Additional *polgD257A* mutation (mutator) does not influence dopaminergic neurodegeneration in aged parkin-deficient mice

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**Abstract**

**Background:** Parkinson's disease is a neurodegenerative disease caused by the loss of dopaminergic neurons in the substantia nigra pars compacta. Among the first identified causes of autosomal recessive Parkinson's disease were mutations in the parkin gene. Independently, we and other groups have developed various parkin knockout mice, and none displayed dopaminergic degeneration in the substantia nigra. Interestingly, dopaminergic degeneration in the substantia nigra has been reported in a parkin knockout line (exon 3 deletion) carrying an additional mutation (D257A) in the mitochondrial DNA polymerase  (polg) gene (mutator). The mutator mice show accelerated mutation rates in mitochondrial DNA resulting in a premature-aging phenotype.

**Methods:** In order to verify this finding, we crossed our parkin-deficient mice with the mutator mice, and characterized phenotypic changes of the parkin/mutator double mutant mice up to one year of age. We examined their locomotion and motor coordination behaviours by using the open field, the rotarod and the pole test, susequently investigating their nigrostriatal axis by counting TH-positive cells in every tenth section throughout the entire substantia nigra pas compacta and their termini in the striatum.

**Results:** The double mutants did not display additional deficiencies in locomotion in our behaviour tests. We could also not detect dopaminergic neurodegeneration in the substantia nigra pars compacta of aged double mutants measured by levels of tyrosine hydroxylase positive neurons in the substantia nigra pars compacta as well as in striatal terminals.

**Conclusion:** Our results do not support the hypothesis that the polgD257A mutation contributes to the age-related vulnerability of dopaminergic neurons in parkin-deficient mice.

**Key words:** parkin; neurodegeneration; polgD257A ; mutator; substantia nigra

**Introduction:**

Parkinson´s disease (PD) is characterized by a progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc), leading to basal ganglia dysfunction and subsequent impairment of movement control. To date 26 PD risk loci have been identified [1], providing substantial PD-research advances via the generation of transgenic mouse models. One of the first genetic variations found in PD patients was the exon 3 deletion in the *parkin* gene, coding for an E3 ubiquitin ligase [2]. However all parkin knockout mouse strains did not show hint of loss of DA neurons in the SNc [3, 4]. The reason for the missing neurodegeneration in those mouse models is not understood. One could assume that the mouse DA neurons in SNc are exposed to less aging, since mice have a shorter lifespan. This explanation is supported by findings that the age-dependent mutation rate of mitochondrial DNA (mtDNA) increases much more in human SNc compared to murine tissue [5]. Such age-dependent accumulation of mtDNA mutations, as well as a broad spectrum of aging-like phenotypes have been observed in the polgD257A mice (mutator; MT) by a knock-in proofreading-deficient version of the mtDNA polymerase  [6]. Utilizing this mouse model, Pickrell et al. 2015 reported age-dependent degeneration of DA neurons in the SNc in aged MT/Parkin KO double mutants [7].

In this study, we intended to verify this finding by crossing MT mice with our parkin-deficient Padel mice [8]. We followed possible phenotypic changes of MT/Padel double mutant mice up to one year of age, but could not find any additional deficiency in locomotion, nor dopaminergic neurodegeneration in the SNc compared to MT and wild type mice. Our results do not support the hypothesis that the polgD257A mutation contributes to the age-related vulnerability of dopaminergic neurons in parkin-deficient mice.

**Materials and methods**

Animals

All animal experiments were conducted and approved using the German guidelines and in accordance with the animal care and use committee of the state of North-Rhine Westphalia. Mice were kept under standard conditions with free access to water and food and a 12:12 hrs day/night cycle.

The parkin knockout Padel carries a deletion for the exon 3 in the *parkin* gene, and the *neo* gene was removed by crossing with the *Deleter* followed by backcrossing with C57BL/6N mice for over 20 generations [8]. The Bl6 congenic Mutator mice (MT) were acquired from the Jackson Laboratory (B6.129S7(Cg)-Polgtm1Prol/J, Strain #:017341). Animals of both sexes were used for all behaviour and histological studies.

Behaviour tests

The rotarod test was conducted on a rod starting with 4 rpm accelerating to 40 rpm within 240 s and the latency to fall was measured (RotaRod, TSE, Germany).

For the pole test mice (12 months old) were placed on top of a metal pole (height: 55 cm; diameter: 8 mm), and the time until the animals reached the ground was measured. Further, it was noted when the animals fell or slipped off the pole.

For the open field tests, the mice were placed in a plastic box (40 x 40 x 40 cm) for 30 min. Their travelled distance, number of stops, time of rest and the time spend in the center of the box (20 x 20 cm) were recorded by the Videomot2 software (TSE, Germany).

Immunohistochemistry

Mice were perfused transcardially with 4% formaldehyde. Brains were removed and dehydrated with ethanol and isopropanol followed by embedding in paraffin. Coronal brain sections (10 µm) of the SN and striatum were made (RM2145, Leica, Germany). These sections were deparaffinized and rehydrated followed by incubation with the primary antibody (anti-TH; 1:1000; Merk Millipore; ab152). Subsequently, sections were incubated with the secondary antibody (biotinylated anti-rabbit IgG; 1:300; Vector Laboratories; BA-1000) followed by incubation with an avidin/biotin complex solution (vectastain® elite ABC-HRP kit; Vector Laboratories) and stained with diaminobenzidine.

Quantifications and statistical analysis

Every tenth consecutive sections containing TH positive cells within the entire SN were counted using the software ImageJ. The staining intensity units of TH positive axonal terminals in the striatum was analysed with CellProfilerTM (Module: MeasureImageIntensity). All statistical analysis with H- and u-tests were performed with the software SigmaPlot 14.0 (Systat Software).

**Results**

Four homozygous genotypes (wild type (WT) littermates (LM): WT/WT; MT: polg-/-; Padel: Padel-/-; MT/Padel: polg-/-/ Padel-/-) were used to examine phenotypic changes up to an age of 12 months, since the median lifespan of homozygous MT is 416 days [6]. We note that MT and MT/Padel mice started to lose body weight when the animals approached 8 months of age (Fig. 1A), consistent with published results [7]. In addition, we also observed spleen enlargement (Fig. 1B) and shorter lifespan (Fig. 1C) of MT/Padel mice, which was not different to that of MT mice. Based on those parameters we confirmed the premature aging phenotype of the MT/Padel mice.

Next, we investigated whether the combination of the polgD257A mutation with the parkin deletion may contributed to additional motor impairments that could indicate loss of DA-neurons in SNc. Six-month-old mice were tested monthly, up to 12 months of age in the rotarod task. Consistent with our previous results [4], the Padel and WT mice showed similar rotarod performance (Fig. 1D). While the MT mice showed low performance constantly, the MT/Padel mice showed an age-dependent, continuous decline in latency to fall (Fig. 1D). However, there was no difference in the rotarod performance between MT and MT/Padel mice at the age from 7 to 12 months, indicating that the motor impairment of the aged MT/Padel mice was dependent only on the polgD257A mutation.

Analogues to the rotarod task, mice were tested in the open field starting at the age of six months to examine locomotor activity and habituation behaviour. Young WT mice exhibited locomotion habituation in the open field over time, showing reduced levels of activity on the second day compared to the first day, while our Padel mice displayed a delayed habituation which was visible on the third day (Fig. 2A, left). The same aged MT and MT/Padel mice exhibited reduced locomotor activity at the first day. However, the delayed habituation behaviour could be observed only in MT/Padel mice, but not in MT mice (Fig. 2A, left). This result indicates that the delayed habituation was caused by the parkin deletion alone, while the polgD257A mutation was responsible for the reduced locomotor activity of the MT/Padel mice. A similar phenomenon was also observed in the second parameter of the open field: The time in the center. Interestingly, the MT/Padel mice seem to spend more time in the center region of the open field compared with the WT mice. However, this effect was dependent on the polgD257A mutation only (Fig. 2A, right). While the WT and Padel mice exhibited relatively constant locomotor activity when they were re-exposed to the open field, two groups of these mice with the polgD257A mutation showed an age-dependent decline in total travelled distance and increased resting time, regardless of the parkin deletion (Fig. 2B). This polgD257A-dependent effect could also be observed in stops, a parameter reflecting initiation of movement (Fig. 2A+B).

So far, our behaviour tests showed severe impairment of MT/Padel double mutant mice in motor coordination and in locomotor activity. However, this impairment seems to be caused by the polgD257A mutation alone, since there was no difference between animals with or without the parkin deletion. These results contradict the finding reported by Pickrell et al., 2015 [7] using the pole test, an alternative behaviour test for dopamine-dependent motor coordination. In order to examine this discrepancy, we performed the pole test using a similar protocol described by Pickrell et al. 2015 [7], and found significantly higher latency time descending the pole for MT/Padel double mutants compared with WT or Padel mice (Fig. 1E). However, MT mice displayed similar higher latency time that was statistically not different to those of MT/Padel double mutants (Fig. 1E). This polgD257A-dependent impairment was also detected in the number of mice that fell off or slid down the pole (Fig. 1F). This result is consistent with those observed in our rotarod and the open field test, and demonstrate impairment of aged MT/Padel double mutant mice in locomotor activity and motor coordination, which is dependent on the polgD257A mutation only.

After the last behavioural test at the age of 12 months, mice were sacrificed to determine the integrity of their nigrostriatal axis by counting TH-positive cells in every tenth section throughout the entire SNc. Mice with all four genotypes displayed similar counts of TH-positive neurons in their SNc (Fig. 3A+C). In order to determine if there was any change regarding the nigrostriatal axis in the MT/Padel mice, we also counted their intensities of the TH-staining in the striatum, and compared them with Padel, MT and wildtype mice. We were not able to find any significant difference of striatal TH staining intensity between all four groups (Fig. 3B+D). Our results demonstrate that those behavioural impairments of the MT and MT/Padel mice in locomotor activity and motor coordination were likely not due to dopamine deficiency in their nigrostriatal axis.

**Discussion**

Pickrell et al., 2015 reported that the MT/Parkin KO mice exhibited age-dependent degeneration of DA neurons in the SNc and motor deficit in the pole test [7]. By crossing MT mice with our Padel mice we intended to verify this finding with an independent mouse strain carrying the same exon 3 deletion of the *parkin* gene. Some characteristic features for premature aging, e.g. lost body weight, spleen enlargement, and short lifespan could also be confirmed in the MT/Padel mice, which were indistinguishable from those of MT mice (Fig. 1A–C). However, there are several discrepancies in motor behaviours between the results of the pole test in both studies. We observed similar impairment of MT mice in latency time descending the pole with or without the parkin deletion (Fig. 1E), while the MT mice did not exhibit such motor impairment in the study of Pickrell et al. [7]. Because their severe aging phenotypes, it seems less likely that the aged MT mice are still able to perform such complex motor task on a wild type level. Indeed, a study by Hauser et al., 2015 demonstrated that the old MT mice tend to slide down or fall off instead of climb down the pole [9], which could be confirmed in our study (Fig. 1F). Additionally, we found that the slide down rates of MT (mean ± SEM: 28.33 ± 10.70 %) and MT/Padel (mean ± SEM: 35.91 ± 5.39 %) mice were significantly higher those of wild type (mean ± SEM: 10.65 ± 4.22 %) and Padel (mean ± SEM: 5.26 ± 2.23 %) mice (H- and u-test, p < 0.05). This higher slide down rate was not dependent on the parkin deletion (H- and u-test, p > 0.05), but on the MT mutation. Furthermore, impairment in locomotion and motor coordination have also been observed in two previous studies, when aged MT mice were tested in the open field and on the rotarod [10, 11]. In accordance with their results, we observed similar motor impairments for the aged MT/Padel mice in both tests, most likely independent from the parkin mutation.

Consistent with our behaviour results, no significant difference was found in the number of the TH positive cell bodies in the SNc and their terminals in the striatum between wild type, Padel, MT and MT/Padel mice in our study (Fig. 3). Those observed motor deficits in the aged MT mice seem not to be related with functions of the nigrostriatal axis. This result is also supported by the fact that L-dopa could not restore normal motor behaviour of the MT mice [9]. Altogether, we were not able to confirm the protective function of parkin against degeneration of DA neuron in the SNc of the MT mice described by Pickrell et al., 2015. Although the Parkin KO strains used in both studies share a similar deletion of the exon 3 in the parkin gene, there are few differences in their genomic region surrounding the deletion site. While a 34 base pair loxP remains in this region in our Padel mouse, the Parkin KO strain used in the study of Pickrell et al. carried the neo-resistant gene with an additional GFP coding sequence [3]. We speculate that the additional big DNA fragment coding for two functional proteins under a strong promoter may cause DA neurons more susceptible to degeneration during aging in the MT background with deficient parkin function. It is also known that the neo-resistant gene can result in altered expression of other genes as much as 100 kilo bases distant in the locus downstream of the pGK NEO insertion, since its excision from the locus results in dramatically different phenotypes [12].

Our results are similar to other studies that have crossed MT mice with other mouse models for PD. The double mutant of MT with DJ-1 KO had no effect on numbers of TH positive cell in the SNc up to 12 months of age [9]. Likewise, treatment of MT mice with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin mouse model for PD, did not result in an additional degeneration of DA neurons in the nigrostriatal axis [13] (Dai et al., 2014). With the same MT/Parkin KO, parkin deficiency has no effect on the cardiac hypertrophy observed in aged MT mice [14]. Several studies have shown that aged MT mice carry mtDNA deletions in the SNc to a similar extent as that found in human, but with no difference between PD patients and controls [15]. Altogether, this suggests that the absence of parkin was unlikely to have a strong effect on survival of SNc DA neurons in aged MT mice, and contradict results found by Pickrell et al., 2015. A difference of genetic background around the exon 3 deletion site in both parkin KO strains seems to be the most likely explanation for these conflicting results. Therefore, at least two independent strains should be included in such investigations in future.

**Conclusion**

Our results do not support the hypothesis that the polgD257A mutation contributes to the age-related vulnerability of dopaminergic neurons in parkin-deficient mice.

**Availability of data and materials:** The data that support the findings of this study are available from the corresponding author, Zhu XR., upon request.

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**Conflicts of interest:** The author declares that there are no conflicts.

**Ethical approval and consent to participate:** Not applicable.

**Consent for publication:** Not applicable

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**Legends**

**Figure 1:** MT and MT/Padel mice displayed similar phenotypical effects. (**A**) Body weight (mean ± SEM) of Padel mice at the age of 6 months was lower than wildtype littermates (LM) (u-test: p < 0.05), increased after 8. Month of age (H-test: p < 0.05), which was then not different from that of LM (u-tests: p > 0.05). MT and MT/Padel mice started to lose body weight when the animals approached 8 months of age (H-tests: p < 0.001), which was different from LM and Padel (u-tests: p < 0.05). There were no differences between MT and MT/Padel mice (u-tests: p > 0.05). (**B**) MT and MT/Padel mice (12 months old) showed similar enlargement of spleen compared to LM and Padel (mean ± SEM; u-test: p > 0.05 for MT vs MT/Padel). (**C**) MT and MT/Padel mice exhibited similar survival rates in Kaplan-Meier analysis (log-rank test: p > 0.05). (**D**) LM and Padel mice exhibited similar constant performance (mean ± SEM) on the rotarod (u-tests: p > 0.05). MT mice showed low performance, compared to LM and Padel (u-tests: p < 0.05). MT/Padel mice showed an age dependent, continuous decline in performances (H-test: p < 0.001). No difference was found between MT and MT/Padel for 8 - 12 months. (u-tests; p > 0.05). (**E**) LM and Padel mice displayed similar latencies (mean ± SEM) in the pole test (u-test: p > 0.05). MT and MT/Padel mice showed reduced performance compared to LM and Padel (u-tests: p < 0.05). No differences were found between MT and MT/Padel (u-test: p > 0.05). (**F**) MT and MT/Padel mice exhibited a higher percentage of trials in which they fell off or slid down the pole during the pole test (mean ± SEM). No difference was found between MT and MT/Padel mice (u-test; p > 0.05). (**A-F**) Numbers in parentheses indicate numbers of analysed animals. H- or u-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

**Figure 2:** MT and MT /Padel mice exhibited similar phenotypical changes in the open field test. (**A**) Naïve mice (6 – 7 months old) were exposed to the open field for 30 min on three consecutive days. (Left) The MT and MT/Padel mice showed reduction of the travelled distance on the day 1, while no difference was found between LM and Padel mice (u-test: p > 0.05). LM and MT mice displayed similar habituation behaviour by showing reduced travelled distance on the day 2 and 3, while a delayed habituation on the day 3 of the test was found for Padel and MT/Padel animals. (Middle) Averaged number of stops within 30 min was lower with MT mice compared to LM. No difference was found between MT and MT/Padel (u-test: p > 0.05). (Right) MT and MT/Padel mice showed similarly increased amounts of time spend in the center compared to LM and Padel. No differences found between MT and MT/Padel (u-test: p > 0.05). (**B**) MT and MT/Padel mice showed similar successive shortening of travelled distances (left), similarly less initiative movements (as stops) (middle), and similar increased resting time (right), when the animals were re-tested monthly in the open field up to 12 months of age. (**A-B**) All data: mean ± SEM, Numbers in parentheses indicate numbers of analysed animals. H- or u-test: \* p < 0.05; \*\*; p < 0.01 \*\*\* p < 0.001.

**Figure 3:** No significant morphological difference in nigrostriatal axis between wildtype (LM), Padel, MT and MT/Padel mice. (**A**) Representative images of TH-stained coronal brain sections containing the substantia nigra pars compacta (SNc), substantia nigra pars reticulata, (SNr) and ventral tegmental area (VTA) at the age of 12-months. (**B**) Representative images of TH-stained coronal brain sections containing the striatum for each of those four genotypes at the age of 12 months. (**C**) Similar TH positive cell counts (mean ± SD) in the SNc (H-test; p > 0.05) (**D**) Similar TH-staining intensity units (mean ± SD) in the striatum between four genetypes (H-Test; p > 0.05). (**A**-**B**) scale bars: 100 µm. (**C**-**D**): Numbers in parentheses indicate numbers of analysed animals.