Molecular Neurobiology (2019) 56:5157–5166
https://doi.org/10.1007/s12035-018-1431-z

Conditional Haploinsufficiency of β-Catenin Aggravates Neuronal Damage in a Paraquat-Based Mouse Model of Parkinson Disease

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Received: 26 September 2018 / Accepted: 13 November 2018 / Published online: 6 December 2018

Abstract

The canonical Wnt pathway is critical for both the development and adulthood survival and homeostatic maintenance of the midbrain dopaminergic (DA) neurons. Expanding evidence has demonstrated that genetic factors associated with familial Parkinson disease (PD) deregulate this important pathway, suggesting that a disturbed canonical Wnt pathway is likely involved in PD pathogenesis; yet, the specific role of this pathway in sporadic PD remains unclear. In this study, we aimed to determine the effects of specific inhibition of the canonical pathway by hemizygous knockout of β-catenin, the obligatory component of the canonical Wnt pathway, on paraquat (PQ)-induced DA neuronal degeneration in the substantia nigra in vivo. We found that while hemizygous conditional knockout of β-catenin in DA neurons did not cause any significant TH+ neuronal loss in the substantia nigra at basal level, it triggered elevated oxidative stress at basal level and further enhanced PQ-induced oxidative damage and loss of TH+ neurons in the substantia nigra and axonal termini in the striatum that manifested as exacerbated motor deficits. These data support the notion that reduced Wnt/β-catenin signaling in sporadic PD likely contributes to DA neuronal loss through an enhanced oxidative stress-response pathway.

Keywords Parkinson disease · Canonical Wnt pathway · β-Catenin · Oxidative stress · Neurodegeneration

Abbreviations

DA	Dopaminergic
DAT	Dopamine transporter
4-HNE	4-Hydroxynonenal
JNKs	c-Jun N-terminal kinases
KO	Knockout
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PQ	Paraquat
PD	Parkinson disease
ROS	Reactive oxygen species
SN	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
Tg	Transgenic
VTA	Ventral tegmental

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Introduction

Parkinson disease (PD) is a chronic progressive neurodegenerative disorder that results in motor impairment including rest tremor, bradykinesia, rigidity, and loss of postural reflexes [1]. It is characterized by the dramatic loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SN) region of the midbrain and the presence of abnormal fibrillary cytoplasmic inclusions rich in misfolded α-synuclein and ubiquitin, called Lewy bodies, in the surviving neurons of both central and peripheral nervous systems [2]. Genetic etiology was well demonstrated in the rare familial forms of the disease with pathogenic mutations being identified in more than a dozen genes which include autosomal dominant mutations in SNCA, LRRK2, and VPS35 as well as autosomal recessive mutations in PINK1, Parkin, DJ-1, etc. [3]. However, the cause of the majority of the disease occurrences, the sporadic form,

remains unknown, although it is believed that a complex interaction between genetic susceptibility and a panel of environmental factors is involved [4]. Indeed, aging and rural living likely due to exposure to a number of neurotoxins such as various pesticide/herbicides are known risk factors for human parkinsonism [5–9]. Characterization of these genes and environmental factors suggests several common cellular mechanisms including mitochondrial dysfunction, oxidative stress, neuroinflammation, protein misfolding, and impairment in the ubiquitin-proteasome and autophagy-lysosome systems [3, 10]. Besides these cellular mechanisms, increasing evidence suggests the involvement of Wnt/β-catenin signaling pathway in the pathogenesis of PD [11], although its exact role remains elusive.

The Wnt/β-catenin-dependent or canonical Wnt pathway is the best characterized among the three known Wnt signaling transduction cascades. In this canonical Wnt pathway, the binding of Wnt ligand to the receptor complex formed by the frizzled receptor and the LRP5/6 activates the intracellular signaling which leads to accumulation and nuclear translocation of β-catenin to form complexes with TCF/LEF and prompt the transcription of target genes [12]. It plays an essential role in the regulation of cell self-renewal, proliferation, and maintenance in a cell type-specific and developmental stage-specific manner [11]. In fact, this pathway has particular importance in specific brain regions as it is required during development of the midbrain dopaminergic system and is also a key pathway for regulating neurogenesis and survival of the midbrain DA neurons during adulthood [13–16]. It is tightly regulated at multiple levels which appears to be either impaired or excessively activated by various genetic and environmental factors involved in PD. For example, LRRK2 acts as a scaffolding protein connecting both membrane and cytosolic components of the canonical Wnt pathway and promotes its activation; however, LRRK2 mutations causing familial PD decrease the ability of LRRK2 to enhance the Wnt signaling [17–19]. Loss of VPS35 prevents the endosome-to-Golgi recycling of Wntless, a protein essential for the secretion of Wnt ligands [20], and it is likely that familial VPS35 mutations impair Wnt signaling. However, on the other hand, Parkin promotes β-catenin ubiquitination/degradation specifically in the ventral midbrain [21], and thus familial Parkin mutations, which cause loss of Parkin function, likely left DA neurons unprotected against excessive canonical Wnt signaling activation. Therefore, it is of importance to understand the exact role of deregulation of canonical Wnt pathway in the pathogenesis of PD.

Given the reports of reduced Wnt signaling in the brain during aging [22, 23] and more specifically in the brain of sporadic PD patients [24], we undertook the current study to determine the specific effect of inhibition of Wnt/β-catenin pathway on paraquat-induced TH+ neuronal loss in the substantia nigra in vivo as a model of sporadic PD. We found that inhibition of the canonical Wnt pathway by hemizygous conditional knockout of β-catenin in the DA neurons sensitizes the nigrostriatal system to the damage induced by PQ treatment likely through enhanced oxidative stress.

Materials and Methods

Animals and Treatment

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University (protocol number 2016-0050). All the mice in this study were maintained under constant environmental conditions in the Animal Research Center of Case Western Reserve University with free access to food and water. DAT-Cre mouse (DAT^{IRES}cre, 006660, Jackson laboratory) and β-catenin floxed strain ($\beta\text{-catenin}^{flox}$, 004152, Jackson laboratory) were purchased to generate experimental mice in this study. Mice were housed 2–4 per cage under a 12-h light and 12-h dark cycle. The development of conditional haploinsufficient β-catenin mice (Cre+) appeared normal compared with the Cre − littermates. Female and male 4-month-old Cre− mice (n = 13) and Cre+ mice (n = 15) were used in all of the experiments and randomly assigned into each group in the experiment. The mice with 20–28-g body weight were selected for this study. No statistical method was used to predetermine sample size in this study. PQ administered to the animals was dissolved in 0.9% sterile saline and injected intraperitoneally. Cre+ mice and Cre− littermate controls received either a dose of 10 mg/kg PQ twice a week for 6 consecutive weeks or an equal volume of vehicle. Seven days after the final injection, motor function of these mice was tested and then the mice were sacrificed for tissue collection. All the injections and measurements of motor function were performed blindly. Half brains were fixed overnight at 4 °C in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry. Striatal tissues were dissected from the other half brains for western blot analysis.

DNA Preparation and PCR Analysis

For the identification of the β-catenin alleles and the DAT-Cre transgene, DNA was isolated from indicated tissues of mice using genomic DNA purification kit (Promega, USA). To detect the β-catenin floxed allele, sense primer (5'AAGGTAGA GTGATGAAAGTTGTT3') and antisense primer (5'CACCATGTCCTCTGTCTATTC3') were used, generating 324 bp and 221 bp products from the floxed and wild-type alleles, respectively [25]. To detect △loxp of β-catenin floxed allele, sense primer (5' GCTATTGGGATTTC CAGGTA 3′) and antisense primer (5'CACCATGT CCTCTGTCTATTC3') were used, which generate a 252-bp

product from △loxp site. PCR was performed at 95 °C for 3 min followed by 45 cycles at 95 °C for 10 s, 50 °C for 15 s, and 72 °C for 30 s.

Antibodies and Chemicals

Primary antibodies used in this study included mouse anti-TH (Millipore, Temecula, CA, MAB318), rabbit anti-GAPDH (Cell Signaling, Danvers, MA, 2118), rabbit anti-4-HNE (Alpha diagnostics, San Antonio, TX, HNE11-S), rabbit anti-HO-1 (Millipore, Temecula, CA, AB1284), rabbit anti-pJNK (Cell Signaling, Danvers, MA, 9251), and rabbit anti-JNK (Cell Signaling, Danvers, MA, 9252). Secondary antibodies used in this study included anti-mouse/rabbit HRP-linked secondary antibody (Cell Signaling, Danvers, MA, 7076 or 7074), Alexa Fluor 488 donkey anti-mouse secondary antibody (Life technologies, Eugene, OR, A21202), Alexa Fluor 568 donkey anti-rabbit secondary antibody (Life technologies, Eugene, OR, A10042), goat anti-mouse or goat anti-rabbit (Millipore, Temecula, CA, AP124 or AP132), and mouse or rabbit PAP antibody (Jackson Immunoresearch, West Grove, PA). PQ dichloride hydrate (Sigma, St. Louis, MO, 856177) was purchased and dissolved in saline for mice treatment.

Immunohistochemistry

Immunohistochemistry was performed as we previously described [26, 27]. Briefly, mouse brains were fixed overnight at 4 °C in 10% neutral buffered formalin. Then, paraffin-embedded brains were sliced into consecutive coronal sections of 14 μm for the midbrain and striatum. The sections were sequentially incubated overnight at 4 °C with appropriate primary antibodies. The sections were then incubated with either goat anti-mouse or goat anti-rabbit antibody, followed by species-specific peroxidase anti-peroxidase complex (Jackson, West Grove, PA, 223005024 or 323005024). 3–3′-Diaminobenzidine (Dako, Carpinteria, CA, K3468) was used as a chromogen. For quantification of DA neurons, multiple sections of the SN region were immunostained for each mouse using tyrosine hydroxylase (TH) antibody. The number of dopamine neurons in the SNpc was estimated by counting TH-positive neurons of five coronal sections per animal that were distributed about every 100 μm along the rostral–caudate axis of the SN (− 3.08 to − 3.64 mm caudal to bregma) [28]. Densitometric analysis of TH immunostained striatum area was performed using image J software.

Western Blot Analysis

Striatal tissues were carefully dissected out and homogenized with RIPA lysis buffer plus protease inhibitor mixture (Roche, Penzberg, Germany, 5892791001/4906837001). Homogenates were centrifuged at 14,000 rpm for 20 min and the supernatants collected and protein level determined using BCA assay (Thermo Fisher Scientific, Waltham, MA, 23225). Equal amounts of total protein extracts were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Temecula, CA, IPVH00010). Following blocking with 10% nonfat milk, appropriate primary and secondary antibodies were applied, and the blots were developed with Immobilon Western Chemiluminescent HRP substrate (Millipore, Temecula, CA, WBKLS0500).

Rotarod Test

A mouse rotarod apparatus (Harvard apparatus, LE 8200) was used to measure motor coordination in these treated mice. The mice were trained to stay on the rotarod for 3 min at each constant speed (4, 10, and 20 rpm) in the week just before PQ treatment. After 3-day training, the motor performance was measured with an accelerating speed from 4 to 40 rpm in 4 min. For each trial, the time until the mice fall off the rod was recorded and a maximum of 120 s was applied in this study. The animals were tested three times with a rest of 5 min between each trial. The motor functions of these mice were tested by rotarod before and after the PQ injections. All the tests were performed during light cycle.

Statistical Analysis

All data represent mean ± standard error of means (SEM). For analysis of statistical difference between three or more groups, one-way analysis of variance with Bonferroni's multiple comparison tests was applied. For rotarod function, the paired *t* test was used. *p* values are indicated by asterisks (**$p < 0.01$; *$p < 0.05$). This study was not pre-registered.

Results

Characterization of the Hemizygous β-Catenin Conditional KO Mice (DAT-Cre$^{+/-}$/CTN$^{f/+}$ Mice)

The Wnt/β-catenin signaling pathway plays an essential role in the development of DA neurons [13, 14] and homozygous β-catenin knockout (KO) mice demonstrated significant loss of DA neurons in the substantia nigra [29]. To avoid developmental complications and specifically assess the potential role of the Wnt/β-catenin in the susceptibility of adult DA neurons in PD-related neurotoxin models, we chose to use hemizygous β-catenin conditional KO mice (DAT-Cre$^{+/-}$/CTN$^{f/+}$) by crossing the floxed β-catenin mice (CTN$^{f/f}$) with hemizygous dopamine transporter (DAT) promotor-driven Cre recombinase mice (DAT-Cre$^{+/-}$). To confirm that Cre recombinase was effective in mediating genomic recombination restricted in DA

neurons in the DAT-Cre$^{+/-}$/CTN$^{f/+}$ mice (i.e., hereafter referred as Cre+ mice or hemizygous cKO mice) but not in the littermate control DAT-Cre$^{-/-}$/CTN$^{f/+}$ mice (i.e., hereafter referred as Cre− mice or control mice), we performed PCR analysis specific to the recombined DNA in different tissues from randomly chosen Cre+ and Cre− mice at 3 months of age. PCR analyses showed Cre-mediated genomic recombination in the midbrain and olfactory bulb, brain areas containing DA neurons, in Cre+ mice but not in Cre− mice (Fig. 1a). Indeed, Cre-mediated recombination only occurred in olfactory bulb and substantia nigra/ventral tegmental (SN/VTA) of midbrain but not in the cortex, cerebellum, striatum, liver, and tail from Cre+ mouse (Fig. 1b). The hemizygous β-catenin cKO mice appear normal as compared to control mice. No significant reduction in the number of TH-positive DA neurons in the SN of hemizygous β-catenin cKO mice, compared to littermate control mice, was noted (Fig. 2b, c). Furthermore, the TH-positive axonal terminals were densely and evenly stained throughout the striatum without noticeable difference between the hemizygous cKO mice and littermate control mice (Fig. 3a, b), indicating that the midbrain DA neurons and their nigrostriatal projections are morphologically normal in the mice with hemizygous deletion of β-catenin in DA neurons.

β-Catenin Haploinsufficiency Exacerbates PQ-Induced Loss of DA Neurons in SN- and TH-Positive Axonal Terminals in Striatum

Cre+ mice and Cre− littermate control mice were intraperitoneally injected with PQ (10 mg/kg) or saline twice a week for 6 weeks starting at 4 months of age, sacrificed 1 week after the last injection, and DA neurons in the SN were analyzed by tyrosine hydroxylase (TH) immunocytochemistry (Fig. 2a). The number of TH-positive neurons was reduced by around 20% in the PQ-treated Cre− mice compared with the saline-treated Cre− mice, but did not reach significance ($p = 0.25$) (Fig. 2b, c). In contrast, the number of TH-positive neurons was significantly reduced by around 40% in the PQ-treated Cre+ mice as compared with that in the saline-treated Cre+ mice ($p < 0.05$). Importantly, there is also a significant reduction in the number of TH-positive neurons in the SN in PQ-treated Cre+ mice compared to that of PQ-treated Cre− littermate controls ($p < 0.05$).

Similar to the effects on DA neurons in SN, PQ caused a trend of decreased density of TH-positive axon termini in the striatum of Cre− mice ($p = 0.19$), as compared to that of saline-treated Cre− mice (Fig. 3a, b). On the contrary, PQ treatment caused a significant decrease in the density of striatal TH-positive fibers in the Cre+ mice as compared to that of saline-treated Cre+ mice ($p < 0.05$).

β-Catenin Haploinsufficiency Exacerbates PQ-Induced Motor Dysfunction

Progressive nigrostriatal injury in PD and neurotoxin-based rodent models result in impairment of motor function [2, 4, 30]. To assess the functional outcome of PQ-induced deficits in the hemizygous β-catenin cKO mice, we measured motor function of saline- and PQ-treated animals by rotarod test before and after the administration of saline or PQ. No significant difference in the latency to fall from the rotating rod was observed between the Cre+ and Cre− littermate controls mice before or after saline treatment (Fig. 4). Six weeks of PQ treatment caused a trend toward decreased latency to fall from rotarod in the Cre− mice as compared to these mice before the treatment ($p = 0.2$). Notably, PQ treatment caused significant decrease in the latency to fall in the Cre+ mice as compared to these same mice before the treatment.

β-Catenin Haploinsufficiency Enhances PQ-Induced Oxidative Stress Signaling in SN

Oxidative stress is believed to play a major role in mediating PQ-induced toxicity. 4-Hydroxynonenal (4-HNE), an α,β-unsaturated aldehyde produced during oxidation of membrane lipid polyunsaturated fatty acids, was suggested to be involved in the pathogenesis of PD- and neurotoxin-induced degeneration of DA neurons [31, 32]. Here, we analyzed levels of 4-

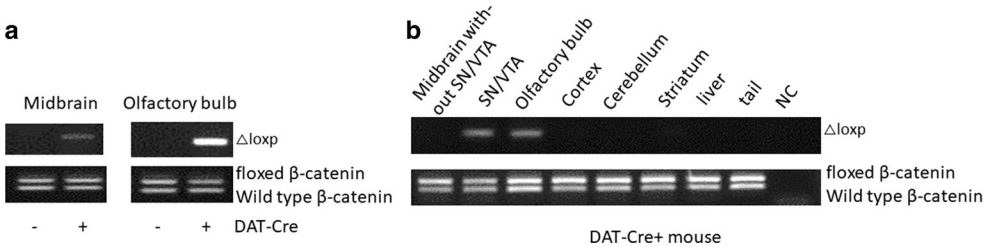

Fig. 1 Cre recombinase specifically deletes the loxp-flanked (floxed) β-catenin gene in dopaminergic neurons of DAT-Cre+/β-catenin± loxp mouse. Genomic DNAs (gDNA) were prepared from the indicated tissues from a DAT-Cre+/ β-catenin± loxp mouse (Cre+ mouse) and a DAT-Cre−/β-catenin± loxp control mouse (Cre− mouse). Special pair of DNA primers was designed for genotyping of deletion of floxed β-catenin. **a** PCR analysis shows DNA recombination is detected in midbrain and olfactory bulb only from Cre+ mouse but not Cre− mouse. There was 200 ng gDNA used in each reaction. **b** β-Catenin deletion (△loxp) is specific to the SN/VTA and olfactory bulb but not other tissues in the Cre+ mouse. There was 60 ng gDNA used in each reaction

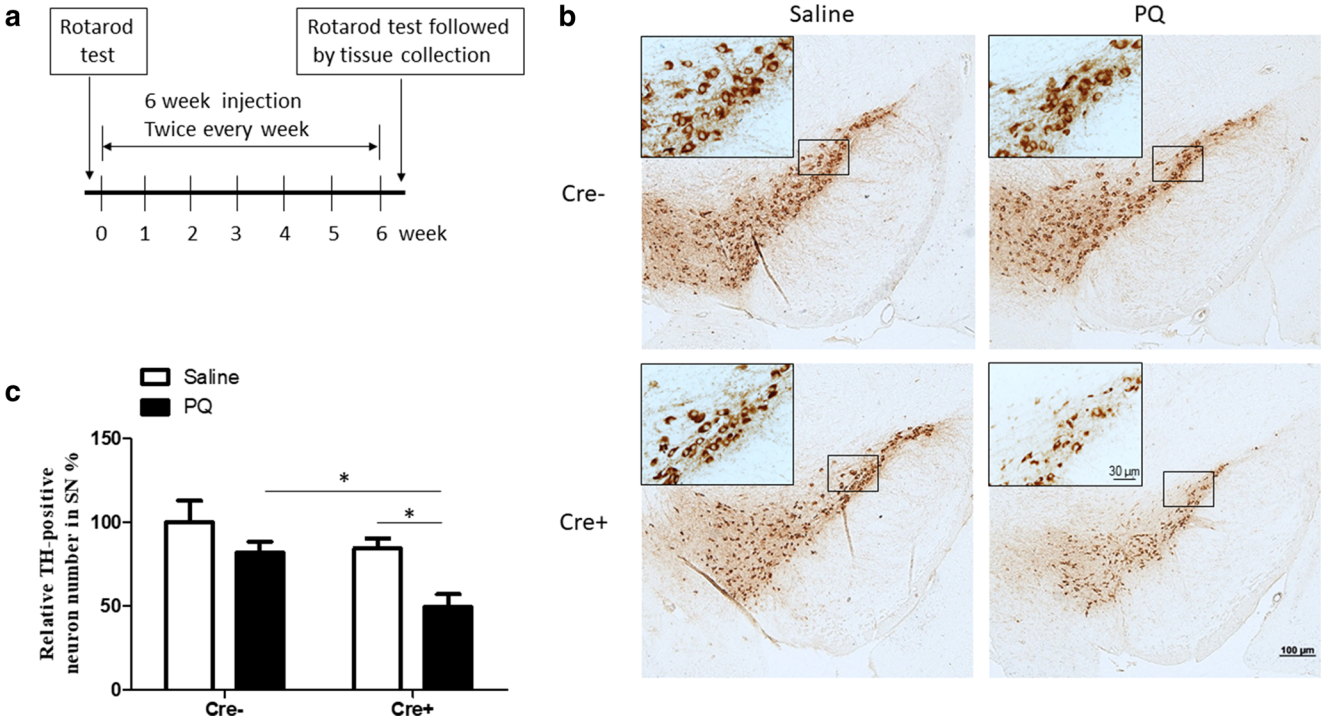

Fig. 2 β-catenin haploinsufficiency aggravates dopaminergic neuronal death in SN caused by PQ treatment. **a** Schematic timeline of experimental paradigm is shown. β-Catenin haploinsufficient (Cre+) and control (Cre−) mice were treated with PQ (10 mg/kg) or vehicle control saline twice a week for 6 weeks. Seven days after the last injection, brain tissue was collected for tyrosine hydrolase (TH) immunostaining. Representative pictures (**b**) and quantification of DA neurons (**c**) in the SNpc were shown. ($N = 5–6$/group, $^*p < 0.05$)

HNE as a measure of oxidative damage in PQ-treated mice (Fig. 5a). Interestingly, in saline-treated mice, a significant increase in 4-HNE immunoreactivity in SN was noted in Cre+ mice compared with that of Cre− mice (Fig. 5a, b), suggesting that β-catenin haploinsufficiency induces elevated basal level of oxidative stress in DA neurons. As expected, PQ treatment led to a significant increase in 4-HNE immunoreactivity in SN of Cre− mice. Importantly, 4-HNE immunoreactivity was further significantly increased in PQ-treated Cre+ mice. Consistently, the immunoreactivity of heme oxygenase-1 (HO-1), antioxidant enzyme that is widely used as an important marker of oxidative stress, was also significantly increased in SN of PQ-treated Cre+ mice compared with that of PQ-treated Cre− mice (Fig. 6a, b). To

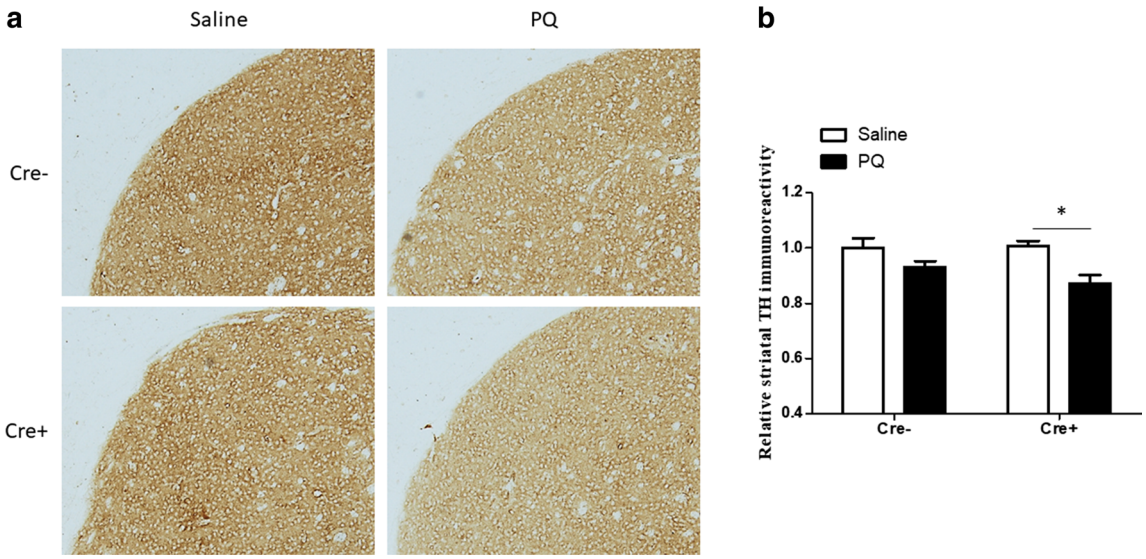

Fig. 3 β-Catenin haploinsufficiency causes significant loss of PQ-induced terminal DA fibers in striatum. Representative pictures of DA terminals (**a**) in the dorsal striatum and quantification of the TH staining intensity (**b**) in this area. ($N = 4$/group, $^*p < 0.05$)

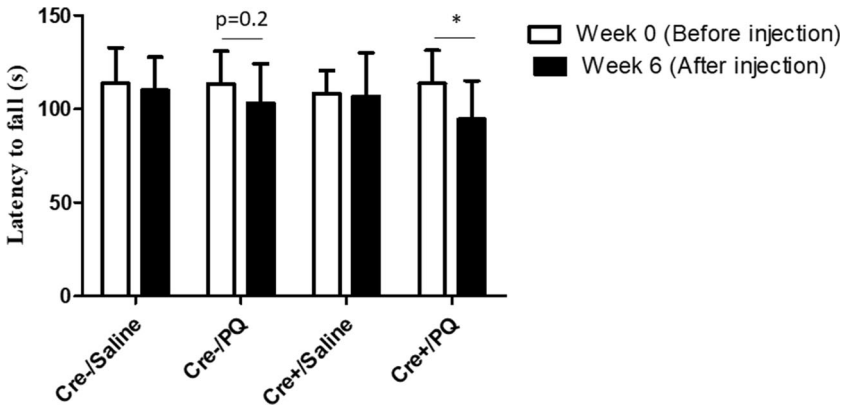

Fig. 4 β-Catenin haploinsufficiency aggravates PQ-induced impairment of motor function. The motor functions of mice were determined before the first injection (week 0) and 1 week after the last PQ injection (week 6). (Paired *t* test, $N = 5–8$/group $^*p < 0.05$)

distinguish the nature of these cells with increased oxidative stress, we performed additional immunofluorescent colocalization study. Immunofluorescence staining of the SN revealed that the PQ-induced immunoreactivities of stress markers, 4-HNE and HO-1, were almost completely localized to TH-positive cell, confirming increased oxidative stress in DA neurons after PQ treatment in both Cre+ and Cre− mice (Fig. 7a, b). These data also confirmed significantly increased numbers of dopamine neurons with 4-HNE or HO-1 immunoreactivity in the PQ-treated Cre+ mice as compared to that of PQ-treated Cre− mice.

As a stress-activated kinase, c-Jun N-terminal kinase (JNK) activation is involved in PQ-induced DA neuronal death [33–35]. In saline-treated mice, consistent with increased oxidative stress in Cre+ mice, there were also significantly increased levels of phosphorylated JNK in the striatum of Cre+ mice as compared to that of Cre− mice (Fig. 8a–c). There was a trend towards increased JNK phosphorylation in PQ-treated Cre− mice as compared to that of saline-treated Cre− mice ($p = 0.09$). Importantly, JNK phosphorylation was significantly increased in the striatum of PQ-treated Cre+ mice as compared to saline-treated Cre+ mice or PQ-treated Cre− mice. Total JNK levels in striatum remain unchanged among different groups in this study (Fig. 8a, c).

Discussion

In this study, we used hemizygous β-catenin cKO to determine whether inhibition of the Wnt pathway exacerbates or protects DA neurons against PQ-induced damage in vivo. We found that while hemizygous conditional knockout of β-catenin in DA neurons did not cause any significant TH+ neuronal loss in the substantia nigra at basal level, it triggered elevated oxidative stress at basal level and further enhanced PQ-induced oxidative damage and loss of TH+ neurons in the substantial nigra and axonal termini in the striatum that manifested as exacerbated motor deficits. These data support the

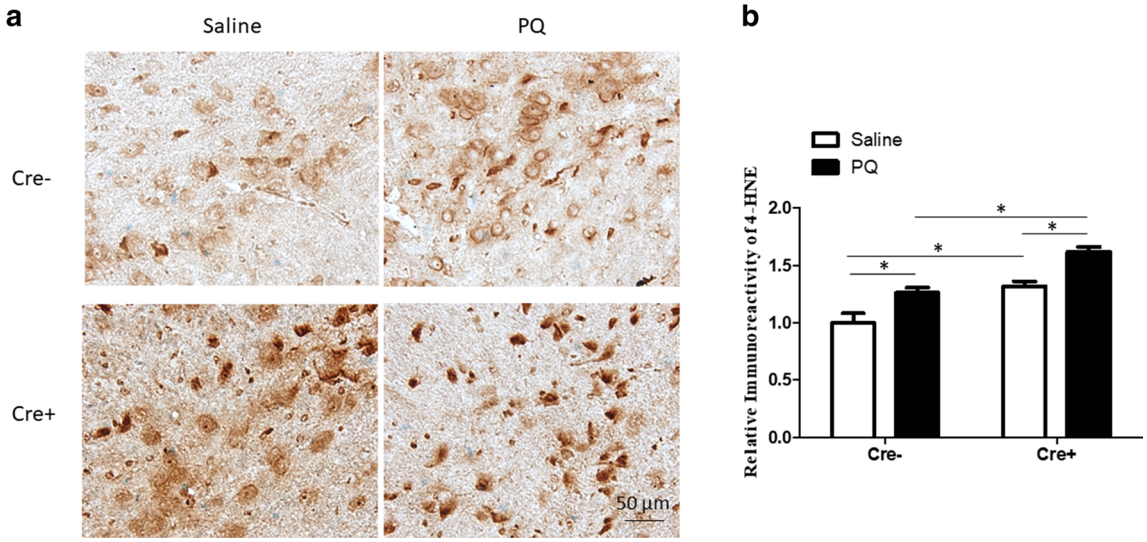

Fig. 5 β-Catenin haploinsufficiency caused increased 4-HNE immunoreactivity in SN at basal level and after PQ treatment. Fixed brains of PQ or saline treated Cre+ and Cre− mice were immunostained for 4-HNE. Representative pictures (**a**) and quantification (**b**) of 4-HNE immunoreactivity in SN. ($N = 5–6$/group, $^*p < 0.05$)

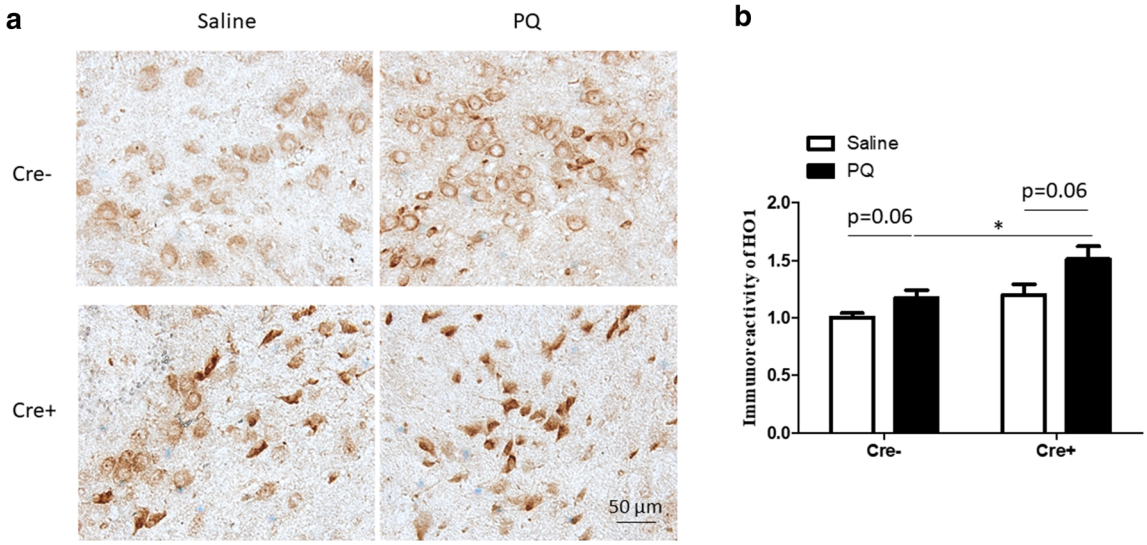

Fig. 6 β-Catenin haploinsufficiency caused increased HO1 immunoreactivity in SN after PQ treatment. Fixed brains of PQ or saline-treated Cre+ and Cre− mice were immunostained for HO1. Representative pictures (**a**) and quantification (**b**) of HO1 immunoreactivity in SN. ($N = 5–6$, $*p < 0.05$)

notion that reduced Wnt/β-catenin signaling in sporadic PD contributes to DA neuronal loss likely through an enhanced oxidative stress response pathway.

While prior studies based on genetic factors associated with familial PD suggest that disturbed Wnt/beta-catenin signaling is likely involved in the pathogenesis of PD, the specific effects of different genetic factors appear different: reduced Wnt signaling likely contributes to mutant LRRK2-induced DA neuronal loss since familial LRRK2 mutations decrease the capability of LRRK2 to enhance the Wnt signaling [17–19] while excessive Wnt signaling is to be blamed in the case of familial loss-of-function Parkin mutation-induced DA neuronal loss since Parkin is critical in the ubiquitination/degradation of β-catenin [21]. Therefore, although reduced Wnt/β-catenin signaling in the DA neurons in substantia nigra of PD patients was noted [24], it remains to be determined

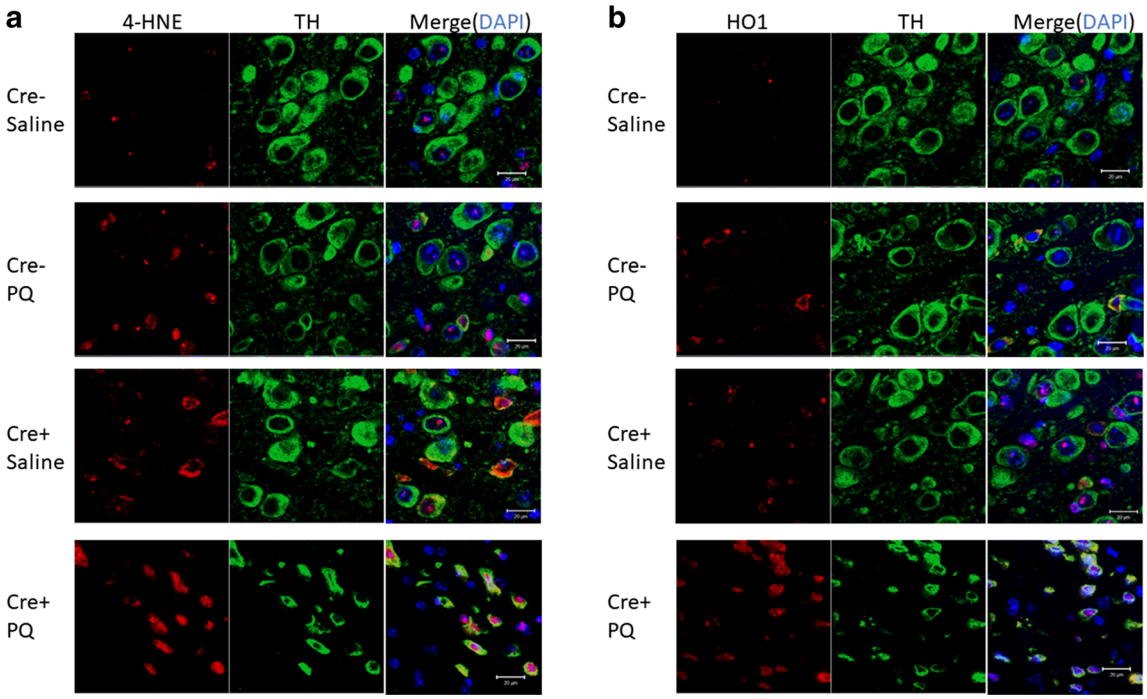

Fig. 7 Immunofluorescence co-localization study confirmed increased oxidative stress in TH+ neurons in SN. Fixed brains of PQ or saline-treated Cre+ and Cre− mice were immunostained for 4-HNE and TH or HO-1 and TH. Representative fluorescent images of 4-HNE and TH (**a**), or HO1 and TH (**b**), in SN ($N = 4$) of PQ or saline-treated mice were shown. Scale, 20 μm

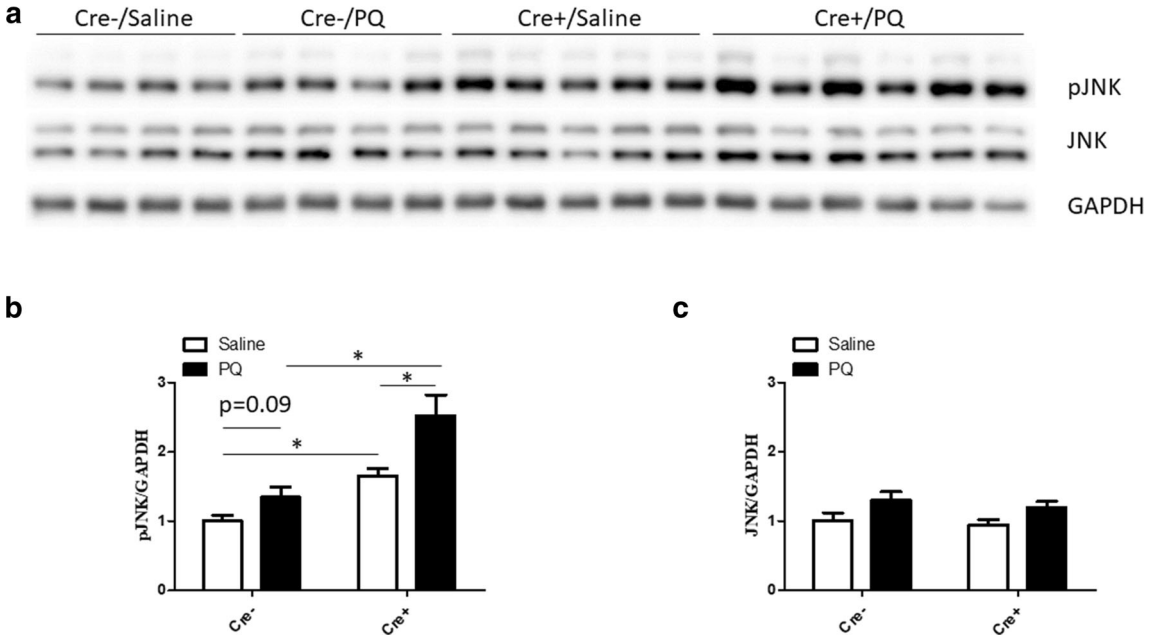

Fig. 8 β-Catenin haploinsufficiency aggravates pJNK activation in the striatum at basal level and after PQ treatment. Cre+ and Cre− mice were treated with PQ or saline. From each half brain, the striatum was dissected for immunoblotting analysis. Representative immunoblot (**a**) and quantification of pJNK (**b**) and total JNK (**c**) in striatum. ($N = 4–6$/group, $^*p < 0.05$)

whether such reduction contributes to neuronal loss or represents an insufficient protective response in sporadic PD pathogenesis. The answer will likely inform future leads for therapeutic developments. Neurotoxins including MPTP and paraquat are widely used to treat DA neuronal cells and animals as models to study sporadic PD. It is of interest to note that PD-relevant neurotoxins including paraquat led to downregulation of canonical Wnt signaling which correlated with neuronal loss both in vitro and in vivo [36–40], implicating that a reduced Wnt signaling likely plays a damaging role in mediating neurotoxin-induced DA neuronal loss. To directly address this important issue, we specifically inhibited the canonical Wnt pathway in the DA neurons by hemizygous knockout of β-catenin. We believe that this is more advantageous than using homozygous knockout because it specifically mimics the reduction but not the total loss of β-catenin in DA neurons in sporadic PD, and avoids the complication of significant loss of DA neurons due to developmental defects caused by total loss of β-catenin in the homozygous β-catenin knockout mice [29]. The treatment of PQ twice a week for 6 consecutive weeks caused only mild neuronal death in dorsal SN of control mice, but significantly more severe DA neuronal degeneration and motor defects in hemizygous β-catenin cKO mice along with significantly reduced TH+ axonal termini in the striatum and motor deficits. Our results thus provide direct evidence to strongly support a pathogenic role of reduced Wnt signaling in sporadic PD models. Furthermore, these data in neurotoxin-based sporadic PD models together support the restoration or activation of Wnt signaling as a promising therapeutic target for PD. Indeed, consistent with this notion, exogenous administration of Wnt1 or LiCl reversed 6-OHDA-induced inactivation of Wnt/β-catenin pathway and attenuated neuronal cell death [38, 41].

How might reduced Wnt/β-catenin signaling contribute/mediate neuronal death in sporadic PD? It is known that oxidative stress plays an important role in the pathogenesis of PD [10]. Early studies demonstrated that β-catenin binds directly to forkhead box O (FOXO) transcription factors and enhances the expression of FOXO target genes critical for resistance to oxidative damage in both *Caenorhabditis elegans* and mammalian cells [42]. Consistently, loss of β-catenin also triggers oxidative stress in liver cells [43]. However, activation of nuclear β-catenin promotes oxidative damage in diabetic cardiomyopathy [44], highlighting the need to verify the specific role of Wnt/β-catenin in cells of target since most Wnt/β-catenin target genes are cell-type and developmental stage-specific. In this regard, our study also demonstrated increased oxidative stress in the DA neurons at basal level in the hemizygous β-catenin cKO mice as evidenced by significantly increased levels of 4-HNE, a lipid peroxidation product, along with significantly increased JNK activation and trends toward increased expression of HO-1, an inducible antioxidant enzyme. Our data thus confirmed the importance of Wnt/β-catenin pathway in maintaining oxidative homeostasis at basal level in the DA neurons. Considering that the higher basal rate of oxidative phosphorylation and thus lower threshold to oxidative damage contributes to the vulnerability of SN DA neurons [45], it was not unexpected to find in our study all these oxidative stress markers were further significantly increased in the hemizygous β-catenin cKO mice exposed to

PQ compared to that of control mice, accompanying significantly increased DA neuronal loss. Our study exemplified a likely situation where reduced Wnt/β-catenin signaling in DA neurons along with aging places these neurons in an adverse situation. Exposure to additional environmental insults such as PQ exposure initiates a downward spiral by further reducing β-catenin signaling and hampering sufficient antioxidant response resulting in enhanced oxidative damage and eventual neuronal loss along with the motor function deficit. Therefore, our study shed new light on the potential neurodegenerative mechanism in sporadic PD. That said, it must be acknowledged that our results do not exclude the possibility that other mechanisms regulated by the canonical Wnt pathway such as neurogenesis [14] may be impaired and involved in the pathogenesis of sporadic PD.

Taken together, our in vivo study revealed that conditional haploinsufficiency of β-catenin caused elevated oxidative stress at basal level in SN DA neurons which made them vulnerable to PQ-induced oxidative damage and neuronal degeneration in nigrotriatal system. Our study provides direct evidence to strongly support the pathogenic role of reduced Wnt/β-catenin signaling in sporadic PD and suggest the restoration/activation of canonical Wnt pathway may be pursued for future therapeutic development for PD.

Author's Contributions FZ designed/carried out experiments, collected data and wrote the manuscript, SLS, SLR QX, BT contributed to interpretation of results and provided feedback on the manuscript. BT and XZ conceived the project. XZ directed the project, interpret results and wrote the manuscript. All authors had final approval of the submitted version.

Funding Information This research was partly supported by the National Institute of Health [grant number NS083498 (to X. Zhu)], the Dr. Robert M. Kohrman Memorial Fund (to X. Zhu), the National Natural Science Foundation of China [No. 81430023 (to B. Tang)], the National Key Plan for Scientific Research and Development of China [No. 2016YFC1306000 (to B. Tang)], and the NSFC-NIH joint program [No. 81361120404 (to B. Tang)].

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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References

1. Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry 79(4):368–376. https://doi.org/10.1136/jnnp.2007.131045
2. Schapira AH (1997) Pathogenesis of Parkinson's disease. Baillieres Clin Neurol 6(1):15–36
3. Domingo A, Klein C (2018) Genetics of Parkinson disease. Handb Clin Neurol 147:211–227. https://doi.org/10.1016/B978-0-444-63233-3.00014-2
4. Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39(6):889–909
5. Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S (2000) A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology 21(4):435–440
6. Van Maele-Fabry G, Hoet P, Vilain F, Lison D (2012) Occupational exposure to pesticides and Parkinson's disease: a systematic review and meta-analysis of cohort studies. Environ Int 46:30–43. https://doi.org/10.1016/j.envint.2012.05.004
7. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC (1997) Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology 48(6):1583–1588
8. Pezzoli G, Cereda E (2013) Exposure to pesticides or solvents and risk of Parkinson disease. Neurology 80(22):2035–2041. https://doi.org/10.1212/WNL.0b013e318294b3c8
9. Freire C, Koifman S (2012) Pesticide exposure and Parkinson's disease: epidemiological evidence of association. Neurotoxicology 33(5):947–971. https://doi.org/10.1016/j.neuro.2012.05.011
10. Yan MH, Wang X, Zhu X (2013) Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic Biol Med 62:90–101. https://doi.org/10.1016/j.freeradbiomed.2012.11.014
11. Garcia-Velazquez L, Arias C (2017) The emerging role of Wnt signaling dysregulation in the understanding and modification of age-associated diseases. Ageing Res Rev 37:135–145. https://doi.org/10.1016/j.arr.2017.06.001
12. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17(1):9–26. https://doi.org/10.1016/j.devcel.2009.06.016
13. Castelo-Branco G, Wagner J, Rodriguez FJ, Kele J, Sousa K, Rawal N, Pasolli HA, Fuchs E et al (2003) Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci U S A 100(22):12747–12752. https://doi.org/10.1073/pnas.1534900100
14. Joksimovic M, Awatramani R (2014) Wnt/beta-catenin signaling in midbrain dopaminergic neuron specification and neurogenesis. J Mol Cell Biol 6(1):27–33. https://doi.org/10.1093/jmcb/mjt043
15. Prakash N, Brodski C, Naserke T, Puelles E, Gogoi R, Hall A, Panhuysen M, Echevarria D et al (2006) A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. Development 133(1):89–98. https://doi.org/10.1242/dev.02181
16. Fukusumi Y, Meier F, Gotz S, Matheus F, Irmler M, Beckervordersandforth R, Faus-Kessler T, Minina E et al (2015) Dickkopf 3 promotes the differentiation of a rostrolateral midbrain dopaminergic neuronal subset in vivo and from pluripotent stem cells in vitro in the mouse. J Neurosci 35(39):13385–13401. https://doi.org/10.1523/JNEUROSCI.1722-15.2015
17. Salasova A, Yokota C, Potesil D, Zdrahal Z, Bryja V, Arenas E (2017) A proteomic analysis of LRRK2 binding partners reveals interactions with multiple signaling components of the WNT/PCP pathway. Mol Neurodegener 12(1):54. https://doi.org/10.1186/s13024-017-0193-9
18. Berwick DC, Harvey K (2012) LRRK2 functions as a Wnt signaling scaffold, bridging cytosolic proteins and membrane-localized LRP6. Hum Mol Genet 21(22):4966–4979. https://doi.org/10.1093/hmg/dds342
19. Berwick DC, Javaheri B, Wetzel A, Hopkinson M, Nixon-Abell J, Granno S, Pitsillides AA, Harvey K (2017) Pathogenic LRRK2

variants are gain-of-function mutations that enhance LRRK2-mediated repression of beta-catenin signaling. Mol Neurodegener 12(1):9. https://doi.org/10.1186/s13024-017-0153-4
20. Eaton S (2008) Retromer retrieves wntless. Dev Cell 14(1):4–6. https://doi.org/10.1016/j.devcel.2007.12.014
21. Rawal N, Corti O, Sacchetti P, Ardilla-Osorio H, Sehat B, Brice A, Arenas E (2009) Parkin protects dopaminergic neurons from excessive Wnt/beta-catenin signaling. Biochem Biophys Res Commun 388(3):473–478. https://doi.org/10.1016/j.bbrc.2009.07.014
22. Hofmann JW, McBryan T, Adams PD, Sedivy JM (2014) The effects of aging on the expression of Wnt pathway genes in mouse tissues. Age 36(3):9618. https://doi.org/10.1007/s11357-014-9618-3
23. Bayod S, Felice P, Andres P, Rosa P, Camins A, Pallas M, Canudas AM (2015) Downregulation of canonical Wnt signaling in hippocampus of SAMP8 mice. Neurobiol Aging 36(2):720–729. https://doi.org/10.1016/j.neurobiolaging.2014.09.017
24. Zhang L, Deng J, Pan Q, Zhan Y, Fan JB, Zhang K, Zhang Z (2016) Targeted methylation sequencing reveals dysregulated Wnt signaling in Parkinson disease. J Genet Genomics 43(10):587–592. https://doi.org/10.1016/j.jgg.2016.05.002
25. Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O et al (2001) Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 128(8):1253–1264
26. Wang W, Wang X, Fujioka H, Hoppel C, Whone AL, Caldwell MA, Cullen PJ, Liu J et al (2016) Parkinson's disease-associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes. Nat Med 22(1):54–63. https://doi.org/10.1038/nm.3983
27. Jiang S, Nandy P, Wang W, Ma X, Hsia J, Wang C, Wang Z, Niu M et al (2018) Mfn2 ablation causes an oxidative stress response and eventual neuronal death in the hippocampus and cortex. Mol Neurodegener 13(1):5. https://doi.org/10.1186/s13024-018-0238-8
28. Fujita K, Seike T, Yutsudo N, Ohno M, Yamada H, Yamaguchi H, Sakumi K, Yamakawa Y et al (2009) Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. PLoS One 4(9):e7247. https://doi.org/10.1371/journal.pone.0007247
29. Dai TL, Zhang C, Peng F, Niu XY, Hu L, Zhang Q, Huang Y, Chen L et al (2014) Depletion of canonical Wnt signaling components has a neuroprotective effect on midbrain dopaminergic neurons in an MPTP-induced mouse model of Parkinson's disease. Exp Ther Med 8(2):384–390. https://doi.org/10.3892/etm.2014.1745
30. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ (1999) Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res 823(1–2):1–10
31. Selley ML (1998) (E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. Free Radic Biol Med 25(2):169–174
32. McCormack AL, Atienza JG, Johnston LC, Andersen JK, Vu S, Di Monte DA (2005) Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. J Neurochemy 93(4):1030–1037. https://doi.org/10.1111/j.1471-4159.2005.03088.x
33. Choi WS, Abel G, Klintworth H, Flavell RA, Xia Z (2010) JNK3 mediates paraquat- and rotenone-induced dopaminergic neuron death. J Neuropathol Exp Neurol 69(5):511–520. https://doi.org/10.1097/NEN.0b013e3181db8100
34. Hunot S, Vila M, Teismann P, Davis RJ, Hirsch EC, Przedborski S, Rakic P, Flavell RA (2004) JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 101(2):665–670. https://doi.org/10.1073/pnas.0307453101
35. Zhao F, Wang W, Wang C, Siedlak SL, Fujioka H, Tang B, Zhu X (2017) Mfn2 protects dopaminergic neurons exposed to paraquat both in vitro and in vivo: implications for idiopathic Parkinson's disease. Biochim Biophys Acta 1863(6):1359–1370. https://doi.org/10.1016/j.bbadis.2017.02.016
36. L'Episcopo F, Tirolo C, Testa N, Caniglia S, Morale MC, Cossetti C, D'Adamo P, Zardini E et al (2011) Reactive astrocytes and Wnt/beta-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Neurobiol Dis 41(2):508–527. https://doi.org/10.1016/j.nbd.2010.10.023
37. L'Episcopo F, Serapide MF, Tirolo C, Testa N, Caniglia S, Morale MC, Pluchino S, Marchetti B (2011) A Wnt1 regulated Frizzled-1/beta-catenin signaling pathway as a candidate regulatory circuit controlling mesencephalic dopaminergic neuron-astrocyte crosstalk: therapeutical relevance for neuron survival and neuroprotection. Mol Neurodegener 6:49. https://doi.org/10.1186/1750-1326-6-49
38. Dun Y, Li G, Yang Y, Xiong Z, Feng M, Wang M, Zhang Y, Xiang J et al (2012) Inhibition of the canonical Wnt pathway by Dickkopf-1 contributes to the neurodegeneration in 6-OHDA-lesioned rats. Neurosci Lett 525(2):83–88. https://doi.org/10.1016/j.neulet.2012.07.030
39. Gollamudi S, Johri A, Calingasan NY, Yang L, Elemento O, Beal MF (2012) Concordant signaling pathways produced by pesticide exposure in mice correspond to pathways identified in human Parkinson's disease. PLoS One 7(5):e36191. https://doi.org/10.1371/journal.pone.0036191
40. Wei L, Ding L, Mo MS, Lei M, Zhang L, Chen K, Xu P (2015) Wnt3a protects SH-SY5Y cells against 6-hydroxydopamine toxicity by restoration of mitochondria function. Transl Neurodegener 4:11. https://doi.org/10.1186/s40035-015-0033-1
41. Wei L, Sun C, Lei M, Li G, Yi L, Luo F, Li Y, Ding L et al (2013) Activation of Wnt/beta-catenin pathway by exogenous Wnt1 protects SH-SY5Y cells against 6-hydroxydopamine toxicity. J Mol Neurosci 49(1):105–115. https://doi.org/10.1007/s12031-012-9900-8
42. Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC (2005) Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science 308(5725):1181–1184. https://doi.org/10.1126/science.1109083
43. Lento W, Ito T, Zhao C, Harris JR, Huang W, Jiang C, Owzar K, Piryani S et al (2014) Loss of beta-catenin triggers oxidative stress and impairs hematopoietic regeneration. Genes Dev 28(9):995–1004. https://doi.org/10.1101/gad.231944.113
44. Liu P, Su J, Song X, Wang S (2017) Activation of nuclear beta-catenin/c-Myc axis promotes oxidative stress injury in streptozotocin-induced diabetic cardiomyopathy. Biochem Biophys Res Commun 493(4):1573–1580. https://doi.org/10.1016/j.bbrc.2017.10.027
45. Pacelli C, Giguere N, Bourque MJ, Levesque M, Slack RS, Trudeau LE (2015) Elevated mitochondrial bioenergetics and axonal arborization size are key contributors to the vulnerability of dopamine neurons. Curr Biol 25(18):2349–2360. https://doi.org/10.1016/j.cub.2015.07.050