# Etablierung eines Tiermodells der Parkinson-Krankheit auf der Grundlage von mitochondrialer Komplex-I Hemmung

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# 1 Introduction

The disabling symptoms in Parkinson's disease (PD) are primarily due to profound deficit in striatal dopamine (DA) content that results from the degeneration of DA-ergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibres in the striatum.

Approximately 5─10% of PD patients have a familial form of Parkinsonism with an autosomal-dominant pattern of inheritance. A very well known mutation in three different genes such as: alpha-synuclein gene, ubiquitin carboxylase-terminal hydroxylase gene and parkin gene are now associated with familial inherited Parkinsonism. However, the genetic form only accounts for a small number of PD cases at most, the major number of patients are 90─95% affected with sporadic PD.

The association of PD syndrome with both rotenone and mutation in different genes suggest that either an environmental or genetic factor can be the cause of PD. However, it is unlikely that in the majority of cases PD will be explained by a single cause. This concept has given rise to the idea that PD is caused by divergent factors which might contribute to destruction of DA-ergic neurons in a convergent pathway. Examples as factors are mitochondrial dysfunction, oxidative stress causing reactive oxygen species (ROS) production and protein mishandling, all of which are tightly linked (Greenamyre and Hastings, 2004).

Several lines of evidence support the hypothesis that mitochondrial dysfunction contributes to the etiology of PD. The mitochondrial electron transport chain produces ATP through oxidative phosphorylation. This process involves the activity of five complexes, namely, I, II, III, IV and V, located along the inner mitochondrial membrane. Protein sub-units of these complexes are nuclear encoded or encoded by the mitochondrial genome. A 30─40% decrease in complex I activity of mitochondrial respiratory chain has been observed in the substantia nigra (SN) but further reports indicated that the complex I defect is systemic in PD, it also has an effect outside the brain, such as on platelets, lymphocytes and muscle (Bind Bindoff et al., 1989, Cardellach et al., 1993, Mizuno et al., 1998, Mann et al., 1991).

Another important pathological feature of PD is the presence of filamentous, cytoplasmic inclusions called Lewy bodies (LB). In PD, LB are present in the DA-ergic neurons of SNpc as well as in other brain regions including the cortex, locus coeruleus and magnocellular basal forebrain nuclei (Braak et al., 1995). Although mutation in the alpha-synuclein gene have been associated with rare familial case of PD, alpha-synuclein is found in all LB, even in the vast majority of sporadic PD cases without alpha-synuclein gene mutation. Native alpha-synucleins are unfolded proteins with little or no ordered structure in physiological conditions. But under unphysiological conditions the conformational transformation of this natively unfolded protein changes into the aggregation component partially folded intermediate. Thus, any intracellular factors that lead to a shift in the equilibrium position between the native unfolded state and the partially folded intermediate will increase the likelihood of alpha-synuclein fibrillation which can cause cellular toxicity and may be involved in PD pathogenesis (Conway 2000, El-Angaf et al., 1998, Goldberg and Lansbury 2000) but the mechanisms causing in vivo aggregation of alpha-synuclein are not fully understood. Mitochondrial complex I inhibition and oxidative stress may be centrally involved, because these two related processes occur in PD and both can promote the aggregation of alpha-synuclein (Betarbet et al., 2000, Hashimoto et al., 1996). The over expression of alpha-synuclein itself can cause oxidative stress, increased inclusion formation and mitochondrial structure abnormalities in cultured neurons (Hsu et al., 2000). Therefore, a link between both mitochondrial dysfunction and oxidative damage as well as protein degradation becomes interestingly prominent in theories about PD pathogenesis.

There is a great importance to develop animal models for PD for better understanding of the pathogenesis and discovery of new therapeutics to treat PD. A number of animal models of PD have been developed to understand the pathogenesis of the disease, as well as to test the appropriate therapeutics. The majority of the established PD models use acute toxin exposure to induce destruction of nigrostrital neurons. Although the relevance of these acute models of Parkinsonism is somehow unclear with the pathogenesis of human PD they however, can be used to screen drugs for symptomatic treatment of the disease. The choice of model to be used depends upon the goals of the particular experimental paradigm and the questions being asked.

Human neurological disorder can be modelled in animals using standardised procedures that create specific pathogenic events and their behavioural outcome. In some of the cases the models are mimicking the symptoms of diseases but they do not recapitulate the construct validity of the diseases. For example the mimicking symptoms of PD can be reproduced instantly by manipulating DA-ergic receptors presynaptically or postsynaptically. The induction of DA hypofunction can be achieved by using reserpine and amphetamine. Both these drugs act on the presynaptic terminals of catecholamine neurons. Their activity is primarily associated to their DA-releasing mechanism. At very high doses, amphetamine has a neurotoxic effect on rodent and non-human primates. Like reserpine, amphetamine administration results in DA depletion at the level of DA-ergic nerve terminals (striatum) with a minimal effect in the nigral cell bodies. Amphetamine has several interactive effects on catecholamine release. Amphetamine acts in at least three ways: 1) reversal of the DA uptake carrier, 2) interference with uptake into the DA vesicle, and 3) inhibition (at higher concentrations) of monoamine oxidase (MAO). Some evidence suggest that amphetamine blocks the vesicular transporter. Some antagonists like MK-801 a N-methyl-D-asparat (NMDA) antagonist, haloperidol a $D_2$ receptor antagonist and also risperidone a mixed serotonin 5-$HT_2$/$D_2$ antagonist, are able to block its toxicity (Schmidt et al.,1985, Sonsalla et al., 1989, George et al., 2004). Furthermore the drugs which block the DA-ergic receptors can also mimic PD like symptoms in human and animals e.g. haloperidol, known as an antipsychotic drug which antagonises the postsynaptic receptors of $D_2$ and also produces catalepsy in rats. The cateplsy in rats reflect the movement disturbance such as akinesia. Animals treated with neuroleptic drugs show a strong rigidity and spontaneous decrease in their behavioural activities (Schmidt, 2000). The DA receptor agonist like L-DOPA or NMDA receptors antagonist could antagonise the neuroleptic induced cataleptic behaviour (Schmidt et al., 1991; Schmidt and Kretschmer, 1997). However, the DA antagonist models for PD which are considered as predictive validity of PD have major drawbacks because in these models, the histological changes of PD, including degeneration of DA-ergic neurons have not been documented. The predictive model can be extensively used for biochemical, physiological and for the studies of neurotransmitter modulating in DA-ergic-depleted striatum to better understand such changes in the PD brain.

The neurodegeneration mimicking models of PD can be produced using toxins such as 6-hydroxydopamine (6-OHDA), 3-nitrotyrosine (3-NT) and 1-methyl-4-phenyl-1.2,3,6-tetrahydropyrodine (MPTP) and rotenone. The prime cause of nigral DA-ergic neurons loss and the consequent extent and pattern of DA depletion in basal ganglia that is seen in PD, can be replicated in animal models by intracerebral injection of 6-OHDA and 3- NT or by the systemic (intraperitoneally or subcutaneous) administration of MPTP and rotenone. Compared to rotenone the mode of action of DA-ergic cell death are different in other toxins because the primary cause of DA-ergic cell death are totally due to specific complex I inhibition in the rotenone model, which is not the case in other toxins.

Most protocols of MPTP administration utilise acute drug treatments and fail to mimic the progressive nature of PD. However, long-term administration of MPTP in smaller doses, has resulted in recovery of motor behaviour deficit in marmosets once the treatment stopped. Additionally, the MPTP model does not directly address the involvement of systemic mitochondrial impairment in PD. The metabolite of MPTP, 1-methyl-4-phenyl-2,3-dihydropyridinium ion ($MPP^+$ ) inhibits complex I activity solely in cells expressing the dopamine transporter (DAT) that is only DA-ergic cells. Thus, this model only tests the hypothesis that complex I dysfunction, limited to DA-ergic neurons, is toxic to DA-ergic neurons.

The 6-OHDA and 3-NT model do not mimic all the clinical and pathological features characteristic of PD. 6-OHDA lesion in the medial forebrain bundle (MFB) or in SNpc does not effect other brain regions, such as locus coeruleus, nor does it result in formation of cytoplasmic inclusion called LB which is the hallmark of PD. Furthermore, the acute nature of the experiment model differs from progressive degeneration of DA-ergic nigral neurons in PD.

In contrast to all other toxins, the chronic and systemic low doses (1.5 – 2.5 mg/kg) of rotenone exposure over a period of (50–60 days) show the behavioural and biochemical features of PD. The rotenone model appears to be an accurate model in that systemic complex I inhibition results in specific, progressive and chronic degeneration of nigrostrital

pathway similar to that observed in human PD. It also produces inclusion of LB and oxidative damage seen in PD. Thus, the rotenone model recapitulates most of the mechanisms thought to be important in PD pathogenesis.

Although an ideal model should reproduce the characteristic clinical and pathological features of PD (i.e., animal model should develop progressive loss of DA-ergic neurons, show deposition of LB-like inclusions in brain, and possess some features of L-DOPA-responsive movement disorder), this seems to be an achievable goal, because the rotenone model shows all the features of the human disease. Therefore, this thesis deals with a PD model based on complex I deficiency. Complex I inhibition is achieved with rotenone.

### 1.1 Parkinson's disease

PD was first formally described in "An essay on the shaking palsy", published in 1817 by a London physician named James Parkinson. It is a chronic progressive, neurodegenerative disorder that may appear at any age, but it is most common in people over 50, effecting 1 to 2% of the population and is rare in those under 30. It is the second most common neurodegenerative disease after Alzheimer's disease (AD). It is not contagious nor is it usually inherited.

Clinically, PD patients suffer from severe motor dysfunction characterised by three cardinal symptoms: resting tremor (most common initial symptom, predominant at rest), akinesia (inability to initiate movement, poverty and slowness of movement e.g. "mask face") and rigidity (increased muscle tone subjectively experienced as muscle pain or stiffness, passive movement reveals "cogwheel phenomenon"). Beside the motor disturbance PD patients suffer from motor habit learning and non-motor habit learning deficit (Schmidt, 2000). Phenomenologically the clinical features of depression and PD overlap psychomotor retardation, attention deficit, day-night sleep reversal, hypophonia, impotence, weight loss, fatigue, preoccupation with health and reduced facial expression are seen in both disorders (Gotham et al., 1986, Poewe, 1999).

As deficits in procedural learning and working memory are a frequent finding in non demented patients with PD, it can be difficult in practice, to determine whether depression is

contributing to cognitive impairment. Severity of depression has been associated with the severity of cognitive impairment in PD and depression has been associated with a significantly increased risk of developing dementia in PD (Marden et al.,1995, Giladi et al., 2000).

Four surveys, one world-wide, have concluded that depression, disability, postural instability, age and cognitive impairment are the major factors having the greatest influence on the quality of life in PD.

The disabling symptoms in PD are primarily due to profound deficit in striatal DA content that result, from the degeneration of DA-ergic neurons in the SNpc and the consequent loss of their projecting nerve fibres in the striatum. DA-ergic cell loss is associated with the presence of eosinophilic intraneuronal inclusions, called LB composed of neuro filaments in SNpc. Neurodegeneration and LB are also found in the locus coeruleus, nucleus basalis, hypothalamus, cerebral cortex and peripheral component of the autonomic nervous system.

The cause of neuro-degeneration in PD remains unknown. Epidemiological studies indicate that there is no relation with one specific factor but there are perhaps a number of factors which increase the risk of developing PD.

## 1.2 Mitochondrial dysfunction and Parkinson's disease etiology

The mitochondrial oxidative phosphorylation system consists of five multimeric enzymes (complex I-V). NADH dehydrogenase or complex I is affected in most of the mitochondrial diseases and in some neurodegenerative disorders like PD.

Mitochondria occupy a pivotal role in metabolic pathways that are critical for both cell survival (oxidative phosphorylation) and cell death (apoptosis). In idiopathic PD, there is a

30 – 40% decrease in complex I activity in the SN. These deficits are associated with increased free – radical production, increased susceptibility to the MPTP metabolite $MPP^+$ and impaired $Ca^{++}$ buffering. However, direct sequencing of the mtDNA complex I and t-RNA genes failed to show homoplasmic mutation, suggesting that complex I defects may be due to heteroplasmic mutation or involve genetic and environmental interaction (Beal et al., 2000).

Mitochondrial oxidative phosphorylation accounts for ~ 90% of the ATP production in neurones. In osteosarcoma lines, rotenone inhibition studies showed that 25 –50% deficiency in complex I activity caused a 5 – 20% decrease in cell respiration, a 48 – 81% increase in ROS generation, an 8 – 27% increase in lipid peroxidation, a 6–13% reduction in mitochondrial membrane potential, an 11–12% decrease in cell growth and a four to five fold increase in cell death. These findings have important implications in interpreting the degree of complex I deficiency found in human disease, particularly the neurodegenerative disorders such as PD in which an approximate 35% decrease in complex I activity has been demonstrated (Schapira et al., 2000).

In a recent study, a complex I defect was found in cybrids carrying mtDNA deriving from PD platelets (Swerdlow et al., 1998, Gu M., et al., 1998). This indicates the presence of a defect in the mitochondria genome that can be transferred through multiple passages. Such a defect could be due to an inherited mutation or to toxic insult, possibly secondary to oxidant stress. mtDNA is particularly susceptible to ROS-induced damage. Furthermore, a genetic defect in energy metabolism may only become significantly effective upon exacerbation by cumulative oxidative degeneration caused by normal ageing. This may help in explaining the late onset and progressive nature of neurodegenerative disorders. Complex I defect might also contribute to the development of apoptosis. Increasing evidence suggests a reduction in the mitochondrial membrane potential (as a result of impaired proton pumping) can lead to the opening of a mitochondrial permeability pore with release of small mitochondrial proteins signalling the onset of apoptosis.

It has been shown recently that although synaptic and non synaptic mitochondria have similar respiratory control, they differ significantly in the control of complex I on oxidative

phosphorylation (Bates et al., 1995; Davey et al., 1997, 1998). In synaptic mitochondria complex I activity was decreased by 25% before oxidative phosphorylation was severely compromised, whereas in non synaptic mitochondria complex I could be inhibited by 70% before major changes in ATP synthesis and oxygen consumption were observed (Davey et al.,1998). These results indicate that complex I has a higher control over oxidative phosphorylation in synaptic than non-synaptic mitochondria. Thus, it would seem that degeneration associated with complex I deficiency preferentially occurs in presynaptic mitochondria, which are more vulnerable to oxidative stress than mitochondria located in the cell body.

There is an open question: is it possible to link the rotenone model with autosomal or familial link mutant (A30Palpha-synuclein, A53T alpha-synuclein, UCH-L1, parkinsonism due to 11778 mitochondrial DNA-mutation that produce a complex I defect or a novel mitochondrial 12sRNA point mutation associated with PD) ? The identification of a specific respiratory chain defect (complex I deficiency) has focused interest on linking this to the aetiology and pathology of PD. Mitochondrial inheritance has been suggested in some familial PD patients (Mizuno et al., 1999). A decrement in the complex I function has the tendency to create oxidative stress through mitochondrial production of reactive oxygen species (ROS). Although rotenone intoxication potently inhibits complex I and leads to the selective degeneration in DA-ergic neurons, it also produces cytoplamic LB inclusion that show ubiquitin, alpha-synuclein positive immuno reactivity.

### 1.3 Animal models

Animals are used to model human disease because they are similar in many respects from the basic cellular level to the whole organ function. Indeed, data from the human genome project indicate that even a mouse shares over 90% of its genes with humans. Research using animals provides useful information despite the obvious differences between animals and humans.

Typically, models are animal preparations that attempt to mimic a human condition or disorder, including human neuropathological or psychological diseases. In developing and assessing an animal model, it is critical to consider the explicit purpose intended for the model, because the intended purpose determines the criteria that the model must satisfy to establish its validity.

The mouse is a principal model organism to link function to genes. The large number of inbred rat models and the vast amount of data available for the rat provides important strengths for the study of human health and disease. In some instances, specific aspects of human disease are duplicated well only in the rat, making these animals an unique resource for studying and identifying genetic pathways relevant to human diseases. The rat is also a model of choice for many physiological studies of cardiac and vascular function, pulmonary circulation, age and gender-related differences and also neurological disorders.

### 1.3.1 Animal models of Parkinson's disease

For PD there are several animal models. Disruption of the nigrostriatal pathway or its target (the striatum) is a method commonly used for creating an animal model of PD. On the basis of experimental and clinical findings, PD was the first neurological disease to be modelled, and, subsequently, to be treated by neurotransmitter replacement therapy. Some DA receptor blockers have been also used as a model or as a model of catalepsy. The predictive validity of catalepsy as a rodent model for observing the hypokinetic, rigidity or akinesia behaviour by blocking the dopamine receptors, haloperidol has mostly been used to screen some new substances and to observe anti-parkinsonian activity (Schmidt et al., 2002). Agents that selectively disrupt or destroy DA-ergic neurons, such as reserpine, 6-OHDA, MPTP have been used to develop PD models. Recently, it has been found that agricultural chemicals, such as rotenone and parquat , when administered systemically, can reproduce specific features of PD in rodents. Transgenic animals that overexpress alpha-synuclein are used to study the role of this protein in DA-ergic degeneration.

### 1.3.2 Models of dopamine depletion and their mode of actions:

**I. Reserpine model**

Reserpine is a naturally occurring alkaloid extracted from several members of the genus *Rauwolfia,* which grows in southern and south-east Asia. It is an anti high blood pressure drug. Systemic administration of reserpine empties relatively all of the monoaminergic vesicles (dopamine, norepinephrine and serotonin) in the peripheral and central nervous system via the inhibition of $Mg^{+}$ / ATPase dependent vesicular transporters. In rabbits administration of reserpine that depletes the monoamines was the first animal model of PD. This model was developed by Professor Arvid Carlsson in Sweden. Reserpine causes a temporary slowness in movement that is reversed by the administration of levodopa (Carlsson et al., 1957).This model has been used to discover new treatments for PD.

However, since the effect of reserpine on the dopamine cells is temporary, the model has limited use. Reserpine doesn't replicate the extensive biochemical and pathological changes seen in PD.

**II. 6-OHDA model**

A toxin specific to dopamine nerve cells is (6-OHDA). This chemical is similar to DA in structure. It is a hydroxylated analogue to the natural DA neurotransmitter. The neurotoxicity of 6-OHDA may largly depend on its incorporation into DA neurons via uptake by the DAT (Pifl et al. 1993, Sundstrom et al. 1986). Using stereotaxic surgery in the rat, 6-OHDA is delivered directly at a number of different points along the nigrostriatal pathway. 6-OHDA was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways (Ungerstedt, 1968; Sachs and Jonsson 1975). Different models have been developed using 6-OHDA, most of them unilateral (as bilateral administration is associated with adipsia and aphagia and high mortality) in various species, mainly rodents and small monkeys (marmosets). 6-OHDA needs to be delivered locally, by stereotactic injection into the nigrostriatal system. Different models can be created by targeting the DA system at different levels: i.e. more restricted lesions result from injection into the SN (cell bodies), more widespread into the nigrostriatal tract (DA axons in the medial forebrain bundle), and more progressive into the striatum (DA terminals). 6-OHDA

models are limited by their acute toxic nature, although following administration into the striatal terminal field, degeneration progresses over several weeks (Sauer and Oertel, 1994, Pederson and Schmidt, 2000). DA neurons die through apoptosis, necrosis and autophagia and they do not form cytoplasmic inclusions. Unilateral loss of DA neurons results in a motor asymmetry that can be easily assessed in vivo using behavioural and imaging techniques. Post mortem evaluation can be performed using immunohistochemistry to quantify the loss of TH positive neurons in the SN and DA fibres in the striatum and using HPLC to quantify the loss of DA in the striatum.

**III. MPTP model**

MPTP was discovered as a result of observations of the PD like syndrome caused by intravenous administration of MPTP contaminated heroin (Davis et al. 1979; Langston et al., 1983) in drug addicts. MPTP crosses the blood brain barrier and is metabolised in the astrocytes to its toxic MPP+, by monoamine oxidase-B (MAO-B). This toxic metabolite is selectively taken up by DA neurons, due to its affinity to the DAT, and concentrated in the mitochondria where it inhibits complex I of the electron transport chain leading to oxidative stress and death of DA neurons.

Rodents show variable susceptibility to MPTP. Rats are resistant to MPTP toxicity (direct intracerebral administration of MPP+ can be used). Mice show variable (and usually transient) DA depletion with also variable behavioural correlation. Susceptibility varies between different mouse strains. Primates are susceptible and develop a motor syndrome closely resembling PD. Different models of parkinsonism are induced by different protocols of MPTP administration. Intracarotid injection of MPTP induces a predominantly unilateral acute degeneration of the DA pathway with 53 – 93% of cell loss in the SNpc and more than 99% loss of DA in the striatum.

Chronic administration of low doses of MPTP to macaques reproduces all the behavioural signs of PD (tremor, bradykinesia, rigidity, hypokinesia and postural impairment) and mimics, to a certain extent, the progressive nature of PD. α-synuclein-positive aggregations

have been reported in baboon nigral neurons following MPTP exposure, although these aggregates where unlike typical LB's. These models are adequate for functional studies and pre-clinical trials.

**IV Rotenone model for PD**

Rotenone can easily cross the blood brain barrier due to its extremely hydrophobic character. Systemic administration acts on complex I uniformly throughout the organism, consistent with its ability to cross biological membranes easily (Betarbet et al.,2000).

The complex I sub-unit encoded by the mitochondrial genome ( ND1-ND6 and ND4L) are all highly hydrophobic and critical for enzyme activity. The ND1 sub-unit has been shown to be the binding side for both rotenone and the photoaffinity-labelled ubiquinone analogue (QH2), suggesting that this is the terminal acceptor for quinone (Q) reduction within the membrane protein of complex I .

To understand a specific role of complex I in neurodegenerative disorder, it will be important to define its regional and cellular distribution and determine how this relates to pathological changes.

Rotenone infusion affects complex I selectively. Histochemical analysis of the enzymatic activities of succinate dehydrogenase (complex II) and cytochrome oxidase (complex IV), revealed that they were not affected by rotenone infusion (Porter, et al., 1994). It does not have homogeneous distribution affinity, in vivo [$^3$H]dihydrorotenone binding assay shows that rotenone distributes heterogeneously in different regions of the brain (Pandykula, 1952). White matter has a very low level binding capacity with levels in areas of pure white matter estimated to be at most 30% (Greenamyre et al., 1992). Maximum levels in the corpus callosum and olfactory tubercle have been shown to be 26% and 20% respectively. Binding ranged between 43 and 72% in the BG with the striatum and core of the nucleus accumbens displaying higher levels. Generally binding affinity in grey matter was higher than in white matter with grey matter displaying more variation than white matter (Deepa, et al., 2000)

The higher content of oxidative enzymes in grey matter probably reflects a higher need for ATP, which is used for ion pumping, mainly by $Na^+$, $K^+$ - ATPase (Mate et al., 1980).

DAT knockout mice are resistant to MPTP. Unlike MPTP, which is also a complex I inhibitor, rotenone is not selective for DA-nergic neurons. Rotenone is a highly lipophilic substance, it can penetrate through the lipid membrane, and therefore it does not require the DA transporter to gain access to the neural interior. Thus, rotenone may provide a more versatile model of environmental toxins than MPP+. Intraperitoneal administration of rotenone results in wide spread uptake and uniform inhibition of complex I, and selective induction of DA-ergic neuronal cell death in the SNpc. The distribution of DA-ergic pathology mimics that of PD, even to the slow time course of neuro- degeneration. In this model, retrograde degeneration of SN neurons is the dominant form of neuronal destruction. Degeneration begins in the nerve terminal in the striatum and progresses towards the SN. Rotenone is a natural product, widely used as an agricultural pesticide. It is possible that traces remain in treated vegetables or fish that we consume. Chronic exposure to rotenone produces selective nigrostriatal degeneration and cytoplasmic inclusions of LBs (Betarbet et al., 2000). In contrast to MPTP which metabolises into $MPP^+$ and is highly charged, making it difficult to pass the membrane, rotenone induction of PD may be proposed as producing a novel animal model due to its lipophilic nature. Both toxins act in different manners, by using a different pathway to reach the SNpc.

The cause of neurodegeneration in PD depends upon two processes, one is a “primary neurodegeneration” and the second is “secondary neurodegeneration”. Primary degeneration is caused by neurotoxins acting directly in the soma (anterograde degeneration) otherwise within the synapses (retrograde transneuronal degeneration). The primary destruction depletes the DA neurotransmitter in the SNpc and causes further glutamate hyperfunction. Through the hypoactivity of DA, a secondary glutamate hyperfunction is caused and thus disturbing the whole homeostasis of the basal ganglia (Pederson and Schmidt, 2000). Their hypothesis makes it easy to emphasise the rotenone toxicity.

## 1.3 Why is a new animal model for PD needed?

Except for the rotenone rat model of PD, there is no comparable rat model, using a chronic and systemic administration of complex I inhibition which produces proteinacious cytoplasmic inclusions, known as LB's. This model also produces slow and progressive nigrostriatal neurodegeneration with formation of LBs, which so far has not been achieved by either 6-OHDA or MPTP. It might therefore, be an invaluable tool for investigating the

molecular basis of LB formation and the link to mitochondrial dysfunction. This model will put forward the paradigm of alpha-synuclein pathology with relation to specific complex I inhibition. Since the appearance of the rotenone model it has been thought that mitochondrial complex I might be one of the causes for LB which has also been recently speculated and reviewed evidence has been given, that mitochondria may play a role in early LB formation (Song et al., 2004). It is obvious that, concerning PD such an ideal model does not exist. So therefore, a better validated animal model is required which resembles all the behavioural, biochemical and proteomic dysfunctional criteria as human PD.

Through the rotenone model more complete understanding of PD pathogenesis will uncover novel targets for rational drug therapy. However, even with our incomplete understanding of PD etiology there are a number of potential avenues for treatment, which include, brain-permeable antioxidants and spin trap agents, glutamate receptors antagonists, and compounds that prevent protein aggregation. Testing in accurate animal models of PD could uncover important clues to the mechanisms that underlie PD neurodegeneration.

## 1.5 Questions and goals of the studies

1. The major goal of the first experiment was to answer the question, whether rotenone (i.p.) treatment could be used to establish a rat model of PD. Until now it has only been possible to produce a relevant model of PD with the toxin MPTP in mice and monkeys, whereas in rats, due to the missing MAO-B enzymes, it was not possible. Rotenone is a toxin which does not need these enzymes to break down and can there-

fore be seen as an independent carrier for self transportation enabling it to penetrate cells unlike MPTP which is carrier dependent on DAT.

2. The second experiment deals with the question as to whether rotenone also showed phenomenological similarities to the 6-OHDA model when infused locally. In this experiment locally administered rotenone together with the vehicle (propylene glycol), which had never been tried before, resulted in the suggestion that it would prove possible to use this as a novel and new method for a locally rotenone induced model of PD.

3. Could a chronic long-term administration of rotenone be capable of inducing nitric oxide (NO) production and lipid peroxidation- like products such as thiobarbituric acid reactive substances ( TBARS ) ? Which roles do NO and LPO processes have in the mechanisms of oxidative damage in the nigro striatal pathway during chronic rotenone administration?

4. This study focused on the fundamental question as to whether systemic administration of rotenone fulfils the criteria of construct validity for a PD model. Are the motor deficit behavioural results shown in the rat due to nigro striatal loss of DA-ergic neurons? To answer this question the rotenone treated rats were challenged with L-DOPA to examine its effectiveness in reversing the motor deficits because in PD the symptoms are reversed or counteracted by L-DOPA treatment.

## 2 Summary of results

***I*** *M. Alam, W.J. Schmidt (2002) Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. Behav Brain Res.136:317-234*

The two different doses (1.5 mg/kg and 2.5 mg/kg) of rotenone over a period of 60 days produced catalepsy in bar and grid test. Further the behaviour observation in open field box showed a significant reduction of spontaneous motor activity like the number of movements, rearing, active sitting, inactive sitting and head dipping as compared to control.

The neurochemistry results which showed depletion of dopamine (DA) in the striatum and prefrontal cortex correlated well with neurobehavioural data, with significant reduction obtained at the 2.5 mg/kg dose for 60 days.

The immunoblot results showed a decreased amount of tyrosine hydroxylase (TH) enzyme in the striatum in dose dependent manner as compared to control.

In summary, the results show that rotenone is capable of destroying DA-ergic neurons and mimics parkinsonian like neurochemical and neurobehavioural deficits in rats. Our findings support the notion of a multi-hit model of PD: a constitutional complex I deficiency and environmental toxin may be implicated in the pathogenesis of PD.

***II*** *M. Alam, A. Mayerhofer, W.J. Schmidt (2003) The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA. Behav Brain Res.151:117-124*

In this study 3 µg of rotenone was administered bilaterally stereotaxically into the medial forebrain bundle (MFB) to observe whether rotenone is capable of destroying DA-ergic neurons and producing parkinsonian like neurobehavioural changes. The neurochemical and behavioural data showed that the level of DA was significantly decreased both in anterior and posterior striatum, a strong increase in catalepsy and a decrease in locomotor activity in rotenone treated rats as compared to control. The rotenone-induced behavioural and neurochemical changes suggest that DA-ergic neurons in the nigrostriatal pathway are sensitive to rotenone toxicity.

If these neurobehavioral changes mimic those found in PD, then L-DOPA should reverse rotenone-induced motor deficits. In this study the chronic effect of two different doses of L-DOPA (5 mg/kg and 10 mg/kg) with a combination of DOPA- decarboxylase inhibitor (Benserazide) to one-quarter dose of L-DOPA were investigated in rotenone-lesioned rats.

The behavioural observation of two different doses of L-DOPA chronically for 30 days showed reversed behavioural deficits in rotenone lesioned rats.

***III*** *Valentina Bashkatova, Mesbah Alam, Anatoly Vanin, and Werner J. Schmidt, (2004). Chronic administration of rotenone increases levels of nitric oxide and lipid peroxidation products in rat brain. Exp Neurol. 186:235-241.*

Excessive generation of nitric oxide (NO) has been implicated in the pathogenesis of a range of neurological disorders such as PD, AD, amyotrophic lateral sclerosis and Multiple sclerosis. Damage to the mitochondrial electron transport chain has also been implicated in these disorders. NO and its toxic metabolite can inhibit the mitochondrial respiratory chain, leading to energy failure and ultimately cell death.

This study has been done to evaluate the mechanism of rotenone toxicity by measuring the levels of nitric oxide (NO) and the lipid peroxidation like product which is thiobarbituric acid (TBARS). Rats were given chronic treatment of rotenone 1.5 mg/kg (i.p) over a period of 1, 10, 20, 30 and 60 days. The first injection of rotenone did not show any difference compared to the control group. Repeated injection of rotenone gradually increased NO levels in the striatum on day 20, 30 and 60 respectively. But the levels of NO in the frontal cortex and nucleus accumbens were significantly higher on the day 30 and 60. The increased levels of NO in striatum, frontal cortex and nucleus accumbens were directly associated with days for lipid peroxidation products (TBARS) in the same regions of brain tissues. The levels of NO and TBARS were increased in a directly proportional manner and were significantly increased in time dependent manner.

Behaviour was assessed on day 30 and 60 for the catalepsy test on horizontal bar and vertical grid. Rotenone treated animals showed an increase in descent latency on day 30 and 60 as compared to control animals.

The goal of this work was to complete and extend our study design by assessing the effect of NO levels and TBARS concentrations on nigrostriatal system. Increased NO levels and oxidative damage of lipid-peroxidation like product TBARS in the dopaminergic neurons also correlate with the behavioural result in this experiment.

***IV*** *M. Alam, W.J. Schmidt (2004) L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone-treated rats. Behav Brain Res.*

This work has been designed to investigate the effect of L-DOPA in rotenone treated rats; because any clinically predictive (PD) model should include a positive response to L-DOPA, which is a standard pharmacotherapy and is generally applicable for PD patients.

Rotenone was administered on a daily basis systemically (i.p.) at a dose of 2.5 mg/kg, over a period of 48 days.

The behaviour was assessed using bar and grid as a catalepsy test and activity box for locomotor activity, rearing and inactive sitting respectively on day 30 and 48.

The behavioural data showed an increase in the descent latency of catalepsy both in bar and grid as compared to control animals. The prolongation of catalepsy also showed a difference between day 30 and 48 in rotenone treated animals but no difference was observed in control animals.

A decrease in locomotor activity (total distance travelled), rearing and increase in inactive sitting were observed on day 30 and 48 in the activity box as compared to control animals.

To investigate the effectiveness of L-DOPA in reversing the motor deficit in rotenone treated rats, a dose of L-DPOA (10 mg/kg) in combination with peripheral amino acid decarboxylase inhibitor benserazide at a dose of 2.5 mg/kg were administered i.p. for a period of 10 days in rotenone treated rats.

On day 49 the rotenone treated animal group was divided into two groups. One of the rotenone treated animal groups received L-DOPA (10 mg/kg) and the other received Vehicle (saline).
Further the control group was also divided into two, one received L-DOPA (10 mg/kg) and the other received saline as vehicle.

In bar and grid the animals were tested on every 3rd day for 10 days (on day 49, 52, 55 and 58). On day 49 first after L-DOPA treatment in rotenone treated animals the descent latency was reduced approximately 40% compared to control animals. On day 52 and 55 in the second and third session of measurement the descent latency reduced to 60%. On the last session of L-DOPA treatment in both bar and grid tests no differences were observed in rotenone treated animals and control animals.

Further in the activity box the three sessions of behaviour assessments which were locomotor activity, rearing and inactive sitting were performed on day 49, 54 and 59. On day 59, the last session of behaviour assessments there were no differences among the control and pre-rotenone treated animals with L-DOPA treatment in all the three parameters (total distance travelled, rearing, and inactive sitting) but there were differences between the pre-rotenone treated animals with saline and control animals treated with saline or L-DOPA.

The amount of DA, serotonin (5-HT) and their metabolites were measured in the anterior striatum, posterior striatum and SN for each group of animals. There was a decrease of DA and its metabolites DOPAC in all the three regions. But no difference of 5-HT and its metabolite 5-hydroxy indole acetic acid (5-HIAA) has been observed.

## 3 Discussions

Animal models are important tools for understanding a disease process, but the majority of models cannot provide enough knowledge to understand the real criteria of the disease. Developing animal models for neurodegenerative disorders is a complicated process as long as we do not know the primary cause of neurodegenerative diseases. It is obvious that, concerning PD such ideal models do not exist and there is a need for better criteria to categorise and validate animal models. For PD different models were produced and these models can be categorised according to pharmacological, behavioural and biochemical criteria in three groups. For example Haloperidol or DA antagonist models for PD show a predictive validity of PD. It is regarded as the lowest level of validity and has similarities to pharmacological isomorphism to PD the generation of symptoms is however totally unrelated to idiopathic PD. The predictive model can be used for a rapid screening approach but not to develop therapeutic approaches to cure the disease. The second categories of animal models are those with face validity. These models assess the phenomenological similarity between the model and the disease. These models have a higher validity than those with predictive validity. The existing model 6-OHDA and MPTP could be categorised as face validity models. They provide similarities to symptomatology but may not mean similar underlying mechanisms. The highest set of validity criteria for animal models deals with construct validity. It should fulfil the criteria for face validity and predictive validity (Ellenbroek et al, 1990).

As outlined in the introduction, complex I deficiency probably contributes to PD. Given this, then rotenone could represent an animal model with construct validity. Considering that the complex I deficiency is the first main cause of cell death in PD, then the rotenone model comes very close to this disease. The mechanisms of cell death, as well as the symptomatic therapy strategies can be developed upon using this model.

The MPTP model is already a similar established model,but the rotenone model distiguishes itself in different aspect from MPTP. MPTP is not toxic by itself, but in the presence of MAO-B enzyme it is metabolised to the active toxin ($MPP^+$). Therefore, MPTP is not able to

produce toxicity in rats due to their absence of MAO-B. In contrast to MPTP, which has to be to be carried through DAT, rotenone can cross the biological membrane easily due to its extremely lipophilic nature and needs no carrier for its transportation. The cells which are damaged by rotenone accumulate cytoplasmic inclusions containing ubiquitine and alpha-synuclein which are remarkably similar to authentic LB's.

Previous studies have found the most causes of PD are due to environmental factors and inhibition of complex I (Di Monte et al 2002). One of the best described environmental related causes of PD is the MPTP. In this study the effects of the complex I inhibitor rotenone were examined, rotenone was administered (i.p.) chronically once per day over a period of 2 months to two groups of rats in two different doses. Catalepsy and open field tests were used as behaviour tests to assess Parkinsonian symptoms. The catalepsy test showed significant cataleptic behaviour and open field test showed a decrease in locomotor activities as compared to control group (Alam and Schmidt., 2002).

In this study a rotenone model of PD in the rat has been established in which further investigation can be carried out to evaluate the biochemical basis for the etiology progression and pathology of PD. The chronic exposure by i.p. injection is comparable to chronic environmental exposure and is more comparable to the "real life" situation and is therefore superior to other existing models, in studying PD.

## 3.1 Chronic and systemic administration of rotenone :

The major aim of this study has been to test whether rotenone i.p. treatment could be used to establish a rat model of PD. In the very first study, rotenone was introduced through osmotic mini pump for five weeks continuously via the route of the jugular vein to produce PD symptoms in rats, further in the initial experiment using rotenone, the study subject (rats) under went no behavioural testing data (Betarbet et al., 2000) for this reason the present study has included all the behavioural assessments which are very close to typical PD symptoms. Similarly, intrafemoral venous infusion of 2.5 mg/kg/day of rotenone for 28 days to male Lewis rat has also

been shown to produce nigrostriatal DA-ergic neurodegeneration (Hoglinger et al., 2003). However, this gives rise to the controversial question of the relevance of these routes for PD patients in whom the disease may have been due to environmental exposure? Therefore it was essential to find a method that is seemly closer to a low level, chronic exposure e.g. orally, inhaling or contact with skin, etc. The chronic exposure by i.p. injection or subcutaneous injection is comparable to chronic environment exposure and is more comparable to the "real life" situation and is therefore superior to other existing models, in studying PD. Therefore this work reproduces the rotenone model by choosing i.p. injection to show a better resemblance to exposure in normal life and this is the first report that showed that chronic i.p. administration of rotenone treatment caused the depletion of dopamine in the posterior striatum and prefrontal cortex and also reduced tyrosine hydroxylase (TH) immunoreactivity in the posterior striatum. Behavioural experiments showed dose-dependent catalepsy in both groups of rats. Data from this study indicated that, in rats, rotenone administrated by intraperitoneal injections is capable of causing degeneration of DA-ergic neurons and an induction of parkinsonian symptoms (Alam and Schmidt 2002).

In summary the rotenone induced metabolic deficiency of DA-ergic neurons in the prefrontal cortex and the striatum proved to be sensitive in this experiment. The neurobehavioural and neurochemical results in this study both provide direct evidence for the vulnerability of the nigrostriatal DA-ergic pathway. All the results showed that rotenone is in fact capable of producing Parkinson like symptoms in rats.

## 3.2 Local administration of rotenone in the medial forebrain bundle produces nigrostrital dopamine deficit.

This study demonstrated for the first time the use of acute itracerebral injection of rotenone to produce a model for PD. Another important novel fact in this study is the solvent which has been used to dissolve the rotenone easily and enabling it to be

injected locally in the brain. In this study propylene glycol was chosen as a solvent for rotenone because rotenone is highly lipophilic in nature and insoluble in water. It is soluble in acetone, carbon disulfide, ethy acetate and chloroform. It was important to choose a solvent to minimise or totally avoid its self toxic effects in neurons when infused in the brain for local lesion. The Food and Drug Administration (FDA) has classified propylene glycol as an additive that is "generally recognised as safe" for use in food. It is used to absorb extra water and maintain moisture in certain medicines, cosmetics, or food products. It is a solvent for food colours and flavours.

The local rotenone infusion in the medial forebrain bundle (MFB) resulted in significant hypokinetic behaviour in the open-field test and rigidity like behaviour in the bar and grid test as compared to sham lesion animals. The neurochemical analysis in the anterior and posterior striatum suggests that, rotenone depletes DA levels in both brain regions and causes the nigrostriatal DA deficit. Further the feasibility of nigrostriatal DA-ergic deficit after locally administered rotenone was challenged with L-DOPA treatment to prove whether the behavioural deficit was counteracted because, mostly DA depleted neuronal behaviour is reversed by L-DOPA. In fact, the behaviour results show an increase in locomotor activity and reversal of the rigidity like behaviour after chronic administration of L-DOPA treatments.

This experiment has shown that local administration of rotenone is able to destroy DA-ergic neurons as it is the case with 6-OHDA. In fact, we found that local rotenone lesioned model rats are susceptible to L-DOPA. These data further support the use of rotenone as a neurotoxin in vivo.

3.3 The mechanism of neurotoxicity after chronic intermittent administration of rotenone.

In summary the study demonstrated, for the first time, that the low dose of rotenone (1.5mg/kg) elevated the concentration of NO and lipid peroxidation in a time depen-dent manner. Increasing lines of evidence have implicated a permanent role for NO

in the pathogenesis of PD. Rotenone has been suggested as an inhibitor of mitochondrial complex I activity uniformly throughout the brain. Therefore the relative selective nigro-striatal DA-ergic injury should be caused by increased sensitivity of this pathway to rotenone. The generation of peroxynitrite has been implicated in DA-ergic neurotoxicity produced by various DA-ergic neurotoxicants such as methamphetamine and MPTP. The increased neuronal NO synthesase activity and increased generation of peroxynitrite in the striatum may explain the possible involvement of NO in the induction of selective nigro-striatal DA-ergic damage by rotenone. The elevated level of NO further generates an increase of peroxynitrite which reacts with cellular lipids in the cells and oxidises them.

The current study also sought to determine the intensity of LPO process during chronic rotenone administration. Our data concerning the increase in the level of TBARS in the studied brain areas of rats following chronic rotenone administration corroborate the results of earlier studies investigating other models of PD (Ogawa et al., 1994; Hung et al., 1998). As far as we know, there are no data concerning the effect of chronic rotenone administration on the concentration of LPO products in the brain. However, it is worth noting, that we did not observe direct correlation between the increase of NO generation and TBARS content in the rotenone model; thus suggesting that the rotenone induced elevation in lipid like oxidative processes is not directly due to the activation of specific NO pathways.

This experiment shows continues metabolism variations after complex I inhibition and how the neuronal NO concentration and dynamically how the behavioural parameters in bar and grid test accord to fluctuation in NO levels and consequent changes in the TBARS production in the different regions of rat brain. The complex I inhibition which is able to modulate NO production in different brain regions, may be at work to modulate mitochondrial properties and thus become useful in disease prevention or treatment.

The results finally provide the molecular cause of DA-ergic cell death via increased levels of NO production.

## 3.4 Validity of rotenone model of Parkinson's disease

The characterisation of rotenone-induced Parkinsonism confirmed its similarities to PD after its validation by challenging with L-DOPA and the important pathology of its LBs inclusions. Peripheral administered rotenone has been proposed as a model of PD but this model still suffers from the lack of validation. Therefore, the specific aim of this work was to investigate whether or not the clinical effect of the anti-parkinsonian drug L-DOPA is able to reverse systemic administered rotenone-induced parkinsonian symptoms; because L-DOPA is one of the most effective and commonly used drugs in the treatment of Parkinsonian symptoms, in the case of successful reversal of the symptoms; then this model would fulfil pharmacological validation for a PD model. In our study, quantitative neurochemical analysis in the anterior and posterior CPu suggests that, rotenone depletes DA content in both areas of brain tissues and causes the metabolite deficiency in the nigrostriatal DA-ergic neurons. Therefore, it seems that complex I inhibition within the nigrostriatal pathway is sufficient to produce neurotoxicity. L-DOPA reversed rotenone-induced parkinsonian symptoms and thus parallels clinical efficacy. The increase in the descent latency in catalepsy, inactive sitting and decrease in locomotor activity and head dips were reversed after chronic administration of L-DOPA.

These results are the first to demonstrate that selective or partial injury of systemic rotenone conforms cellular and behavioural supersensitivity to L-DOPA, the phenomology of L-DOPA induced reversal motor deficit can be accounted for by the topography of DA denervation within the striatum.

Before the rotenone model can be used as an appropriate model to investigate a new drug for PD therapy the criteria need to be fulfilled which has been shown as valid after the returns of L-DOPA administration up to this experiment.

Finally this thesis discusses the advantages and disadvantages of the various animal models of PD and considers their potential role in revealing the mechanisms responsible for PD pathogenesis, and for testing experimental therapeutics (Table1 and 2).

**Table 1. A comparison between human PD and rotenone models of PD**

| Human PD | Rotenone-induced effect |
|---|---|
| ***Behaviour*** | ***Behaviour*** |
| Resting tremor, akinesia and rigidity. In PD the behavioural deficit response to L-DOPA. | Akinesia, rigidity and movement deficit. Rotenone-induced rats are responsive to L-DOPA. |
| ***Neurochemical*** | ***Neurochemical*** |
| 70-80% DA deficit in striatum | 50% DA deficiency has been observed as compared to control |
| ***Biochemical*** | ***Biochemical*** |
| Decrease in nigrostriatal TH-positive Immunoreactivity and depletion of NA in locus ceruleus neurons. | Decrease in nigrostriatal TH-positive Immunoreactivity and depletion of NA in locus ceruleus neurons. |
| Post-mortem brain shows α-synuclein inclusion reminiscent of LB | Formation of ubiquitin and α-synuclein positive inclusions which are similar to LB of PD. |

**Table 2. Characteristics of animal models of PD**

| Pros | Cons |
|---|---|
| ***6-OHDA*** | |
| Selective for monoaminrgic neurons taken up by monoamine-transporters | Does not penetrate the blood Brain barrier, local infusion required |
| ***3-NT*** | |
| Produces oxidative stress | Not yet extensively investigated/ described; requires intracerebral injection. |
| ***MPTP*** | |
| Crosses blood brain barrier | Works only in primates, some mice strains but not in rats. |
| ***Rotenone*** | |
| Crosses the blood brain barrier<br>Works in rats and produce LB | Unselective in high doses |

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## 5 Abbreviations

| | |
|---|---|
| AD | Alsheimer's disease |
| 6-OHDA | 6-hydroxydopamine |
| DA | Dopamine |
| DAT | Dopamine transporter |
| LB | Lewy body |
| LPO | Lipid peroxidation |
| MAO-B | Monoamine oxidase-B |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| NADH | Nicotinamide adenine dinucleotide |
| NO | Nitric oxide |
| PD | Parkinson's disease |
| ROS | Reactive oxygen species |
| SNpc | Substantia nigra pars compacta |
| TBARS | Thiobarbituric acid |
| TH | Tyrosine hydroxylase |

## 6 Declaration to personal contribution and realisation in each publication

I Complete conceptional and textual plan, complete design and experimental realisation including total analysis and summary of results and writing of the manuscript.

II Complete conceptional and textual plan, complete design and experimental realisation, measurements performed 100%, including analysis, summary and writing of the manuscript.

III Experimental realisation, analysis, summary and writing of the manuscript by M.Alam. NO measurements performed by V.Bashkatova. Conception, experimental design, textual plan proposed by Professor W.J. Schmidt

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**APPENDIX: ORIGINAL PUBLICATIONS I – IV**

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Behavioural Brain Research 136 (2002) 317–324

BEHAVIOURAL BRAIN RESEARCH

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Research report

# Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats

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**Abstract**

Rotenone (an inhibitor of mitochondrial NADH dehydrogenase, a naturally occurring toxin and a commonly used pesticide) appears to reproduce the neurochemical, neuropathological and behavioural feature of Parkinson's disease (PD) in the rat. In this study, rotenone was administrated on a daily basis systemically by intraperitoneal injection of two different doses: 1.5 mg/kg (low dose) and 2.5 mg/kg (moderate dose), over a period of 2 months. This treatment caused depletion of dopamine in the posterior striatum (CPu) and prefrontal cortex and also reduced tyrosine hydroxylase-immunoreactivity in CPu. Behavioural experiments showed dose-dependent catalepsy in the two treatment groups of rats. Data from this study indicate that in rats rotenone is capable of causing degeneration of dopaminergic neurons and induction of parkinsonian symptoms. It is concluded that the causal mechanisms of neuronal degeneration implicate a complex I deficiency in the aetiology of rotenone-induced and perhaps in some cases of sporadic PD.


*Keywords:* Complex I; Mitochondria; Parkinson's; Rotenone; NADH dehydrogenase; Dopamine; Substantia nigra; Tyrosine hydroxylase

## 1. Introduction

It is well known that in Parkinson's disease (PD) dopaminergic (DA-ergic) neurons degenerate, but the primary cause for this degeneration is still unknown. The disabling symptoms in PD are primarily due to a profound deficit in striatal dopamine (DA) content that results from the degeneration of DA-ergic neurons in substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibres in the striatum.

Probably there is not one single factor responsible for neurodegeneration; it appears that several factors are acting in concert. One of these factors is mitochondrial complex I deficiency [9].

DA-ergic neurons are especially vulnerable to complex I inhibitors. MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine), for example, which represents a widely used toxin to induce parkinsonian symptoms in animals, is a potent inhibitor of complex I. Also, rotenone is a complex I inhibitor and has been reported to have a rather selective toxicity on DA-ergic cells in vitro [7] and in vivo. However, there are differences between MPTP and rotenone. Rotenone facilitates the formation of alpha-synuclein fibrils [1] and crosses the blood–brain barrier as well as the cell membrane because of its lipophilic structure.

Tentatively, rotenone treatment may represent an animal model that shares basic processes with the disease. Non-familiar sporadic PD is characterised by 15–30% reduction of complex I activity [10]. Given that complex I deficiency persisting over the lifespan of a human being causally contributes to PD, rotenone treatment may mimic the processes that develop in humans and thus represents a unique model with construct validity (i.e. the highest degree of validating criteria for animal models [2]).

The selective toxicity of rotenone is especially relevant because it is widely used as a herbicide in private gardens and in several powders for delousing humans or animals, and thus a real threat that an environmental substance can cause PD does seems to exist.

In a previous study of Betarbet et al. [1], rotenone was administered continuously through minipumps, 2–3 mg/

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kg per day for 5 weeks, and it was found that the rats developed motor and postural deficits characteristic of PD. Immuno-assays also showed that ubiquitin and alpha-synuclein inclusions in brain shared the features of Lewy bodies.

The present study was designed to assess rotenone toxicity after intraperitoneal (i.p.) injection daily for 2 months at two different doses: 1.5 mg/kg (low dose) and 2.5 mg/kg (medium dose). Pulsatile administration was chosen because it may be similar to exposure in normal life, such as through inhalation, dermal contact and oral ingestion of pesticide residues and in foods such as vegetables, fruits and fish.

To assess quantitatively the transmitter concentrations, we measured both DA and its metabolites in the prefrontocortical and striatal tissue by means of high-performance liquid chromatography with electrochemical detection (HPLC–ECD) and tyrosine hydroxylase-immunoreactivity (TH-immunoreactivity) level in the striatum using Western blot analysis. Parkinsonian symptoms were assessed in behavioural tests (catalepsy and open field).

## 2. Material and methods

### 2.1. Animals

Thirty-six male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) aged 7 weeks were chosen for the experiment. At the beginning of the experiment, the rats weighed 220–240 g. They were divided into three groups, each containing 12 rats. Rats were housed in cages and kept in a room maintained at a constant temperature of 22 °C, 50–60% humidity and a 12/12 h light–dark cycle (7:00–19:00). They obtained dry food (Altromin, 1324) 12 g/day/rat, and water was available ad libitum. All experiments were performed in compliance with international ethical standards and the German Animal-Protection Law and have been approved by the local animal care committee (Tierschutzkommission, Regierungspräsidium Tübingen, ZP 5/01).

### 2.2. Chemicals

Rotenone and all solvents for HPLC–ECD were purchased from Sigma (St. Louis, MO).

### 2.3. Treatment

Rotenone emulsified in sunflower oil at 1.5 mg/ml and 2.5 mg/ml was given intraperitoneally once a day at 1 ml/kg for 60 days to two groups. Oil was injected as vehicle to the control rats (1 ml/kg).

### 2.4. Neurochemistry

#### 2.4.1. Sample dissection

Rats were killed by decapitation with the use of a guillotine. The brains were removed rapidly within 25–50 s and put into ice-chilled 0.9% NaCl solution for 1 min. After that, the brain was cut into coronal sections using the method described by Hefner et al. [6]. Tissue samples were taken bilaterally to include cortex, striatum anterior, posterior striatum (CPu) and nucleus accumbens. The tissue was immediately weighed and stored in liquid nitrogen until analysis.

#### 2.4.2. Sample preparation

Frozen tissue was homogenised for 20 s using a tapered motorised pestle in 500 μl of ice-cold mobile phase (50 mM sodium acetate buffer, pH 4.3, containing 35 mM citric acid, 0.13 mM disodium EDTA, 0.45 mM 1-octansulphonate and 10% methanol) containing known amount of 3,4-dihdroxybenzylamine (DHBA) as an internal standard. The homogenised tissue suspension was centrifuged at 10,000*g* for 60 s, and the supernatant was removed and filtered through a 0.2 μm Teflon syringe filter (Schleicher and Schuell, Dassel, Germany) for immediate HPLC analysis.

#### 2.4.3. HPLC analysis

Levels of biogenic amines, DA and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in the reverse-phase HPLC coupled to ECD. First, the mobile phase was oxidised by a guard cell (V = +450 mV). The guard cell serves to perform a partial clean up of the mobile phase prior to sample injection. The samples were introduced into the autosampler carrousel (CMA 200, CMA Microdialysis, Solna, Sweden) with the flow rate of mobile phase being 0.8 ml/min (ESA 2200 Analytic Pump, Bischoff, Leonberg, Germany). Separation of the neurotransmitters was carried out by means of reverse-phase column (Prontosil 33 × 4.0 mm, pore diameter: 3.0 μm; Bischoff). The substrates were detected by an ESA detector (ESA Coulochem 5100A, Analytical cell ESA 5010). Typical values of applied potential used in the present experiment were +20 mV at Electrode 1 and +320 mV at Electrode 2.

#### 2.4.4. Data handling

Chromatograms were analysed with the aid of chromatographic data system (Axxiom 727, Sykam, Gilching, Germany) and peak areas were quantified using internal standard method. The peaks were classified with the retention times of reference substances. Data were expressed as the concentration of neurotransmitters or its metabolites per unit wet weight of tissue in picograms per milligram.

### 2.5. Estimation of TH level in the striatum using Western blot analysis

#### 2.5.1. Tissue preparation

Dissected tissue (free of blood and meninges) was homogenised immediately (within 10–20 s) in the same HPLC mobile phase (50 mM sodium acetate buffer, pH 4.3, containing 35 mM citric acid, 0.13 mM disodium EDTA, 0.45 mM 1-octansulphate and 10% methanol). Total tissue homogenate was centrifuged at 10,000*g* for 1 min to remove cell debris. The separated supernatant was concentrated by a *Speed vac* centrifuge (Bachoffer, Reutlingen, Germany) to a final volume of 60 μl at 4 °C.

#### 2.5.2. Protein estimation

Total protein concentration was measured by a Bio-Rad Kit (Bio-Rad Lab, Hercules, CA) method in microtiter plate following the manufacturer's protocol. Total protein concentration (yield) of 124–255 μg was estimated depending on the wet weight of tissue (18–30 mg striatum tissue).

#### 2.5.3. Sample preparations and gel electrophoresis

Known amount of samples (25 μg) was diluted in ratio of 1:1 in sample treatment or diluting buffer (2 × SDB, pH 6.8), soft vortexed and denatured in a PCR thermocyler at 100 °C for 3 min. Samples were loaded on 10% polyacrylamide gel in discontinuous gel and buffer system, i.e. 5% stacking gel (pH 6.8) and 10% separating gel (pH 8.8). High-molecular mass pre-stained markers (Bio-Rad Lab) were also loaded to assess the molecular weight of unknown protein band. Electrophoresis was carried out in Tris–glycine buffer, pH 8.3 at 140 V for approximately 1.5 h.

#### 2.5.4. Immuno-detection of TH (Western blotting)

After transferring the gel to nitrocellulose, the membrane was rinsed and washed twice in 1 × phosphate-buffered saline (PBS) containing 0.05% Tween20 (pH 7.5) for 2 min on shaker at room temperature (RT). The membrane was incubated for 1 h in blocking solution (5% casein in 0.05% Tween20–PBS, pH 7.5) at RT on shaker. The band corresponding to TH was probed by incubating the membrane in monoclonal TH anti-mouse antibodies (1:1000 dilution, Santa Cruz Biotechnology, Heidelbery, Germany Inc.) in the same blocking solution on shaker for 1 h at RT. After washing three times (5 min per wash) in washing buffer (0.05% Tween20–PBS, pH 7.5), a further incubation with secondary antibody, rabbit anti-mouse Ig-AP, conjugated with alkaline phosphatase (1:1500 dilution, Santa Cruz Biotechnology, Inc.) was carried out for 1 h at RT on shaker. The membrane was washed further three times for 5 min in washing buffer and incubated at RT in detection buffer (100 mM Tris–HCl, pH 9.5, containing 100 mM NaCl and 50 mM MgCl). Colour detection was carried out by incubating the AP substrate NBT/BCIP (Boehringer Mannheim, Mannheim, Germany) at RT in the same detection buffer. Reaction was stopped manually with water.

#### 2.5.5. Data analysis

Western blots were analysed by determining OD using a Lumi® imager (Boehringer Mannheim) and percentage intensities of individual bands of TH calculated. The molecular mass of TH was extrapolated from the standard protein marker.

### 2.6. Behaviour tests

After 2 months of treatment with rotenone or vehicle, the animals were tested 1 week after the final injection for catalepsy and the next day following this in the open-field box for locomotor activities.

#### 2.6.1. Catalepsy

Each rat was hung by the paws on a vertical grid (25.5 cm wide and 44 cm high with a space of 1 cm between each wire). The time taken for the rats to move their paws or any sort of first movement was noted.

The second part of the test was the bar test. The rats were placed with both forepaws on a bar which was 9 cm above and parallel from the base. The rats were placed with both forepaws on the bar in a half-rearing position. Latency with removal of the paw was noted. The maximum descent latency was fixed at 180 s for both tests.

#### 2.6.2. Open-field test

Locomotor activity was measured after 1 and 2 week intervals post injection. Both sessions were measured to confirm whether the animals showed any sign of recovery.

Each animal was placed in the open field, a square wooden box with a fan to provide ventilation and measuring 69 × 69 cm and lit by 1 × 25 W red bulb. The floor of the box was divided into 16 small equal-sized squares, with each square having a small hole in the centre (diameter 4 cm, depth 3 cm). The hole board was situated 3 cm above the ground floor of the open-field box. A video camera was fixed on the top of the box. The movement and behaviour of the rats were recorded on the video and the film was later observed for off-line measurements with the use of a DOS programme.

Five parameters were measured in this experiment:

- active sitting (time in seconds),
- inactive sitting (time in seconds),
- head dipping (number of dips),
- rearing (number of rears),
- line crossing (number of lines crossed).

### 2.7. Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were carried out using the GB-STAT V5.4 software (Dynamic Microsystem, Inc., Silver Spring, MD). The data from behaviour test, neurochemical analysis and TH-immunoreactivity assay were evaluated using non-parametric analysis (multifactorial Kruskal–Wallis one-way ANOVA followed by Mann–Whitney $U$-test). $P < 0.05$ and $P < 0.01$ were taken as levels of statistical significance.

## 3. Results

### 3.1. Neurochemistry

The amount of DA and its metabolites was measured in the prefrontal cortex (PFC) and CPu for each group of animals. There was a decrease of DA in all these two regions of the brain in rotenone-treated animals.

In CPu, the amount of DA and its metabolites DOPAC and HVA was decreased in medium-dose-treated animals—DA: $\chi^2 = 5.1635$, df = 2, $P = 0.0756$; DOPAC: $\chi^2 = 3.3147$, df = 2, $P = 0.1485$; and HVA: $\chi^2 = 5.5893$, df = 2, $P = 0.0611$—as shown in Fig. 1a.

DA was decreased in low- and medium-dose-treated animals in PFC ($\chi^2 = 15.1802$, df = 2, $P = 0.0005$). The DA metabolite DOPAC was also decreased in low- and medium-dose-treated animals ($\chi^2 = 5.5978$, df = 2, $P = 0.0609$). HVA was decreased by medium doses ($\chi^2 = 8.354$, df = 2, $P = 0.0153$) as shown in Fig. 1b.

### 3.2. Quantitative analysis of TH-immunoreactivity

Western blot analysis of TH protein in the rat's CPu is shown in Fig. 2a,b only for two animals from control group and four animals from each low-dose- and medium-dose-treated groups. The rest of the control group animals and two remaining animals from low-dose and medium-dose group are not shown in the figure. Striatal samples were isolated 2 months after the last treatment of rotenone. Samples were taken from six rats each from the low (1.5 mg/kg) and medium (2.5 mg/kg) group. Dose-dependent reduction of TH-immunoreactivity in both groups is shown in Fig. 3. The graph is plotted by taking the percentage of intensities of TH bands in individual groups. Rotenone-treated animals showed a dose-dependent decrease in TH-immunoreactivity. Kruskal–Wallis one-way ANOVA—$\chi^2 = 15.15$, df = 2, $P = 0.0005$. Significance at $P < 0.05$ and significance at $P < 0.01$ have been observed when each group was compared with control group.

### 3.3. Behaviour

#### 3.3.1. Catalepsy

Rotenone-treated animals showed a prolonged descent latency as compared to vehicle-treated control group in bar and grid test (Fig. 4a,b).

The results showed significant differences when compared with control group by two-way repeated measures ANOVA followed by Mann–Whitney $U$-test. Bar: Krukal–Wallis one-way ANOVA—$\chi^2 = 24.80$, df = 2, $P < 0.0001$; and grid—$\chi^2 = 26.40$, df = 2, $P < 0.0001$.

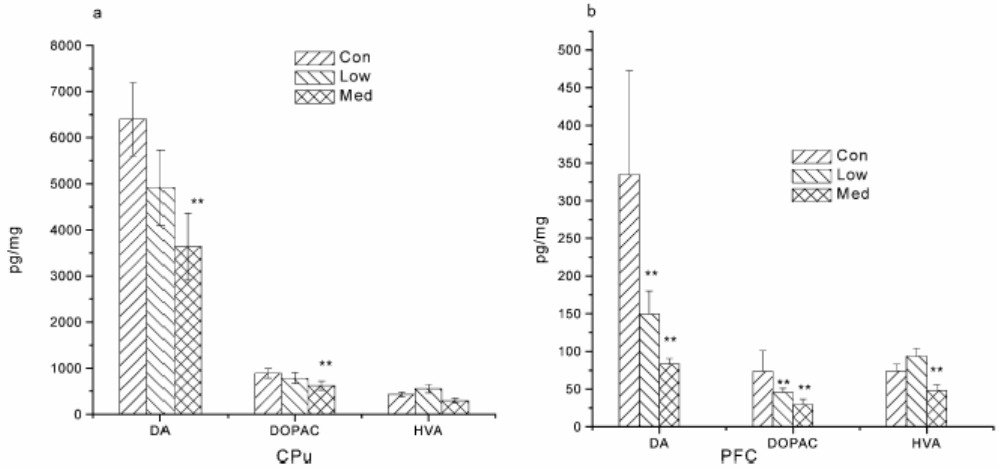


Fig. 1. Levels of DA and its metabolites DOPAC and HVA in the CPu and PFC tissues. Control group $N = 10$, compared with low dose (1.5 mg/kg) $N = 9$, medium dose (2.5 mg/kg) $N = 7$. Mann–Whitney $U$-test—*significant at $P < 0.05$; and **significant at $P < 0.01$, compared to respective control.

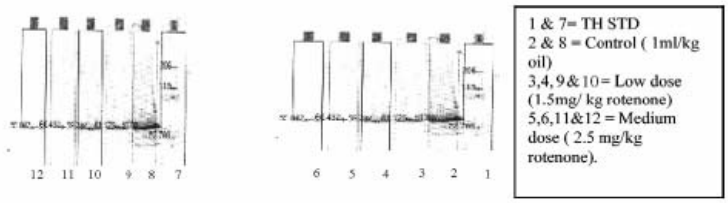


Fig. 2. Western blot of TH protein in rat's CPu. For each sample, 25 μg of striatal protein was loaded onto separate *lanes* of the gel. In addition, a known amount of purified TH protein standard (TH STD) was loaded onto the right lane and was used to normalize density units between the gels.

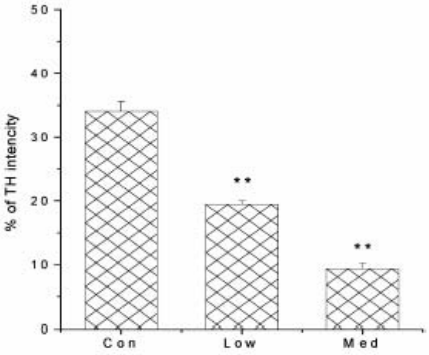


Fig. 3. Group mean (±SEM) levels of TH protein levels (plotted as percent of control group values) were measured by Western blot analysis in CPu. Control group $N = 6$, compared with low dose (1.5 mg/kg) $N = 6$ and medium dose (2.5 mg/kg) $N = 6$. Mann–Whitney *U*-test—*significant at $P < 0.05$; and **significant at $P < 0.01$, compared to respective control.

### 3.3.2. Open field

The behavioural parameters of active sitting, line crossing, rearing, and head dips were found to be reduced by rotenone as compared to control group (Fig. 5). The duration of inactive sitting was significantly high in rotenone-treated animals as compared to control. $N = 12$ for control group, $N = 12$ for low dose (1.5 mg/kg) and $N = 8$ for medium dose (2.5 mg/kg). Active sitting, inactive sitting, rearing were expressed in time (s). Kruskal–Wallis one-way ANOVA—$\chi^2 = 4.6$, df = 2, $P = 0.1$; $\chi^2 = 23.54$, df = 2, $P = 0.0001$; $\chi^2 = 19.82$, df = 2, $P = 0.0001$, respectively. The number of line crossing and head dip showed $\chi^2 = 21.86$, df = 2, $P = 0.0001$ and $\chi^2 = 7.39$, df = 2, $P = 0.02$, respectively.

## 4. Discussion

### 4.1. Neurochemistry

HPLC analysis of homogenised brain tissue shows that rotenone depletes DA and reduces the concentration of DOPAC and HVA. NA and 5HT were not affected, showing the selectivity of rotenone toxicity (data not shown). The depletion of DA was sensitive to the action of rotenone in the posterior part of the striatum and PFC, suggesting the existence of a constitutive metabolic deficiency in the nigrostriatal DA-ergic neurons. This methodology also provides a good monitor of energy metabolism. In order to maintain a low sodium and high potassium intracellular concentration, $Na^+/K^+$-ATPase uses 40–45% of the total ATP available [3]. Thus, impairment of ATP production due to complex I inhibition disrupts the ionic gradients required for the transmembrane transport of neurotransmitters. In vitro experiments on mesencephalic neuronal cultures have shown that the uptake of DA is much more sensitive to inhibition by rotenone than the uptake of GABA, NA and 5HT [2]. The profound striatal DA depletion observed in rotenone-treated rats might be due to an energy-related impairment in the presynaptic end terminal of DA-ergic neurons and their dendrites. Many studies have shown that DA-ergic neurons are more sensitive to environmental and endogenous insults because they receive direct glutamatergic input from different pathways, e.g. cortex, subthalamic nucleus (STN) and prepontine tegmentum (PPTg). Glutamate inputs result in raised intracellular $Na^+$ concentration in DA-ergic neurons in SNpc requiring more $Na^+/K^+$-ATPase and high energy consumption due to the increased activity.

In order to keep the DA-ergic neurons in a constant tonically active condition, DA-ergic synaptosomes require increased mitochondrial activity with a consequent increase in ROS levels. This slight oxidative insult may play a role in making this group of neurons more prone to damage. Furthermore, the vesicular uptake of DA also requires energy to protect the cells against oxidative stress induced by DA. However, due to mitochondrial inhibition, the uptake of DA in SNpc does not take place, resulting in the metabolisation of this neurotransmitter in the extracellular milieu.

The rotenone-induced metabolic deficiencies observed in this study suggest that DA-ergic synapses in SNpc and in the nigrostriatal pathway are sensitive to the action of rotenone. In contrast, Betarbet and colleagues [1] have recently found that continuous intravenous

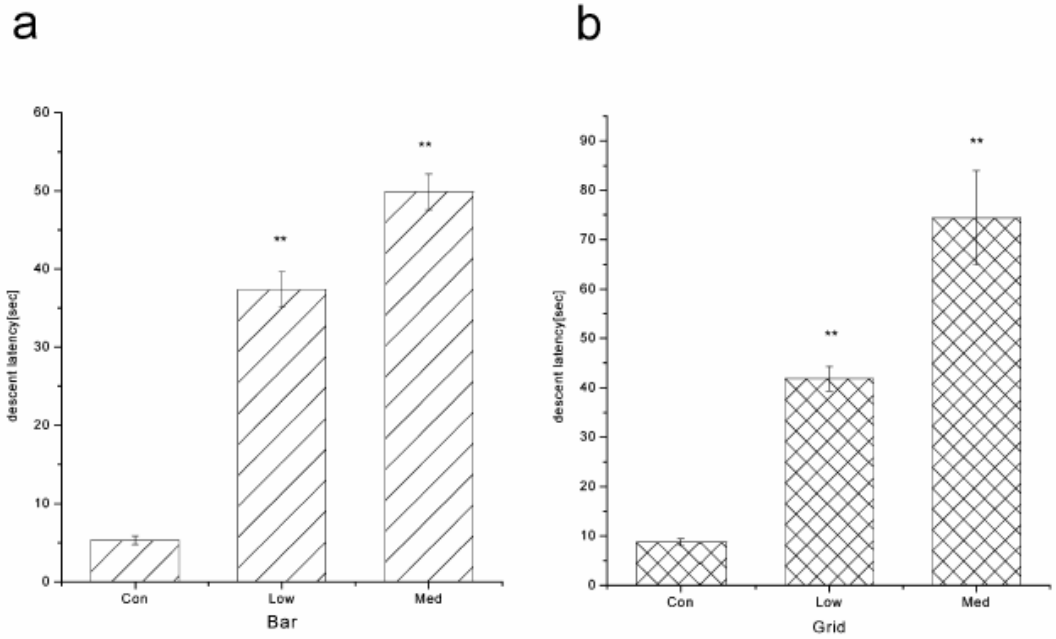


Fig. 4. Catalepsy tests of rotenone-treated animals in horizontal bar and vertical grid. Control rats ($N = 12$), low-dose group ($N = 12$) treated with 1.5 mg/kg of rotenone and medium-dose group ($N = 8$) treated with 2.5 mg/kg of rotenone. Mann–Whitney $U$-test—*significant at $P < 0.05$; and **significant at $P < 0.01$, compared to respective control.

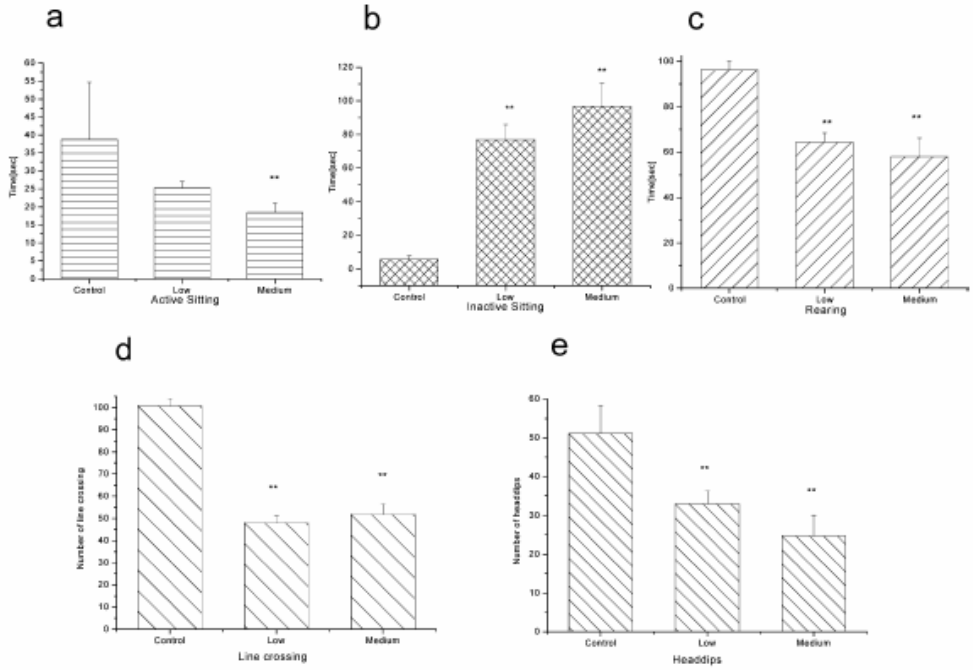


Fig. 5. Effect of two different doses, low (1.5 mg/kg) and medium (2.5 mg/kg), of rotenone as compared to control group in the spontaneous locomotion and exploration—(a) active sitting, (b) inactive sitting, (c) rearing, (d) line crossing and (e) head dips in the open field. Control group ($N = 12$), low-dose group ($N = 12$), medium-dose group ($N = 8$). Mann–Whitney $U$-test—*significant at $P < 0.05$; and **significant at $P < 0.01$, compared to respective control.

infusion of rotenone causes highly selective DA-ergic lesions, but the striatal nerve terminals were affected earlier and more severely by rotenone than SN which occurred later in a retrograde manner. Ferrante and colleagues [5] reported that rotenone produced selective damage in the striatum and globus pallidus; however, SN was not spared. In our experiment, we observed that the selectivity of rotenone (i.p.) in rats greatly diminished the endogenous DA content in striatum and PFC. These results provide direct evidence for the vulnerability of the nigrostriatal DA-ergic pathway.

### 4.2. The effect of rotenone exposure on behaviour

#### 4.2.1. Catalepsy

It is a known fact that reduced DA activity causes parkinsonian symptoms in humans such as rigidity and akinesia. This state is shown as catalepsy in the rat. With decreasing DA-ergic activity, the descent latency of catalepsy increases as well [11]. Catalepsy tests revealed an increase in cataleptic behaviour in the low-dose (1.5 mg/kg) and medium-dose (2.5 mg/kg) rotenone-treated groups as compared to vehicle (1.0 ml/kg) treated control group.

#### 4.2.2. Locomotion

A 50–70% depletion of DA in the striatum leading to a 50% neuronal loss in SNpc causes spontaneous loss of locomotor activities [4]. In the open-field test, hypoactivity was observed in active sitting, rearing, head dips and line crossing behaviour. In summary, it is apparent that the results from the collective findings of behaviour test show that the rotenone-treated rats display an increase in cataleptic behaviour and a decrease in locomotor activity as compared to control rats. This correlated closely with levels of deficit in DA in the nigrostriatal region.

## 5. Conclusion

The major goal of this study has been to test whether rotenone i.p. treatment could be used to establish a rat model of PD. Other studies have introduced rotenone either stereotactically or via intrajugular or intramuscular routes to produce PD symptoms. This gives rise to the controversial question of the relevance of these routes for PD patients in whom the disease may have been due to environmental exposure.

In this study a rotenone-based rat model of PD has been established: The chronic exposure by i.p. injection which is comparable to chronic environmental exposure and is comparable to the "real life" situation.

Animal models are important tools for understanding the disease process. For PD, different models were produced, and these models can be categorised according to pharmacological, behavioural and biochemical criteria in three groups. For example, haloperidol or DA antagonist model for PD shows a predictive validity of PD. It is regarded as the lowest level of validity and has similarities to pharmacological isomorphism to PD. The generation of symptoms is, however, totally unrelated to idiopathic PD. The second category of animal models is those with face validity. These models assess the phenomenological similarity between the model and the disease. These models are hierarchically higher than those with predictive validity. The existing models (6 OHDA and MPTP) could be categorised as face validity. They provide similarities to symptomatology but may not mean similar underlying mechanisms. The highest set of validity criteria for animal models deals with construct validity. It should fulfil the criteria for face validity and predictive validity and should share the same underlying mechanism as the disease does. Rotenone-induced complex I inhibition that leads to LB occurrence [1] and to a selective degeneration of DA-ergic neurons may represent an animal model with construct validity.

Previous studies have found that most causes of PD are due to environmental toxins and inhibition of mitochondrial complex I [8,10]. One of the best-described environmental causes of PD is MPTP. In this study, the effects of the complex I inhibitor rotenone were examined. Rotenone which was administered i.p. leads to a dose-dependent reduction of DA and TH-activity which correlates with parkinsonian symptoms.

These findings support the notion of a multi-hit model of PD: a constitutional complex I deficiency and environmental toxins may be implicated in the pathogenesis of PD. Rotenone, as an environmental toxin which is in close contact to our food, gardeneries, agriculture and water supplies, remains a prime suspect for such a risk factor. Chronic exposure to low residual levels of this toxin could quite conceivably be a cause of neurodegenerative disease like Parkinson's in human beings.

Little evidence exists regarding levels of human exposure to these types of pesticides and the relative efficiencies of the routes of ingestion, although it is likely that the major routes would be through inhalation, dermal contact and oral ingestion of pesticide residues on and in foods (e.g. vegetables, fruits and fish). This area requires urgent evaluation. The lowest dose used in this study is more relevant to that anticipated in chronic human exposure. The low and medium doses did not affect the body weight in these groups of rats, whereas a 20% loss was observed in the high-dose group (3 mg/kg, unpublished data). Changes in the motor activity, in the level of DA depletion and the descent latency of catalepsy were, however, observed in the low- and medium-dosage groups. Therefore, it is plausible that

the chronic exposure to such environmental toxins may be able to produce PD, even in the absence of other physiological symptoms.

It is also likely that individual susceptibility to chronic toxin exposure may have a genetic determinant, for example the expression of paraoxanase protein—which metabolises a number of pollutants. The risk for individual homozygous for the implicated protein was, however, one–six times greater than those homozygous for the alternative version, thus emphasising the critical influence of the environment.

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Research report

# The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA

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**Abstract**

Rotenone (an inhibitor of mitochondrial complex I) has been proposed as a model of Parkinson's disease (PD) as it induces nigrostriatal degeneration associated with α-synuclein inclusions. So far, only peripherally administered rotenone has been used as a model of PD. There has not been any investigation on the neurobehavioral changes induced by bilateral lesions of dopaminergic neurons by rotenone in rats. In the present study, rotenone (3 μg) was administered bilaterally stereotaxically into the medial forebrain bundle (MFB) to produce parkinsonian symptoms. Behavioural and biochemical data showed a strong increase in catalepsy, a decrease in locomotor activity and a significant depletion of dopamine levels in the striatum as compared to sham-lesioned animals. If the locomotor deficits are caused by the depletion of dopaminergic neurons, then L-DOPA should counteract motor deficits because L-DOPA therapy reverses mostly all motor deficits in human Parkinsonian patients. To examine the effectiveness of L-DOPA in reversing the motor deficit in rats, two different doses of L-DOPA (5 and 10 mg/kg) in combination with the peripheral amino acid decarboxylase inhibitor benserazide were daily administrated intraperitonially for a period of 31 days lesioned animals. L-DOPA plus benserazide counteracted catalepsy dose-dependently and increased locomotor activity. The results indicate that rotenone infused into the MFB destroys dopaminergic neurons, induces pakinsonian symptoms that are reversed by the clinically effective anti-parkinsonian drug L-DOPA. Therefore, sterotaxically infused rotenone may be useful for screening drugs for the treatment of PD.


*Keywords:* Rotenone; Complex I; Parkinson's; L-DOPA; Substantia nigra

## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder which is characterised by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and a decrease of dopamine (DA) in the striatum. The etiopathogenesis of PD is diverse and complex. Some genetic mutation may be a cause of PD, but most cases of PD are sporadic. Epidemiological studies indicate that there is no relation to one specific factor but there are perhaps a number of factors which increase the risk for development of the disease. Recent work suggests that environmental toxins could be one cause of PD [10]. A leading hypothesis claims that oxidative stress is an inducer of cell death in PD [8]. But all factors have in common that they compromise mitochondrial functions. Mitochondria occupy a pivotal role in metabolic pathways that are critical for both survival (oxidative phosphorylation) and cell death (apoptosis). There is significant and specific reduction of mitochondrial complex I activity in the substantia nigra of patients with PD [11]. The majority of the findings in PD and the mode of action of rotenone suggest that complex I deficiency in PD may be related to the primary disease process. Thus, exposure to rotenone in rats or mice provides a valuable model for studying mechanisms of toxicant-induced dopaminergic cell injury and α-synuclein pathology.

Till now, the toxin 6-OHDA is most useful and most frequently applied for local lesion technique namely uni-lateral lesion because animals with bilateral lesion have a higher postoperative risk of lethality and require specific care (especially tube-feeding). In our study we used the environmental toxin rotenone locally, which is a specific mitochondrial complex I inhibitor and lipophilic in nature. This toxin does not need any transporter or carrier to enter dopaminergic neurons. Rotenone appears to reproduce the neurochemical, neuropathological and behavioural feature of PD in rats [1].

The present study was designed to assess rotenone toxicity after stereotaxic injection of 3 μg in 4 μl. The bilateral

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administration of rotenone in the MFB resulted in catalepsy (hypokinesia and rigidity) very similar to those including 6-hydroxy-dopamine (6-OHDA) or in human Parkinsonism. To date only one study has been published which produced a MFB lesion by rotenone in rats [6] and a 80% decrement of DA content in the neostriatum was observed. However for the first time our study provides a detailed account on the behavioural deficits which are produced by rotenone lesion in MFB in rats. In the present study, rotenone lesioned animals have been compared to sham treated animals with respect to their motor response to L-DOPA. L-DOPA was administrated chronically 5 and 10 mg/kg, respectively for 31 days and motor performance was assessed after 31 days of L-DOPA application.

## 2. Materials and methods

### 2.1. Animals

Twenty-five adult male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) aged 7 weeks were chosen for the experiment. At the beginning of the experiment, the rats weighed 230–240 g. Animals were housed in cages under a 12:12 h light/dark cycle in a room maintained at constant temperature of 22 °C and 50–60% humidity with food and water available ad libitum.

All treatments used in this experiment adhere to the international ethical standards and the German Animal-Protection Law and have been approved by the local animal care committee (Tierschutzkommission, Regierungspräsidium Tübingen, Z P 2/03).

### 2.2. Drugs

L-DOPA methyl ester (Sigma-Aldrich, Germany) was dissolved in saline containing 0.2% ascorbic acid. Benserazide hydrochloride (Sigma; one -quarter dose of L-DOPA) a peripheral DOPA-decarboxylase inhibitor was dissolved in physiological saline. The injection volume was 1 ml/kg body weight for all the drugs. The rotenone, obtained from Sigma was dissolved in propylene glycol at 0.75 μg/μl.

### 2.3. Surgery

Animals were anaesthetised with sodium pentobarbital (60 mg/kg, i.p., Narcorane; Abbott Laboratories, Germany) and placed in a sterotaxic device (Trent Wells Inc., So. Gate. Calif.) using the 45° earbars. After the rats were fixed in the sterotaxic device, the scalp was retracted to expose the skull, and two holes were drilled over the right and left medial forebrain bundle (MFB) at the following co-ordinates: AP −3.5, LAT ±1.5 and DV −7.8 from dura [9]. The injection volume in each case was 4 μl, resulting in 3 μg rotenone/side. The sham operated animals received 4 μl propylene glycol. The injection was given by automatic infusion pump (TSE Injection pump, Model 540220, Germany), over a 20-min period using a 5-μl Hamilton syringe. The infusion rate was 0.2 μl/min. After infusion, the incision was closed by stitching and sprinkled with antibiotic powder. The animals were returned to their homecage to recover.

### 2.4. Experimental procedures

On the 21st day after stereotaxic surgery, rats were observed in neurobehavioral tests at intervals of 14 days over of a 49 day period. The two week pause between the tests was given to minimise behavioural sensitisation. On the 50th day L-DOPA treatment (i.p.) combined with benserazide hydrochloride (one-quarter dose of L-DOPA) was started on a daily basis in three groups (lesioned group A, $n = 6$, 10 mg/kg; lesioned group B, $n = 10$, 5 mg/kg; sham-lesioned control group, $n = 6$, 5 mg/kg) for 31 days. Benserazide hydrochloride was administered 30 min before the treatment with L-DOPA and 30 min after the L-DOPA administration behaviour was tested.

### 2.5. Behavioural testing

The behavioural tests were started 3 weeks after lesion (Fig. 1). The experiment was performed between 09:00 and 14:00 always in the same context and at standard conditions. Catalepsy test and open-field apparatus were chosen for the assessment of the behavioural changes. Starting on the first day of L-DOPA treatment, catalepsy and open-field was measured and further catalepsy tests were performed every third day, and open-field took place on every sixth day.

### 2.6. Catalepsy

Each rat was hung by all four paws on a vertical grid (25.5 cm width and 44 cm high with a space of 1 cm between each wire) and a stopwatch was started as soon as the rat held onto the grid. Just as they moved their paws or showed first movement the stopwatch was stopped and the time noted as descent latency.

The second part of the test was the bar test. The rats were placed with both front paws on a bar which was 9 cm above, and parallel from the base. The rats were placed with both front paws on the bar in a half rearing position. Here they were timed with the stopwatch. When the animals removed one paw from the bar the stopwatch was stopped and the time noted. The maximum descent latency for grid and bar was fixed at 180 s.

### 2.7. Open-field test

After lesion, the first three measurements took place at intervals of two weeks but after the treatment of L-DOPA open-field tests were done on a weekly basis (Fig. 1).

Each animal was placed into the open field, a square wooden box measuring 69 cm × 105 cm × 69 cm, a fan pro-

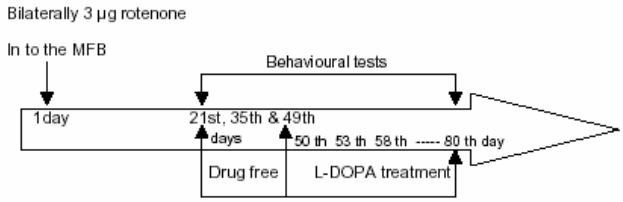


Fig. 1. Experimental design.

vided ventilation and it was lit by a 1 × 25 W red bulb. The floor of the box was divided into 16 small equal-sized squares (5 cm × 5 cm) with each square having a small hole in the centre (diameter 4 cm, depth 3 cm). The hole board was situated 3 cm above the ground floor of the open-field box. A video camera was fixed on the top of the box. The movement and behaviour of the rats was recorded on the video which was later observed for off-line measurements with the use of a DOS programme.

Four parameters were measured in this experiment:

- inactive sitting (time in seconds were measured);
- head dipping (number of dips into holes were counted);
- rearing (time in seconds was measured);
- line crossing (number of lines crossed counted).

### 2.8. Neurochemistry

One week after the behaviour test, animals were sacrificed and brains were taken out rapidly within 25–40 s and put into ice-chilled 0.9% NaCl solution for 1 min. The brains were cut into cronal sections using the method described by Hefner [5]. The tissue samples of anterior and posterior striatum were taken bilaterally and immediately weighed and stored in liquid nitrogen until assay.

Frozen tissue was homogenised for 20 s using a motorised pestle in 500 µl of ice-cold mobile phase (50 mM sodium acetate buffer, 35 mM citric acid, 0.13 mM disodium EDTA, 0.45 mM 1-octansulphonate and 10% methanol) containing known amount of 3,4-dihyroxybenzylamine (DHBA) as internal standard.

Dopamine was quantified by reverse-phase HPLC coupled to an electrochemical detector (EC). The filtered supernant of samples and an external standard were introduced into the autosampler carrousel (CMA 200, CMA Microdialysis, Solana, Sweden) with flow rate 0.8 ml/min (Bischhoff pump, Germany) using a C 18 column (Prontosil, 33 mm × 4.0 mm, pore diameter: 3.0 µm). The coulometric detector (ESA Clouchem II Multi-Electrode Detector model 5200/5200A, Bedford, MA) consisted of an analytical cell (model 5010) and a guard cell (model 5020). The potential applied in the present experiment were +20 mV at electrode 1 and +320 mV at electrode 2. The chromatograms were analysed with the aids of a chromatographic data system (AXXiom 727, Sykam, Gilching, Germany) and peak areas of DA were quantified using a standard curve generated by determining the ratio between the known amounts of amine and a constant amount of internal standard (DHBA) and represented as pg/mg of tissue.

### 2.9. Statistical analysis

All data were expressed as mean ± standard error of the mean (mean ± S.E.M.). Statistical analysis was carried out using the GB-Stat V5.4 software (Dynamic Microsystems, Inc., Silver Spring, MD). The data from behavioural tests were evaluated using non-parametric analysis. Within a group the data were submitted to the non-parametric Mann–Whitney *U*-test. The different groups were compared by the non-parametric (multifactorial Kruskal–Wallis one-way ANOVA). A $^{*}P$-value $<0.05$ and $^{**}P$-value $<0.01$ were taken as levels of statistical significance.

## 3. Results

### 3.1. Rotenone-induced catalepsy

Bilateral MFB administration of rotenone resulted in a significantly prolonged descent latency as compared to sham-lesioned control group both in the bar and grid test (Fig. 2a and b) in all the three measurements which took place at intervals of two weeks. The results showed highly significant differences when compared with control group by Kruska-Wallis ANOVA followed by Mann–Whitney *U*-test test but there were no differences between two rotenone-lesioned groups which we devided as groups A and B. The first three measurements after 21 days on the bar were: Kruskal–Wallis one-way ANOVA—chi-square ($\chi^2$) = 12.23, d.f. = 2, $P < 0.002$; $\chi^2 = 13.09$, d.f. = 2, $P < 0.001$; and $\chi^2 = 10.71$, d.f. = 2, $P < 0.004$. In the grid: $\chi^2 = 11.50$, d.f. = 2, $P < 0.003$, $\chi^2 = 12.64$, d.f. = 2, $P < 0.001$ and $\chi^2 = 12.71$, d.f. = 2, $P < 0.001$, respectively.

### 3.2. Effect of L-DOPA on behavioural changes

#### 3.2.1. Catalepsy

On the first day of treatment with L-DOPA the descent latency was reduced approximately by 40% showing a significant difference to control group in bar tests, however the

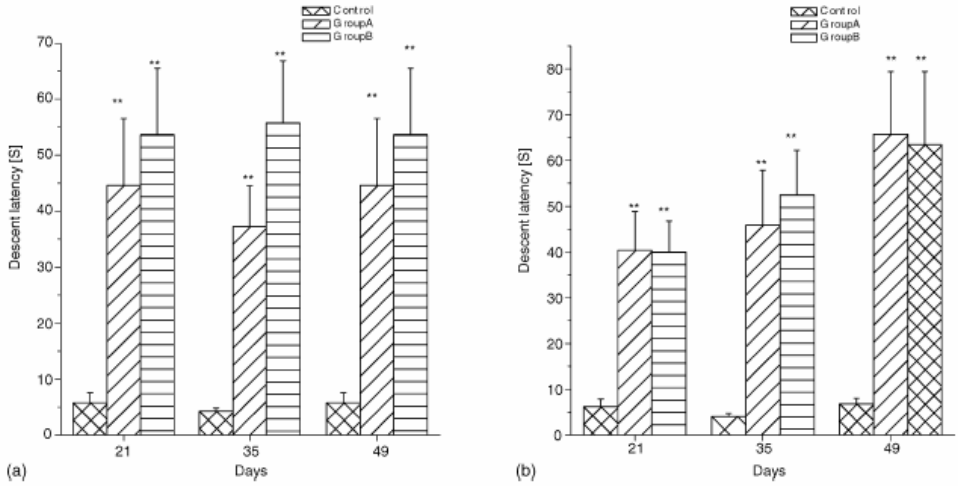


Fig. 2. Catalepsy tests (a) bar and (b) grid were done after the lesion on 21st, 35th and 49th day on all three groups of animals. Rotenone-lesioned group A ($N = 6$) and group B ($N = 10$) and sham-lesioned as a control ($N = 6$). Mann–Whitney $U$-test: significant at $^{*}P < 0.05$; and significant at $^{**}P < 0.01$, compared to respective control.

grid test showed no differences compared to control or between the two different dose of treatment groups. In the last two weeks of L-DOPA treatment groups A and B showed a significant reversal in the descent latency (Fig. 3a and b) in both bar and grid tests and statistically there were no differences among all the three groups.

### 3.2.2. Open field

In the Fig. (4a and b) the decrease in locomotor activity (line crossing) and head dipping induced by bilateral rotenone lesions were significantly reversed by L-DOPA (5 and 10 mg/kg). The time of inactive sitting was also significantly reduced after L-DOPA treatment (Fig. 4d). But in the rearing (Fig. 4c) parameter no statistical differences were observed. The first three measurements for locomotor activity were taken on 21st, 35th and 49th day, Kruskal–Wallis one-way ANOVA—chi-square ($\chi^2$) = 12.53, d.f. = 2, $P < 0.001$; $\chi^2 = 11.01$, d.f. = 2, $P < 0.004$ and $\chi^2 = 11.01$, d.f. = 2, $P < 0.004$. Head dipp: $\chi^2 = 8.31$, d.f. = 2, $P < 0.01$, $\chi^2 = 9.13$, d.f. = 2, $P < 0.01$ and $\chi^2 = 12.30$, d.f. = 2, $P < 0.002$. Inactive sitting in second were $\chi^2 = 12.31$, d.f. = 2, $P < 0.002$, $\chi^2 = 9.21$, d.f. = 2, $P < 0.01$ and $\chi^2 = 12.00$, d.f. = 2, $P < 0.002$. Rearing does not show statistical difference in rotenone-lesioned and sham-lesioned animals. After treatment of L-DOPA no difference was observed among all the three groups.

### 3.2.3. Neurochemistry

The amount of DA was measured in the anterior striatum (Ant. Cpu) and posterior striatum (Post. Cpu) for each group of animals. In rotenone treated animals, DA concentrations were significantly depleted in both striatal regions.

There was no statistical difference between groups A and B. The amount of DA in rotenone treated and control animals in Ant. Cpu-DA $\chi^2 = 7.3801$, d.f. = 2, $P = 0.025$ and post. Cpu-DA $\chi^2 = 11.4152$, d.f. = 2, $P = 0.0033$ as shown in Fig. (5a and b).

## 4. Discussion

In this study, bilateral MFB lesion with rotenone resulted in a significant increase of the descent latency in catalepsy as compared to sham-lesioned rats and also in a significant decrease in locomotor activity, headdips and in increased inactive sitting. The strong hypokinetic behaviour in rotenone lesioned animals was caused by a 50% decrease of striatal dopamine. It has been reported that 50% neuronal loss in the SNpc leads to 80% loss in striatal DA content and causes loss of locomotor activity [3] and an increase in the descent latency of catalepsy [12].

The rotenone-induced behavioural deficiencies observed in this study suggest that dopaminergic neurons in the nigrostriatal pathway are sensitive to the action of rotenone. Previously two well-known models 1-methyl-4-phenyl-1; 2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) have most frequently been used as models of PD. Both toxins are taken up by the DAT and selectively destroy catacolaminergic neurons. Rotenone, is not DAT dependent, and is believed to destroy dopaminergic neurons due to complex I inhibition. In comparison to 6-OHDA, rotenone seems to be more potent which only requires a dose of 2.5 μg to achieve 80% loss of DA content in the striatum [6], compared to 6–8 μg of 6-OHDA to achieve

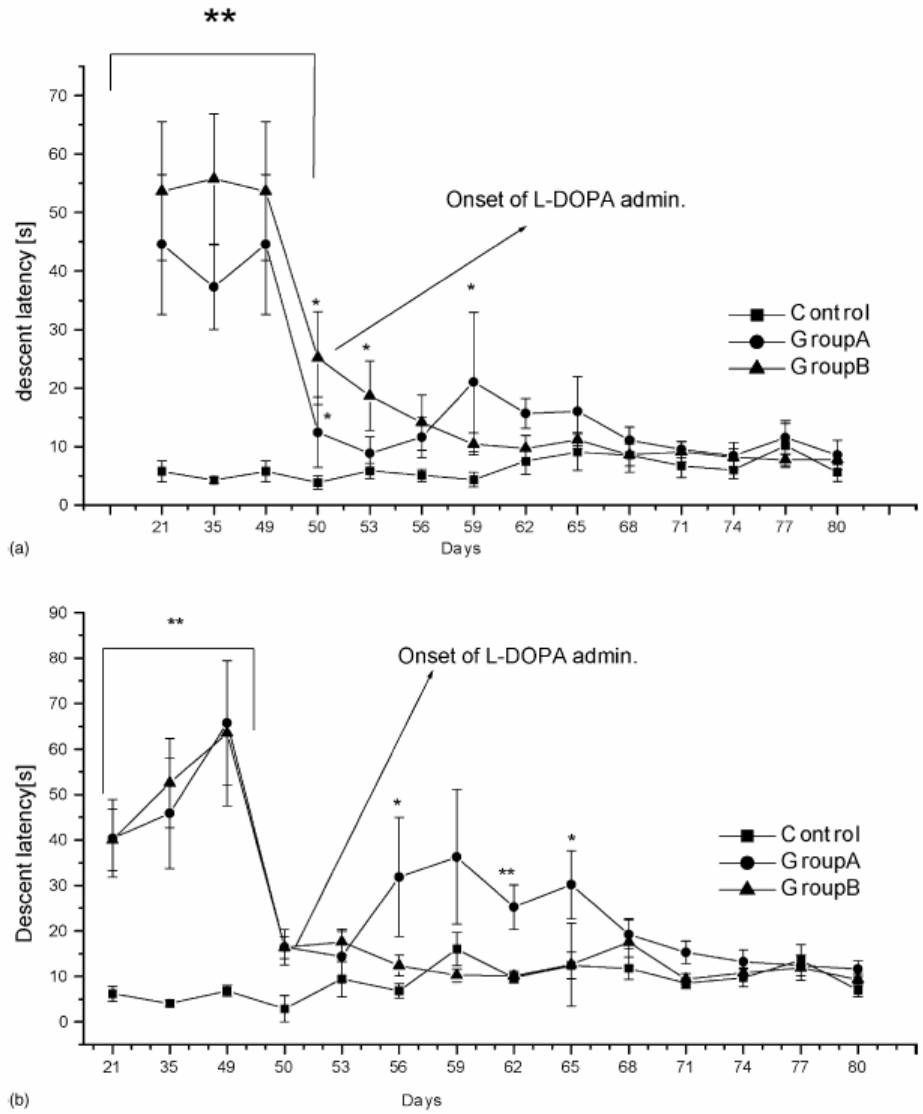


Fig. 3. Catalepsy tests both in bar (a) and grid (b) were performed after the lesion on 21st, 35th and 49th day on all three groups of animals, group A ($N = 6$) and group B ($N = 10$) both were rotenone-lesioned and control group ($N = 6$) was sham-lesioned. Subsequently on 50th day L-DOPA was administered on group A, 10 mg/kg; group B, 5 mg/kg; and control group 5 mg/kg every day for 31 days and further catalepsy were measured on every second consecutive days for each group. Mann–Whitney $U$-test: significant at $^{*}P < 0.05$; and significant at $^{**}P < 0.01$, compared to respective control.

the same. In many of the most recent works [4,7,13] on rotenone model histological data have been shown for the degeneration of dopaminergic neurons. In our study, quantitative neurochemical analysis in the anterior and posterior CPu suggests that, rotenone depletes DA content in both areas of brain tissues and causes the metabolite deficiency in the nigrostriatal dopaminergic neurons. Therefore, it seems that complex I inhibition within the nigrostriatal pathway is sufficient to produce neurotoxicity.

Because L-DOPA is one of the most effective and commonly used drugs in the treatment of Parkinsonian symptoms, we assessed whether L-DOPA administration

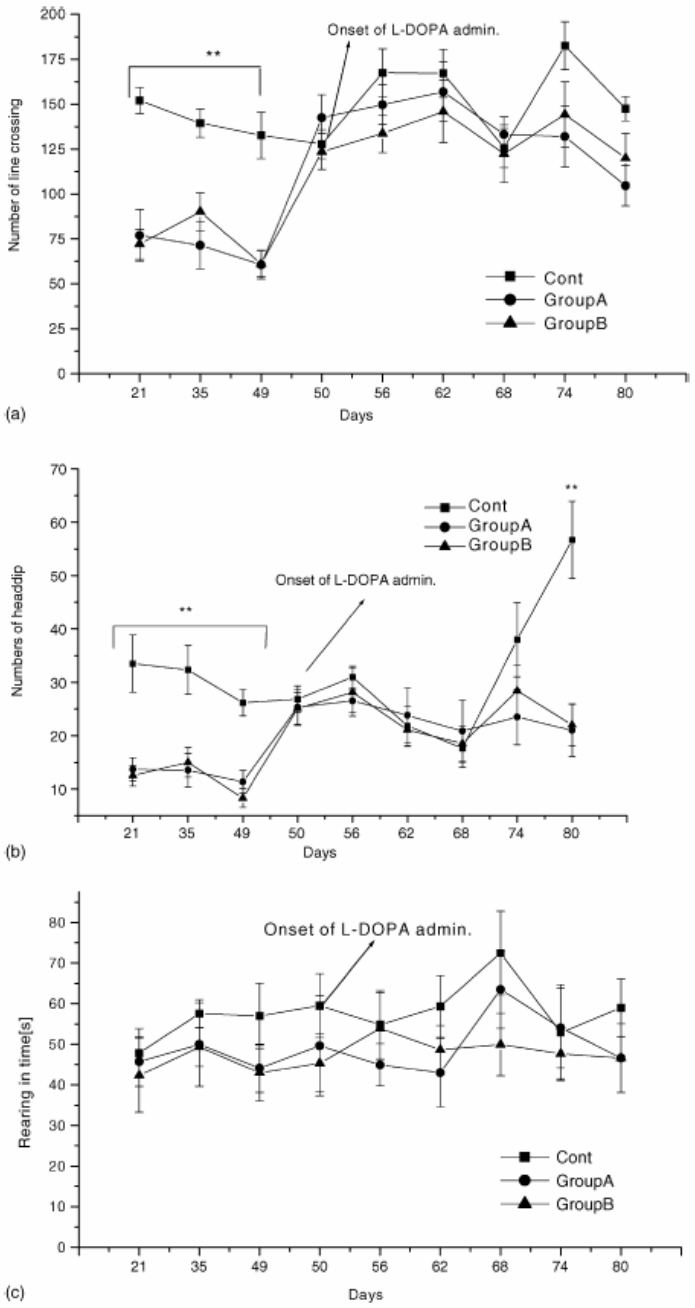


Fig. 4. (a–d). Open-field tests were performed after the lesion on 21st, 35th and 49th day in both rotenone lesioned animals groups A ($N = 6$) and group B ($N = 10$) and sham-lesioned animals as a control ($N = 6$). Subsequently on 50th day chronic L-DOPA was administered on group A, 10 mg/kg; group B, 5 mg/kg and control group, 5 mg/kg for 31 days and line crossing, headdips, and active sitting were measured on every 6th day in open-field. Mann–Whitney $U$-test: significant at $^{*}P < 0.05$; and significant at $^{**}P < 0.01$, compared to respective control.

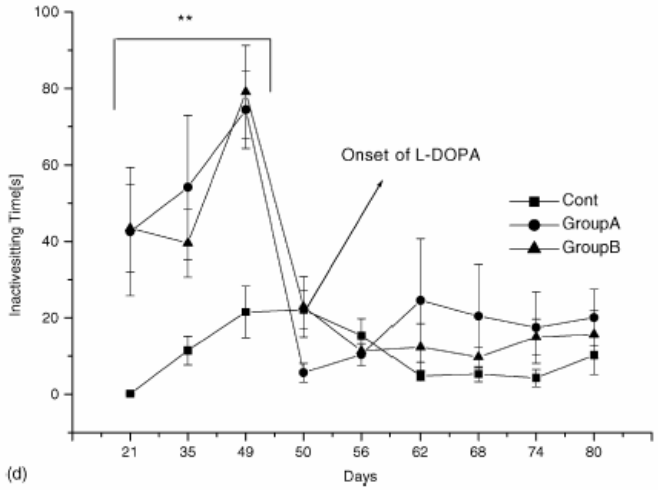


Fig. 4. (*Continued*).

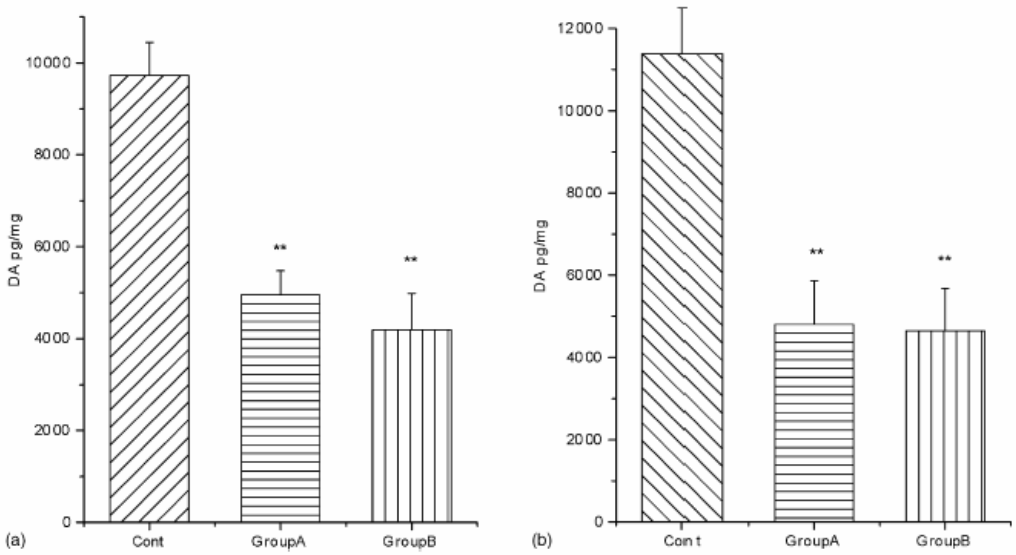


Fig. 5. (a and b). Anterior (Ant. Cpu) and posterior (Post. Cpu) striatal DA in rotenone lesioned group A ($N = 6$) and group B ($N = 6$) and sham-lesioned as a control group ($N = 6$). Mann–Whitney $U$-test: significant at $^{*}P < 0.05$; and significant at $^{**}P < 0.01$, compared to respective control.

reversed the rotenone-induced behavioural changes. L-DOPA reversed rotenone-induced parkinsonian symptoms and thus parallels clinical efficacy. The increase in the descent latency in catalepsy, inactive sitting and decrease in locomotor activity and headdips were reversed after chronic administration of L-DOPA.

## 5. Conclusion

Peripheral administration of rotenone has been proposed to represent a potential model of PD [2,13]. Here it is shown that local administration of rotenone is also able to destroy dopaminergic neurons as it is the cas wit 6-OH DA. In fact,

we found that local rotenone lesioned model rats are susceptible to L-DOPA. These data further support the use of rotenone as a neurotoxin in vivo.

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# Chronic administration of rotenone increases levels of nitric oxide and lipid peroxidation products in rat brain

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**Abstract**

The complex I inhibitor rotenone is a neurotoxin that has been proposed to induce Parkinson-like degeneration. As the mechanisms of rotenone toxicity are not fully understood, the present study addresses the question of whether rotenone induces NO production and lipid peroxidation-like products, that is, thiobarbituric acid reactive substances (TBARS). Rotenone at a dose of 1.5 mg $kg^{-1}$ ip was administered to rats daily for 10, 20, 30, and 60 days, and NO and TBARS were measured in the frontal cortex and in the striatum. On the 1st and 10th day, there were no increases in NO and TBARS levels, after 20 days, the NO and TBARS levels were increased in the striatum. After 30 and 60 days, NO and TBARS levels were increased in striatum and frontal cortex. Behaviorally, on days 30 and 60, the rats exhibited akinesia and rigidity in the catalepsy test. These results show that chronic administration of rotenone over a long period is capable of increasing NO and TBARS in the cortex and striatum and mimics Parkinson's disease (PD)-like behavioral symptoms that are akinesia and rigidity in rats.


*Keywords:* Rotenone; Neurotoxicity; Nitric oxide (NO); Thiobarbituric acid (TBARS)

## Introduction

Parkinson's disease (PD) is one of the most widespread neurodegenerative diseases. Pathologically, the hallmark of idiopathic PD is the loss of dopaminergic neurons in the substantia nigra (SN), leading to major clinical abnormalities that characterize this disease. But in the absence of nigral involvement, non-catecholaminergic neurons are also affected (Del Tredici et al., 2002).

The cause of neuronal loss in the brain is still unknown. A reduction of complex I activity has been demonstrated in the mitochondria of PD patients (Mizuno et al., 1989), and complex I inhibitors such as environmental toxins are involved in some cases of toxic, but not in the majority of sporadic PD. It is well known that the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPP) after its conversion to one of the most prominent mitochondrial complex I inhibitors 1-methyl-4-phenylpyridinium (MPP+) produces PD symptoms with severe motor symptoms, striatal dopamine depletion, and loss of tyrosine hydroxylase immunoreactivity in humans, monkeys, and various other species (Beal, 2001; Schmidt and Ferger, 2001). However, till now, there is no adequate experimental model of PD. Previous studies demonstrate that chronic systemic exposure to rotenone (an inhibitor of mitochondrial NADH dehydrogenase, a naturally occurring toxin and a commonly used pesticide) through jugular vein cannulation reproduces many features of PD in rats, including nigrostriatal dopaminergic degeneration and formation of alpha-synuclein-positive cytoplasmic inclusions in nigral neurons (Betarbet et al., 2000). Although novel and conceptually important, the rotenone model of PD suffered from being extremely labor-intensive. Findings concerning the effects of different doses of rotenone on the nigrostriatal system are controversial. The recent data demonstrate that these same features could be reproduced by chronic, systemic exposure to rotenone following implantation of subcutaneous osmotic pumps. Chronic subcutaneous exposure to low doses of rotenone (2–3 mg $kg^{-1}$ $day^{-1}$) caused highly selective nigrostriatal dopaminergic lesions (Sherer et al., 2003). In a drug regimen of 1.5–2.5

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mg $kg^{-1}$ $day^{-1}$ rotenone-treated animals showed a rather selective degeneration of dopamine (DA) in the striatum and prefrontal cortex (PFC), and the reduction of DA was dose dependent; further, the behavioral experiments showed dose-dependent catalepsy (Alam and Schmidt, 2002). A study has shown that rotenone produced selective damage in the striatum and the globus pallidus, but the substantia nigra was spared (Ferrante et al., 1977). In this, the doses used by Ferrante were similarly high (10–18 mg $kg^{-1}$ $day^{-1}$). It was further demonstrated that 2.2 mg $kg^{-1}$ $day^{-1}$ showed a significant loss of intrinsic striatal neurons without a significant loss of dopaminergic fibers (Hölinger et al., 2003). They both reported extra-nigral–striatal damage. However, it is known that beside nigrostriatal dopaminergic neurons, other neurons are also affected in PD (Braak et al., 2003). A loss of striatal cells was functionally not evident in our experiments because the expression of cataleptic behavior is critically dependent from striatal cells, lesioning the striatum abolishes catalepsy (Calderon et al., 1988). Data from this study indicate that in rats, rotenone is capable of causing degeneration of dopaminergic neurons and produces parkinsonian symptoms. However, while the behavioral effects of rotenone administration are well characterized, the mechanisms underlying rotenone action are unclear. It is hypothesized that PD is accompanied by several neurochemical, cellular, and molecular disturbances. These include enhanced generation of free radicals including excessive production of nitric oxide (NO); these, in turn are thought to result in the damage of dopaminergic neurons. There is a growing number of recent studies concerning the role of NO, a molecule which is regarded as a universal neuronal messenger in the central nervous system, in the pathophysiology of neurodegenerative diseases. Several findings demonstrate a marked increase in the number of reactive macrophages which generate NO and reactive oxygen species (ROS) from L-arginine in parkinsonian substantia nigra (Irvani et al., 2002). NO and its derivatives may cause the death of mesencephalic dopaminergic neurons. The aim of this project is to study the mechanisms underlying oxidative damage of the various brain areas of rats produced by rotenone and to investigate a possible role of NO and lipid peroxydation (LPO) processes during chronic rotenone administration.

## Materials and methods

### *Animals*

Experiments were carried out on 60 male Sprague–Dawley rats (Charles River, Sulzfeld, Germany). All experiments were performed in compliance with the German Animal Protection Law and have been approved by the local committee (Tierschutzkomission, Regierungsprasidium, Tuebingen, ZP 5/01). At the beginning of the experiments, the rats weighed 220–240 g. They were kept in cages in a room at a constant temperature of 20 °C in 12-h light–dark cycle (light on at 7.00 am). The rats obtained dry food (Altromin, 1324) 12 g $day^{-1}$ $rat^{-1}$, and tap water was available ad libitum. All experiments were conducted during the light cycle in awake freely moving rats.

### *Chemical*

Rotenone and all substances for NO determination were purchased from Sigma (St. Louis, MO). The sterile natural oil (middle chain triglycerides, MCT; Miglyol 812) was obtained from clinical pharmacy, University Tuebingen.

### *Treatment*

Rotenone emulsified in natural oil at 1.5 mg $kg^{-1}$ was given intraperitoneally (ip) once a day at 1 ml $kg^{-1}$ during 60 days. Oil was injected as vehicle to the control rats (1 ml $kg^{-1}$). For the determination of NO and thiobarbituric acid-reactive substances (TBARS), two groups of rats were used, namely vehicle ($N = 6$) and rotenone ($N = 6$)-treated group. Control groups received oil for 1, 10, 20, 30, and 60 days, and rats were treated with rotenone for 1, 10, 20, 30, and 60 days.

### *NO content measurement*

For NO determination, diethyldithiocarbamate (DETC, Sigma; 500 mg $kg^{-1}$, ip) and a mixture of FeSO4 (37.5 mg $kg^{-1}$, sc) and sodium citrate (165 mg $kg^{-1}$, sc) were simultaneously injected 1 day after rotenone injection. DETC penetrates the blood–brain barrier and forms a complex with intracellular non-haem iron. This scavenger traps NO in the tissue, thereby generating a paramagnetic monoitrosyl-iron DETC complex (Obolenskaya et al., 1994). Animals were decapitated 30 min after DETC injection. Cerebral cortices (FC), prefrontal cortex (PFC), striatum (CPu), and nucleus accumbences (NAc) were quickly removed and immediately frozen in liquid nitrogen. The electron paramagnetic resonance (EPR) spectra were recorded at 77 K using a Brucker ESR 300E spectrometer at a frequency of 9.33 kHz, hf-modulation frequency 0.5 mT, microwave power 20 mW and time constant 0.05 s. The concentration of trapped NO was calculated from the intensity of the third ultrafine splitting line of the resonance at $g\perp = 2.035$ of the $O\text{-}Fe(DETC)^2$ complex (Mikoyan et al., 1994). DETC was dissolved in sterile water. The mixture of $FeSO^4$ and sodium citrate was prepared immediately before using.

### *Measurement of lipid peroxidation secondary products*

Determination of lipid peroxidation in the brain tissue was performed by measuring thiobarbituric acid reactive

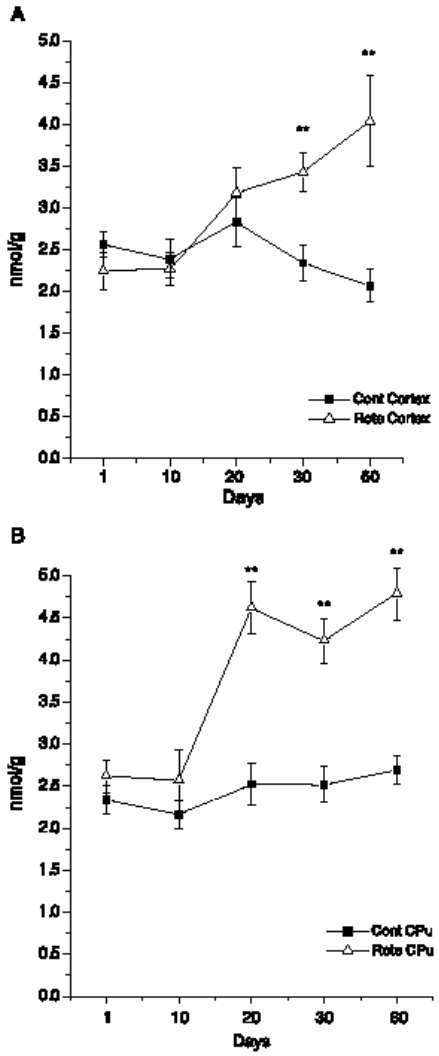


Fig. 1. Levels of NO in FC (A) and CPu (B) tissues. Control group $N = 6$, compared with rotenone-treated animals (1.5 mg kg$^{-1}$) $N = 6$, on every 10th consecutive days till the 60th day. Values are expressed as mean ± SEM. Mann–Whitney $U$ test; significant at $^{*}P < 0.05$, $^{**}P < 0.01$, compared to respective control.

substances (TBARS). Briefly, 10% (w/v) tissue homogenate was mixed with sodium dodecyl sulfate (Sigma), acetate buffer (pH 3.5), and aqueous solution of thiobarituric acid (Sigma). After heating at 95°C for 60 min, the red pigment was extracted with $n$-butanol–pyridine mixture and determined by the absorbency at 532 nm (Ohkawa et al., 1979).

*Behavior*

After 30 and 60 days of treatment with rotenone or vehicle (oil), six control and six rotenone-treated rats were tested for catalepsy.

*Catalepsy*

Each rat was hung by all four paws on a vertical grid (25.5 cm width and 44 cm high with a space of 1 cm between each wire) and a stopwatch was started as soon as the rat held onto the grid. Just as they moved their paws, the stopwatch was stopped and the time noted as descent latency.

The second part of the test was the bar test. The rats were placed with both front paws on a bar which was 9 cm above the surface in a half rearing position. Here they were timed with the stopwatch. When the animals removed one paw from the bar the stopwatch was stopped and the time noted. The maximum descent latency for the grid and bar was fixed at 180 s.

*Statistical analysis*

Data are expressed as means ± SEM. Statistical analyses were carried out using the GB-STAT V5.4 software (Dynamic Microsystem, Inc., Silver Spring, MD). The data from behavior test, nitric oxide content, and lipid peroxidation assay were evaluated using nonparametric analysis (multifactorial Kruskal–Wallis One-Way ANOVA followed by Mann–Whitney U Test). $^{*}P < 0.05$ and $^{**}P < 0.01$ were taken as levels of statistical significance.

## Results

Repeated injections of rotenone (1.5 mg kg$^{-1}$ injected every day, ip, 30 and 60 days) led to a pronounced and permanent increase in NO generation in all studied brain areas. The signal which reflects NO generation was greatly increased after 30 and 60 days of rotenone administration in both FC and CPu regions (Fig. 1). In the control animals, the basal level of NO in CPu was 2.16–2.69 nmol g$^{-1}$. The NO level in all studied brain structures of rats after the first rotenone injection was not different from that of the control group. NO formation was increased after 20 days of rotenone administration in the CPu. The PFC and NAc

Table 1
The prefrontal cortex (PFC) and nucleus accumbens (NAc) of six control and six rotenone-treated animals were pooled and nitric oxide (NO) generation was measured

| After the treatment | Control oil treated (NO, nmol g$^{-1}$) | | Rotenone treated (NO, nmol g$^{-1}$) | |
|---|---|---|---|---|
| | PFC | NAc | PFC | NAc |
| Day 30 | 1.95 | 2.65 | 3.82 | 5.42 |
| Day 60 | 2.45 | 2.16 | 4.25 | 6.22 |

regions of six rats were pooled separately for the estimation of NO. The NO level reached its maximum in dopaminergic structures, prefrontal cortex (PFC), and NAc 60 days after administration of rotenone (Table 1). In FC, the level of NO

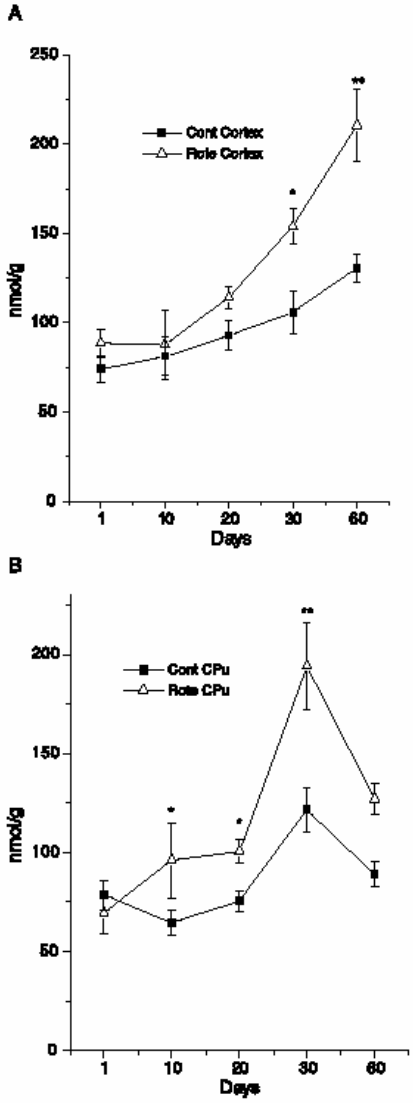


Fig. 2. Concentration of TBARS in FC (A) and CPu (B) tissues. Control group $N = 6$, compared with rotenone-treated animals (1.5 mg kg$^{-1}$) $N = 6$, on every 10th consecutive day till the 60th day. Values are expressed as mean ± SEM. Mann–Whitney $U$ test; significant at $^{*}P < 0.05$, $^{**}P < 0.01$, compared to respective control.

Table 2
The prefrontal cortex (PFC) and nucleus accumbens (NAc) of six control and six rotenone treated animals were pooled and TBARS was measured

| After the treatment | Control oil treated (TBARS, nmol g$^{-1}$) | | Rotenone treated (TBARS, nmol g$^{-1}$) | |
|---|---|---|---|---|
| | PFC | NAc | PFC | NAc |
| Day 1 | 56 | 67 | 77 | 81 |
| Day 20 | 109 | 85 | 97 | 125 |

was increased on days 30 and 60. Kruskal–Wallis one-way ANOVA–$\chi^2 = 5.7692$, df = 1, $P < 0.016$ and $\chi^2 = 5.5$, df = 1, $P < 0.019$ but in the CPu the level of NO increased on days 20, 30, and 60. Kruskal–Wallis one-way ANOVA–$\chi^2 = 7.4103$, df = 1, $P < 0.0065$, $\chi^2 = 8.307$, df = 1, $P < 0.0039$ and $\chi^2 = 6.54$, df = 1, $P < 0.010$.

The basal levels of TBARS in the FC and CPu of Sprague–Dawley rats were 56–83 nmol g$^{-1}$ (Fig. 2). An elevation of TBARS formation was observed in FC on days 30 and 60. But in Cpu, the elevation of TBARS had taken place on days 10, 20, 30, and 60 as compared to control. Following one injection of rotenone, the levels of TBARS in all studied brain areas were found to be indistinguishable from control animals. PFC and NAc of six rats were pooled separately and TBARS were measured. On days 1 and 20, there were slight increases of TBARS in the PFC and Nac, but on days 30 and 60, the amount of TBARS was two times greater as compared to control in this region (Table 2).

Catalepsy was tested by measuring the descent latency after 30 and 60 days using a bar and grid test. Rotenone-treated animals showed a prolonged descent latency as compared to control animals. An increase of descent latency from days 30 to 60 was not observed (Fig. 3).

The results show differences when compared with the control group on day 30 in the bar and grid, respectively. Kruskal–Wallis one-way ANOVA–$\chi^2 = 13.23$, df = 1, $P < 0.0003$ and $\chi^2 = 11.8008$, df = 1, $P < 0.0006$. On day 60 in bar and grid—$\chi^2 = 8.3077$, df = 1, $P < 0.0039$ and $\chi^2 = 8.300$, df = 1, $P < 0.003$.

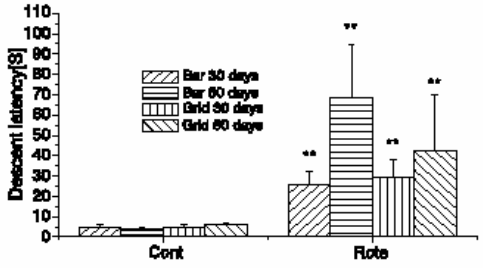


Fig. 3. Catalepsy test of rotenone and vehicle-treated animals on the horizontal bar and vertical grid. On day 30 vehicle-treated rats ($N = 12$) and rotenone-treated rats ($N = 12$). On day 60 vehicle-treated rats ($N = 6$) and rotenone-treated rats ($N = 6$). Mann–Whitney U test; significant at $^{*}P < 0.05$, $^{**}P < 0.01$, compared to respective control.

## Discussion

The findings indicate that rotenone at the low dose of 1.5 mg $kg^{-1}$ enhances NO generation in all studied brain areas after both 30 and 60 days of administration and in the NAc and CPu already after 20 days. Also, TBARS content is significantly increased in the studied brain areas following 30 and 60 days of treatment with rotenone. For methodological reasons, it was not possible determine NO and TBARS levels in the substantia nigra but in terminal fields of DA (NAc and CPu). In addition to the biochemical alterations, neurological disturbances occurred 20–60 days after rotenone administration.

There may be a feed-forward cycle wherein the mitochondrial dysfunction causes *N*-methyl-D-aspartate (NMDA) receptor activation, which leads to further mitochondrial impairment (Greenamyre et al., 1999). It has been shown that metabolic impairment depletes ATP, depresses Na+/K(+)-ATPase activity, and causes graded neuronal depolarization. This relieves the voltage-dependent Mg2+ block of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor, which is highly permeable to Ca2+. Consequently, innocuous levels of glutamate become lethal via secondary excitotoxicity. Moreover, the facilitation of NMDA receptor function leads to further mitochondrial dysfunction. Activation of excitatory amino acid receptors leads to activation of neuronal NO synthase by an increase in intracellular calcium concentrations. NO may inhibit key enzymes of energy metabolism, damage DNA, deplete intracellular glutathione, and react with superoxide to form peroxynitrite (Schulz et al., 1997).

It has been shown that besides its role as a mediator of several physiological functions, NO appears to be a neurotoxin under conditions of excessive production, which suggests a role for NO in neurodegenerative diseases (Prezedborski et al., 1996; Schulz et al., 1995). NO, generated by the inducible form of nitric oxide synthase (iNOS) in glial cells (Liberatore et al., 1999) or the neuronal form (nNOS) (Castagnoli et al., 1999), can play a key role in the cascade of events leading to the degeneration of neurons. NO-induced catecholamine oxidation could also explain neurotoxicity in PD by the inherent production of reactive oxygen and nitrogen species (Daveu et al., 1997).

NO is cytotoxic, partly due to its effects on the mitochondria. The targets include iron–sulfur proteins. It appears that species derived from NO such as peroxynitrite may be responsible. Addition of peroxynitrite to the mitochondria leads to depletion of the EPR-detectable iron–sulfur clusters (Cammack et al., 1998). In brains from victims of PD, a nitrosyl species, identified as nitrosyl hemoglobin, has been observed in the substantia nigra. This is an indication for the involvement of NO or a derived species in the damage to this structure (Cammack et al., 1998).

NO levels were measured by the EPR method, thus providing the first direct measurement of this reactive molecule in the brain tissue following chronic rotenone administration. Our results demonstrate an enhancement of NO level in the CPu and NAc after 20 days of treatment with rotenone while the NO level was not elevated yet in the FC. This data can be interpreted that dopaminergic neurons may be intrinsically susceptible to oxidative damage as compared to other neurons. The results of our study are consistent with our previous data using this same technique and demonstrating significant increases in NO generation in the cortex and CPu of rats injected with neurotoxic dosage of amphetamine (Bashkatova et al., 1999).

Although NO may act independently, it may also act cooperatively with other reactive oxygen species (ROS) to induce neuronal damage. Repeatedly, it has been suggested that increased formation of free radicals are implicated in the pathogenesis of PD, and dopaminergic neurons may be intrinsically susceptible to oxidative damage (Nakamura et al., 2001). Studies (Sherer et al., 2002) indicate that chronic low-grade complex I inhibition caused by rotenone exposure induces accumulation and aggregation of alpha-synuclein and ubiquitin, progressive oxidative damage, and caspase-dependent cells death, mechanisms that may be central to PD pathogenesis. It was recently demonstrated that a moderate level of complex I inhibition characteristic for PD leads to significant ROS formation (Sipos et al., 2003). On the other hand, it was shown that dopaminergic neurons have intrinsically lower levels of superoxide than nondopaminergic neurons (Nakamura et al., 2001). Dopaminergic neurons are also likely to face a higher baseline level of oxidative stress due to ROS formed from the degradation of dopamine. Thus, monoamine oxidase metabolizes dopamine to $H_2O_2$, which may be detoxified primarily by the tyrosine hydroxylase (TH) through partial uncoupling of the hydroxylation reaction (Haavik et al., 1977). Therefore, in order for normal dopaminergic neurons to survive, they may require more intrinsically increased antioxidant capacity than other neurons.

The current study also sought to determine the levels of TBARS during chronic rotenone administration. Our data concerning the increase in the level of TBARS in the studied brain areas of rats following chronic rotenone administration corroborate the results of earlier studies investigating other models of PD (Hung and Lee, 1998; Ogawa et al., 1994). As far as we know, there are no data concerning the effect of chronic rotenone administration on the concentration of LPO products in the brain. However, it is worth noting that we did not observe a direct correlation between the increase of NO generation and TBARS content in the rotenone model; thus suggesting that the rotenone-induced elevation in lipid-like oxidative processes is not directly due to the activation of specific NO pathways.

Parkinsonian syndromes in rats, that is, akinesia and rigidity (catalepsy), are thought to represent PD in the

animal model (Schmidt and Kretschmer, 1997). In our study, catalepsy tests revealed an increase in cataleptic behavior in rotenone-treated animals as compared to the control group already after 30 days of administration, which is in accordance with our previous study (Alam and Schmidt, 2002). Thus, the behavioral data point to decreased dopamine activity.

In conclusion, rotenone (1.5 mg $kg^{-1}$ once a day for 60 days) changes motor activity, depletes dopamine, and enhances catalepsy (Alam and Schmidt, 2002). It might be suggested that oxidative stress associated with partial inhibition of complex I is a crucial factor for neurodegeneration occurring under rotenone administration and may be an important factor in neuronal cell death observed in neurodegenerative disorders such as PD. The results of our study provide the first direct evidence that there is no acute toxic effect of rotenone, only chronic administration of rotenone increases NO tissue level. The findings also show that treatment with rotenone during 30 and 60 days increases TBARS levels which points to toxicity. These results may contribute to the understanding of the mechanism of pesticide action in the pathogenesis of PD, in particular to a role of oxidative damage in the pathophysiology of PD.

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Research report

# L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone-treated rats

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**Abstract**

Peripherally and locally administered rotenone (an inhibitor of mitochondrial complex I) has been proposed as a model of Parkinson's disease (PD) as it induces nigrostriatal degeneration associated with α-synuclein inclusions. If rotenone-induced symptoms represent a model of PD, than they should be counteracted by L-DOPA. To answer this question, rats were treated with rotenone 2.5 mg/kg over 48 days. Behavioural data showed a strong increase in catalepsy, a decrease in locomotor activity and biochemical data showed a significant depletion of dopamine levels in the striatum (Cpu) and substantia nigra in rotenone treated animals compared to vehicle. To examine the effectiveness of L-DOPA in reversing the motor deficit in rats, a dose of L-DOPA (10 mg/kg) in combination with the peripheral amino acid decarboxylase inhibitor benserazide were daily administrated intraperitonially for a period of 10 days in the rotenone-treated rats. This treatment counteracted catalepsy and increased locomotor activity and number of rearings but decreased inactive sitting. In this animal model (rotenone model), catalepsy tests and motor activities showed that the clinically used anti-parkinsonian drug L-DOPA substitutes rotenone-induced dopamine (DA) deficiency.


*Keywords:* Rotenone; Complex I; Parkinson's disease; L-DOPA; Substantia nigra; Dopamine

## 1. Introduction

Parkinson's disease (PD) is a multifactorial, late-onset, neurodegenerative disease with both genetic and acquired etiologies. It is characterised by the degeneration of dopaminergic neurons of the substantia nigra and a decrease of dopamine (DA) in the striatum. Substantia nigra is not the only site in the brain which under goes a neurodegenerative process in PD but also extranigral components of the motor system, as well as numerous limbic structures and autonomic centres also undergo serious damage [5,4]. There is growing evidence that mitochondrial damage, particularly to the respiratory chain of complex I (NADH/ubiquinone oxido reductase) resulting in oxidative stress, underlies the pathology of PD. A 30% decrease in complex I activity has been detected in post-mortem brain tissue from PD patients [18] and evidence of markedly decreased complex I subunits has also been reported [14]. The extent of damage to complex I and other key enzymes of energy metabolism that trigger the onset of neurodegeneration and the characteristic symptoms of PD is not well established. However, it is pertinent to note that complex I activity in the brain is the highest of any tissue, such that it requires only a modest degree of inhibition of oxidative phoshorylation to cause neuronal damage [7]. Two well-known models 1-mythyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxy-dopamine (6-OHDA) have most frequently been used as models of PD and both toxin selectively destroy catecolamine neurons specifically dopaminergic neurons due to their carrier-dependent transport. Therefore the vulnaribility of dopaminergic neurons for both these toxin is due to dopamine transporter (DAT). However, rotenone is capable of destroying dopaminergic neurons without DAT. Further, the mode of the action of both toxins is not similar to rotenone. Rotenone toxicity is specifically related to complex I inhibiton, but 6-OHDA and 1-methyl-4-phenylpyridinium ($MPP^+$) toxicity may not primarily be due to complex I inhibition. $MPP^+$ typically produces $10^{-4}$ to $10^{-6}$ fold less complex I inhibition than rotenone [9,15,17]. In vivo, the conversion of MPTP to $MPP^+$ may represent a significant source of ROS including $H_2O_2$ and superoxide anions [13]. This implies that

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$MPP^+$-induced ROS formation is not of mitochondrial in origin but a consequence of intracellular DA oxidation. In contrast to in vitro studies $MPP^+$ kills Rho 0 cells that lack functional mitochondria at doses equivalent to those that kill normal cells [12]. Recently it has been shown that another complex inhibitor, rotenone, also damages dopaminergic neurons but additionally rotenone induces the formation of α-synuclein inclusion bodies [3,11,19]. The toxicity of rotenone in the nigrostriatal dopaminergic system depends on the drug regimen because acute and high dose is toxic to virtually any cell of an organism [8,20] but, a low dose given chronically can be tolerated by most cells except the dopaminergic neurons. This treatment depletes dopamine in the striatum and induces parkinsonian-like symptoms [1,19].

Thus, it is documented that rotenone mimics the neuronal damage and the resulting behaviour occurring in PD, but it has not been proven so far whether or not rotenone treated animals respond to an established anti-parkinsonian drug.

Therefore, in the present study rats were treated with rotenone in order to induce parkinsonian symptomatology. Thereafter they were treated with L-DOPA. The observed reversal of cataleptic and hypolocomotor behaviour supports the view, that rotenone-induced neurodegeneration represent a valid animal model for PD.

## 2. Materials and methods

### 2.1. Animals

Forty adult male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) aged 7 weeks were chosen for the experiment. At the beginning of the experiment, the rats weighed 240–250 g. Animals were housed in cages under a 12/12 (h) light/dark cycle in a room maintained at constant temperature of 22 °C and 50–60% humidity with food and water available ad libitum.

All treatments used in this experiment adhere to the international ethical standards and the German Animal-Protection Law and have been approved by the local animal care committee (Tierschutzkommission, Regierungspräsidium Tübingen, Z P 2/03).

### 2.2. Drugs

L-DOPA methyl ester (Sigma-Aldrich, Germany) was dissolved in saline containing 0.2% ascorbic acid. Benserazide hydrochloride (Sigma Chemical Co.; one-quarter dose of L-DOPA) a peripheral DOPA-decarboxylase inhibitor was dissolved in physiological saline. The injection volume was 1 ml/kg body weight for all the drugs. The rotenone, obtained from Sigma (St. Louis, MO, USA) was dissolved in sterile natural oil (middle chain triglycerides, MCT; Miglyol 812, clinical pharmacy, University of Tuebingen) and animals received 2.5 mg/kg rotenone per day for up to 48 days. Control animals received vehicle, oil at 1 ml/kg.

### 2.3. Animals grouping and experimental procedures

The first behavioural tests were performed after 30 days of rotenone treatment and on Day 48 after the last injection of rotenone. On Day 49 the rotenone treated group was divided into two groups, one of the groups received saline and another L-DOPA combined with benserazide hydrochloride (i.p.) and the same was done with the control group. One of the control groups received saline and another control group received L-DOPA. The behavioural assessment was done 30 min after the L-DOPA and saline administration.

### 2.4. Behavioural testing

The tests were carried out between 0900 and 1400 h always in the same context and at standard conditions. Catalepsy test and activity box were chosen for the assessment of the behavioural changes. Starting on the first day of L-DOPA treatment, catalepsy and locomotor activity were again tested. Catalepsy tests were performed every third day, and acivity box tests took place on every fifth day after challenging with anti-parkinsonian drug L-DOPA.

### 2.5. Catalepsy

The catalepsy test serves to qualify and further quantify the specific parkinsonian symptoms in rats. Catalepsy can be seen as, bradykinesia (slower movement), akinesia (inability to move) and rigidity (specific muscle tension). All these symptoms, together with tremor (shaking) are essential symptoms of PD. Each rat was hung by all four paws on a vertical grid (25.5 cm width and 44 cm high with a space of 1 cm between each wire) and a stopwatch was started as soon as the rat held onto the grid. Just as they moved their paws or showed first movement the stopwatch was stopped and the time noted as descent latency.

The second part of the test was the bar test. The rats were placed with both front paws on a bar which was 9 cm above, and parallel from the base. The rats were placed with both front paws on the bar in a half rearing position. Here they were timed with the stopwatch. When the animals removed one paw from the bar the stopwatch was stopped and the time noted. The maximum descent latency for grid and bar was fixed at 180 s.

### 2.6. Activity assessment

After the rotenone treatment over a period of 48 days, the first two measurements took place on Days 30 and 48 without L-DOPA challenging. On Day 49 all animals were chronically treated with L-DOPA for 10 days and further activity was assessed on the first on set of L-DOPA and

the next two measurements took place every 5th day after treatment of L-DOPA (Fig. 3).

Automated locomotor activity boxes (TSE moti; Technical & Scientific Equipment System, Bad Homborg, Germany) were used to quantify locomotor activity. Each animal was placed into the activity box, a square plastic box measuring (47 cm × 47 cm × 44 cm) spontaneous locomotor activities parameter such as total distance moved, numbers of rearing and the time of inactive sitting were monitored for 10 min for each rat in the box that automatically recorded by interruptions of two orthogonal light beams (3.5 and 13.0 cm above the floor of activity box) which were connected to automatic software (MOTI 4 software).

Three parameters were measured in this experiment:

distance moved (in meters),
rearings (number of rears),
inactive sitting (time in seconds).

### 2.7. Neurochemistry

#### 2.7.1. Tissue preparation

Two weeks after the behaviour test, animals were sacrificed and brains were taken out rapidly within 25–40 s and put into ice-chilled 0.9% NaCl solution for 1 min. The brains were dissected into coronal sections [10] and tissue samples of anterior, posterior striatum and substantia nigra were taken bilaterally and immediately weighed and stored in liquid nitrogen until assay.

Frozen tissue was homogenised for 20 s using a motorised pestle in 500 μl of ice-cold mobile phase (50 mM sodium dihydrogen phosphate dihydrate, 0.13 mM disodium EDTA, 0.27 mM 1-octansulphonic acid sodium monohydrate, 6.2% methanol, 3.5% acetonitril and finally 162 μl of 10% phosphoric acid were added to obtain PH 4.48) containing known amount of 3,4-dihyroxybenzylamine (DHBA) and 5 hydroxyindole (5-HI) as internal standards.

Dopamine (DA), dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were quantified by reverse-phase HPLC coupled to an electrochemical detector (EC). The filtered supernatant of samples and an external standard were introduced into the autosampler carrousel (CMA 200, CMA Microdialysis, Solana, Sweden) with flow rate 0.8 ml/min (Bischhoff pump, Germany) using a C 18 column (Prontosil, 33 mm × 4.0 mm, pore diameter, 3.0 μm). The coulometric detector (ESA Clouchem II Multi-Electrode Detector, Model 5200/5200A, Bedford, MA, USA) consisted of an analytical cell (Model 5010) and a guard cell (Model 5020). The potential applied in the present experiment were +20 mV at electrode 1 and +320 mV at electrode 2. The chromatograms were analysed with the aids of a chromatographic data system (AXXiom 727, Sykam, Gilching, Germany) and peak areas of DA, 5-HT and it metabolites were quantified using a standard curve generated by determining ratio between the known amounts of amine and a constant amount of internal standards (DHBA and 5-HI) and represented as pg/mg of tissue.

### 2.8. Statistical analysis

All data were expressed as mean ± standard error of the mean (mean ± S.E.M.). Statistical analysis was carried out using the GB–Stat V5.4 software (Dynamic Microsystems, Inc., Silver Spring, MD, USA). The catalepsy data were evaluated using non-parametric analysis. Within a group the data were submitted to the non-parametric Mann–Witney *U*-test. The different groups were compared by the non-parametric (multifactorial Kruskal–Wallis one-way ANOVA). The behavioural data without L-DOPA treatment in the activity boxes requiring group comparison on different time periods (Days 30 and 48) were analysed with two factors, ANOVA and Newman–Keuls test but after the onset of L-DOPA treatment, the overall effect of L-DOPA treatment on behaviour in the activity box were analysed by three-way ANOVA followed by Newman–Keuls test. The neurochemical data were analysed by one-way ANOVA followed by Newman–Keuls test. A $P$-value $< 0.05$* and $P < 0.01$** was taken as levels of statistical significance.

## 3. Results

### 3.1. Body weight assessment

Rats in the dose 2.5 mg per kg per day rotenone-treated groups showed a minimum treatment-related effects in their body weights as compared to vehicle (oil 1 ml per kg per day) treated control group. However, concerning the qualitative observation in Sprague–Dawley rats in our laboratory we have observed that they required minimum 25–30 days to exhibit their hypokinetic movement and less intake of food as compared to control (vehicle treated). We have also measured the body weight on every seven consecutive days. All the surviving animals till the last administration of rotenone lost 8% as compared to vehicle-treated animals. In the first week the average weight loss was approximately 4–5% in all rotenone-treated rats which further rose to 8–9% till the end of the experiment.

### 3.2. Rotenone-induced catalepsy

Chronic administration of rotenone resulted in a significantly prolonged descent latency as compared to the vehicle-treated control group both in the bar and grid test (Fig. 1a and b) on Days 30 and 48. The results also revealed highly significant differences when compared with the control group by Kruskal–Wallis ANOVA followed by Mann–Witney *U*-test test and also differences between the Days 30 and 48 in rotenone-treated animals. The two measurements after 30 and 48 days on the bar and grid were: Kruskal–Wallis one-way ANOVA, bar—$\chi^2 = 49.0518$,

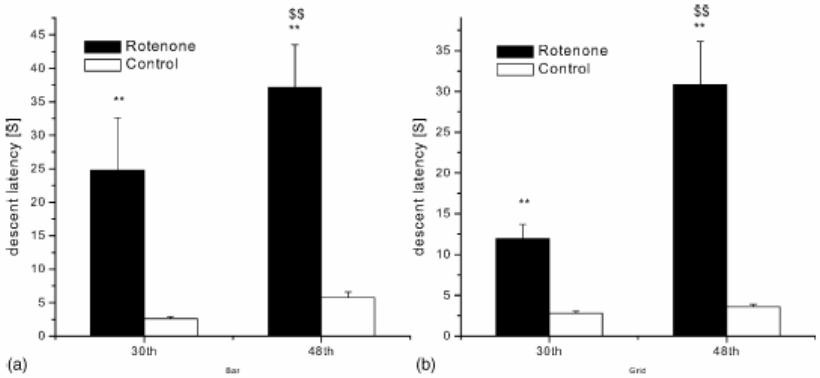


Fig. 1. On the Days 30 and 48 catalepsy tests on (a) bar and (b) grid were carried out on rotenone-treated ($N = 12$) and vehicle-treated ($N = 20$) groups of animals. Values are shown as mean ± S.E.M. $P$ represent statistical analysis: *significant at $P < 0.05$; and **significant at $P < 0.01$ (compared to respective control within the group, Kruskal–Wallis ANOVA followed by Mann–Whitney $U$-test). $P < 0.05$ ($) and $P < 0.01$ ($$) in comparison between two different time periods, on Days 30 and 48.

d.f. = 3, $P < 0.0001$; and grid—$\chi^2 = 45.4115$, d.f. = 3, $P < 0.0001$, respectively.

### 3.3. Rotenone-induced locomotor activity

In Fig. 2a–c the decrease in locomotor activity (total distance travelled), rearing and increase in the inactive sitting induced by chronic rotenone treatment were significantly different compared to the control group (vehicle-treated animals). A significant effect of treatment was found in both groups in both sessions (on Days 30 and 48) of measurements. The total distance travelled in 10 min were $F(1, 30) = 77.54$, $P < 0.0001$, number of rearing $F(1, 30) = 43.586$, $P < 0.001$ and inactive sitting $F(1, 30) = 186.83$, $P < 0.0001$.

### 3.4. Effect of L-DOPA on behavioural changes

#### 3.4.1. Catalepsy

On the first day of treatment with L-DOPA the descent latency was reduced approximately by 40% showing a significant difference to rotenone-induced animals with saline in bar tests, on Days 52 and 55 the second and third session of measurements for the descent latency reduced to 60% compared to pre-rotenone-treated animal with saline treatment. The last assessment of L-DOPA treatment showed a significant reversal in the descent latency (Fig. 3a and b) in both bar and grid tests and statistically there were no differences among all the three groups on Day 58. L-DOPA administration on Day 49 showed a significant difference compared to Days 52, 55 and 58, but in the

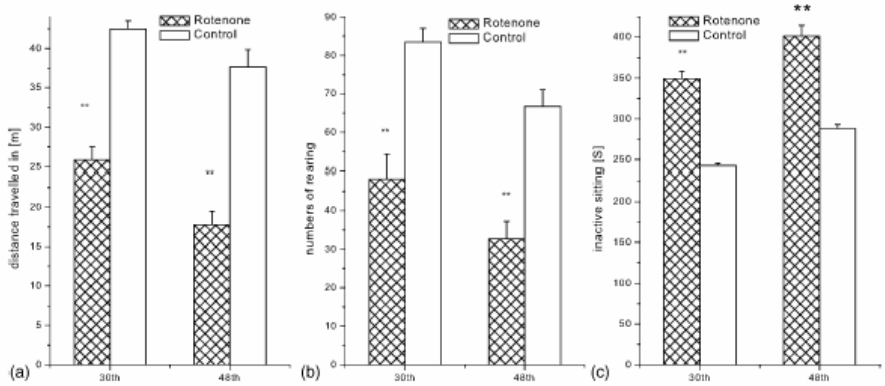


Fig. 2. (a–c) Activity box tests were carried out on Days 30 and 48 in both rotenone-treated animals group ($N = 12$) and vehicle-treated control group ($N = 20$) and locomotor activity, rearing and inactive sitting were measured. Values are shown as mean ± S.E.M.: *significant at $P < 0.05$; and **significant at $P < 0.01$ (compared to respective vehicle, two-way ANOVA followed by Newman–Kleus test).

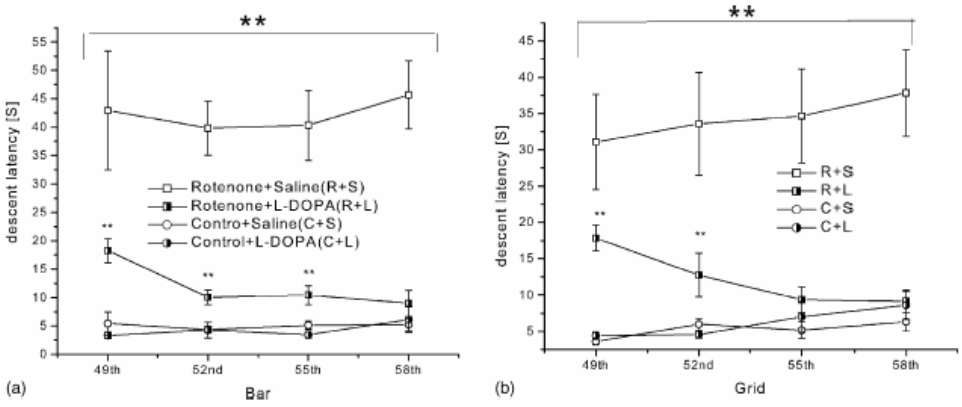


Fig. 3. Catalepsy tests both in bar (a) and grid (b) were performed after Day 49 on all four groups of animals, rotenone-treated animals with saline treatment ($N = 6$) and with L-DOPA treatment (10 mg/kg) ($N = 6$) and further control group ($N = 10$) with saline and L-DOPA (10 mg/kg) ($N = 10$) for 10 days. Catalepsy were measured on every third consecutive day for each group. Mann–Whitney $U$-test: *significant at $P < 0.05$; and **significant at $P < 0.01$ (compared to respective control).

session between Days 52 and 58 no significant change was observed.

### 3.4.2. Activity box

In Fig. 4a–c the decrease in locomotor activity (total distance travelled), rearing and inactive sitting time were significantly reversed after onset of L-DOPA (10 mg/kg). The first three measurements for locomotor activity were taken on Days 49, 54 and 59, three factorial ANOVA—for distance $F(1, 28) = 5.686$, $P < 0.0001$; for rearing $F(1, 28) = 14.400$, $P < 0.0001$ and for inactive sitting $F(1, 28) = 31.896$, $P < 0.0001$, respectively. Subsequently using Newman–Keuls test, each group was compared in the same time period or different groups with different time periods. On Day 59, the last behavioural assessments in the activity box took place. Statistically there were no differences among the control and pre-rotenone animals with 10 mg/kg L-DOPA treatment in all the three parameters (total distance travelled, rearing and inactive sitting). But there was a significant difference in all the three parameters of pre-rotenone-treated animals with saline compared to L-DOPA treatment animal groups.

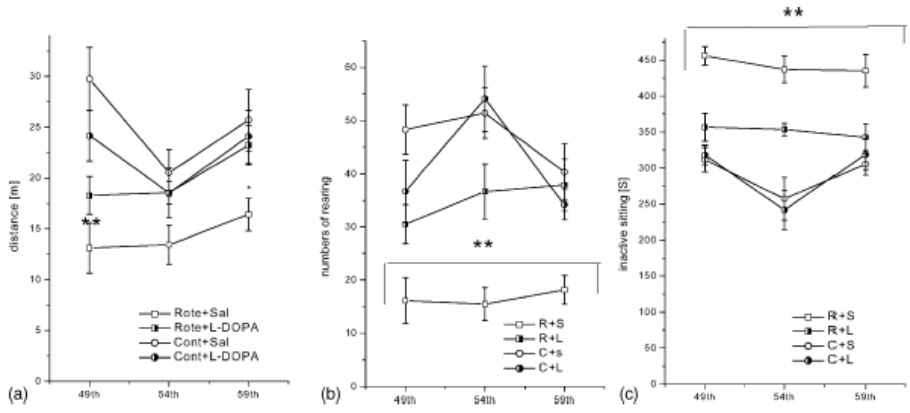


Fig. 4. (a–c) Activity box tests were carried out subsequently on Day 49 after the first onset of chronical L-DOPA treatment, then further after every fifth day on all four groups of animals, rotenone-treated animals with saline treatment ($N = 6$) and L-DOPA treatment (10 mg/kg) ($N = 6$) and further control group ($N = 10$) with saline and L-DOPA (10 mg/kg) ($N = 10$) for 10 days. Locomotor activity, rearing and inactive sitting were measured for each group. Subsequent three factorial ANOVA for each group confirmed significant treatment effect for the three different time periods (on Days 49, 54 and 59). Newman–Keuls test shows for each time period differences (*significant at $P < 0.05$; and **significant at $P < 0.01$ compared with vehicle + saline).

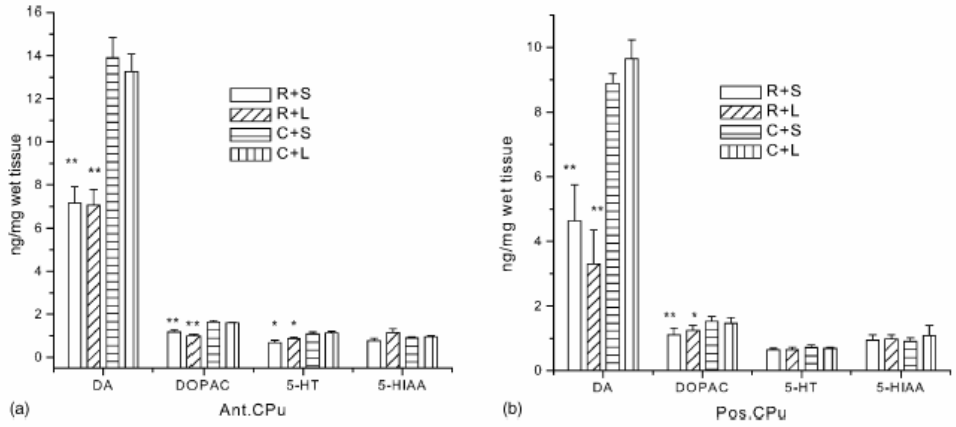


Fig. 5. (a and b) Anterior (Ant.CPu) and posterior (Post.CPu) striatal DA, 5-HT and their metabolites in rotenone-treated group with saline ($N = 6$) and L-DOPA ($N = 6$) and vehicle-treated with saline and L-DOPA as a control group ($N = 6$). Data are given as mean ± S.E.M. $P$ represent statical analysis: *significant at $P < 0.05$; and **significant at $P < 0.01$ (compared with vehicle + saline, one-way ANOVA followed by Newman–Keuls test).

### 3.4.3. Neurochemistry

The levels of DA, 5-HT and their metabolites DOPAC and 5-HIAA were measured in the anterior striatum (Ant.CPu) and posterior striatum (Post.CPu) for each group of animals as shown in Fig. 5a and b. In rotenone-treated animals, DA concentrations were significantly depleted in both striatal regions. The concentration of DA and DOPAC in the Ant.CPu $F(3, 15) = 24.64$, $P < 0.0001$; $F(3, 15) = 25.05$, $P < 0.001$ and Post.CPu $F(3, 15) = 17.94$, $P < 0.001$; $F(3, 15) = 6.52$, $P < 0.004$were detected. The concentration of serotonin (5-HT) and its metabolite 5-hydroxy indole acetic acid (5-HIAA) were Ant.CPu $F(3, 15) = 4.87$, $P < 0.01$; $F(83, 15) = 1.87$, $P < 0.17$, respectively.

The concentrations of DA, DOPAC, 5-HT and 5-HIAA in the sustantia nigra $F(3, 15) = 22.01$, $P < 0.0001$; $F(3, 15) = 6.99$, $P < 0.003$; $F(3, 15) = 2.97$, $P < 0.06$ and $F(3, 15) = 2.30$, $P < 0.111$, respectively, were detected. The DA turnover ratio DOPAC/DA, $F(3, 15) = 1.79$,

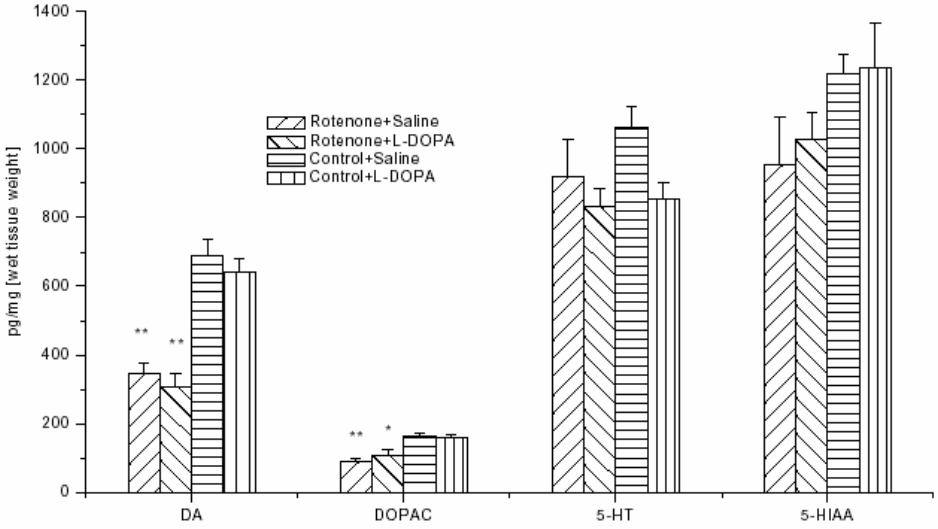


Fig. 6. Levels of DA, 5-HT and their metabolites in the substantia nigra's tissues. Control group treated with saline ($N = 6$) and L-DOPA ($N = 6$) and rotenone-treated group with saline ($N = 6$) and L-DOPA ($N = 6$). Data are given as mean ± S.E.M. $P$ represent statistical analysis: *$P < 0.05$ and **$P < 0.01$ (compared with vehicle + saline, one-way ANOVA followed by Newman–Keuls test).

$P = 0.191$ and in the 5-HT turnover, 5-HIAA/5-HT ratio $F(3, 15) = 1.76$, $P = 0.197$ were detected. The 50% decrease of DA, 47% DOPAC, 13% 5-HT and 23% 5-HIAA have been detected compared with control value (Fig. 6). Statistically there was no change in the level of 5-HT, 5-HIAA and also in 5-HIAA/5-HT ratio in the substantia nigra.

## 4. Discussion

Administration of rotenone 2.5 mg per kg per day over a period of 48 days caused a decrease in DA and its metabolite DOPAC in both parts of striatum (anterior and posterior) and also in the substantia nigra. 5-HT and its metabolite 5-HIAA were unchanged in posterior striatum and in the substantia nigra. However, the levels of 5-HT were decreased in the anterior striatum as compared to control. On the behavioural level, hypokinetic behaviour was observed such as enhancement of the descent latency in catalepsy, both in bar and grid tests, reduced locomotor activity, reduced rearing and increased inactive sitting in the activity box.

From our observation the rotenone susceptibility in the same strain of rats was individual. Some rats refused food and water intake after 20–22 days. It is difficult for rats to survive bilaterally lesioned dopaminergic neurons. Thus, without breaking the treatment regimen we continued administration of rotenone. The disadvantage of this model is its labour-intensive nature and variability [2]. So, it is difficult to get neurodegeneration at the same time in every animal. From our behaviour analysis we could say that, perhaps each and every individual start to develop DA-nergic neurons degeneration between 25 and 35 days.

The rotenone-induced neurochemical and behavioural deficiencies observed in this study suggest that dopaminergic neurons in the nigrostriatal pathway are sensitive to the action of rotenone. Acute administration of rotenone 5–10 mg/kg increases DA turnover after 24 h in mice but after 7 days it remains unchanged. But low and chronic intermittent dose (1.5 mg/kg) three times a week does not show an increase in DA turnover [20]. Findings of this study support the view that only acute dose is capable to produce increased DOPAC/DA ratio turnover but with chronic doses over a longer time the compensatory mechanism might be fully disrupted because dopaminergic neurons are destroyed and there is less extracellular DA and thus less DA metabolites. Our neurochemistry results indicate that systemic chronic administration of 2.5 mg/kg over a period 48 days is not able to increase DA turnover ratio in striatum and substantia nigra. Our data are in accordance with findings [11] which have already revealed that under rotenone, to some extent, also serotonergic neurons degenerate and 5-HT levels in the striatal region were decreased. Therefore, it is questionable as to whether rotenone-infused animals parallels idiopathic PD. Striatal cell loss is typical for Huntington's disease (HD). However, usually in typical cases of HD there is no positive response to anti-parkinson medication. In most cases these agents may worsen choreic movement. Mostly the lesion of 3-NP or quinolic acid injury in striatum produces spontaneous locomotor hyperactivity, adipsia and aphagia-like behaviour [6]. Thus, to summarise, there can be no doubt that rotenone destroys dopaminergic cells, the specificity to which extent other transmitter systems may also be affected, remains to be investigated.

The mechanism leading to degeneration is not ultimately substantiated but most probably involves the complex I inhibition. In our study, quantitative neurochemical analysis seems to show that complex I inhibition within the nigrostriatal pathway is sufficient to produce neurotoxicity. The fact that also in human PD complex I deficiency plays a crucial role makes the animal model especially attractive.

All these findings support the view that rotenone-induced neuronal and behavioural deficiencies resemble those found in Parkinson's disease. The specific aim of this work was to investigate whether or not the clinically effective anti-parkinsonian drug L-DOPA is able to reverse rotenone-induced parkinsonian symptoms. The rotenone-induced increase in the descent latency in catalepsy, inactive sitting and decrease in locomotor activity and rearings were reversed after chronic administration of L-DOPA over a period of 10 days.

These results are the first to demonstrate that hypokinetic behaviour, which mimics parkinsonian behavioural symptoms, e.g. akinesia and rigidity (catalepsy) in rats, was reversed by L-DOPA.

## 5. Conclusion

Peripheral administration of rotenone has been proposed as a potential model of PD [2,16] but this model still suffers from the lack of validation. Chronic treatment with a low dose of rotenone is able to induce neuronal degeneration and behavioural deficits that resemble PD very closely. Our results demonstrate that a low dose of L-DOPA combined with decarboxylase inhibitor selectively reversed rotenone-induced parkinsonian symptoms.

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