

PHASE VALIDATION OF NEUROTOXIC ANIMAL MODELS OF
PARKINSON'S DISEASE

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ABSTRACT

PHASE VALIDATION OF NEUROTOXIC ANIMAL MODELS OF PARKINSON'S DISEASE

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Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic nigral neurons and striatal dopamine resulting in serious motor deficits but also some non-motor anomalies. Animal models of human neurodegenerative diseases are essential for better understanding their pathogenesis and developing efficient therapeutic tools. There are many different PD models, however, none of them is fully reproducing all the symptoms of the disease. In addition, different investigators use different behavioral measures which makes even more difficult to compare and evaluate the results. The aim of the present study was to

compare motor and cognitive deficits in two most common models of PD: the Rotenone and 6-OHDA model, using a large battery of neurological tests and a probabilistic learning task. To the best of our knowledge, this is the first study to examine the effects of bilaterally induced Rotenone and 6-OHDA through behavioral test batteries assessing the cardinal motor symptoms and the cognitive abnormality of Parkinson's Disease in the same rat population. Also, the present study is unique on the basis of providing both longitudinal observations of behaviour in the same treatment group and the cross-sectional comparisons of the behavioural responses between different groups. In the current study, the neurotoxins were applied at relatively low doses of 3-4 μ g, bilaterally to the substantia nigra pars compacta (SNpc). Experiments were conducted on 50 young-adult male Sprague–Dawley rats randomly assigned to five experimental groups: Rotenone, 6-OHDA, vehicle (DMSO/Saline), and the intact control. The neurological tests included locomotor activity, catalepsy, rearing, stepping, and rotarod/accelerod tests. They were applied prior to, and on days 4-7-10-20-40-150 while the learning task was applied 49 days after drug infusion. During the first 2 postoperational months, both neurotoxins produced progressive deterioration in motor performance but showing no effect on cognitive functions. Five months after the surgery, regression of bradykinesia but persistence of sensorimotor deficits was noted. The tests' results suggest different susceptibility of different motor functions to the degeneration of nigro-striatal (N-S) pathway. So, different tests were demonstrated to have different power in detecting similar motor deficits.

Key words: Parkinson's Disease, animal models, Rotenone, 6-OHDA, probabilistic learning.

ÖZ

PARKİNSON HASTALIĞININ NÖROTOKSİK HAYVAN MODELLERİNDE FAZ VALİDASYONU

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Parkinson hastalığı, dopaminerjik nigral nöronların ve striatal dopaminin progresif kaybı ile ortaya çıkan motor davranışlarda ciddi yetersizlik ve de motor-olmayan anomaliler ile karakterize bir hastalıktır. İnsanlardaki bu nörodejeneratif hastalığın hayvan modelleri, hastalığın patogenezi için daha iyi anlamada ve yeni terapötik araçlar geliştirmekte oldukça önemli. Birçok Parkinson hayvan modeli olmasına karşın hiç biri tam olarak hastalığın tüm semptomlarını oluşturamamaktadır. Ayrıca, farklı araştırmacılar farklı davranış ölçümleri

kullanmakta bu da sonuçları karşılaştırmayı ve değerlendirmeyi daha da zorlaştırmaktadır. Mevcut çalışmanın amacı, oldukça yaygın kullanılan iki PD modeli olan Rotenon ve 6-OHDA modellerinde birçok nörolojik test ve olasılıksal öğrenme testini kullanarak motor ve kognitif bozuklukları karşılaştırmaktır. Bildiğimiz kadarıyla mevcut çalışma aynı popülasyonda ve birçok test baterisi ile, bilateral verilen Rotenon ve 6-OHDA'nin Parkinson hastalığının en önemli motor semptomları ve kognitif anormallikleri üzerindeki etkisini çalışmak adına bir ilktir. Ayrıca, mevcut çalışma aynı müdahale grubunun zaman içindeki davranışlarının değişimini ve farklı gruplar arasındaki davranışsal tepkilerin karşılaştırılmasını incelemek bakımından da bir ilktir. Nörotoksinler, 3-4 µg olacak şekilde düşük dozlarda, bilateral olarak substantia nigra pars compacta (SNpc) bölgesine uygulandı. Deneyler, 50 adet, genç-erişkin, erkek Sprague-Dawley cinsi sıçanın rastgele beş deney grubuna ayrılması ile gerçekleştirildi. Gruplar: Rotenon, 6-OHDA, araç kontroller (DMSO/Saline) ve hiç bir şey uygulanmamış kontrol olarak ayrıldı. Nörolojik testler, lokomotor aktivite, katalepsi, rearing (dikelme), adım atma ve rotarod/accelerod testi olarak seçildi. Tüm bu testler, ameliyatlardan önce ve ameliyattan 4-7-10-20-40-150 gün sonra; öğrenme testi ise ameliyattan 49 gün sonra yapıldı. Ameliyattan sonraki ilk iki ay boyunca nörotoksinler, motor performansında progresif bir bozulma yaratırken kognitif fonksiyonlarda bir etki göstermediler. Ameliyattan beş ay sonra ise bradikineziye gerileme görülürken duyuşal-motor bozukluklarda devamlılık not edildi. Test sonuçları göstermektedir ki farklı motor fonksiyonları nigro-striatal (N-S) yolaktaki dejenerasyona farklı oranlarda hassasiyet göstermiştir. Yani, farklı testler benzer motor bozuklukları tespit etmede farklı miktarda etkili olmuşlardır.

Anahtar Kelimeler: Parkinson Hastalığı, hayvan modelleri, Rotenon, 6-OHDA, olasılıksal öğrenme.

To My Family

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LIST OF ABBREVIATIONS

6-OHDA	6-Hydroxydopamine
Ach	Acetylcholine
AP	Antero-Posterior
AS	Adjusting Step
ATP	Adenosine Triphosphat
BBB	Blood-Brain Barrier
CGT	Cambridge Gamble Task
D1	Dopamine -1 Receptor
D2	Dopamine -2 Receptor
DA	Dopamine
DAT	Dopamine Transporter
DMSO	Dimethyl Sulfoxide
DV	Dorso-Ventral
ETC	Electron Transfer Chain
GABA	Gaba-Aminobutyric Acid
GP	Globus Pallidus
GPe	Globus Pallidus externa
GPi	Globus Pallidus interna
HD	Huntington Disease
IGT	Iowa Gambling Task
IT	InitiationTime

L	Lewis Rat
LB	Lewy Body
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO-B	Monoamine Oxidase-B
MB	Methylene Blue
METH	Methamphetamine
MFB	Medial Forebrain Bundle
ML	Medio-Lateral
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
N-S	Nigro-Striatal Pathway
PD	Parkinson's Disease
PQ	Paraquat
ROS	Reactive Oxygene Species
SD	Sprague Dawley
SL	Stepping Length
SNc	Substantia Nigra compacta
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata
STN	Subthalamic Nucleus
STR	Striatum
VMAT2	The Vesicular Monoamine Transporter 2
VTA	Ventral Tegmental Area

CHAPTER 1

INTRODUCTION

1.1 Anatomy and Functions of The Basal Ganglia with Special Focus on The Nigro-Striatal Dopaminergic System

The loss of dopaminergic neurons in SNc and DA depletion in striatum is closely related to the complex motor dysfunctions which depend on the duration of disease and extent of dopamine loss (Meredith & Kang, 2006). PD patients suffer from the four cardinal features of motor disability which are bradykinesia, muscular rigidity, resting tremor and postural instability with gait abnormalities. For example, PD patients show hypokinesia (reduced bodily movements) with poor coordination (Jankovic, 2008). In this regard, PD animal models should mimic these motor symptoms. Furthermore, the other motor symptoms (sudden, unpredictable freezing) and nonmotor symptoms (motivational disturbances and learning and memory impairments) preferably should be reflected by these models (Ferro et al., 2005; Jankovic, 2008). Since the motor behaviour of rodents is based on sensory and motivational signals which may differ from those in humans, it is necessary to take a glance at the dopamine-mediated basal ganglia circuitry in rodents (Blandini, Nappi, Tassorelli, & Martignoni, 2000; Jankovic, 2008).

The basal ganglia are a group of nuclei located at the base of the vertebrate brain and linked to a variety of functions, including voluntary motor control, procedural learning relating to routine behaviours or "motor habits", and cognitive functions (Deumens et al., 2002; Frank et al., 2006; Knowlton, Squire, & Gluck, 1994). Figure 1.1 presents the anatomical components of the basal ganglia and their organization. The main components of the basal ganglia are the striatum, or neostriatum (composed of the caudate and putamen), the globus pallidus, or pallidum (composed of globus pallidus externa (GPe) and globus pallidus interna (GPi)), the substantia nigra (composed of both substantia nigra pars compacta (SNpc) and substantia nigra pars reticulata (SNpr)), and the subthalamic nucleus (STN). As the major

input structure of the basal ganglia circuit, the striatum mainly receives excitatory input through glutamatergic projections from virtually all cortical areas, the midline and intralaminar nuclei of the thalamus and from limbic structures, particularly the amygdala, and additionally, receives dopaminergic input from SNpc neurons and VTA. Interestingly, despite of having such a rich input, striatum sends output only to other components of the basal ganglia (Blandini et al., 2000).

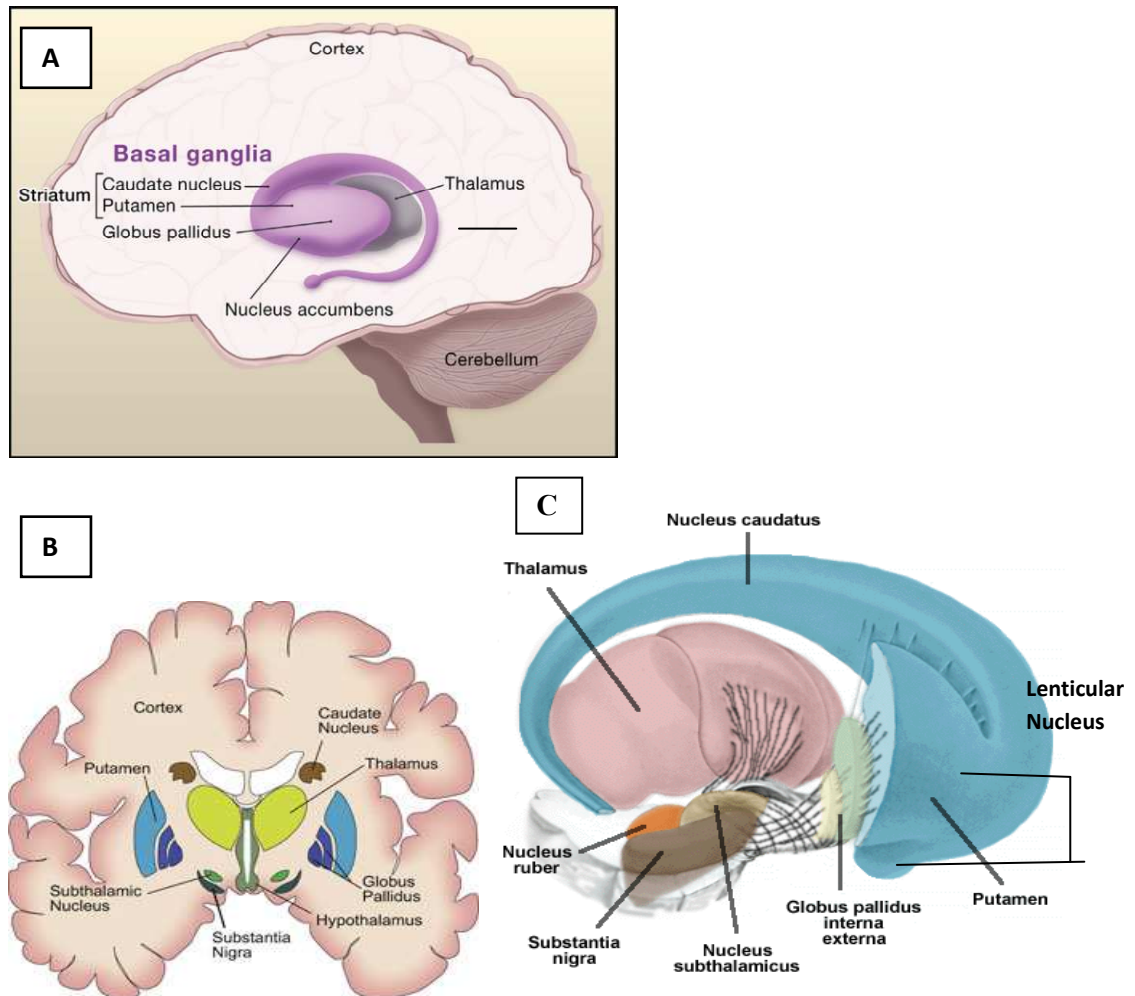


Figure 1.1. Anatomical components of basal ganglia: A. Sagittal view; B. Cross-sectional view; C. Model (Adopted from Lieberman, 2009; <http://www.dnalc.org/view/833-Basal-Ganglia-and-Learning.html>, http://drugline.org/img/term/putamen-12424_1.gif, respectively).

The nigro-striatal pathway (NS), which contains about 75% of the dopamine in the brain and suffers damage in Parkinson’s disease, constitutes an important part of basal ganglia circuitry belonging to the extrapyramidal motor system. Dopaminergic fibers originate in the

substantia nigra pars compacta also referred to as mesencephalic A9 area. They primarily project to striatum (nucleus caudatus/putamen) to largely terminate on its cholinergic and GABAergic interneurons, controls striatal release of acetylcholine (ACh) that in turn controls (generally inhibits) GABAergic striatal output to the globus pallidus (GP) and substantia nigra pars reticulata. As seen from the Figure 1.2 (normal circuitry diagram), the GP sends inhibitory output to motor-related areas, including the part of the thalamus that projects to the motor-related areas of the cortex. Normally, striatal DA is damping striatal GABAergic output through the direct pathway to the GP increasing inhibitory control of GP over the upstream motor structures (Bergman et al., 1998; Blandini et al., 2000; Shohamy, Myers, Kalanithi, & Gluck, 2008).

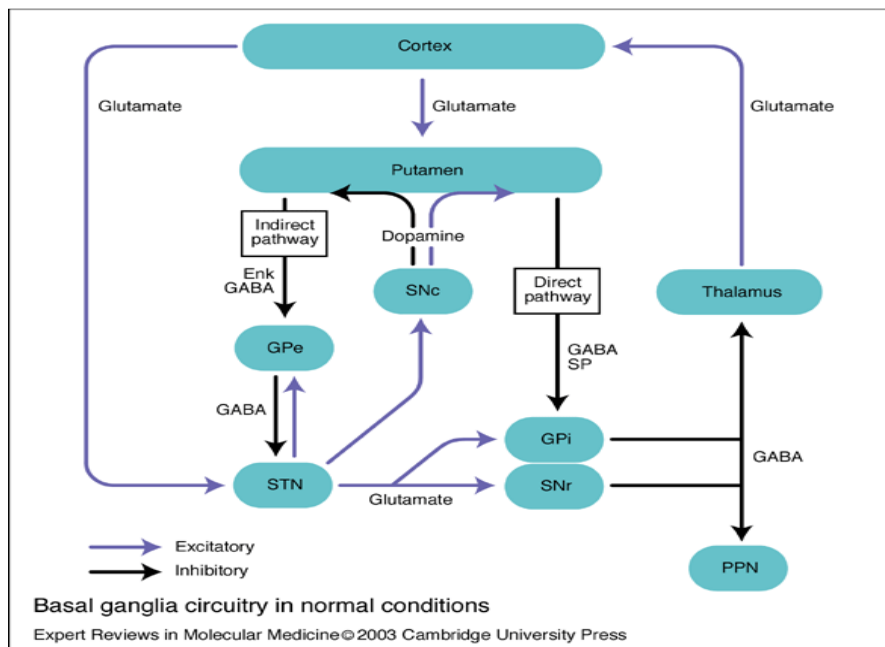


Figure 1.2. The basal ganglia circuitry (Adopted from Cambridge University Press, 2003).

The substantia nigra pars reticulata (SNpr) functions similarly to the globus pallidus. The subthalamic nucleus (STN) receives input mainly from the striatum (indirect pathway) and cortex, and projects to a portion of the pallidum (pars interna or GPi). This neurotransmitter balance is disturbed with low levels of striatal DA in PD.

As an important role of the striatum, it is the initiation point for the direct and indirect pathways of the basal ganglia motor circuit (Blandini et al., 2000; Zinger, Barcia, Herrero, & Guillemín, 2011). The direct pathway is the pathway between SNpc and striatum, and

between striatum and the internal segment of globus pallidus (GPi) and the substantia nigra pars reticulata (SNpr). These two latter structures are the projecting to thalamus and through thalamus to cortex. On the other hand, the indirect pathway consists of the striatal connections with the the subthalamic nucleus through external segment of the globus pallidus (GPe) (Zinger et al., 2011). Both pathways are heavily innervated by the dopaminergic neurons of the SNpc (Meredith & Kang, 2006). In PD, because of the degeneration of dopaminergic neurons in SNpc, DA input from SNpc into striatum decreases which results in reduction in direct pathway signal but increase in the indirect pathway signal. Decrease activity in direct pathway leads to decreasing of the disinhibition on thalamus which in turn prevents certain motor and cognitive functions. Simultaneously, the activity change in the indirect pathway increases inhibition on the thalamic neurons, as a result, motor cortex receives decreased glutamatergic input from the thalamus leading to decrease in movement which in turn results increased muscle tonus, rigidity and bradykinesia in PD patients (Zinger et al., 2011). The depletion of DA in striatum leads also to an overall decline in sensorimotor functions including a postural imbalance, deficits in forepaw and digit use, and a complete disruption of syntactic grooming (Meredith & Kang, 2006).

1.2 Parkinson Disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson as a peculiar form of progressive motor disability (Samii, Nutt, & Ransom, 2004). Today, PD is the second most common neurodegenerative disorder after Alzheimer's dementia and the incidence of the disease, according to the 2005 report of World Health Organization, has been arising day by day along with the increasing life expectancy (Bezard & Przedborski, 2011). Epidemiological studies show the increment in the prevalence of Parkinson's disease in the industrialized countries having the percentage of 0.3% of general population and about 1% of population over the age 60 years (Samii et al., 2004). Today, Parkinson's disease can be seen not only in elderly people but also in young adults and even children (von Bohlen Und Halbach, 2005). For the young people with Parkinson's disease, the initial symptoms can be observed at the ages 21-40 years while it can be even before 20 years for the juveniles (Samii et al., 2004). The first signs of the disease are sensorimotor disabilities (Meredith & Kang, 2006).

Parkinson's disease is characterized by four cardinal features including tremor at rest, muscular rigidity, akinesia (or bradykinesia) and postural instability. Also, flexed posture and freezing can be considered among the classic features of PD. Resting tremor is detected mostly in the distal part of an extremity such as hands, legs, jaw, even lips and is noted as the most frequently seen symptom of PD (Jankovic, 2008). Muscular rigidity is characterised by increased resistance to passive movements of the limb, neck, or trunk (axial rigidity). Bradykinesia is generally defined as the slowness of movement. Likewise, akinesia refers to a deficiency in the spontaneous movements such as the lack of facial expression, weight adjusting movements during sitting or difficulty with initiating a movement. In general, PD patients show decreased bodily movements (hypokinesia) and poor coordination in these movements (Meredith & Kang, 2006). As the fourth clinical feature of PD, postural instability is based on the loss of postural reflexes, and along with the freezing which is the loss of movement usually appears at the late stages of PD after other cardinal features (Jankovic, 2008).

In addition to these motor impairments, the non-motor impairments, also, should be taken into consideration in PD. Patients suffer from autonomic dysfunction, cognitive and/or neurobehavioral disorders, and sensory and sleep abnormalities (Jankovic, 2008). The most prominent non-motor impairments occurring in PD with a high incidence rates (40-50%) are the cognitive dysfunctions including impairments of learning and memory, abstract thinking, and language skills as well as emotional disorders such as depression (Deumens et al., 2002; Miyoshi et al., 2002; Santiago et al., 2010; Swainson, Rogers, Sahakian, Summers, & Polkey, 2000).

1.2.1 Pathology of Parkinson's Disease

1.2.1.1 Nigrostriatal Dopaminergic Degeneration

One of the major distinguishing pathological characteristic and seemingly the most important underlying cause of PD is the selective degeneration of nigrostriatal dopaminergic pathway (Greenamyre, Betarbet, & Sherer, 2003). This neural pathway provides a connection between substantia nigra and striatum and has an important role in controlling motor activities (Greenamyre et al., 2003; Uversky, 2004). The cell bodies of dopaminergic neurons in this pathway are located in substantia nigra pars compacta (SNpc) and their axons and nerve terminals make projections into striatum (Prou & Przedborski, 2005). Normally,

striatum receives dopamine influx from dopaminergic neurons in SNpc, however, progressive neurodegeneration of these dopaminergic neurons cause a dopamine depletion in striatum (Uversky, 2004). First, dopaminergic nerve terminals in striatum deteriorate and progressively perikaryons of dopaminergic neurons in SN degenerate (Greenamyre et al., 2003). According to neuropathological studies, the clinical symptoms of PD can be recognized when about 80% of dopaminergic neurons in SNpc disappears (Betarbet, Sherer, & Greenamyre, 2002a).

At the later phases of the disease, in addition to the SNpc pathology, a more widespread neurodegeneration is seen in the brain (Moore, West, Dawson, & Dawson, 2005). The noradrenergic neurons in the locus coeruleus and dorsal vagal nucleus, serotonergic neurons within the raphe nucleus, and cholinergic neurons within the substantia innominata and in the pedunculopontine nucleus become also affected. Eventually, degeneration in these neural structures leads to non-motor symptoms of PD such as cognition and depression (Cicchetti, Drouin-Ouellet, & Gross, 2009; Deumens et al., 2002).

1.2.1.2 Lewy Body (LB) Formation

Although there are some exceptional PD forms (such as characterized by parkin mutations somal recessive juvenile PD) with lacking Lewy Body formation (von Bohlen Und Halbach, 2005), LB formation is accepted another important pathological feature of PD (Betarbet et al., 2002a). LBs are spherical eosinophilic cytoplasmic inclusions and composed of aggregated proteins such as α -synuclein, parkin, ubiquitin, and neurofilaments (Dauer & Przedborski, 2003; von Bohlen Und Halbach, 2005). All LB aggregates include protein α -synuclein, with or without α -synuclein gene mutations (Betarbet et al., 2002a). Thus, it seems that especially the presynaptic protein α -synuclein has an important role in LB formation in PD (Uversky, 2004). Lewy bodies are commonly located in the dopaminergic neurons of SN, locus coeruleus, the dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert, but also in neocortex, diencephalon, spinal cord, and even peripheral autonomic ganglia (Zigmond & Burke, 2002).

The actual mechanisms for transformation of normal soluble α -synuclein to insoluble α -synuclein in the LBs are still not known, nevertheless α -synuclein visualization techniques are used to detect LB formation in PD cases (von Bohlen Und Halbach, 2005).

1.2.1.3 Misfolding and Aggregation of Proteins

In various age-related neurodegenerative disease, abnormal protein aggregation in and/or between neurons is seen, and it is considered as a toxic event for the brain tissue through several possible mechanism:

- Cell formation may be directly impaired by protein aggregates or
- Protein deposits may impair intracellular trafficking and cause damage on neurons
- Important proteins for cell survival may be sequestered by these inclusions

However, many of the studies, especially on the Huntington disease (HD) and other neurodegenerative diseases, show that there is no direct correlation between formation of protein aggregates and cell death (Dauer & Przedborski, 2003). Thus, it is suggested that the actual reason of cytoplasmic aggregates may come from the sequestered soluble misfolded proteins not from the simply precipitated misfolded proteins. At this point, the cellular and molecular mechanism for the active sequestering processes should be considered (Dauer & Przedborski, 2003).

Normally, misfolded proteins are first sent to chaperons and if they still lack a proper folding they are sent to proteasomes for degradation through several pathways such as ubiquitination. The formation of Lewy bodies in PD brains has revealed the question of whether there is a relationship between these cytoplasmic protein aggregates and proteasome activity, since high amounts of ubiquitin are found in LB deposits (Dauer & Przedborski, 2003; Greenamyre et al., 2003). Also, the mutations of some proteins normally involving in proteasome ubiquitination system are observed in the familial PD. Likewise, there are studies that inhibition of proteasome activity cause neuronal loss in SN. All the studies so far support the important role of proteasomal activity in PD, however, the exact relationship between proteasome and α -synuclein aggregation is still unknown (Greenamyre et al., 2003).

1.2.1.4 Mitochondrial Dysfunction and Oxidative Stress

Dysfunction in mitochondria which is a very important organelle in metabolic pathways like oxidative phosphorylation or apoptosis has been showed in many neurodegenerative disorders (Saravanan, Sindhu, & Mohanakumar, 2005). Particularly, a systemic decrease in

the complex I activity of the mitochondrial electron transfer chain (ETC) was reported in the tissues of brain, skeletal muscle, and platelets of PD patients (Alam, Mayerhofer, & Schmidt, 2004; Greenamyre et al., 2003; Sherer, Betarbet, Testa, et al., 2003). As another biochemical abnormality in pathology, damage in SN neurons of PD patients caused by free radicals like reactive oxygen species (ROS) and peroxynitrite has been observed. Also, it has been indicated that there are certain polymorphisms in the genes of complex I and mutations in some nuclear genes like PINK1 and DJ-1, which are encoding mitochondrial proteins, in developing familial PD (Greenamyre et al., 2003; Schapira, 2002).

1.2.1.5 Glial Cell Activation

Microglia are the brain's resident immune cells. In the case of an immunological stimuli or neuronal injury, microglia go into a morphological alteration and produce cytokines (Schapira, 2009) and potentially neurotoxic ROS (Sherer, Betarbet, Kim, & Greenamyre, 2003). In PD, a high microglia activation is observed in SNpc, the area with elevated levels of cytokines and the highest degeneration (Le et al., 2001; Schapira, 2009). It may be concluded that activation of microglia may contribute to the oxidative damage through ROS mechanisms (Greenamyre et al., 2003). Although the actual reasons of emergent responses and the exact role of the immune/inflammatory components are not known, it is quite probable that microglia have a role in neurodegeneration in PD (Schapira, 2009).

1.2.2 Etiology of Parkinson's Disease

Today, the exact cause of PD is unknown, however, studies indicate several factors having a potential in developing PD (Dauer & Przedborski, 2003). These factors include environmental factors, genetic factors and aging (Zigmond & Burke, 2002).

1.2.2.1 Environmental Factors

The environmental toxins were the most popular consideration in the field, especially after the discovery of the MPTP and its effects on developing parkinsonism in humans (Dauer & Przedborski, 2003). Environmental toxin hypothesis is attributed to the toxin-induced dopaminergic neurodegeneration either by chronic exposure or short-time exposure as an

initiator of deleterious events (Dauer & Przedborski, 2003). Furthermore, the studies on twins reinforced the important role of environmental factors by excluding genetic factors (Samii et al., 2004; Zigmond & Burke, 2002). Epidemiological studies on humans show that living in a rural areas, drinking well water, exposure to pesticides, herbicides, industrial chemicals, wood pulp mills, and farming increase the risk of developing PD. Trace metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide as well as tetrahydroisoquinolines and beta-carbolines are shown as the risk factors of PD (Gao, Hong, Zhang, & Liu, 2003; Schapira, 2009). However, a specific toxin causing sporadic PD in humans is still not known (Dauer & Przedborski, 2003).

1.2.2.2 Genetic Factors

Genetic factors may also have contribution to the development of PD, however, only about 15% of PD patients have familial PD history and generally, there is a lack of a significant inheritance pattern (Alam et al., 2004). Twin studies found concordance at very low rates and the further studies on twins in the context of age and disease onset suggest that genetic susceptibility plays a significant role primarily in the early-onset of the disease (Samii et al., 2004).

Most recent studies indicate the importance of specific mutations in the disease development and pathogenesis. Based on the hypothesis of mitochondrial complex I impairment in the SNpc of PD patients, there were conducted many experiments on the mutations in the mitochondrial genome. However, no specific mitochondrial mutation or in other words a maternal pattern of inheritance was detected in PD. Although studies discovered the importance of α -synuclein in PD, especially in the LB formation, no particular mutations in the α -synuclein gene have been identified in patients with sporadic PD. It suggests that not the gene mutation but rather the accumulation of the gene product may play a central role in the PD development. It is necessary to note that PD may be caused not only by a single environmental or genetic factor but by interactions of both factors, which is called the “double hit hypothesis” (Zigmond & Burke, 2002).

1.2.2.3 Aging

PD is seen in the late middle age and its prevalence getting higher at the older ages. However, aging itself is not the main actor in the neurodegeneration of the nigro-striatal dopaminergic pathway. This claim may be supported by two findings:

- I. The specific regions of cell losses differ in the aging and PD brain. For example, in the aging brain cell losses mainly belong to the dorsal tier of the SNpc while in the PD brain neurodegeneration is commonly seen in the lateral ventral tier.
- II. A microglia activation is much higher in PD than during physiological aging.

In conclusion, aging is an obvious risk factor for PD development through a non-direct way, however, this exact role in the disease pathogenesis has not been illuminated yet (Samii et al., 2004; Zigmond & Burke, 2002).

1.3 Animal Models of Human Neurological and Psychiatric Disorders

1.3.1 Purpose of Animal Models

To build an animal model, the primary step is to determine and define the purpose of the model (van der Staay, Arndt, & Nordquist, 2009). For example, animal models are very important in studies of pathogenesis, mechanism or therapeutic approaches of human diseases (Betarbet, Sherer, & Greenamyre, 2002b). It should provide elucidative aspects about, for example, underlying mechanisms in normal and impaired behaviour or molecular and cellular cascades of the target disease. Also, the model should bridge between preclinical animal studies and clinic studies of patients and provide new approaches for drug action targets, pathways and mechanisms, and approaches for treatments with extensive advantageous and disadvantageous (van der Staay et al., 2009).

1.3.2 Validation of Animal Models

After the process of defining purpose(s), which mostly depends on relation between brain and behaviour in these studies, the models is developed and tested. In the evaluation stage, model must encapsule several scientific criteria such as replicability/reliability (internal validity), face validity, predictive, construct and external validity/generalizability of the

model (van der Staay et al., 2009). At this point, it is good to provide a definition for the validity of an animal model.

Validity is the evaluation process of model based on the interpretation of the results revealed from the model. Of note, none of the animal models are considered totally valid in all parameters but limited to a specific aspect. The model should be flexible in the sense of discussion and new assessment. Here below, a brief presentation of validation criteria of animal models of human psychiatric and neurological disorders:

- **Reliability and Replicability, Internal Validity:** Replicability is the reproducibility of measurements or results obtained in an animal model. In other words, it is the corresponding of the results achieved from the same experiment by independent and/or different laboratories. Reliability is to assess an instrument which means how much a device or method evaluated or tested is reliable/consistent. In this regard, internal validity is the quality of the experimental evaluation of the animal model. For example, it looks for an answer for how well a study was performed or how strictly putative confounding variables were controlled and so on. Of note, it would be nonsense to talk about an external validity/generalizability of a study outside the laboratory unless it is not proved having valid results within lab (internal validity) (van der Staay et al., 2009).
- **Face Validity:** Face validity is simply to value the similarity of behaviour of modelled animal to the behaviour revealed in humans (Brooks & Dunnett, 2009). For example, in a PD animal model, a bradykinesia test should reflect a similarity between the abnormal movements exhibited by modelled animal and humans having specific behavioural disorder (van der Staay et al., 2009). Even if face validity seems a desirable criterion to evaluate a model, there are several drawbacks that eliminate the necessity of it. For example, similar behaviours may implicate different functions, different behaviours may implicate the same function or there may be different physiological states underlying the same behavioural dysfunction in which all depend on the animal (van der Staay et al., 2009). Face validity present a superficial similarity in symptomatology between the model and the disorder. Therefore, it is not an obligation to evaluate a model.

- **Predictive Validity**: It provides a prediction about the expected behaviour in the modeled animal considering the similar response in human under the same manipulation or let's say situation. For example, it can make estimations about the effects of a particular experimental manipulation on different species, different conditions or different time points of manipulation. Therefore, development of animal models and the clinical measures should continue in parallel for fair comparisons (van der Staay et al., 2009).
- **Construct Validity**: It reflects the evaluation on the mechanism underlying behavior in the model and in the disease (Brooks & Dunnett, 2009; van der Staay et al., 2009). Actually, it addresses how much of the theoretical hypotheses tested fit to the practical results (manipulations and measurements) derived from the model. It is collectively accepted as the most important criterion for animal models (van der Staay et al., 2009).
- **External validity/Generalizability**: Here the idea is application of an animal model across different species, environments or with different parameters. It is not just to repeat the previous studies but to extend the scope, deepen the knowledge, and to allow the generalisation about obtained results. The external validity depends on the experimental process to be evaluated. Beside, internal validity limits the range of test conditions and enhances the explanatory power, still compromise external validity/generalizability. Whether internal validity or external validity is used, generalizability/external validity should be taken into consideration in model building process (van der Staay et al., 2009).

1.3.3 Models of Parkinson's Disease

Parkinson's disease does not spontaneously occur in animals, therefore there is a necessity to create animal models of PD which allow to investigate and better understand the etiology and pathogenesis of this disease and to improve the therapeutic approaches (Jackson-Lewis, Blesa, & Przedborski, 2012; Uversky, 2004).

In order to constitute an ideal animal PD model, three important hallmarks of human PD should be reproduced. These are (1) the selective loss of dopaminergic neurons, (2) DA depletion in striatum, and (3) formation of LB-like inclusions in DA neurons (Uversky,

2004; von Bohlen Und Halbach, 2005). Also, the behavioral hallmarks of human PD including akinesia, rigidity and resting tremor should be generated (von Bohlen Und Halbach, 2005). Unfortunately, up to now, none of the animal models is covering all the features of human PD. Therefore, it is important to carefully analyze the advantages and disadvantages of already existing animal models of PD and to choose the ones which are the most beneficial for the studies on PD pathophysiology.

There are two general categories for the currently available animal models of PD, namely neurotoxic models and genetic models (Duty & Jenner, 2011). Neurotoxic models for a variety of uses are further divided into several sub-categories such as pharmacological models, classical toxin-induced models, and pesticide/herbicide-induced models (Duty & Jenner, 2011). Below a brief description of all models is provided with a special focus on two models: 6-OHDA and rotenone models, which have been used in the present study.

1.3.3.1 Neurotoxic Models

There are different toxin-induced animal models which work either through Complex I inhibition and reactive oxygen species (ROS) production or through the modulation of DA neurotransmission, both mechanisms finally leading to dopaminergic neuron degeneration (Meredith, Sonsalla, & Chesselet, 2008). All the models have advantages and disadvantages which will be discussed below.

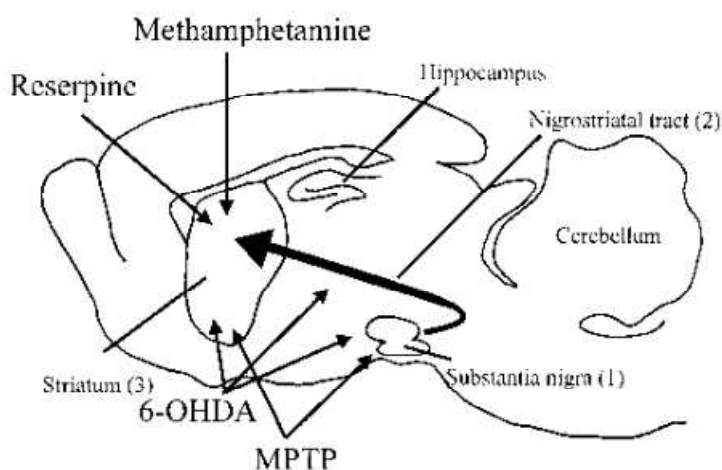


Figure 1.3. Neuroanatomic lesion sites of rodent brain in PD animal models induced by different toxins. The dopaminergic neurons of the substantia nigra project axons and their neurotransmitter dopamine (large filled arrow) to the striatum (Adopted from Tolwani, Jakowec, Petzinger, Green, & Waggie, 1999).

1.3.3.1.1 Pharmacological Models

1.3.3.1.1.1 Reserpine

In PD research, reserpine was the earliest pharmacological PD model and the pioneer of displaying the reversing effect of L-DOPA on PD-induced akinesia. Hereby, the importance of striatum dopamine in the motor behavior was discovered. This model also contributed to the drug-development studies against the PD symptoms, specifically, to the reduction of the monoamine depletion effect.

Reserpine inhibits monoamine transporter of synaptic vesicles (VMAT2) through magnesium- and ATP-dependent mechanisms and in this way blocks the uptake and storage of monoamines such as dopamine, noradrenaline and serotonin in synaptic terminals. Thus, it is possible to say that reserpine model has a resemblance with the biochemistry of PD. Administration of reserpine depletes dopamine in SNpc and striatum by ~85% and ~95%, respectively. Behavioural symptoms observed after reserpine administration in rats include akinesia and hind limb rigidity which are similar to the motor symptoms in human PD patients, yet the effects of reserpine are short-lasting due to DA renewals in 24 h. This acute effect of reserpine application is not associated with dopaminergic cell degeneration in SN therefore the reserpine model is not a favored PD model (Duty & Jenner, 2011; Tieu, 2011).

1.3.3.1.1.2 Haloperidol

Haloperidol is dopamine D2 and D1 receptors blocker commonly used as a tranquilizer and/or antipsychotic drug. At high doses, within an hour after injection, it causes muscle rigidity and catalepsy. Long-term drug administration is required to produce PD-like motor disorders (Duty & Jenner, 2011).

1.3.3.1.1.3 Methamphetamine

The amphetamines are psychostimulatory drugs having high addiction potential (Betarbet et al., 2002a). Methamphetamine (METH) is an amphetamine derivative of increased psychostimulant potential (Tieu, 2011). METH alike amphetamine by interacting with both, the specific neuronal synaptic vesicle uptake transporter (VMAT2) and the dopamine transporter (DAT) in presynaptic membrane, triggers reverse transport of DA from the vesicle to the

cytosole and from the cytosole to the synaptic cleft which leads to highly increased DA release into the extracellular space and in turn, causes acute effects on behaviour like increased locomotor activity. In the long term it may cause DA depletion and PD-like symptoms. However, there is a lack of dopaminergic neuron degeneration and LB-like inclusions formation which makes that this model is not a preferable one in PD studies (Betarbet et al., 2002a; Tieu, 2011).

1.3.3.1.2 Classical Toxin-induced Models

1.3.3.1.2.1 6-OHDA

6-Hydroxydopamine (6-OHDA) is an analog of dopamine and is endogeneously present in human brain and urine samples (Tieu, 2011). 6-OHDA model was the first neurotoxin-induced animal model of PD causing neurodegeneration of dopaminergic neurons in SNpc (Dauer & Przedborski, 2003). 6-OHDA selectively induces neurotoxicity in the monoaminergic neurons because these kind of neurons contain dopaminergic and noradrenergic transporters on the plasma membrane having high affinity to 6-OHDA which makes them open to neural damage (Tieu, 2011). Once 6-OHDA enters the neuron, it accumulates in the cytosol and being oxidized leads to increased ROS and quinines production which, in turn, through oxidative stress mechanisms, inactivate biological macromolecules, reduce antioxidant enzyme levels in striatum and increase iron levels in SN (Dauer & Przedborski, 2003; Duty & Jenner, 2011; Tieu, 2011). This elevated iron interacts with the Complex-I and Complex-IV of mitochondria and leads to an inhibition of the respiratory chain and further oxidative stress. These mechanisms of 6-OHDA toxicity are considered as the pathological events of human PD, therefore, it makes the model applicable (Duty & Jenner, 2011).

With systemic (peripheral) administration, 6-OHDA does not induce a nigrostriatal damage but destroys cells in the peripheral nervous system. The main reason of this is the inability of 6-OHDA to cross the blood-brain barrier (BBB) (Prou & Przedborski, 2005). For that reason, it must be directly administered into the brain by stereotaxic injection. The injection can be made into the SN, medial forebrain bundle (MFB), which contains the dopaminergic nigrostriatal fibers and the striatum (Blesa, Phani, Jackson-Lewis, & Przedborski, 2012; Dauer & Przedborski, 2003). However, direct injection of the toxin requires the stereotaxic

surgical instruments and a special training for the surgery, thus these factors become the drawbacks of the model (Duty & Jenner, 2011).

The main factors determining the lesion magnitude and temporal pattern of degeneration are the dose, the site, and the way of injection of 6-OHDA, as well as the animal species used in the model (Blesa et al., 2012). For instance, by adjusting the dose of 6-OHDA more than 90% of DA neurons can be destroyed (Duty & Jenner, 2011). Injections into SN or MFB produce complete and rapid (within 24 h) degeneration of dopaminergic neurons lacking apoptotic morphology, while injections into striatum lead to a partial retrograde neurodegeneration of neurons in nigrostriatal pathway in a slower, progressive manner throughout 1-3 weeks (Dauer & Przedborski, 2003; Tieu, 2011). It is considered that the second route of administration is more similar to human PD with its progressive and partial lesion features, and producing non-motor symptoms. Among the most important model characteristics is the way of injection which can be bilateral or unilateral. The bilateral injection of 6-OHDA may produce severe aphagia, adipsia, and seizures which ultimately cause death. Therefore, unilateral injections which produce less severe brain damage and give an opportunity to use the intact side as an internal control have been more commonly used. Unilaterally lesioned animals demonstrate asymmetric circling behaviour confirming the lesion accuracy (Bezard & Przedborski, 2011). However, bilateral damage to the N-S pathway better simulates the neurodegeneration in human PD patients. 6-OHDA model has been performed on many species such as mice, rats, cats, dogs and non-human primates, yet, the best simulated and the most commonly used species is the rat (Emborg, 2004).

The 6-OHDA model provides dopamine and tyrosine hydroxylase depletion in the striatum which is consistent with the biochemical features of human PD. In the 6-OHDA model alike in human PD patients, an activation of microglia and ongoing inflammation are also observed (Blesa et al., 2012). In contrast to human PD pathology, 6-OHDA PD model does not include any effect on the regions like lower brain stem areas or locus coeruleus. Most importantly, it does not produce protein aggregates or Lewy body-like inclusions which are the pathological hallmarks of PD (Betarbet et al., 2002a; Blesa et al., 2012). Despite all its drawbacks, 6-OHDA PD model has been utilized for many purposes such as cell transplantation and neurotrophic factor studies (Betarbet et al., 2002a).

1.3.3.1.2.2 MPTP

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) is a lipophilic protoxin, therefore it can rapidly cross the BBB in systemic administration. It is called protoxin because MPTP needs to be metabolized into 1-methyl-4-phenylpyridinium (MPP⁺) by the enzyme monoamine oxidase-B (MAO-B) to reveal a toxic effect (Emborg, 2004; Tieu, 2011). This conversion takes place in the astrocytes of SN and striatum and the MPP⁺ product is taken up by the dopaminergic neurons and terminals in these regions through DA transporter system (Tieu, 2011). Once in the cytoplasm of the neuron, MPP⁺ leads to ROS production. However, its main toxic effect is based on the accumulation within the mitochondria and inhibiting Complex I of the electron transport chain which causes deficiency in mitochondrial respiration.

MPTP model has been tried on many species, however many of these species including rat were found insensitive to this neurotoxin. Only specific strains of mice such as black C57 and Swiss Webster are found suitable for the model (Duty & Jenner, 2011). Today, monkey is approved as a gold standard MPTP-induced PD model used for preclinical and therapeutic studies (Tieu, 2011). Considering in terms of PD, the MPTP model has many advantageous like providing nigrostriatal dopaminergic neurodegeneration, behavioral deficits, decrement in striatal dopamine levels and some other biochemical changes seen in PD. Also, with this model there is no need to use high-skilled stereotaxic surgery. However, lack of Lewy body formation and induction of an acute not a progressive type of PD are shown as limitations of this model (Duty & Jenner, 2011).

1.3.3.1.3 Pesticide/Herbicide-induced Models

The epidemiological studies show that there is a direct relationship between emergence of PD and environmental factors including living in rural areas, farming, drinking well water and exposure to agricultural chemicals. It has been shown that some commonly used pesticide/herbicide may trigger late-onset PD. This became a reason for developing pesticide- and herbicide-induced PD models. The prominent pesticide/herbicide models used so far are rotenone, paraquat and maneb (Uversky, 2004).

1.3.3.1.3.1 Rotenone

Rotenone is not only a herbicide but it is also an insecticide. As a pesticide, it is used for poisoning unwanted fish in lakes and for killing insects (Blesa et al., 2012; Tieu, 2011). Rotenone is naturally found in the plants of Leguminosa family, hence it has been used in organic farming. It has a 3-5-day long half-life and under exposure to the sunlight it rapidly decays in the soil or in the water which eliminates it from the list of ground/water pollutants.

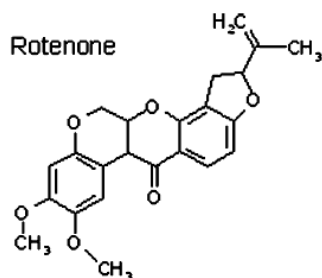


Figure 1.4. Chemical structure of rotenone (Adopted from Uversky, 2004).

Similar to the neurotoxin MPTP, rotenone is highly lipophilic, that's why it can easily pass through the BBB (Tieu, 2011). However, unlike MPTP or 6-OHDA (DAT), it does not depend on dopamine transporters for its action and as such causes a uniform inhibition of mitochondrial Complex-I in the entire brain (Betarbet et al., 2002a). Following its accumulation within mitochondria, it triggers ATP depletion, ROS production and glutathione depletion, all of which lead to the oxidative stress. In rats, rotenone-mediated oxidative damage was reported in striatum but also in midbrain, olfactory bulb, and cortex (Duty & Jenner, 2011). Rotenone-induced neurodegeneration was reported to have a progressive character (Duty & Jenner, 2011) opposite to the rapid depletion of DA by 6-OHDA (Betarbet et al., 2002a). These results well replicate the human PD symptoms. Another important feature of this model is an extensive microglial activation in SNpc and striatum and inhibition of proteasome activity, both characteristic to the PD in man (Duty & Jenner, 2011). In contrast to 6-OHDA or MPTP models, in rotenone model of PD, nigrostriatal dopaminergic neurodegeneration is accompanied by formation of α -synuclein- and ubiquitin-positive Lewy body-like cytoplasmic inclusions (Uversky, 2004). Rotenone model supports the idea that environmental factors may have a role in PD pathogenesis (Uversky, 2004). Although some researchers classify this feature as a limitation of the model, there is a high variability between animals in the magnitude of the lesion produced by rotenone administration. Actually, this individual variation in the susceptibility to rotenone

arises from genetic differences and it points towards the interplay between genetic and environmental factors in PD induction (Tieu, 2011; Uversky, 2004).

Administration of rotenone via i.v. mode causes decrements in DA levels in striatum especially in the Sprague Dawley (SD) and Lewis (L) rats, but at unknown percentages (Cicchetti et al., 2009). Still, i.v. rotenone administration generates substantial neurochemical and behavioral deficits (Blesa et al., 2012). It has been demonstrated that chronic i.v. injections of rotenone cause degeneration of dopamine terminals in striatum, formation of LB-like structures (Duty & Jenner, 2011) and in most of the studies, model has induced motor impairments (Cicchetti et al., 2009). Similarly, the i.p. administration of rotenone, also, leads to DA decreasing, but this time it has been showed that the decrement is about 25-50% in striatum. Also, it has been showed dopaminergic terminals in striatum (Duty & Jenner, 2011). The studies on motor deficits come from the experiments conducted especially in the SD and L rats (Cicchetti et al., 2009) and indicate clear motor abnormalities such as reduced mobility, flexed posture, muscle rigidity and even catalepsy (Prou & Przedborski, 2005). This route of administration, also, produce α -synuclein and polyubiquitin aggregates. Eventhough there is no enough comprehensive and consistent results for the oral and intranasal routes in the animal models of PD (Cicchetti et al., 2009), studies show that oral rotenone administration cause little neurotoxicity (Tieu, 2011). Absorption in the stomach and intestine is slow and not complete, additionally, a break down occurs in the liver. All together, this route is not very effective in inducing any behavioral or pathological features of PD (Prou & Przedborski, 2005). On the other hand, s.c. administration has been studied in many species and strains by numerous labs. While all the studies indicate producing of motor deficits and dopaminergic neuron loss in SN, plus DA decrements in striatum and α -synuclein and polyubiquitin aggregates, the outcomes of the percentage in these disruptions are inconsistent (Cicchetti et al., 2009). Unfortunately, the most common and the important disadvantageous for these systemic route of administrations is that large variations occur in animal sensitivity and variations in motor response (Meredith et al., 2008). Additionally, systemic injection of rotenone as the weak point of the model produces systemic adverse effects including cardiac, stomach and liver problems, thereby high mortality rates (Cicchetti et al., 2009). Conjunctively, there are controversial claims that the changes in the motor behaviour in these models may not be induced by a specific nigrostriatal dopaminergic degeneration, but it may be this systemic complications (Cicchetti et al., 2009). Differently, the intracranial route of administration provide a site-specific injection of the toxin, by this way, it offers a site-specific degeneration in the brain.

Consistently, rotenone injection into SN, MFB, STR and N-S regions in SD rats induce striatal DA decrements more than 50% percentage (Cicchetti et al., 2009), also, studies of the intracerebral injection of rotenone into MFB or SNc show neurochemical and neuropathological features of hemiparkinsonism in rats (Xiong et al., 2009). Particularly, bilateral MFB lesion result in increases of the descent latency in catalepsy and significant decreases in locomotor activity, headdips and increased inactive sitting (Alam et al., 2004). Moreover, stereotaxic infusion of rotenone produces histopathological features of PD, including Lewy bodies structures (Xiong et al., 2009).

In the intracerebral infusions, bilateral or unilateral lesion models can be generated. However, the bilaterally lesioned animals develop PD that is more similar to human PD with respect to biochemistry eventhough unilaterally lesioned models present behavioural impairments that can be well identified and evaluated such as drug-induced rotations (Sindhu et al., 2006). However, it is the fact that human PD affects the brain bilaterally. Also, in bilateral lesion models, target regions at both sides of the brain are influenced by the toxin, so there is no compensatory site (intact) for the affected site. Although the rotational behaviour is considered as the gold standard test in unilateral PD models, studies show that bilateral 6-OHDA lesions cause motor deficits that can be evaluated by more behavioral motor paradigms than unilateral 6-OHDA lesions (Deumens et al., 2002). Why is that not possible in the bilaterally lesioned rotenone models? As a good opportunity, bilateral models give chance to investigate higher cognitive tasks (e.g. choice reaction-time task).

As mentioned earlier, the severity of the rotenone effects depends on the rout of its administration. It naturally depends also on the rotenone dose and duration of application (Duty & Jenner, 2011; Prou & Przedborski, 2005; Tieu, 2011). The strain of rats used in this model also seems to play an important role. Among different rat strains, Wistar, Spraque Dawley and Lewis strains were found more susceptible compared to other strains to the rotenone adverse effects.

1.3.3.1.3.2 Paraquat and Maneb

Paraquat (PQ) or N,N'-dimethyl-4,4'-bipyridinium is a widely used herbicide and structurally very similar to MPP⁺. However, unlike MPP⁺ which is a Complex-I inhibitor, PQ executes oxidative stress through revealing ROS such as superoxide radical, hydrogen

peroxide and hydroxyl radical, consequently exerts a harmful effect (Jackson-Lewis et al., 2012).

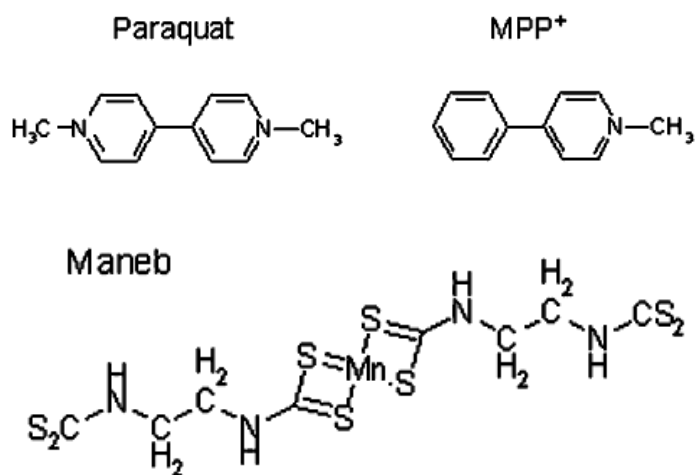


Figure 1.5. Chemical structures of paraquat, MPP⁺ and maneb (Adopted from Uversky, 2004).

By using neutral amino acid transporters, PQ can cross the BBB, but not as rapidly as MPTP (Betarbet et al., 2002a; Tieu, 2011). Noteworthy, BBB permeability to PQ changes with age in such a way that BBB permeability is seen higher in young and old animal (Tieu, 2011). While some researchers claim the absence of changes of DA levels in striatum, systemic administration into mice was reported to induce dopaminergic neuron loss in SN and deteriorated motor performance (Jackson-Lewis et al., 2012).

Many different herbicides and pesticides are being used in agriculture, and during search for their proparkinsonian effects, the attention has been directed to the fungicide maneb (manganese ethylenebisdithiocarbamate) which is used in the same areas as PQ (Betarbet et al., 2002a; Uversky, 2004). It has been found that maneb can reduce the locomotor activity (Betarbet et al., 2002a). Indeed, administration of both PQ and maneb together causes much greater damage in the dopaminergic neuron terminals in striatum and cell bodies in SN and results in changes in the striatal DA levels. Also a greater impairment in locomotor activity is observed (Uversky, 2004). These results indicate synergistic effects of environmental toxins in PD pathogenesis (Betarbet et al., 2002a).

1.3.3.2 Genetic Models

Genetic models of PD are the newest members of the PD research. The most common form of PD referred to as a sporadic PD is not associated with any genetic defects. However, about 10% of all PD cases referred to as familial type of PD shows genetic mutations (Bezard & Przedborski, 2011). Up to now, there have been discovered 13 loci, and 9 genes related with both autosomal dominant and autosomal recessive forms of parkinsonism. Basing on this knowledge, different genetic animal models of PD can be generated. Both the sporadic and familial PD is characterized by formation of alpha-synuclein containing LBs and degeneration of DA nigrostriatal pathway resulting in DA depletion in striatum (Bezard & Przedborski, 2011; Blesa et al., 2012).

It needs to be addressed that mutations using in the models are not overexpressed or knocked out in the human PD. The reason of using them in the models is to investigate the relation between the expression amount of a specific protein and the effect on its function. For example, transgenic mice knocking out alpha-synuclein does not show any effect on dopaminergic neuron development or maintenance (Jackson-Lewis et al., 2012). On the other hand, transgenic mice with overexpressing human alpha-synuclein exhibits many features of PD like loss of dopaminergic terminals in striatum, formation of LB-like structures and motor abnormalities (Betarbet et al., 2002a). Nonetheless, more excessive research on the actual role of alpha-synuclein in PD is required (Jackson-Lewis et al., 2012).

Briefly, there are various animal models of PD developed to better understand the pathogenesis and to investigate novel therapeutic approaches for the disease (Blesa et al., 2012). Each model has its own advantageous and disadvantageous. For example, among the toxin-induced models, some offers pathological symptoms like Lewy Body inclusions or some offers DA depletion in progressive manner while genetic models offer specific contributions of genes or proteins. Through the toxin-induced destruction of the nigro-striatal pathway in PD models, screening drugs for symptomatic treatment of the disease (Duty & Jenner, 2011) or novel dopaminergic approaches to treatment is possible (Blesa et al., 2012); and by the genetic models such as transgenic or knockout models, further evaluation of the genetic basis in disease is achievable (Blesa et al., 2012). However, there has been found no exact animal model of PD reflecting the progressive nature of the illness and its complexity in terms of the extent of pathology and biochemical changes (Duty & Jenner, 2011).

Therefore, it is needed much more progress to make an ideal model with the features of combination of neurotoxin-induced and genetically induced models to mimic Parkinson's Disease and further investigation of its nature.

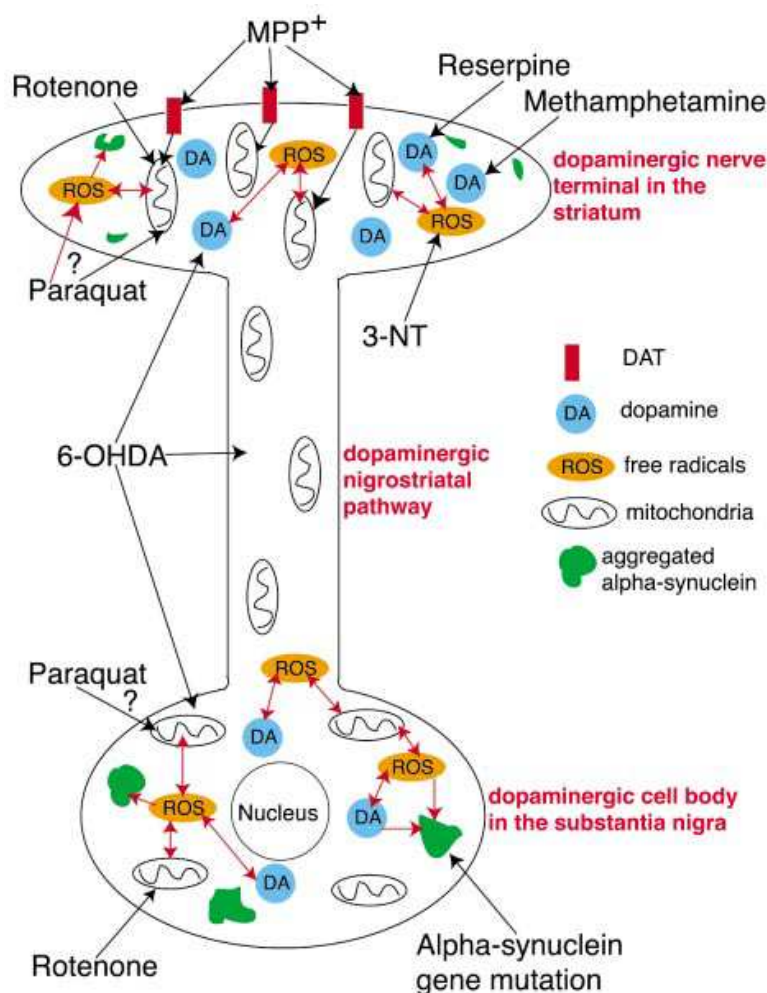


Figure 1.6. Molecular mechanisms of pharmacological agents and genetic manipulations used to develop rodent PD models through nigrostriatal degeneration and striatal DA depletion (Adopted from Betarbet et al., 2002).

1.4 Behavioral Analysis

1.4.1 Locomotor Activity Test

By definition, the locomotor behaviour of animal is the all acting of moving from one place to another. It includes the activity of movement initiation, turning and climbing, exploratory behaviour, walking and swimming and circadian activity (Whishaw, Haun, & Kolb, 2004). Among these, the simplest test of locomotor activity is putting an animal in a small field or an open environment and observing and recording the movements in this arena which is, also, called as open-field test with minor differences (Brooks & Dunnett, 2009; Whishaw et al., 2004). When the animal is removed from its home cage and placed in to a new arena, it, first, pauses then starts exploring the new environment by turning, rearing, and walking. This exploratory behaviour begins from the edge of the arena (tigmotaxi) then normally spreads towards the center of the arena. During this exploratory test, the number of trips, duration of trips, their total distance, velocity of movements, number of rears, etc. can be measured (Whishaw et al., 2004).

On the other hand, another feature of the test comes up in the course of time which is habituation. In this context, the habituation means the reduction in the locomotor activity due to exposing new environment, plus reducement in anxiety levels revealing more activity toward center of the arena (Brooks & Dunnett, 2009, Jakubowska-Dogru, 2006). In the studies of toxin-induced PD models, the locomotor activity test is the most preferred one to detect the alterations of behavioral activity (Sedelis, Schwarting, & Huston, 2001).

1.4.2 Catalepsy Test

Catalepsy is defined as being unable to correct an externally imposed posture which expresses itself as frozen or motionless postures, akinesia, bradykinesia, and/or tonic grasping (Alvarez-Cervera et al., 2005; Sanberg, Bunsey, Giordano, & Norman, 1988). A normal animal corrects its unusual posture within seconds while a cataleptic animal keeps this unusual posture for a much longer time (Sanberg et al., 1988). Since catalepsy is a commonly seen, strange symptom in pathologies of many states such as catatonic schizophrenia, some forms of brain damage (e.g., basal ganglia damage) and parkinsonism, and also, it is a highly preferred behavioural tool in the studies of behavioural mechanisms of

neurochemical systems, it has been used in the most of the pharmacological animal models of PD (Sanberg et al., 1988). For example, in these models, catalepsy was used to study the neurochemical mechanisms of extrapyramidal function through catecholaminergic damage. It has been showed that striatal DA depletion is closely related to the increasing cataleptic behaviour and/or akinesia. Particularly, it was observed that catecholamine depletion or blockade induce inactivation in horizontal displacement, locomotion, head-orienting, head-scanning, and mouthing behaviours (biting and licking) (Sanberg et al., 1988; Schallert, Whishaw, De Ryck, & Teitelbaum, 1978).

1.4.3 Rearing Test

The rearing test (or cylinder test) was first developed to test the forelimb impairments in the 6-OHDA-induced rat model of PD (Brooks & Dunnett, 2009). These studies proved that the rearing test to be a simple and efficient test for the forelimb use after unilateral lesions to the N-S system (Brooks & Dunnett, 2009). The aim of using a cylinder-shaped box is to promote vertical exploration of the walls with the forelimbs (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). The test is short-lasting (5 min) and does not require a special training, thus, it is easy to perform.

1.4.4 Stepping Test

Stepping test was introduced by Schallert et al. (1992) as a detection test for motor initiation deficits in the forelimbs, similar to limb akinesia and gait problems such as those in PD patients (Fang et al., 2006; Olsson et al., 1995, Olsson, Nikkhah, Bentlage, & Björklund, 1995). The stepping test measures initiation of movement, the length of each step during locomotion, and the number of adjusting steps under postural imbalance (Fang, Sugiyama, Akamine, & Namba, 2006; Olsson et al., 1995). This test results were reported to show a close correlation with the deficiency in DA levels and the generated motor abnormality (Paillé, Henry, Lescaudron, Brachet, & Damier, 2007).

1.4.5 Rotarod / Accelerod Test

The rotarod is considered as the most popular test for the gross motor function. It is based on staying of an animal on a revolving rod and gross motor skills and coordination are analyzed by measuring the latency to falling off the rod (Avoli, 2002, Sedelis et al., 2001). The rotarod with stable speed was first introduced by Dunham and Miya (1957) to test neurological deficits in rodents, then test was developed to an accelerated rod to remove the extensive training for the animal. The use of rotarod test is widespread, especially in the rodent models of human disease including Parkinson's Disease (Monville, Torres, & Dunnett, 2006).

Deficits in sensorimotor behaviour induced by progressive DA cell loss in SN or striatal DA depletion was previously shown to negatively influence the performance of the animal in this test (Gambhir, Mathur, & Behari, 2011; Sedelis et al., 2001). It has been used in the studies examining:

- The effects of lesions of dopaminergic systems on motor behaviour (Meredith & Kang, 2006);
- Evaluation of pharmacological therapies of Parkinson's disease (Rozas, Guerra, & Labandeira-García, 1997);
- Effects of tissue grafts in PD models (Meredith & Kang, 2006; Rozas, Guerra, & Labandeira-García, 1997).

1.4.6 Probabilistic Learning Test

Decision-making is very important to maintain our daily life. However, some of the psychiatric disorders like substance abuse, pathological gambling, schizophrenia, eating disorders, attention-deficit hyperactivity disorder, obsessive-compulsive disorder, chronic pain and Parkinson's disease occur impairments in decision-making which prevent making profitable long-term decisions (de Visser et al., 2011).

The main feature of the decision-making is its necessity of evaluation of multiple response options and then selection of the optimal response. Two outcomes as reward and punishment can be used to characterize the response option. More specifically, the response option may be shaped by the factors like;

- the magnitude of reward and punishment,

- the probability of receiving reward or punishment,
- the delay to reward or punishment (Clark, Cools, & Robbins, 2004).

The decision-making impairments may be manifested as an increased sensitivity to reward or reduced sensitivity to punishment, inability to avoid rewards with long-term disadvantageous or the preference for a small immediate reward over a larger but delayed reward. In this context, there are two behavioural tests being conducted in humans to investigate neuropsychological basis of the decision-making which are the Iowa Gambling Task (IGT) and the Cambridge Gamble Task (CGT) (Clark et al., 2004). Yet, the mostly used test among these two is the IGT because it mimics the complexity of the human choices made in everyday life. At first, IGT was used for the cognitive deficits in the people with prefrontal cortex damaged, then it has been found that damage in other brain regions like amygdale and insula may also cause similar cognitive deficits (de Visser et al., 2011, Maddox & Filoteo, 2001). Neuroimaging studies also showed an increased activity in striatum in healthy individuals, and decreased activity in this structure in PD patients subjected to the probabilistic classification learning task (Maddox & Filoteo, 2001).

In the Iowa Gambling Task, the advantageous behaviour is the preference of low immediate rewards but having a higher net gain in the long run over high immediate rewards with higher net loss in the long term (Clark et al., 2004). In brief, the IGT is designed in such a way that it contains lowered predictability of the consequences of a choice, the need to weigh short- and long-term gains and losses, and the necessity to exert behavioural control to maximize gains in the long-term (de Visser et al., 2011).

1.5 Aim of The Study

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic nigral neurons and striatal dopamine. Animal models of PD are very important because they allow studies which are impossible to perform in patients and which are essential for better understanding the etiology and pathology of PD. They are also used for the preclinical testing of candidate therapies. As discussed earlier, there are many different PD models, however, none of them is fully reproducing the disease symptoms. In addition, different behavioral measures are used by investigators which makes even more difficult to compare the results. The aim of the present study was to compare the

motor and cognitive deficits (if any) in two most common pharmacological models of PD: the Rotenone and 6-OHDA model, using a large battery of neurological tests and a probabilistic learning task. Neurological tests applied in this study screened deficits in animals' motor performance and sensorimotor coordination. Motor impairments are considered the primary behavioral criterion for the animal PD models. The probabilistic learning task tested potential deficits in animals' learning skills and decision making claimed to be also affected in PD. To avoid peripheral effects, both neurotoxins were applied at relatively low doses, bilaterally, directly into the SNpc brain area.

CHAPTER 2

MATERIALS AND METHODS

2.1 Subjects

Experiments were conducted on 50 young-adult (3-months old) male Sprague–Dawley rats weighing 264–380g at the beginning of the experiment, obtained from Kobay Test Animals Laboratory Ltd., Ankara. Throughout the experiments, rats were kept in the animal house at METU Biological Sciences Department under constant temperature ($22\pm 2^{\circ}\text{C}$) and $60\pm 5\%$ humidity with 12/ 12-hour light/dark cycles (lights on at 07:00 a.m. and lights off at 07:00 p.m.).

Animals were provided food (laboratory chow) and water *ad libitum* except during the cognitive tests. One week before the learning training, rats were subjected to a food deprivation until their *ad libitum* body weight was reduced by 15%. In the course of cognitive tests, rats were receiving their daily food portion (3 standard food pellets) once a day, 20-30 min after the completion of the training session. Throughout the experiments, animal's body weights were recorded on the daily basis.

The experimental protocol was pursued in accordance with the ethic rules in Helsinki Declaration and Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health, USA and approved by the METU Local Ethic Committee (*Protocol No: 2010/05*).

2.2 Apparatus

In the course of experiments, rats were subjected to a battery of neurological tests checking their sensori-motor coordination and muscle strength. Additionally, they were tested in the probabilistic learning task.

2.2.1 Neurological Tests

2.2.1.1 Locomotor Activity Boxes

The locomotor activity test was measured in the activity apparatus (MAY ACT 508 Model, Animal Activity System, Commat Ltd,TR) (please see Figure 2.1). This system comprises of 45cm x 45cm square arena surrounded by 30 cm high wall made of transparent plexiglass equipped with two rows infrared photocells located at 1cm and 13cm above the floor on each side of the Plexiglas cage walls 2.5cm apart from each other.

Rats' ambulatory, vertical (rearing) and horizontal movements executed without place change (i.e. grooming) were sensed by photocells and recorded by a computer based "special motion recognition software at 0.1 s sensitivity To assess ambulatory activity several measures were taken including total travelled distance, total movement time, average velocity of the motion and total number of motions during the testing period. The total number of vertical movements, total time and average time of a single motion were also recorded. All those recorded data were stored as "activity score" with the help of the software.



Figure 2.1. Locomotor Activity Test Apparatus

2.2.1.2 Catalepsy Tests

2.2.1.2.1 Bar Test Apparatus

Catalepsy is the condition of muscle rigidity and inability to correct an unusual posture. It is one of the symptoms of Parkinson's disease and thus it is a good behavioral tool to investigate dopaminergic functions in animal models of this disease. The most common catalepsy test is the standard bar test which was originally described by Kuschinsky &

Hornykiewicz (1972). The apparatus consists of 37 cm long, 26 cm wide, and 13 cm high white box made of rigid foam. A wooden bar 1 cm in diameter is placed 9 cm over the box floor (see Figure 2.2). The experimenter puts animal's forelimbs on the bar and measures the time until animal removes one or both paws from the bar. The procedure was adopted from Alam & Schmidt, 2002; Alvarez-Cervera et al., 2005; Sanberg et al., 1988.



Figure 2.2. Bar Test Apparatus (left) and Grid Test Apparatus (right).

2.2.1.2.2 Grid Test Apparatus

The apparatus consists of a 50 cm wide and 85cm high metal grid with 0.5cm² grid size (Figure 2.2). During testing it was placed on the wall perpendicular to the ground. The time until the first movement of any paw was noted was measured (Alam & Schmidt, 2002; Sanberg et al., 1988).

2.2.1.3 Rearing Test Apparatus

The apparatus consisted of a cylinder 20 cm in diameter and 30 cm high made of transparent Plexiglass (Figure 2.3). Placing rat into such cylinder encourages exploratory rearing behavior. To facilitate observation of rearing activity at all angles, a mirror was put behind the cylinder. The number of rears was recorded throughout the testing period. This procedure has been adopted from Cannon et al., 2010; Schallert et al., 2000.



Figure 2.3. Rearing Test Apparatus

2.2.1.4 Stepping Test Apparatus

Stepping test is generally used to detect the forelimb akinesia, which includes motor deficits such as failure in walking initiation and postural instability. To examine these deficits, there were used particularly three types of tests which were the initiation time (the time elapsed until the initiation of stepping by each forelimb), stepping length (length of the step measured for each forelimb independently), and the adjusting step (the number of steps executed by the free paw used to keep balance). To conduct these tests, a 110 cm long wooden ramp was used. It was placed between the table and the rat's home cage of subject (see Figure 2.4). To ensure a precise measurement, the ramp was scaled on its right side. The procedure was adopted from Fang et al., 2006.



Figure 2.4. Stepping Test Apparatus

2.2.1.5 Rotarod / Accelerod

This test was conducted by an automatic Rotarod / Accelerod apparatus (MAY RR 0711, Commat Ltd, TR) to monitor the muscle strength and the sensorimotor coordination of rats. The apparatus consisted of a 5 cm diameter revolving rod driven by a small motor and having 4 separated lanes (Figure 2.5). The speed of the rotation was constant or accelerated over a range of different speeds. In the rotarod mode, revolving rod was kept at a steady speed of 20 rpm while in the accelerod mode it was accelerated from 0 to 80 rpm either within 10 or within 4 min. In order to keep the rat on the rod, a metal grid was placed under the revolving rod with mild electrical current passing through. The total time spent on the rod before falling down was recorded automatically by the device.



Figure 2.5. Rotarod / Accelerod Apparatus

2.2.1.6 Y-Maze

A system similar to Y-maze was specifically designed to measure potential deficit in the probabilistic learning task. The apparatus was constructed of three identical 50 cm long and 10 cm wide arms surrounded by 20 cm high Plexiglass walls (Figure 2.6). One of the arms including its floor was covered with black while the other arm with white lining. The floor of the third arm was painted grey and the walls remained transparent. The white and black arms were separated by a 120° angle. At the ends of white and black arms food cups were found wherein the chocolate flavored rice puffs were placed as a reinforcement. In order to prevent the smell of the food pellets from guiding the animal's arm choices, the food cups had double floor made of metal wire and a few food pellets were always placed under it in both arms. At the entrance to the white and black arms there was a guillotine door which could be manually open or closed. The maze was positioned 50 cm above the ground and surrounded

by white curtains to minimize the potential distraction of the animal by the external cues belonging to the experimental room. The tests were conducted under day light conditions.

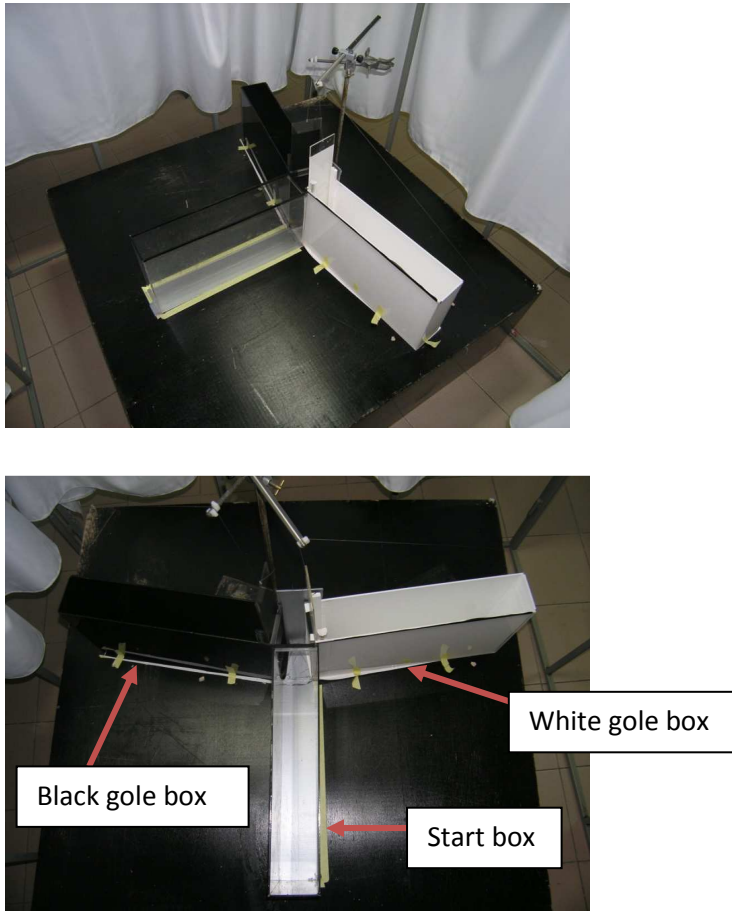


Figure 2.6. Y-Maze Apparatus (from different views)

2.3 Experimental Procedure

Among the available PD animal models, 6-OHDA as a widely studied neurotoxin model with its acute induction of catecholaminergic neuron damage and Rotenones as the most recent and least studied animal model with its similar progressive manner of human PD were chosen to compare and evaluate their neurobehavioral effects in PD (Meredith & Kang, 2006). The behavioral effects of these toxins are also compared with respect to the different action mechanism such as 6-OHDA is taken up by the dopamin transporter (DAT) and induces a rapid depletion of DA while rotenone does not depend on dopamine transporters for its action and as such causes an uniform inhibition of mitochondrial Complex-I in the entire brain (Betarbet et al., 2002a). Also, Rotenone PD animal model has not been studied as much as MPTP or 6-OHDA neurotoxins, so wide variety of behavioral tests, especially in

bilateral lesions, have to be individually tested and investigated (Meredith & Kang, 2006). More than 13 years, the neurodegenerative effect of rotenone on striatal neurons through systemic injection has been known. However, systemic injection becomes the main weak point of the models because it produces high levels of toxicity and mortality (Mulcahy, Walsh, Paucard, Rea, & Dowd, 2011) and causes large variations in animal sensitivity and in motor response (Meredith et al., 2008). Also, there are controversial claims that the changes in the motor behaviour in these models may not be induced by a specific nigrostriatal dopaminergic degeneration, but it may be due to systemic complications (Cicchetti et al., 2009). From the 6-OHDA view, systemic (peripheral) administration does not induce a nigrostriatal damage but destroys cells in the peripheral nervous system. The main reason for this is the inability of 6-OHDA to cross the blood-brain barrier (BBB) (Prou & Przedborski, 2005). Therefore, the intracranial route of administration was preferred with the advantage of a site-specific injection of the toxin, by this way, a site-specific degeneration in the brain. Additionally, one-time low dose infusion was favored to prevent acute, sudden neural damage, following a high mortality rate. Also, it was considered that highly severe symptoms would retain an extensive behavioral analysis and comparison and prevent an expected learning performance in the long run. By considering probabilistic learning tasks at the end of the neurological tests, unilateral infusions of toxins were eliminated. Although there is a gold-standard behavioral test to detect lesion accuracy in unilaterally-lesioned PD rodents, which is the asymmetric circling behaviour (Bezard & Przedborski, 2011), bilateral damage to the nigrostriatal pathway better simulates the neurodegeneration in human PD patients (Emborg, 2004) and the possibility of compensation of the non-lesioned side in the learning process. Considering all these, to generate the PD animal models, 3 µg rotenone dissolved in 1 µl DMSO and 4 µg 6-OHDA dissolved in 1 µl saline containing 0.2% ascorbic acid were bilaterally injected into the target site substantia nigra pars compacta (SNpc).

2.3.1 Experimental Design

Prior to the experiments, rats were randomly assigned into the five treatment groups: Rotenone group (n=15) received bilateral intranigral infusion of this drug dissolved in dimethyl sulfoxide (DMSO); 6-OHDA group (n=15) was bilaterally infused with 6-hydroxydopamine (6-OHDA) dissolved in saline; DMSO group (n=7) was the vehicle control for the rotenone group and as such received bilateral intranigral infusion of DMSO, while Saline group (n=7) was the vehicle control for the 6-OHDA and was infused with saline. The fifth group was an intact control (IC, n=7). However, 2 rats from the Rotenone group, 1 rat from the DMSO group, 2 rats from the Saline group, and 3 rats from the IC

group died either during or shortly after the surgery, or later in the course of experiments. The final number of rats in each group was as follows: Rotenone: n=11; 6-OHDA: n=13; DMSO: n=6; Saline: n=5; IC: n=4.

Animals were individually housed throughout the experiment. In the recovery period after surgery, the fighting and injuries are seen in very high rates (Jackson-Lewis & Przedborski, 2007). So, animals were housed individually throughout the whole experiment to maintain the consistency between preoperative and postoperative period. Also, the previous studies have showed that bilaterally infused 6-OHDA into SNpc induces aphagia, adipsia and akinesia (Sakai & Gash, 1994). Of course the severity of these symptoms depends on the given dose of the toxin. Since the striatal DA depletion is closely related to the feeding impairments, we closely monitored all the animals throughout the experiment with a higher caution immediately after the surgery. Especially in the recovery period, individual housing provided an accurate control of animals with respect to water and food consumption and rapid response to extreme body weight loss. Another advantageous of individual housing to us was to control of food diet including 3 standard food pellets per animal during cognitive tests.

Table 2.1 presents the schedule of the experiments. Before the stereotaxic surgery, all animals were handled for 5 days and then for a week subjected to a battery of neurological tests including locomotor activity, catalepsy, rearing, stepping, and rotarod/accelerod tests to conceive the preoperational sensorimotor conditions of the animals. Five days after the completion of neurological tests, Rotenone, 6-OHDA, DMSO, and Saline groups received bilateral intranigral drug infusions. It took four days to complete the stereotaxic surgeries. After the three days recovery period, all groups including IC group, were again subjected to the neurological tests. The tests were repeated six times: between the postoperative days 4-6, 7-9, 10-12, 20-23, 40-42, and 150-152. Fourty six days after the surgery, cognitive tests were carried out. The cognitive task consisted of three days of habituation and shaping training, 7 days of probabilistic learning, a 3-week-break, and the 7 days of the reversal of the original learning task.

In the following days, two rats from each Rotenone and 6-OHDA groups were chosen according to the best and the worst activity scores and were used for the histological verification of the infusion site.

Table 2.1. The time schedule of the experiments

TESTS & TREATMENTS	DAYS
Handling	5 consecutive days
Preoperative neurological tests	7 consecutive days
Surgery	Rotenone and 6-OHDA → 3 consecutive days DMSO, Saline and IC → within a day
Recovery	3 consecutive days
Postoperative Neurological Tests:	
Activity Test	Postoperative Days 4 th , 7 th , 10 th , 20 th , 40 th , 150 th
Catalepsy	Postoperative Days 4 th , 7 th , 10 th , 20 th , 40 th , 150 th
Rearing	Postoperative Days 4 th , 7 th , 10 th , 20 th , 40 th , 150 th
Activity, catalepsy and rearing tests were conducted at the same day with 2h intervals	
Stepping	Postoperative Days 5 th , 8 th , 11 th , 21 th , 41 th , 151 th
Rotarod/Accelerod	Postoperative Days 6 th , 9 th , 12 th , 22 th , 42 th , 152 th
Habituation and Shaping Training in Y maze	Postoperative Days 46-48
Probabilistic Learning (PL)	Postoperative Days 49-55
Three-weeks Rest Period	Postoperative Days 56-77
Reversal Training in PL Task	Postoperative Days 78-84
Decapitation for DA measurement	

2.3.2 Chemicals

The Rotenone, dimethyl sulfoxide (DMSO), 6-hydroxydopamine (6-OHDA), and ascorbic acid were purchased from Sigma (St. Louis, MO). Saline and sucrose solutions were prepared in the lab. Rotenone was dissolved in DMSO and 6-OHDA was dissolved in saline containing ascorbic acid. 3 µg Rotenone/1µl DMSO per side and 4 µg 6-OHDA/1µl saline containing 0.2% ascorbic acid per side were bilaterally injected into SNpc.

2.3.3 Surgery

Rats were anesthetized with a mixture of ketamin/ xylazine (80 mg/kg / 10 mg/kg i.p.). The animal was placed on a cotton bed within a stereotaxic frame (TAXIC-653 Dual Manipulator Stereotaxic Frame, World Precision Instruments Inc., USA). The incisor bar was adjusted to flatten the rat's skull which was further confirmed by the equal height of bregma and lambda marks on the skull. After making a midline approximately 1-1,5 cm long skin incision on the scalp and cleaning the skull of the connective tissue, two holes, one on each side, were drilled with high performance coreless micromotor (Nakanishi Inc., Japan) according to the stereotaxic antero-posterior (AP-5.0 mm from bregma) medio-lateral (ML-2.0 mm from midline) and dorso-ventral (DV -8.0 mm from the top of the skull) coordinates for SNpc adopted from Xiong *et al.*, (2009) and Lima *et al.*, (2010) and confirmed by Rat Brain Atlas (Paxinos and Watson, 2007). To make the infusion, 3 µg of rotenone (Sigma, St. Louis, MO, USA) was dissolved in 1µl DMSO (Sigma, St. Louis, MO, USA) and injected at a flow rate of 0.2 µl/min via 22 gauge, 50 µl volume Hamilton microsyringe using UltraMicroPump III and SYS-Micro IV Controller (World Precision Instruments Inc., USA). Drug infusions were made bilaterally at the depth of 8.0 mm from the skull and after stopping the infusion of the toxin, the probe was kept in the same position for a further 5 min for complete diffusion of the drug and then slowly retracted.

The 6-OHDA group was bilaterally administered 4 µg 6-OHDA (Sigma, St. Louis, MO, USA) dissolved in 1µl saline containing 0.2% ascorbic acid (Sigma, St. Louis, MO, USA) into SNpc. Sham-operated animals were injected 1µl DMSO or 1µl saline. The same flow rate and the needle retention were used during all these injections. At the end of the 5-min retention, needle was withdrawn and the holes were coated by a small amount of bone wax to assure a fast recovery of the skull. The incision was closed by stitching and animals were put back into their cages placed near the heater to prevent any hypothermia. During the 3-day recovery period, all animals were closely monitored and provided proper postoperative care such that animals showing weight loss were supplied 10% sucrose solution in addition to the solid food diet.



Figure 2.7. Stereotaxic Surgery (from different views).



Figure 2.8. Stereotaxic Surgery Area

2.3.4 Behavioral Procedures

Handling

Before starting any behavioral tests, all the subjects were daily handled on regular basis such that they were taken out of their cages, weighed, allowed to move on the table and touched for a while to get use to the experimenter. The handling was carried out for five consecutive days.

Rats were subjected to the behavioral test in the same order and at about the same time during the daily hours. All the neurological (sensorimotor) tests were performed once before the surgery and 6 times, at different times windows, after the surgery. This allowed us to

evaluate the animal performance after the drug administration on the individual basis comparing it to that displayed prior to the drug administration. In addition, the cross-sectional comparisons have been made between different treatment groups and their controls.

2.3.4.1 Locomotor Activity Test

When the animal is placed into a new arena, it, first, pauses then starts rearing, turning, grooming behaviours and following an exploration the field from edge of wall toward rest of the arena. During this exploratory test, the number of home bases, number of trips, kinematics of excursions and returns, number of stops, number of rears, incidence of grooming, duration of trips, total distance of trips, etc. can be measured (Whishaw et al., 2002). In the studies of toxin-induced PD models, the locomotor activity test is the most preferred one to detect the alterations of behavioral activity (Sedelis et al., 2001). In this regard, we tested the locomotor activity in 15 min period with 5-min intervals, but only analyzed the first 5-min values because of habituation. The habituation means the reduction in the locomotor activity due to exposing new environment, plus reduction in anxiety levels revealing more activity toward center of the arena (Brooks & Dunnett, 2009). In our study, the locomotor activity measurements were performed once before the surgery and six times after surgery for each group independently. The locomotor activity test was chosen the first test of the day in order to preclude performance of the subjects from being negatively influenced from other behavioral tests. For the measurement, subject was taken from his cage and put into the activity apparatus. At each testing day, animals were placed into the activity box at the same time and in the same order and activity measurements were automatically recorded over 15 min in the consecutive 5 minutes intervals.

2.3.4.2 Catalepsy Test

In Parkinson's Disease, catalepsy expresses itself as akinesia, bradykinesia or failure to correct an unusual posture. In the present study, it was aimed to record the time needed for rat to correct such unusual postures. For this purpose, two components as the bar test and the grid test were used.

In grid test, rat was hung on a metal grid by all four paws with its head up (see Figure 2.9). Time from the first moment that rat was put on the grid till the first movement of any of four paws was recorded.

At the second part, each rat was placed in the catalepsy test chamber in a half rearing position, so that hindlimbs of the rat were touching the floor of the chamber and forelimbs were grabbing the bar (see Figure 2.9). Time until the removal of at least one paw from the bar was recorded. The maximum descent latency was determined as 180 s for both tests. Tests were repeated three times for each animal at each testing day in an inter-trial manner and the mean scores were calculated. This procedure was adopted from Alam et al., 2004; Alvarez-Cervera et al., 2005.

There are several factors need to take into consideration in bar test in rodents such as apparatus, animal weight, maximal test duration, strain of animal and auditory and visual environment (Sanberg et al., 1988). Here, the most controversial parameter is the diameter and height of the bar. A thin bar generally resulted in lower catalepsy scores, regardless of height of the bar (Sanberg et al., 1988). On the other hand, too high bar prevents animal to hold and rest on it for a determined time. In this context, animal weight is another factor: a low bar (6 cm) should not be used for a 340-g rat. We decided to use 1 cm as the bar diameter, 9 cm as a bar length according to the studies by Alvarez-Cervera et al., 2005; Moss, McMaster, & Rogers, 1981; Sanberg et al., 1988 who suggest 8-9 cm height for 250-350 g weighted rats with a maximum test duration of 180 sec.



Figure 2.9. The Grid Test (on the left) and the Bar Test (on the right)

2.3.4.3 Rearing Test

An exploratory behavior is seen as soon as a rat is put into a cylinder, and a major part of this behavior is rearing. Rearing behavior includes rising on the hindlimbs, initial contact of the forelimbs with the wall, changing the posture to rebalance the center of gravity, lateral movements across the wall, and finally landing. These criteria can be examined for each forelimb separately or for both forelimbs simultaneously. Because, in the present study, a

bilateral lesion model was attempted to be generated, a rearing was classified with respect to the use of both forelimbs. Rearing movement was counted only when both paws made contact with the cylinder wall and only when the contact was above the shoulder level (see Figures 2.10 and 2.11). If both paws touched to the cylinder floor after a rising movement like mentioned above, it was determined as the end of the rearing. Rats were tested for forelimb use by placing into a clear cylinder with 5min-long observation and the total number of rearings was recorded. After each animal, the cylinder was cleaned and prepared for the next measurement. The procedure was adopted from Cannon et al., 2010; Tillerson et al., 2001.



Figure 2.10. Rearing (top view)

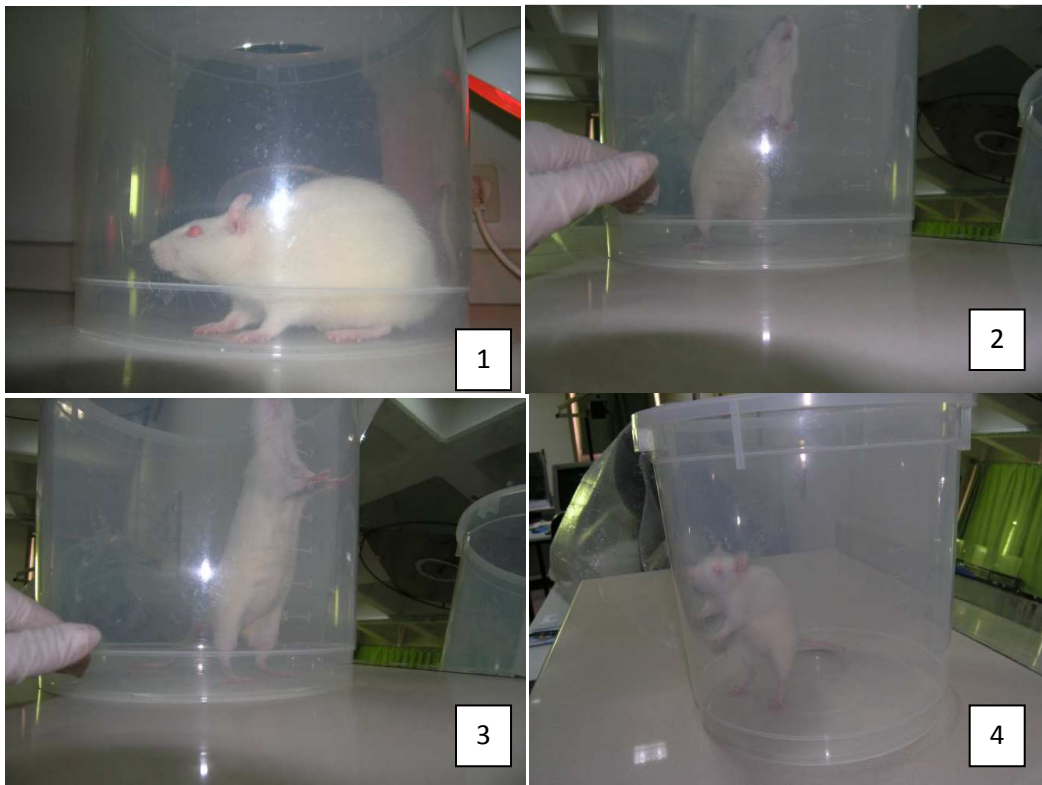


Figure 2.11. Rearing (side views)

2.3.4.4 Stepping Test

Stepping test is used for the purpose of testing the stiffness of the limbs which is expressed itself as rigidity in PD (Fang et al., 2006) and used to test the akinesia (Singh, Ahmed, Sagar, & Krishana, 2006). There are different parameters of which it was used in the present study such as;

- I. Initiation time
- II. Stepping length
- III. Adjusting steps

One day before the preoperational testing, rats were allowed to walk freely on the wooden ramp to become familiar with the system and the environment. In the first part of the test, the experimenter held the rat by his hindlimbs with one hand and one of the forepaws was held by the other hand, therefore animal's weight was given onto the free-paw side and the captured forelimb was let to rebalance the body (see Figure 2.12). Also, the hind part of the body was lifted a bit from the ramp. Time until initiation of any movement by the free paw was recorded.

In the stepping length, rat was placed at the zero point of the scaled ramp and allowed to move towards the cage at the end of the ramp. The total length of six steps was recorded and the length of a single step was calculated. In the case of any lateral movement on the ramp the test was repeated.

After the six step made by the rat, rat was again held by experimenter as mentioned in the first part, and moved along the ramp by the distance of 90 cm in 5s in, once forward and then backward. While rat is moved at a speed of 90cm/5s, it must keep walking and adjusting his balance with the free paw touching the ramp. The number of steps made by the free paw was measured.

Each test was run with the right and the left forelimb independently. The maximum descent latency for all tests was noted as 180s and tests were repeated three times one hour apart. This procedure was adopted from Fang et al., 2006; Olsson et al., 1995.



Figure 2.12. Stepping Test

2.3.4.5 Rotarod / Accelerod Test

The procedure of this test was adopted from Dursun *et al.*, (2006). This test examines muscle strength and sensorimotor coordination in small rodents such as rats and mice. Rats were put on the revolving rod, which was faster than normal walking speed, in the opposite direction to the rotation and the time spent on the rod until falling down was automatically recorded. Animals remained on the rod until they fell down or after 10 min elapsed. Unlike the procedure of Dursun *et al.*, (2006), entire experiment was separated into two steps like shaping and testing. In the shaping day, the speed of the rod was stable and fixed at 20 rpm. To assure that the rat will make an effort to remain on the rod as long as possible, an electrical current (1 mA) was applied to the metal grid floor beneath the rod. Once the animal fell down and got in touch with grid it received a mild electric shock. This step was conducted only once before the surgical operations. No scores were recorded. In the second part, test was repeated over three consecutive sessions in a day with different conditions such as rotation stable at 20 rpm, acceleration-I and acceleration-II:

- I. Stable 20 rpm: For this step, all the conditions in the shaping day remained same, except that the time of staying on the rod was recorded.
- II. Acceleration-I: The rotation speed of the rod was not stable but accelerated from 0 to 80 rpm within 10 min.
- III. Acceleration-II: The speed of the rod was accelerated from 0 to 80 rpm within 4 min.

All 4 steps were performed on the same day, 2-3h apart.



Figure 2.13. Rotarod / Accelerod Test

2.3.4.6 Probabilistic Learning Test

To investigate decision making in rats, Iowa Gambling Task (IGT), a task designed to model real-life choices in humans, was modified for the rats. In this task, the preference between immediate gain of high rewards but higher loss in the long-term and higher gain in long-term with low immediate rewards was tested. Immediate high-reward-arm was labeled as “disadvantageous” and contained 3 food pellets (coco pops) twice over 20 choices (trials). On the other hand, the arm labeled as “advantageous” contained a single food pellet per choice 14 times over 20 choices. For the punishment, nothing special like quinine-treated pellet was used, but 6 empty choices for the advantageous arm and 2 for the disadvantageous arm as a punishment with no reward were arranged. To increase uncertainty, the sequence of presentation food rewards in “advantageous” and “disadvantageous” arms was semi-random and changing between daily sessions although the same sequence was applied in all groups on a given daily session. The number of entries into each arm and the number of consumed pellets were recorded. The ultimate gain ratio between advantageous and disadvantageous choices was 70:30 or 14:6 which became 2.33.

Throughout this experiment, all animals were subjected to a food deprivation in order to increase their motivation in the learning task based on food reinforcement. In their home cages, animals were receiving 3 food pellets only, delivered 20-30 min after the completion of a daily training session. On the very first day of training (habituation session), subjects were allowed to explore the maze for 5 min. For each animal, the numbers of spontaneous entries to black and white arm was recorded and the side preference (left vs, right), if any was, observed. For an individual rat, the arm with less preferred color and/or position was

assigned as “advantageous”. Basing on their performance during the first testing session, rats were divided into 4 subgroups:

- 1) Advantageous arm black on the right
- 2) Advantageous arm black on the left
- 3) Advantageous arm white on the right
- 4) Advantageous arm white on the left

This was followed by 3 daily shaping sessions. On the first shaping day, 7 food pellets were scattered throughout maze. On the second shaping day, the number of pellets was reduced to 5 and they were placed in the arms close to the food cups. On the last shaping day, 2 pellets were placed into the food wells of the goal (black and white) arms.

During the learning stage, rat was placed in the transparent arm (starting arm) and the guillotine doors to the goal arms were open. As soon as the rat made a choice between black and white goal arms, the door was closed and it was confined to the chosen arm for 20s (see Figure 2.14). If there was food in the food cup, rat was allowed to collect the food pellets. 20s after the entry to one of the goal arms and consumption of food pellets (if there were any), rat was taken into his home cage and the maze was cleaned. 20 trials per day with approximately 20-30 min inter-trial interval was applied. Training lasted for 7 consecutive days.



Figure 2.14. Making a choice in Probabilistic Learning Task.

During the reversal training, the same procedure was applied except that the left/right position of the black and white goal arms was reversed. It means that a subgroup having as an “advantageous” arm white arm on the left side, now on had a white arm on right side as

an “advantageous” arm. By this way, it was examined whether a rat made its choices depending on the color of the arm or rather was guided by its left/right position. This procedure was adopted with some modifications from Adriani et al., 2006; Stevens & Cowey, 1973; de Visser et al., 2011.

Table 2.2. Pellet number and time table of probabilistic learning task

Color (white/black) and Position (right/left) Preference Test	1 day without pellet use
Shaping	3 consecutive days (7, 5, 2 pellets, respectively)
Probabilistic Learning Test	7 consecutive days (14 pellets in advantageous arm and 6 pellets in disadvantageous arm per trial)
Reversal Probabilistic Learning Test	7 consecutive days (14 pellets in advantageous arm and 6 pellets in disadvantageous arm per trial)

2.3.5 Brain Tissue Studies

2.3.5.1 Methylene Blue Staining

Before the actual experiments, methylene blue (MB) injection was conducted to standardize the target position of SNpc in the rat brain. Same surgical protocol with the injection of rotenone was followed for methylene blue stain. 1,5 µl MB / side was infused and as soon as the surgery was over, rats were decapitated, brains were removed in 15 min and stored at -20°C. Later on, brain slices were investigated.

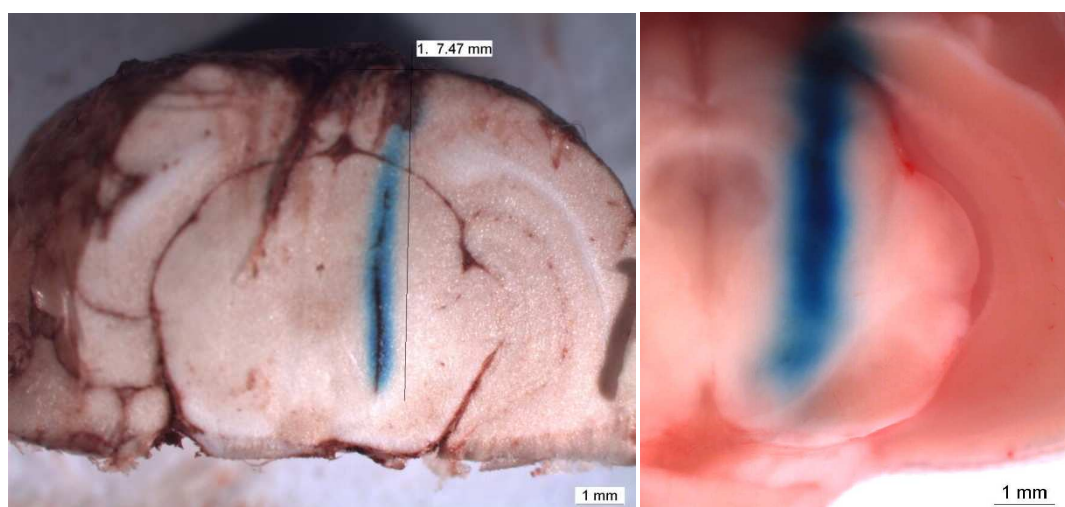


Figure 2.15. Injection of MB into SNpc with 8mm from skull.

2.4 Data Analysis

The mean group values (\pm SEM) have been calculated from all the measures taken. The main effect of treatment was examined by two-way repeated measures ANOVA (group x time) with a LSD post hoc test, when the ANOVA reached significance. One-way ANOVA with group as an independent factor was performed for each test and for each post-operative testing day, separately. Behavioral comparisons of pre- and post-operative data were analyzed by a paired Student's *t*-test. For all tests, $p < 0.05$ was deemed significant. The statistical packages SPSS 17.0 was used.

CHAPTER 3

RESULTS

3.1 Body Weights

The mean body weight (\pm SEM) has been calculated for each group separately for the 5-day preoperative handling period and the, selected days during the 5-months long, postoperative period (see Figure 3.1.). As seen from the Fig.3.1, no substantial weight loss was recorded in any of the treatment groups during the post-operative period. The data were analyzed by the two-way repeated measures of ANOVA (group x days) confirmed a significant group effect ($F_{(4,44)} = 16.950, p=0.000$), significant day effect ($F_{(45,1980)} = 26.011, p=0.000$) and significant day x group interaction ($F_{(180,1980)} = 8.087, p=0.000$). In all groups, an increase in the body weight was noted with age, however, at the beginning of the experiments the mean body weight of the intact control group was significantly lower and the mean body weight of the saline control group was significantly higher comparing to the remaining treatment groups which were reflected by a significant group effect ($F_{(4,49)} = 28.663, p=0.000$) depending on one-way ANOVA analysis, and post hoc analysis of LSD reveals a significant difference between saline and all treatments groups ($p=0.000$ for all) and IC and all treatments groups ($p=0.000$ for all, too).

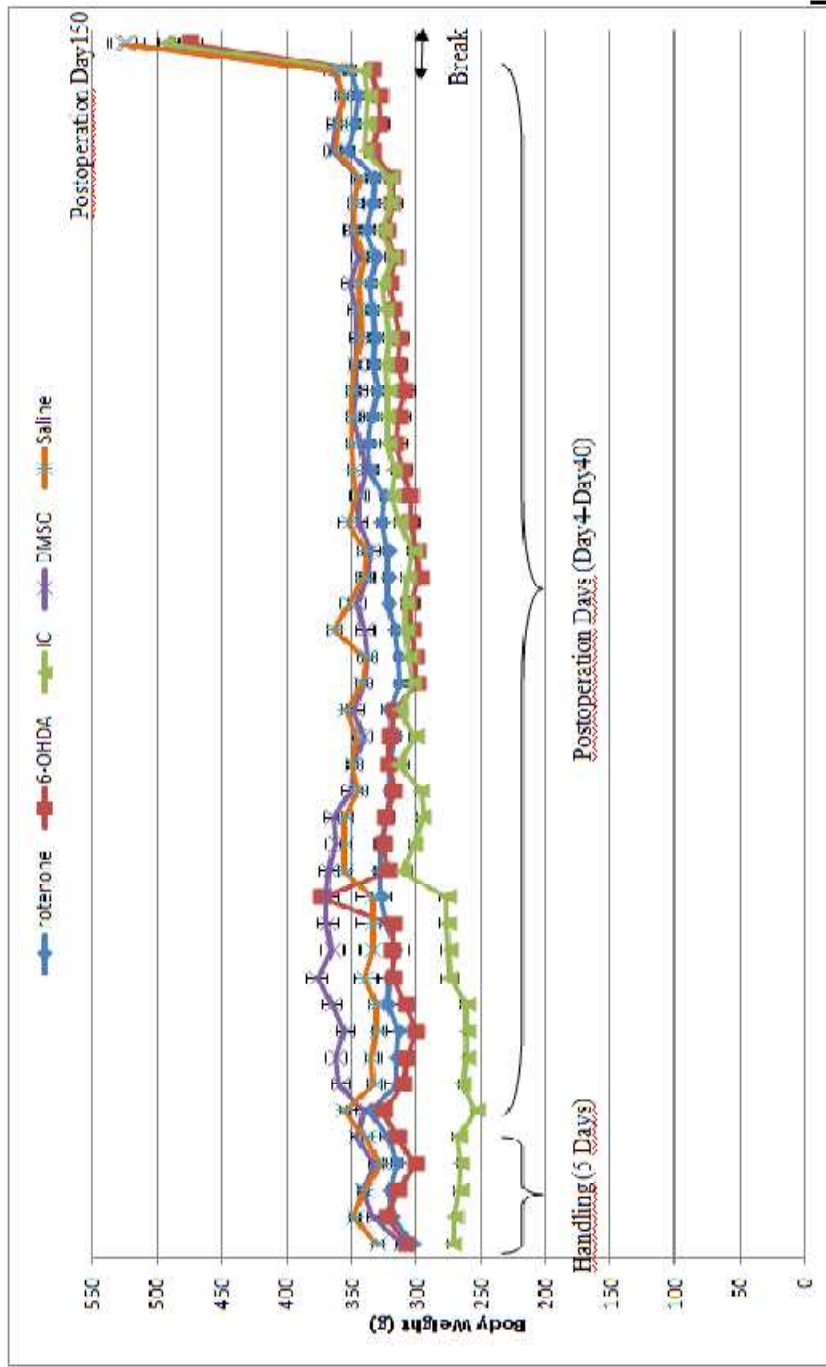


Figure 3A. Changes in the groups' mean body weights occurring in the course of the experiments.

3.2 Results of Behavioral Tests

3.2.1 Locomotor Activity Test

Figure 3.2. shows the ambulatory activity in the Rotenone and DMSO groups before and at different time points after the surgery. For comparison the ambulatory activity in the IC is presented. The activity scores presented in the graph are confined to the first 5 min of the 15 min testing period when the animals' exploratory activity was the highest. As seen from the figure 3.2, during the period covering the first 40 postoperational days locomotor activity in the IC group was higher than that in infusion groups. Two-way repeated measure ANOVA (group x day) yielded significant day effect ($F_{(6,108)} = 11.079, p=0.000$), significant day x group interaction ($F_{(12,108)} = 2.340, p=0.010$) and only marginally significant group effect ($F_{(2,18)} = 2.711, p=0.093$).

One-way ANOVA applied to pre-operational data confirmed a lack of significant between-group differences in the level of ambulatory activity ($F_{(2,20)} = 1.471, p=0.256$). One-way ANOVA performed on the data recorded 4 days after the surgery (the 4th day) yielded a significant group effect ($F_{(2,20)} = 9.089, p=0.002$). Post-hoc comparisons of simple effects revealed significant differences between IC and both Rotenone and DMSO groups ($p=0.002$ and $p=0.001$, respectively) with no significant difference between the infusion groups themselves which may suggest an adverse nonspecific effect of surgery and/or infusion on animals' behavior. On the 7th day after the surgery, the main effect of group remained significant ($F_{(2,20)} = 2.753, p=0.091$), with the difference between IC and DMSO groups significant at $p=0.042$, and the difference between IC and Rotenone groups only marginally significant ($p=0.082$). These differences disappeared on 10 and 20 days after the surgery, and on the 40th day marginally significant worse performance with the main group effect of $F_{(2,20)} = 2.006, p=0.163$ was recorded in Rotenone group as compared to both IC and DMSO control groups ($p=0.104$ and $p=0.147$, respectively) which might have indicated towards the minimal adverse effect of rotenone administration itself on the animals' locomotor activity. However, the average locomotor activity observed in Rotenone group 40 days after the surgery was not significantly lower from that recorded prior to the operation in this group. This confirms lack of Rotenone effect on the locomotor activity under the present experimental conditions. Five months after the surgery no between-group differences in the animals' ambulatory activity were noted.

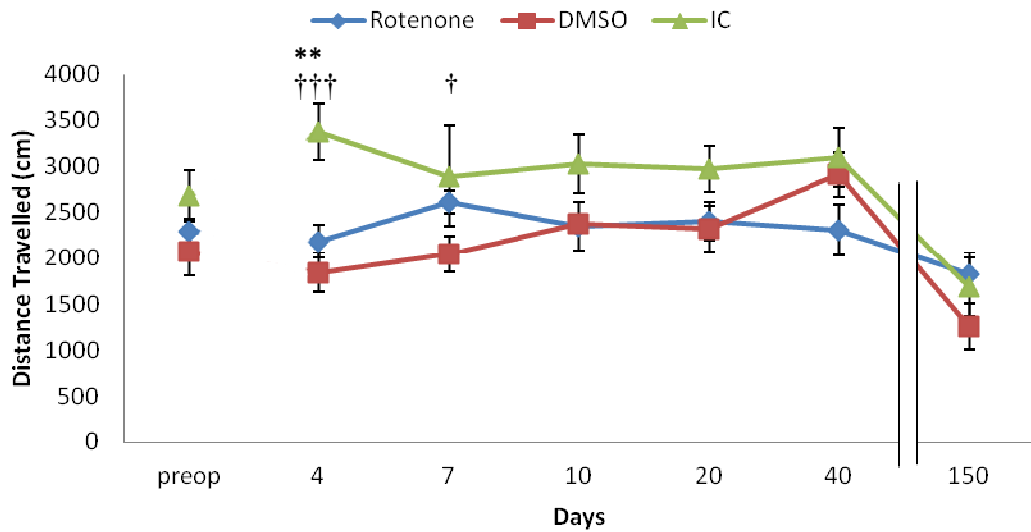


Figure 3.2. Locomotor activity presented as mean (\pm SEM) distance travelled (cm) calculated for the first 5-min interval of the total 15-min testing period at seven time points for Rotenone, DMSO and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; † denotes the level of significance for DMSO - IC groups comparison: † $p < 0.05$, ** $p < 0.01$, and ††† $p < 0.001$.

Figure 3.3 shows the locomotor activity results for the 6-OHDA group, its vehicle control Saline group and IC. Two-way repeated measures ANOVA (group \times day) yielded a significant main effect of group ($F_{(2,19)} = 3.631$, $p = 0.046$). The post-hoc comparisons revealed significant difference between IC and the two operated groups: 6-OHDA and Saline control ($p = 0.030$ and $p = 0.020$, respectively). A significant main effect of day ($F_{(6,114)} = 8.455$, $p = 0.000$) was also found showing a general decrease in overall locomotor activity throughout the testing period probably due to the animals' habituation to the activity box. The day \times group interaction was also significant ($F_{(12,114)} = 1.909$, $p = 0.040$).

No significant between-group difference was found on the preoperation day ($p = 0.399$). Here too a significant main effect of group was found on day 4 ($F_{(2,21)} = 5.882$, $p = 0.010$). This result reflected a significantly worse performance in 6-OHDA and Saline groups as compared to IC group ($p = 0.003$ and $p = 0.017$, respectively). Again, a significantly worse performance observed in operated groups compared to intact control on day 4 can be attributed to the lack of full recovery after the operation by the postoperative day 4. No between group differences were noted on days 7 and 20 but the a significant main effect of group was found on day 10 and 40 ($F_{(2,21)} = 3.556$, $p = 0.049$ and $F_{(2,21)} = 6.537$, $p = 0.007$, respectively). The differences in performance in 6-OHDA and Saline groups were significantly lower compared to IC group

at the day 10 and 40 ($p=0.025$ and $p=0.026$, and $p=0.003$ and $p=0.006$, respectively). There was no significant difference between groups on day 150 with a general decrease in each group.

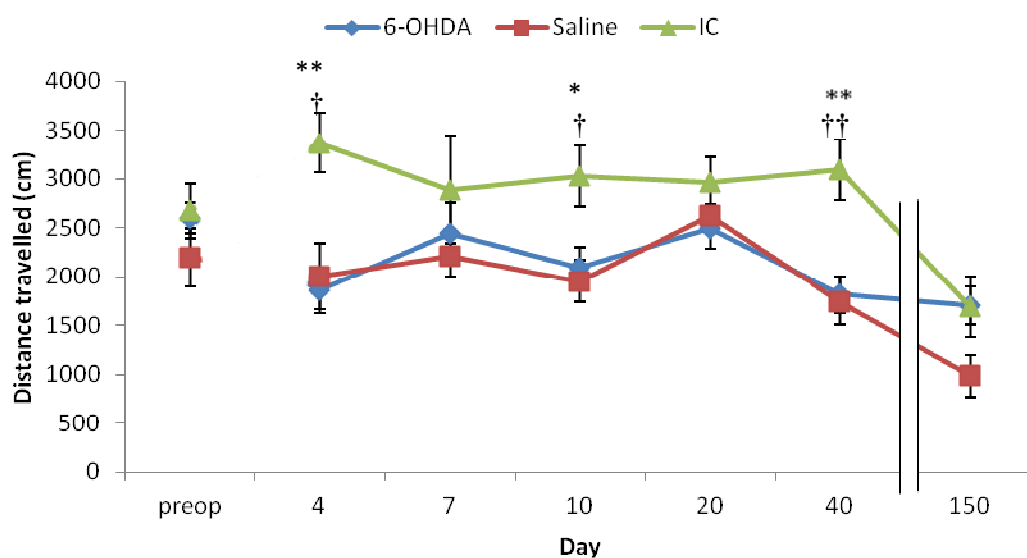


Figure 3.3. Locomotor activity presented as mean (\pm SEM) distance travelled (cm) calculated for the first 5-min interval of the total 15-min testing period at seven time points for 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison; † denotes the level of significance for Saline - IC groups comparison: */† $p<0.05$, **/ †† $p<0.01$.

Figure 3.4 shows the differences in locomotor activity between preoperation day and the postoperation day 40, when the greatest locomotor deficits were observed both in Rotenone and 6-OHDA groups. These data were evaluated by the student-t test for paired comparisons. As seen from the Figure 3.4, only in 6-OHDA group there was a significant deterioration in animals performance ($p=0.005$). Also, one-way ANOVA analysis revealed no significant differences between Rotenone, 6-OHDA and IC group on both preoperation day and the postoperation day 40 in the distance travelled in first 5-min.

Locomotor Activity

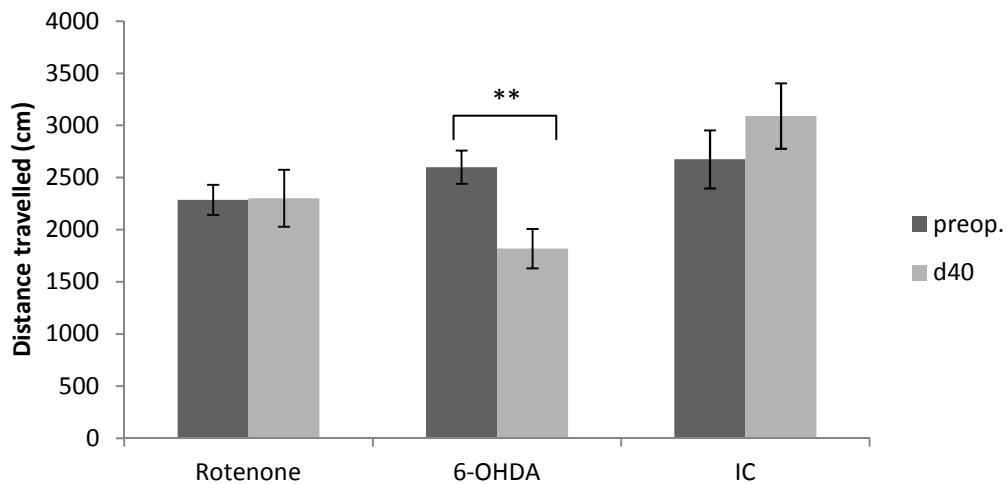


Figure 3.4. Comparison of the locomotor activity between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) distance travelled (cm) calculated for the first 5-min interval of the total 15-min testing period. Error bars denote \pm SEM. ** denotes the level of significance at $p < 0.01$.

3.2.2 Catalepsy Tests

For the catalepsy measurement, both the bar test and the grid test were conducted.

3.2.2.1 Bar Test

Figure 3.5 shows results of the bar test for the Rotenone, DMSO and IC groups. As seen from the graph, there was no substantial between- group difference prior to the surgery. During the postoperative period, Rotenone group showed longer descent latency compared to the control groups. However, two-way repeated measures ANOVA (group \times day) yielded no significant day effect ($F_{(6,108)} = 1.259$, $p = 0.283$) and the main group effect only marginally significant ($F_{(2,18)} = 3.216$, $p = 0.064$). The day \times group interaction was also insignificant ($F_{(12,108)} = 2.036$, $p = 0.028$).

One-way ANOVA confirmed lack of a significant difference between groups prior to the surgery. During the postoperation period a significant group effect was yielded only on day 40 ($F_{(2,20)} = 9.385$, $p = 0.002$) with significantly worse performance in the Rotenone group compared to DMSO and IC groups ($p = 0.004$ and $p = 0.002$, respectively) with no difference between control groups.

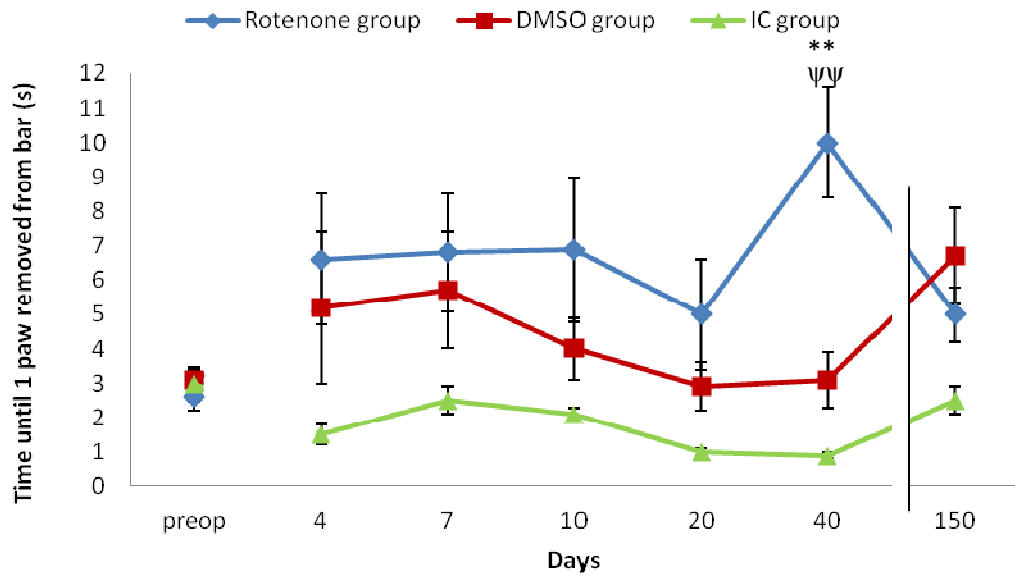


Figure 3.5. Mean time (\pm SEM) until at least one paw removal in the catalepsy bar test prior to the operation and at different time points after the operation for Rotenone, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; and ψ denotes the level of significance for Rotenone - DMSO groups comparison: **/ ψ ψ $p < 0.01$.

Similar data analysis performed for 6-OHDA treatment group and its controls confirmed lack of a significant between-group difference before the operation. The preoperation bar test scores in 6-OHDA and Saline groups were consistent with similar scores in Rotenone and DMSO groups. During the first 40 postoperative days, 6-OHDA group showed longer descent latency compared to the control groups (Fig. 3.5). Two-way repeated measures ANOVA (group x day) yielded a significant day effect ($F_{(6,114)} = 3.246$, $p = 0.006$), significant group effect ($F_{(2,19)} = 5.166$, $p = 0.016$) and significant day x group interaction ($F_{(6,114)} = 2.918$, $p = 0.001$). Post hoc analysis of simple effects showed a significant difference between 6-OHDA and both Saline and IC groups ($p = 0.039$ and $p = 0.011$, respectively).

On postoperative day 4, one-way ANOVA revealed a significant group effect ($F_{(2,21)} = 8.786$, $p = 0.002$) reflecting significant differences between 6-OHDA and both Saline and IC groups ($p = 0.008$ and $p = 0.002$, respectively). Also on the postoperative day 20, the main group effect was highly significant ($F_{(2,21)} = 9.710$, $p = 0.001$) with significantly worse performance of 6-OHDA group as compared to both saline and IC groups ($p = 0.012$ and $p = 0.001$,

respectively). On the days 7 and 40 the main group effect approached but did not reached the required significance level ($F_{(2,21)}=2.874, p=0.081$ and $F_{(2,21)}=3.199, p=0.063$, respectively).

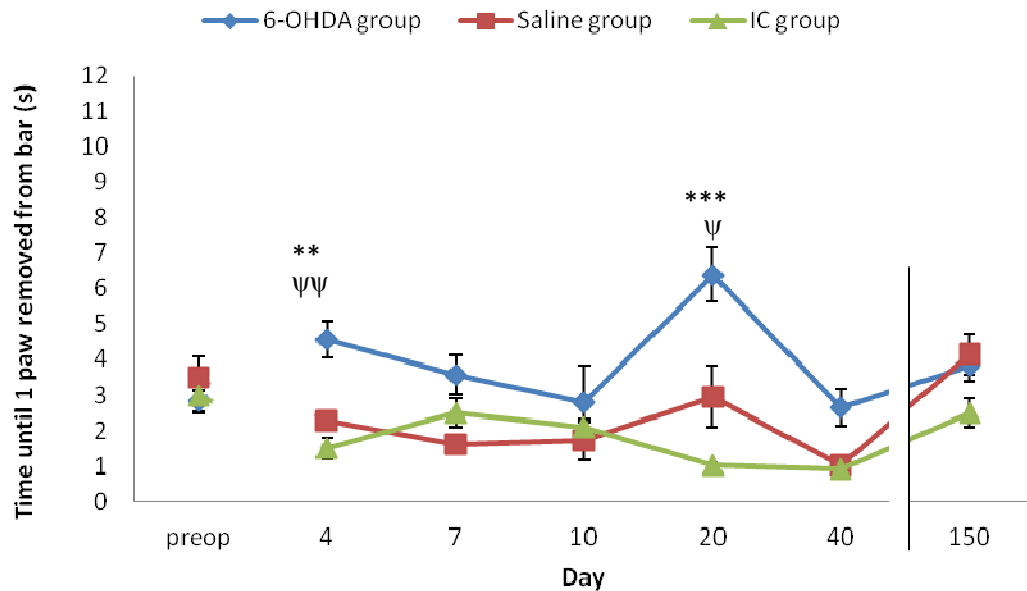


Figure 3.6. Mean time (\pm SEM) until at least one paw removal in the catalepsy bar test prior to the operation and at different time points after the operation for 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: ψ $p<0.05$, $**/\psi\psi$ $p<0.01$, and $***$ $p<0.001$.

The scores of the catalepsy bar test in 6-OHDA group were worse than the scores recorded in this group during the preoperative testing, however they did not reveal a significant level ($p=0.790$). As seen in Figure 3.7, student-t test for paired comparisons revealed a significant difference between preoperation day and the postoperation day 40 in group Rotenone (increase in descent latency) and IC (decrease in descent latency) ($p=0.001$, and $p=0.043$, respectively), while no significant difference was found for group 6-OHDA. However, in 6-OHDA group, the longest descent latencies were recorded on day 20 and the performance of this group on day 20 was significantly worse than the preoperative one ($p=0.002$).

One-way ANOVA analysis found no significant difference between these groups on the preoperation day, however it revealed a significant main group effect for the bar test performance on postoperation day 40 ($F_{(2,27)}= 15,163, p=0.000$). Post hoc analysis of simple effects showed the significant difference between Rotenone and both 6-OHDA and IC groups ($p=0.000$ and $p=0.000$, respectively).

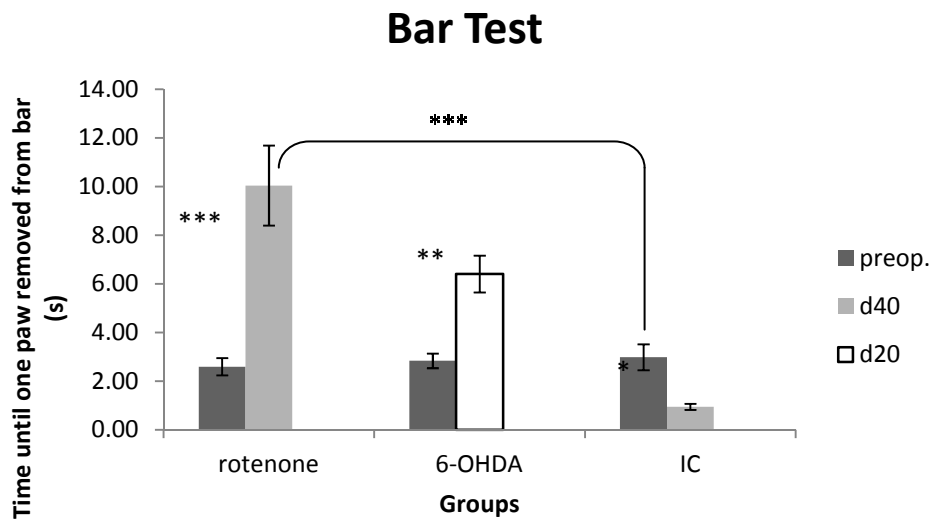


Figure 3.7. Comparison of the bar test scores between preoperation day and the postoperation day 40 for Rotenone and IC group, and comparison of the bar test scores between preoperation day and the postoperation day 20 for 6-OHDA group, independently. Data were presented as mean (\pm SEM) time until one/both paws removed from bar. Error bars denote \pm SEM. * denotes the level of significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.2.2.2 Grid Test

The results of the the grid catalepsy test are showed in the Figures 3.8-10. Similarly, to the bar test results, Rotenone group demonstrated longer latencies to the first movement compared to other two groups. Two-way repeated measures ANOVA (group \times day) yielded a significant day effect ($F_{(6,108)} = 2.154$, $p = 0.053$) reflecting an increase in descent latency in the infusion groups post-operation. The main group effect was statistically significant ($F_{(2,18)} = 4.798$, $p = 0.021$). Post hoc comparison of simple effects confirmed highly significant difference between Rotenone and IC groups ($p = 0.006$) while the differences between Rotenone and DMSO group ($p = 0.257$) and DMSO and IC group ($p = 0.077$) were insignificant.

One-way ANOVA revealed significant main group effect on days 7, 20 and 40 ($F_{(2,20)} = 4.012$, $p = 0.036$, $F_{(2,20)} = 3.250$, $p = 0.062$, $F_{(2,20)} = 4.158$, $p = 0.033$, respectively). The group effect on days 4 and 10 was only marginally significant ($F_{(2,20)} = 1.941$, $p = 0.172$ and $F_{(2,20)} = 1.784$, $p = 0.196$, respectively). The comparison of simple effects revealed significant differences between Rotenone and IC groups on days 7, 20 and 40 ($p = 0.012$, $p = 0.026$, and $p = 0.014$, respectively), and marginally significant difference on days 4 and 10 ($p = 0.068$ and

p=0.075, respectively). However the differences between Rotenone and DMSO and between DMSO and IC were found insignificant.

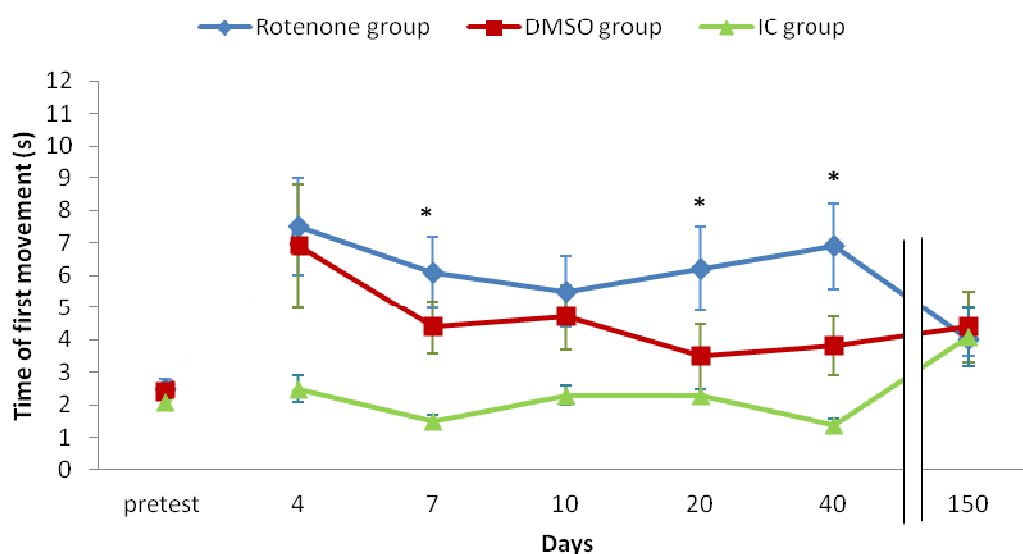


Figure 3.8. Mean time (\pm SEM) until the first movement in the catalepsy grid test prior to the operation and at different time points after the operation for Rotenone, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison at * $p < 0.05$.

As seen from the Fig. 3.9, in 6-OHDA and Saline groups, the scores of the catalepsy grid test recorded prior to the surgery are similar to those from the Rotenone and DMSO groups. After the surgery, in 6-OHDA group, the time to the first movement on the grid is longer compared to both control groups throughout all 40 days after the surgery. On the other hand, the performance scores in Saline and IC groups are (except day 20) very similar. The two-way repeated measures ANOVA showed a significant main effect of group ($F_{(2,19)} = 15.341$, $p = 0.000$). The main effect of day was insignificant but day x group interaction was yielded significant ($F_{(12,114)} = 2.314$, $p = 0.011$). The post hoc comparison of simple effects confirmed significant differences between 6-OHDA and Saline group ($p = 0.000$) and between 6-OHDA and IC group ($p = 0.000$).

One-way ANOVA showed no significant between-group difference prior to the surgery and 150 days after the surgery. However, highly significant treatment effect has been yielded on postoperative days 4, 7, 20 and 40 ($F_{(2,21)} = 23.459$, $p = 0.000$, $F_{(2,21)} = 10.686$, $p = 0.001$, $F_{(2,21)} = 5.086$, $p = 0.017$, and $F_{(2,21)} = 8.623$, $p = 0.002$, respectively). Post hoc comparison of simple effects confirmed significant differences between 6-OHDA group and both Saline

and IC control groups on days 4, 7, and 40 ($p = 0.000$, $p = 0.001$, $p = 0.004$ and $p = 0.000$, $p = 0.002$, $p = 0.004$, respectively). On day 20, a significant difference was also found between 6-OHDA and IC group ($p = 0.005$) but not Saline group which was significantly worse than the IC group ($p = 0.047$).

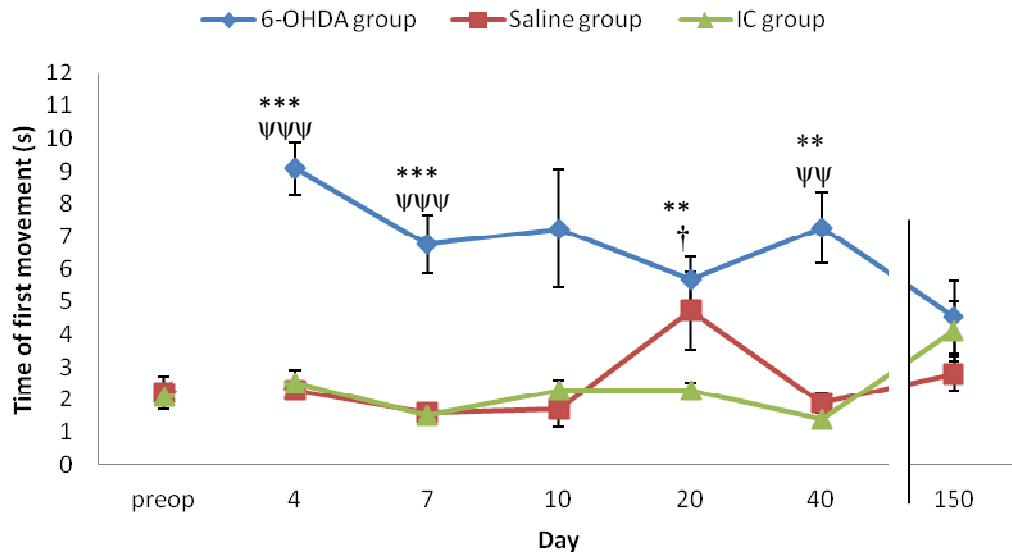


Figure 3.9. Mean time (\pm SEM) to the first movement in the catalepsy grid test prior to the operation and at different time points after the operation for 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison; † denotes the level of significance for Saline - IC groups comparison; and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: † $p < 0.05$, **/ $\psi\psi$ $p < 0.01$, and ***/ $\psi\psi\psi$ $p < 0.001$

One-way ANOVA was applied to compare the preoperative performance and performance on day 40 after the surgery between toxin-induced groups and IC, and a significant main groups effect was yielded ($F_{(2,27)} = 3.761$, $p = 0.037$) on day 40 but not on the preoperation day.

Post hoc comparison of simple effects confirmed differences between IC and both, Rotenone and 6-OHDA groups ($p = 0.022$ and $p = 0.013$) but not between the toxin groups. As seen in the Fig.3.10, the paired t-test showed that the postoperative performance in both, Rotenone and 6-OHDA groups was significantly worse ($p = 0.010$ and $p = 0.000$, respectively) than before surgery with no difference in the IC group.

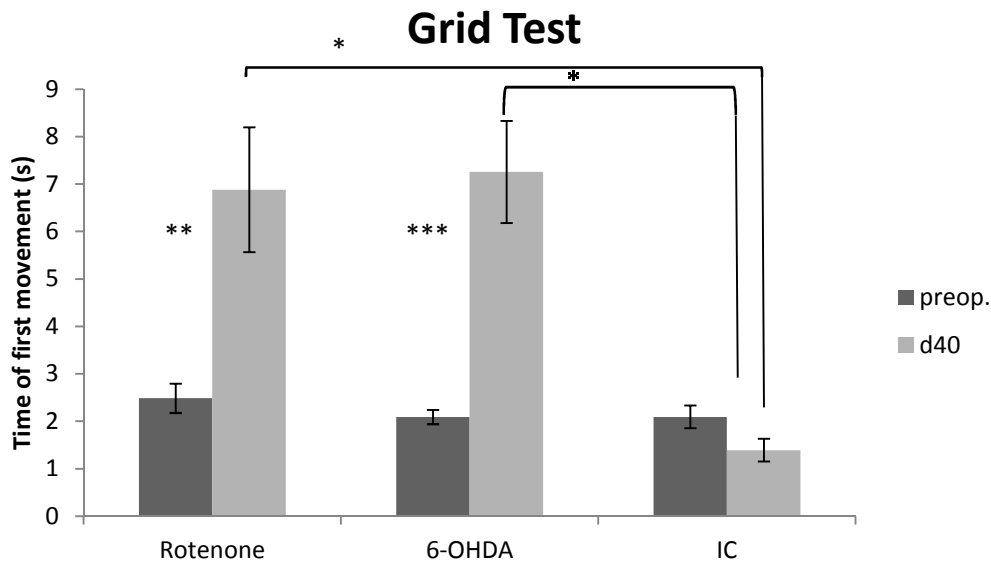


Figure 3.10. Comparison of the grid test scores between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) time of the first movement. Error bars denote \pm SEM. * denotes the level of significance: ** $p < 0.01$, and *** $p < 0.001$.

3.2.3 Rearing Test

Figure 3.11 shows the results of rearing test (number of rearings during 5 min in transparent cylinder) in the Rotenone, DMSO and IC groups. It can be clearly seen from this figure that the number of rearings was substantially higher in IC group compared to both Rotenone and DMSO infusion groups on postoperational days 4-40, with no between-group differences on preoperative testing day and 5 month after the surgery (day 150). The two-way repeated measures ANOVA yielded the main group effect highly significant ($F_{(2,18)} = 14.665$, $p = 0.000$). Post hoc comparison of single effects confirmed significant differences between IC group and both, Rotenone and DMSO infusion groups ($p = 0.000$ for both). The day effect and the group x day interaction were also significant ($F_{(6,108)} = 9.355$, $p = 0.000$ and $F_{(12,108)} = 5.830$, $p = 0.000$, respectively). The day effect reflected a declining trend in the incidents of rearing occurring in all groups with time, probably due to the habituation to the experimental environment since the rearing is part of an exploratory behaviour.

One-way ANOVA performed on these data confirmed a statistical significant group effect on the postoperative days 4-40 ($F_{(2,20)} = 22.104$, $p = 0.000$, $F_{(2,20)} = 7.731$, $p = 0.004$, $F_{(2,20)} = 5.635$, $p = 0.013$, $F_{(2,20)} = 24.694$, $p = 0.000$, and $F_{(2,20)} = 12.341$, $p = 0.000$, respectively). On all these days, a statistically significant ($p = 0.000$, $p = 0.001$, $p = 0.005$, $p = 0.000$, $p = 0.000$ and $p = 0.000$,

p=0.010, p=0.010, p=0.000, p=0.000, respectively) differences were found between IC and both infusion groups Rotenone and DMSO which may claim the presence of toxic or harmful effects of DMSO itself.

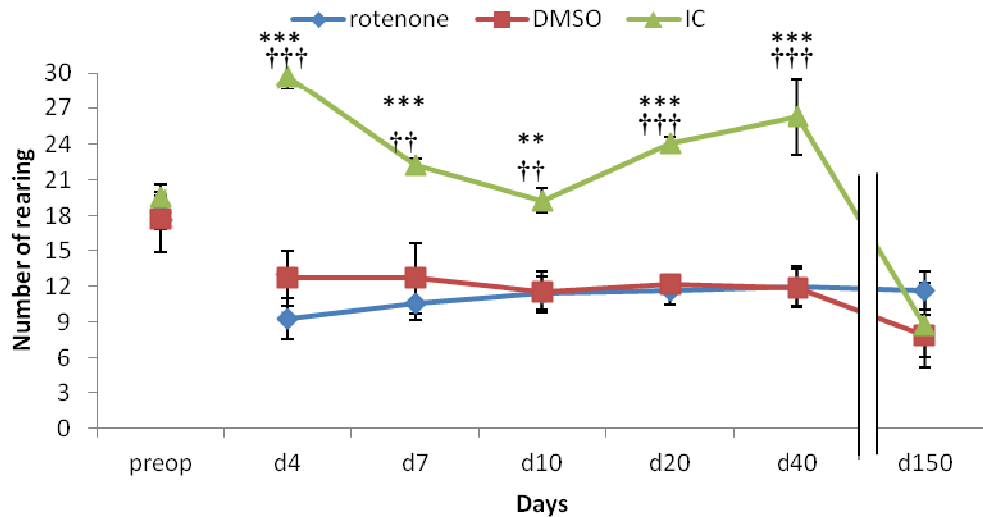


Figure 3.11. Mean number of spontaneous rearings (\pm SEM) prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; † denotes the level of significance for DMSO - IC groups comparison: **/ †† p<0.01, and ***/ ††† p<0.001.

Figure 3.12 shows the rearing scores of 6-OHDA, Saline and IC groups. Here too except the preoperative testing day and postoperative 150th day, neurotoxin group showed lower number of rearings than IC and the vehicle control. Two-way repeated measures ANOVA yielded a significant main effect of group ($F_{(2,19)}=3.802$, p=0.041) and significant effect of day ($F_{(6,114)}=9.092$, p=0.000) as well as significant day x group interaction ($F_{(12,114)}=3.487$, p=0.000). The day effect reflected a steady decrease in the rearing incidents taking place in all groups across the time. The post hoc comparison of single effects revealed a significant difference between 6-OHDA and IC group (p=0.017) but no significant difference between 6-OHDA and Saline group.

One-way ANOVA applied to these data confirmed a significant main group effect for the postoperative days 4 and 40 ($F_{(2,21)}=8.977$, p=0.002 and $F_{(2,21)}=7.677$, p=0.004, respectively). However, it yielded no significant difference between these groups on preoperation day. Post hoc comparisons of simple effects revealed significant differences

between 6-OHDA and both, Saline and IC controls ($p=0.027$ and $p=0.001$, respectively) on day 4, and a significant difference between 6-OHDA group and IC control ($p=0.001$) on day 40. The difference between the 6-OHDA group and the Saline control remained at $p=0.175$ level of significance.

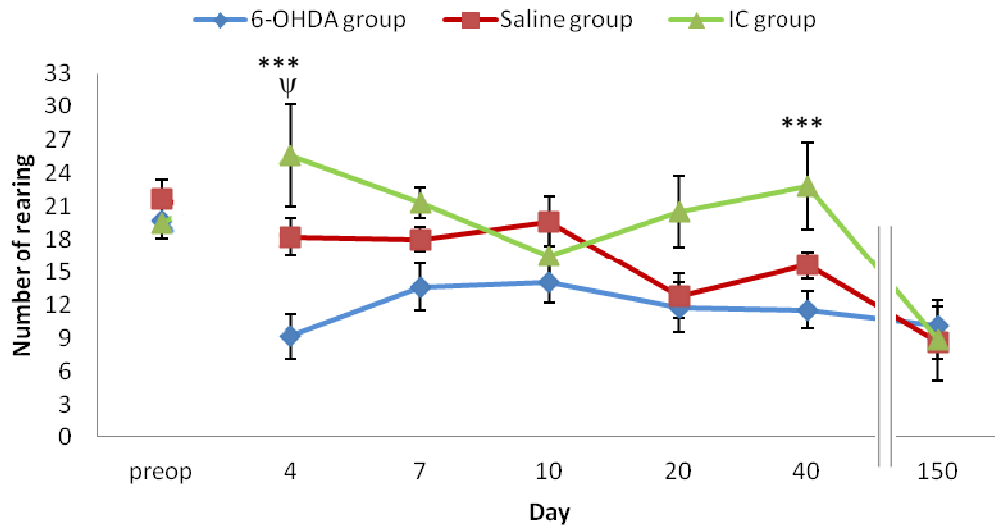


Figure 3.12. Mean number of spontaneous rearings (\pm SEM) prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: ψ $p<0.05$ and *** $p<0.001$.

Figure 3.13 shows the comparison of rearing scores between the preoperation day and the postoperation day 40 for all 3 groups. No significant difference was seen in rearing behavior between groups on the preoperation day, however one-way ANOVA revealed a significant main group effect for the postoperation day 40 ($F_{(2,27)} = 5,109$, $p=0.014$). Post hoc comparisons of simple effects yielded a significant difference between IC and both Rotenone ($p=0.010$) and 6-OHDA ($p=0.005$). The paired t-test analysis revealed significant difference (a decrease in the number of spontaneous rearings) for the 6-OHDA group ($p=0.000$) and the Rotenone group ($p=0.007$) and insignificant difference for IC group ($p=0.367$).

Interestingly, despite of the lack of a significant difference between Rotenone and its vehicle group on the postoperative testing days, the paired t-test comparisons of pre- and postoperative performance in this group showed that the number of rearings in Rotenone groups was significantly lower on all postoperative days compared to the preoperative testing ($p=0.006$, $p=0.024$, $p=0.000$, $p=0.006$, $p=0.007$ and $p=0.005$, respectively) while in DMSO group, the number of rearings being also lower on the postoperative days compared

to the preoperative testing, never reached the significant level of $p = 0.05$ except for the day 150 ($p=0.019$). The numbers of rearing incidents of 6-OHDA group on the postoperative days were also significantly lower than the rearings' number recorded on the preoperative testing day ($p=0.002$, $p=0.031$, $p=0.032$, $p=0.002$, $p=0.000$, and $p=0.000$, respectively). For the Saline group, the paired t-test comparisons of pre- and postoperative performance showed that the number of rearings in Saline groups was significantly lower on postoperative days 20, 40 and 150 compared to the preoperative testing ($p=0.011$, $p=0.022$, and $p=0.000$, respectively).

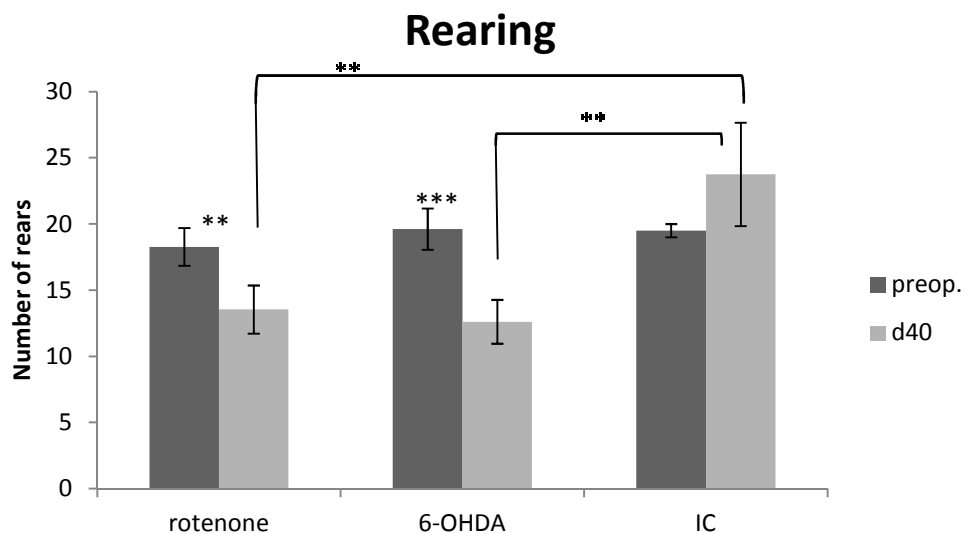


Figure 3.13. Comparison of the rearing test scores between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) number of rearing in 5-min period. Error bars denote \pm SEM. * denotes the level of significance: ** $p < 0.01$, *** $p < 0.001$.

3.2.4 Stepping Test

In the Stepping test, 3 different measurements were taken: the movement initiation time, the stepping length and the number of adjusting steps. All these measurements were collected for the right and left forelimbs independently and the results were analyzed by student t-test for the presence of a difference between right and left side. Since there were found no significant differences between the responses from the left and right forelimbs, these data have been pooled for the further statistical analysis.

3.2.4.1 Initiation Time

As seen from the Figure 3.14, no between-group differences are manifested on the preoperative testing day and 150 days after the surgery. On the remaining postoperative days, except the day 4, the longest time to initiate free limb movement was observed in the Rotenone group. Two-way repeated measures ANOVA (group x day) confirmed significant main effect of group ($F_{(2,18)} = 3.682, p=0.046$), the main effect of day ($F_{(6,108)} = 4.692, p=0.000$), and the day x group interaction ($F_{(12,108)} = 2.642, p=0.004$).

One-way ANOVA confirmed a significant main group effect for the postoperative days 20 and 40 ($F_{(2,20)} = 4.621, p=0.024$ and $F_{(2,20)} = 7.742, p=0.004$, respectively). Post hoc comparison of simple effect confirmed significant differences between Rotenone group and both DMSO and IC control groups on day 20 ($p=0.023$ and $p=0.026$, respectively) and day 40 ($p=0.006$ and $p=0.005$, respectively).

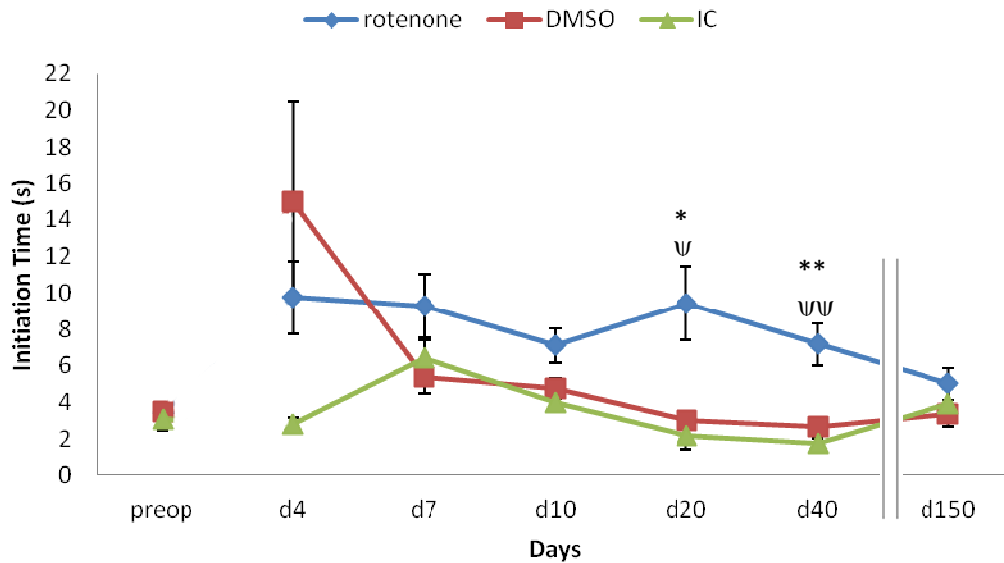


Figure 3.14. Mean initiation time \pm SEM for the Stepping Test part I calculated along the testing days for the treatment groups Rotenone, DMSO and IC independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison and ψ denotes the level of significance for Rotenone - DMSO groups comparison: */ ψ $p<0.05$ and **/ $\psi\psi$ $p<0.01$.

Figure 3.15 shows the mean movement initiation time for the groups 6-OHDA, Saline and IC. Two-way repeated measures ANOVA (group x day) confirmed a significant main effect of group ($F_{(2,19)} = 9.769, p=0.001$), day ($F_{(6,114)} = 3.584, p=0.003$), and significant day x group

interaction ($F_{(12,114)} = 3.364, p = 0.000$). The post hoc tests showed the differences between 6-OHDA and both, Saline and IC controls significant ($p = 0.001$ and $p = 0.005$, respectively).

One-way ANOVA confirmed significant between-group differences on the postoperative days 4-40 ($F_{(2,21)} = 6.729, p = 0.006$, $F_{(2,21)} = 5.343, p = 0.014$, $F_{(2,21)} = 4.816, p = 0.020$, $F_{(2,21)} = 9.688, p = 0.001$ and $F_{(2,21)} = 16.334, p = 0.000$, respectively) with significantly longer movement initiation time in the 6-OHDA group compared to both control groups. The significance level of the differences between 6-OHDA and Saline group on the postoperative days 4-40 were as follows: $p = 0.015$, $p = 0.004$, $p = 0.009$, $p = 0.005$, and $p = 0.000$, respectively; and the significance level of the differences between 6-OHDA and IC group on postoperative days 4, 20 and 40 were $p = 0.006$, $p = 0.001$, and $p = 0.001$, respectively. The largest significant difference was noted on the postoperative day 40.

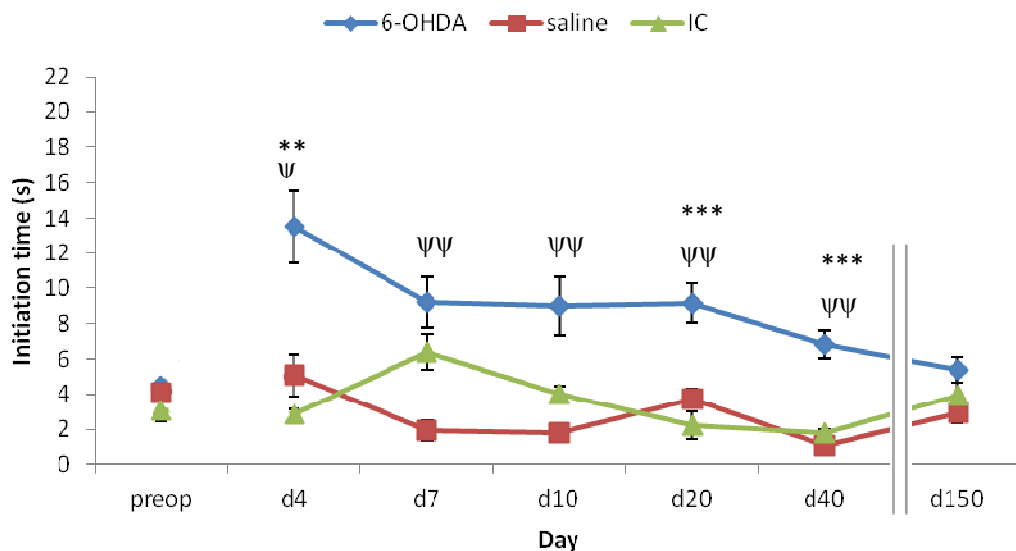


Figure 3.15. Mean initiation time \pm SEM prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: ψ $p < 0.05$, $**/\psi\psi$ $p < 0.01$, and $***$ $p < 0.001$.

Figure 3.16 shows the comparisons between preoperation day and postoperation day 40 in Rotenone, 6-OHDA and IC groups. The data have been evaluated by t-test for paired comparisons. The analysis revealed significant differences between pre- and postoperational animals' performance in Rotenone (0.014) and 6-OHDA group (0.013) but not in IC group.

The postoperative scores in Rotenone group were higher than those recorded during preoperative testing on days 4 through 150 ($p=0.011$, $p=0.005$, $p=0.004$, $p=0.016$, $p=0.014$, and $p=0.073$, respectively). Also, in 6-OHDA group, initiation time was significantly longer on postoperative days 4-40 as compared to the preoperative testing ($p=0.001$, $p=0.006$, $p=0.017$, $p=0.001$, $p=0.013$, respectively) Additionally, the one-way ANOVA yielded significant group difference on both pre- and post-operative day 40 ($F_{(2,27)}=4.967$, $p=0.015$ and $F_{(2,27)}=3.746$, $p=0.038$, respectively) between toxin groups on preoperation day ($p=0.031$); and IC and both Rotenone and 6-OHDA group on the day 40 ($p=0.006$ and $p=0.008$, respectively).

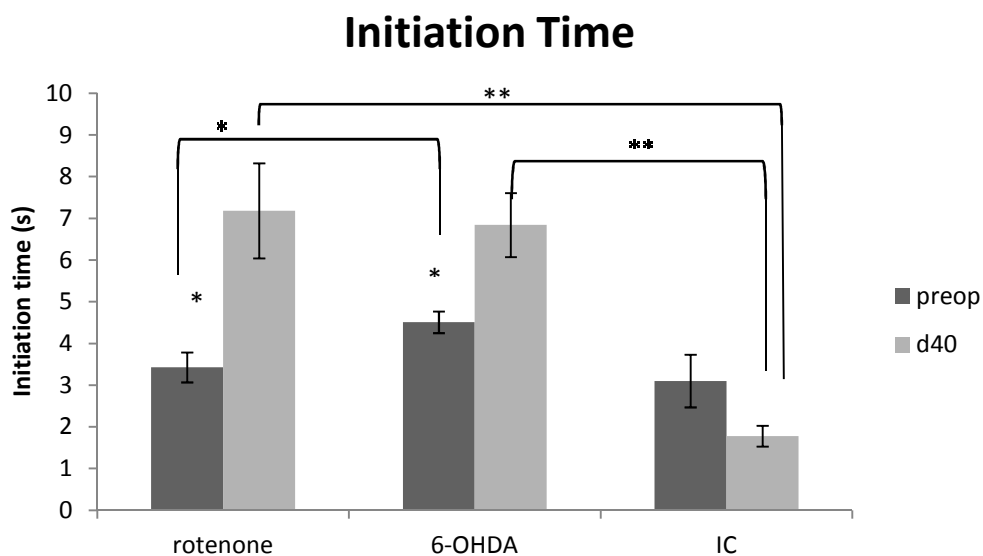


Figure 3.16. Comparison of the initiation time test scores between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) time to initiate movement. Error bars denote \pm SEM. * denotes the level of significance: $*p<0.05$.

3.2.4.2 Stepping Length

Figure 3.17 demonstrates the stepping lengths for Rotenone, DMSO and IC groups on the preoperative testing and throughout the postoperative period. Repeated measures ANOVA (group x day) revealed a significant main effect of group ($F_{(2,18)}=6.685$, $p=0.007$), significant day effect ($F_{(6,108)}=5.947$, $p=0.000$), and significant day x group interaction ($F_{(12,108)}=1.837$, $p=0.051$). The post hoc comparisons confirmed significant difference between Rotenone and IC groups ($p=0.002$) and marginally significant difference between Rotenone and DMSO group ($p=0.064$).

One-way ANOVA yielded a significant group effect on postoperative days 20 and 40 ($F_{(2,20)}=7.596$, $p=0.004$ and $F_{(2,20)}=23.441$, $p=0.000$, respectively). The step length was significantly shorter in Rotenone group as compared to DMSO and IC controls on both day 20 ($p=0.005$ and $p=0.006$, respectively) and day 40 ($p=0.000$ and $p=0.000$) with no significant difference between control groups.

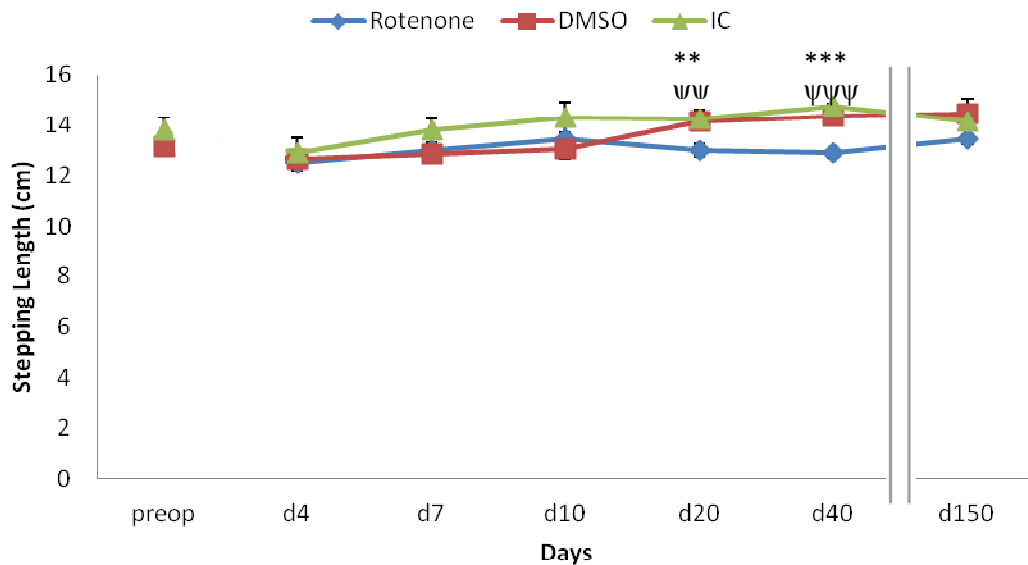


Figure 3.17. Mean step length \pm SEM prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison and ψ denotes the level of significance for Rotenone - DMSO groups comparison: **/ $\psi\psi\psi$ $p<0.01$, and ***/ $\psi\psi\psi\psi$ $p<0.001$.

Figure 3.18 shows the results of the stepping lengths for 6-OHDA, Saline and IC groups. Repeated measures ANOVA (group x day) revealed a significant main effect of group ($F_{(2,19)}=7.100$, $p=0.005$), significant day effect ($F_{(6,114)}=9.974$, $p=0.000$), and marginally significant day x group interaction ($F_{(12,114)}=1.751$, $p=0.065$). The post hoc comparisons confirmed significant difference between 6-OHDA and both IC group ($p=0.005$) and Saline group ($p=0.013$).

However, one-way ANOVA analysis, unlike before, asserted a significant group effect on preoperative day ($F_{(2,21)}=4.134$, $p=0.032$) which precluded an accurate between group comparisons after the surgery. A significant main group effect was also found on all the

postoperation days 4-to-40 ($F_{(2,21)}=4.368$, $p=0.027$, $F_{(2,21)}=3.755$, $p=0.042$, $F_{(2,21)}=4.830$, $p=0.020$, $F_{(2,21)}=5.599$, $p=0.012$, and $F_{(2,21)}=10.805$, $p=0.001$, respectively), but not on day 150. On the postoperative days 4-40, except day 20, the step length was significantly shorter in 6-OHDA group as compared to the Saline group ($p=0.026$, $p=0.016$, $p=0.012$, and $p=0.001$, respectively). Comparing to IC control, the significant difference was revealed both on the preoperative testing day ($p=0.013$) and postoperative days 4-to-40 without day 7 ($p=0.036$, $p=0.054$, $p=0.004$, and $p=0.007$, respectively). However, the postoperation days between 4-40 had no significant difference between control groups despite a significant difference on preoperation day ($p=0.026$).

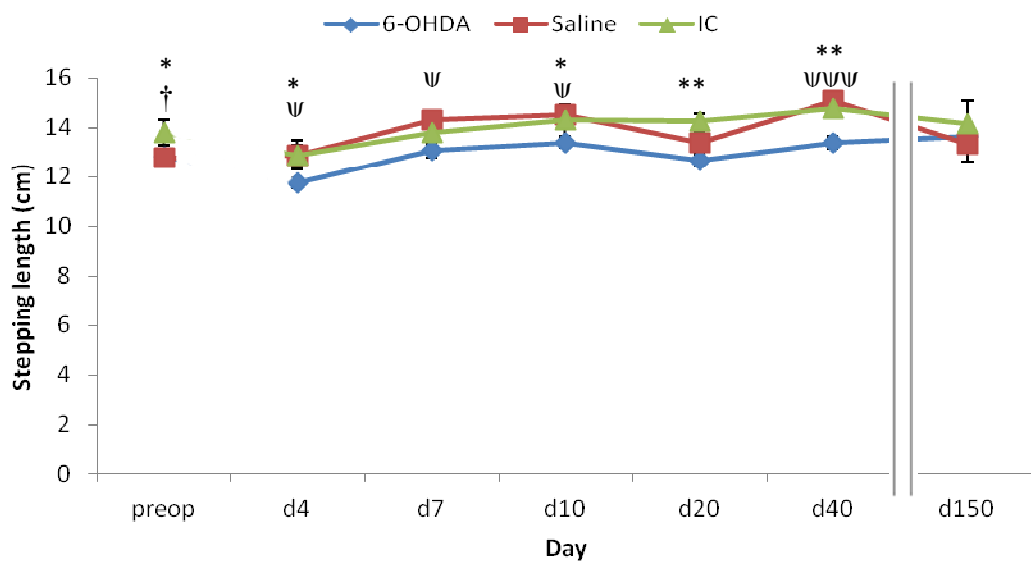


Figure 3.18. Mean step length (\pm SEM) prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison; † denotes the level of significance for Saline - IC groups comparison; and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: */†/ ψ $p<0.05$, **/††/ $\psi\psi$ $p<0.01$, and ***/†††/ $\psi\psi\psi$ $p<0.001$.

When there are observed between-group differences during the pre-operative testing, as it was in this case, the evaluation of a potential motor impairment on individual basis gains special importance. Figure 3.19 shows the comparisons between preoperation day and postoperation day 40 for groups Rotenone, 6-OHDA and IC. The paired t-test analysis of data revealed no significant differences in either Rotenone or IC, and a marginal difference in 6-OHDA group (0.069) indicating towards a decrease in the step length after 6-OHDA administration only. One-way ANOVA resulted, on the other hand, a significant group effect

on the day 40 ($F_{(2,27)} = 9.803, p = 0.001$) between IC group and both Rotenone ($p = 0.000$) and 6-OHDA ($p = 0.003$).

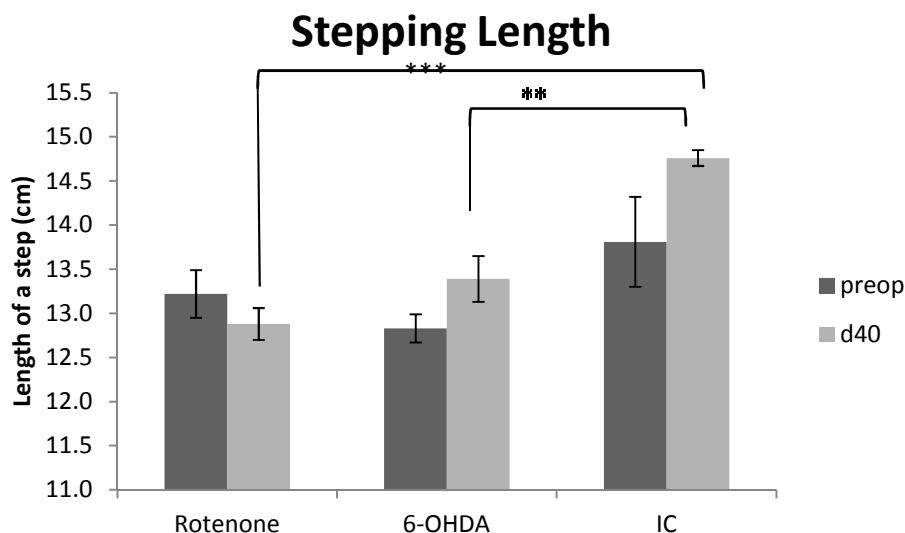


Figure 3.19. Comparison of the stepping length test scores between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) time to initiate movement. Error bars denote \pm SEM. * denotes the level of significance: * $p < 0.05$.

3.2.4.3 Adjusting Steps

As the last part of the stepping test, the number of steps made by the free paw touching the ground with the rat moved at a speed of 90cm/5s was counted. The results are presented in Figure 3.20. Two-way repeated measures ANOVA yielded significant group effect ($F_{(2,18)} = 12.397, p = 0.000$), significant day effect ($F_{(5,90)} = 42.933, p = 0.000$) and significant day x group interaction ($F_{(10,90)} = 5.813, p = 0.000$). According to the results of post hoc between group comparisons, significant differences between Rotenone and both DMSO and IC control groups were found ($p = 0.007$ and $p = 0.000$, respectively).

One-way ANOVA yielded significant between group differences on postoperative days 4, 7, 10, and 40 ($F_{(2,20)} = 6.109, p = 0.009$, $F_{(2,20)} = 15.423, p = 0.000$, $F_{(2,20)} = 14.681, p = 0.000$, and $F_{(2,20)} = 16.909, p = 0.000$, respectively). The number of adjusting steps was significantly lower in Rotenone group as compared to both DMSO and IC controls on day 7 ($p = 0.001$ and $p = 0.000$, respectively), day 10 ($p = 0.000$ and $p = 0.000$, respectively), and day 40 ($p = 0.000$ and $p = 0.003$, respectively). In addition to these, there was found a significant difference between Rotenone and IC ($p = 0.046$) and DMSO and IC group only on day 4 ($p = 0.003$).

which may be the result of the surgery effect. On the day 150, no data could be recorded because most of the animals were reluctant and resistant to this test.

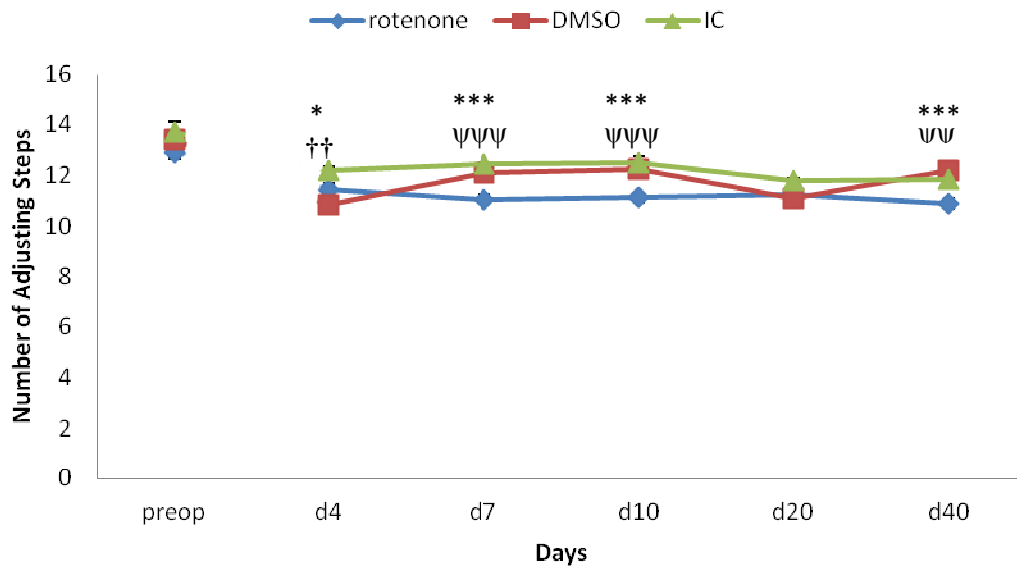


Figure 3.20. Mean number of adjusting steps (\pm SEM) prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; † denotes the level of significance for DMSO - IC groups comparison; and ψ denotes the level of significance for Rotenone - DMSO groups comparison: * $p < 0.05$, ††/ $\psi\psi$ $p < 0.01$, and ***/ $\psi\psi\psi$ $p < 0.001$.

Figure 3.21 is showing the numbers of adjusting steps for the groups 6-OHDA, Saline and IC groups on prior to the operation and throughout the postoperational days 4-40. Two-way repeated measures ANOVA yielded significant group effect ($F_{(2,19)} = 7.100$, $p = 0.005$), significant day effect ($F_{(5,95)} = 9.974$, $p = 0.000$) and a marginal day x group interaction ($F_{(10,95)} = 1.751$, $p = 0.065$). According to the results of post hoc between group comparisons, significant differences between 6-OHDA and both control Saline and IC groups were found ($p = 0.013$ and $p = 0.005$, respectively).

One-way ANOVA yielded significant between group differences both on the preoperation day ($F_{(2,21)} = 4.927$, $p = 0.019$) and postoperative days 4-40 ($F_{(2,21)} = 6.046$, $p = 0.009$, $F_{(2,21)} = 59.892$, $p = 0.000$, $F_{(2,21)} = 14.893$, $p = 0.000$, $F_{(2,21)} = 19.546$, $p = 0.000$ and $F_{(2,21)} = 32.468$, $p = 0.000$, respectively) which precluded an accurate between-group comparison on postoperational testing days. The number of adjusting steps was significantly lower in 6-OHDA group as compared to IC control on preoperation day ($p = 0.005$). The number of

adjusting steps was also significantly lower in 6-OHDA group as compared to both Saline and IC controls on day 4 ($p=0.005$ and $p=0.049$, respectively), day 7 ($p=0.000$ and $p=0.000$, respectively), day 10 ($p=0.080$ and $p=0.005$, respectively), day 20 ($p=0.000$ and $p=0.001$, respectively), and day 40 ($p=0.000$ and $p=0.000$, respectively). However, a significant difference was found between the two control groups, the Saline and IC, on days 7, 10, and 40 ($p=0.000$, $p=0.005$, and $p=0.009$, respectively) with lower performance in Saline group compared to IC control.

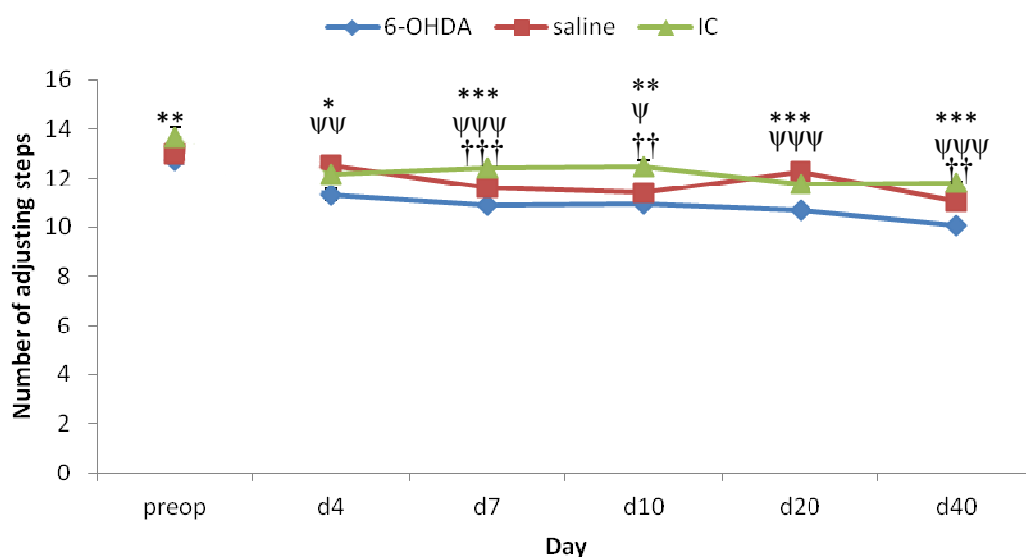


Figure 3.21. Mean number of adjusting steps (\pm SEM) prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison; † denotes the level of significance for Saline - IC groups comparison; and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: * $p<0.05$, **/ ††/ $\psi\psi$ $p<0.01$, and ***/†††/ $\psi\psi\psi$ $p<0.001$.

Figure 3.22 shows the comparisons between the animals' performance on preoperation day and postoperation day 40 in Rotenone, 6-OHDA and IC groups. As mentioned earlier, when between-group differences are observed during the pre-operative testing, as it was in this test, the evaluation of a potential motor impairment on individual basis is especially important. However, the student t-test for paired comparisons applied to these data revealed significant differences between pre- and postoperative (Day 40) animals' performance in all 3 groups: Rotenone, 6-OHDA and IC group ($p=0.000$, $p=0.000$, and $p=0.007$, respectively). Also, one-way ANOVA analysis yielded a significant main group effect on day 40 ($F_{(2,27)}=23.630$, $p=0.000$), and the post hoc LSD analysis showed significant difference

between all 3 groups as follows Rotenone versus 6-OHDA : $p=0.000$, Rotenone vs IC : $p=0.002$, and 6-OHDA vs IC : $p=0.000$.

To compare the results obtained in all three stepping tests, it must be acknowledged that these tests are examining different aspects of motor behavior. The first test measuring initiation time of the paw movement is a test for bradykinesia due to catalepsia, and is not correlated with two other stepping tests. In the present study, the results of this test are consistent with the results of two other tests aimed to screen catalepsia (the bar and the grid tests) showing elongation of the movement initiation time in neurotoxin groups. On the other hand, the tests measuring the step length and the number of adjusting steps are screening the gait and postural balance and are correlated with each other. Normally, the number of steps negatively correlates with the number of steps executed over a certain distance. In the present study such correlation was observed in control and 6-OHDA groups. Only in the Rotenone group, the decrease in the number of adjusting steps was accompanied by a decrease in the step length suggesting stronger effect of Rotenone as compared to 6-OHDA.

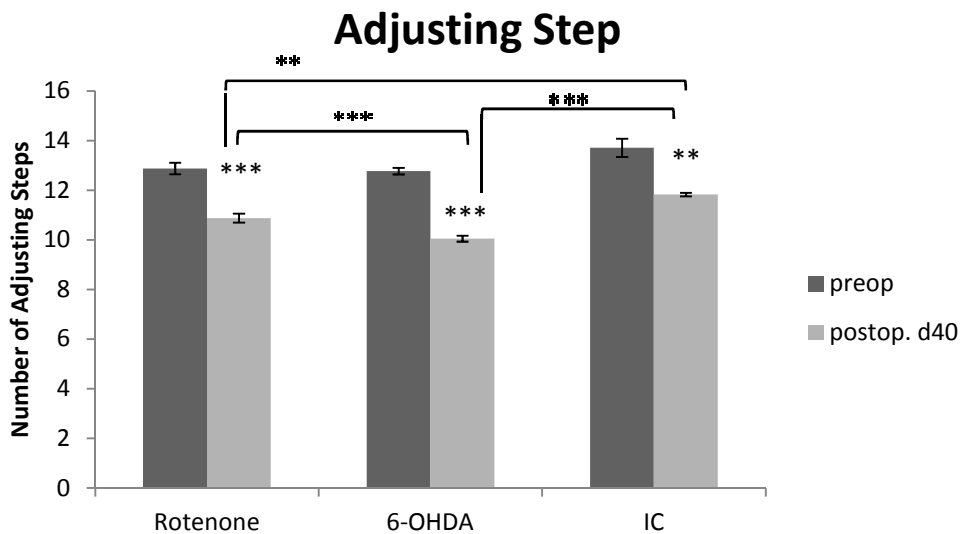


Figure 3.22. Comparison of the adjusting step test scores between preoperation day and the postoperation day 40 for Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) number of adjusting steps. Error bars denote \pm SEM. * denotes the level of significance: ** $p<0.01$, and *** $p<0.001$.

3.2.5 Rotarod / Accelerod Test

For the rotarod/accelerod test covering 3 different sessions, the length of time that each animal was able to stay on the rotating rod was recorded as the latency to fall.

3.2.5.1 Rotarod

Figure 3.23 presents the results of the rotarod test with a stable speed of 20 rpm. As seen from this figure, no substantial between-group differences were observed on a preoperative day and on 150th day after the surgery. On the remaining postoperative days 4-40, the latency to fall was higher in IC group as compared to both infusion groups with little difference between Rotenone group and its vehicle control, the DMSO group. Two-way repeated measure ANOVA showed a highly significant main effect of group ($F_{(2,18)}=14.612, p=0.000$), significant day effect ($F_{(6,108)}=6.209, p=0.000$) and significant day x group interaction ($F_{(12,108)}=4.147, p=0.000$). The significant day x group interaction is reflecting different temporal patterns of the changes in the latency to fall observed over time in different treatment groups: increase in the IC group (probably reflecting motor learning across the repeated trials), decrease in the Rotenone group (potentially related to a drug-induced progressive deterioration of motor skills), and no substantial change in DMSO group. Post-hoc LSD test yielded significant the differences between IC group and both infusion ($p=0.000$ and $p=0.001$, respectively).

One-way ANOVA revealed significant group effect on the postoperative days 4, 7, 20, and 40 ($F_{(2,20)}=11.390, p=0.001, F_{(2,20)}=8.337, p=0.003, F_{(2,20)}=15.075, p=0.000$, and $F_{(2,20)}=9.855, p=0.001$, respectively). Post hoc comparison of simple effects confirmed significantly shorter fall latencies in Rotenone group ($p=0.000, p=0.001, p=0.000$, and $p=0.000$) and DMSO group ($p=0.003, p=0.021, p=0.000$, and $p=0.002$) as compared to IC control, with no difference between Rotenone and DMSO groups.

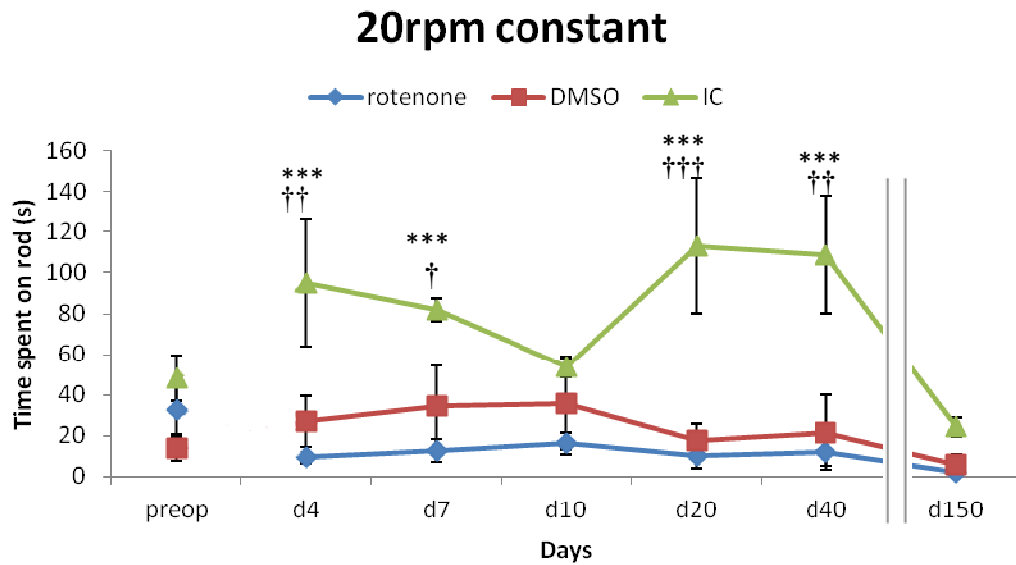


Figure 3.23. Mean latency to fall (\pm SEM) on the rotarod prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenone - IC groups comparison and † denotes the level of significance for DMSO - IC groups comparison: † $p < 0.05$, †† $p < 0.01$, and ***/††† $p < 0.001$.

Figure 3.24 shows the results of the rotarod test in 6-OHDA, Saline, and IC groups. As seen from the figure, there was no between-group difference during the preoperative testing, however, on the postoperative testing days distinct group differences appeared with the longest fall latencies in IC control, intermediate scores in Saline control, and the shortest latencies in 6-OHDA group. Two-way repeated measures ANOVA confirmed a highly significant main effect of group ($F_{(2,19)} = 60.811$, $p = 0.000$), significant day effect ($F_{(6,114)} = 8.615$, $p = 0.000$), and significant day x group interaction ($F_{(12,114)} = 3.822$, $p = 0.000$). The day x group interaction, indicates different temporal patterns of the changes in the fall latency in different treatment groups over the postoperative period: an increase in both IC and Saline control groups (an evidence of motor learning over the repeated trials) and decrease in 6-OHDA group. The post hoc comparisons by LSD test confirmed significant difference between 6-OHDA group and both Saline and IC control groups ($p = 0.000$ and $p = 0.000$, respectively) but also between Saline and, IC controls ($p = 0.000$).

One-way ANOVA, yielded a significant group effect on all 6 postoperative days from 4 day throughout day 150 ($F_{(2,21)} = 14.526$, $p = 0.000$, $F_{(2,21)} = 14.089$, $p = 0.000$, $F_{(2,21)} = 14.377$, $p = 0.000$, $F_{(2,21)} = 14.792$, $p = 0.000$, $F_{(2,21)} = 14.704$, $p = 0.000$ and $F_{(2,21)} = 27.243$, $p = 0.000$,

respectively). The post hoc comparison of simple effects revealed significant differences between 6-OHDA and IC group on all 6 postoperative days ($p=0.000$, $p=0.000$, $p=0.000$, $p=0.000$, $p=0.000$, and $p=0.000$, respectively), between the Saline and IC group on days 4, 7, 20, and 150 ($P=0.001$, $p=0.047$, $p=0.006$, and $p=0.000$, respectively), and between 6-OHDA and Saline group on days 7, 10, and 40 ($P=0.011$, $p=0.007$, and $p=0.002$, respectively). On days 7, 10, and 40, the performance in the 6-OHDA group was significantly lower from that in both IC and vehicle control.

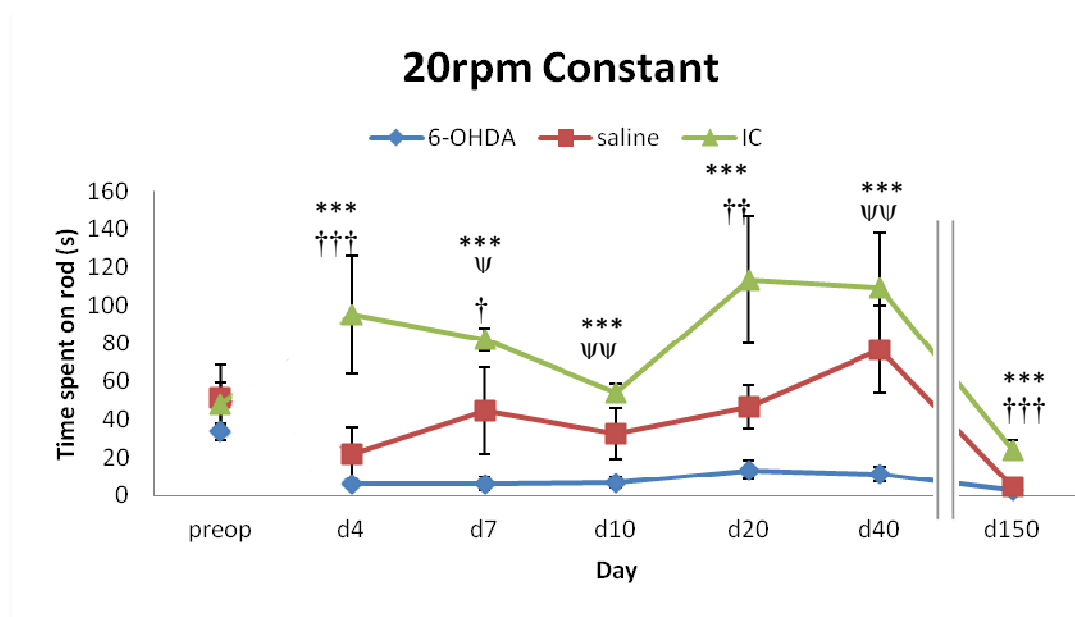


Figure 3.24. Mean latency to fall (\pm SEM) on the rotarod prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC groups comparison; † denotes the level of significance for Saline - IC groups comparison; and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: ψ $p<0.05$, ††/ $\psi\psi$ $p<0.01$, and ***/††† $p<0.001$.

When evaluating drug effects on the individual basis (longitudinal comparisons), all postoperative values of the fall latency in Rotenone group were lower than the fall latency recorded in this group prior to the surgery, however the paired t-test yielded marginal difference between preoperation day and the day 40 ($p=0.166$) while yielded significant difference only between the preoperation day and the day 150 ($p=0.029$). The difference between preoperation day and the rest of the postoperative days such as 4, 7, 10, and 20 were found marginally significant ($p=0.076$, $p=0.169$, $p=0.127$, and $p=0.154$, respectively). Also, the latency values recorded in 6-OHDA group at different postoperative days were lower than the latency observed on the preoperative testing day. The student t-test for paired comparisons revealed significant difference for all postoperative days in 6-OHDA ($p=0.000$, $p=0.001$, $p=0.000$, $p=0.018$, $p=0.001$, and $p=0.000$, respectively). For the IC group, the

student t-test for paired comparisons revealed marginal difference for postoperative day 40 ($p=0.104$) while revealed a significant difference on day 150 ($p=0.024$) compared to preoperative day scores.

As seen from the Figure 3.25, one-way ANOVA yielded no significant group effect on the preoperation day, however showed a significant main group effect on the postoperative day 40 ($F_{(2,27)}=21.909$, $p=0.000$) between the IC group and both toxin groups Rotenone ($p=0.000$) and 6-OHDA ($p=0.000$).

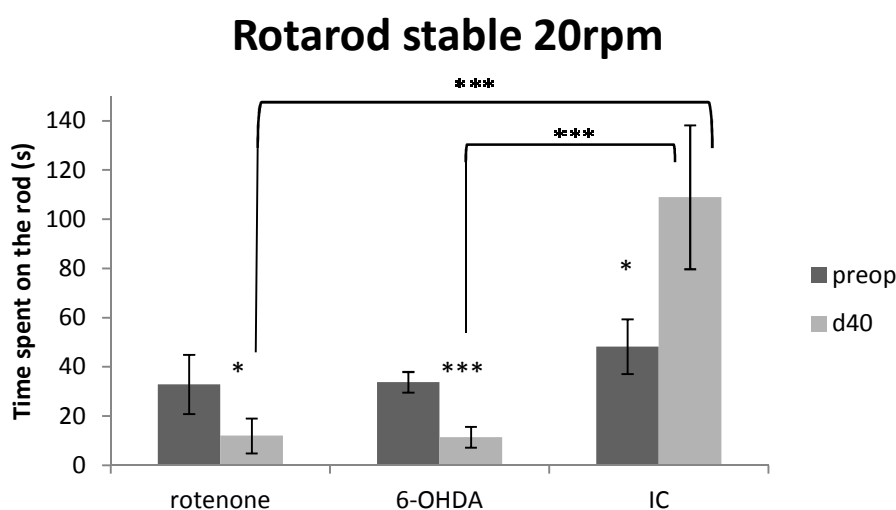


Figure 3.25. Comparison of the latency to fall on the rotarod between preoperation day and the postoperation day 40 in Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) times spent on the rod. Error bars denote \pm SEM. * denotes the level of significance: * $p<0.05$ and *** $p<0.001$.

3.2.5.2 Accelerod-I

In this step, the rotation speed of the rod was not stable but accelerated from 0 to 80 rpm within 10 min. Figure 3.26 displays the results of this test in Rotenone, DMSO, and IC groups. As seen from this figure, in all the postoperative tests, the fall latency was longer in IC group compared to both infusion groups. Also the performance of the DMSO control group was generally higher than the performance recorded in Rotenone group. Two-way repeated measure ANOVA (day x group) showed a significant main effect of group ($F_{(2,18)}=18,113$, $p=0.000$), significant effect of day ($F_{(6,108)}=8.115$, $p=0.000$), and significant day x group interaction ($F_{(12,108)}=1.851$, $p=0.049$). The post hoc comparisons confirmed significant differences between IC and both, Rotenone and DMSO group ($p=0.000$ and $p=0.001$, respectively). As in the previous tests, the day x group interaction indicates different

temporal patterns of the changes in the fall latency in different experimental groups over the postoperative period: an increase in both IC and DMSO control groups and decrease in Rotenone group.

One-way ANOVA yielded the main group effect on the preoperative day insignificant but highly significant on all postoperative days, 4-150 ($F_{(2,20)}=11.515$, $p=0.001$, $F_{(2,20)}=10.155$, $p=0.001$, $F_{(2,20)}=8.693$, $p=0.002$, $F_{(2,20)}=8.276$, $p=0.003$, $F_{(2,20)}=11.040$, $p=0.001$ and $F_{(2,20)}=12.698$, $p=0.000$, respectively). The post hoc comparisons revealed significantly worse performance in both infusion groups, Rotenone and DMSO, as compared to IC group on all postoperative days 4, 7, 10, 20, 40, and 150 ($p=0.031$, $p=0.000$, $p=0.001$, $p=0.001$, $p=0.000$, and $p=0.000$, respectively for the Rotenone group, and $p=0.041$, $p=0.003$, $p=0.013$, $p=0.025$, $p=0.025$, and $p=0.001$, respectively for the DMSO group). Among the postoperative day scores, the post hoc comparisons yielded a significant difference between infusion groups Rotenone and DMSO only on day 40 ($p=0.040$).

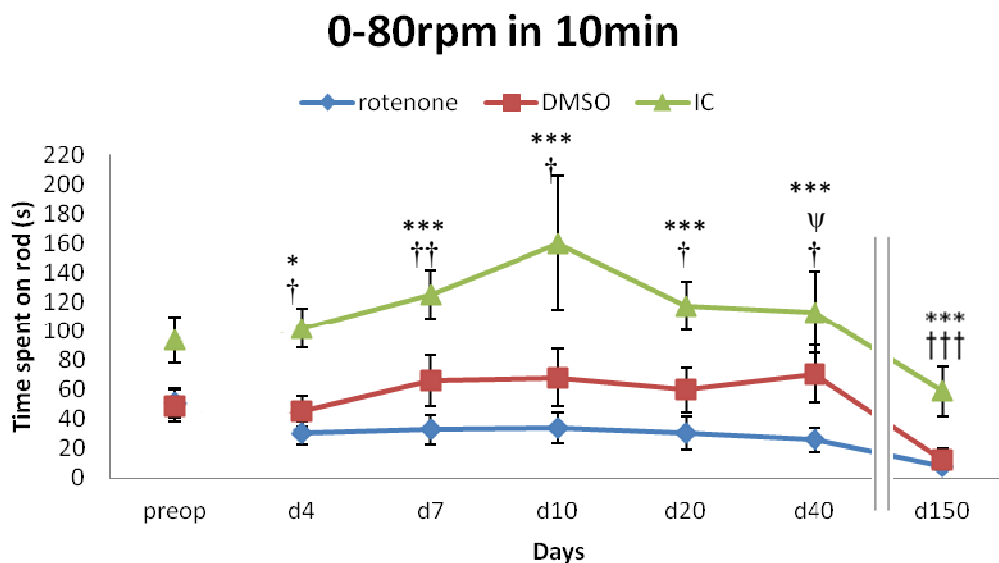


Figure 3.26. Mean latency to fall (\pm SEM) on the accelerod (80 rpm/10 min) prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; † denotes the level of significance for DMSO - IC groups comparison; and ψ denotes the level of significance for Rotenone - DMSO groups comparison: */†/ ψ $p<0.05$, ††/††† $p<0.01$, and ***/†††† $p<0.001$.

Figure 3.27, compares the results of the accelerod task with the increasing rotation speed of the rod up to 80 rpm in 10 min between 6-OHDA, Saline and IC groups. As seen from the

figure, both control groups showed longer fall latencies than the 6-OHDA group throughout the postoperative days 4-40. While the performance of the control groups was improving in the course of the experiment, the performance of the 6-OHDA group was declining. Repeated measures ANOVA revealed significant group effect ($F_{(2,19)}=46.771, p=0.000$), significant day effect ($F_{(6,114)}=9.233, p=0.000$) and significant day x group interaction ($F_{(12,114)}=2.982, p=0.001$).

One-way ANOVA yielded significant group effect on all postoperative days 4, 7, 10, 20, 40, and 150 ($F_{(2,21)}=10.383, p=0.001, F_{(2,21)}=21.967, p=0.000, F_{(2,21)}=15.164, p=0.000, F_{(2,21)}=24.225, p=0.000, F_{(2,21)}=17.856, p=0.000, and F_{(2,21)}=11.940, p=0.000$, respectively) with the significant differences between the 6-OHDA and Saline groups ($p=0.003, p=0.001, p=0.007, p=0.000, and p=0.001$, respectively for days 4-40) and between the 6-OHDA and IC groups ($p=0.001, p=0.000, p=0.000, p=0.000, p=0.000, and p=0.000$, for days 4-150, respectively). However, in this task, a significantly worse performance was observed in 6-OHDA group compared to both Saline and IC controls also on the preoperative testing day ($p=0.040$ and $p=0.012$, respectively), the evaluation of the neurotoxin-induced motor impairment is mainly based on longitudinal comparisons using student t-test for paired comparisons. According to the results of this test, the postoperative performance in the neurotoxin group was significantly worse from its preoperative performance on all the postoperative days 4, 7, 10, 20, 40, and 150 ($p=0.010, p=0.005, p=0.005, p=0.008, p=0.012, and p=0.000$, respectively).

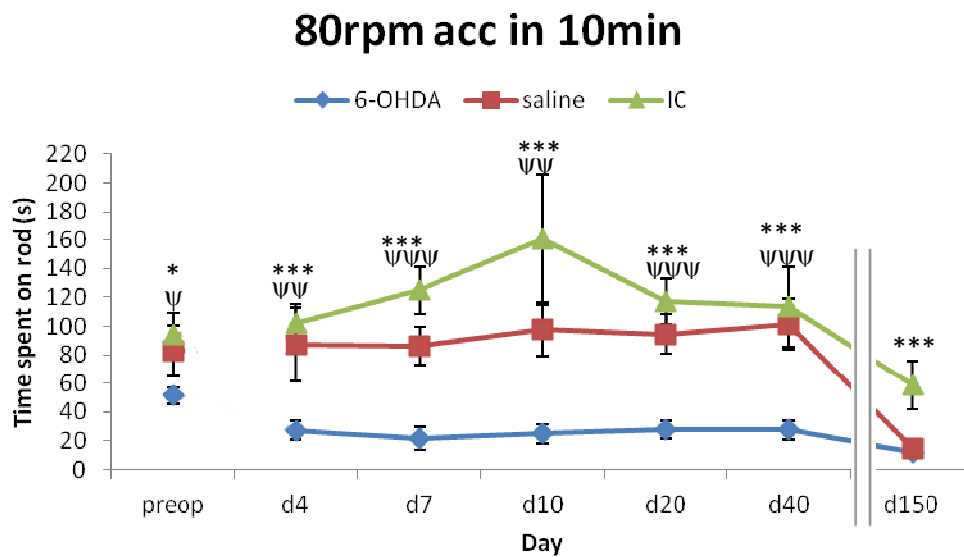


Figure 3.27. Mean latency to fall (\pm SEM) on the accelerated rod (80 rpm/10 min) prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC group comparison and ψ denotes the level of significance for 6-OHDA - Saline group comparison: */ ψ $p < 0.05$, $\psi\psi$ $p < 0.01$, and $***/\psi\psi\psi$ $p < 0.001$.

Figure 3.28 shows the comparisons of fall latency on accelerated rod (80rpm/10min) between the preoperation testing day and postoperation day 40 for the Rotenone, 6-OHDA and IC groups. The t-test for paired comparisons revealed a marginally significant difference in Rotenone group ($p=0.029$), a significant difference in 6-OHDA group ($p=0.007$) and nearly a significant difference in IC group ($p=0.059$). The one-way ANOVA yielded significant main group effect on both preoperation day ($F_{(2,27)}=4.163$, $p=0.028$) and the postoperation day 40 ($F_{(2,27)}=20.024$, $p=0.000$) with differences between groups IC and both Rotenone ($p=0.013$ and $p=0.012$, respectively) and 6-OHDA ($p=0.000$ and $p=0.000$, respectively).

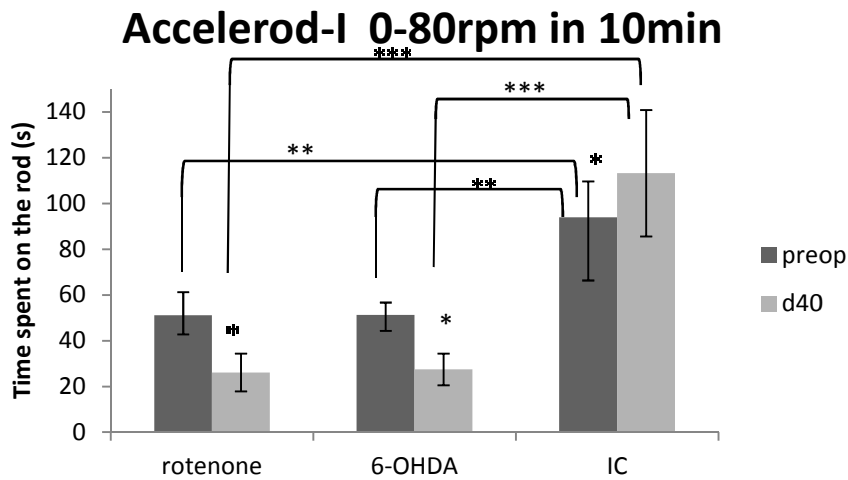


Figure 3.28. Comparison of the latency to fall on the accelerod (80 rpm/10 min) between preoperation day and the postoperation day 40 in Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) time spent on the rod. Error bars denote \pm SEM. * denotes the level of significance: * $p < 0.05$, ** $p < 0.010$, *** $p < 0.001$.

3.2.5.3 Accelerod-II

The last step of the accelerod testing measured fall latency under the rod acceleration from 0 to 80 rpm within 4 min. The results are presented in Fig. 3.29 and Fig. 3.30 for the Rotenone and 6-OHDA groups and their controls, respectively. As seen from the Figure 3.28, in all the postoperative tests, the fall latency was longer in IC group compared to both infusion groups. Also the performance of the DMSO control group was generally higher than the performance recorded in Rotenone group. Two-way repeated measure ANOVA (day x group) showed a significant main effect of group ($F_{(2,18)} = 18,113$, $p = 0.000$), significant effect of day ($F_{(6,108)} = 13.591$, $p = 0.000$), and significant day x group interaction ($F_{(12,108)} = 3.109$, $p = 0.001$). The post hoc comparisons confirmed significant differences between IC and both, Rotenone and DMSO group ($p = 0.000$ and $p = 0.001$, respectively). As in the previous tests, the day x group interaction indicates different temporal patterns of the changes in the fall latency in different experimental groups over the postoperative period: an increase in both IC and DMSO control groups and decrease in Rotenone group.

One-way ANOVA, yielded the main group effect on both the preoperative day ($F_{(2,20)} = 4.262$, $p = 0.031$) and all postoperative days, 4-150 ($F_{(2,20)} = 7.349$, $p = 0.005$, $F_{(2,20)} = 8.188$, $p = 0.003$, $F_{(2,20)} = 7.067$, $p = 0.005$, $F_{(2,20)} = 14.701$, $p = 0.000$, $F_{(2,20)} = 20.529$, $p = 0.000$ and $F_{(2,20)} = 7.377$, $p = 0.005$, respectively). The post hoc comparisons revealed that the animal's performance in

both infusion groups was significantly worse than that in IC group indicating significant differences between IC and both Rotenone and DMSO groups on preoperation day ($p=0.019$ and $p=0.014$, respectively) and all postoperative days 4-to-150 ($p=0.001$, $p=0.001$, $p=0.002$, $p=0.000$, $p=0.000$, and $p=0.001$, respectively for the Rotenon group and $p=0.029$, $p=0.079$, $p=0.062$, $p=0.001$, $p=0.001$, and $p=0.014$, respectively for the DMSO group). A significant difference between infusion groups Rotenone and DMSO was found only on days 7 and 40 ($p=0.045$ and $p=0.020$, respectively).

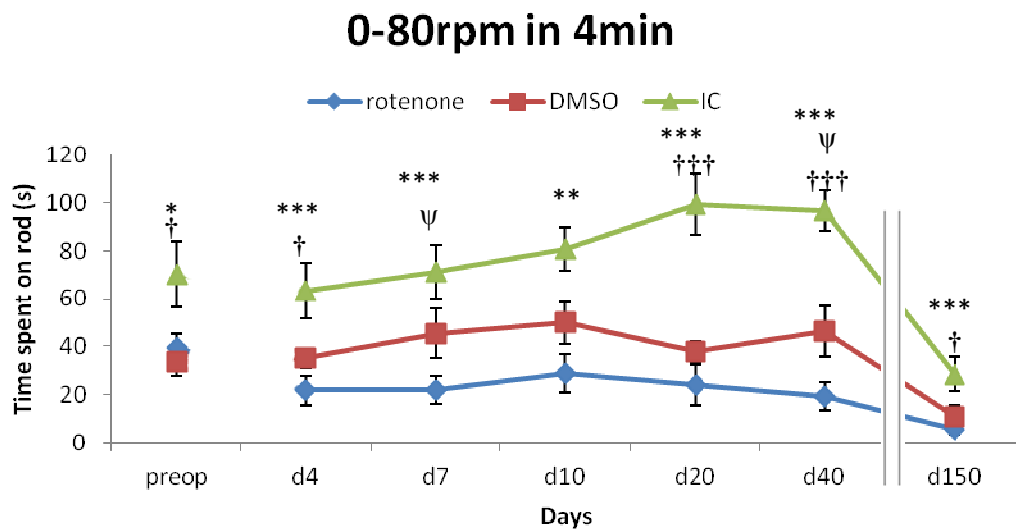


Figure 3.29. Mean latency to fall (\pm SEM) on the accelerated rod (80 rpm/4 min) prior to the operation and at different time points after the operation in Rotenon, DMSO, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for Rotenon - IC groups comparison; † denotes the level of significance for DMSO - IC groups comparison; and ψ denotes the level of significance for Rotenone - DMSO groups comparison: */†/ ψ $p<0.05$, ** $p<0.01$, and ***/††† $p<0.001$.

Figure 3.30, compares the results of the accelerated task with the increasing rotation speed of the rod up to 80 rpm in 4 min between 6-OHDA, Saline and IC groups. As seen from the figure, both control groups showed longer fall latencies than the 6-OHDA group throughout the postoperative days 4-40. While the performance of the control groups was improving in the course of the experiment, the performance of the 6-OHDA group was declining. Repeated measures ANOVA revealed significant group effect ($F_{(2,19)}=38.037$, $p=0.000$), significant day effect ($F_{(6,114)}=15.432$, $p=0.000$) and significant day x group interaction ($F_{(12,114)}=3.692$, $p=0.000$).

One-way ANOVA, yielded the group effect on both the preoperative day ($F_{(2,21)} = 5.018$, $p=0.018$) and all postoperative days, 4-150 ($F_{(2,21)} = 7.3024$, $p=0.005$, $F_{(2,21)} = 22.724$, $p=0.000$, $F_{(2,21)} = 11.851$, $p=0.000$, $F_{(2,21)} = 19.564$, $p=0.000$, $F_{(2,21)} = 28.196$, $p=0.000$ and $F_{(2,21)} = 6.283$, $p=0.008$, respectively). The post hoc comparison of simple effects revealed significantly worse performance in 6-OHDA group compared to IC control on all postoperative days 4-150 ($p=0.009$, $p=0.000$, $p=0.002$, $p=0.000$, $p=0.000$, and $p=0.002$, respectively) and between Saline and IC groups on days 20 and 150 ($p=0.022$ and $p=0.015$, respectively). On the postoperative days 4, 7, 10, 20, and 40, a significant difference between infusion groups 6-OHDA and Saline was found ($p=0.007$, $p=0.000$, $p=0.001$, $p=0.004$, and $p=0.000$, respectively).

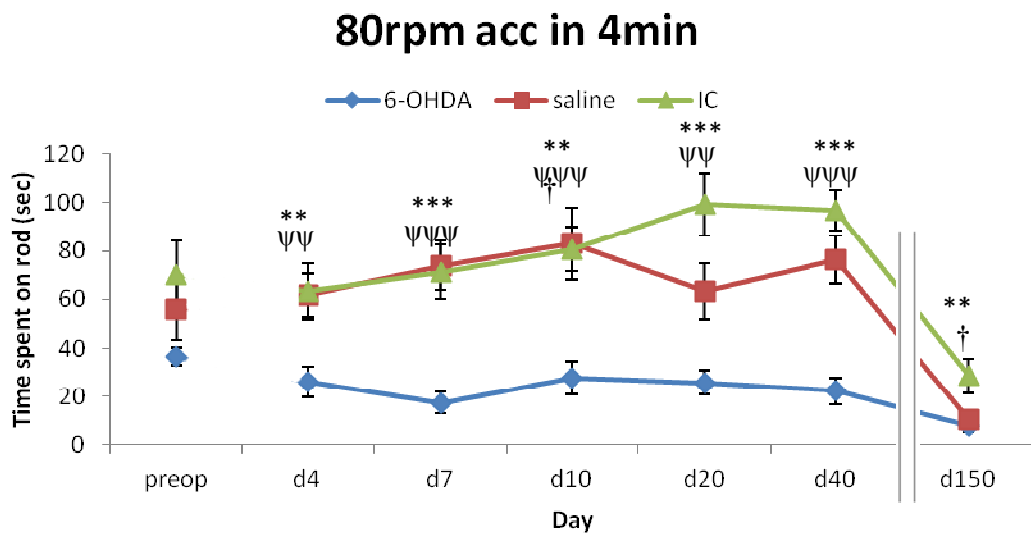


Figure 3.30. Mean latency to fall (\pm SEM) on the accelerated rod (80 rpm/4 min) prior to the operation and at different time points after the operation in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM. * denotes the level of significance for 6-OHDA - IC group comparison; † denotes the level of significance for Saline - IC groups comparison; and ψ denotes the level of significance for 6-OHDA - Saline groups comparison: † $p<0.05$, **/ $\psi\psi$ $p<0.01$, and ***/ $\psi\psi\psi$ $p<0.001$.

Figure 3.31 shows the comparisons of latency time scores on accelerated rod (80rpm/4min) between the preoperation day and the postoperation day 40 in Rotenone, 6-OHDA and IC groups. The t-test for paired comparisons revealed a significant differences in Rotenone and 6-OHDA groups ($p=0.001$ and $p=0.028$, respectively) and no significant difference in IC group. Similar to previous results, one-way ANOVA yielded a significant group effect on both pre- and post-operative day 40 ($F_{(2,27)} = 5.299$, $p=0.012$ and $F_{(2,27)} = 27.573$, $p=0.000$, respectively). The post hoc comparisons revealed the significant difference for these days

between IC and both toxin groups Rotenone ($p=0.008$ and $p=0.000$, respectively) and 6-OHDA ($p=0.004$ and $p=0.000$, respectively).

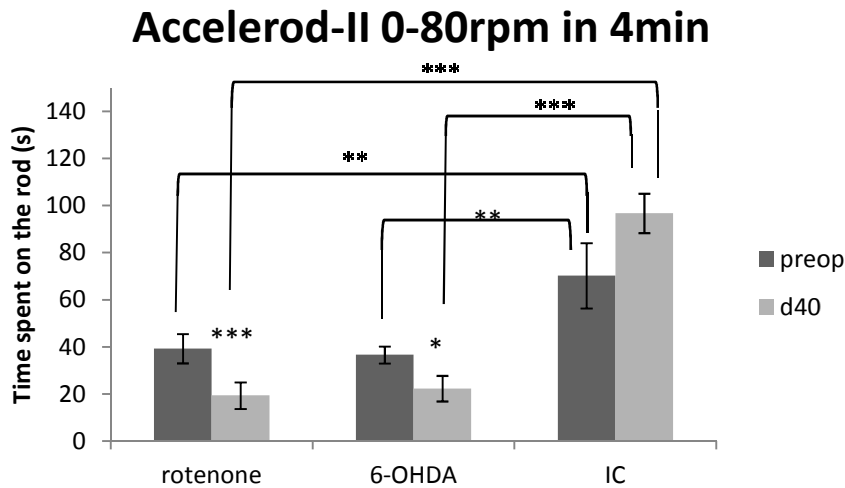


Figure 3.31. Comparison of the latency to fall on the accelerod (80 rpm/4 min) between preoperation day and the postoperation day 40 in Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) distance travelled (cm) calculated for the first 5-min interval of the total 15-min testing period. Error bars denote \pm SEM. * denotes the level of significance: * $p<0.05$, ** $p<0.010$, and *** $p<0.001$.

3.2.6 Probabilistic Learning

In the probabilistic learning task, choices of the advantageous arms in 20 trial-block/day for 7 days were recorded. As described in the Material and Methods chapter, at the beginning of experiments, animals were randomly assigned into 4 sub-groups to counteract the nonspecific effects of potential direction (right/left) or color (white/black) preference by the subjects. The data from these groups were pooled and analyzed for the frequency of advantageous and disadvantageous arm choices.

The Figure 3.32 presents the learning curves for Rotenone, DMSO, and IC groups showing in all three groups, an increase in the number of the advantageous arm choices over the seven consecutive days of training. After applying a normality test (Shapiro-Wilk) to these data confirming normal data distribution, the two-way repeated measures ANOVA (group \times day) was performed. It revealed a marginal main effect of group ($F_{(2,18)}=2.841$, $p=0.085$), a significant main effect of day ($F_{(6,108)}=20.485$, $p=0.000$) and no day \times group interaction.

However, one-way ANOVA performed for each training day separately did not revealed a

significant main group effect on any of 7 days precluding a potential deteriorating effect of Rotenon or DMSO on the animals cognitive performance.

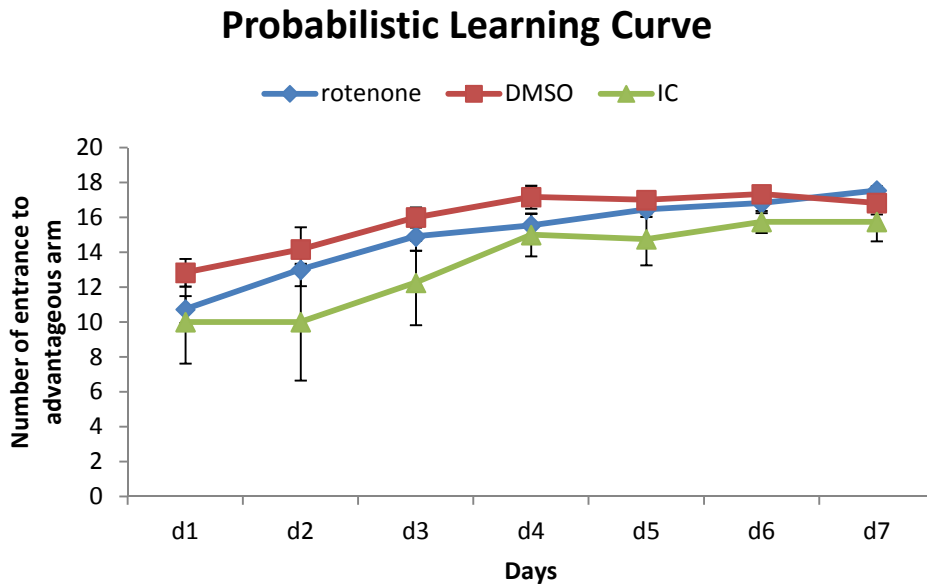


Figure 3.32. Mean number of advantageous arm choices (\pm SEM) in the probabilistic learning task on seven consecutive training days in Rotenone, DMSO, and IC groups, independently. Error bars denote \pm SEM.

Figure 3.33 presents the learning curves for 6-OHDA, saline and IC groups. Two-way repeated measure ANOVA (group x day) revealed significant only the day effect ($F_{(6,108)} = 13.661, p=0.000$) confirming the lack of deteriorating effect of 6-OHDA and/or saline infusions to SNpc on the animals' cognitive status.

Figure 3.34 shows the comparisons of learning scores on in PL task between preoperation day and postoperation day 40 in groups Rotenone, 6-OHDA and IC depending on student t-test for paired comparisons. The analysis revealed a high significant difference in Rotenone ($p=0.000$), 6-OHDA group ($p=0.000$) and nearly significance in IC group ($p=0.059$). The one-way ANOVA yielded significant group difference not for the preoperation day but day 40 ($F_{(2,27)} = 3.457, p=0.047$) between Rotenone and both 6-OHDA and IC group depending on post hoc test LSD ($p=0.031$ and $p=0.049$ on day 40).

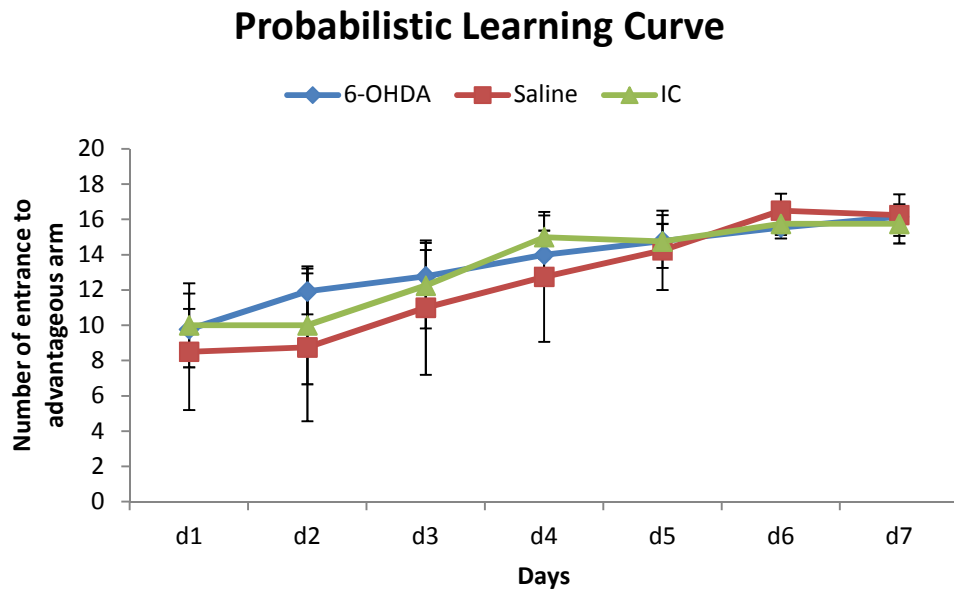


Figure 3.33. Mean number of advantageous arm choices (\pm SEM) in the probabilistic learning task on seven consecutive training days in 6-OHDA, Saline, and IC groups, independently. Error bars denote \pm SEM.

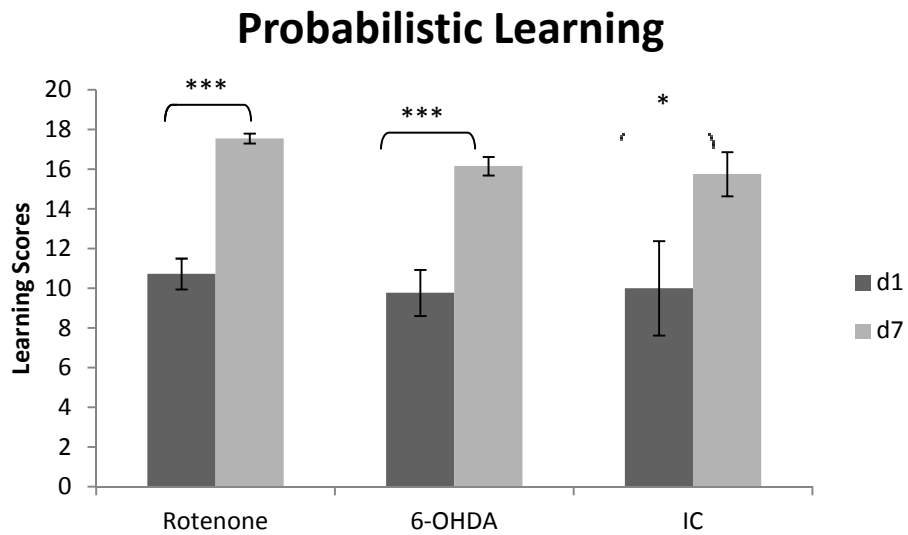


Figure 3.34. Comparison of the learning scores in PL task between preoperation day and the postoperation day 40 in Rotenone, 6-OHDA and IC group, independently. Data were presented as mean (\pm SEM) number of advantageous arm choices. Error bars denote \pm SEM. * denotes the level of significance: * $p < 0.050$ and *** $p < 0.001$.

CHAPTER 4

DISCUSSION

To the best of our knowledge, this is the first study comparing effects of the bilateral, intranigral administration of two neurotoxins, Rotenone and 6-OHDA, on the development of PD-like symptoms in the same rat population using a large battery of behavioral tests. Also, the present study is unique because it provides detailed, both cross-sectional and longitudinal analysis of the behavioral data and thus enables the investigation of induction and progression of the PD-like symptoms on the individual basis. The applied behavioral tests are evaluated for their diagnostic potential.

In the present study, all infusion groups showed significantly lower behavioral performance on Day 4 which indicated a lack of a full recovery from the surgery and manifested the negative, nonspecific effect of the surgery on animals' physical conditions and thus their performance in the behavioral tasks applied. However, in our pharmacological PD models with intranigral infusions of low doses of toxins, no mortality was recorded among toxin-induced groups except one subject from the Rotenone group. Instead, we have recorded few deaths in the control groups (3 from IC, 1 from DMSO and 2 from Saline) which, however, were not caused by the toxin infusion. After the surgeries, no explicit sign of severe health problems were detected in operated animals, but especially during the recovery period (postoperative days 1- 4), some animals showed decreased food consumption and the liqued sucrose solution diet was supportively provided to prevent an excessive loss of body weight. In the long run, the housing animals in separate cages to better control food intake by individual subjects could be the reason of weight gain towards the end of experiments and could effect the results on day 150, five months after the surgery.

As assessed by both cross-sectional (between different treatment groups on a particular testing day) and longitudinal (between pre- and the postoperative performance in the same treatment group) comparisons of the results recorded in a battery of behavioral tests, the low doses of Rotenone and 6-OHDA applied bilaterally to SNpc showed adverse effects on the

animals' motor performance with the greatest impairment observed between days 20-40 after the drug infusion suggesting progressive development of the motor symptoms in case of both neurotoxins. Despite PD animal model of Rotenone (an agricultural chemical) is relatively new and has not been studied as much as MPTP or 6-OHDA models, the mechanisms of toxicity of these two neurotoxins are largely known. As mentioned in the introduction part classical toxin-induced models (1.3.3.1.2), 6-OHDA competitively uptaken by the dopamine transporter (DAT) was shown to cause a rapid and selective damage to DA neurons. It easily forms free radicals and is a potent inhibitor of the mitochondrial respiratory chain complexes I and IV both leading to the reduction of intracellular net ATP levels and ATP/ADP ratios (Glinka et al., 1997; Lehmensiek et al., 2006). On the other hand, Rotenone does not depend on dopamine transporters for its action but due to its fat solubility enters the cells by solubility diffusion. It has been postulated that intrinsic metabolic properties of the nigrostriatal dopaminergic neurons explain the strong Rotenone effect on these neurons including inhibition of striatal dopamine uptake (Semper et al., 1993), inhibition of mitochondrial Complex-I (Betarbet, Sherer, & Greenamyre, 2002) and microtubule depolymerization disrupting transport of neurotransmitters vesicles and formation in mesencephalic neurons cytoplasmic aggregates containing γ -tubulin and α -synuclein proteins (Eisenhofer et al., 2004; Floor et al. 1995). Due to selective uptake of 6-OHDA by catecholaminergic neurons and wider and more uniform uptake of Rotenone by different types of neurons in the brain, 6-OHDA was reported to produce acute toxicity and Rotenone progressive chronic changes especially with systemic administration of these neurotoxins (Meredith & Kang, 2006). However, with local microinjection to SNpc, as in the present study, the temporal profile of Rotenone and 6-OHDA toxicity was similar. The severity of behavioral deficits observed in these two models was also similar what is not surprising considering similarity in the major mechanisms of their neurotoxicity converging on the inhibition of the mitochondrial respiratory chain and DA uptake.

Compared to the intact control, intranigral, low-dose Rotenone infusion showed only marginal effect on animals' locomotor activity restricted to Days 7 and 40. This observation was consistent with the lack of a significant difference between pre- and postoperational performance in the Rotenone group. Conversely, similar infusion of low dose of 6-OHDA produced significant reduction in locomotor activity on days 10 and 40 with significant difference between pre- and postoperational performance. However, the effects of vehicle infusions showed effects similar to the effects of neurotoxins which may suggest that the observed behavioral deficits were not specific to the neurotoxin-induced damage. The

decrease in locomotor activity over repeated test trials could be related to habituation to experimental situation and decrease of exploratory drive.

In the literature, systemic administration (i.p., i.v., and s.c.) of 6-OHDA (Rodríguez Díaz, Abdala, Barroso-Chinea, Obeso, & González-Hernández, 2001) and Rotenone (Alam & Schmidt, 2002; Duty & Jenner, 2011; Fleming et al., 2004) were reported as causing decrease in locomotor activity with dependence on many different factors such as dose of the neurotoxin, timing and way of its administration, the age and even the strain of the animals (Sanberg et al., 1988). There are also studies showing the adverse effects of unilateral infusion of both 6-OHDA and Rotenone in SNc or MFB regions on locomotor behavior (Klein et al., 2011; Meredith & Kang, 2006). Sakai & Gash (1994) showed the effect of low dose (4µg/1µl saline) bilateral 6-OHDA infusion into SNpc which revealed significant decrease 20 and 27 days after surgery; or Alam, Mayerhofer, & Schmidt (2004) displayed the low dose (3µg/4µl propylene glycol) rotenone infusion bilaterally into MFB effect on locomotor activity as the studies above in a decreasing manner. On the other hand, in the literature, there are no specific reports about Rotenone effects on locomotor behavior in rats after SNpc-targeted Rotenone infusions. In opposition to these effective results, Rodríguez Díaz, Abdala, Barroso-Chinea, Obeso, & González-Hernández, (2001) showed that 5, 11 and 60 days after the infusion of various doses of 6-OHDA into the third ventricle, no significant decrease of locomotor activity in the low dose-given groups were observed. Similarly, de Meira Santos Lima et al., (2006) showed no significant decrease in the locomotion frequency in the SNpc-targeted bilaterally 6-OHDA-induced rats. To make an inference from these, in the present study, the mild effect of neurotoxins on locomotor activity may be due to the low dose of the drug and lower vulnerability of this behavior to S-N damage.

Several previous studies on PD patients (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003) and animals (Tillerson et al., 2002; Muralikrishnan and Ebadi, 2001; Uthayathas et al., 2007) reported close relation between neurodegeneration of nigrostriatal system and such motor symptoms as akinesia, muscle rigidity and catalepsy. In the current study, elongation of the movement initiation time (bradykinesia) was observed in both neurotoxin groups, in the catalepsy tasks, however, more profound impairment was noted in the grid task than in the bar task. In the bar task, the elongation of the time to the first movement was observed only on Day 20 in 6-OHDA and on Day 40 in Rotenone group (which may indicate the more prominent neurotoxic effect of 6-OHDA), while in the grid task, the behavioral deficits were recorded from Day 7 through Day 40. In both tasks, the animals showed deficient performance as compared to both controls which would suggest specific effect of the neurotoxins rather than the side effect of

the infusion itself. Similar results as in the bar test were obtained when the initiation time of the free limb movement was recorded in the stepping test. Here too, both neurotoxin groups, as compared to intact and vehicle controls, manifested elongation of the movement initiation time on days 20 and 40. These results are consistent with the results of previous studies wherein bradykinesia was reported after administration of Rotenone and 6-OHDA to rats. Particularly, bilateral administration of 6-OHDA (Ferro et al., 2005; Sakai & Gash, 1994) and rotenone (Alam et al., 2004) into SNpc; various mode of administration of rotenone such as intracranial commonly into SN and MFB sites (Abdulwahid Arif & Ahmad Khan, 2010; Alam et al., 2004; Sindhu, Saravanan, & Mohanakumar, 2005); i.v. delivery via osmotic mini-pumps (Abdulwahid Arif & Ahmad Khan, 2010); s.c. or i.p. (Alam & Schmidt, 2002) especially on Sprague-Dawley and Lewis rats all produced a cardinal motor symptom of PD bradykinesia. Our results suggest that slowing down the movements of the whole body (just like in the grid test) appears earlier after the nigrostriatal damage than slowing of the limb movements (just like in the bar and the stepping tests).

Rearing behavior allows to evaluate a rodent's spontaneous forelimb use and body balance when standing on the hind limbs. During the postoperational tests starting from day 4 through day 40, in both neurotoxin groups, the number of rearings significantly decreased as compared to intact control and preoperational performance bias. However, there was no difference between the Rotenone group and its vehicle control which may indicate the toxic effect of DMSO itself. The comparison of the behavioral scores in two vehicle groups (DMSO and Saline) also suggests the presence of the adverse behavioral effects of DMSO which are manifested apart from the potential, short-lasting effect of the surgery/drug infusion per se. Nevertheless, our results from the rearing (cylinder) test are consistent with the results of previous studies where bilateral nigro-striatal lesions produced by either Rotenone or 6-OHDA administration caused decrease in the rearing activity proportional to the striatal DA depletion (Alam et al., 2004; Sakai & Gash, 1994).

The tasks measuring length of a step, and the number of adjusting steps executed by a free paw touching the ground when the rest of the body is lifted and moved along the ramp are testing stiffness of the limbs which is expressed itself as rigidity in PD (Fang et al., 2006) and motor initiation deficits in the forelimbs, similar to limb akinesia and gait problems in PD patients (Olsson et al., 1995). In the present study, the step length was significantly shorter in both neurotoxin groups compared to both IC and vehicle controls, on days 20-40. On individual basis, a significant change in the step length was found both in the Rotenone and 6-OHDA group after the drug infusion which would suggest that the gait was affected at a certain level by the neurotoxins administration. Similarly, the number of adjusting steps

executed to keep body balance was significantly decreased in both neurotoxin groups with respect to both controls starting from day 7 through day 40 after the surgery. The postoperative decrease in the number of adjusting steps was also significant when compared with the preoperative records. However, this trend was observed also in the intact control group which undermines the potential neurotoxin effect in this test at the same time questioning the value of the test in screening deficits in posture and body balance regulation. Stepping length in the unilaterally 6-OHDA-induced rats (into various sites) was observed as significantly shorter than the unlesioned side (Fang et al., 2006). Another nigrostriatal unilateral lesion study in rats reinforced the stride step length in normal subject while significantly shorter steps in lesioned rats (Metz, Tse, Ballermann, Smith, & Fouad, 2005). Similar study but with mice and 6-OHDA injection into MFB showed the same results (Iancu, Mohapel, Brundin, & Paul, 2005). While a study of i.v. and s.c. route of administration of rotenone showed the prolonging initiation times in stepping test and decreasing adjusting steps at the third week of injection, no assertion about the step length was suggested (Sindhu et al., 2005). Interestingly, only a single study displayed an increase in the step length in unilaterally MFB-lesioned rats over a 5-week period (Klein et al., 2011). In the context of adjusting step, similar results were showed in the literature such that bilaterally 6-OHDA-injected (MBF) rats yielded significantly decreased stepping scores measured 6 weeks after surgery compared to controls (Paillé et al., 2007). Unilateral studies of 6-OHDA, also, showed reduced number of adjusting steps compared to controls (Fang et al., 2006; Meredith & Kang, 2006). Besides, unilateral intrastriatal infusion of rotenone caused a dose- dependent impairment in adjusting step test to detect the forelimb akinesia with highly reduced number of steps (Kirik, Rosenblad, & Björklund, 1998). By inferring from these studies, it might be claimed that results of the present study with respect to step length and adjusting step tasks highly correspond to the results in literature in the sense of decreasing step length and number of adjusting steps in bilaterally lesioned rats with 6-OHDA and rotenone. As it has been mentioned in the previous chapters, PD patients suffer from tremor, slowness of movements and motor-planning disturbances which are totally affecting the fine motor skills. Therefore, it is very important to mimic these symptoms in rat models. Also in this context, these stepping tests are supporting a high relevance to human PD (Emborg, 2004).

The rotarod/accelerod tests are also screening animal's sensorimotor coordination and the maintenance of the body balance. The both neurotoxins significantly impaired animals' performance as compared to intact control and Saline vehicle control. The deteriorating effect of toxins administration was also observed on individual basis when pre- and

postoperative performance was compared in the same subjects. The decrease in motor skills in medicated animals contrasted with the performance improvement observed over the repeated trials in the intact subjects apparently manifesting the motor learning. Interestingly DMSO group also demonstrated deficit in sensorimotor coordination which again suggests an adverse behavioral effect of the vehicle alone. The decrease in the motor skills in animals which received the neurotoxins infusions appeared shortly after the surgery, and persisted throughout the whole postoperative period suggesting that sensorimotor coordination and postural adjustments are especially sensitive to the nigrostriatal damage. Similar but more pronounced effects were observed when more difficult accelerated task was applied. Considering all these results, it can be suggested that accelerated test is much more sensitive tool than the stable rotarod test for screening the changes in motor behaviour induced by nigro-striatal lesions. By comparison, in one study, rats with unilateral 6-OHDA injection into MFB and STR were sequentially tested in 12-38 rpm stable rotarod and 4-40 rpm acceleration in 5 min along a 6-week period and it resulted that rats with lesion indicated highly reduced latency to fall in both high speeds stable rotarod (28 and 38 rpm) and accelerated (Monville et al., 2006). Another unilateral 6-OHDA model (MFB targeted and mice selected) showed a linear correlation between cell loss in SN and reduced scores on rotarod on 15 rpm stable rotarod 12 weeks after surgery and through a training period between 5-15 rpm in this time (Iancu et al., 2005). Similarly, a unilateral 6-OHDA model with SNc target lesion indicated a significant decrease in time spent on 10 rpm stable rod again with a 14-day long training on the rod (Gambhir et al., 2011). On the other hand, i.p. injection of rotenone into rats revealed significantly loss of balance and muscle strength which in turn a decrease in latency period of accelerating rod from 4-40 rpm (Sonia Angeline, Chatterjee, Anand, Ambasta, & Kumar, 2012). Oral administration of rotenone along a 28-day period into mice, also, indicated significant decrease in endurance time and time spent on the accelerating rod with 2-20 rpm in 5 min (Inden et al., 2009). A 30-day s.c. injection of rotenone in mice consistently showed decrease latency to fall on the accelerated with 4-40 rpm in 5 min (Richter, Hamann, & Richter, 2007). However, no relevant SNpc-target bilateral rotenone rat model testing both motor behaviour on stable (rotarod) and accelerating (accelerated) rod was found. Still, results of the present study shows the supportive and consistent results with the literature such that neurotoxin-induced groups reflected decreasing latency to fall over the time after surgery which meant they could not manage to stay on the both rotarod and accelerated for a long time while intact control revealed a significant amount of increased motor skill on the revolving rod throughout the experiment. Therefore, rotarod/accelerated test provides a highly effective tool to detect and analyze the dopaminergic lesions in rats.

In most of the tests, the greatest impairment was noted on day 40 indicating progressive worsening of the animals' conditions. However, the analysis of behavioral scores on day 150 (five months after the drug infusions) shows regression of some symptoms such as bradykinesia although some motor deficits such as (sensorimotor coordination and postural adjustment) still persisted compared to the preoperative bias.

As described in the Introductory part, human and animal point towards the importance of basal ganglia not only in motor behaviour but also for non-declarative learning process and memory (Shohamy et al., 2008). However, in contrast to the deficits in the motor skills, with low doses of neurotoxins administered directly to SNpc area no significant impairment in the animals' cognitive status was observed as assessed by the probabilistic learning test.

Human studies showed that PD patients obviously with disrupted basal ganglia were highly bad at the probabilistic learning tasks like 'weather prediction' compared to controls (Packard & Knowlton, 2002; Shohamy et al., 2008). An electrophysiological study on monkeys showed that dopaminergic neurons in SNpc and VTA were highly involved in the reward probability (Burke & Tobler, 2011). de Visser et al. (2011) investigated various experimental rodent models of Iowa Gambling Task (IGT) to understand the decision-making mechanisms and found that dopamine has a critical role in associative learning, time perception and signaling within the reward system which in turn necessary for decision-making. Also, they found the important role of DAT in decision-making such that DAT-lacking rats yielded higher amount of disadvantageous choices in the IGT (de Visser et al., 2011). On the other hand, a study of IGT in normal humans revealed poor performance in the learning scores by claiming an immature prefrontal cortex effect (Li, Lu, D'Argebeau, Ng, & Bechara, 2010). Still, it is showed that there are factors having a crucial role in guiding choice: wins and losses, probability and time, and effects of dopamine and serotonin on the integration of this information (de Visser et al., 2011). In our case, the reason may be that this modified version of IGT may not be enough sensitive to N-S damage, or the task may be too easy to reveal tiny deficits, if there are any, or deficits to develop require more time, or the applied dose was insufficient.

To be able to prove a causal relation between the neurological symptoms observed and the N-S damage the DA levels in striatum, prefrontal cortex and hippocampus should be estimated.

The exceptional feature of the present study is to provide a broad range of neurobehavioral tests together which were performed in the same rat population, and it allows to compare the neurobehavioral effects of catecholaminergic neurotoxin 6-OHDA and herbicide/pesticide

Rotenone among different individuals of this population. In another words, by presenting pre- and post-operative behavioral data of the same animal (cross-sectional) and data between different treatment groups on a particular testing day (longitudinal), present study allows investigation of time-dependent and drug-induced degeneration. Bilateral SNpc infusion of rotenone, a recent approach in PD modeling, in the present study may further enhance the understanding about the mechanism of progressive, Complex-I inhibitor, dopaminergic neuron selective rotenone under specific conditions. It will be informative both to compare a highly studied model 6-OHDA to the similar previous 6-OHDA studies and to compare two different models (6-OHDA and Rotenone) under the same conditions. The modifications performed in the motor behaviour tests and the unique version of probabilistic learning paradigm may open a new door. Briefly, this study may contribute to better understanding of the environmental toxin- and catecholaminergic neurotoxin-induced animal models of PD, hereby it may elucidate the neurobehavioral and cognitive processes of Parkinson's Disease at some points and procure new pharmacological agents and treatment strategies for the disease.

However, it should not be forgotten that up-to-now, none of the animal models of PD, including models investigated in the present study fully reproduced the clinical symptoms of this disease. Even so, the data obtained from animal models of PD could be successfully translated into clinics. However, translation of the animal data into the clinics should be done with the reserve and the behavioral, neuropsychological, and genetic differences between model animals and human should be taken into consideration (Potashkin, Blume, & Runkle, 2010)

CHAPTER 5

CONCLUSION

1. As assessed by both cross-sectional (between different treatment groups on a particular testing day) and longitudinal (between pre- and the postoperative performance in the same treatment group) comparisons of the results recorded in a battery of behavioral tests, the low doses of both neurotoxins applied bilaterally to SNpc showed deteriorating effects on the animals' motor performance but not on the cognitive functions.
2. In both neurotoxic groups, in most of the neurological tests applied, the greatest behavioral impairment was observed within time window between day 20 and day 40 after the drug infusion indicating progressive development of the motor symptoms.
3. Five month after the surgery (day 150), regression of some symptoms such as bradykinesia was noted although some motor deficits such as (sensorimotor coordination and postural adjustment) still persisted as compared to the preoperative bias.
4. The severity of behavioral deficits in different neurological tests screening different aspects of motor performance varied indicating different susceptibility of different motor functions to the degeneration of N-S pathway. According to the results obtained in this study, at the initial stage of the disease, movement initiation and body balance/postural adjustments are more affected than the gait and locomotor activity. Also the initiation of movements of the whole body seems to be earlier affected than the initiation of the limb movements.
5. Not all tests have the same power in detecting similar motor deficits: in scanning body balance and postural adjustments stepping test has relatively low, rotarod intermediate, and accelerod the highest diagnostic power.

6. In the common Rotenone model of PD, DMSO used as a vehicle control was shown to have by itself an adverse effect on animals' motor performance and thus aggravating the Rotenone effects.

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**ODTÜ
ENFORMATİK ENSTİTÜSÜ**

YAZARIN

Soyadı : TELKES

Adı : İLKNUR

Bölümü : SAĞLIK BİLİŞİMİ BÖLÜMÜ TIP BİLİŞİMİ PROGRAMI

TEZİN ADI (İngilizce) : PHASE VALIDATION OF NEUROTOXIC ANIMAL MODELS
OF PARKINSON'S DISEASE

TEZİN TÜRÜ : Yüksek Lisans **X**

Doktora

- 1) Tezimden fotokopi yapılmasına izin vermiyorum X
- 2) Tezimden dipnot gösterilmek şartıyla bir bölümünün fotokopisi alınabilir X
- 3) Kaynak gösterilmek şartıyla tezimin tamamının fotokopisi alınabilir

Yazarın imzası

Tarih 08.10.2012