BRAIN COMMUNICATIONS

To be or not to be pink(1): contradictory findings in an animal model for Parkinson's disease

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The PTEN-induced putative kinase 1 knockout rat ($Pink1^{-/-}$) is marketed as an established model for Parkinson's disease, characterized by development of motor deficits and progressive degeneration of half the dopaminergic neurons in the substantia nigra *pars compacta* by 8 months of age. In this study, we address our concerns about the reproducibility of the $Pink1^{-/-}$ rat model. We evaluated behavioural function, number of substantia nigra dopaminergic neurons and extracellular striatal dopamine concentrations by *in vivo* microdialysis. Strikingly, we and others failed to observe any loss of dopaminergic neurons in 8-month-old male $Pink1^{-/-}$ rats. To understand this variability, we compared key experimental parameters from the different studies and provide explanations for contradictory findings. Although $Pink1^{-/-}$ rats developed behavioural deficits, these could not be attributed to nigrostriatal degeneration as there was no loss of dopaminergic neurons in the substantia nigra and no changes in neurotransmitter levels in the striatum. To maximize the benefit of Parkinson's disease research and limit the unnecessary use of laboratory animals, it is essential that the research community is aware of the limits of this animal model. Additional research is needed to identify reasons for inconsistency between $Pink1^{-/-}$ rat colonies and why degeneration in the substantia nigra is not consistent.

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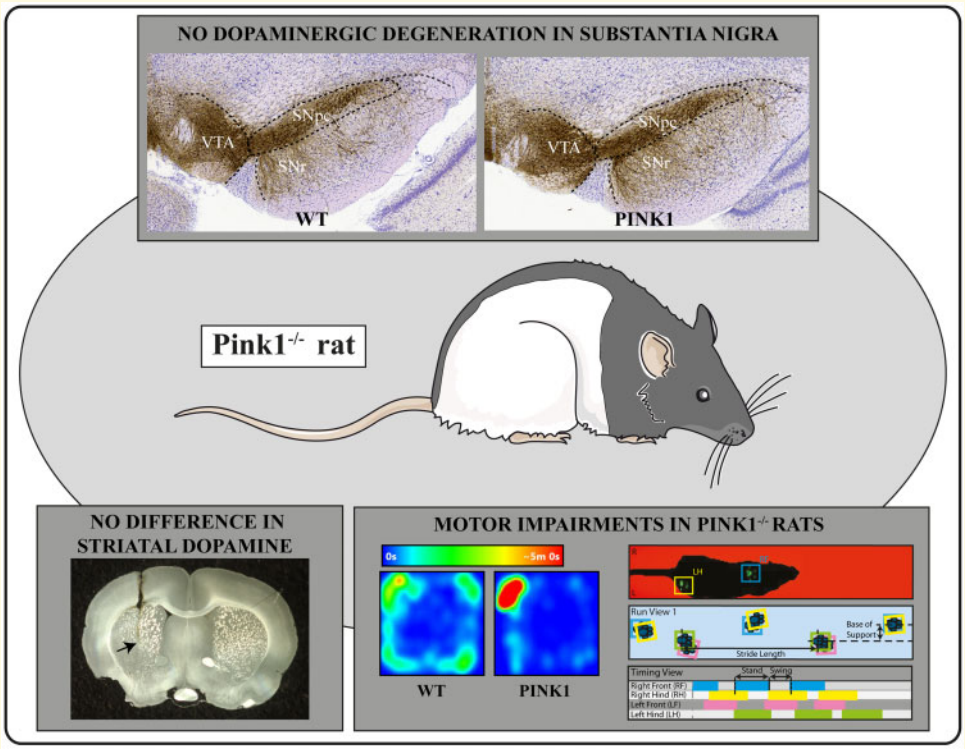
Abbreviations: DA = dopamine; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; PINK1 = PTEN-induced putative kinase 1; SNpc = substantia nigra *pars compacta*; TH = tyrosine hydroxylase; WT = wild type

Graphical Abstract



Introduction

Parkinson's disease is a progressive and disabling neurological disorder for which only symptomatic treatments are available (Kalia *et al.*, 2015). The preferential loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* (SNpc) is a defining feature. Familial forms of Parkinson's disease can be caused by mutations in several genes, including PTEN-induced putative kinase 1 (PINK1), which encodes a mitochondrial serine/threonine-protein kinase that regulates mitochondrial quality control (Youle and van der Bliek, 2012; Hernandez *et al.*, 2016; Kasten *et al.*, 2018). Failure of the mitochondrial quality control system and increased levels of reactive oxygen species are reported to cause neurodegeneration (Lin and Beal, 2006; Schon and Przedborski, 2011; Rugarli and Langer, 2012; Pathak *et al.*, 2013). To study the pathophysiology of Parkinson's disease and to explore new therapeutic approaches, there is an urgent need for suitable genetic rodent models (Creed and Goldberg, 2018). The commercially available Pink1 knockout rat model (Pink1$^{-/-}$ rat) generated by Zinc Finger Nuclease Technology is (together with the DJ-1 knockout rat) the only available genetic rodent model reported to show selective loss of DA neurons in the SNpc and, when created, was expected to be of tremendous value to the field (Baptista *et al.*, 2013; Dave *et al.*, 2014). Dave *et al.* (2014) performed phenotypic characterization of the Pink1$^{-/-}$ rat and showed significant age-related decreases in the number of tyrosine hydroxylase (TH) positive neurons in the SNpc by TH immunohistochemistry. Compared with healthy wild type (WT) rats, at the age of 6 and 8 months, there was an average reduction in neurons of 25% and 50%, respectively. Pink1$^{-/-}$ rats also exhibited significant behavioural dysfunction, which manifested as reduced rearing and locomotor activity in the open field, impaired hindlimb strength, an increased number of foot slips on the balance beam and abnormal gait (Dave *et al.*, 2014). However, unlike Parkinson's disease, levels of dopamine and serotonin were both significantly increased in the striatum at the age of 8 months, seemingly at odds with the model's claimed characteristic of DA cell loss. This was described as a possible functional presynaptic compensatory effect by a yet unknown mechanism. A recent imaging study hypothesized that a reorganization of the anatomical connectivity in the brain occurs to compensate for the loss of DA neurons in the SNpc (Cai *et al.*, 2019; Ferris *et al.*, 2018). In this study,

we investigated the reproducibility of the Pink1$^{-/-}$ rat model by evaluating behavioural dysfunction, loss of DA neurons in the SNpc and extracellular striatal dopamine concentrations in 8-month-old Pink1$^{-/-}$ male rats. Our and others' results call into question the reproducibility of the DA-specific neurodegeneration phenotype initially observed in these rats.

Material and Methods

Animals

Initial breeding pairs of homozygous Pink1$^{-/-}$ knockout rats were purchased by Horizon Discovery SAGE (LE-*Pink1*$^{em1Sage-/-}$) and Long-Evans (Crl:LE) rats were purchased from Charles River Laboratories International, Inc. Breeding was sustained in our central animal facility (CDL, Nijmegen, The Netherlands). Animals were group housed (two to three animals per cage) under controlled conditions (temperature 20–22°C and humidity 50–70%) with free access to standard food (Ssniff GmbH, Soest, 76, Germany, V1534 R/M-H, 10 mm pellet) and water. Animals were randomly divided (by using http://www.randdom.org) in either the behavioural group (housed on a reversed 12 h light/dark cycle, lights off at 7 am) or microdialysis group (housed on a normal 12 h light/dark cycle, lights on at 7 am). Randomization of the housing conditions was performed by manual randomization of the location of the cages within the animal room (rack/shelf). Only male rats at the age of 8 months were used (body weight range 550–650 g). The experiments carried out in this study (including sampling, outcome assessment and analysis) were performed blinded. Animal care givers were also blinded during day-to-day animal care concerning genotype and animal group. Ethical approval was obtained by the Committee for Animal Care and Experimental Use of the Radboud University Medical Center Nijmegen, The Netherlands (ref. no. 2015-0132). Experiments were carried out in agreement with the Dutch laws, ARRIVE guidelines and the European Communities Council Directive (2010/63/EU).

Genotyping

Ear punches were taken and used for genotyping to confirm the deletion of the Pink1 gene. The method was performed according to the previously described procedure (Dave *et al.*, 2014). Detailed information can be found in the Supplementary material.

Immunohistochemistry

Brains were dissected ($n = 8$ per genotype) and post fixed overnight in 4% paraformaldehyde. Using MultiBrain technology (NeuroScience Associates), brains were embedded into a gelatine block, flash frozen and cryosectioned to obtain 40 µm coronal sections through the entire striatum and substantia nigra. Immunohistochemistry was performed with anti-TH antibody (Pelfreeze Cat#P40101-0, dilution 1:6000) and counterstained with Thionine every eighth section spaced at 320 µm intervals. Quantification of TH-positive neurons in the SNpc was performed by two independent methods: unbiased stereology (performed by Charles River Discovery Research Services Finland Ltd.) and total counts per section using ImageJ software in our laboratory. Both methods were performed blinded with respect to genotype.

For stereology, the optical fractionator method was used to estimate the total number of TH-positive cells throughout the SNpc (between −4.56 and −6.60 mm from bregma). TH-positive cells were counted manually with Stereo Investigator software (MicroBrightField, VT, USA). The counting frame was 50 × 50 µm and the grid was 200 × 200 µm. Counting was performed bilaterally ($n = 4$ per genotype). For each animal, a total of five stained sections covering the entire SNpc were used for counting.

For counts on ImageJ software, TH-positive cells were quantified in five to seven sections encompassing the entire SNpc (Bregma −4.56 to −6.60 mm). The SNpc was identified at a low magnification (4×) and bilateral counts for each section were averaged over the total number of sections per animal ($n = 8$ per genotype).

In vivo microdialysis

Pink1$^{-/-}$ ($n = 5$) and WT rats ($n = 6$) underwent stereotactic surgery under isoflurane gas anaesthesia for the implantation of a guide cannula (AG-6; with dummy probe AD-6 and cap nut AC-5, Eicom, USA) in the dorsal striatum (coordinates: 0.5 mm anterior to bregma, 3.0 mm lateral to midline and 4.0 mm lower from the skull). After a minimum recovery period of 1 week, the dummy probe was replaced by a microdialysis probe (A-I-6-02), with 2-mm membrane length. Microdialysis was performed during their inactive phase (housed on a normal 12 h light/dark cycle, lights on at 7 am) according to the previously described procedure (Homberg *et al.*, 2007; Verheij *et al.*, 2008). Extracellular DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations were measured by liquid chromatography with mass spectrometry (LC-MS/MS) (details can be found in the Supplementary material).

Behavioural paradigms

Pink1$^{-/-}$ and WT rats ($n = 8$ per group) were tested during their active phase (housed on a reversed 12 h light/dark cycle, lights off at 7 am) in three tests of motor behaviour and performance: the tapered balance beam, open field activity and CatWalk. Each rat was tested daily for three consecutive days. The tapered balance beam test was performed according to the experimental

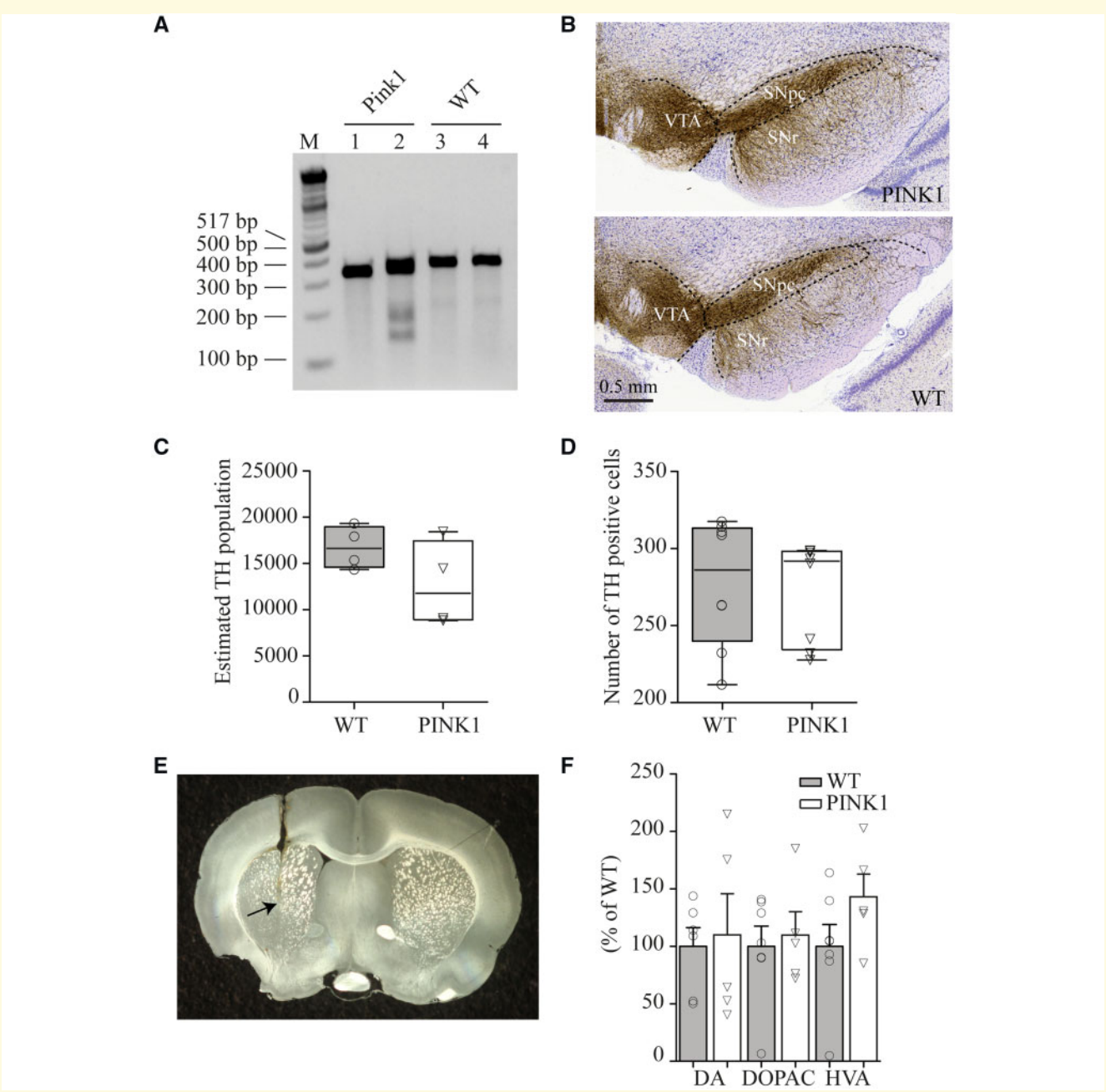


Figure 1 Pink1$^{-/-}$ rats do not show DA degeneration at 8 months of age. Genotyping confirmed absence of the Pink1 gene in Pink1$^{-/-}$ rats (**A**). Pink1 and WT amplicons were annealed either alone (lanes 1 and 3) or in combination with the wild-type amplicon (lanes 2 and 4). Immunohistochemistry showed no difference in TH reactivity in the SNpc between Pink1$^{-/-}$ and WT rats (**B**). Quantification of the TH-positive cells were performed by stereology [one-way ANOVA $F(1,6) = 2.446$, $P = 0.169$] (**C**) and counting using Image J [one-way ANOVA $F(1,14) = 0.088$, $P = 0.771$] (**D**) (each boxplot includes: minimum, first quartile, median, third quartile and maximum). Extracellular neurotransmitters concentrations were measured in the dorsal striatum (**E**) and showed no difference between Pink1$^{-/-}$ and WT animals [multivariate ANOVA, DA: $F(1,9) = 0.072$, $P = 0.795$, DOPAC: $F(1,9) = 0.076$, $P = 0.790$, HVA: $F(1,9) = 1.459$, $P = 0.258$) (**F**). Neurotransmitter concentrations are expressed relative to WT (bars indicate mean ± SEM, circles and triangles indicate individually value per animal).

set-up and procedure previously described (Dave *et al.*, 2014). The mean number of foot slips and mean run duration of three consecutive trials were determined by video analysis. Distance moved (cm) during 1 h in a 50 × 50 cm open field arena was measured by using the behavioural tracking software ANY-maze. Grooming and rearing frequency were determined by blinded observers using video recordings. Gait analysis was performed by using the CatWalk (Noldus, Wageningen, The Netherlands), an automated system to assess gait

abnormalities objectively in rodents (de Haas *et al.*, 2016, 2017). Animals were habituated to the task for 3 days prior to the testing day. Data from six consecutive test runs were averaged for each animal. The following gait parameters were evaluated: stand duration (duration of contact of a paw with the glass plate), stride length (distance between successive placements of the same paw), swing duration (duration of no contact of a paw with the glass plate), swing speed, maximum contact [maximum contact at (s) relative to stand], maximum intensity [maximum intensity at (s) relative to stand] and base of support (average width between either the front paws or the hind paws).

Statistical analysis

Data were analysed by IBM SPSS Statistics 25 software. Univariate and multivariate ANOVAs were used for group comparisons.

Data availability

The data that support the findings of this study are available from the corresponding author, upon request.

Results

Genotyping and neurochemical findings

PCR genotyping confirmed the deletion of the Pink1 gene in our colony of Pink1$^{-/-}$ rats (Fig. 1A). Mutant (Pink1$^{-/-}$) and WT amplicons were annealed either alone (lanes 1 and 3) or in combination with the WT amplicon (lanes 2 and 4). In lane 2, no homo-duplex between Pink1$^{-/-}$ and WT could be formed and two cleavage products of approximately 206 and 133 base pairs were present, confirming the absence of Pink1. Immunohistochemistry was performed to examine TH reactivity in the SNpc of Pink1$^{-/-}$ and WT rats at the age of 8 months. Quantification was performed blinded by two independent methods, stereology and counting using Image J. Both methods showed no statistical difference in the number of TH-positive neurons in the SNpc between Pink1$^{-/-}$ and WT (Fig. 1B–D).

Extracellular DA, DOPAC and HVA concentrations in the dorsal striatum were determined by *in vivo* microdialysis followed by LC-MS/MS in Pink1$^{-/-}$ and WT animals at the age of 8 months. Position of the microdialysis probe was confirmed by histology (Fig. 1E). No differences in DA, DOPAC or HVA concentrations were found between the two genotypes (Fig. 1F).

Behavioural findings

Despite observing no loss of DA neurons in the SNpc, the Pink1$^{-/-}$ animals did show behavioural deficits assessed by open field, balance beam and gait analysis. Pink1$^{-/-}$ rats displayed significantly reduced rearing frequency and locomotor activity in the open field (Fig. 2A–C). Run duration on the balance beam was significantly increased in Pink1$^{-/-}$ animals and they showed a trend for more footslips ($P = 0.10$; Fig. 2D and E). To investigate gait abnormalities in more detail, the animals were tested on the CatWalk, an automated gait analysis system (Noldus, Wageningen, the Netherlands). Explanation of the gait parameters can be found in the Material and Methods section and the schematic overview (Fig. 2F). Pink1$^{-/-}$ animals had decreased run speed (Fig. 2G) with increased stand and swing duration, maximum contact and base of support for both front and hindlimbs (Table 1). Stride length and swing speed were also decreased in Pink1$^{-/-}$ rats (Table 1).

Discussion

In this study, we were unable to replicate the loss of DA neurons in the SNpc or changes in neurochemical levels in the striatum of male Pink1$^{-/-}$ rats at 8 months of age. However, behavioural deficits measured by open field, balance beam and gait analysis were in line with previous findings (Dave *et al.*, 2014).

Recently, an *in vivo* microdialysis study showed significant age-dependent decreased basal dopamine levels in the striatum of Pink1$^{-/-}$ rats at 12 months compared with 4 months of age (Creed *et al.*, 2019). However, in line with this report, no significant differences in basal DA, DOPAC and HVA striatal levels were found between 8-month-old Pink1$^{-/-}$ and WT animals (Creed *et al.*, 2019). These findings align with the equal numbers of TH-positive neurons in the substantia nigra shown here of Pink1$^{-/-}$ and WT rat at the age of 8 months.

During the course of our study three other publications reported contrary results concerning the loss of DA neurons (Dave *et al.*, 2014; Grant *et al.*, 2015; Villeneuve *et al.*, 2016; Orr *et al.*, 2017). The paper by Villeneuve *et al.* (2016) was in support of dopamine neuron loss in the SNpc in Pink1$^{-/-}$ rats, whereas the other two studies were at variance with this observation (Grant *et al.*, 2015; Orr *et al.*, 2017). Grant *et al.* (2015) reported early and progressive ultrasonic vocalization and oromotor deficits in the Pink1$^{-/-}$ rats. At the age of 8 months, TH-positive soma numbers were significantly reduced in the locus coeruleus. However, no loss of TH immunoreactivity was observed in the SNpc or the striatum. Likewise, Orr *et al.* (2017) showed no difference in either the total number of dopamine neurons in the SNpc, or in the density of dopamine terminals in the striatum in Pink1$^{-/-}$ versus WT rats at 8 months of age.

To understand the variable levels of DA neuron loss in different colonies of Pink1$^{-/-}$ rats, we compared key experimental parameters from these studies including source of animals, age and immunohistochemistry methodology

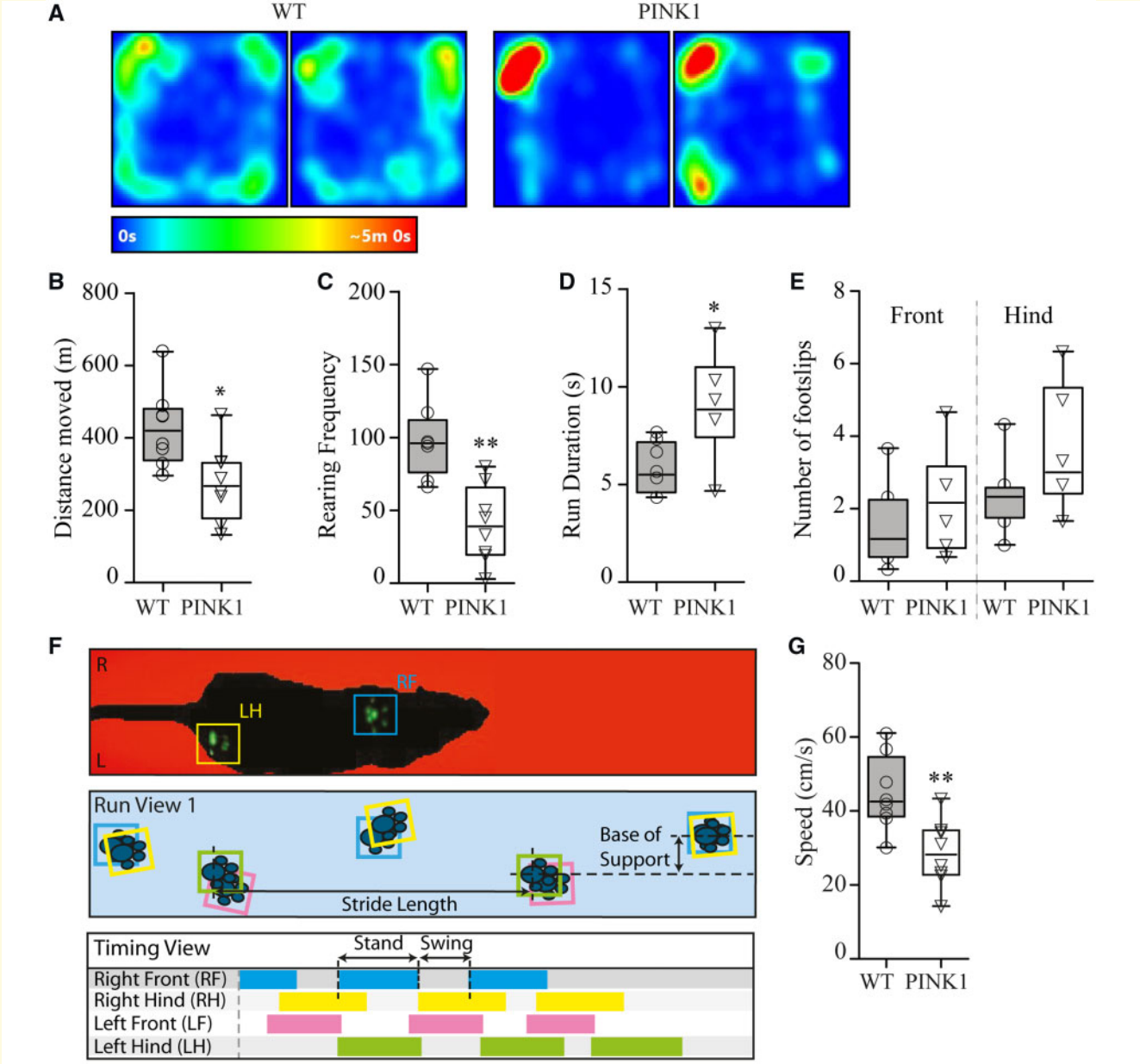


Figure 2 Pink1$^{-/-}$ rats have motor impairments at 8 months of age. Pink1$^{-/-}$ rats showed significantly reduced locomotor activity in the open field indicated by the representative heat maps and total distance moved [one-way ANOVA $F(1,14) = 8.256$, $P = 0.012$] (**A**, **B**). Rearing frequency was significantly reduced in Pink1$^{-/-}$ rats compared with WT rats [one-way ANOVA $F(1,14) = 19.574$, $P = 0.001$) (**C**). Run duration on balance beam was significantly longer in Pink1$^{-/-}$ compared with WT rats (**D**) and number of footslips (front and hind were scored separately) showed no significant differences (**E**) [multivariate ANOVA, run duration: $F(1,12) = 8.439$, $P = 0.013$, front: $F(1,12) = 1.114$, $P = 0.312$, hind: $F(1,12) = 3.135$, $P = 0.102$). A schematic overview depicting the CatWalk parameters (**F**). CatWalk run speed was significantly reduced in Pink1$^{-/-}$ compared with WT rats [one-way ANOVA $F(1,14) = 11.378$, $P = 0.005$] (**G**). Each boxplot includes: minimum, first quartile, median, third quartile and maximum, *P*-values *≤0.05, **≤0.01.

(Table 2). The study by Villeneuve *et al.* did not specify the origin or gender of the rats that were studied, or the source of the anti-TH antibody used. Comparing the studies, it is unlikely that differences in immunohistochemistry or quantitation methods resulted in their discrepancies. Antibody source can be ruled out since our study, Dave *et al.* and Orr *et al.* all used the same anti-TH antibody, yet conflicting results were obtained. Similarly, both this report and that of Dave *et al.* used NeuroScience Associates for tissue processing (Table 2). Further, we validated our quantification of DA cell numbers by two independent methods: TH-positive cell counting using ImageJ in our laboratory and unbiased stereology (Fig. 1C and D), the two methods employed in

Table 1 CatWalk data expressed as mean (SD) for WT and Pink1$^{-/-}$ rats at the age of 8 months for right front (RF), right hind (RH), left front (LF) and left hind (LH) limb or for both front and hind limbs (ANOVA *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

	WT, mean (SD)				PINK1, mean (SD)			
CatWalk Parameter	**RF**	**RH**	**LF**	**LH**	**RF**	**RH**	**LF**	**LH**
Stand (s)	0.19 (0.03)	0.23 (0.06)	0.20 (0.04)	0.22 (0.05)	0.36 (0.19)*	0.35 (0.09)**	0.35 (0.17)*	0.32 (0.11)*
Stride length (cm)	17.5 (1.7)	17.4 (2.0)	17.5 (1.5)	17.1 (2.4)	14.5 (2.2)**	14.3 (2.0)**	14.5 (2.3)**	13.5 (3.5)*
Swing (cm/s)	134 (20)	146 (24)	127 (15)	143 (22.4)	100 (16)**	133 (43)	102 (13)**	130 (45)
Swing (s)	0.14 (0.03)	0.12 (0.01)	0.15 (0.02)	0.12 (0.02)	0.18 (0.08)	0.14 (0.09)	0.17 (0.07)	0.15 (0.08)
Max. contact (s)	41 (5)	27 (5)	40 (4)	27 (2)	51 (7)**	35 (6)**	55 (7)***	32.6 (7.01)*
Max. intensity (a.u.)	61 (5)	56 (4)	61 (4)	54 (6)	58 (6)	60 (5)	59 (4)	54 (10)
	Front		Hind		Front		Hind	
Base of support (cm)	2.2 (0.4)		3.38 (0.39)		3.33 (0.51)***		3.97 (0.60)*	

Table 2 Relevant information from studies investigating dopamine loss in Pink1$^{-/-}$ rats by TH stain

References	Animal origin	Gender	Age	TH primary antibody	Coordinates	Quantification method	Loss of DA neurons
Dave *et al.* (2014)	WT: Charles River (Crl:LE) PINK1: Sage labs	M	8 months	Pelfreeze Cat#P40101-0, dilution 1:6000	−2.54 to −3.88 mm from bregma	Stereology	Yes
Grant *et al.* (2015)	WT: Sage Labs PINK1: Sage labs	M	8 months	EMD Milipore AB152, AB_390204, dilution 1:2000	−4.56 mm from bregma	Image J Cell counter	No
Villeneuve *et al.* (2016)	WT: Long-Evans Hooded (LEH) PINK1: Origin not specified	Gender not specified	9 months	Specific TH antibody (Abcam), no further information	−4.36 to −6.72 mm from bregma	Stereology	Yes
Orr *et al.* (2017)	WT: Sage Labs PINK1: Sage labs	M	8 months	Pelfreeze Cat#P40101-0, dilution 1:1000	−4.5 to −6.5 mm from bregma	Stereology (blinded)	No
de Haas *et al.* (current study)	WT: Charles River (Crl:LE) PINK1: Sage labs	M	8 months	Pelfreeze Cat#P40101-0, dilution 1:6000	Between −4.56 and −6.60 mm from bregma	Stereology and counting by Image J (blinded)	No

the other studies. Notably, the quantification of TH cell counts was performed blinded with respect to genotype only in our studies (this report and Orr *et al.*). All studies used similar coordinates for counting TH cells except for Dave *et al.*, but this is likely an error since their reported coordinates match those for the SN in mouse (Paxinos, George and Keith B.J. Franklin. The mouse brain in stereotaxic coordinates: hard cover edition). Based on the similarities in quantification methodologies among the studies, it is unlikely that the majority could have missed an expected 50% reduction in TH-positive cells.

Despite these comparisons, we were unable to pinpoint a clear reason for the discordant findings using this model, though we can suggest a few possible explanations. One is that only a subpopulation of the Pink1$^{-/-}$ rats develops dopamine neuron loss in the SNpc. It is also unclear whether the Pink1$^{-/-}$ rats originate from the same source colony or whether this colony is large enough to minimize genetic drift, which can cause spontaneous mutations. There could have also been variation in the progression of neurodegeneration within the strain. It may, therefore, be necessary to evaluate SNpc degeneration beyond the age of 8 months. Notably, the Villeneuve *et al.* study used 9-month-old rats and observed a 50% loss of TH-positive neurons in the SNpc. Environmental and internal factors can play a role in the progression of neurodegeneration, such as housing conditions, stress, microbiological status, microbiome and food. Importantly, in our study, Pink1$^{-/-}$ rats did show abnormal motor behaviour; however, this is not controlled exclusively by SNpc function. This abnormal motor behaviour may occur in advance of the neuronal loss, possibly due to synaptic dysfunction. The underlying mechanism of the behavioural dysfunction remains unclear at present and deserves further investigation.

In conclusion, the Pink1$^{-/-}$ rat model does not reliably result in the loss of SNpc DA neurons, an important hallmark of Parkinson's disease. Because it is marketed as an established model for motor impairments and DA cell loss observed in Parkinson's disease, it is important for investigators interested in the neurodegenerative phenotype to be aware of the varying results obtained by different research groups. We encourage researchers to validate the model in their own laboratories and

potentially age the animals beyond the commonly used 8-month timepoint to verify reproducible SNpc degeneration. In addition, we believe a comprehensive effort assaying degeneration at older ages and across sites should be undertaken to resolve discrepancies in dopamine neuron loss in the SNpc. Though time-consuming, this effort will limit unnecessary costs and prevent the needless use of laboratory animals. If consistent age-dependent degeneration can be demonstrated, the Pink1$^{-/-}$ rat model would likely become a valuable tool for studying both motor dysfunction and DA degeneration in Parkinson's disease.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests. J.A.M.S. is the founding CEO of Khondrion B.V.

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