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Ogg1 null mice exhibit age-associated loss of the nigrostriatal pathway and increased sensitivity to MPTP

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Abstract

Cumulative damage to cellular macromolecules via oxidative stress is a hallmark of aging and neurodegenerative disease. Whether such damage is a cause or a subsequent effect of neurodegeneration is still unknown. This paper describes the development of an age-associated mild parkinsonian model in mice that lack the DNA repair enzyme 8-oxoguanine glycosylase 1 (Ogg1). Aged *OGG1* knock-out (*OGG1* KO) mice show a decreased spontaneous locomotor behavior and evidence a decrease in striatal dopamine levels, a loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (SN), and an increase in ubiquitin-positive inclusions in their remaining SN neurons. In addition, young *OGG1* KO mice are more susceptible to the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) than their wild-type (WT) counterparts. Age-associated increases in 7,8-dihydro-2'-deoxyguanine (oxo⁸dG) have been reported in brain regions and neuronal populations affected in Parkinson's disease (PD), toxin-induced parkinsonian models, and mice harboring genetic abnormalities associated with PD. Because of these increased oxo⁸dG levels, the *OGG1* KO mouse strain could shed light on molecular events leading to neuronal loss as a consequence of cumulative oxidative damage to DNA during aging and after toxicological challenge.

Keywords

DNA Damage; DNA Repair; 7,8-dihydroxy-2'-deoxyguanine; Ogg1; Aging; Parkinson's disease; MPTP

1 Introduction

Parkinson's disease (PD) is an age-associated movement disorder, characterized by a loss in the number of dopaminergic neurons in the substantia nigra (SN) and the formation of Lewy bodies, ubiquitin and synuclein positive eosinophilic aggregates (Alves-Rodrigues et al., 1998; Wakabayashi et al., 1998). There is growing evidence that reactive oxygen species

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(ROS) play a major role in the neurodegenerative process and targeting their formation or deleterious effects can serve as a therapeutic approach (Fukae et al., 2007; Mancuso et al., 2007).

All major macromolecules are targets for ROS-induced damage (Halliwell and Gutteridge, 1986). Among the four DNA bases, guanine has the lowest oxidation potential (Devasagayam et al., 1991; Milligan et al., 2001; Steenken and Jovanovic, 1997); thus, 7,8-dihydro-2'-deoxyguanine (oxo⁸dG) is the most prevalent form of oxidative base modifications produced (Dizdaroglu et al., 2002). Oxo⁸dG levels have become biomarkers of oxidative stress associated with aging and diseases ranging from cancer to neurological deficits (Chiou et al., 2003; Proteggente et al., 2002; Wang et al., 2005). Oxo⁸dG formation and accumulation are associated with deleterious cellular effects such as altering of telomere ends stability and maintenance (Opresko et al., 2005), activation of the cell cycle and apoptosis in neurons (Kruman et al., 2004).

Oxo⁸dG has been shown to be significantly higher in PD postmortem brain samples than in age-matched controls (Alam et al., 1997; Sanchez-Ramos, 1994; Zhang et al., 1999). Oxidative damage to DNA in PD is not restricted to the nigrostriatal pathway as oxo⁸dG has been found to be elevated in lymphocytes (Migliore et al., 2002; Petrozzi et al., 2001), cerebrospinal fluid, and serum (Abe et al., 2003). Urinary oxo⁸dG levels have also been found to be elevated in PD patients correlating with the progression of the disease (Sato et al., 2005). Also, the expression of enzymes that repair or remove oxidized guanine bases are found to be upregulated in PD brains (Nakabeppu et al., 2007). Recently, it has been shown that alterations in 8-oxoguanine glycosylase 1 (Ogg1) are also a component in AD brains (Shao et al., 2008).

High oxo⁸dG levels are not restricted to clinical cases of PD. Increased oxidative damage to DNA has been found after exposure to the dopaminergic toxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone (Beretta et al., 2006; Betarbet et al., 2000; Chen et al., 2005; de Lima et al., 2005; Mandavilli et al., 2000). Despite its association with both neuronal loss in the nigrostriatal system and the effect of dopaminergic neurotoxins, oxo⁸dG accumulation has been regarded as a marker for but not as an initiator of neurodegenerative processes.

Mice lacking Ogg1, the enzyme responsible for the removal of oxo⁸dG from DNA, accumulate oxo⁸dG at a faster rate than wild-type (WT) mice (Klungland et al., 1999; Osterod et al., 2001). The mice age normally without a significant phenotype (Friedberg and Meira, 2006). Oxo⁸dG accumulation in *OGG1* deficient mice is accentuated in organs with reduced cell proliferation as well as in mitochondria (Minowa et al., 2000; Stuart et al., 2005). Recent studies indicate that the *OGG1* deficient mice are more susceptible to a challenge with methamphetamine during development (Wong et al., 2008), and primary cortical neurons from these mice are more susceptible to ischemic episodes (Liu et al., 2011). Thus, supporting a role of accumulation of oxo⁸dG in neurodegenerative cascades. Studies looking at biological systems involved in reducing oxidative burden in the nucleotide triphosphate pools indicate that mice lacking the enzyme MTH1, responsible for removing free radical-damaged nucleotides, exhibit increase damage to RNA and DNA and are more susceptible to a neurotoxic challenge with MPTP. These results lend support to the role of DNA damage in neurodegeneration of the nigrostriatal system (Yamaguchi et al., 2006). However, despite the overwhelming evidence of oxo⁸dG as a major component of the pathology in PD no study has directly determined the role of increased DNA damage in the nigrostriatal system in relationship to aging and neurotoxicological susceptibility.

We present data demonstrating that mice lacking *Ogg1* evidence age-associated spontaneous motor behavior deficiencies paralleled by neurochemical and histological changes in the nigrostriatal system, loss of striatal DA, loss of TH-positive neurons in the SN, and the development of ubiquitin-positive inclusions in surviving SN neurons. In addition similar to published results of increased toxicological susceptibility in these mice, mice lacking *Ogg1* are more susceptible to a challenge with MPTP. The age-dependent changes in striatum are neurochemically specific, striatal glutamate levels are unaffected and no or less drastic histopathological changes are seen in non-SN neuronal populations. This study evidences the role of oxidative stress and the susceptibility of the nigrostriatal pathway. Specifically, the role increased oxidative damage to DNA plays in neuronal vulnerability and how deficiencies in repair capacity can alter neuronal fate.

2 Materials and methods

2.1 Animals and Behavioral Tests

All animal use was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with protocols approved by the Institutional Animal Care and Use Committee.

All chemicals were purchased from Sigma (Sigma, St. Louis, MO), unless otherwise indicated. Male and female WT, heterozygous (HZ), and *OGG1* knock-out (KO) mice all on 129/SvJ background were used for these studies. Experimental groups consisted of littermates with ages at either 3 (young) to 26 (aged) months old. Original colony founders were kindly donated by Dr. Tomas Lindahl.

Motor behavior was evaluated by a 10 minutes single-session open field test. The tests were performed in a 30 cm. square box, under a 20 LUX light intensity and during the light hours of the day. All the animals were evaluated at the same time, after a 1-hour conditioning period.

2.2 MPTP treatment

MPTP hydrochloride was dissolved in PBS at a concentration of 10 mg/mL and injected at a volume of 1.25 μ L/g for a total dose of 25 mg/Kg (2 i.p. injections of 12.5 mg/Kg 8 hours apart). MPTP treated mice were compared to saline treated control littermates of each genotype. All mice were sacrificed by cervical dislocation followed by rapid decapitation 7 days post-injection. Safety precautions for the handling of MPTP and MPTP-treated animals were carefully followed (Przedborski et al., 2001) <http://www.nih.gov/od/ors/ds/pubs/mptp/>.

2.3 Measurement of Ogg1 enzymatic activity

The extraction and activity of Ogg1 was performed as described elsewhere (Bolin et al., 2004; Cardozo-Pelaez et al., 2000).

2.4 Neurotransmitter Analysis by RP-HPLC

Levels of DA, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in caudate/putamen (CP) were measured using reverse-phase high performance liquid chromatography (RP-HPLC) with electrochemical detection (HPLC-ECD) as previously described (Cardozo-Pelaez et al., 2005; Cardozo-Pelaez et al., 1999). Briefly, CP were weighed and homogenized by sonication in 500 μ L of perchloric acid (0.05M) containing 3,4-dihydroxybenzylamine (DBA, 31 ng/mL). Homogenates were centrifuged for 20 minutes at 14000 g, 4°C and filtered through a 0.45 μ m filter. Sample filtrates were injected into the HPLC using an ESA Model 542 autosampler (ESA Chelmsford, MA). The mobile phase consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12

M sodium hydroxide, 0.60 mM EDTA, and 1.30 mM sodium octyl sulfate. An ultrasphere ODS column with a length of 25 cm and internal diameter of 4.6 mm was used for separation (Beckman Instruments, San Ramon, CA). The flow rate was kept at 1 mL/min (ESA Model 582 Solvent Delivery Module, Chelmsford, MA) and the column eluent was analyzed with an electrochemical detector (ESA Model 5600A CoulArray Detector, 3 ESA Model 6210 four channel electrochemical cells, Chelmsford, MA). All RP-HPLC data was recorded, stored and analyzed on a PC Pentium computer using CoulArray for Windows ³²Software (ESA Chelmsford, MA). DA, DOPAC, and DBA were monitored at 100 mV; HVA was monitored at 300 mV. The ratio of the peak heights produced by DA and its metabolites (DOPAC and HVA) to the peak height produced by DBA (internal standard) in the samples were used to obtain the CP analyte levels from a calibration curve. Data was expressed as micrograms of analyte per gram of wet tissue weight ($\mu\text{g/g wt}$).

Glutamate levels were measured in CP homogenates using a precolumn α -phthalaldehyde/ β -mercaptoethanol (OPA/ β ME) derivatization method as previously described (Donzanti and Yamamoto, 1988). Derivatization was performed by mixing 20 μl of sample with 20 μl of the OPA/ β ME working derivatizing reagent. After 1 minute, 20 μl of the reaction mixture was injected into the HPLC. This process was automated by the ESA Model 542 autosampler. The mobile phase consisted of 100mM disodium hydrogen phosphate, 20% methanol, and 3.5% acetonitrile; the pH was adjusted to 6.7 with phosphoric acid. A Waters XterraTM MS C18 (3.0 $\times$ 50 mm, 2.5 μL ; Milford, MA) column was used for separation, the flow rate was kept at 0.3 mL/min. The OPA/ β ME glutamate derivative was monitored at 380 mV. Data was expressed as micrograms of analyte per gram of wet tissue weight ($\mu\text{g/g wt}$).

2.5 Isolation and analysis of Oxo⁸dG and 2-deoxyguanosine in caudate putamen

The procedures for extraction, purification, and enzymatic hydrolysis of DNA were based on previously published methods with minor modifications (Bolin et al., 2004; Cardozo-Pelaez et al., 1999). Analysis of the levels of oxo⁸dG, expressed as the ratio of oxo⁸dG/ 2-deoxyguanosine (2-dG) was performed as follows. Oxo⁸dG and 2-dG were resolved by HPLC with a reverse phase YMCbasic column (4.6 $\times$ 150 mm; particle size 3-micron) (YMC Inc., Wilmington, NC) and quantified using a CoulArray electrochemical detection system (ESA, Inc., Chelmsford, MA). An isocratic mobile phase consisting of 100 mM sodium acetate, pH 5.2, 4% Methanol (HPLC Grade) diluted in water polished with C18 Sep-Pak cartridges (Waters Corp., Milford, MA) was utilized to elute the nucleosides from the column. The mobile phase was filtered using 0.2 m Nylon filters and degassed by sonication before use with the HPLC. Potentials of the four coulometric analytical cells of the CoulArray system, placed in series, were as follows: 50, 125, 175, 200, 250, 380, 500, 700, 785, 850, 890, 900 mV. Calibration curves were generated from standards of 2-dG ranging from 100 ng to 2 μg (0.350 nmol to 7.01 nmol) and oxo⁸dG (Cayman Chemical Company, Ann Arbor, MI) ranging from 5 pg to 100 pg (17.6 fmol to 353 fmol). The calibration curve for oxo⁸dG was created based on the peak area of the oxo⁸dG standard, eluting at 9.0 min, in the 250 mV channel. The calibration curve for 2-dG was created based on the sum of peak areas of the 2-dG standard, eluting at 7.8 min, in the 850, 890, and 900 mV channels. The electrochemical cells were allowed to equilibrate and wake up standards (three times the highest concentration of calibration standards) were injected before calibration standards. The amount of oxo⁸dG and 2-dG was calculated by comparing the peak area and sum of peak areas respectively from the 10 μL injection of reconstituted enzymatic hydrolysate of the DNA sample with the calibration curves for oxo⁸dG and 2-dG. Levels of oxo⁸dG in the samples were expressed relative to the content of 2-dG (e.g. the molar ratio of oxo⁸dG to 2-dG [fmol of oxo⁸dG/ nmol of 2-dG]). Data were recorded,

analyzed, and stored using CoulArray for Windows data analysis software (ESA Inc., Chelmsford, MA).

2.6 Histology, tissue preparation, and immunofluorescence labeling

Midbrains and forebrains were fixed by immersion in fresh 4% paraformaldehyde/100 mM phosphate buffer (pH 7.4) for 4 days at 4°C. Tissues were then paraffin embedded and sectioned coronally at 10 µm. Every third section through the SN was immunohistochemically stained for tyrosine hydroxylase (TH). Adjacent sections were later used for ubiquitin and 8-hydroxydeoxyguanosine (8-OHdG) immunoreactivity, as well as hematoxylin and eosin (H&E) staining.

Single chromogenic immunolabeling studies for TH (1:600, rabbit polyclonal, Chemicon, Temecula, CA) and ubiquitin (1:100, rabbit polyclonal, Abcam, Cambridge, MA) were performed according to the biotin-avidin peroxidase method. All sections were incubated in 3% H₂O₂ for 10 minutes to eliminate endogenous peroxidase activity. All rinses were done with 0.1 M Tris buffer (pH 7.4). Non-specific binding was reduced by using 0.5% bovine serum albumin in 0.1 M Tris buffer. Blocking was performed by incubating sections in 4% normal goat serum for 20 minutes. Slices were then exposed to a primary antibody, anti-TH 1:600 or anti-ubiquitin 1:100 for 18 hours at 21°C. Sections were rinsed and incubated with a biotinylated goat anti-rabbit IgG, 1:400 (Vector Laboratories, Burlingame, CA) for 1 hour at 21°C. The antibody signal was amplified using the Vectastain Elite ABC Kit (Vector Laboratories), 1 hour at 21°C. Color was developed by a 3,3'-diaminobenzidine (DAB) hydrogen peroxide reaction for 10 minutes. A sodium-citrate buffer antigen retrieval method was used prior to ubiquitin immunolabeling. After deparaffinization and rehydration, sections were immersed in a solution of 10 mM sodium citrate and 0.05% Tween 20 (pH 6.0) for 25 minutes at 95-100°C.

Single immunofluorescence labeling studies for oxo⁸dG and ubiquitin were performed as follows: Midbrains were fixed by immersion in fresh 4% paraformaldehyde/100 mM phosphate buffer (pH 7.4) for 4 days at 4°C. Tissues were then paraffin embedded and sectioned coronally at 10 µm. All rinses were done with 0.1 M Tris buffer (pH 7.4). Non-specific binding was reduced by using 0.5% bovine serum albumin in 0.1 M Tris buffer. Sections through the SN were incubated with an anti oxo⁸dG (1:200, goat polyclonal, Chemicon) antibody for 18 hours at 21°C. Blocking was performed with 4% normal donkey serum. Sections were then incubated with an AlexaFluor®-488 (1:400, donkey anti-goat IgG, Molecular Probes, Eugene, OR) secondary antibody for 1 hour at 21°C. Adjacent SN sections were incubated with an anti-ubiquitin (1:100, rabbit polyclonal, Abcam) antibody for 18 hours at 21°C. Blocking was performed with 4% normal goat serum. Sections were then exposed to a Texas Red® (1:400, goat anti-rabbit IgG, Molecular Probes) secondary antibody for 1 hour at 21°C.

A green NeuroTrace™ (Molecular Probes) fluorescent Nissl stain, 1:300 and 20 minutes at 21°C, was used as a counterstain to reveal the location of intranuclear ubiquitin-positive inclusions. A sodium-citrate buffer antigen retrieval method, as mentioned above, was used prior to ubiquitin immunolabeling. Fluorescence was visualized and imaged with a Nikon TE300 (Melville, NY) inverted microscope equipped with a Bio-Rad Radiance 2000 confocal laser scanning system (Hercules, CA) and Lasersnap 2000 imaging software. A Plan Aplanachromat 60× 1.4 oil immersion objective was used for collecting images. Laser parameters and pinhole sizes were kept constant for each respective study.

H&E staining was performed with a Varistain™ 24-4 Automatic Slide Stainer (Shandon Scientific Limited, Cheshire, England).

2.7 Neuronal quantitation

Tissue sections were viewed with a Nikon E-800 microscope attached to a Cohu videocamera connected to a Pentium PC computer. The following anatomical features were used to define the borders of the SN. *Rostral*: the first coronal section moving from rostral to caudal in the midbrain region that contains TH-positive neurons in the A9 (SN) cell group; *Caudal*: the last coronal section that includes TH-positive in the A9 cell group; *Medial*: The appearance of TH-positive neurons that belong to A10 cell group (ventral tegmental area, VTA); *Lateral*: the disappearance of TH-positive neurons of the A9 cell group. Based on these defined boundaries, numbers of TH-positive neurons in the substantia nigra were determined. Neuron counts were obtained from one focal plane of non-overlapping images. Within each section counted, the field of view was focused on the dorsal region of the SN using a 40X objective. Sequential images were captured and saved as the field of view moved towards the VTA. Counting was performed by assistants blind to genotype using the public domain NIH Image J program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Mean cell number per plane and SEM were then calculated for each group.

2.8 Statistical analysis

For the aging study group, spontaneous motor activity was recorded by using ANYMAZE software. Means and SEMs were determined for DA, DOPAC and HVA. Quantitative data from histological analysis (number of TH-positive neurons) are expressed as number of TH-positive neurons per section and SEMs. Two-way ANOVA tests were used to determine *OGG1* genotype and age effect in the status of the nigrostriatal system (DA levels or TH-positive neurons). One-way ANOVA tests were used to compare measured parameters in CP followed by the student Newman Keuls test for significant differences for DA levels, *OGG1* genotype, and age. Student t-test was used to evaluate parameters that include a single condition (age or treatment).

The toxicological effects of MPTP in the 3 month old mice (young) were analyzed as follows: All group values were expressed as mean $\pm$ SEM. Between group differences for striatal concentrations of DA evaluated for treatment group by one-way ANOVA tests followed by the student Newman Keuls test for significant differences for DA levels. The significance level of all evaluations was set at $p < 0.05$. Statistical tests were performed with Graphpad Prism® statistical software (Sorrento, CA).

3 Results

It has previously been established that as *OGG1* KO mice age they accumulate oxo⁸dG at a faster rate than age matched WT mice (Klungland et al., 1999). Although the age-dependent accumulation of oxo⁸dG has been demonstrated in whole brain, no reports have aimed to demonstrate the same phenomena in discrete brain regions. Using HPLC-ECD and oxo⁸dG histological assessment, we identified higher levels of the damaged base in CP (Figure 1A) and in SN (Figure 1B) of aged *OGG1* KO mice when compared with age-matched WT. Both approaches indicate that oxo⁸dG levels are increased in the *OGG1* KO mice.

3.1 Spontaneous motor behavior

Aged *OGG1* KO mice exhibited decreased line crossings (Figure 2A), speed (Figure 2B), and number of rearings (Figure 2C), while time immobile was longer compared to age-matched WT mice (Figure 2D). These differences found in the aging group were not found in young mice (Corresponding inset for each Figures 2A, 2B, 2C, and 2D).

3.2 Effects of aging in the nigrostriatal system of the *OGG1* KO mice

Given these findings, along with reports of increased oxo⁸dG levels in brains of PD patients, we opted to evaluate the status of the nigrostriatal system in aged WT and *OGG1* KO mice. Our data indicates that at 3 months of age there are not significant differences between WT and *OGG1* KO mice (Figure 3A). Also, no significant differences were detected in the DA index of turnover between young WT and *OGG1* KO mice (not shown). However, aged *OGG1* KO mice have a striatal DA reduction of 35% as compared to age-matched WT mice (Figure 3B). This loss of striatal DA is paralleled by a reduction in the number of TH-positive neurons in the SN of the *OGG1* KO (Figures 3C and 3D). It is worth noting that the difference in striatal DA between the WT and the *OGG1* KO is only significant in the aged mice, indicating an age-dependent phenomenon that is related to the deficiency in repair of oxo⁸dG.

Neuronal degeneration is typically accompanied by many histological changes. High magnification of TH-positive neurons in the SN from Figure 3D reveals that the neurons in the *OGG1* KO mice exhibit an increased level of atrophy, vacuolization, cytosolic condensation, and nuclear displacement (Figure 4).

H&E staining was employed to identify histopathological differences between aged WT and *OGG1* KO mice. Gross pathological changes between aged WT and aged *OGG1* KO mice were assessed in the SN (Figure 5A and 5B), CP (Figure 5C and 5D), and HP (Figure 5E and 5F). *OGG1* KO SN neurons exhibit signs of nuclear condensation and displacement, shrinkage of the cytoplasm (arrow head in Figure 5B) not seen in the WT SN. No evidence of chromatolysis or nuclear displacement is evident in the CP (Figures 5C and D). However, HP neurons in the aged *OGG1* KO (Figure 5F) mice exhibit a reduced cell volume but retain their cytoarchitectural organization. Lower magnification picture indicates areas used for histological assessments in HP and SN (supplemental Figure 1).

Histological changes in the SN, the decreased number of TH-positive neurons and signs of nuclear condensation, are paralleled by a reduction in striatal levels of the neurotransmitter DA (Figure 3A). To test the specificity of this neurochemical deficiency, levels of striatal glutamate were determined in both WT and *OGG1* KO mice at 3 and 26 months of age. No significant difference was detected between the genotypes and no age related changes were evident (Figure 6).

In idiopathic PD, the striatal DA loss is accompanied by the formation of proteinaceous inclusions in the SN. These eosinophilic inclusions stain positive for ubiquitin and -synuclein (Alves-Rodrigues et al., 1998; Gibb et al., 1991; Wakabayashi et al., 1998). Given the histological changes seen in the SN of the aged *OGG1* KO mice, we opted to evaluate brain sections for the presence of protein aggregations. In addition to a reduced number of TH-positive neurons in the SN of the aged *OGG1* KO mice and to morphological changes in the surviving neurons, there is a development of ubiquitin-positive inclusions (Figure 7B, arrowheads). These inclusions are not present in the SN of the age-matched WT mice (Figure 7A). Also, the ubiquitin-positive inclusions appear to be restricted to the SN as indicated by a lack of staining in the VTA (Figure 7C WT, Figure 7D *OGG1* KO). Despite the positive staining for ubiquitin we found little -synuclein reactivity in the SN of adjacent sections in the aged WT and *OGG1* KO mice (Data not shown).

In order to establish the time associated events with ubiquitin aggregate formation, we stained the SN of WT and *OGG1* KO mice at 3, 16, and 26 months of age. We found ubiquitin-positive staining to be age dependent in the SN of the *OGG1* KO mice. No ubiquitin-positive inclusions were visible in the SN of WT mice at any age (Figure 8 A, C, and E); nor positive ubiquitin accumulation was found in the *OGG1* KO at 3-months of age

(Figure 8B). Inclusions were few and diffuse in the 16-month-old *OGG1* KO SN (Figure 8D), and they became more prominent in the SN of the 26-month-old *OGG1* KO mice (Figure 8E).

High magnification of NeuroTrace fluorescent Nissl stain (green) followed by immunofluorescence ubiquitin staining in the SN of a 26 month old mice indicate that the ubiquitin-positive inclusions are located inside of SN neurons (Figure 9).

3.3 Neurotoxic response in the *OGG1* KO mice

The objective of this study is to establish a relationship between increased oxidative burden to DNA due to deficient DNA repair and the susceptibility of the nigrostriatal system to an acute challenge with MPTP. Two-way ANOVA analysis identified an MPTP effect ($F=10.72$; $df=1,48$; $p<0.05$) and an interaction with age effect ($F=8.50$; $df=1,48$; $p<0.05$) in DA and DA index of turnover. One-way ANOVA analysis followed by student Newman's Keul post-hoc test indicate a significant reduction ($P<0.05$) in DA levels and a significant elevation in DA index of turnover in the MPTP treated cohort, as compared to saline treated mice. The dose used in these studies is known to produce small changes in the nigrostriatal system of the 129/SvJ mice (Dauer et al., 2002). Young WT mice did not present DA reduction in striatum one week after a challenge with 25 mg/Kg of MPTP (two 12.5 mg/Kg i.p. injections 8 hours apart) (Figure 10A). However, we found that young *OGG1* KO mice had an increased vulnerability with a 35% reduction in striatal DA levels one week after MPTP exposure (Figure 10 B). The reduction in DA is paralleled by a reduction in TH-positive staining in SN of MPTP-treated *OGG1* KO mice (Figure 10 D) as compared to TH-positive staining in SN of MPTP-treated WT mice (Figure 10 C)

4 Discussion

Our results suggest that elevated levels of oxo⁸dG due to deficient DNA repair capacity are a major player in the vulnerability of the nigrostriatal pathway in relationship to aging and to a challenge with MPTP. The notion that accumulation of oxidative damage to DNA is deleterious to specific neuronal populations, particularly those found in the nigrostriatal pathway, is supported by the age-dependent loss of TH-positive neurons, reduction in striatal dopamine, histopathological evidence, and the evidence of intraneuronal ubiquitin-positive inclusions only found in the aged *OGG1* KO mice. The decreased spontaneous motor behavior found in aged *OGG1* KO mice suggests that the modulatory effect of DA neurotransmission is affected by nigrostriatal pathway damage. Further support comes from the *OGG1* expression-dependent susceptibility to MPTP.

Relevant clinical findings in PD patients are not restricted to the nigrostriatal pathway. For instance, systemic deficiencies such as decreased mitochondrial complex I activity have been found in patients with PD (Mizuno et al., 1989; Schapira et al., 1989). The mitochondrial complex I deficiency seen in PD was exploited to generate an animal model that reproduces deficits seen in PD. Rats chronically treated with the complex I inhibitor rotenone show neurochemical and histological changes similar to those seen in PD (Betarbet et al., 2000). Our results, attained by using a mouse deficient in the ability to remove the oxidatively modified base oxo⁸dG, fall into this category of whole system impact coupled with specific vulnerability of the nigral neurons, suggesting a role for oxo⁸dG accumulation prior to neuronal loss. Clinical findings of systemic increased oxo⁸dG levels in cases of PD (Abe et al., 2003; Alam et al., 1997; Sanchez-Ramos, 1994; Sato et al., 2005) are in agreement with our findings and lend support to the hypothesis that increased oxo⁸dG levels are precursors in neuronal loss. Reproduction of some of the deficiencies seen in PD using the DNA repair deficient mouse, *OGG1* KO, suggests that accumulation of oxo⁸dG is a primary event in dopaminergic neuronal loss. Relevant to the results we have presented, *in*

vitro studies show that chronic treatment with the mitochondrial complex I inhibitor rotenone leads to free radical production and oxidative damage to DNA (Beretta et al., 2006; de Lima et al., 2005).

Although Ogg1 has been extensively studied in the context of cancer, little is known of its activity in the central nervous system as a function of aging, neuropathological changes, or as a result of exposure to neurotoxicants. Recent reports demonstrate that Ogg1 is neuroprotective in neurotoxicological paradigms that impact the nigrostriatal pathway (Cardozo-Pelaez et al., 2005; Wong et al., 2008) similar to the results presented in our studies. Also, mice lacking the nucleotide-cleansing enzyme MTH1 exhibit increased levels of oxidative damage to RNA and DNA and are more susceptible to a challenge with MPTP (Yamaguchi et al., 2006). However, no assessment of the impact of aging has been done in the context of increased oxidative burden to DNA. Other investigators point that under pathological conditions that mimic ischemia in cellular and animal models Ogg1 activity ameliorates the neurodegenerative process and acts as neuroprotective mechanism (Liu et al., 2011). In conjunction with our results, all these studies support a major role for DNA damage in neurodegenerative processes.

The histological assessment and the lack of a gene- or age-dependent loss of striatal glutamate in the aged mice points to a specific impact to the dopaminergic neurons of the SN. It has been established that the metabolic pathway involved in DA removal leads to increased oxidative stress (Chiueh et al., 1993; Fahn and Cohen, 1992). In addition, we have previously shown that during aging the nigrostriatal pathway accumulates more oxo⁸dG than other brain regions (Cardozo-Pelaez et al., 1999). Consequently, this normal oxidative stress endogenous to the nigrostriatal pathway, coupled to impaired DNA repair in *OGG1* KO, could render a population of dopaminergic neurons more susceptible to aging and toxicological challenge.

Results from genetic manipulation studies attempting to reproduce genetic anomalies found in familial cases of PD lend additional support to the role of oxidative stress in the neurological deficit. In particular, there is evidence of increased levels of oxo⁸dG in some of these models. Two -synuclein mutations, A30P and A53T, have been associated with the development of PD (Kruger et al., 1998; Polymeropoulos et al., 1997). Mice and cell cultures overexpressing the most toxic A53T form show evidence of oxidative stress, and in cell cultures its toxicity correlates with increased levels of oxo⁸dG (Giasson et al., 2002; Lee et al., 2001; Lee et al., 2002). The lack of overt -synuclein-positive aggregates in the aged *OGG1* KO mice and the increased oxo⁸dG levels found in models harboring -synuclein mutations suggest that -synuclein aggregates are upstream of oxo⁸dG accumulation in the neurodegenerative cascade. Future studies of mice with *Ogg1* deficiencies and -synuclein mutations could help to evaluate this possibility.

An understanding of the mechanisms associated with neuronal loss after an MPTP challenge has aided the development of PD therapeutics and PD animal models (Greenamyre et al., 2003; MacMahon and Bland, 1996; Olanow et al., 1996). We have shown that *OGG1* KO mice are more susceptible to the toxic effects of MPTP than WT mice. This suggests a direct relationship between burden of damage to DNA and neuronal vulnerability. These findings could help to understand neurodegenerative mechanisms not previously addressed by other models and facilitate novel therapeutic advancements.

The susceptibility to toxic agents in the *OGG1* KO mice is not restricted to MPTP. We have previously reported that Ogg1 plays an important role in protecting dopaminergic neurons against a neurotoxicological challenge with manganese (Cardozo-Pelaez et al., 2005). Exposure to manganese has been associated with the development of Parkinson-like

deficiencies; however, most exposure protocols have failed to reproduce the effects of manganese in rodents (Newland, 1999; Pappas et al., 1997). Our previous manganese experiments contrast such findings and point to oxidative stress, specifically oxo⁸dG levels, as an indicator of neuronal health and susceptibility to toxins.

5 Conclusion

Mice lacking the enzyme Ogg1 show pathologic changes in the nigrostriatal system. This deficiency is age-dependent and appears to be localized in dopaminergic neurons of the SN. In addition, these mice show an age-dependent bradykinetic phenotype and an increased susceptibility to a challenge with MPTP. These results support the hypothesis that free radical mechanisms are heavily involved in the process of neurodegeneration and that oxidative damage to DNA affects neuronal populations, specifically the dopaminergic neurons of the SN. This mouse model of mild PD could be used to propose novel mechanisms of neurodegeneration and for the development of antioxidant therapies affording neuronal protection. Thus, our findings of increased vulnerability of the nigrostriatal system to neurotoxins such as manganese and MPTP, along with the age-dependent loss of SN neurons and intraneuronal ubiquitin accumulation in the *OGG1* KO, lead us to infer that the *OGG1* KO mice are a unique model for the neuronal loss seen in PD. Alternatively, it is possible to speculate that genetic or environmentally induced changes in *OGG1* expression or activity can predispose an individual to the effects of other environmental or genetic factors, eventually leading to neuronal loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Mice Lacking the DNA repair enzyme Ogg1 present an age-dependent Parkinson Disease phenotype

Dopaminergic neurons in OGG1 KO mice have a higher susceptibility to the neurotoxin MPTP

Loss of dopamine neurons during aging is accompanied by the increased formation of ubiquitin positive inclusions

This reports supports the role of oxidative damage to DNA and the risk of Parkinson's disease

Fig. 1.

(A) oxo⁸dG levels in DNA from caudate putamen (CP) of aged (26 month old) WT (white bar) and *OGG1* KO (black bar) mice. Data expressed as mean $\pm$ SEM. *Significant difference as compared to age-matched control values ($p < 0.05$; $n = 8-9$). (B) Microphotograph of oxo⁸dG immunostaining in the substantia nigra (SN) of aged (26 months old) WT and *OGG1* KO mice.

Fig. 2. Open field test in aged WT (white bars) and *OGG1* KO (black bars) mice. A) Line crossings; B) Mean speed; C) Number of rearings; D) Time immobile. * denotes significant difference from WT mice ($p < 0.05$; $n = 10$). Insets are data for same analysis in young WT (white bars) and *OGG1* KO (black bars) mice.

Fig. 3.

A) Striatal DA levels in young WT (white bar) and *OGG1* KO (black bar); **B)** Striatal DA levels in aged WT (white bar) and *OGG1* KO (black bar) mice. **C)** number of TH-positive neurons in SN aged WT (white bar) and *OGG1* KO (black bar) mice. **D)** Low magnification of TH-positive neurons in SN of WT (left) and *OGG1* KO (right) aged mice. Data expressed as mean $\pm$ SEM. *Significant difference as compared to age-matched control values ($p < 0.05$; $n = 6$).

Fig. 4. High magnification of TH-positive neurons in the substantia nigra (SN) of WT (left) and *OGG1* KO (right) aged mice. Neurons in the aged *OGG1* KO mice exhibit increased levels of atrophy, vacuolization, and cytosolic condensation (arrow head). Long arrow shows a typical healthy TH-positive neuron in the SN of the aged WT mice. Scale Bar 50 μ m.

Fig. 5. Representative pictures of hematoxylin and eosin staining in the substantia nigra (**A, B**), caudate putamen (**C, D**) and hippocampus (**E, F**) of aged WT (**A, C, E**) and *OGG1* KO (**B, D, F**) mice. Long arrows show nuclear location, indicating nuclear condensation in the SN of the *OGG1* KO. Open arrowhead shows a chromatolytic neuron with a displaced nucleus. Scale Bar 75 μ m.

Fig. 6. Glutamate levels in the caudate putamen of WT (white bars) and *OGG1* KO (black bars) mice at 3 and 26 months of age. Data expressed as mean $\pm$ SEM (n=5-7).

Fig. 7. Representative microphotographs of the substantia nigra (**A, B**) and ventral tegmental area (**C, D**) of aged WT (**A, C**) and *OGG1* KO (**B, D**) stained for ubiquitin. Long arrows denote neuronal localization; arrowheads identify intraneuronal ubiquitin aggregates. Scale Bar 50 μ m.

Fig. 8. Microscopic assessment of age-associated accumulation of ubiquitin-positive intraneuronal inclusions in the WT and *OGG1* KO mice. Representative photographs of the substantia nigra of WT (A,C,E) and *OGG1* KO (B,D,F) mice. Arrow heads identify intraneuronal ubiquitin aggregates. Scale Bar 50 m.

Fig. 9.
Representative confocal micrograph of green Nissl NeurTrace staining (green) and ubiquitin positive immunostaining (red) in the SN of aged *OGG1* KO mouse.

Fig. 10. Assessment of MPTP susceptibility in 3 month old wild-type (**A**) Striatal DA levels in young WT treated with saline (white bar) or with MPTP (black bar) (**B**) Striatal DA levels in young *OGG1* KO treated with saline (white bar) or with MPTP (black bar). Data expressed as mean $\pm$ SEM, *Significant differences from saline treated control. ($p < 0.05$; $n = 6-8$) (**C**) TH-positive staining in young WT after MPTP treatment (**D**) TH-positive staining in young *OGG1* KO after MPTP treatment