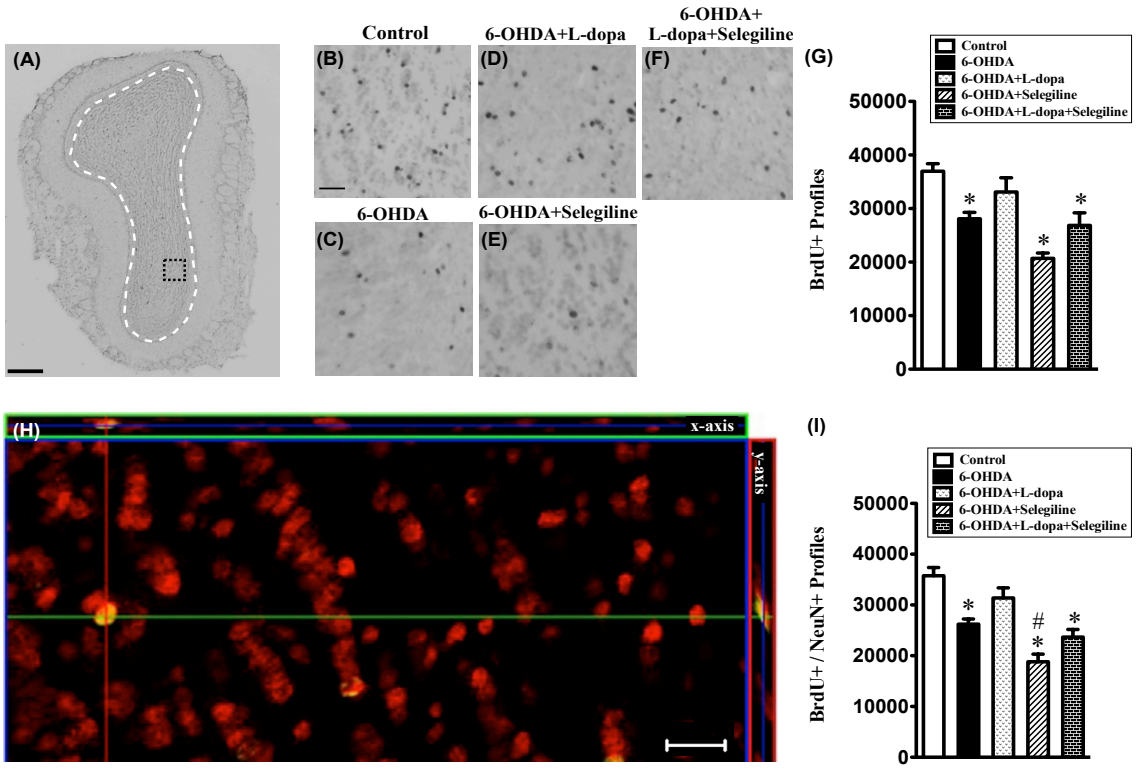


Fig. 10. Number of BrdU+ and BrdU+/NeuN+ cells in the GCL of the OB. There was a significant decrease in the number of BrdU+ NPC after 6-OHDA injection, as compared to controls (**A-C, G**). While Selegiline tended to further decrease the number of BrdU+ cells in the GCL, there was no significant decrease with L-DOPA (**D, E, G**). The number of co-labeled BrdU+/NeuN+ cells was counted in the GCL (**H, I**), showing a similar effect by the different treatment regimens with the difference between 6-OHDA and 6-OHDA + Selegeline reaching significance. Scalebars in A, B and H represent 500, 50 and 20 µm, respectively (G and I: One-way ANOVA followed by Tukey`s post-hoc test, n = 5-8, * $p < 0.01$, compared to controls; # $p < 0.05$, compared to 6-OHDA).

1.5 Modulation of NPC in the PGL of the OB following 6-OHDA lesion and subsequent L-DOPA and Selegiline treatment

The number of BrdU+ cells in the PGL of the OB was significantly reduced in the 6-OHDA lesioned animals (5024 ± 237) in comparison to controls (6522 ± 359). As in the GCL, this reduction in the PGL was restored by L-DOPA treatment for 4 weeks (6480 ± 488), while Selegiline further decreased the BrdU+ cell number in the PGL to 4347 ± 149 cells. The combination of L-DOPA and Selegiline increased the number of cells (5516 ± 188) compared to Selegiline only, but did not reach the effect of the L-DOPA treatment (**Fig. 11A-G*)***.

As in the GCL, we assessed the neuronal fate by BrdU-NeuN co-localization in the PGL (**Fig. 11H-I**). We again randomly selected 50 BrdU+ cells per group, and assessed the percentage of newborn NeuN+ neurons in control animals (**Table 2**). This percentage was not changed after a 6-OHDA lesion. Both L-DOPA and Selegiline treatment showed a trend to increase the percentage of newborn neurons, whereas the combination did not.

Accordingly, the absolute cell number showed a significant decrease of newborn neurons (BrdU+/NeuN+ cells) in 6-OHDA lesioned animals in relation to controls. Similarly, a reduction was observed in the Selegiline only group. In contrast, L-DOPA treatment normalized the number of newborn neurons (**Fig. 11I**).

In addition, we investigated the BrdU+ cells for their expression of TH (**Fig. 11H, J; Table 2)**. The percentage of TH+ cells was significantly higher following 6-OHDA, increasing from 16.9 ± 1.4% in controls to 40.1 ± 2.6% in the 6-OHDA lesioned animals (**Table 2)**. A significant increase was also seen in the group treated with either L-DOPA alone (47.4 ± 5.8%), or the combination of L-DOPA and Selegiline (36.2 ± 2.3%). In the animals treated with Selegiline alone the percentage of TH+ profiles equaled the level of control animals (18.8 ± 2.5%). The number of newborn TH+ cells (2015 ± 169 cells) showed a significant increase in the 6-OHDA lesioned animals compared to controls (1107 ± 91; **Fig. 11J**). This number was significantly increased further after L-DOPA treatment (3022 ± 332). The Selegiline only group, showed a number of TH+ neurons in the PGL not different from control (814 ± 110; **Fig. 11J**). Combining the two drugs resulted in a number compared to that after 6-OHDA (2010 ± 172).

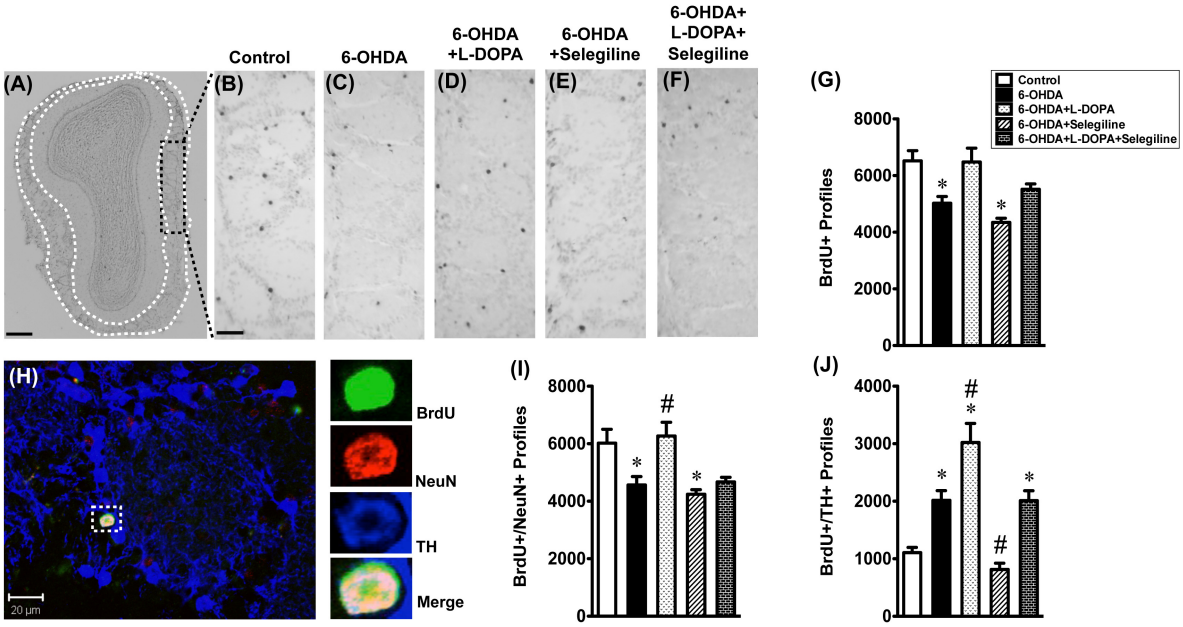


Fig. 11. Number of BrdU+, BrdU+/NeuN+ and BrdU+/TH+ cells in the PGL of the OB. Following 6-OHDA injection, the number of BrdU+ cells was significantly decreased as compared to controls (**A-C, G**). With L-DOPA there was no change compared to control, while Selegiline treatment tended to further decrease the number of BrdU+ cells (**A, D, E, G**). The combination of L-DOPA + Selegiline showed an intermediate response (**A, F, G**). Co-labeling of BrdU, NeuN and TH is shown in **H**. The number of BrdU+/NeuN+ cells (**I**) and BrdU+/TH+ cells (**J**) was evaluated in the PGL. The number of newborn neurons (BrdU+/NeuN+) showed a significant decrease after 6-OHDA injection, which was reversed by L-DOPA treatment (**I**). The number of newborn dopamine neurons (BrdU+/TH+) showed a significant increase or decrease following L-DOPA or Selegiline, respectively, compared to a 6-OHDA lesion alone (**J**). The scalebar in A represents 500 µm, in B 50 µm and in H 20 µm, respectively (G, I and J: One-way ANOVA followed by Tukey`s post-hoc test, n = 5-8, * $p < 0.05$, compared to control; # $p < 0.05$, compared to 6-OHDA).

1.6 Modulation of neuronal proportion in the PGL following 6-OHDA lesion and subsequent L-DOPA and Selegiline treatment

To better understand these changes in the ratio of newborn TH+ neurons in the PGL after a 6-OHDA lesion and subsequent drug treatment, the number of newborn dopamine neurons (BrdU+/TH+) was divided by the total number of newborn neurons (BrdU+/NeuN+) in the PGL (**Fig. 12C**). This ratio was significantly higher in the 6-OHDA lesioned animals (44.6 ± 3.0%), and this about 2-fold increase was sustained in the L-DOPA treated animals (46.7 ± 5.0%), as compared to control animals (18.8 ± 1.7%). The Selegiline treated group in contrast had a normal rate (19.3 ± 2.6%), but not when Selegiline was combined with L-DOPA (40.5 ± 3.0%; **Fig. 12C**).

This increased ratio of newborn TH+ neurons is reflected by an increased total number of TH+ cells in the PGL of the OB (**Fig. 12A, B**). The 6-OHDA injection alone and L-DOPA treatment increased the total number of TH+ cells to 37958 ± 1907 and 41468 ± 1864, respectively, compared to 29481 ± 960 in the control group. Selegiline normalized the cell number (27533 ± 1308). However, Selegiline, when given in combination with L-DOPA, was not able to counteract the increase seen by L-DOPA administration (37380 ± 1769; **Fig. 12B**).

1.7 Changes in TUNEL staining following 6-OHDA lesion and subsequent L-DOPA and Selegiline treatment

To investigate if Selegiline is regulating the number of newborn neurons in the OB by reducing the number of migrating NPC, TUNEL staining was performed (**Fig. 12D-H).** The 6-OHDA lesioned group showed a non-significant trend to a decrease in the number of TUNEL+ cells, which was not changed by L-DOPA treatment, as compared to control (**Fig. 12H)**. In contrast, Selegiline alone and in combination with L-DOPA showed a highly significant upregulation of TUNEL+ cells in the OB.

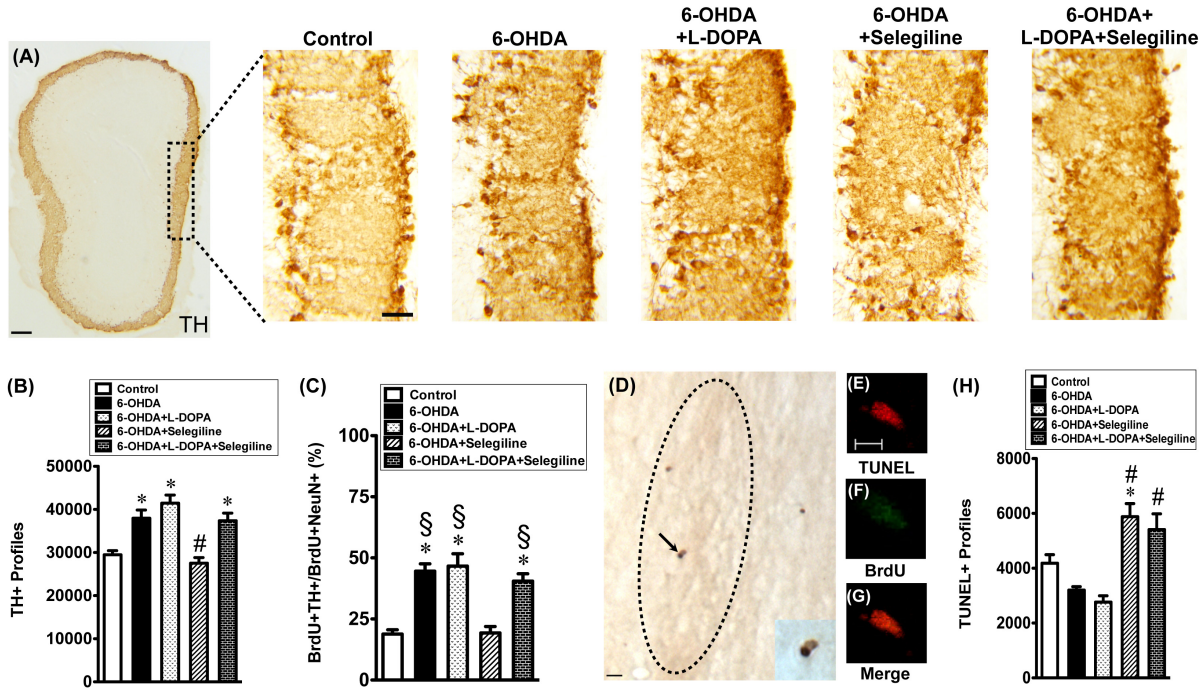


Fig. 12. Total number and proportion of TH+ cells, and number of TUNEL+ cells in the PGL of the OB. The total number of TH+ neurons was increased by the lesion alone or with L-DOPA treatment, as compared to controls. In comparison to the lesioned animals Selegiline decreased the number of TH+ neurons to the level of control (**A, B**). The ratio of newborn TH+ neurons to total newborn neurons in the PGL is significantly increased following 6-OHDA lesion, and sustained by L-DOPA treatment. Selegiline in contrast normalized it (**C**). TUNEL+ cells, shown in (**D**) (indicated by an arrow with a high power inset), are mainly detected near the RMS (outlined region) and panel (**E-G**) indicate co-expression of TUNEL/BrdU by immunofluorescence. Selegiline alone and in combination with L-DOPA increased the TUNEL+ cells. A 6-OHDA-lesion with or without L-DOPA, in contrast tended to decrease the number of TUNEL+ cells (**E**). Scalebar in A represents 25 µm, in D 200 µm and in E 5 µm (B, C and H: One-Way ANOVA followed by Tukey posthoc test, n = 5-7, * $p < 0.05$, compared to control; # $p < 0.01$, compared to 6-OHDA; § $p < 0.001$, compared to 6-OHDA + Selegiline).

2 Behavioral study

2.1 Establishment of six animal models

According to the finding in the histological part of this study, Selegiline normalizes the number of newborn DA neurons and the total number of DA neurons in the PGL of the OB in an acute 6-OHDA mouse model. In previous reports, odor discrimination seemed not to be affected while NPC were eliminated by antimitotic agents or genetic manipulation (Lazarini and Lledo, 2011). However, there is no report so far concerning the relationship between an increased number of DA neurons in the PGL and olfactory discrimination. In order to characterize whether Selegiline could improve olfactory discrimination, we established six different animal models to perform habituation-dishabituation of odor discrimination (by using male-female odor): (1) Control animals with sham operation (Control); (2) Unilateral intranigral 6-OHDA lesion (Uni); (3) Unilateral bulbectomy (UniBX); (4) Unilateral bulbectomy plus contralateral intranigral 6OHDA lesion (UniC); (5) Bilateral intranigral 6-OHDA lesion (Bil6OHDA); (6) Bilateral bulbectomy (BilBX) **(Fig. 6A)**.

2.2 Novel open field behavioral assessment

Novel open field (NOF) assessment was performed at 3 weeks, 12 weeks post operation and after 12 weeks of Selegiline treatment. To monitor general activity the duration (s) the animals spent in the central area of the area, a 36.5 cm x 36.5 cm square within the arena (50 cm x 50 cm) and the tracklength (cm) were measured. The central tracklength showed the highest value in the control group (3738 ± 271). Tracklength was significantly lower in the BilBX group (2336 ± 283). The UniBX group (3058 ± 336) and the UniC group (2662 ± 200) had a slightly lower tracklength than control, but did not show a significant difference 3 weeks post op (**Fig. 13A**). The time spent in the central area and the ratio of time spent in the central area to total time was lower in the Bil6OHDA group (305 ± 31, 19 % of total time) and the BilBX group (349 ± 58, 17 % of total time) compared to the control group (463 ± 32, 26 % of total time), but there was no statistical significance between groups (**Fig. 13B**).

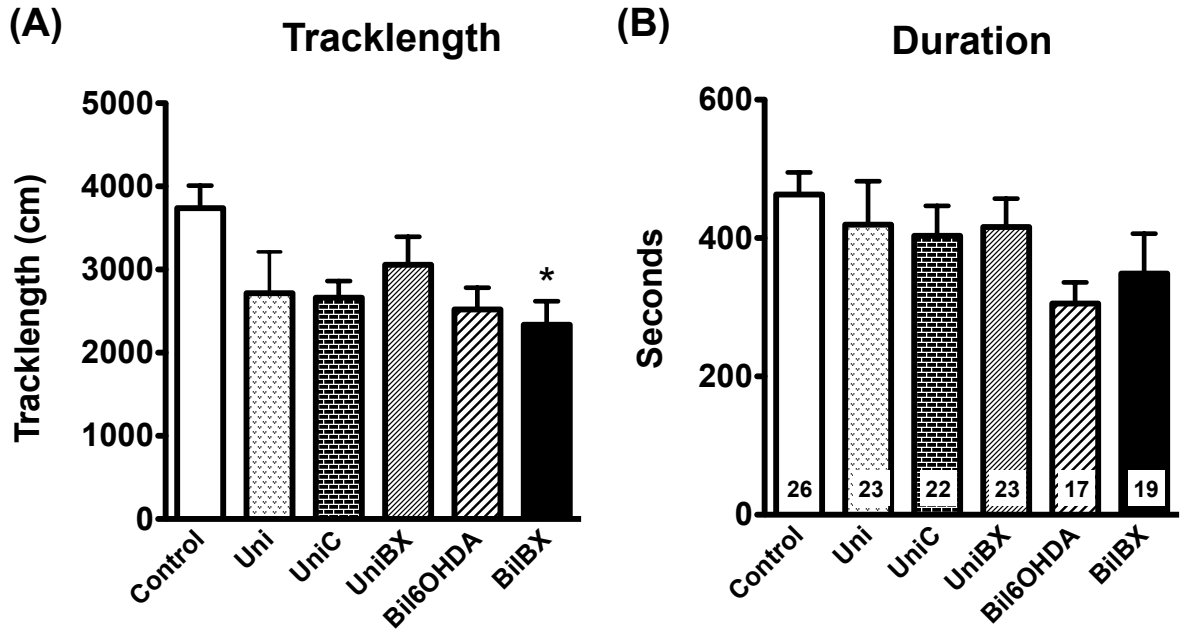


Fig. 13. Novel open field test. Tracklength and time spent in the central area. **(A)** Tracklength (cm) in the central area. **(B)** Time spent of total time the in the central area, the number on the bottom of each column represents the percentage of total time the animals spent in the central area (total test time 30 min) (*One-way ANOVA, *p<0.05, n=5-8*).

At twelve weeks post op and before treatment, there was no statistical difference in central tracklength and time spent in the central area between groups. However, UniBX (3663 ± 526) and UniC (3760 ± 471) showed higher values of central tracklength compared to the control group (2722 ± 451), Uni (2320 ± 543) and BilBX (3015 ± 565) (**Fig. 14A**). For the time spent in the central area, there was a similar pattern regarding the different experimental groups (**Fig. 14B**).

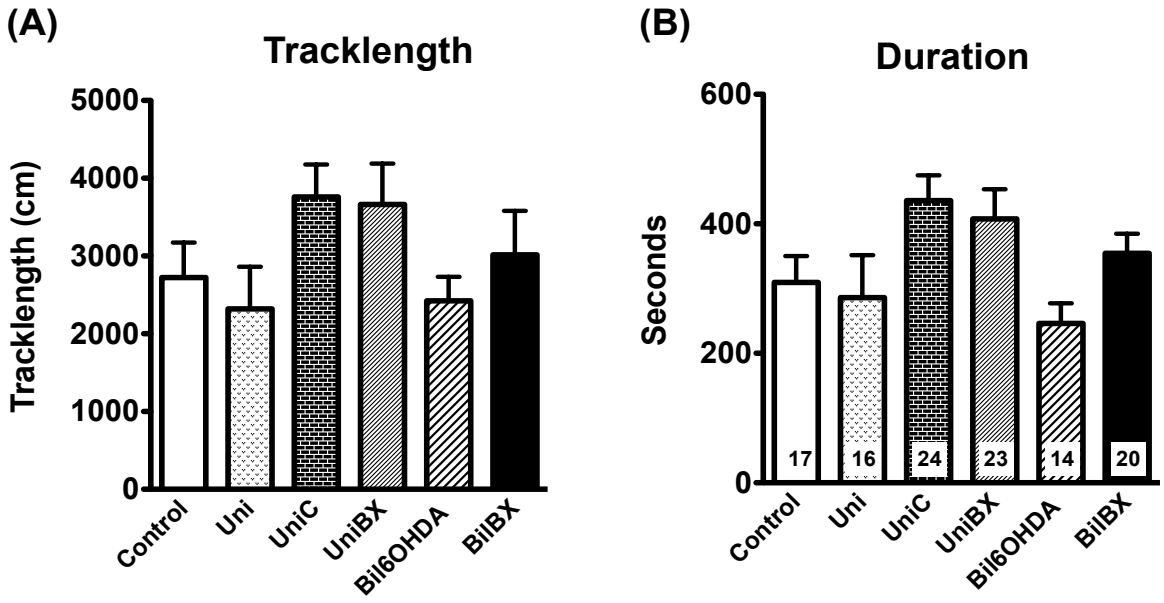


Fig. 14. Novel open field test. Tracklength and time spent in the central area. **(A)** Tracklength (cm) in the central area. **(B)** Time spent in the central area, the number on the bottom of each column represents the percentage of total time the animals stayed in the central area (total test time 30 min) (*One-way ANOVA, p>0.05, n=5-8).*

After 84 days of Selegiline treatment in the NOF assessment there was no significant difference between groups regarding tracklength and duration spent in the central area **(Fig. 15)**. The Uni (3951 ± 452) and UniBX (3807 ± 543) groups showed higher values of the tracklength compared to the groups of control (2704 ± 446), the Bil6OHDA (2933 ± 427) and the BilBX (2817 ± 567) **(Fig. 15A)**. The UniC group (518 ± 102, 29 % of total time) showed the longest duration spent in the central area compared to the shortest one in the Bil6OHDA group (399 ± 51, 22 % of total time) **(Fig. 15B)**.

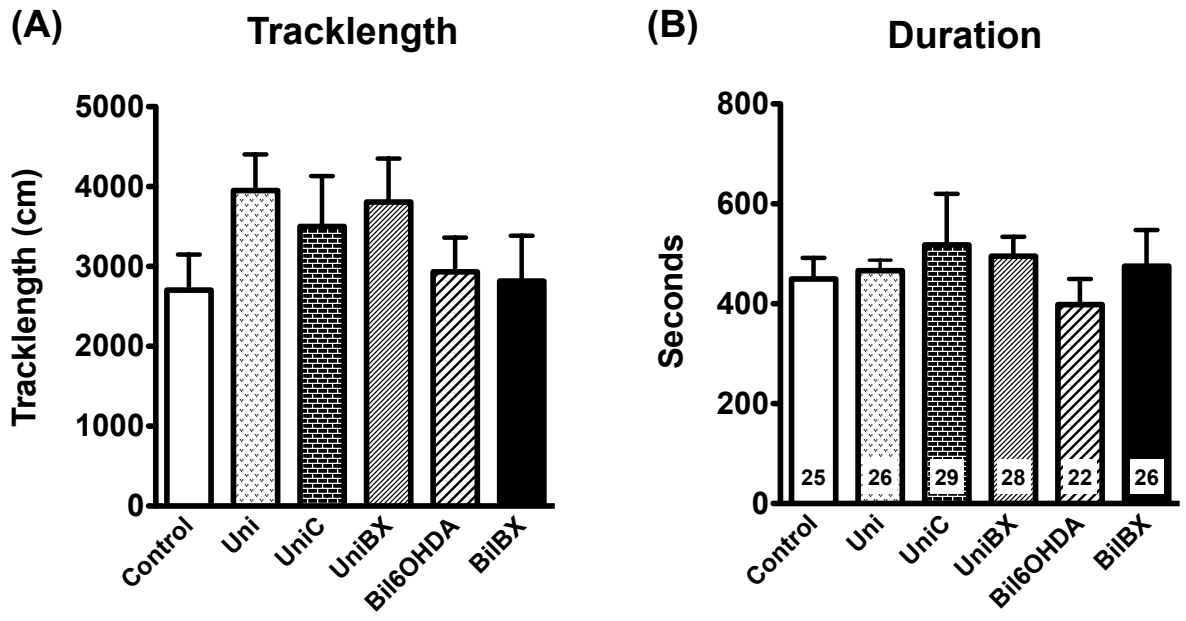


Fig. 15. Novel open field test. Tracklength and time spent in the central area. **(A)** Tracklength (cm) in the central area. **(B)** Time spent in the central area, the number on the bottom of each column represents the percentage of total time the animals stayed in the central area (total test time 30 min) (*One-way ANOVA, p>0.05, n=5-8)*.

2.3 Olfactory discrimination test

In order to detect the time point of a beginning olfactory deficit in all models, we performed the olfactory discrimination test every 3 weeks. There was a significant increased duration of time spent on the experimental block in the 6th trial (female's odor) in comparison to the 5th trial (other male's odor) in all groups, except the BilBX group 3 weeks post op **(Fig. 16)**. The time spent investigating the block carrying with the experimental odor in the 5th versus the 6th trial is shown in **Fig. 16**, [percentage of time spent at the experimental block compared to the time spent on both blocks (%)]: Control: 44.96 ± 18.86 (5th trial) versus 79.38 ± 9.30 (6th trial); Uni: 47.09 ± 9.33 versus 76.90 ± 20.93; UniC: 50.93 ± 19.70 versus 73.39 ± 11.75; Bil6OHDA: 46.12 ± 24.80 versus 76.78 ± 15.36; UniBX: 47.99 ± 14.82 versus 72.64 ± 23.64; BilBX: 50.22 ± 25.71 versus 61.15 ± 10.60.

The time spent on the experiment block in the 6th trial in comparison to the 5th trial was significantly increased in control, Uni and UniBX animals whereas UniC, Bil6OHDA and UniBX groups showed no significant difference at 12 weeks post op **(Fig. 17)**. Control: 45.10 ± 28.04 versus 72.30 ± 12.94; Uni: 28.93 ± 12.79 versus 64.58 ± 19.79; UniC: 34.39 ± 17.19 versus 48.15 ± 10.90; Bil6OHDA: 48.48 ± 25.40 versus 58.42 ± 22.88; UniBX: 41.11 ± 25.05 versus 69.80 ± 11.16; BilBX; 37.07 ± 29.95 versus 46.32 ± 25.25.

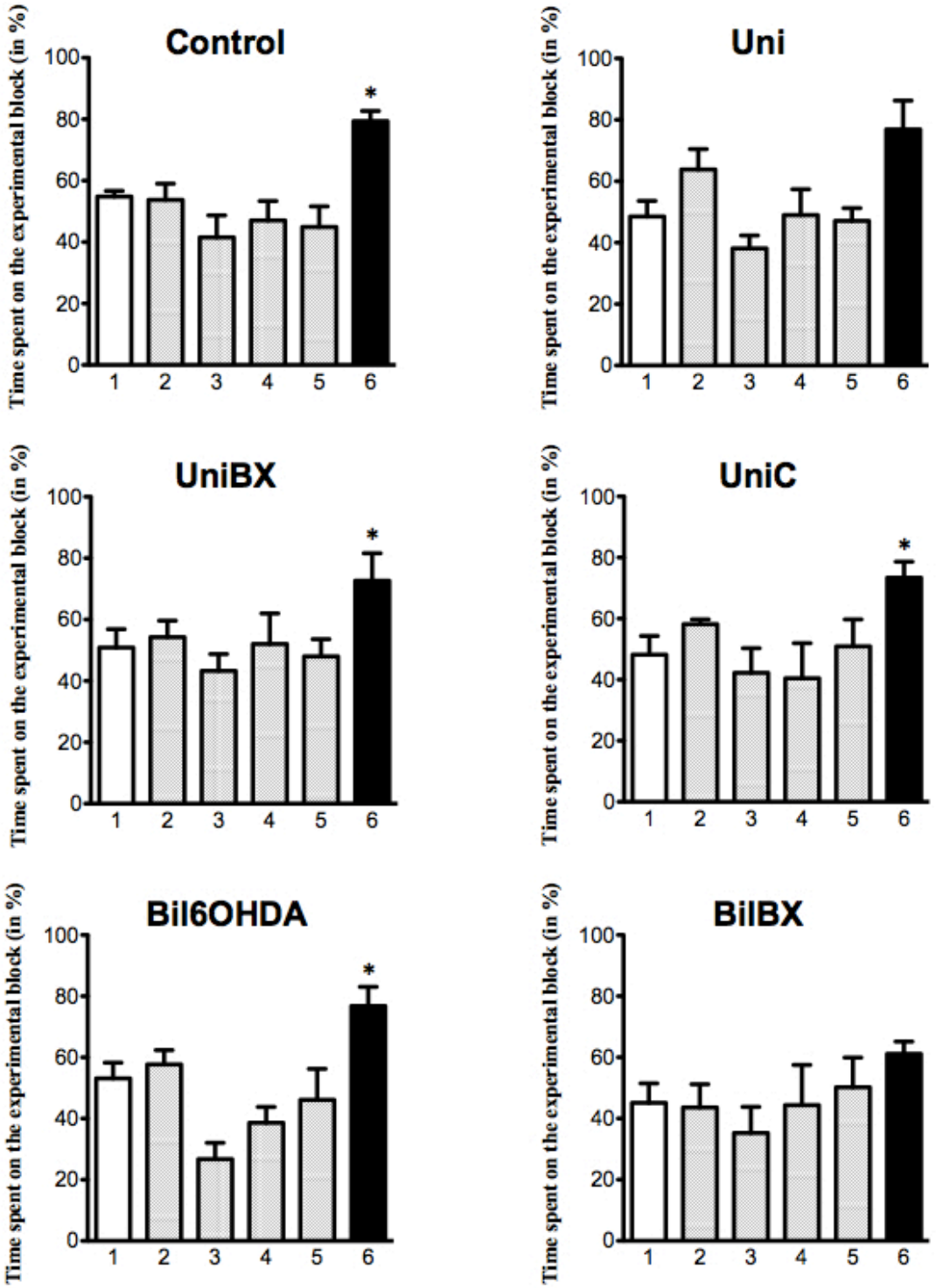


Fig. 16. Olfactory discrimination test at 3 weeks in six experimental groups. X-axis represents the exposure to another male's odor (2nd-5th trial) and to female's odor (6th trial); Y-axis represents the percentage of time spent on the experimental block compared to the overall time spent on both blocks). *$p<0.05$, *t-test* (5th trial versus 6th trial).

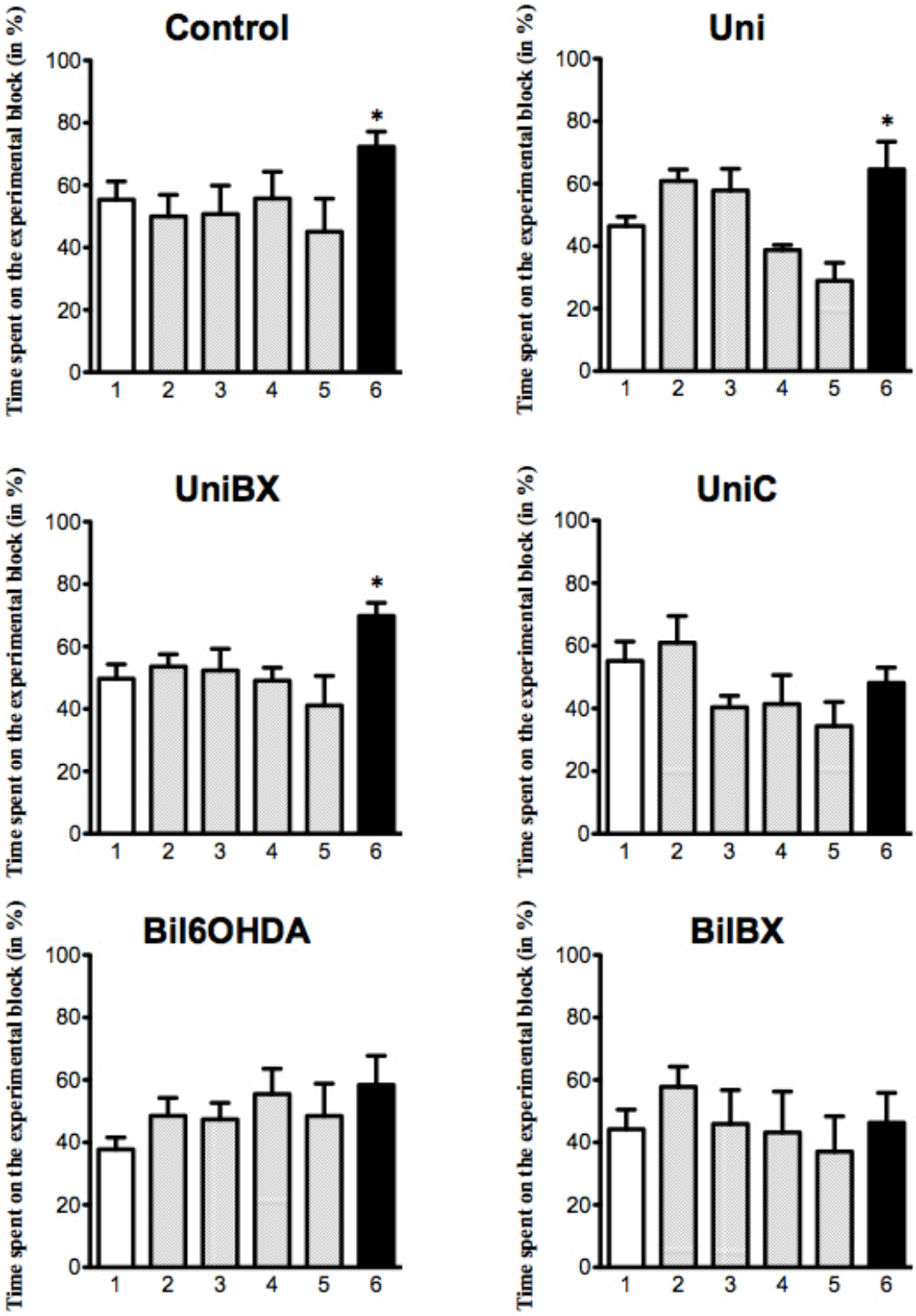


Fig. 17. Olfactory discrimination test at 12 weeks in six experimental groups. *p<0.05, *t-test* (5th trial versus 6th trial).

2.4 Effect of Selegiline treatment on olfactory discrimination test

After the appearance of an olfactory deficit observed at 12 weeks post operation in the groups of UniC and Bil6OHDA, a subcutaneous daily Selegiline injection was performed in all groups for 12 weeks. In the UniC, Bil6OHDA, and BilBX group, the time spent on the experimental block carrying female odor increased **(Fig. 18)**. In the Bil6OHDA group, however, the increase in time spent on the experimental block in 6th trial did not show statistical significance. The percentage of time spent investigating the experimental odor compared to the time spent in total blocks: Control: 45.27 ± 20.08 (5th Trial) versus 72.54 ± 12.99 (6th Trial); Uni: 39.81 ± 10.84 versus 58.97 ± 17.88; UniC: 50.45 ± 5.50 versus 69.83 ± 6.55; UniBX: 47.87 ± 14.81 versus 63.36 ± 10.72; Bil6OHDA: 35.98 ± 16.00 versus 57.94 ± 19.15; BilBX: 25.01 ± 21.86 versus 68.29 ± 9.92.

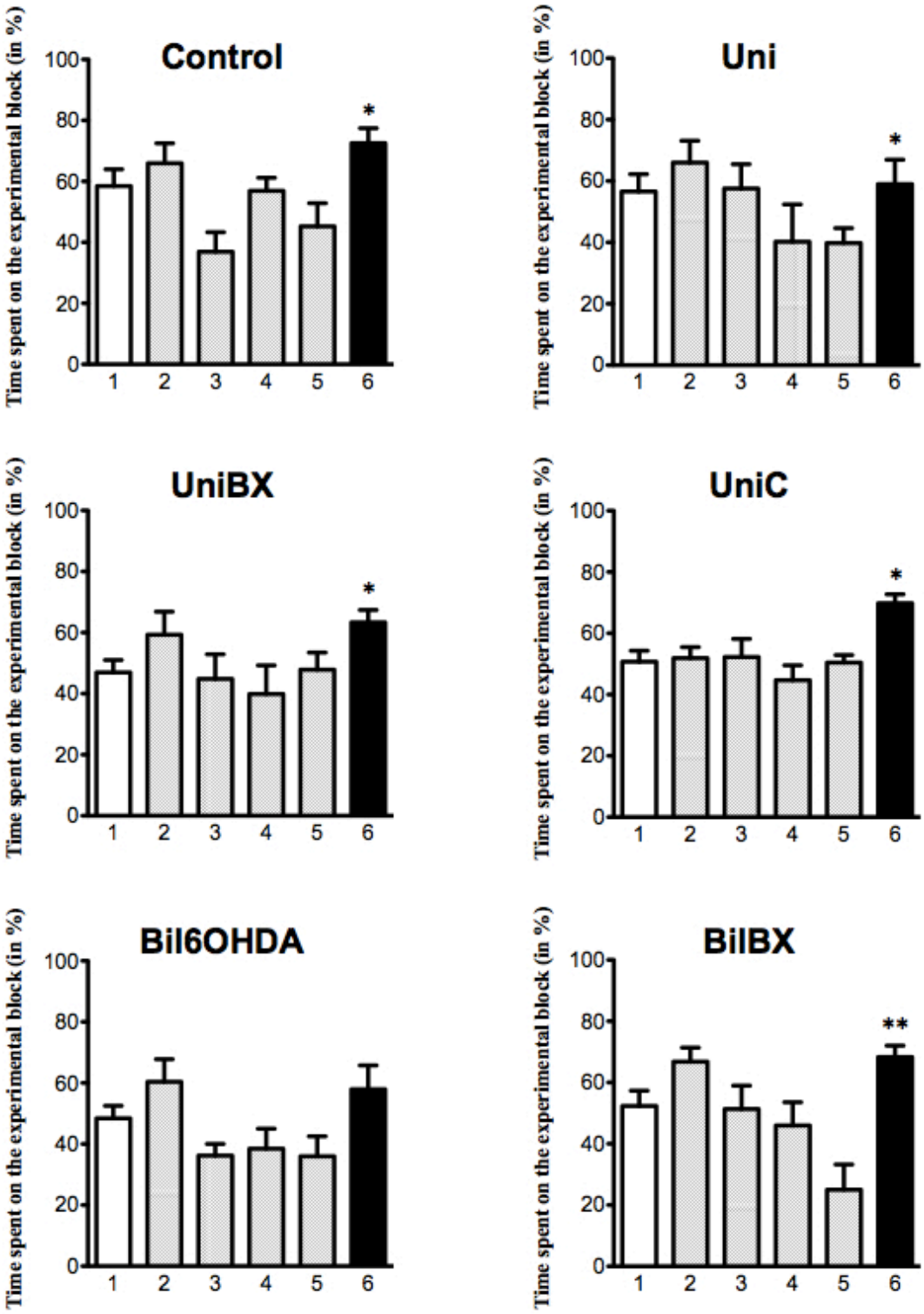


Fig. 18. Olfactory discrimination test after 12 weeks of Selegiline treatment in six experimental groups. *p<0.05 and **p<0.01, *t-test* (5th trial versus 6th trial).

2.5 Olfactory discrimination after withdrawal of Selegiline treatment

To determine whether the improvement of olfactory function still continued after Selegiline treatment, treatment was terminated and the behavioral test was performed again 4 weeks after the withdrawal of Selegiline. Interestingly, comparing to the 5th and 6th trial, Uni, Bil6OHDA, UniC and BilBX no longer showed a significant increase in time investigating the experimental odor **(Fig. 19)**. In control and UniBX, they showed an increase time spent on the experimental odor as shown in previous tests. Control: 28.26 ± 19.52 (5th Trial) versus 74.87 ± 15.41 (6th Trial), Uni: 49.15 ± 10.28 versus 59.95 ± 15.55, UniC: 42.99 ± 15.65 versus 59.50 ± 20.26, UniBX: 38.00 ± 10.98 versus 65.31 ± 18.90; Bil6OHDA: 51.83 ± 23.36 versus 61.30 ± 13.66; BilBX: 60.32 ± 19.71 versus 69.01 ± 21.60.

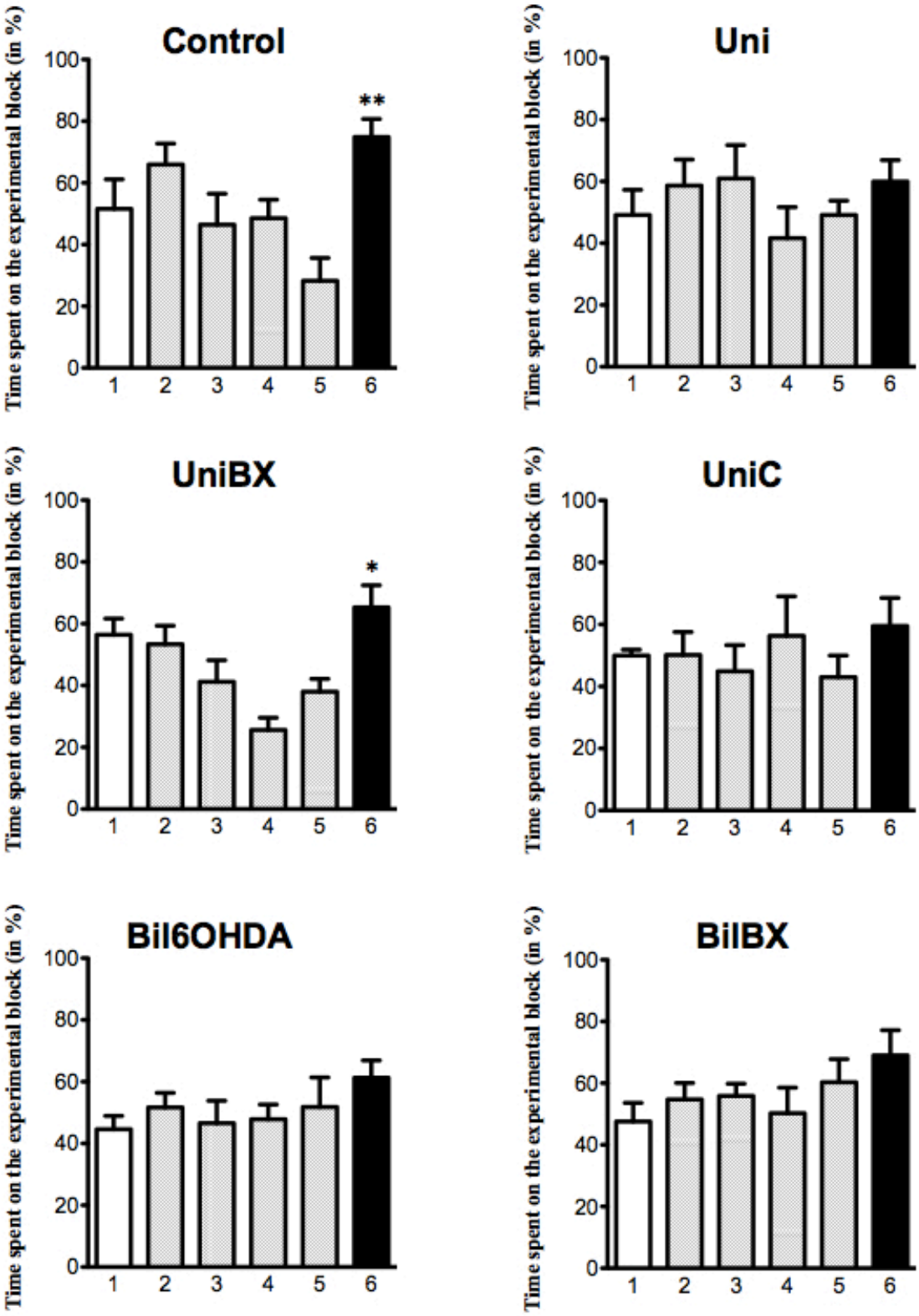


Fig. 19. Olfactory discrimination test 4 weeks after withdrawal of Selegiline in six experimental groups. *p<0.05 and **p<0.01, *t-test* (5th trial versus 6th trial).

To conclude in the olfactory discrimination test, only the BilBX group showed an olfactory deficit (red dash square in **Fig. 20**) at the time points 3 weeks post op and 12 weeks post op. The UniC group started to develop an olfactory deficit at 12 weeks post op (**Fig. 20**) After 12 weeks of Selegiline treatment, the olfactory deficit seen in UniC and BilBX were reversed to normal. The olfactory deficit appeared again after 4 weeks of Selegiline withdrawal in UniC and BilBX. The control and UniBX maintained a normal olfactory function at all time points of the test.

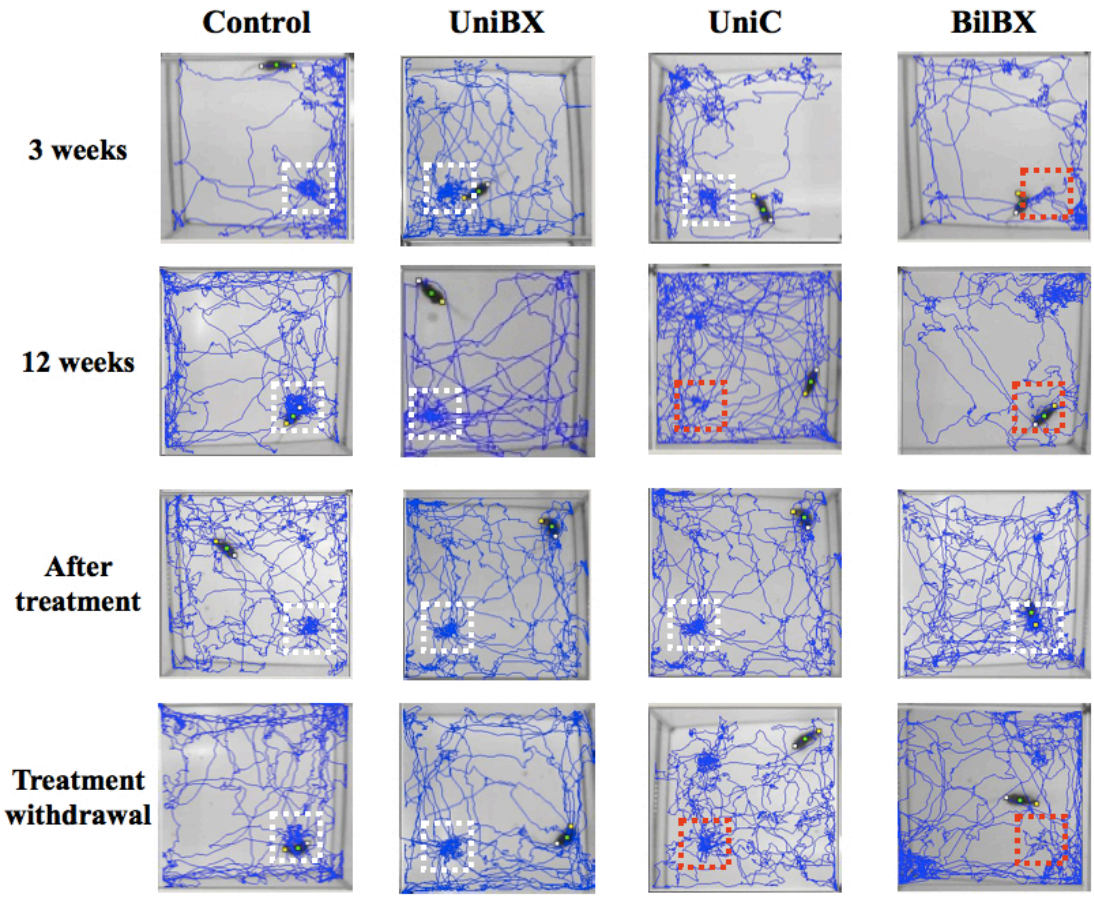


Fig. 20. Summary of the olfactory discrimination test. White dash square represents animal spent most of the time on the position of the wooden block with the female odor; Red dash square represents animal did not prefer the wooden block with the female odor.

V Discussion

1. Histological study

In the current study, we demonstrate that L-DOPA and Selegiline show a similar modulation of NPC proliferation in the SVZ in an unilateral 6-OHDA mouse model of PD, but distinct modulation of the number of newborn TH+ neurons in the OB, most likely due to a differential regulation of their survival.

An intranigral injection of 6-OHDA in rodents was first described and characterized by Ungerstedt et al. (Ungerstedt, 1968). Because of the electroactive properties of 6-OHDA, 6-OHDA is rapidly oxidized in neurons to generate free radicals and hydrogen peroxidase, which induces neurodegeneration (Schwarting and Huston, 1996). Besides the SN, the striatum and the median forebrain bundle (MFB) are also used to perform a 6-OHDA lesion in rodents (Alvarez-Fischer et al., 2008; Carlsson et al., 2011a; Deumens et al., 2002; Ungerstedt, 1968). The striatum and the MFB were not considered to inject 6-OHDA, in order to avoid physical damage caused by surgery, direct toxicity from 6-OHDA to NPC residing in the lateral wall of the ventricle, and a higher rate of mortality after 6-OHDA injection into the MFB of mice. In addition, an injection of 6-OHDA into the striatum and the MFB causes an inevitable lesion of VTA neurons, while injecting 6-OHDA into the SN causes a more specific lesion to SN neurons (Deumens et al., 2002; Kirik et al., 1998). Therefore, the SN was chosen for injection in our study.

Following nigrostriatal dopamine depletion, the number of NPC was significantly decreased in the SVZ. Pharmacological restoration of dopaminergic transmission in 6-OHDA lesioned mice restored proliferation of NPC to the level seen in control animals. This is in accordance with previous reports (Baker et al., 2004; Höglinger et al., 2004; O'Keeffe et al., 2009b). A partial recovery in the number of PCNA+ cell was also observed in Selegiline treated animals (alone or in combination with L-DOPA). The proliferation of C cells located in the SVZ is stimulated by dopaminergic projection via D2-like receptors, (Höglinger et al., 2004). Furthermore, dopamine D2 and D3 receptors are also expressed on migrating NPC (Van Kampen et al., 2004; Winner et al., 2009), and a lack of dopaminergic stimulation on these cells may lead to a decreased number, while increased dopamine receptor stimulation would increase

the proliferation of PCNA+ cells in the SVZ. Similar to L-DOPA, we show here for the first time that Selegiline alone can partly normalize proliferation of NPC in the SVZ. Selegiline, which is an irreversible MAO-B inhibitor, indirectly increases extracellular dopamine in the brain by reducing its degradation. In addition, its metabolite (R)-(-)-methylamphetamine is reported to inhibit the re-uptake and to promote the release of dopamine from the terminals (Magyar, 2004). Today, Selegiline is commonly used as anti-parkinsonian treatment, especially in the early stages of PD to delay the need for L-DOPA treatment, and in later stages combined with L-DOPA to reduce motor fluctuations in PD patients (Fernandez and Chen, 2007; Lo and Jain, 2006; Pålhagen et al., 1998; The-Parkinson-Study-Group, 1993). The exact mechanism of Selegiline to increase the number of NPC in the SVZ after nigrostriatal lesion is unknown, but indirect dopamine receptor stimulation would be a plausible theory.

Höglinger et al. showed in a postmortem study a significant downregulation of NPC proliferation in brains of PD patients as compared to control subjects. Consistently, O'Sullivan and colleagues investigated the role of dopaminergic treatment, confirming a positive impact of chronic L-DOPA administration on the number of NPC in the SVZ (O'Sullivan et al., 2011). Van den Berge et al. recently observed no change in the proliferation of NPC in the SVZ between control subjects, PD patients treated with L-DOPA, or patients with incidental Lewy body disease (LBD) without L-DOPA treatment (van den Berge et al., 2011). However, the cases with incidental LBD did not show a significant reduction in TH expression in the striatal area underneath the SVZ. Despite these controversial findings in human brains, rodent 6-OHDA models of PD rather consistently show a downregulation of NPC proliferation in the SVZ after dopamine depletion.

The NPC migrate from the SVZ to the OB where they differentiate into interneurons (Altman, 1969; Lois and Alvarez-Buylla, 1994). In order to better understand survival and fate of these cells following dopamine depletion, we analyzed the NPC in the OB, using BrdU labeling. In line with other investigators, the number of surviving BrdU+ NPC in PGL and GCL decreased after a 6-OHDA lesion (O'Keeffe et al., 2009a; O'Keeffe et al., 2009b). This may readily be explained by the evident downregulation of NPC proliferation in the SVZ. However, also here contradicting data have recently been published showing an increased survival of BrdU+ cells in the GCL and the

PGL (Sui et al., 2012), and an increase in the PGL, but no change in the GCL (Winner et al., 2006), at 6 weeks post 6-OHDA injection. The time point of the first BrdU injection may explain these discrepancies. A decreased proliferation of NPC in the SVZ has been seen in rodent 6-OHDA models, three weeks post lesion at the earliest (Baker et al., 2004; Höglinger et al., 2004). As Winner et al. and Sui et al. administered the first BrdU-injection at day 7, as compared to day 21 in the study by O'Keefee, and day 19 in our study, there may not yet have been a definitive decrease in proliferation established in the SVZ in the former studies. BrdU injections at an earlier time point, as performed by Winner et al. and Sui et al., might therefore label proliferating NPC of a rather intact SVZ and miss to detect a decreasing number of BrdU+ cells in the OB. It is also worth noting, that NPC need about 30 days to migrate from the SVZ to the OB and to differentiate into interneurons (Lois and Alvarez-Buylla, 1994). In our study, the four week-treatment regimen of dopamine modulating drugs (L-DOPA and Selegiline) is within this time window.

In order to further understand whether increased apoptosis is involved in the modulation of the survival of NPC following L-DOPA and Selegiline treatment, TUNEL staining was performed to evaluate the number of apoptotic cells in the OB. Our data clearly demonstrate that Selegiline alone or in combination with L-DOPA, significantly increased the number of TUNEL+ cells. This fact might contribute to the decreased number of newborn neurons detected in these two groups. About 30 000 NPC migrate from the SVZ to the OB every day (Lledo et al., 2006). However, only half of them will reach the OB and differentiate into neuronal or glial phenotypes (Winner et al., 2002). The other NPC will undergo apoptosis during migration, or will be incapable to integrate into the neural circuitry of the OB. Selegiline is metabolized to amphetamine derivatives, which recently have been shown to inhibit the neuroprotective actions of both Selegiline and desmethyl selegiline *in vivo* (Jenner and Langston, 2011). The increase in amphetamine derivatives following Selegiline treatment have in fact recently been a matter of debate as potentially harmful (Yasar et al., 2006). This has, however, not been reported in PD patients. It is therefore possible that the decreased survival of NPC found in the OB after Selegiline treatment, reported in our study, may be influenced by the interaction with amphetamine derivatives, causing an increase in the number of TUNEL+ cells (Tian et al., 2009).

Evidence of a decreased number of NPC was also found in the OB in human postmortem tissue of PD patients compared to controls (Bedard and Parent, 2004; Höglinger et al., 2004; Liu and Martin, 2003). Due to different experimental methods, there are diverging opinions concerning the existence and structure of RMS and whether NPC migrate from the SVZ to the OB via a SVZ-RMS-OB route in the human brain (Curtis et al., 2007; Sanai et al., 2004).

In order to investigate the neuronal fate of the NPC in the OB, the frequency of newborn BrdU+ cells co-stained with NeuN to confirm a neuronal phenotype, and with TH as a dopaminergic marker, was analyzed. The modulation of adult-born neurons was determined by the frequency of neuronal differentiation and the number of surviving NPC in the OB. We, and others, show that the frequency of neuronal differentiation is stably sustained in the GCL and PGL in both control and 6-OHDA lesioned animals (Winner et al., 2008; Winner et al., 2006). In addition, we show that L-DOPA and Selegiline treatment has no significant effect on the frequency of neuronal differentiation in the GCL and PGL. In regard to the number of surviving NPC and adult-born neurons, we show a substantial restoration following L-DOPA treatment, in line with the study of O'Keefee and colleagues (O'Keeffe et al., 2009b). The decreased number of surviving NPC and adult-born neurons after Selegiline treatment might be explained by a significant increase in TUNEL+ profiles. The effect of the combination of L-DOPA and Selegiline on the survival of NPC and adult-born neurons may rather suggest a modulation by the drug itself, than an effect of the elevated dopamine concentration in the OB.

An increased number of dopamine neurons in the PGL of the OB in toxin-based animal models has previously been reported (Belzunegui et al., 2007; Winner et al., 2008; Winner et al., 2006; Yamada et al., 2004). In this study we have investigated the effect of L-DOPA and Selegiline on the number of adult-born neurons, their dopaminergic differentiation and total number of dopaminergic neurons in the PGL of the OB. We show that dopaminergic differentiation is 2-fold higher and the number of adult-born dopaminergic neurons is increased after 6-OHDA-injection. We believe that our data show for the first time a differential modulation of L-DOPA and Selegiline on adult-born dopaminergic neurons in the PGL of the OB after dopamine depletion. L-DOPA seems to restore not only the survival of NPC, but also sustains a 2-fold increase of dopaminergic differentiation (**Table 2**) and the pathological shift in the proportion of newborn dopamine neurons after 6-OHDA injection (**Fig. 12C**).

This might explain why L-DOPA treatment cannot only maintain, but even increase the total number of dopamine neurons. In contrast, the decreased number of surviving NPC and newborn neurons observed with Selegiline treatment, combined with a normal dopaminergic differentiation and neuronal proportion normalizes the upregulation of dopamine neurons (**Fig. 12**; **Table 2)**.

Although adult-born neurons in the OB are thought to relate to olfactory function, the impact of these adult-born neurons integrating into OB circuitry on functional outcome remains uncertain (Imayoshi et al., 2008; Jaskelioff et al., 2011; Lazarini et al., 2009; Mouret et al., 2009; Sakamoto et al., 2011). Dopamine neurons are one of the major subtypes of interneurons, which can be marked by TH in the PGL (Parrish-Aungst et al., 2007). Our data suggest that the generation and differentiation of adult-born dopamine neurons respond to pathological impacts (e.g. 6-OHDA) and pharmacological treatment.

In patients, olfactory dysfunction is not improved by dopaminergic therapy (Quinn et al., 1987; Roth et al., 1998). An increased number of dopaminergic neurons can be seen in the PGL of the OB in postmortem studies of PD patients (Huisman et al., 2004; Mundiñano et al., 2011a), with a conflicting report by Huisman and colleagues (Huisman et al., 2008). The increased number of inhibitory dopaminergic neurons in the PGL maintained by L-DOPA, but not Selegiline, in the current study might explain that olfactory dysfunction is still a common symptom in drug naïve PD patients and patients receiving L-DOPA therapy (Doty et al., 1992b).

2. Behavioral study

In this study, we discovered for the first time, a delayed olfactory deficit in an acute mouse model of intranigral 6-OHDA lesion. The olfactory deficit was reversed following long-term Selegiline treatment.

The NOF assessment was used to evaluate general motor activity and the anxiety status of the animals by measuring the tracklength and the time spent in the central area of the arena. In our experimental design, wooden blocks were positioned in the central area (36.5 cm x 36.5 cm). The groups showed no severe motor deficits or anxiety, as the animals were able to perform the olfactory discrimination task in the central area. BilBX and Bil6OHDA showed a lower tracklength and time spent in the central area among groups. Comparing the results after 3 weeks and 12 weeks post op, the tracklength of the BilBX group was significantly decreased at 3 weeks post op, but there was no significant difference at 12 weeks post op compared to control, suggesting that the BilBX group might require a longer period to recover after the surgery compared to other groups. The surgery of bilateral bulbectomy showed local bleeding and edema, the disruption of the local blood supply together with retrograde, anterograde and transneuronal degeneration (Song and Leonard, 2005).

Rat models of BilBX and Bil6OHDA were used to study anxiety and depression-like behavior (Rodriguez-Gaztelumendi et al., 2009; Tadaiesky et al., 2008). Olfactory bulbectomy caused not only anosmia but also a loss in detection of pheromones regulated by VNO and the accessory olfactory bulb, which connected to the amygdala and other brain areas, and were responsible for gender recognition, aggressive behavior and social dominance (Song and Leonard, 2005). It has been shown that in a Bil6OHDA rat floating time in the forced swim test increased and the time spent in the open arm of an elevated plus maze decreased, indicating an anhedonic-depressive like behavior (Tadaiesky et al., 2008). The group of Bil6OHDA showed the lowest duration spent in the central area at 3 weeks and 12 weeks post op, which might be related to depressive symptoms. However, the difference did not reach significance. There was no significant behavioral change in the mouse model of BilBX at 12 weeks post op in the current study, which might be due to species differences.

After Selegiline treatment, the groups of Uni and UniC showed an increase, but no significant difference of the tracklength compared to control. The time spent in the

central area in all groups was slightly increased. These data might suggest that Selegiline has no effect on general motor activity. However, the increased time spent in the central area in all groups might be explained by the fact that Selegiline has an anxiolytic effect as shown in previous reports (Imamura et al., 2012; Riederer and Laux, 2011).

In the 1st trial of odor discrimination, two wooden blocks carrying the animal's own odor were placed in the arena. Animals with normal olfactory function explored the whole arena and wooden blocks, where they spent approximately the same time on each wooden block. In the 2nd trial, one of the wooden blocks was replaced by one carrying another male's odor. The time spent at that block was slightly elevated. In the following trials (3rd to 5th), the time at the experimental block slowly decreased, which is called "habituation", caused by an elimination in part of mitral cells responding to an odor with increased firing rates in the absence of any paired reinforcement (Lazarini and Lledo, 2011). Moreover, habituation is required in non-associative olfactory learning and short-term olfactory memory (Breton-Provencher et al., 2009). In the 6th trial, a wooden block scented the odor of a female was introduced; the duration of time at that wooden block increased significantly. The sex discrimination might be mixed regarding the function of the main OB and VNO system (Zufall and Leinders-Zufall, 2007). However, the olfactory discrimination of social odor in the groups of UniBX and BilBX (from 3 to 12 weeks post op) suggest that the main OB still plays a major role to identify odor of different genders. At 3 weeks post op, the BilBX group was the only group, showing a deficit to discriminate odors. Olfactory bulbectomy in both sides (BilBX) might cause a destruction of the olfactory pathway from the olfactory epithelium to the olfactory cortex or higher brain areas. In contrast, the olfactory discrimination was preserved due to the intact olfactory bulb in the UniBX group, not different from control.

An olfactory deficit in the UniC and Bil6OHDA was shown at 12 weeks post op (**Fig. 17**), but not at 3 weeks post op, which suggests a delayed effect of the pathological alteration to show as a behavioral impact. Even though there are no histological data to support at 12 weeks time point (refer to Part 1: Histological study), we have shown an impaired proliferation in the SVZ until 7 weeks post op. Therefore we believe that there is no recovery in the SVZ after an intranigral 6-OHDA lesion (**Fig. 8-9**). Recently, a deficit of innate olfactory function was shown after NPC depletion with mice displaying behavioral deficits in male-male aggression and male sexual behavior

toward females (Masayuki Sakamoto, 2011). In the embryonic stage olfactory discrimination was clearly impaired by insufficient neurogenesis, but not later in adulthood (Lazarini and Lledo, 2011). We show for the first time in a 6-OHDA mouse model a delayed deficit of innate olfactory discrimination (male-female odors) at 12 weeks post op, which suggests that innate odor discrimination is possibly related to abnormal NPC modulation in the OB after 6-OHDA lesion.

In order to understand whether Selegiline can reverse the olfactory deficit shown in the UniC group, animals of all groups received Selegiline treatment for 12 weeks and the treatment was withdrawn thereafter for 4 weeks. The groups of control and UniBX still maintained a constant olfactory discrimination. However, the olfactory function of UniC, Bil6OHDA and BilBX groups was reversed after Selegiline treatment. But the groups showed again an olfactory deficit following Selegiline withdrawal. First, the data indicate that Selegiline has no impact on positive control groups, such as the control, Uni and UniBX, suggesting that a specific therapeutic effect of Selegiline, also in the case of a loss of olfactory discrimination cannot be detected in our experimental design. Second, in the groups of UniC, Bil6OHDA and BilBX olfactory dysfunction was recovered only by Selegiline treatment, suggesting a continuous and chronic therapeutic effect of Selegiline on olfactory function. In a recent report a complementary role of the main olfactory system and the vomeronasal system has been documented. Sensory nerves from the main OB and the VNO responded to social chemosignals and reacted with extraordinary high sensitivity in an overlapping manner (Brennan and Zufall, 2006). In our study, despite both main OB were removed surgically in the BilBX group (anterior parts from AP: +3.56 relative to bregma), parts of the accessory OB (posterior part from AP: +3.56 relative to bregma) were still remaining in the caudal part of the brain and the olfactory signal might possibly be reinforced and transferred via the vomeronasal neurons. Selegiline might increase the sensitivity and indirectly enlarge the signal transmission in the accessory OB-VNO system, which might replace the function of the main OB in the BilBX animals. This might explain an uncovered olfactory function in the BilBX group with Selegiline treatment. In the groups of UniC and Bil6OHDA the accessory OB-VNO system remained intact, therefore the effects of Selegiline treatment could be on NPC in the main OB and/or in the accessory OB-VNO system. A continuous and chronic Selegiline treatment can reverse an increased number of DA neurons in the PGL in 6-OHDA lesioned animals, which might be the reason that the groups of UniC and

Bil6OHDA regained normal olfactory function. Moreover, Selegiline in addition might have an unknown effect on activating the accessory OB-VNO system. Further investigations are required to understand the details regarding the relationship between the effects of Selegiline on the main OB and the VNO system in 6-OHDA lesioned animals.

VI. Summary

Hyposmia, often preceding the cardinal motor symptoms, such as bradykinesia, rigidity, tremor at rest and postural instability, is frequently reported in PD. This symptom appears to be related to an increased number of dopamine neurons in the periglomerular layer (PGL) of the OB. In the histological study, we have investigated the survival and neuronal differentiation of NPC in the OB, following L-DOPA and/or Selegiline in a unilateral, intranigral 6-OHDA lesion model in mice. Our data show that the number of NPC in the SVZ is decreased after 6-OHDA lesion, while there is no difference from control in lesioned mice with Selegiline or L-DOPA treatment. Moreover, the monoamine oxidase-B inhibitor, Selegiline, is able to normalize the number of dopamine neurons by increasing the number of TUNEL+ cell and reverses the dopaminergic differentiation to control in the PGL, while L-DOPA treatment sustains the increased number, by increasing the survival of NPC and upregulating 2-fold dopaminergic differentiation observed in 6-OHDA lesioned animals. In the behavioral study, to understand the olfactory function after 6-OHDA lesion and the effects of Selegiline treatment, six different experimental groups were investigated and performed an olfactory discrimination test. The data show that a delayed olfactory deficit was first appeared 12 weeks post 6-OHDA lesion and the recovery of olfactory function followed a daily, chronic treatment of Selegiline for additional 12 weeks. However, the olfactory dysfunction of the animals relapses again after a 4-week withdrawal of Selegiline treatment.

To combine the findings of our studies, we conclude that there is a distinct modulation of newly generated dopamine neurons of the OB after L-DOPA and/or Selegiline treatment. Furthermore, a delayed olfactory deficit was detected after a downregulation of adult neurogenesis, and only a chronic, continuous treatment of Selegiline can reverse the olfactory dysfunction in an acute mouse model of PD.

Our findings provide practical implications and stimulate further clinical and preclinical research, bridging the basic knowledge of hyposmia of PD from bench to bedside. Although the relationship of hyposmia and an increased number of dopamine neurons in the OB of PD patients is not well defined yet, the evidence in this study may offer the explanation, that the dysregulation of adult neurogenesis might be one of reasons to cause hyposmia of PD. Moreover, the effects of Selegiline on olfactory

function could be a consideration for clinical application. However, further studies are needed to show whether the interaction of dopamine depletion and drug treatment plays a role in olfactory function in PD patients.

VII. Abbreviations

6-OHDA	6-hydroxydopamine
AADC	aromatic-L-amino-acid decarboxylase
AP	antero-posterior
AAV	adeno-associated virus
Bil6OHDA	bilateral intranigral 6-OHDA injection
BilBX	bilateral bulbectomy
BrdU	bromodeoxyuridine
DA	dopamine
DAB	3,3´-diaminobenzidine
DBS	deep brain stimulation
DAergic	dopaminergic
DG	dentate gyrus
DV	dorso-ventral
EGFR	epidermal growth factor receptor
GABA	gamma aminobutyric acid
GAD	glutamic acid decarboxylase
GCL	granule cell layer
GDNF	glial derived neurotropic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescence protein
GPi	globus pallidus internus
HPLC	high perfomance liquid chromatography
iLBD	incidental Lewy body disease
LB	Lewy bodies

L-DOPA	$_{L}$-3,4-dihydroxyphenylalanine
LN	Lewy neurites
M/T cells	mitral and tufted cells
MAO-A	monoamine oxidase-A
MAO-B	monoamine oxidase-B
MFB	median forebrain bundle
ML	mediao-lateral
MPTP	1-methyl-4phenyl-1,2,3,6-tetrahydropyridin
MRI	magnetic resonance imaging
NOF	novel open field
NPC	neural progenitor cell
OB	olfactory bulb
ODT	olfactory discrimination test
OSN	olfactory sensory neuron
PB	phosphate buffer
PCNA	proliferating cell nuclear antigen
PD	Parkinson's disease
PET	positron emission tomography
PFA	paraformaldehyde
PGL	periglomerular layer
RBS	rapid eye movement sleep behavior disorder
RMS	rostral migratory stream
SGZ	subgranular zone
SN	substantia nigra
SSC	saline sodium citrate
STN	subthalamic nucleus

STR	striatum
SVZ	subventricular zone
TF	transcriptional factor
TH	tyrosine hydroxylase
TUNEL	TdT-mediated dUTP-biotin nick end labeling.
Uni	unilateral intranigral 6-OHDA injection
UniBX	unilateral bulbectomy
UniC	unilateral intranigral 6-OHDA injection plus contralateral bulbectomy
UPSIT	university of Pennsylvania smell identification test
VMAT2	vesicular monoamine transporter 2
VNO	vomeronasal organ
VTA	ventral tegmental area

VIII. References

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X. Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren die Damen/ Herren,

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XI. Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel "Modulation of Adult Neurogenesis in the Olfactory Bulb in an Acute Mouse Model of Parkinson's Disease" in der Klinik für Neurologie unter der Leitung von Prof. Dr. Oertel und PD Dr. Ries ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, den

Wei-Hua CHIU