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A rat model of Parkinsonism shows depletion of dopamine in the retina

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Abstract

The retinal dopamine (DA) deficiency is an important feature of the pathogenesis in Parkinson’s disease (PD) visual dysfunction. Systemic inhibition of complex I (rotenone) in rats has been proposed as a model of PD. In this study, we investigated whether systemic inhibition of complex I can induce impairment of DA-ergic cells in the retina, similar to the destruction of retinal cells found in PD patients. Rotenone (2.5 mg/kg i.p. daily) was administered over 60 days. Neurochemically, rotenone treated rats showed a depletion of DA in the striatum and substantia nigra (SN). In addition, the number of retinal DA-ergic amacrine cells was significantly reduced in the rotenone treated animals.

This study is the first one giving highlight towards a deeper understanding of systemic complex I inhibition (rotenone as an environmental toxin) and the connection between both, DA-ergic degeneration in the nigrostriatal pathway, and in the DA-ergic amacrine cells of the retina.

1. Introduction

Parkinson’s disease (PD) is not only a disease which leads to a decrease of dopamine (DA) in the nigrostrital regions of the brain, but it is a widespread degenerative illness in which the central, peripheral, somatomotor and visual systems become particularly severely damaged. The visual system defects show similarities to the basal ganglia (BG) system defects due to depletion of the neurotransmitter DA in the amacrine cells of the retina. Previous studies have shown that visual abnormalities are also observed in an animal model of PD using MPTP (1-methyl, 4-phenyl, 1-2-3-6- tetrahydropyridine) where the retina shows dopaminergic deficiency with loss of a subset of retinal amacrine cells (Bodis-Wollner, 1990; Tatton et al., 1990). Rotenone model rats of Parkinson’s disease (PD) provide evidence for the plausibility that the pathophysiology of this disease is not limited to the mid-brain region of the SN pars...
compacta, but that most of the peripheral disturbances at the early stage of PD occur in the same way as in human patients. As the disease progresses, components of the limbic and somatomotor system become partially damaged. The wide occurrence of Lewy bodies in the peripheral autonomic nervous system in patients with PD also accompanies autonomic dysfunction. Histological evidence shows that Lewy bodies and Lewy neurites appear in the dorsal nucleus of the vagus in the early stage of the disease and later, in the third stage, extend upwards to the substantia nigra (Braak et al., 2002), and also postganglion sympathetic and intrinsic neurons in the heart are involved in the PD process (Iwanaga et al., 1999). Thus, all of these data suggest that PD is a systemic degenerative disease that is not only affecting tissues in the BG, but also non cerebral tissues. This is consistent with the multiple-hit hypothesis (Cory-Slechta et al., 2005; Carvey et al., 2006) which predicts that multiple concurrent insults occurring at different target sites within a system (here nigrostriatal dopamine) may constrict the range and flexibility of compensatory mechanisms, thereby compromising the integrity and viability of the system.

The decrease of DA in the BG and in the retina causes movement and spatial working memory disturbances, and an alteration of the visuocognitive process, respectively. The DA-ergic projections from both, the SNpc and from retinal amacrine interneurons share the property of spontaneous firing (Yung et al., 1991; Feigenspan et al., 1998) thereby suggesting that tonic DA release is a common function and characteristic of DA-ergic neurons throughout the nervous system. Both, DA producing cells in the SNpc and amacrine cells in the retina possess a pacemaker activity (Grace & Bunney, 1983; Grace & Onn, 1989; Yung et al., 1991).

Different studies demonstrate that PD patients express modest, but reproducible, reductions in complex I activity in tissues including brain and blood platelets cells (Mizuno et al., 1989; Parker et al., 1989; Schapira, 1998). On average, in platelets, there appears to be about a 25% decrease in complex I activity (Greenamyre et al., 2001). But the retinal complex I activity in
PD is not well investigated. However, the pathology of Leber’s herediatry (LHON) optic neuropathy and mutations in the mitochondrial DNA (mtDNA) of three different genes (ND1, ND4 or ND6) of complex I are well investigated (Huoponen, 2001). The main pathological alteration the LHON retina is severe nerve fiber layer (NFL) thinning. Additionally, numerous patients, especially those with a mutation of gene ND6, show accompanying neurological symptoms such as tremor, Parkinsonism, dystonia etc. As PD patients do also show NFL thinning (Inzelberg et al., 2004) together with the neurological systems of PD, complex I dysfunction is very likely to cause retinal and brain dysfunction in both diseases, LHON and PD.

Here, we show that systemic inhibition of complex I by rotenone induces impairment of DA-ergic cells in both, the striatum and substantia nigra, and the retina.

2. Experimental procedures

2.1. Animals

Twenty male Sprague-Dawley rats (Charles River), weighing 260–300 g at the beginning of the experiment were used. Animals, housed in groups of six in cages under a 12: 12h light/dark cycle in a room maintained at constant temperature (22°C) and humidity (55–60%). The rats obtained dry food (Altromin, 1324) 15 mg/day/rat and tap water was available ad libitum. All the animals were maintained in facilities fully accredited by the German Animal Protection Law. The experiments were approved by the local ethical committee (Tierschutzkomission, Regierungspräsidium Tübingen, ZP 2/03).

2.2. Chemicals

Rotenone was purchased from Sigma (St. Louis, MO, USA) and was dissolved in sterile natural oil (middle chain triglycerides, MCT; Miglyol 812, clinical pharmacy, University
Tuebingen). Rotenone, dissolved in natural oil at 2.5 mg/ml, was given i.p. daily at 1 ml/kg for 60 days. Oil was injected as vehicle to control rats (1 ml/kg).

2.3. Body weight assessment and mortality
The body weight was measured on every day before the drug treatment. During the first 30 days the average body weight decreased 4-5% compared to control but till the end of the experiment average loss of body weight was 8-10% compared to control. The mortality began to take place after 30 days of treatment and 4 rotenone treated animals died during the last 30 days of rotenone treatment. The rate of mortality in rotenone treated animals was approximately 33%.

2.4. Experimental design
This study was comprised of two different groups of rats using systemic chronic i.p. administration of rotenone in one group (N = 12) and vehicle (oil) treatment i.p. (N = 8) in the other group. 10 days after the last injection of rotenone i.e. on day 70 all the rats were decapitated. Brain regions (striatum and SN) were collected as described by Heffner and coworkers (1980) for neurochemical and immunohistochemical analysis, respectively. The tissue samples of striatum and SN were taken bilaterally and immediately weighed and stored in liquid nitrogen until assay.

Because of the limitation of retinal tissue, we did not perform any HPLC on this tissue but decided to use as much retinas as possible for the analysis of the altered retinal morphology. Thus, the eyes were enucleated, the anterior chamber was removed, and the eyecups were fixed for 45 min in 4% PFA phosphate-buffered saline (PBS; 50 mM). Before further processing, the specimens were washed 3 times for 10 min in PBS.
2.5. Neurochemistry

Dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) 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 x 4.0 mm, pore diameter: 3.0µm). 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, 5-HT and its 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.

2.6. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was carried out using the GB–Stat V5.4 software (Dynamic Microsystems, Inc., Silver Spring, MD, USA). The neurochemical data were analysed by one-way ANOVA followed by Newman-Keuls post hoc test. A **$P < 0.01$ and *$P<0.05$ were considered statistically significant.

2.7. Retinal Histology

2.7.1. Standard histology

For standard histology, eyecups were dehydrated in a graded series of ethanol-water mixtures and embedded in plastic (Technovit 7100; Kulzer, Wehrheim, Germany) for high structural preservation of the tissue. Microtome sections (3 µm) were prepared and mounted on
superfrost slides (Menzel-Gläser, Braunschweig, Germany). Sections were then air dried at 60°C, stained with Richardson solution (1% azur, 1% methylene blue, and 1% borax in deionized water), and coverslipped with Entellan Medium (Merck, Darmstadt, Germany).

2.7.2. Immunocytochemistry

Eyecups were cryoprotected in 30% sucrose for at least 4h, then embedded in Cryomatrix (Tissue Freezing Medium; Jung-Leica, Nussloch, Germany) and rapidly frozen in liquid N2; 20 µm thick transversal sections were cut at -20°C, mounted on superfrost slides, and air dried at 37°C for at least 2h. Slides were stored at -20°C. Before further use, slides were thawed and washed three times in PBS, and treated with blocking solution (BS; 20% normal goat serum, 2% bovine serum albumin in PBS containing 0.3% Triton X-100) for 1h. Sections were then incubated overnight in BS containing 1:250 mouse anti-tyrosine hydroxylase monoclonal antibody (Immunostar, Wisconsin, USA) at 4°C. The immunoreaction was visualized by using Alexa Fluor 488 antimouse (Molecular Probes, Leiden, Netherlands) 1:1000 as a secondary antibody. For all immunocytochemical experiments, negative controls were carried out in the same way but without using the first antibody. Slides were viewed a Zeiss Axioscope2 MOT microscope and pictures were taken with Axiovision 4.1 software using an Axiocam camera.

2.7.3. Whole-mount immunohistochemistry.

After fixation the eyecups were washed five times 10 min in PBS, then, retinae were dissected out of the fixed eyecups and four radial cuts were made to allow flattening. The retinae were dehydrated in ascending ethanol concentrations (50, 70, 80, 90, 95, and 100%, for 10 min each), incubated for 10 min at room temperature (RT) in xylene, rehydrated in a descending alcohol series, and washed three times for 20min in PBS. Retinae were then incubated overnight in blocking solution (20% NGS, 1% BSA in PBS with 0.1% Triton X-100) with 7
continuous agitation at RT. Following this, retinæ were incubated in blocking solution containing 1:250 mouse anti-tyrosine hydroxylase (Immunostar, Wisconsin, USA) for 5 days at 4°C with continuous agitation and for 2 d at 4°C in the same buffer containing a 1:1000 dilution of Alexa488 labelled goat anti-mouse (Molecular Probes, Leiden, Netherlands) with continuous agitation. Retinæ were mounted flat, with the retinal ganglion cell layer oriented upward, and coverslipped with a 9:1 mixture of glycerol/PBS. The slides were stored at 4°C between examinations.

2.7.4. Morphometric analysis.

TH-labelled cells were counted on pictures taken with Axiovision 4.1 using an Axiocam at a Zeiss Axioscope2 MOT microscope with a 20× objective. For each rat retina, the sample consisted of two to three adjacent 0.37 mm² fields in the central area of the retina at the focal plane of the DA-ACs (type I). The differences between rotenone treated and untreated retinæ were tested statistically with a paired Student's *t* test using GraphPad Prism. Additionally, the mean cell size was estimated by tracing their outlines and analyzing the resulting surfaces with NIH ImageJ 1.34l. The sampled areas were the same as described above. The differences between rotenone treated and untreated retinæ were tested statistically with a Wilcoxon matched pairs test (data did not pass normality test) using GraphPad Prism.

3. Results

3.1. DA and its metabolites

Treatment with rotenone significantly decreased the levels of DA and DOPAC in both parts of striatum. In the anterior striatum (Ant. CPu) the levels of DA 49%, DOPAC 38%, and in the posterior striatum (Post. CPu) DA 40%, DOPAC 28% were depleted as compared to control animals. The levels of DA, DOPAC and HVA in the Ant.CPu F(1, 16) = 16.49,
P<0.0009; F(1,16) = 57.88, P<0.0001; F(1,16) = 1.20, P<0.289 and Post.CPu F(1, 16) = 10.96, P<0.004; F(1, 16) = 9.01, P<0.008. In the SN the levels of DA, F(1, 16) = 6.89, P<0.01; DOPAC, F(1, 16) = 4.03, <P0.06 and HVA, F(1, 16) = 4.09, P<0.06, respectively.

In the SN the depletion of DA levels was 38% and DOPAC 23% as shown in Fig. 1(a -c). There was a tendency to a decrease in the level of DOPAC and HVA in the SN but statistically they were not significant.

3.2. Morphology of retinal DA-ergic neurons is unaffected in rotenone treated animals

In order to identify whether the general morphology of the retina was altered as an effect of the rotenone treatment, we analyzed the retinas of control and rotenone injected animals with standard histologic techniques (Fig. 2). In the transverse sections all five retinal layers were clearly identifiable and no pathological alterations, like pyknotic nuclei or reduced cell numbers, could be identified.

Additionally, a specific labelling of dopaminergic amacrine cells (DA-ACs) with anti-TH (tyrosine hydroxylase) antibody showed that DA-ACs are labelled to the same extent in the rotenone treated animals (Fig.3 B) and in the controls (Fig.3 A). In both groups DA-AC somata were clearly labelled and the DA-ACs showed the classical stratification in stratum 1 of the IPL (Nelson et al., 1981). Thus, there was no morphological difference discernible in the transverse sections of the rotenone treated animals and their retinas appeared to be completely healthy.

3.3. The density of retinal DA-ergic cells is decreased in rotenone treated animals

As DA-ACs are rather sparsely distributed throughout the retina (usually only one to three cells are visible per cross-section), it is very difficult to do a reliable quantification of their density on the basis of transversal sections and the very limited sample size. Therefore, we
prepared retinal whole mounts for TH immunohistochemistry in order to be able to quantify the cell density in defined areas of the retina in both rotenone and control injected animals.

In adult mammals, two morphological classes of dopaminergic cells can be differentiated (Mariani & Hokoc, 1988): scarce cells with large somata displaying intense TH immunoreactivity (type I), and a more numerous population of smaller cells with faint TH immunoreactivity (type II). In our preparation we could only clearly identify type I cells (Fig. 4 A+B, arrows) and thus, we did only quantify this cell type. These DA-ACs were sparsely distributed throughout the retina in both, control (Fig. 4 A) and rotenone (Fig. 4 B) treated animals. The labelled cells in the rotenone rats appeared to have a larger cell size (Fig. 4 D) than in the controls (Fig. 4 C). However, even though the quantification of the cell size revealed a difference of approx. 10% in between the mean cell body size of the rotenone animals (124.6 ± 7.2 SEM) compared to the controls (112.3 ± 4.7 SEM), the difference was not significant (p > 0.05). In addition, maximum intensity projections of respective DA-ACs in confocal stacks showed (Fig. 4 C+D) that the general morphology, including cell body shape, ramification, and bouton density, remained unchanged after rotenone treatment. In contrast, the retinas of treated rats differed with high significance (p < 0.001) from control rat retinas in that the density of DA-ACs was reduced by approx. 28% from 14.4 ± 0.5 SEM in the control to 10.3 ± 0.6 SEM in the rotenone rats (Fig. 5; compare Fig. 4A and B).

Thus, we suggest that the rotenone treatment lead to a significant decrease in the number of dopaminergic cells in the retina.

\section*{4. Discussion}

Chronic and very low-level environmental exposures of rotenone may be more relevant to sporadic PD but difficult to detect because the individual genetic variations may explain the
development of PD only in a subset of individuals for the susceptibility to similar doses or
levels of toxins to produce the symptoms in the same time.

In the present study, two principle findings which cause symptoms as in PD patients have
been identified after systemic and chronic administration of rotenone in rats. A relation
between complex I inhibition and depletion of both nigrostriatal DA and a decrease of retinal
da-ergic amacrine cells has been found. The quantitative neurochemical analysis in the
anterior, posterior striatum and SN showed significant depletion of DA levels in rotenone
treated rats as compared to vehicle (oil) treated rats.

Rotenone treated rats show emerging extranigral neurodegeneration of the visual system i.e.
decrease of tyrosine hydroxylase positive cell density in the retina. Contrarily to the decrease
in the number of retinal DA-ergic amacrine cells, other retinal cell populations, like Muller
cell glia (specifically labelled with glutamine synthetase immunostainings; data not shown), or
specific bipolar cell populations (labelled with PKCβ immunostainings; data not shown) are
not affected by the rotenone treatment. Therefore, it can be concluded that a systemic
inhibition of complex I preferentially destroys dopaminergic cells both in the basal ganglia
and in other parts of the brain, like the retina as a part of the diencephalon. Additionally, other
catecholaminergic neuron groups, such as the locus coeruleus and other neurotransmitter
systems, including cholinergic neurons in the nucleus basalis, are also affected as the disease
progresses. Additionally, recent in vivo studies clearly show that chronic systemic application
of rotenone combined with oxidative stress leads to serious dysregulation of neurotransmitter
release with hypofunction of dopaminergic neurons and consequent hyperfunction of
cholinergic interneurons, rather than nonselective, extended impairment of neurotransmission
(Milusheva et al., 2005). Furthermore, Milusheva and coworkers (2003) showed that
oxidative stress can lead to a flood of non-synaptic noradrenaline from cytoplasmic stores,
which consequently provides an additional source of highly reactive free radicals if combined
with mitochondrial dysfunction (caused by rotenone) and thus initiating a fatal self-
amplifying cycle leading to neuronal degeneration. However, in \textit{in vitro} studies, it has been shown that rotenone preferentially kills dopaminergic neurons in the SN, whereas noncatechominergic neurons, such as those in the perifornical nucleus, were more resistant to rotenone toxicity (Bywood & Johnson, 2003).

In addition to these effects on PD related areas of the BG, we found that specifically the dopaminergic amacrine cells of the retina are degenerating as a result of the chronic rotenone application. Given the fact that the remaining dopaminergic amacrine cells show normal morphology, it seems that the degenerating dopaminergic amacrine cells die very rapidly once affected by the rotenone treatment. The reduced number dopaminergic amacrine cells in the treated animals fits nicely to previous data described in human patients, where post mortem measured DA levels in the retina were low in comparison to control individuals (Harnois & Di Paolo, 1990). This primary deficiency of DA is thought to be due to a reduced activity of retinal dopaminergic amacrine cells, therefore eventually leading to impaired vision in PD which had first been shown in psychophysical and visual evoked potential measuring experiments in both, rat (Dyer et al., 1981; Onofrj & Bodis-Wollner, 1982) and human (Bodis-Wollner & Yahr, 1978). This assumption is furthered by more recent experimental evidence which links a deficiency of dopaminergic, preganglionic amacrine cells and visual processing by showing reduced ganglion cell activity (Tagliati et al., 1994; Tagliati et al., 1996; Bodis-Wollner & Tzelepi, 1998). Furthermore, morphological evidence shows significant nerve fiber layer thinning in PD patients (Inzelberg et al., 2004), as well as significant reductions in both the thickness and cell numbers of the retinal ganglion cell layer in mice with rotenone injected eyes (Zhang et al., 2006). Both findings again suggest a degenerative process in the retina of PD affected subjects as well as in rotenone treated animals. Resulting from the retinal alterations, PD subjects have reduced contrast sensitivity and reduced colour discrimination which is progressive over time but can be improved with L-DOPA (Kupersmith et al., 1982; Smith et al., 1985; Price et al., 1992; Barbato et al., 1994).
As a decrease in the number of DA amacrine cells has already been described in other animal models of PD, like MPTP treated monkeys (Ghilardi et al., 1988), we suggest that chronic rotenone application is a suitable model to mimic PD that has been – at least partially-induced by environmental toxins.

5. References


Figure 1. Levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) in the anterior striatum (A), the posterior striatum (B), and in the substantia nigra (C). The rotenone treated animals (N = 8) and the control i.e. the vehicle (oil) treated animals (N = 10). Data are given as mean ± SEM. P – represent statistical analysis. **P <0.01 (compared with rotenone treated animals with saline, one-way ANOVA followed by Newman-Keuls test) value less than 0.05 was accepted as a significant.

Figure 2. Transverse sections of control injected (A) and rotenone treated animals (B). Overall retinal histology remains unchanged in control and rotenone injected animals. Outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL). Scale bar: A+B 50 µm.

Figure 3. Dopaminergic amacrine cells (DA-ACs) expressing tyrosine hydroxylase (TH) in transverse sections of control injected (A) and rotenone injected animals (B). The morphology of DA-ACs is identical in both, control (A), and rotenone injected (B) animals. DA-ACs send numerous, long processes throughout the entire IPL and the somata are strongly labelled. Scale bar: A+B 100 µm.

Figure 4. Effects of rotenone injections on TH immunoreactivity on retinal whole-mount preparations. Fluorescence picture with focus at the level of the DA-AC somata (arrows) in the INL (A+B): There are more cells labelled in the control injected retina (A) than in the rotenone injected (B). Maximum intensity projection of a confocal stack (C+D): The cell size of the DA-AC somata is slightly larger in the rotenone injected animals (D) compared to the control animals (C). However, general morphology, including cell body shape, ramification,
and bouton density do not show any identifiable difference. Scale bars: A+B 100 µm; C+D 30 µm.

**Figure 5.** Quantitative analysis of the retinal density of dopaminergic neurons in control injected and rotenone injected rats. The numbers indicate the mean of labelled cells counted in adjacent 0.37 mm² fields (as shown in Figure 4) in the central retina. The number of DA-ACs is reduced with high significance in rotenone injected animals (p < 0.001) compared to the control rats. Error bars represent SEM.
Figure 1

1a

1b

1c
Figure 2

A  Control

ONL
OPL
INL
IPL
GCL

B  Rotenone

ONL
OPL
INL
IPL
GCL
Figure 3

A  Control

B  Rotenone
Figure 4

(A, C) Control

(B, D) Rotenone
Figure 5

![Diagram showing the number of dopaminergic cells in Control and Rotenone conditions with a significant difference indicated by ***]

- Control
- Rotenone

Number of dopaminergic cells