



Dopamine neuron dysfunction and loss in the *Prkn*R275W mouse model of juvenile parkinsonism

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Mutations in the *PRKN* gene encoding the protein parkin cause autosomal recessive juvenile parkinsonism (ARJP). Harnessing this mutation to create an early-onset Parkinson's disease mouse model would provide a unique opportunity to clarify the mechanisms involved in the neurodegenerative process and lay the groundwork for the development of neuroprotective strategies. To this end, we created a knock-in mouse carrying the homozygous *Prkn*R275W mutation, which is the missense mutation with the highest allelic frequency in *PRKN* patients. We evaluated the anatomical and functional integrity of the nigrostriatal dopamine (DA) pathway, as well as motor behaviour in *Prkn*R275W mice of both sexes.

We report here that *Prkn*R275W mice show early DA neuron dysfunction, age-dependent loss of DA neurons in the substantia nigra, decreased DA content and stimulus-evoked DA release in the striatum, and progressive motor impairment. Together, these data show that the *Prkn*R275W mouse recapitulates key features of ARJP. Thus, these studies fill a critical need in the field by introducing a promising new Parkinson's disease model in which to study causative mechanisms of the disease and test therapeutic strategies.

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Introduction

Mutations in the *PRKN* gene, encoding for the protein parkin, are the most common cause of autosomal recessive juvenile parkinsonism (ARJP) (*PRKN*, OMIM 600116).¹ The disease is characterized by the progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc),² leading to dopamine (DA) depletion in the striatum and motor symptoms such as bradykinesia, resting tremor and rigidity.¹ Median age at onset is ~31 years.¹ More than 100 different *PRKN* mutations have been found, the most frequent being exon rearrangements resulting in premature stop codons, transcript degradation and absence of parkin protein. Disease-causing mutations also include single-nucleotide substitutions resulting in missense, nonsense, or splice-site mutations.³ (<https://www.mdsgene.org>).

Five mouse lines carrying *Prkn* exon deletions have been described.^{4,5} These mice show mitochondrial dysfunction and proteomic changes but no overt DAergic neuron loss or a parkinsonian phenotype.^{4,5} The lack of a phenotype is surprising since selective *Prkn* deletion in the midbrain of adult mice or expression of the human variant parkinQ311X in DAergic neurons leads to age-dependent DAergic neuron degeneration in the SNc.^{6–8} It is therefore possible that germline *Prkn* deletion leads to genetic compensation, a phenomenon frequently observed in null germline mutations.^{9–11}

To reproduce the human ARJP condition without modifying the DNA copy number and thereby avoid genetic compensation, we created a knock-in mouse carrying the p.Arg275Trp (R275W) mutation, i.e. the missense mutation with the highest allelic frequency in *PRKN* patients (<https://www.mdsgene.org/d/1/g/4>).³ The mutation introduced in the murine DNA allows the expression of missense parkin variants observed in ARJP patients under the endogenous promoter. Here, we evaluated the anatomical and functional integrity of the nigrostriatal pathway of homozygous *Prkn*R275W mice.

Materials and methods

The full methodology and the study limitation section are provided in the [Supplementary material](#).

Results

Generation of the *Prkn*R275W mouse

Using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology, we created a knock-in mouse model expressing the *PRKN* mutation p.Arg275Trp (R275W). The R275 amino acid in the human cDNA corresponds to the R274 amino acid in the *Prkn* mouse orthologue (Ensembl gene ID: ENSMUSG00000023826), thus we inserted the analogous R274W mutation in the mouse *Prkn* gene (Fig. 1A–C).

Homozygous *Prkn*R275W mice were born at expected Mendelian frequencies, appeared normal, were viable and fertile. Real-time PCR on brain lysates at 1 month of age revealed similar *Prkn* mRNA levels in wild-type (WT) and *Prkn*R275W mice, indicating that the mutation did not induce *Prkn* mRNA downregulation

(Fig. 1D). However, brain lysates of *Prkn*R275W mice showed significantly reduced levels of endogenous parkin protein when compared with WT (Fig. 1E and [Supplementary Figs 1–3](#)), in agreement with previous studies showing that the R275W mutation results in a conformational alteration in parkin protein and a loss-of-function phenotype.¹² Levels of other brain proteins, including those involved in glutamatergic and GABAergic synaptic transmission, evaluated in cortical, cerebellar and hippocampal lysates, did not differ significantly between *Prkn*R275W and WT mice ([Supplementary Figs 2 and 4–6](#)).

Dysfunction of substantia nigra pars compacta dopaminergic neurons in young *Prkn*R275W mice

We counted the number of DAergic neurons (tyrosine hydroxylase positive, TH+) and total neurons (identified by Nissl staining, Nissl+) in the SNc using unbiased stereology.

At 1 month of age, WT and *Prkn*R275W mice had similar TH+ and Nissl+ neuron counts (Fig. 2A) and showed no motor deficits ([Supplementary Fig. 7](#)). However, examination of SNc DAergic neurons from *Prkn*R275W mice at higher magnification revealed an increased number of cytoplasmic vacuoles. The average number of vacuoles per SNc DAergic neuron section was 0.4 (range 0–2) in WT and 2.8 (range 0–10) in *Prkn*R275W mice (Fig. 2B and [Supplementary Figs 8 and 9](#)). As a negative control, we analysed DAergic neurons in the ventral tegmental area (VTA), a brain area that does not undergo extensive degeneration in Parkinson's disease.¹³ One to two vacuoles per cell section were observed in a small subset of DAergic neurons in the VTA from WT and *Prkn*R275W mice at 1 month of age, with no difference between genotypes ([Supplementary Fig. 10](#)). Therefore, cytoplasmic vacuolization is a feature of SNc DAergic neurons of *Prkn*R275W mice. Similar cytoplasmic vacuolization has been found in isogenic human DAergic neurons bearing *PRKN* mutations¹⁴ and in SNc DAergic neurons of the parkinQ311X mouse.⁷ To investigate morphological abnormalities in SNc DA neurons of *Prkn*R275W mice, we analysed mitochondria in SNc DAergic neurons using transmission electron microscopy (TEM). In WT specimens, most mitochondria showed physiological tubular morphology, whereas *Prkn*R275W samples displayed disruptions in mitochondrial ultrastructure, including inner membrane herniation, cristae enlargement and outer membrane disruption (Fig. 2C). These abnormalities suggest early dysfunction of SNc DAergic neurons in *Prkn*R275W mice.

To confirm dysfunctional SNc DAergic neurons, *in vivo* recordings of the spontaneous activity of SNc DAergic neurons were carried out in 1-month-old mice. The tonic firing activity tended to be lower in *Prkn*R275W mice than in WT mice, but the difference did not reach statistical significance (Fig. 2D and [Supplementary Fig. 11](#)). However, the percentage of spikes that occurred in bursts was lower in *Prkn*R275W mice (Fig. 2D).

These results point to early pathophysiological changes in SNc DAergic neurons of *Prkn*R275W mice.

Catecholaminergic neuron degeneration, striatal DA loss and motor deficits in adult *Prkn*R275W mice

To test the hypothesis that the changes in DA neuron morphology and function observed in young mice precede DAergic neuron death,

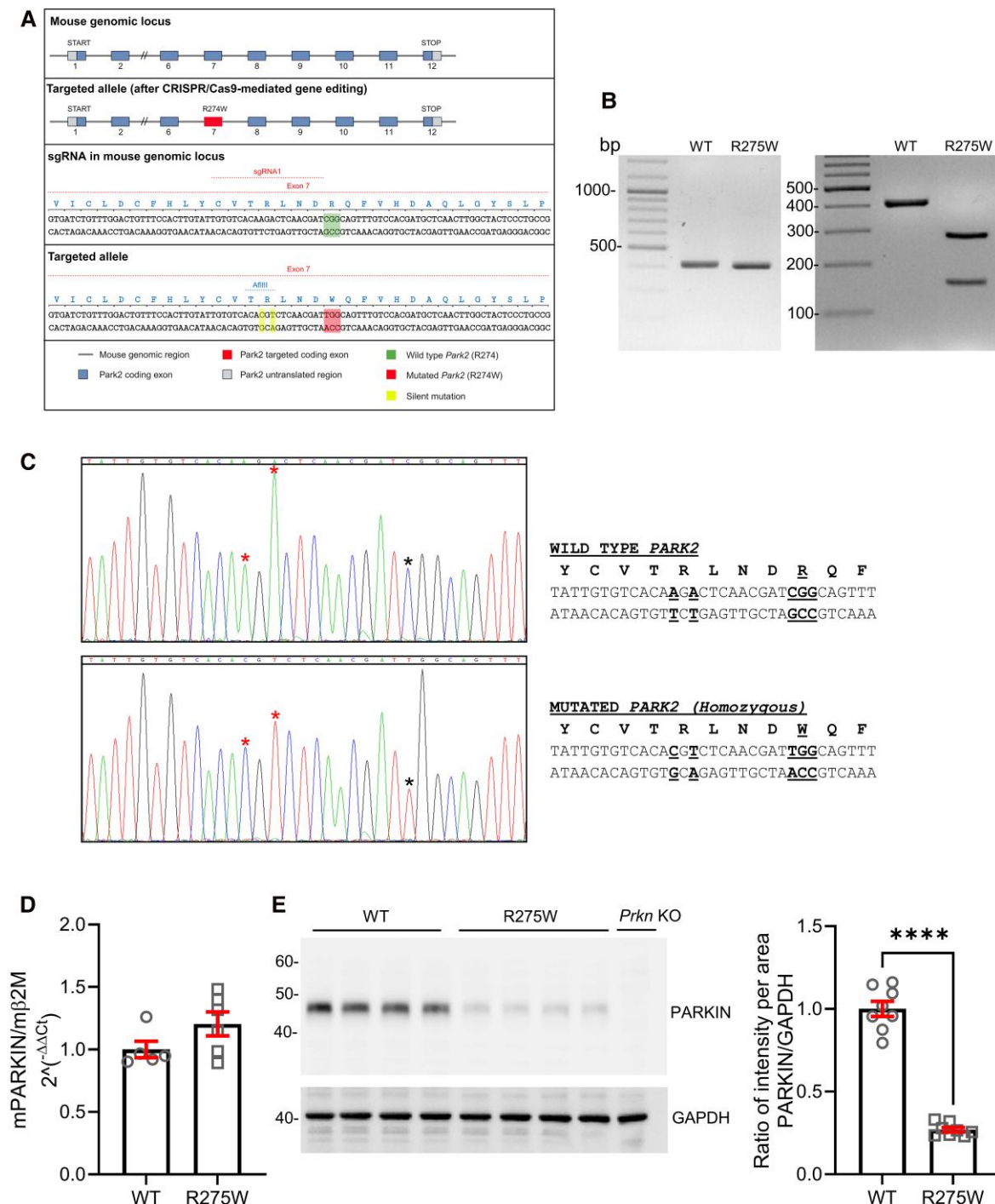


Figure 1 Generation of the *Prkn*R275W mouse. (A) The image shows the strategy used for generating a constitutive knock-in of a point mutation in the *Prkn* gene using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology. In the *Prkn* mouse orthologue (Ensembl gene ID: ENSMUSG00000023826), we introduced the R274W missense mutation because in the *Prkn* mouse orthologue, the amino acid R274 corresponds to human R275. Alongside the exchange of the first nucleotide in the R274 codon, two silent mutations were introduced close to the site of the mutation to create a novel AflIII restriction site that allows for genotyping of the mutated allele. Image was created with Biorender.com. (B) Left: Representative image of the result of a PCR with primers that amplify exon 7 in the *Prkn* locus containing the mutated codon. The expected fragment was 424 bp. Right: AflIII digestion of this PCR product resulted in cleavage of the 424 bp PCR product in two fragments (279 and 145 bp) selectively in the mutated allele. DNA ladder is 100 bp. (C) Sanger sequence analysis of exon 7 in the *Prkn* locus in genomic DNA from wild-type (WT) mouse and mouse homozygous for the R275W mutation. The black asterisk labels the missense mutation. Red asterisks indicate the silent mutation introduced to create the AflIII restriction site. (D) Quantitative real-time PCR performed on mRNA prepared from total mouse brains at 1 month of age with TaqMan probes specific for *Prkn* mRNA and for the housekeeping *Beta-2 microglobulin* ($\beta 2 M$) mRNA. The level of endogenous *Prkn* mRNA was normalized on $\beta 2 M$. The levels of *Prkn* mRNA in WT and homozygous *Prkn*R275W mice were virtually identical ($n = 5$ brains from WT mice, $n = 6$ brains from *Prkn*R275W mice, two-tailed unpaired t-test; $P > 0.05$). (E) Representative western blot performed on lysates from mouse brains at 1 month of age with an antibody specific for parkin (monoclonal antibody P6248—Sigma-Aldrich). *Prkn*R275W mice showed significantly reduced endogenous parkin protein levels when compared with WT [1-month-old (1 mo) WT 1.00 ± 0.05 versus 1 mo *Prkn*R275W 0.27 ± 0.01 , two-tailed unpaired t-test; **** $P < 0.0001$; $t = 15.22$, $df = 14$, $n = 8$ brains from WT mice, $n = 8$ brains from *Prkn*R275W mice]. Negative control is a brain protein lysate from *Prkn* knockout mouse.

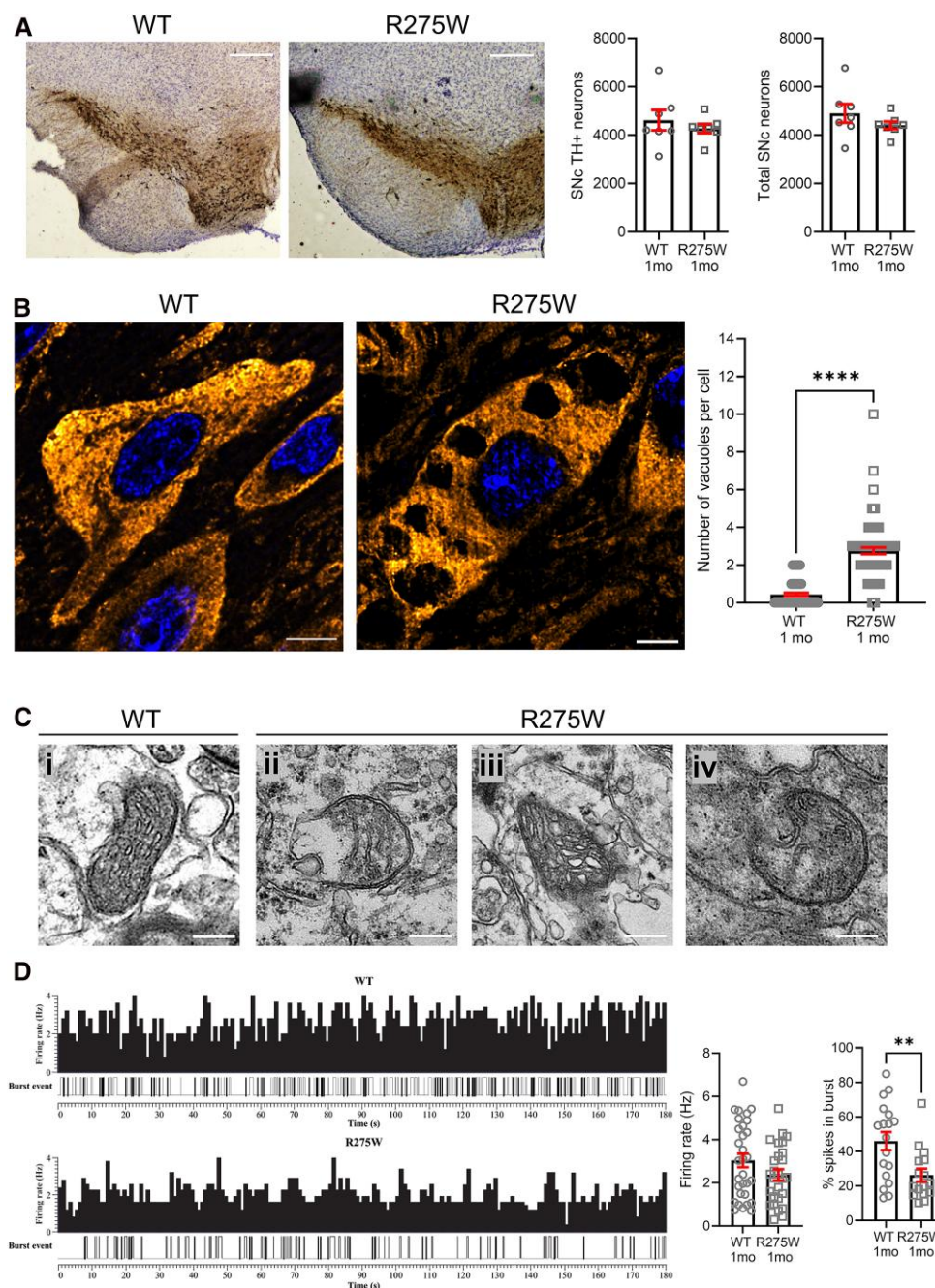


Figure 2 Dopaminergic neuron dysfunction in young (1-month-old) *Prkn*R275W mice. (A) Representative images showing immunoperoxidase (tyrosine hydroxylase, TH) labelling in the substantia nigra pars compacta (SNc) of wild-type (WT) and *Prkn*R275W mice at 1 month of age (scale bar = 100 μ m). Dopaminergic (DAergic) neuron quantification performed by unbiased stereology counting of TH+ neurons and Nissl+ neurons is shown on the right. At 1 month of age, the number of SNc DAergic neurons was similar in WT and *Prkn*R275W mice (WT 4618 ± 419 versus *Prkn*R275W 4276 ± 190 , $n = 7$ mice analysed for each genotype. Nissl+ neurons WT 4903 ± 386 versus *Prkn*R275W 4407 ± 159 , $n = 7$ mice analysed for each genotype; two-tailed unpaired t-test; $P > 0.05$). (B) Representative DeepSIM super-resolution images of DAergic neurons (TH labelling in OrangeHot) in the SNc of WT and *Prkn*R275W mice at 1 month of age at $\times 100$ magnification (scale bar = 5 μ m). The graphs show the number of vacuoles counted per cell ($n = 75$ cells analysed for each genotype, three mice for each genotype, Mann–Whitney test, $P < 0.001$). [C(i–iv)] Representative transmission electron microscopy images showing mitochondria morphology in 1-month-old WT and *Prkn*R275W SNc neurons (scale bar = 1 μ m). Most mitochondria in WT specimens showed normal tubular morphology (i), whereas mitochondria of *Prkn*R275W neurons displayed ultrastructure disruptions, including inner membrane herniation (ii), cristae enlargement (iii) and outer membrane disruption (iv). Data quantification showed that 80% of mitochondria in WT SNc neurons displayed normal morphology versus 48% in *Prkn*R275W neurons; 12% of mitochondria in WT SNc neurons displayed inner membrane damage versus 18% in *Prkn*R275W neurons; 8% of mitochondria in WT SNc neurons displayed outer membrane damage versus 34% in *Prkn*R275W neurons. Data derive from $n = 5$ sections analysed for each mouse, $n = 3$ mice for each genotype, chi-squared = 48.16, $P < 0.0001$, $df = 2$. (D) Representative histograms of spontaneous single-neuron firing rate activity in WT and *Prkn*R275W mice at 1 month of age. The data quantification is represented in the histograms on the right. The tonic firing activity tended to be lower in *Prkn*R275W mice than in WT mice, but the difference did not reach statistical significance (WT 3.04 ± 0.32 Hz versus *Prkn*R275W 2.37 ± 0.26 Hz, $n = 31$ SNc DAergic neurons from eight WT mice, $n = 28$ neurons from seven *Prkn*R275W mice, $P = 0.117$ unpaired Student's t-test). The percentage of spikes in burst was significantly lower in *Prkn*R275W than in WT mice (WT $46.0 \pm 5.2\%$ versus *Prkn*R275W $26.3 \pm 3.7\%$; $P = 0.0053$, $t = 2.996$, $df = 32$, unpaired Student's t-test). For the data illustrated, ** $P < 0.01$, **** $P < 0.0001$.

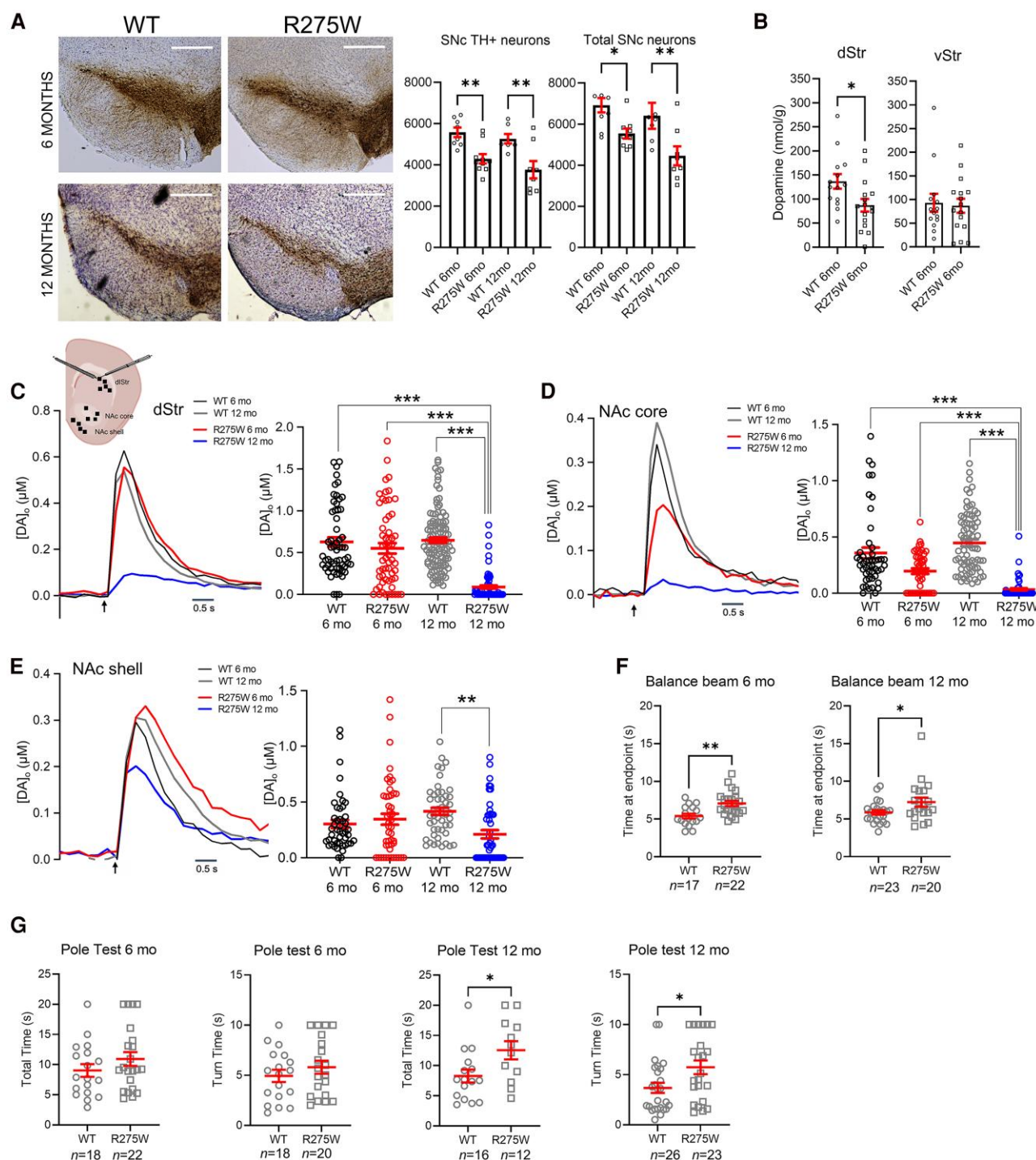


Figure 3 Dopaminergic neuron loss, decreased striatal dopamine content and release, and motor impairment in adult (6–12-month-old) *PrknR275W* mice. **(A)** Representative images showing immunoperoxidase (tyrosine hydroxylase, TH) labelling in the substantia nigra pars compacta (SNc) of wild-type (WT) and *PrknR275W* mice at 6 and 12 months of age (scale bar = 100 μm). The graphs on the right show TH+ neuron quantification performed by unbiased stereology [6-month-old (6 mo) WT 5578 ± 235 versus 6 mo *PrknR275W* 4298 ± 228, $P = 0.0055$, $n = 8$ WT and $n = 10$ *PrknR275W* mice; 12 mo WT 5266 ± 231 versus 12 mo *PrknR275W* 3764 ± 417, $P = 0.0029$, $n = 7$ WT and $n = 8$ *PrknR275W* mice, one-way ANOVA followed by Šidák's multiple comparisons test] and the number of Nissl+ neurons (6 mo WT 6919 ± 350 versus 6 mo *PrknR275W* 5543 ± 242, $P = 0.0426$, $n = 8$ WT and $n = 10$ *PrknR275W*; 12 mo WT 6404 ± 635 versus 12 mo *PrknR275W* 4457 ± 461, $P = 0.0079$, $n = 7$ WT and $n = 8$ *PrknR275W* mice, one-way ANOVA followed by Šidák's test). **(B)** Representative histograms of dopamine (DA) tissue content in dorsal striatum (dStr) and ventral striatum (vStr) of WT and *PrknR275W* mice at 6 months of age. *PrknR275W* mice showed a reduction of tissue DA content in dStr (nmol/g: 6 mo WT 136.7 ± 15.2 versus 6 mo *PrknR275W* 87.2 ± 13.2, $t = 2.464$, $df = 28$, $n = 14$ –16 samples from six mice per genotype, unpaired t -test, $P = 0.0201$), whereas no difference was seen in vStr (6 mo WT 93.1 ± 19.1 nmol/g versus 6 mo *PrknR275W* 87.0 ± 14.8 nmol/g, $P = 0.8861$, unpaired U -test; $n = 14$ –16 samples from six mice per genotype, $P > 0.05$). **(C)** Top: Representation of section of mouse brain showing typical level of forebrain slices used to study axonal DA release (modified from Franklin and Paxinos, ¹⁵). At this level, local electrical stimulation can be used to evoke DA release in dorsolateral striatum (dStr) or in nucleus accumbens

(continued)

we counted the number of TH+ neurons in the SNc of adult WT and *PrknR275W* mice. At 6 and 12 months of age, *PrknR275W* mice showed a 23% and 28% decrease in DAergic neurons, respectively (Fig. 3A). The decrease in DAergic neurons was confirmed by stereological count of Nissl+ neurons in the SNc (Fig. 3A). Since recent studies showed that SOX6 expression distinguishes DAergic neuron populations more vulnerable to Parkinson's disease-related degeneration in the SNc,^{16,17} we analysed the number of SNc SOX6+ neurons. At 12 months of age, *PrknR275W* mice showed a statistically significant reduction in double SOX6+/TH+ neurons, with 39% co-labelled DAergic neurons in WT mice and 21% in *PrknR275W* mice (Supplementary Fig. 12). Cytoplasmic vacuolization also increased significantly in SNc but not VTA DA neurons in 6- and 12-month-old *PrknR275W* versus WT mice (Supplementary Figs 8 and 10). TEM analyses confirmed mitochondrial abnormalities in DA neurons of adult *PrknR275W* mice (Supplementary Fig. 13). To characterize mitochondrial dysfunction further, transcriptomic analysis was performed by bulk RNA sequencing of isolated SNc tissue from 6-month-old WT and *PrknR275W* mice. Combined analysis showed 130 protein-coding differentially expressed genes (DEGs), with 73 that were upregulated and 57 downregulated. Gene ontology (GO) analysis revealed an enrichment of sets related to mitochondrial function (Supplementary Fig. 14). Thus, the *PrknR275W* transgenic strain recapitulates the degeneration and dysfunction of nigrostriatal DAergic neurons typical of ARJP patients.

Since neuropathological analyses of ARJP brains at autopsy showed an early loss of noradrenergic neurons in the locus coeruleus (LC),² we counted LC neurons in the brains of *PrknR275W* mice. Stereology indicated a 25%–30% loss of LC neurons (Supplementary Fig. 15). Additionally, as previously observed in human ARJP brains,^{2,18,19} SNc and LC degeneration occurred in the absence of α -synuclein accumulation or Lewy body formation (Supplementary Figs 16 and 17). Thus, these results confirm that *PrknR275W* mice recapitulate the neuropathology seen in ARJP.^{1,2,20}

To assess whether SNc DAergic neuron degeneration resulted in decreased tissue DA levels in target regions, we determined DA content in dorsal striatum (dStr) and ventral striatum (vStr) from 6-month-old *PrknR275W* and WT mice using high-performance liquid chromatography (HPLC). Consistent with the significant decrease in SNc DA neuron number, total tissue DA content in the dStr was significantly lower in *PrknR275W* mice compared to that in WT mice (Fig. 3B). No difference was observed in DA content of

the vStr, which contains nucleus accumbens (NAc) core and shell (Fig. 3B).

We also examined the influence of the *PrknR275W* mutation on dynamic DA release in *ex vivo* corticostriatal slices from 6- and 12-month-old mice. Fast-scan cyclic voltammetry (FSCV) was used to quantify evoked [DA]_o in the dorsolateral striatum (dlStr) and NAc core and shell (Fig. 3C, inset). Peak evoked [DA]_o in the dlStr did not differ between *PrknR275W* and WT mice at 6 months of age. At 12 months, however, significantly lower evoked [DA]_o was seen in the dlStr of *PrknR275W* versus WT mice, and was also lower than in 6-month-old *PrknR275W* mice (Fig. 3C). A similar pattern was also seen in the NAc core (Fig. 3D) and NAc shell (Fig. 3E), with no significance in evoked [DA]_o between WT and *PrknR275W* mice at 6 months, but significantly lower evoked [DA]_o in *PrknR275W* mice at 12 months. Consistent with the relative preservation of VTA neuron density in Parkinson's disease,¹³ DA release was better maintained in the NAc shell in 12-month-old *PrknR275W* mice than in either dlStr or NAc core in which evoked [DA]_o was often below detection limits (Fig. 3C–E).

To evaluate consequences of DAergic neuron loss and striatal DA depletion in adult *PrknR275W* mice, we analysed motor function in 6- and 12-month-old mice. *PrknR275W* mice were significantly impaired in the balance beam test (Fig. 3F). In the pole test, *PrknR275W* mice performed similarly to WT mice at 6 months, whereas at 12 months *PrknR275W* were impaired (Fig. 3G). In the rotarod test, 6- and 12-month-old *PrknR275W* mice performed similarly to their WT counterparts (Supplementary Fig. 18). No sex effect was observed in motor tests (Supplementary Fig. 19). These behavioural studies indicate that *PrknR275W* mice have deficits in fine balance and motor coordination already by 6 months of age.

Dopaminergic neuron loss and overt motor impairment in aged *PrknR275W* mice

By 18 months of age, *PrknR275W* mice showed a 40% decrease in DAergic neuron number in the SNc but not in VTA (Fig. 4A and Supplementary Fig. 10). Quantification of cytoplasmic vacuolization in SNc TH+ cells at 18 months showed a reduction in vacuolization compared to that seen at earlier ages (Supplementary Fig. 8), suggesting that DAergic neurons with vacuoles may be the primary population that dies. Eighteen-month-old *PrknR275W* mice performed significantly worse than WT mice in the rotarod and

Figure 3 (Continued)

(NAc) core and shell in the same *ex vivo* slice. The curve shows the average evoked [DA]_o (single-pulse stimulation) in dlStr. Arrow indicates time of stimulation. In 6-month-old mice, peak evoked [DA]_o did not differ between the two genotypes (6 mo WT: $0.63 \pm 0.05 \mu\text{M}$ versus 6 mo *PrknR275W*: $0.55 \pm 0.06 \mu\text{M}$, $P = 0.1026$, Kruskal-Wallis with Dunn's test; $n = 59$ –57 release records from six mice per genotype). Evoked [DA]_o differed between dlStr of 12-month-old *PrknR275W* mice and age-matched WT mice, as well as 6-month-old mice of both genotypes (12 mo *PrknR275W*: $0.09 \pm 0.02 \mu\text{M}$, *** $P < 0.001$ versus 12 mo WT: $0.64 \pm 0.03 \mu\text{M}$; *** $P < 0.001$ versus 6 mo WT; *** $P < 0.001$ versus 6 mo *PrknR275W*; Kruskal-Wallis with Dunn's test; $n = 58$ –116 release records from 6–10 mice per genotype). (D) In NAc core, average evoked [DA]_o (single pulse stimulation) did not significantly differ between the two genotypes at 6 months of age (6 mo WT: $0.36 \pm 0.05 \mu\text{M}$ versus 6 mo *PrknR275W*: $0.20 \pm 0.03 \mu\text{M}$, Kruskal-Wallis with Dunn's test, $P = 0.063$). However, in NAc core of 12-month-old *PrknR275W* mice evoked [DA]_o was lower as compared to age-matched WT mice and 6-month-old mice of both genotypes (12 mo *PrknR275W*: $0.035 \pm 0.013 \mu\text{M}$, *** $P < 0.001$ versus 12 mo WT: 0.44 ± 0.03 ; *** $P < 0.001$ versus 6 mo WT; *** $P < 0.001$ versus 6 mo *PrknR275W*; Kruskal-Wallis with Dunn's test; $n = 46$ –77 release records from 6–10 mice per genotype). (E) In NAc shell, average evoked [DA]_o (100 Hz 5-pulse stimulation) did not differ between the two genotypes at 6 months of age (6 mo WT: $0.30 \pm 0.04 \mu\text{M}$ versus 6 mo *PrknR275W*: $0.35 \pm 0.05 \mu\text{M}$, $P > 0.05$). In 12-month-old *PrknR275W* mice average evoked [DA]_o was decreased compared to age-matched WT mice and 6-month-old mice of both genotypes (12 mo *PrknR275W*: $0.42 \pm 0.03 \mu\text{M}$, Kruskal-Wallis with Dunn's test; $n = 46$ –50 release records from 6–10 mice per genotype). (F) Six- and 12-month-old *PrknR275W* mice were significantly impaired in the balance beam test (6 mo WT: 5.4 ± 0.3 versus 6 mo *PrknR275W*: 7.1 ± 0.3 , unpaired t -test, $t = 3.456$, $df = 37$, $P = 0.0014$, data from 17 WT and 22 *PrknR275W* mice; 12 mo WT: 6.0 ± 1.1 s versus 12 mo *PrknR275W*: 12.6 ± 1.5 s, unpaired t -test, $t = 1.836$, $df = 41$, $P = 0.0431$, data from 23 WT and 20 *PrknR275W* mice). (G) Results from pole test were similar between WT and *PrknR275W* mice at 6 months of age, whereas 12-month-old *PrknR275W* mice showed an impairment as compared to WT mice of the same age (total time pole test 12 mo WT: 8.3 ± 0.3 s versus 12 mo *PrknR275W*: 7.2 ± 0.6 , unpaired t -test, $t = 2.367$, $df = 26$, $P = 0.0256$, data from 16 WT and 12 *PrknR275W* mice; turn time pole test 12 mo WT: 3.7 ± 0.5 s versus 12 mo *PrknR275W*: 5.7 ± 0.7 s, unpaired t -test, $t = 2.462$, $df = 47$, $P = 0.0176$, data from 26 WT and 23 *PrknR275W* mice). For the data illustrated, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

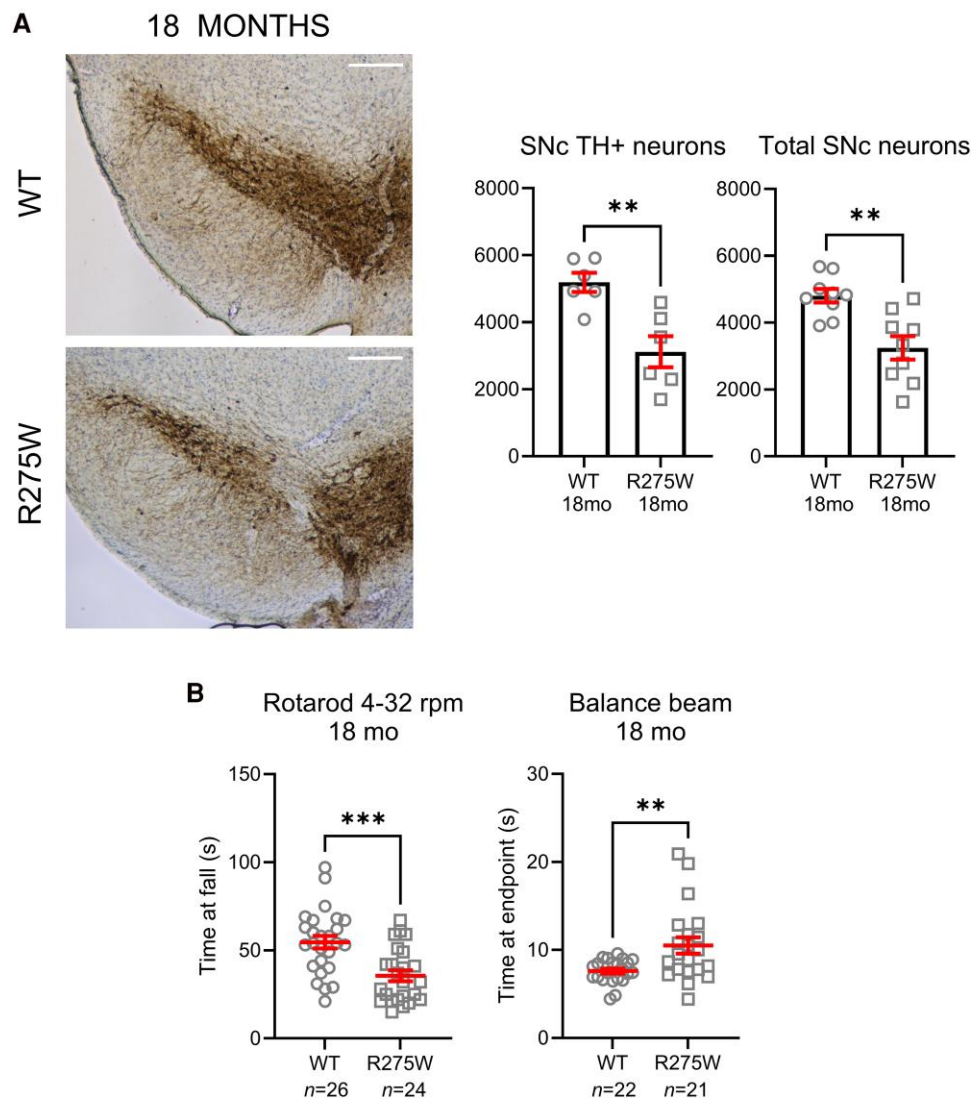


Figure 4 Dopaminergic neuron loss and impairment in the motor balance and coordination in aged (18-month-old) *PrknR275W* mice. (A) Representative images showing immunoperoxidase (tyrosine hydroxylase, TH) labelling in the substantia nigra pars compacta (SNc) of wild-type (WT) and *PrknR275W* mice at 18 months of age. *PrknR275W* mice showed a reduction in the levels of staining (scale bar = 100 μ m). The graphs on the right show the dopaminergic (DAergic) neuron quantification performed by unbiased stereology by analysing the number of TH+ neurons [18-month-old (18 mo) WT 5190 ± 285 versus 18 mo *PrknR275W* 3120 ± 464 , unpaired t-test, $t = 3.804$, $df = 10$, $P = 0.0035$, data from six WT and six *PrknR275W* mice]. The progressive decrease in DAergic neurons was confirmed by stereological count of Nissl+ neurons in SNc (18 mo WT 4805 ± 204 versus 18 mo *PrknR275W* 3245 ± 349 , unpaired t-test, $t = 3.861$, $df = 16$, $P = 0.0014$, data from nine WT and nine *PrknR275W* mice). (B) The graph shows the performance at rotarod and balance beam test of 18-month-old mice. Eighteen-month-old *PrknR275W* mice performed significantly worse in both the rotarod and the balance beam test (rotarod test: 18 mo WT 54.6 ± 3.6 s versus 18 mo *PrknR275W* 35.5 ± 3.2 s, unpaired t-test, $P = 0.0002$, data from 26 WT and 24 *PrknR275W* mice; balance beam: 18 mo WT 7.6 ± 0.3 s versus 18 mo *PrknR275W* 10.6 ± 0.9 s, unpaired t-test with Welch's correction, $P = 0.0063$, data from 22 WT and 21 *PrknR275W* mice). Pole test results are not shown because 18-month-old *PrknR275W* mice were too impaired to perform the task. For the data illustrated, ** $P < 0.01$, *** $P < 0.001$.

balance beam test (Fig. 4B). No sex effect was observed in either of these motor tests (Supplementary Fig. 19). Pole test results are not shown because 18-month-old mice were too impaired to accomplish the task. These data confirm that *PrknR275W* mice display progressive, age-dependent motor impairments.

Discussion

Many pathogenic variants in *PRKN* gene, including exonic rearrangements and missense, nonsense, and frameshift mutations, cause juvenile parkinsonism. The present work describes the phenotype

of the knock-in mouse model expressing the *PRKN* pathogenic variant p.Arg275Trp (R275W).

There is no clear genotype-phenotype correlation for the various pathogenic variants in the *PRKN* gene. This is in line with the idea that all the pathogenic gene variants in the *PRKN* gene cause either a substantial loss-of-protein function or the virtual absence of protein.¹² Consistently, we found a 75% decrease in parkin protein levels in the brains of *PrknR275W* mice. This downregulation of endogenous parkin caused by the missense *PrknR275W* mutation could explain why this model exhibits a parkinsonian phenotype, whereas *Prkn* KO mice do not. Indeed, genetic compensation is a feature of KO models but not of knockdown and mutant models.⁹

Patients carrying *PRKN* mutations display loss of nigrostriatal DAergic neurons and loss of noradrenergic neurons in LC that are not associated with α -synuclein neuronal inclusions.² In stereological counts, we found a 23% loss of DAergic neurons in the SNc in *Prkn*R275W mice as young as 6 months. Considering that 6 months of murine age correspond to ~30 years of age in humans,²¹ this finding correlates well with the median age at symptom onset in humans (31 years).^{1,20} In 12- and 18-month-old *Prkn*R275W mice, we found a loss of 28% and 40%, respectively, of TH+ neurons, which correlates with the slow progression of symptoms in ARJP patients.^{1,20,22} A further confirmation of the DAergic neuron loss comes from the labeling for SOX6, a marker of DA neuron vulnerability in Parkinson's disease.^{16,17} The early loss of noradrenergic neurons in LC and the absence of α -synuclein neuronal inclusions further show that this mouse model mirrors human neuropathology.

The loss of DAergic neurons is also in agreement with the decrease of total DA content in the dlStr seen as early as at 6 months of age in *Prkn*R275W mice. Interestingly, in these mice DA release assessed by FSCV was relatively preserved. This finding is consistent with previous studies in 6-OHDA lesioned rats, showing relatively constant peak evoked [DA]_o in vivo in spite of progressively larger DA loss.²³ This process reflects loss of DA uptake sites as well as of release sites, leading to maintenance of net evoked [DA]_o. This maintenance of dynamic DA release levels is also a likely contributing factor to the relative preservation of motor function seen in 6-month-old *Prkn*R275W mice. Other compensatory mechanisms might however also play a role, such as the concurrent release of acetylcholine (ACh) from striatal cholinergic interneurons, which promotes DA release acting at nicotinic ACh receptors on DA axons.²⁴ Conversely, partial inhibition of striatal cholinergic interneurons might decrease ACh release and thereby minimize ACh desensitization of nAChRs on DA axons to maintain DA homeostasis. These hypotheses will be tested in future studies.

Strikingly, in striatal slices from *Prkn*R275W mice, evoked [DA]_o was significantly lower at 12 months than at 6 months of age in the dlStr and NAc core, which is consistent with the progressive loss of DA neurons and progressive motor behaviour impairment between 6 and 18 months. Evoked [DA]_o in the NAc shell was largely preserved, consistent with the relative sparing of DA neurons of the NAc shell in these mice, as well as in post-mortem samples from patients with Parkinson's disease,²⁵ and in animal models of Parkinson's disease.²⁶ Our *Prkn*R275W mice recapitulate this phenotype, meeting a key criterion to judge the validity and value of animal models of Parkinson's disease.²⁶

Finally, it is interesting to note that in the *Prkn*R275W model histological and functional alterations, including cytoplasmic vacuolization, mitochondrial abnormalities and DAergic neuron firing changes, already appear at 1 month of age, i.e. long before the loss of DAergic neurons. Since cytoplasmic vacuolization is a morphological phenomenon that often accompanies cell dysfunction and precedes cell death,²⁷ these findings reveal early pathology, consistent with observations in both human and mouse DA neurons expressing parkin mutations.¹⁴ Another marker of dysfunction seen in 1-month-old *Prkn*R275W mice is a change in DAergic neuron firing pattern. In line with the proposed synaptic function of parkin,²⁸ early DAergic neuron firing changes might compound changes in DA release. Decreases in the number of spikes in a burst at 1 month of age in the *Prkn*R275W model might reflect changes in a potassium (K⁺) conductance. Indeed, ATP-sensitive K⁺ (K-ATP) channels control burst firing in SNc neurons via multiple mechanisms.²⁹ However, excitability changes of DAergic neurons may also depend on changes

in the amplitude of the afterhyperpolarization-associated current (I_{AHP}), as recent papers described in other Parkinson's disease models.³⁰

In conclusion, the *Prkn*R275W mouse recapitulates key features of ARJP, including DAergic neuron loss in the midbrain, noradrenergic neuron loss in LC, DA depletion in the striatum, and progressive motor impairment. We therefore propose the *Prkn*R275W mouse as a tool for the study of the causal mechanisms of Parkinson's disease and the development of neuroprotective therapies.

Data availability

All data generated in this study are available upon reasonable request. Raw data are available at San Raffaele Open Research Data Repository DOI: 10.17632/wgddvkjdrb.1.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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